Antimicrobial chromogranin A derived peptides and Staphylococcus aureus : from host pathogen interaction analysis to development of antimicrobial polymer coating

Rizwan Aslam

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Présentée par :

Rizwan ASLAM

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Les peptides antimicrobiens dérivés de la chromogranine A et *Staphylococcus aureus*: de l'analyse de l'interaction hôte-pathogène au développement de revêtement de polymère antimicrobien

THÈSE dirigée par :

Mme METZ BOUTIGUE Marie Hélène	Directrice de Recherche, Université de Strasbourg
M. PREVOST Gilles	MCU-PH, Université de Strasbourg

RAPPORTEURS :

M. DRIDER Djamel	Pr, Université de Lille-1
M. BERJEAUD Jean-Marc	Pr, Université de Poitiers
M. BECHINGER Burkhard	Pr, Université de Strasbourg

AUTRES MEMBRES DU JURY :

M. JACQUES Philippe	Pr, Université de Lille-1

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ABBREVIATIONS

A. fumigatus: *Aspergillus fumigatus*

AFM: Atomic force microscopy measurements

ATCC: American Type Culture Collection

bCAT: Bovine catestatin

C. albicans: *Candida albicans*

C. glabrata: *Candida glabrata*

C. tropicalis: *Candida tropicalis*

CBS: Centraalbureau voor Schimmelcultures

CFU: Colony forming unit

CgA: Chromogranin A

CgB: Chromogranin B

CgC: Chromogranin C

Cgs: Chromogranins

CHI/HA: Chitosan/Hyaluronic acid

CHI: Chitosan

CHR: Chromofungin

CLSM: Confocal laser scanning microscopy

CTL: Cateslytin

DDT: Dichloro-Diphenyl-Trichloroethane

DMEM: Dulbecco's Modified Eagle Medium

E. coli: *Escherichia coli*

ECL:

ECM: Extra Cellular Matrix

EDC: N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride

EDTA: Ethylene-Diamine-Tetra-Acetic Acid

EGTA: Ethylene glycol-bis-(L-aminoethyl ether) N, N, N´, N´-tetraacetic acid

ETs: Exfoliative toxins (A, B)

FITC: Fluorescein Isothiocyanate

Fmoc: Fluorenylmethoxycarbonyl

FnBPA: Fibrinectin binding protein A

FnBPB: Fibrinectin binding protein B

FPLC: Fast performance liquid chromatography

HA: Hyaluronic acid

hCAT: Human catestatin

HCl: Hydrochloric acid

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGFs: Human gingival fibroblasts

Hlg: Gamma hemolysin (A, B, C)

IgG: Immunoglobulin G

IgM: Immunoglobulin M

KCl: Potassium chloride

LbL: Layer-by-Layer

MALDI-TOF: Matrix-assisted laser desorption/ionization-Time Of Flight

MH: Mueller Hinton

MRSA: Methicillin-resistant *S. aureus*

MSCRAMMs: Microbial surface components recognizing adhesive matrix molecules

MSSA: Methicillin-susceptible *S. aureus*

N. crassa: *Neurospora crassa*

NaCl: Sodium chloride

NaOH: Sodium hydrooxide

NH_4HCO_3: Ammonium bi-carbonate

NMR: Nuclear magnetic resonance

OD: Optical density

PAH/PAA: poly-allylamine hydrochloride/poly-acrylic acid

PAH/PAAm: Poly-alanine hydrochloride/polyacrylamide

PAH: Poly-allylamine hydrochloride

PDB: Potato Dextrose Broth

PDMS: Polydimethylsiloxane

PEC: Polyelectrolyte complex

PEI: Polyethyleneimine

PEM: Polymer electrolyte membrane

PEMs: Polyelectrolyte multilayers

PLL / PGA-g-PEG: Poly-L-lysine/Poly-glutamic acid -poly-ethylene glycol

PLL/PGA: Poly-L-lysine/Poly-glutamic acid)

PMNs: Polymorphonuclear neutrophils

PSS: Poly-sodium styrene sulfonate

PTH-aas: Phenylthiothiohydantoine-amino acids

PVDF: Polyvinylidene Fluoride

PVL: Panton Valentine leucocidin

QCM: Quartz crystal microbalance

RP-HPLC: Reverse phase-high performance liquid chromatography

S.aureus: *Staphylococcus aureus*

SDS: Sodium dodecyle sulphate

SDS-PAGE: Sodium dodecyle sulphate polyacrylamide gel electrophoresis

SERAM: Secretable expanded repertoire adhesive molecule

SEs: Staphylococcal enterotoxins

SP sepharose: Sulfopropyl sepharose

Sulfo-NHS: 3-sulfo-N-hydroxysuccinimide ester

T. mentagrophytes: *Trichophyton mentagrophytes*

TFA: Trifluoroacetic acid

TRIS: 2-Amino-2-hydroxymethyl-propane-1, 3-diol

TSSTs: Toxic shock syndrome toxins (0, I)

YCP: Yeast extract-casamino acids-pyruvate

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RÉSUME

L'étude que j'ai menée dans le cadre de ma thèse de l'Université de Strasbourg, a porté principalement sur l'analyse de l'interaction hôte-pathogène et le développement de polymères de recouvrement antimicrobiens par conjugaison de peptides antimicrobiens sur les matériaux.

Dans une première partie, nous avons d'une part évalué l'interaction de la leukotoxine LukE/D de *Staphylococcus aureus* avec les neutrophiles polynucléaires (PMNs) et d'autre part nous avons examiné le rôle des protéases de *S. aureus* sur la dégradation des peptides antimicrobiens dérivés principalement de la chromogranine A (CgA). Dans la seconde partie de ma thèse, nous avons préparé un revêtement de polymère antimicrobien biodégradable par conjugaison de la cateslytine (CTL), peptide correspondant à la séquence de la CgA bovine 344-358, à l'acide hyaluronique dans un modèle de multi-couches acide hyaluronique/chitosan.

➢ LEUKOTOXINE LUKE/D ET PMNS

Staphylococcus aureus (*S. aureus*), est une bactérie présente dans 20-30% de la population et responsable de nombreuses infections nosocomiales. Elle est la cause de nombreuses maladies allant d'infections cutanées bénignes à des pathologies mettant en danger la vie du malade, telles que le sepsis, la pneumonie, l'endocardite, la méningite et l'ostéomyélite. En géneral, les infections à *S. aureus* démarrent par une étape d'adhésion de la bactérie au tissu de l'hôte et se pousuivent par l'étape de colonisation. La colonisation est associée aux protéines de la famille "Microbial Surface Components Recognizing Adhesive Matrix Molecules" (MSCRAMMs) qui sont importantes pour l'attachement de la bactérie à la fibronectine. De plus, *S. aureus* produit un grand nombre de facteurs de virulence incluant des toxines, telles que les entérotoxines (SEs), la toxine-1 du syndrome de choc toxique (TSST-1), les toxines exfoliatives A et B (ETA et ETB), la leukotoxine de Panton-Valentine (PVL) et la leukotoxine LukE/D. Cette dernière est composée de deux sous-unités: LukE et LukD qui se lient à la membrane des leucocytes en induisant la formation de pores membranaires à Ca2+ qui provoquent ensuite la lyse cellulaire. L'expression de LukE/D est associée à

plusieurs pathologies. Ainsi, elle a été isolée avec les deux épidermolysines A et B dans 78% des cas d'impetigo. Par ailleurs, LukE/D a été isolée, associée aux enterotoxines chez 93.6% de patients avec des diarrhées post-antibiotiques. Cependant, actuellement aucune étude n'a examiné le rôle de LukE/D sur les interactions hôte-pathogènes.

Les neutrophiles sont des constituants importants de la première ligne de défense contre les pathogènes, parce qu'ils participent à l'élimination des pathogènes et contribuent à l'activation de l'immunité adaptative. Des résultats précédents ont indiqué que chez l'Homme, les Cgs (CgA et CgB) sont présentes dans les granules de sécretion des neutrophiles et sont surexprimées dans le plasma de patients avec le syndrome de réponse inflammatoire systémique (SIRS) ou un sepsis. Plusieurs peptides endogènes dérivés des CgA et CgB sont décrits comme présentant d'intéressantes propriétés antimicrobiennes. Par ailleurs, en plus de leurs effets directs contre les pathogènes, ils activent les neutrophiles, en induisant un influx de calcium extra-cellulaire, via une liaison à la calmoduline cytoplasmique, puis une sécrétion par exocytose des cytokines et des molécules de l'immunité innée, comme les peptides antimicrobiens (PAMs).

La compréhension des mécanismes de régulation de la réponse des neutrophiles à une agression par des pathogènes peut être obtenue par l'analyse protéomique des sécretions des neutrophiles activés. En utilisant les techniques d'analyse protéomique, nous avons identifié les protéines sécrétées par les neutrophiles activés par LukE/D puis, discuté les effets de cette leukotoxine sur la regulation dynamique des neutrophiles. Nous avons montré que LukE/D est capable de stimuler les PMNs (Manuscrit 1: Figure 2), comme ce qui est décrit pour le LPS bactérien par une rapide augmentation du calcium intracellulaire (Manuscrit 1: Figure 2). Le facteur C3 du complément (CD35) est détecté en tant que marqueur de l'activation des PMNs. Il est retrouvé après traitement par la PVL, ce qui indique une activation des neutrophiles. Dans ce travail, nous avons démontré qu'en plus de la toxicité induite, Luk E/D peut aussi stimuler les PMNs. L'identification dans les sécretions de PMNs de proteines provenant des différents types de granules, confirme le rôle de LukE/D dans la dégranulation de ces différents compartiments.

Ainsi, les PMNs actives produisent le materiel contenu dans les granules azurophiles et spécifiques très enrichies en PAMs. L'analyse protéomique de ces sécrétions a été réalisée en collaboration avec le laboratoire de spectrométrie de masse bio-organique de Strasbourg (CNRS 7178, Dr A. Van Dorsselaer). La comparaison avec les banques de données

protéiques, qui a été obtenue par utilisation du Mascot Ion Score, a permis d'identifier les nombreux facteurs et de les classer suivant leurs fonctions physiologiques par le "gene ontology term mapper" (Manuscrit 1: Figure 4). Nous avons ainsi répertorié un grand nombre de protéines impliquées dans les mécanismes d'activation de l'immunité adaptative (34%), les mécanismes inflammatoires (8%), les protéines anti-oxydantes (3%) et les enzymes (13%) etc... (Manuscrit 1: Figure 4). En complément de cette analyse, par Western blot et à l'aide d'anticorps spécifiques de CgA et CgB, nous avons identifié plusieurs fragments dérivés montrant leur expression lors de la réponse immunitaire à une infection à Straphyloccoques. Parmi tous les fragments identifiés, en utilisant les techniques de tests antimicrobiens, nous avons caractérisé plusieurs peptides actifs contre *S. aureus, Micrococcus luteus, Candida albicans, Candida tropicalis, Neurospora crassa* et *Aspergillus fumigatus*.

L'ensemble de ces résultats souligne l'importance de la stimulation des PMNs par LukE/D en relation avec les défenses immunitaires (innées et adaptatives).

➢ Les protéases de *S. aureus* et les peptides antimicrobiens de la chromogranine A

Dans la deuxième partie, nous avons analysé l'activité de plusieurs peptides antimicrobiens dérivés de la CgA contre plusieurs souches de *S. aureus*. Les Cgs sont les protéines majoritaires des granules de sécrétion des cellules chromaffines (Cgs) et sont exprimées dans les cellules nerveuses, endocrines et immunitaires. Elles sont naturellement maturées pour produire de nombreux peptides aux propriétés biologiques très variées, mais intervenant toutes pour produire le retour à l'homéostasie après la réponse au stress.

Pendant les dix dernières années, notre groupe a caractérisé de nouveaux peptides antimicrobiens dérives des CgA et CgB. Les séquences de ces peptides sont très conservées au cours de l'évolution, ce qui suggère l'importance de leur rôle dans l'immunité innée. Dans les situations de sress et de sepsis les peptides dérivés des Cgs sont produits par les cellules chromaffines, entérochromaffines et les neutrophiles. En plus de leurs effets antimicrobiens directs, quelques peptides tels que la sécrétoneurine (un fragment dérivé de la CgC) établissent un lien entre les systèmes endocrine et immunitaire.

La CgA contient deux domaines antimicrobiens, la vasostatine I (1-76/78) et la prochromacine (79-431). Le domaine actif de la vasostatine-1 correspond à la chromofungine (CgA47-66), qui est à la fois antibactérienne et antifongique, à une concentration de l'ordre du micromolaire. La prochromacine inclut la séquence de la catestatine (CgA344-364) et du fragment raccourci cateslytin (CgA344-358) qui est à la fois antibactérien et antifongique à une concentration de l'ordre du micromolaire. En 2009, il a été montré par notre laboratoire que la chromofungine (CHR, CgA47-66) et la catestatine (CAT, CgA344-364), activent les PMNs en induisant un influx de calcium extra-cellulaire.

S. aureus est le pathogène à Gram-positif le plus fréquemment isolé dans les cas de sepsis, souvent mis en cause dans les problèmes liés à la formation de caillots et à la destruction du tissu cardiaque. *S. aureus* a développé plusieurs mécanismes pour éviter la réponse immunitaire. En résistant à l'action des PAMs, en évitant le recrutement des phagocytes, en interférant avec le complément, en évitant les pièges des neutrophiles liés à leur lyse et en resistant aux conséquences de l'oxydation et aux liaisons non spécifiques des imunoglobulines. Les mécanismes mis en place par *S. aureus* pour déjouer les PAMs comprennent la dégradation protéolytique par les métallo-protéases, les protéases à sérine et à cystéine. L'expression des enzymes protéolytiques est controlée directement par les régulateurs des facteurs de virulence tels que *agr*, *sar* et indirectement par ailleurs, SarA est aussi un régulateur du facteur de résistance à la méthicilline (fmtA). Précédemment, il a été indiqué que la métallo-protéase aureolysine peut cliver la cathélicidine LL-37 pour l'inactiver et contribuer ainsi à l'échappement du système immunitaire.

Notre étude se focalise sur les effets antimicrobiens des catestatine humaine et bovine (bCgA344-364 et hCgA352-372) et du fragment cateslytine bovine (CTL, bCgA344-358) contre *S. aureus* qui est connu pour coloniser la peau et l'épithélium. Parce que la CTL se révèle beaucoup plus active que la CAT contre la croissance de *S. aureus*, nous avons analysé la dégradation potentielle de ces deux peptides par les protéases de cette bactérie en utilisant les techniques d'HPLC, séquençage et spectrométrie de masse. Ainsi, nous avons décrit (1) une relation entre la séquence des deux peptides et leur sensibilité aux protéases bactériennes et (2) la possibilité de les utiliser comme agents antimicrobiens en combinaison avec des antibiotiques conventionnels.

En comparaison avec les autres peptides testés, CTL se révèle être le peptide le plus actif contre les différentes souches testées ((ATCC49775, ATCC25923, S1, and S2). Les

souches S1 et S2 sont isolées à partir de patients de l'hôpital: S1 est une souche MRSA (résistante à la méthicilline) et S2 est une souche MSSA (sensible à la méthicilline). Les activités de ces deux peptides sont comparées avec celle de LL-37 (fragment C-terminal de hCAP-18) (Manuscrit 2: Tableau 1). L'activité de CTL est comparable à celle de LL-37 contre S1 et S2, mais significativement différente contre ATCC49775 et ATCC25923. Les valeurs des concentrations minimales d'inhibition de bCAT et hCAT sont 2-4 fois plus élevées que pour le court fragment bCTL (Manuscrit 2: Tableau 1).

Afin de préciser l'activité antimicrobienne de CTL, nous avons déterminé la cinétique de l'activité antibactérienne contre la souche ATCC 25923. CTL agit très rapidement et à une concentration de 2 x CMI, CTL tue toutes les bactéries en 40 minutes et avec la CMI, le même résultat est obtenu en 60 minutes et les bactéries ne repoussent plus pendant 24h (Manuscrit 2: Figure 2AB). Parce que CTL est le peptide le plus actif, nous posons l'hypothèse que ce fragment est plus résistant aux protéases produites par *S. aureus*.

Pour démontrer la stabilité de CTL par comparaison avec celles des CAT humaine et bovine nous avons incubé les trois peptides avec les surnageants des quatre souches différentes de *S. aureus*. Les facteurs de *S. aureus* agr et sar régulent l'expression des protéases, qui sont souvent modulées par le régulateur SarA pendant la formation du biofilm. Ces protéases sont principalement exprimées dans la dernière phase de croissance et sécrétées dans le milieu extra-cellulaire qui facilite la dispersion bactérienne. Les peptides synthétiques ont été incubés par les surnageants de *S. aureus* et séparés par RP-HPLC. Les profils chromatographiques obtenus pour les deux CAT et CTL sont comparés avec le profil du milieu MHB et ceux des surnageants bactériens. Les pics chromatographiques correspondant à la dégradation bactérienne ont été analysés par séquençage automatique d'Edman et en spectrométrie de masse par la technique MALDI-TOF. Les résultats obtenus montrent que contrairement aux CAT bovine et humaine, CTL n'est pas dégradé.

L'HPLC de bCAT indique deux pics majeurs élués à 38.7 et 40 min (Manuscrit 2: Figure 2A), correspondant au peptide complet, d'après les résultats de séquençage et MALDI-TOF (2426 Da) (Manuscrit 2: Figure 3). La présence de ces deux formes peut s'expliquer à la conformation induite par le résidu de proline en position 360 et des états d'isomérisation (cis/trans). Dans le milieu MHB seul bCAT n'est pas dégradé, tandis qu'en présence des surnageants des cultures des souches S49775, S25923 et S1, bCAT est mature pour produire les fragments élués à 38.3 et 38.6 min, correspondant aux isoformes de bCgA349-364 (1782

Da) (Manuscrit 2: Figure 3), ce qui montre le rôle de la bactérie dans la dégradation du peptide.

En présence du surnageant de S2, bCAT est fortement dégradé pour produire des fragments élués à 28.0, 36.6 et 37.5 min (Manuscrit 2: Figure 2A). le séquençage et l'analyse par MALDI-TOF indiquent que ces fragments correspondent à bCgA350-356 (826 Da) et les deux isoformes de bCgA357-364 (887 Da) (Manuscrit 2: Figure 3).

De plus, le profil HPLC de hCAT montre un pic majoritaire qui correspond au peptide complet d'après le séquençage et l'analyse en MALDI-TOF (2327 Da) (Manuscrit 2: Figure 3). En presence du milieu MHB, hCAT n'est pas clivée (Manuscrit 2: Figure 2C). Cependant, après incubation avec les surnageants des souches S49775, S25923 et S1, hCAT est partiellement dégradé pour générer un fragment élué à 36 min (Manuscrit 2: Figure 2C). Le séquençage et la spectrométrie de masse MALDI-TOF indiquent que ce fragment correspond à hCgA357-372 (1780 Da) (Manuscrit 2: Figure 3). En présence du surnageant S2, hCAT est complètement dégradé pour générer des fragments élués à 28.5 et 33 min (Manuscrit 2: Figure 2C). Le séquençage et l'analyse MALDI-TOF indiquent que ces fragments correspondent à hCgA358-364 (840 Da) et hCgA365-372 (871 Da) (Manuscrit 2: Figure 3).

De plus, le court peptide bCTL (bCgA344-358), est élué comme un peptide unique et n'est pas dégradé par les souches de *S. aureus* testées (Manuscrit 2: Figure 2E).

Nous avons alors testé l'effet d'inhibiteurs de protéases sur la dégradation des peptides par les surnageants bactériens. Les trois peptides testés bCAT, hCAT et bCTL ne sont plus dégradés en présence des surnageants bactériens (Manuscrit 2: Figure 3B/D/E), démontrant ainsi que la dégradation des CAT est due aux protéases du *S. aureus*.

Il est intéressant de noter que les points de clivage des CAT par les surnageants des souches S49775, S25923 et S1 sont identiques. Ils correspondent aux liaisons L348-S349 et L356-S357 respectivement pour bCAT et hCAT (Manuscrit 2: Figure 3). Avec la souche S2, ils correspondent aux liaisons S349-F350+G356-F357 et S357-F358+G364-F365, respectivement pour bCAT et hCAT (Manuscrit 2: Figure 3). Il est important de noter que le court peptide bCTL (bCgA344-358) (Manuscrit 2: Figure 3) n'est pas clivé par les protéases de S. aureus ce qui démontre que sa structure primaire lui permet de résister au mélange protéolytique produit par *S. aureus*.

A cause du développement de la résistance de *S. aureus* aux antibiotiques conventionnels, l'intérêt pour les peptides antimicrobiens est croissant. Par ailleurs, certains peptides antimicrobiens sont toxiques pour les cellules de l'hôte, ce qui n'est pas le cas pour les peptides antimicrobiens dérivés des chromogranines. Jusqu'à présent, aucun résultat concernant des activités antibactériennes contre *S. aureus* a été rapporté pour les peptides dérivés des chromogranines. CAT est décrit comme étant actif contre plusieurs souches bactériennes au niveau du micromolaire et CTL est bien caractérisé pour ses activités antibactériennes contre Micrococcus luteus, des levures et des souches de champignons. De plus il n'est pas lytique contre les érythrocytes. Au cours de ma thèse, pour la première fois, nous avons caractérisé l'activité de CTL contre plusieurs souches de S. aureus avec une CMI de 37-45 µg/mL (Manuscrit 2: Tableau 1).

Au cours d'une infection, *in vivo* il s'établit une communication dynamique entre les cellules de l'hôte et les bactéries. De nombreuses souches bactériennes expriment une grande variété de protéases qui dégradent de nombreuses proteines jouant un rôle dans l'immunité innée. L'absence de dégradation par *S. aureus* pour le peptide bCTL par comparaison avec bCAT et hCAT peut expliquer la différence d'activité (d'un facteur 3-4) entre les peptides testés (Manuscrit 2: Tableau 1). En effet, vis-à-vis des quatre souches testées, bCAT et hCAT sont degradés, tandis que bCTL résiste à la dégradation protéolytique.

Dans une autre série d'expériences, en utilisant la protéase Glu-C de S. aureus et le mélange complexe des proteines sécrétées par les cellules chromaffines stimulées, nous avons identifié plusieurs sites de clivage identifiés après traitement (Manuscrit 3). Les protéines chromogranine A (CgA) et chromogranine B (CgB) possèdent très peu de régions antimicrobiennes qui ne soient pas dégradées par la protéase Glu-C. Les nouveaux fragments générés sont purifiés par RP-HPLC et analysés pour leurs propriétés antimicrobiennes. Les activités antibactériennes sont perdues, mais de manière intéressante les peptides présentent des activités antifongiques contre *Neurospora crassa*. Parmi les fractions d'HPLC actives nous avons identifié deux peptides de la CGA correspondant à CgA (47-60 et 418-426) (Manuscrit 3: Figure 2) et trois peptides de la CgB (279-291, 450-464 et 470-486) (Manuscrit 3: Figure 3). Les activités antimicrobiennes de ces fragments sont validées par l'utilisation des peptides synthétiques correspondants avec une CMI de 2µM à 80µM contre différents Candida et des souches de champignons filamenteux (Manuscrit 3: Tableau 1).

La biosynthèse de CTL résulte de l'action de la prohormone convertase PC1/2, présente dans la matrice des granules chromaffines. La prohormone thiol protease (PTP) est aussi primordiales pour la formation de CAT (bCgA344-364) par clivage de D-R et L-R. De plus, dans les vésicules de sécrétion des cellules chromaffines la cathepsine L (CTSL) produit CTL par un clivage supplémentaire R-G de la CAT. La CAT est capable d'activer les neutrophiles en induisant un influx de calcium extra-cellulaire via la calmoduline. Dans mon travail de thèse, j'ai montré la résistance de CTL en présence de S. aureus, ce qui renforce son rôle dans l'immunité innée.

Les séquences de CAT et CTL sont très conservées au cours de l'évolution (Manuscrit 2: Figure 4). Le taux d'arginine est important puisqu'il module l'interaction avec les charges négatives de la membrane du microorganisme. Ainsi, il a été démontré que les résidus d'arginine ont une forte tendance à interagir avec les lipides comme il a été suggéré pour d'autres peptides tels la protéine Tat, activateur transcriptionnel de HIV-1. Pour hCAT, bCAT, et bCTL les taux d'arginine sont, respectivement de 15%, 23%, and 33%. Le fort pourcentage d'arginine pour bCTL est en faveur d'une forte interaction avec les phospholipides de la bicouche. La relation structure-activité de CTL avec la membrane bactérienne est aussi démontrée par des expériences récentes concernant le peptide FLE-CTL dans lequel le peptide CTL a été rallongé à l'extrémité N-terminale de FLE. FLE-CTL (FLE-RSMRLSFRARGYGFR). En utilisant une combinaison de l'HPLC et des tests antimicrobiens, nous avons montré que ce peptide synthétique est inactif contre *S. aureus* à 400 µg/mL et interagit fortement avec la membrane bactérienne. Au contraire le peptide raccourci auquel il manque l'extrémité C-terminale (YGFR): FLE-RSMRLSFRARG, possède une activité antibactérienne avec une CMI de 200 µg/mL. Ce dernier résultat montre que CTL correspondant à une séquence avec les critères requis pour obtenir une activité antibactérienne.

Concernant la structure secondaire de CTL, une structure en feuillets β aggrégés a été décrite en utilisant des expériences de dichroïsme circulaire et d'ATR (Attenuated total reflectance). Au contraire , hCAT et bCAT forment de courtes structures en hélice (résidu 7 à résidu 11) en présence de fortes concentrations de DPC, ce qui a été confirmé par des expériences de dichroïsme circulaire et de RMN. CTL adopte majoritairement un caractère en feuillets β en présence de membranes chargées négativement, tandis qu'il est principalement non structuré dans l'eau. Les structures en feuillets β donnent une plus grande stabilité que les structures en hélices décrites pour CAT. De plus, l'analyse (HR-MAS) 1H NMR de CTL

indique que l'arginine et les résidus hydrophobes sont localisés à proximité induisant ainsi une pénétration profonde des résidus chargés dans la membrane. Ainsi, les interactions électrostatiques entre les résidus d'arginine chargés positivement et les lipides chargés négativement seraient responsables de la liaison de CTL à la bicouche et les résidus arômatiques stabiliseraient l'interaction lipide-peptide.

Dans notre groupe, nous avons récemment évalué l'effet synergique de trois peptides derivés de la CgA (CAT, CTL et CTL amidée) avec des antibiotiques conventionnels. Nous avons montré que ce co-traitement induit une diminution de la concentration en antibiotiques et potentialise leurs activités. Les tests antimicrobiens sont réalisés à des concentrations inférieures à la CMI et des tests contrôles sont réalisés avec le peptide et l'antibiotique à des doses similaires. Pour toutes ces expériences, nous avons évalué la « Fractional Inhibitory Concentration » (FIC) des peptides dérivés de la CgA en combinaison avec la Minocycline et le Voriconazole. FIC correspond à un effet synergique, s'il est ≤ 0.5, à un effet additif, s'il est >0.5 et <2, et à un effet antagoniste s'il est >2. Dans le cas de la combinaison de la CTL amidée et de la Minocycline, nous obtenons un FIC de 0,37 contre *S. aureus*, et pour la combinaison de CTL et du Voriconazole, nous obtenons un FIC de 0,25, et 0,5 respectivement, contre *Candida albicans* et *Candida tropicalis*. D'après ces résultats, on peut imaginer un mécanisme dans lequel les peptides pourraient favoriser la déstabilisation de la membrane bactérienne permettant ainsi, une meilleure pénétration des antibiotiques pour atteindre leurs sites d'action. Ces résultats obtenus in vitro pourraient se produirent in vivo pendant les états d'inflammation systémique.

En effet, au cours du sepsis on observe de nombreux changements dans les activités des protéases et de leurs inhibiteurs. Ces régulations sont fortement reliées avec la gravité du sepsis. Dans des situations de stress oxydatif, se produisant au cours du sepsis, CAT et CTL seront produites plus difficilement à partir de la CgA complète. L'oxydation produit des modifications structurales de la CgA tels que l'oxydation des résidus de méthionine, des résidus arômatiques, des glycanes, des phosphorylations et aussi l'aggrégation de la protéine complète qui pourraient empêcher la dégradation de la protéine pour produire les peptides bénéfiques.

La structure des peptides est très importante pour l'expression des activités antimicrobiennes. Il a été montré qu'une courte séquence hydrophobe terminale peut augmenter l'activité. Dans notre étude nous avons ajouté un résidu de cystéine séparément

aux deux extrémités de CTL et nous avons montré une augmentation de l'activité lorsque le résidu C est positionné à l'extrémité C-terminale et une diminution de l'activité lorsqu'il est positionné à l'extrémité N-terminale. Le peptide CTL-C a alors été utilisé pour developper des revêtements de polymères via le depôt alternatif de polyélectrolytes conjugués à CTL-C. L'activité de CTL n'est pas altérée lorsqu'il est inséré dans le biomatériau. De plus il n'est pas toxique pour les cellules de l'hôte (fibroblastes gingivaux).

Finalement pour la première fois, nous présentons l'importance de l'interaction des protéases bactériennes sur la dégradation des peptides antimicrobiens et montrons le caractère très résistant de la CTL qui représente un peptide intéressant pour des études futures.

➢ Revêtements multicouches de polyélectrolytes antimicrobiens

Dans cette dernière partie, nous avons décidé d'utiliser CTL pour préparer des matériaux antimicrobiens qui recouvriront des intruments médicaux et chirurgicaux.

Les implants sont très utilisés en chirurgie, non seulement pour remplacer des tissus altérés ou perdus, mais aussi dans les services de soins intensifs pour l'administration de gaz ou fluides par cathéters ou tube trachéal. Ces implants constituent une porte d'entrée pour l'invasion microbienne. La prévention de la colonisation microbienne des implants est une importante question médicale et financière, puisque les infections nosocomiales représentent une des plus sérieuses complications après la chirurgie et les soins intensifs. En effet chaque année en Europe, 5% des patients admis à l'hôpital souffrent d'infections acquises lors de leur séjour à l'hôpital et conduisant à 10% de mortalité.

S. aureus, une bactérie à Gram-positif est responsable des infections acquises lors d'hospitalisations et plus particulièrement dans les cas de patients immuno-déprimés. C'est une des bactéries les plus virulentes conduisant à de forts pourcentages d'infections systémiques dues aux implants et au décès. Une étude récente a caractérisé du point de vue génétique les souches de *S. aureus* responsables des infections dues à la pose de cathéters et démontré que 82% de ces souches sont résistantes à la méthicilline et contiennent plusieurs gènes impliqués dans la formation du biofilm et la dispersion bactérienne.

C. albicans, la levure pathogène pour l'homme la plus répandue, a la capacité de former des biofilms qui sont une source d'infections locales et systémiques. De plus, les biofilms colonisés par *C. albicans* permettent la formation de microcolonies de *S. aureus* à leur surface et d'augmenter la résistance de *S. aureus* aux antibiotiques. Quand elles sont associées aux infections bactériennes, les infections par champignons augmentent la gravité de l'état du patient. La résistance récente de *C. albicans* aux thérapies antifongiques et de *S. aureus* aux antibiotiques soulève le besoin de revêtements multifonctionnels assurant à la fois la protection contre les bactéries et les champignons.

Plusieurs approches basées sur l'immobilisation et la libération de substances bactéricides, utilisant des assemblages de monocouches ou des structures de polymères ont été explorées et développées. Les films de multicouches de polyélectrolytes (PEM), basés sur le dépôt alterné de polycations et polyanions sur la surface solide, représentent une méthode simple et efficace pour fonctionnaliser les surfaces de manière contrôlée. Les premiers films antibactériens ont été obtenus par insertion de nanoparticules d'argent. Ensuite, des bactéricides hydrophobes et des liposomes contenant de l'argent sont insérés dans des films PEM pour obtenir des revêtements bactéricides efficaces.

Les films PEM à base de chitosan sont antibactériens contre *Escherichia coli* et *Enterococcus faecalis.* Les antibiotiques tels que la gentamicine ont été insérés dans des films PEM pour être ensuite libérés. Malgrés une augmentation du pouvoir bactéricide des films, l'utilisation d'antibiotiques et de particules d'argent présente des limites dues à leur toxicité ou à leur rôle dans l'émergence de pathogènes multi-résistants.

Les peptides antimicrobiens naturels (PAMs), sécrétés par de nombreux organismes vivants contre les pathogènes, suscitent l'intérêt par leur large spectre d'activité antimicrobienne et leur faible cytotoxicité. Ils causent principalement une destruction de la membrane plasmique des agents pathogènes et les empêchent de développer des mécanismes de résistance. Les PAMs chargés positivement sont utilisés comme partie intégrante des structures de PEM pour obtenir l'effet antibactérien par contact ou après libération.

Guyomard et al. 2008 ont réussi à intégrer dans des films PEM un complexe de PAMs hydrophobes. Ces films sont antibactériens contre les bactéries à Gram-positif. Des exemples de films PEM intégrant des PAMs antifongiques sont aussi rapportés. Il est urgent de developper des revêtements portant à la fois les activités antibactériennes et antifongiques.

Jusqu'à présent peu de revêtement possèdent les deux propriétés. Ils sont principalement basés sur les revêtements d'argent ou les groupements ammonium quaternaires et cationiques, les polymères synthétiques et les silanes.

A notre connaissance, aucun revêtement de base sur les PAMs possèdent les deux propriétés. Dans le but de créer un tel matériau, nous avons utilisé le peptide cateslytine (CTL), un peptide dérivé de la chromogranine A (CGA), protéine sécrétée pendant les infections avec de nombreux peptides dérivés par les cellules nerveuses, endocrines et immunitaires dans le cadre de l'immunité innée.

CTL, un PAM correspondant à CGA344-358, agit à l'échelle du micromolaire avec un large spectre d'activités antimicrobiennes contre les bactéries à Gram-positif, les champignons filamenteux et les levures, il est sans activité cytotoxiques contre les cellules de l'hôte. De plus, il est très stable vis-à-vis des protéases bactériennes. Nous avons utilisé des films d'acide hyaluronique, comme polyanions fonctionnalisés par le peptide CTL-C dans lequel un résidu Cystéine a été rajouté à l'extrémité C-terminale du peptide CTL, et de chitosan comme polycations (HA-CTL-C/CHI) qui sont disposés sur une surface plane afin d'être efficace contre les bactéries et les champignons. L'acide hyaluronique et le chitosan sont biodégrédables par hydrolyse enzymatique, respectivement par la hyaluronidase et la chitosanase. Ces deux molécules sont déjà largement utilisées pour des applications biomédicales à cause de leurs propriétés intrinsèques. La capacité de *S. aureus*, *M. luteus* et les *Candida* de dégrader HA en produisant la hyaluronidase, permet la libération de CTL-C par les films PEM, exclusivement en présence des pathogènes. La liberation des composés antimicrobiens (PAMs ou antibiotiques conventionnels) est habituellement obtenue par diffusion passive des films à pH physiologique ou par dégradation des films induite par variation du pH.

Pavlukhina et al. 2010 ont publié la libération des agents antimicrobiens en fonction de la variation de pH liée à la pousse microbienne. Cependant, la libération des agents antimicrobiens par variation du pH reste limitée étant donné que le pH devient acide au site de l'infection quand la survie du patient est déjà engagée. A notre connaissance, nous avons développé ici le premier revêtement d'auto-défense par dégradation du film induite par le pathogène lui-même. La masse des polysaccharides absorbée est déterminée par Surface Plasmon Resonance (SPR). La construction et la topographie des films ont été caractérisés par microscopie à force atomique (AFM). Les activités antibactériennes et antifongiques des

solutions de HA-CTL-C et des films de HA-CTL-C/CHI ont été testées contre deux souches de bactéries Gram-positif (*S. aureus* et *M. luteus*) et une souche de levure *C. albicans*, en utilisant le test liquide par micro-dilution.

La microscopie confocale par laser (CLSM) permet de suivre par fluorescence la pénétration de HAFITC-CTL-C (en solution ou intégré dans le film PEM) dans la membrane de *C. albicans*. Par la suite, la cytotoxicité des films de HA-CTL-C/CHI a été testée sur des fibroblastes gingivaux humains (HGFs).

Le peptide CTL-C a été couplé de façon covalente à HA par deux étapes successives comprenant le greffage d'une fonction maléimide sur HA et le couplage d'un thiol maléimide pour greffer CTL-C sur le HA modifié. Ces réactions sont décrites dans le document complémentaire de la thèse (manuscrit 4). Après dialyse, séchage puis congélation, un rapport de 5% de couplage a été determiné par RMN du proton, correspondant au greffage de 5 molécules de CTL-C pour 100 dimères de HA (Manuscrit 4: Figure 1A).

Les activités antimicrobiennes de CTL et CTL-C sont testées en solution contre deux souches bactériennes *M. luteus* et *S. aureus* (ATCC 25923) ainsi que la levure *C. albicans*. La concentration minimale d'inhibition (CMI) de CTL, CTL-C et HA-CTL-C a été determinée après analyse des tests antibactériens et antifongiques par la technique de microdilution (Voir la partie complémentaire de la thèse). Ainsi, le peptide CTL-C est antimicrobien à une concentration de l'ordre du micromolaire (< 100 µM) et présente une plus forte activité antibactérienne contre *M. luteus* par rapport au peptide CTL seul (sans C).

La comparaison avec le peptide CTL-C non greffé, la CMI de HA-CTL-C augmente de 35 à 45 µM pour *S. aureus* et de 20 à 25 µM pour *C. albicans* (Manuscrit 4: Tableau 1). Dans le cas des essais avec *M. luteus*, la CMI de HA-CTL-C (5 µM) est cinq fois plus élevée que celle de CTL-C (1 µM).

Dans le cas de *M. luteus*, la CMI de HA-CTL-C (5 µM) est cinq fois plus élevée que celle de CTL-C (1 µM). Bien qu'il y ait une diminution de l'efficacité, le peptide CTL-C lié de façon covalente au polymère HA est encore antimicrobien à une concentration de l'ordre du micromolaire. La construction du film HA-CTL-C/CHI est suivie par SPR. Une augmentation linéaire de la masse absorbée est observée à chaque depôt de couche de polysaccharide indiquant que la construction se déroule normalement (Manuscrit 4: Figure 1B).

Nous avons recherché la topographie des films de HA-CTL-C/CHI avec différents niveaux de depôts par ZFM en état sec (Manuscrit 4: Figure S1). Il est difficile d'obtenir une bonne qualité des images d'AFM dans l'état sec, à cause de la viscoélasticité des films. Les images AFM permettent de mesurer l'épaisseur des films. Avec 5 bicouches, la surface est presque entièrement couverte avec un film de 5 nm d'épaisseur et une rugosité de 1,6 nm. Le film atteint une épaisseur de 52 nm et une rugosité de 16.5 nm avec 30 bicouches (Manuscrit 4: Figure S2).

Après caractérisation de la construction du film HA-CTL-C/CHI, les activités antibactériennes et antifongiques des films fonctionnalisés ont été évaluées contre *M. luteus* et *S. aureus.*

Après la caractérisation de la construction couche par couche de HA-CTL-C/CHI, les activités antibactériennes et antifongiques du film fonctionnalisé ont été évaluées contre deux souches de bactéries *M. luteus* et *S. aureus* ainsi que la levure *C. albicans* (Manuscrit 4: Figure 2A-C). L'influence du nombre de couches fonctionnalisées est analysée par la construction de différents fims avec des nombres croissants de couches et pour lesquels on évalue les activités antimicrobiennes. Ainsi, des films PEI-(HA/CHI)15-n-(HA-CTL-C/CHI)n avec n= 0, 5, 10, 15 et d'autres avec PEI-(HA-CTL-C/CHI)30 ont été élaborés. Les pathogènes ont été incubés 24h avec les films de HA-CTL-C/CHI à 37°C pour les souches bactériennes et à 30°C pour *C. albicans* (Manuscrit 4: Figure 2C). Pour chaque pathogène, la croissance microbienne est mesurée à différents temps (1 h, 4 h, 6 h et 24 h) par détermination de la densité optique à 620 nm de la suspension bactérienne en présence du film (Manuscrit 4: Figure 2).

Les résultats ont été normalisés et exprimés comme des pourcentages de croissance détectés à une Densité optique de 620 nm. Pour chaque pathogène testé, nous observons qu'en augmentant le nombre de couches de HA-CTL-C/CHI, la croissance microbienne diminue. Une diminution importante de la croissance microbienne est obtenue pour au moins 5 bicouches pour *M. luteus* et *C. albicans* et 15 couches pour *S. aureus* (Manuscrit 4: Figure 2). Après 6h d'incubation, au moins 70% d'inhibition est obtenue pour 15 couches pour tous les pathogènes testés (Manuscrit 4: Figure 2).

Après 24h d'incubation la croissance de *M. luteus, C. albicans* et *S. aureus* est complètement inhibée avec 5, 15 et 30 couches de HA-CTL-C/CHI. Ces résultats sont en

accord avec les CMI de HA-CTL-C en solution. Afin de vérifier si les films peuvent être réutilisés plusieurs fois comme revêtement antimicrobiens, la croissance de 3 pathogènes a été controlée après incubation pendant 24h de films de PEI-(HA-CTL-C/CHI)15 pour *M. luteus* et *C. albicans,* ainsi que des films PEI-(HA-CTL-C/CHI)30 pour *S. aureus* (Manuscrit 4: Figure 2D). Dans ce but, une suspension de pathogène a été mise en contact du film fonctionnalisé. Après une incubation de 24h, le surnageant a été retiré et recouvert d'une suspension fraîche. Après chaque retrait, la DO 620 nm du surnageant a été mesurée pour déterminer la croissance du pathogène après 24h d'incubation. Quand les suspensions de *M. luteus* et *C. albicans sont* en contact avec le film de (HA-CTL-C/CHI)15 et réutilisé chaque 24h, une inhibition complète a été observée , respectivement pour 2 et 3 cycles (Manuscrit 4: Figure 2D). Une diminution significative de l'efficacité du film (HA-CTL-C/CHI)15 est détectable après le renouvellement de la suspension du pathogène. La croissance de *S. aureus* est inhibée de 40% par la seconde utilisation du film (HA-CTL-C/CHI)30 et il devient complètement inefficace pour la troisième utilisation (Manuscrit 4: Figure 2D). En parallèle nous avons incubé les surnageant retirés avec des suspensions fraîches de pathogènes pendant 24h et la DO 620 nm a aussi été mesurée (Manuscrit 4: Figure S3).

Nous n'avons pas observé d'inhibition, ce qui indique l'absence (ou une très faible quantité) de CTL-C relargué en solution.

Pour clarifier le mécanisme d'inhibition de HA-CTL-C, nous avons synthétisé HA-FITC-CTL-C et HA-FITC pour analyser leurs interactions avec *C. albicans* lorsqu'ils sont en solution ou intégrés dans des films multicouches (Manuscrit 4: Figure 4).

Une étude a montré par microscopie confocale que les peptides pénètrent dans les membranes plasmiques et s'accumulent dans les levures. Après 45 minutes d'incubation à 30°C avec HA-FITC ou HA-FITC-CTL-C en solution (Manuscrit 4: Figure 4A-B), *C. albicans* est observé et HA-FITC-CTL-C a été détectable dans le cytoplasme sans provoquer de lyse cellulaire. Au contraire, HA-FITC est clairement détecté autour des cellules de levure, s'accumulant sur les membranes et conduisant à une structure en nid d'abeille. Ceci suggère que CTL-C peut traverser la membrane plasmique, même quand il est couplé à HA et s'accumuler à l'intérieur du cytoplasme. Les films ont été traités à la paraformaldéhyde (PFA) et cette procédure n'induit pas de changement dans le cas des films de HA-FITC/CHI, au contraire des films de HA-FITC-CTL-C/CHI qui apparaissent hétérogènes (Manuscrit 4: Figure 4C-D).

C. albicans a été incubé pendant 45 min à 30°C au contact de film de PEI-(HA-FITC-CTL-C/CHI)15 et observé ensuite par microscopie confocale (Manuscrit 4: Figure 4). Parmi les hétérogénéités dues au traitement par PFA, une forte fluorescence verte est observée principalement à l'intérieur de la levure. Dans le cas de films de HA-FITC/CHI, une faible fluorescence est localisée à l'intérieur des levures et seulement quelques cellules paraissent fortement fluorescentes. Même inséré dans le film de PEM, CTL-C permet la pénétration de HA-FITC-CTL-C à l'intérieur des levures, justifiant ainsi l'activité antimicrobienne des films.

S. aureus and *C. albicans* sont connus pour sécréter de la hyaluronidase, une classe d'enzymes capables d'hydrolyser HA. Le découpage d'un des partenaires du film peut conduire à sa destruction comme cela a été observé par Etienne et al 2005.

Nous avons alors examiné par microscopie confocal des films de PEI-(HA-FITC/CHI)15 avant et après 24h de contact avec les pathogènes (Manuscrit 4: Figure 3). Les trois pathogènes induisent la dégradation des films HA/CHI avec cependant quelques différences dans la morphologie résultante des films. Après 24h d'incubation avec *S. aureus*, les films de HAFITC/CHI films sont presque tous dégradés (Manuscrit 4: Figure 3A). Le film apparaît peu homogène avec des taches fluorescentes après incubation avec *M. luteus*. et *C. albicans* (Manuscrit 4: Figure 3B-C) induisant la formation de structures en nid d'abeilles dans le film, par dégradation de HA. La dégradation de HA peut conduire à la libération de CTL-C dans le surnageant et à la promotion de l'interaction entre les peptides CTL-C et les pathogènes.

Pour vérifier cette hypothèse, nous avons construit des films résistants à la hyaluronidase, CTL-C étant greffé sur la poly(allylamine hydrochloride) pour des matériaux de (PAA/PAH-CTL-C). Après 24h d'incubation les films de (PAA/PAH-CTL-C)15 ne montrent pas d'inhibition contre *C. albicans*. Ceci renforce le fait que l'activité antimicrobienne des films de HA-CTL-C/CHI est due à la dégradation par la hyaluronidase. Ainsi, les pathogènes initient leur propre mort lorsqu'ils sont au contact du film HA-CTL-C/CHI. De manière intéressante, même si le film est dégradé avec le temps en présence du pathogène, il peut être utilisé au moins 2 à 3 fois sans perdre son activité contre *M. luteus* et *C. albicans*.

Il est ensuite important de s'assurer que le film n'est pas toxique pour les cellules de l'hôte. Les fibroblastes sont les premières cellules qui viennent se déposer sur la surface de l'implant pendant le processus de cicatrisation.

La viabilité des fibroblastes gingivaux humains (HGFs) cultivés sur des films PEI-(HA-CTL-C/CHI)15 , comparés avec des films PEI-(HA/CHI)15 et le substrat en verre, a été évaluée au travers de l'activité mitochondriale, suivi par un test Alamar blue™. Après une journée déjà, l'activité métabolique mesurée sur un film d'HA-CTL-C/CHI est comparativement inférieure au film d' HA/CHI ainsi qu'au substrat non coaté. La bonne compatibilité du film de HA/CHI pour HGFs, pour le récepteur CD44 , a été rapporté lors de notre travail précédent. Après sept jours en culture, le nombre d'HGFs viables sur le film HA-CTL-C/CHI représente 25% des HGFs viables déposées sur le film HA/CHI (Manuscrit 4: Figure 5A). La fonctionnalisation de HA par le peptide CTL-C induit une faible adhésion des HGFs qui semblent ralentir la prolifération des cellules. Sachant que HA-CTL-C en solution à 100 μM n'est pas cytotoxique, un tel comportement suggère que les multicouches ne sont pas cytotoxiques.

Pour faire la différence entre la cytotoxicité et une faible propriété d'adhésion initiale d'HA-CTL-C/CHI, nous avons effectué deux expériences.. Premièrement après 24 h de contact, nous avons récupéré la suspension de cellules du surnageant du film HA-CTL-C/CHI et nous les avons passées sur une nouvelle plaque de culture. Après 24 h de culture, plusieurs cellules facilement attachées se propagent comme des cellules fraîches. Deuxièmement, nous avons analysé la biocompatibilité des films avec un test complémentaire d'étallement des cellules avec un réarrangement du cytosquelette quand les cellules sont ensemencées sur un substrat en verre à moitié coaté par (HA-CTL-C/CHI)15. Après 24 h, les HGFs adhèrent sur le substrat en verre (Manuscrit 4: Figure 5BI), mais à un degré moindre sur un film (HA-CTL-C/CHI)15 (Manuscrit 4: Figure 5BII). Après 24 h de culture, à confluence, on observe une couche de cellules fibroblastiques typiques et des fibres polymérisées d'F-actine, sur le substrat en verre. Sur le film d'HA-CTL-C/CHI, apparaissent des cellules moins allongées ayant une distribution d'actine à leur périphérie. Ceci nous indique que le film d' (HA-CTL-C/CHI)15 n'est pas cytotoxique pour le HGFs mais, semble être plutôt anti-adhérent. L'échec de l'implantation peut être du à des fonctions des fibroblastes en relation avec la formation des tissus fibreux. Ces résultats qui diminuent l'adhésion des fibroblastes sur les substrats fonctionnalisés avec les films d'HA-CTL-C/CHI . Ces résultats nous incitent à développer ces matériaux pour des implants.

En conclusion de cette dernière partie de notre étude, nous avons choisi une nouvelle surface recouverte avec des films de multicouches de polysaccharides contenant de l'HA fonctionnalisé avec 5% de CTL-C, un peptide possédant les deux propriétés , antibactérienne et antifongique. Les propriétés antimicrobiennes de CTL-C sont préservées quand elles sont greffées sur HA en solution ou quand elles sont enfouies dans des films PEM. Après 24 h d'incubation, les films d'HA-CTL-C/CHI inhibent le développement de *S. aureus* et *C. albicans*, qui sont des agents pathogènes communs et virulents rencontrés dans les maladies.

La présence du peptide CTL-C sur HA permet la pénétration du polysaccharide modifié à l'intérieur de *C. albicans* après 45 min de contact. La sécrétion de hyaluronidase pour tous les agents pathogènes testés, semble être responsable de la libération d' HA-CTL-C du film et de son activité. Le film garde son activité durant trois cycles d'utilisation avec des suspension fraîches de *C. albicans*. Par ailleurs, l'adhésion limitée de fibroblastes, sans cytotoxicité, sur les films d' HA-CTL-C/CHI met en évidence une application clinique pour prévenir les infections sur des cathéters ou des tubes trachéaux, où les encapsulations de tissu fibreux sont indésirables.

PART : I

INTRODUCTION

STAPHYLOCOCCUS AUREUS

1 STAPHYLOCOCCUS AUREUS

*Staphylococcus aureu*s belongs to genus *Staphylococcus* and family *Micrococcaceae*. This genus is composed of gram positive, non-sporulated and aerobic bacteria. Most of the members of the genus are opportunistic pathogens, but few are pathogenic. *S. aureus* was first isolated by Scottish surgeon Alexander Ogston in 1880, from the pus (Ogston, 1881). Later, he observed microscopically and named as "clustered micrococci" (Staphylococci) in 1882 (Ogston, 1882). *S. aureus* is one of the most commonly isolated human pathogen. *S. aureus* is distinguished from other staphylococcal species because it produces gold pigmentation of colonies, coagulase positive as well mannitol fermenter and gives positive results for the deoxyribonuclease test (Wilkinson, 1997). It is commensal, mostly found in many biological fluids including skin, throat, nares and many more. Mostly, it is found extracellular, but can be observed intra cellular in some cases (Sendi and Proctor, 2009). *S. aureus* colonizes at multiple sites in human beings but anterior nares are the most common site of colonization (about 30% of general population) (Rich, 2005; Wertheim et al., 2005), extra-nares that typically harbor *S. aureus* colonization include skin, perineum, pharynx (Wertheim et al., 2005). Other sites, harboring less colonization as compared to skin or nares may include the oral cavity, gastrointestinal tract, vagina, axillae etc. (Wertheim et al., 2005; Williams, 1963).

The transmission is mostly through contact of infected skin between two persons, ruptured skin can provide favorable environment for progressive colonization of pathogen. Innate immune system controls the colonization of skin pathogens, but horizontal transmission between two persons can disturb the equilibrium between innate immune control of host and skin pathogens (Dethlefsen et al., 2007). Once the favorable conditions are furnished such as rupture of skin barrier or inadequate immune strength, Staphylococcus can rapidly colonize resulting in an invasive growth deep into skin. Followed by the bacteremia and once Staphylococci is in blood it can penetrate into vital organs of body, resulting in mortal pathogenesis such as endocarditis, toxic shock, pneumonia and septicemia (Lina et al., 1997).

S. aureus is responsible for infections, very diverse in nature: from minor skin infections (pimples, impetigo, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and skin abscesses) to life threatening diseases such as sepsis, bacteremia, pneumonia, osteomyelitis, toxic shock syndrome, endocarditis, and meningitis, (Freeman-cook and Freeman-cook, 2006). This diverse variability of infections indicates that the pathogen is well adapted to the

different environmental conditions. Moreover, *S. aureus* has developed resistance against various families of antibiotics that is progressing day by day, often due to non rational use of antibiotics during the last two decades (Campion et al., 2004; Denis et al., 2003; Hiramatsu, 2001; Hiramatsu et al., 2001; Zhang et al., 2008). This day by day increasing resistance is provoking hindrance in effective treatment of infections.

Meanwhile, *S. aureus* is capable of forming biofilm, which helps to protect it, against the adverse environmental conditions like action of detergents and even can interfer process of sterilization. Then, it becomes difficult to eliminate pathogen by some conventional sterilization process used in the hospitals, which results in about 30% more nosocomial infections caused by MRSA each decade (Klevens et al., 2006). Finally, the increasing frequency and the gravity of infections caused by *S. aureus* is becoming an adverse public health problem.

S. aureus growing pathogenicity is due to production of a large number of virulence factors, which are expressed at the site of infection (Foster, 2004; Holden et al., 2004; Novick, 2003). These virulence factors are responsible for infection severity; these include surface proteins, exo-proteins, toxins, and cell adherence proteins. All these virulence factors not only help to combat against the immune system but also to settlement and nutrition (Novick, 2003). Most of the adherence proteins are synthesized by *Staphylococcus* during the initial growth phase while exoproteins are synthesized later during the exponential phase (Dunman et al., 2001; Vandenesch et al., 1991). This model of different stages of virulence factors production is well explained *in-vivo*, where first attempt is to well stick at the site of infection which is helped by the production of various adhesion proteins during initial phase of growth. After well settlement next step is to get nutrient, to colonize and to combat against the gathering immune cells, which is favored by the production of exo-toxins. These help to invade deep into tissue and later disperse through blood to other organs and to colonize at new sites of infection.

1.1 Virulence factors of the S. aureus

S. aureus is capable of producing a large number of virulence factors, can be broadly divided into two categories: surface proteins and the secretory proteins.

1.1.1 Surface proteins

Adhesion to skin or connective tissue surface is more important for the beginning of division cycles of *S. aureus* and then to invade into host tissue. Generally, *Staphylococcus aureus* follows two types of mechanisms to settle at the site of infection depending on the environment: un-specific and a specific pathway. Un-specific pathway is generally the production of normal surface factors mostly hydrophobic in nature and in specific pathway, it has the ability to sense the environment and to modify the nature of surface proteins accordingly (Carruthers and Kabat, 1983). Depending on the nature of surface proteins, it can be broadly categorized into two classes: **MSCRAMM** (Microbial surface components recognizing adhesive matrix molecules) and **SERAM** (Secretable expanded repertoire adhesive molecules) (Chavakis et al., 2005; Menzies, 2003; Patti et al., 1994; Schwarz-Linek et al., 2006). Various surface and secretory proteins are presented in figure 1.

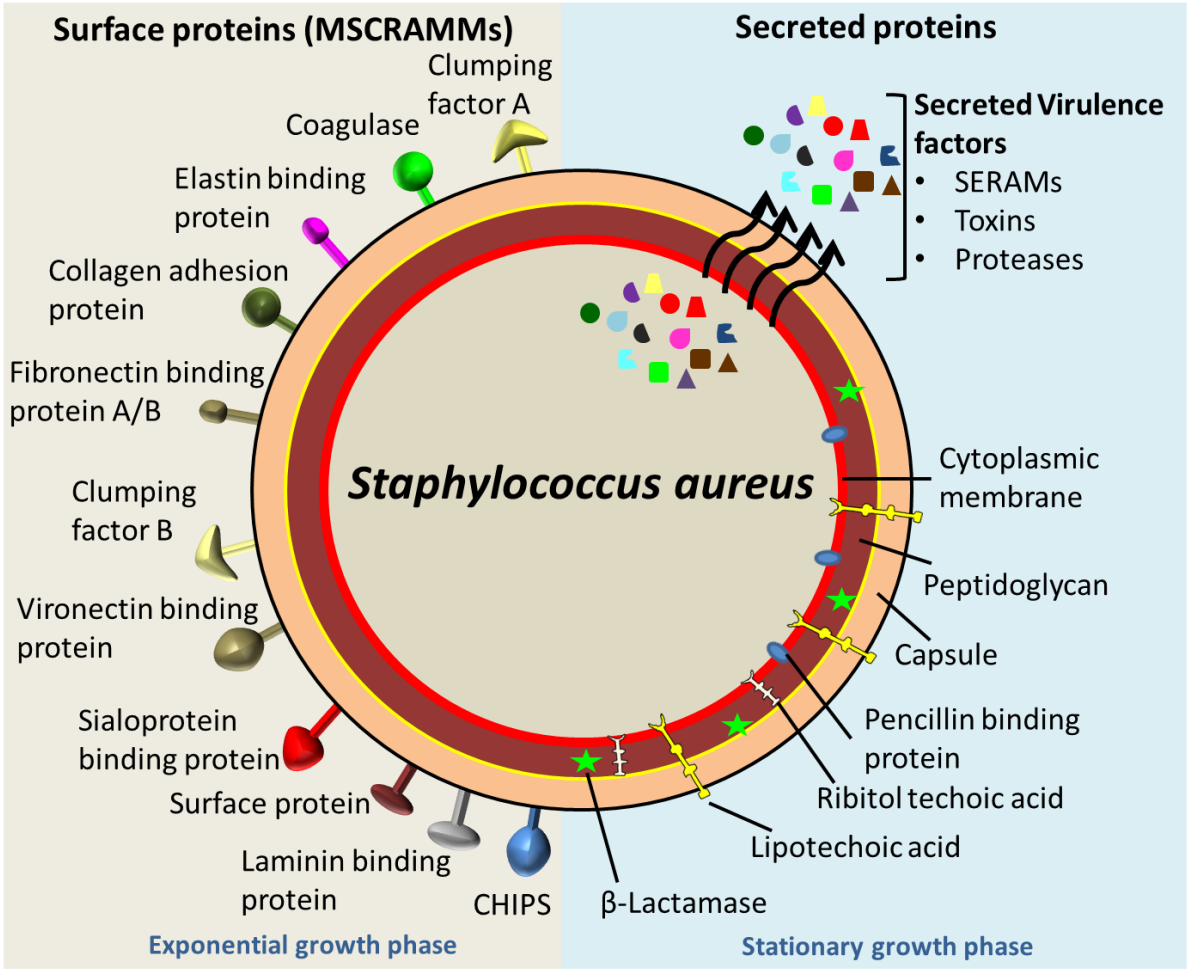

Figure 1: Structure of S. aureus

Left panel presents the different surface and secreted proteins. The synthesis of these proteins is controlled by regulatory genes locus (*agr*) and is dependent on growth phase. Lower right Panels presents the cross sections of the cell envelope.

1.1.1.1 Cell wall and capsular factors

The staphylococcal cell wall is composed of peptidoglycan which consists of alternating polysaccharide subunits of *N*-acetylmuramic acid and *N*-acetylglucosamine (Figure 1). Peptidoglycan may have endotoxin-like activity, which can stimulate release of cytokines by neutrophils and macrophages, leading to activation of complement system and aggregation of platelets (Matsui and Nishikawa, 2012; Wang et al., 2000). Staphylococcal strains results in variation in disseminated intravascular coagulation, which is due to differences in the peptidoglycan structure (Kessler et al., 1991). Most of Staphylococci produce microcapsules. Capsule is composed of polysaccharides, 11 different serotypes based on the capsular antigens have been recognized out of which serotypes 5 and 8 are mainly identified in human infections. Most of methicillin-resistant *S. aureus* (MRSA) isolates are type 5 (Lee, 1996).

1.1.1.2 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM)

MSCRAMM can be classified as molecule of interest that helps to localize pathogen and help to recognize a macromolecular ligand in host, which can be found in the ECM (Extra Cellular Matrix). These macromolecular ligands are exclusively found in the ECM such as collagen, laminin, fibronectin, fibrinogen and vitronectin. These ligands are recognized as MSCRAMM ligands. Third important thing is the MSCRAMM's affinity towards these ECM ligands. Some molecules that recognize carbohydrate determinants exclusively present in many macromolecules do not fall under the category of MSCRAMM such as lectin type adhesion molecules. MSCRAMMs adhesion proteins are attached to the microbial membrane by covalent interaction (Foster and Hook, 1998).

Usually, a single MSCRAMM can bind to several ECM ligands and inversely pathogen can produce several MSCRAMMs to bind a single ECM ligand. For example as *S. aureus* is capable of several fibrinogen binding proteins at a time (Boden and Flock, 1989;

McDevitt et al., 1994). Several MSCRAMMs produced by *S. aureus* are listed in the table below (Table I).

Microbial MSCRAMMs may be virulence factors that mediate host tissue colonization, which in many cases is a prerequisite for infection development. *Staphylococcus aureus* and many other microorganisms have developed several adhesion proteins (Table I, Figure 1). These factors can be operated in combination or independently of each other. This scenario appears to be particularly important when microbes invade a host tissue. Some of the more important MSCRAMMs are discussed below briefly (Table I).

Table I: MSCRAMMs produced by *Staphylococcus aureus*.

MSCRAMM	ECM ligand	Gene	MW	Reference
FnBPA	Fibronectin A	*fnbA*	110 kDa	(Wann et al., 2000)
FnBPB	Fibronectin B	*fnbB*	101 kDa	(Howden et al., 2010)
Protein A	V_h3 region of IgM	*spa*	55 kDa	(Sjodahl, 1977)
Collagen adhesion protein	Collagen	*cna*	133 kDa	(Allon et al., 2012)
Vironectin binding protein	Vironectin		60 kDa	(Liang et al., 1995)
Laminin binding protein	Laminin	*eno*	47 kDa	(Carneiro et al., 2004)
Surface protein	Plasmin	*pls*	174 kDa	(Hilden et al., 1996)
	Thrombospondin			(Rennemeier et al., 2007)
Elastin binding protein	Elastin	*ebpS*	53 kDa	(Downer et al., 2002)
Sialoprotein binding protein	Bone sialoprotein	*bbp*	124 kDa	(Yacoub et al., 1994)
Clumping factor A	Fibrinogen	*clfa*	97 kDa	(McDevitt et al., 1994)
Clumping factor B	Fibrinogen	*clfb*	97 kDa	(Ni Eidhin et al., 1998)

1.1.1.2.1 Fibronectin binding proteins (A and B)

Most of *S. aureus* strains specifically bind to the fibronectin. Fibronectin is a 440 kDa glycoprotein serves as an ECM ligand for the bacterial MSCRAMMs, found in the extracellular matrix and body fluids. The interaction between fibronectin binding protein (FnBP) and its ligands is highly specific. A signal peptide at the N-terminal of FnBP is

followed by a unique sequence that is interrupted by the 35 amino acids long unit and another domain of 38 amino acids is repeated, which is rich in proline residues. The 38 amino acids domain contains a motif "LPXTGX" is found in most of surface proteins produced by gram positive bacteria; it represents the cell wall domain. The cell wall domain is followed by the membrane spanning domain rich in hydrophobic residues (Fischetti et al., 1990). C-terminal extremities mostly contain positively charged residues which is probably the cytoplasmic domain.

1.1.1.2.2 Protein A

Protein A is 55 kDa surface protein of *S. aureus,* present in more than 95% *S. aureus* strains and represents about 7% of total proteins (Patel et al., 1992). It has multiple functions either serves as MSCRAMM or in immune modulation of the host. Its anti-opsonic function is very potent, which help to modulate macrophages based immune clearance. It binds to the Fc region of the immunoglobulin G (King and Wilkinson, 1981). Due to this binding ability; it inhibits the specific detection of antigen and blocks macrophage phagocytosis (Foster, 2005). Moreover, it specifically binds to the Vh3 region of the IgM molecules that is located adjacent to the antigen binding domain. The cells bearing Vh3 region of IgM are readily exposed to B lymphocytes. Finally, stimulated to proliferate and undergo apoptosis (Goodyear and Silverman, 2004). Protein A can also activate the complement system and can stimulate the secretion of histamine by the basophils (Gomez et al., 2004; Nguyen et al., 2000).

1.1.1.2.3 Collagen adhesion proteins

Collagen based proteins are the major ECM constituents, which represent the major target for most of gram positive pathogens. These proteins follow the pattern of FnBPs, containing a signal peptide and followed by the 187 amino acids long domain which is repeated three times. C-terminal contains a motif of 64 amino acids rich in lysine and proline residues, and hydrophobic motif of representing the membrane domain. Collagen binding MSCRAMM ligands on *S. aureus* is both necessary and sufficient for adhesion of pathogens to cartilage. Only cells expressing the MSCRAMMs can adhere to cartilage (Speziale et al., 1986).

1.1.1.2.4 Clumping factors (A and B)

Clumping factors are also called fibrinogen binding proteins due to its specificity to bind fibrinogen. Fibrinogen is found in high concentration in blood plasma, and plays an important

role in the formation of blood clot structures. Clumping factors are composed of a stretch of 15 amino acids in the carboxy terminal of the protein that helps to anchor fibrinogen monomers (Strong et al., 1982). The fibrinogen-binding proteins contain the typical feature of gram-positive surface proteins such as the LPXTGX motif.

1.1.1.3 Secretable expanded repertoire adhesive molecules (SERAMs)

These adhesion proteins are identified, having the property to adhere to various proteins present in the extra-cellular matrix for example fibrinogen, collagen, prothrombin (Chavakis et al., 2005). Two hypotheses are established either this family of proteins is secreted directly into the extracellular matrix or they form bonds with membrane proteins (Chavakis et al., 2005). Although, SERAMs and MSCRAMMs are structurally unrelated but functionally these are same. In addition, these do not form covalent interaction with the bacterial membrane as premeditated in MSCRAMMs. Two important functions are designated with SERAMs, to bind to ECMs present in the host and to bind to a large array of host proteins, which results in modulation of host response against infection. A list of few well studied SERAMs and their ECM ligands are presented in Table II.

Most important and most studied SERAM is coagulase which is somehow characteristic of *Staphylococcus spp* and was used for the detection of *Staphylococcus* infections, despite now MALDI-TOF assited bacterial identification gives a direct identification of these bacteria. Coagulase interacts with thrombin and form a staphylothrombin complex, this complex has the capacity to polymerize with fibrin and finally inhibit agglutination (Palma et al., 1998). Moreover, these proteins can act as immunoregulators and can provoke vascular pathies (Chavakis et al., 2002). For example protein Eap (Extracellular adherence protein) (Hussain et al., 2001; Hussain et al., 2002) and Efb (Extracellular fibrinogen binding protein) (Palma et al., 1998) can modulateimmune responsese by facilitating internalization in phagocytes and demolish inflammatory response (Haggar et al., 2003; Hansen et al., 2006). Moreover, Efb can effectively block complement system, as these are homologous to C3 convertases of the complement system (Jongerius et al., 2007). As Eap blocks inflammatory process, thereby it can affect pro-angiogenic activity of inflammatory cells and so indirectly modulate neovascularization. Moreover, Eap interacts with proteins of wound repair that form a provisional adhesive fibrillar network thereby regulating diverse functions of tissue and vascular cells that contribute to repair angiogenesis (Plow et al., 2000; Springer, 1994). Another secreted protein Emp (Extracellular matrix

binding protein) have been shown to bind to fibronectin, collagen, vibronectin (Hussain et al., 2001).

Table II: SERAMs produced by *Staphylococcus aureus.*

SERAM	ECM ligands	Gene	MW	Reference
FbpA	Fibrinogen	*FbpA*	34 kDa	(Cheung et al., 1995)
Coagulase	Prothrombin/fibrinogen	*Coa*	70 kDa	(Heilmann et al., 2002)
Emp	Collagen/vironection	*Emp*	40 kDa	(Hussain et al., 2001)
Efb	Fibrinogen/complement C3	*Efb*	19 kDa	(Palma et al., 1998)
Eap	Prothrombin/ICAM-1	*Eap*	90 kDa	(Geisbrecht et al., 2005)
Von Willebrand factor binding protein	Von Willebrand factor		66 kDa	(Bjerketorp et al., 2002)

1.1.2 Secreted proteins

S. aureus secreted virulence factors mostly include enzymes, superantigens, cytotoxins, exotoxins and exfoliative toxins. These virulence factors mainly play important role to evade from the host defense and to convert host components into nutrients that are used by the bacteria for their growth.

1.1.2.1 Toxins

S. aureus produces several toxins broadly divided into two categories either single or bi-component toxins. They are mostly implicated in modulating the immune response, which is achieved by cell lysis or tissue destruction. Few are named as superantigens such as enterotoxins, toxic shock syndrome toxins. Exfoliative toxins are specialized proteases with a weak panel of molecular targets including a desmosomal protein, melatonin-stimulating hormone, and few MHC class II variants. The left over are involved in cell lysis such as hemolysins that are associated to red blood cell lysis.

1.1.2.1.1 Staphylococcal Enterotoxins

Staphylococcal enterotoxins are members of a family of about 30 different exotoxins. Some of these bacterial protein toxins are mostly tangled in pyrogenic diseases such as food

poisoning and enteritis. These enterotoxins are sometimes named as superantigens and are abbreviated as SE (Staphylococcal enterotoxins). SEs coding genes are mostly located on the mobile elements such as plasmids and pathogenicity islands (Lindsay et al., 1998; Varshney et al., 2009), thus they can easily be horizontally transferred to other non-virulent strains. Out of these distinct staphylococcal enterotoxins, few most commonly isolated SEs are SEA, SEB, SED, SEE, SEF/TSST-1, SEG, SEH, and SEI (Table III).

Table III: Superantigens of *S. aureus.*

Superantigens	Pathology	Gene	MW	*agr*	Reference
Enterotoxin A	Enteritis, food poisoning	*sea*	28 kDa	-	(Tremaine et al., 1993)
Enterotoxin B	Enteritis, food poisoning	*seb*	29 kDa	+	(Gaskill and Khan, 1988)
Enterotoxin C	Enteritis, food poisoning	*sec*	28 kDa	+	(Regassa et al., 1991)
Enterotoxin D	Enteritis, food poisoning	*sed*	27 kDa	+	(Zhang and Stewart, 2000)
Enterotoxin E	Food poisoning	*see*	28 kDa	+	(Morris et al., 1972)
Enterotoxin G	Food poisoning	*seg*	26 kDa	+	(Chen et al., 2004)
Enterotoxin H	Food poisoning	*she*	27 kDa	+	(Chen et al., 2004)
Enterotoxin I	Food poisoning	*sei*	27 kDa	+	(Ikeda et al., 2005)
ET-A	SSSS, Dermatitis	*eta*	30 kDa	+	(Sheehan et al., 1992)
ET-B	SSSS, Dermatitis	*eta*	30 kDa	+	(Sheehan et al., 1992)
TSST-0	Toxic shock syndrome	*tst*	22 kDa	+	(Recsei et al., 1986)
TSST-1	Toxic shock syndrome	*tst*	22 kDa	+	(Recsei et al., 1986)

SEA and SEB are most commonly implicated with staphylococcal related food poisoning. SED and SEE are the second most important in the list of food poisoning toxins (Morris et al., 1972), but SEF/TSST-1 is mostly involved in toxic shock syndrome (Bergdoll et al., 1981). SEC, SEG, SEH and SEI have also been reported to be associated with food poisoning in few cases and are recognized as an emetic (Table III) (Chen et al., 2004; Ikeda et al., 2005). Enterotoxins are also known as thermostable toxins, but this is not the case with SEF which is also categorized as TSST-1. SEs (SEA and SEB) and TSST-1 have no relation

with the apoptosis of neutrophils but these can act indirectly via the production of T-cell-derived and monocyte-derived cytokines such as gamma interferons (Moulding et al., 1999).

SEs mostly share same structural features with few variations, SEA, D and E share about 70-90 % sequence homology and about 50-60 % with the other members of the family including TSST-1 (Balaban and Rasooly, 2000). Most the members of the family are about 240 amino acids long; in tertiary structure they are almost similar to each other (Baker and Acharya, 2004; Schlievert et al., 1995). Most food borne diseases associated with *S. aureus* are resulting from consumption of SEs contaminated food and are the second most important cause of food poisoning (Archer and Young, 1988; Bunning et al., 1997). *Staphylococcal* food poisoning is mostly characterized by a short incubation period generally 2-6 h after ingestion of contaminated food. The amount of the toxin required to cause is very small ranging from 5-20 µg per person or animal, even in some cases a probable dose of 200 ng have caused sufficient intoxication, certainly in particular to individual's sensitivity (Evenson et al., 1988; Meyrand et al., 1998).

1.1.2.1.2 Exfoliative toxins

Exfoliative toxins (ETs) are usually not directly involved in disease but they can cause complication by acting in a group with other virulence factors such as SEs and TSSTs (Dinges et al., 2000). These are also known as epidermolytic toxins. These particularly cleave desmoglein 1, present in the upper layers of skin, in a similar mechanism as serine proteases (Amagai and Stanley, 2012; Hanakawa and Stanley, 2004; Nishifuji et al., 2008). This mechanism is accountable for the clinical demonstration of staphylococcal scalded skin syndrome (SSSS).

Exfoliative toxins exist in two different serotypes ETA and ETB (Kondo et al., 1974; Wiley and Rogolsky, 1977) and are proteins of about 30 kDa in nature (Table III) (Dimond and Wuepper, 1976; Johnson et al., 1975). ETA is more prevalent than ETB in Europe, USA and Africa and is expressed in about 80% of *S. aureus* strains (Becker et al., 2003; Ladhani and Evans, 1998; Peters et al., 1998). Exfoliative toxins belong to chymotrypsin family of serine proteases as they share structural similarities with V8 protease (Glu-C protease of *S. aureus*) (Figure 2). ETs expression is controlled by an accessary gene regulatory system (*agr*) as described for most of the other virulence factors. In humans, ETs primarily affects neonates by causing SSSS and sometimes it is involved in severe kidney diseases. ETs production varies with species variation as ETs producing strains are usually more species specific.

About 1.5 % and 0.5 % human infecting strains produce ETA and ETB respectively (Becker et al., 2003).

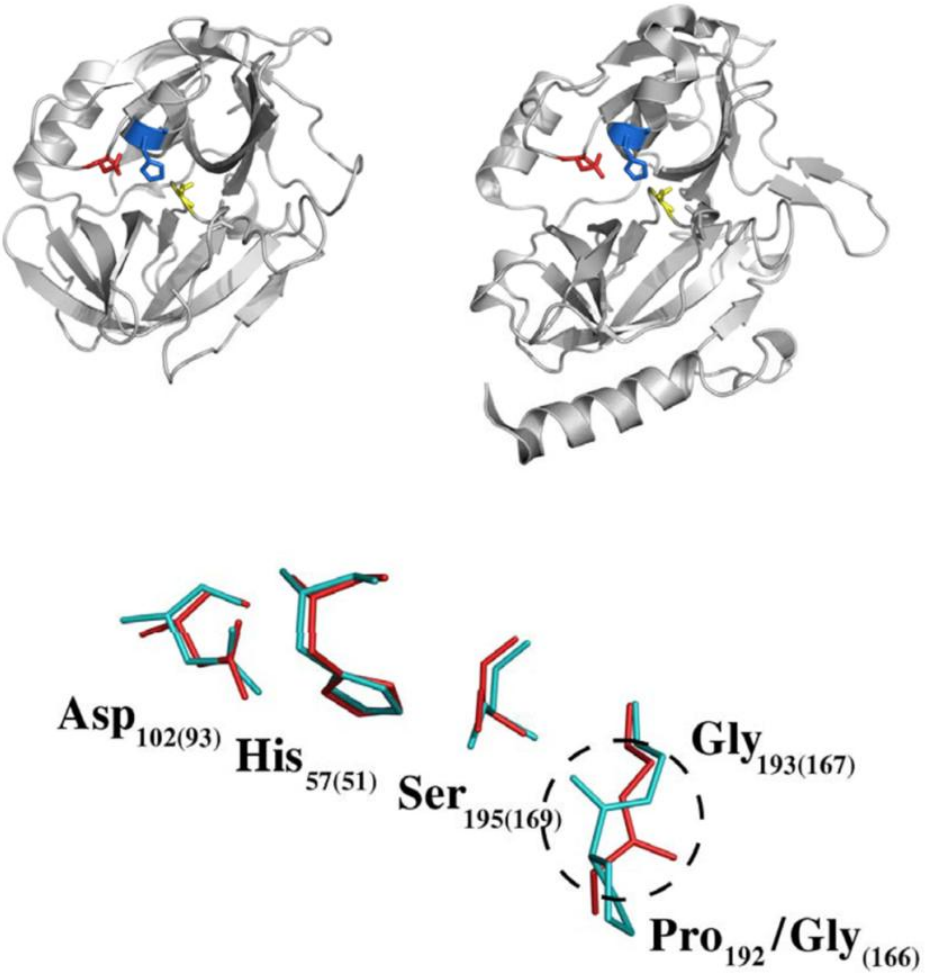

Figure 2: Ribbon presentation of exfoliative toxin A (ETA) sharing structural similarities with glutamylendopeptidase V8 protease (Glu-C protease of S. aureus).

Exfoliative toxins belong to the chymotrypsin family of serine proteases as they share the structural similarities with V8 protease (Glu-C protease of *S. aureus*). A structural comparison of glutamylendopeptidase (left) and ETA (right) is presented. In red, blue and yellow, the catalytic triad residues (Asp, His, Ser) are presented respectively. The superimposition of Asp-His-Ser catalytic triad is presented below. (Dinges et al., 2000).

1.1.2.1.3 Toxic shock syndrome toxins

Toxic shock syndrome toxins (TSSTs) are mainly involved in toxic shock syndrome (TSS) and mostly with pyogenic conditions. TSS is characterized by the acute onset of fever, rashes, hypotension, and ultimately leading to multiple organ failure (Chesney, 1989). TSS is associated with excessive colonization of *S. aureus*, capable of producing TSST-1 (Gaventa et al., 1989; Todd et al., 1985). TSSTs are also controlled by the *agr* regulatory system as the

majority of other virulence factors (Recsei et al., 1986). Two variants are most commonly isolated TSST-0 and TSST-1 (Table III). These superantigens are of 22 kDa and are produced by approximately 20-25 % of the *S. aureus* isolates (Lozano et al., 2011). TSST-1 is isolated from 40-60% of the non-menstrual cases and about 90 % of the menstrual cases (Descloux et al., 2008). TSST-1 is most common toxin of such category that is excessively isolated from pyogenic disorders. TSST-1 is a superantigen as it can trigger high production of cytokines and gamma interferon, through stimulation of monocytes and T cells (Marrack and Kappler, 1990; Mourad et al., 1989). It binds to MHC class II and simultaneously to T cell receptors (*hVβ* chain) which can induce T-cell proliferation at an accelerated rate (Figure 3). This TSST-1 and MHC-II complex can also hinder B-cell proliferation ultimately diminishing immune system (Andre et al., 1994). TSST-1 can also result in accumulation of cAMP in B cells which ultimately lead to cell apoptosis (Fuleihan et al., 1991; Hofer et al., 1996).

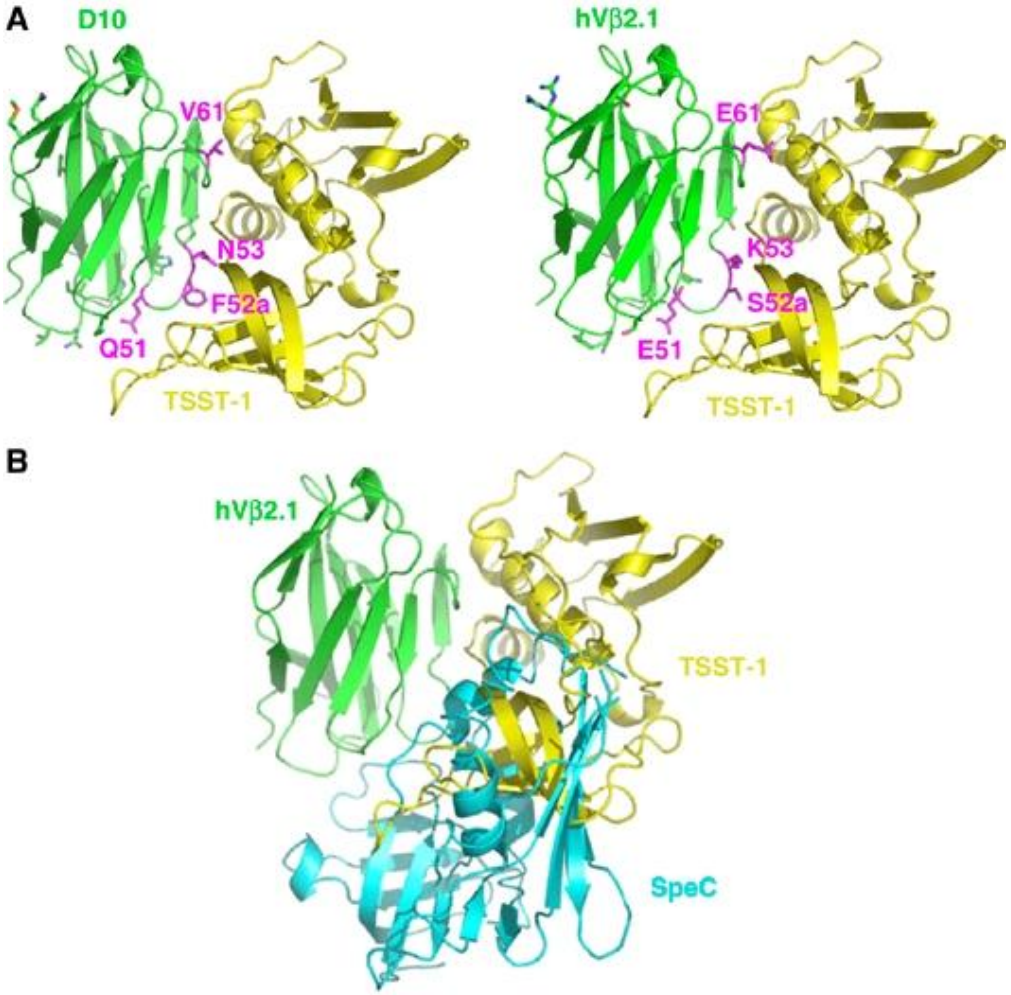

Figure 3: Crystal structure representation of the binding site of TSST-1 with hVβ chain of T-cell receptor (D-10 varient and wild type) and SpeC (Streptococcus pyogenic exotoxin C).

(**A**) Crystal structure of the TSST-1 complex with D-10 variant (left) and of the wild-type hVβ (right). TSST-1 is in yellow and hVβ is in green. (**B**) Superposition of the TSST-1–hVβ2.1 and SpeC–hVβ complexes. The SpeC is in cyan. (Moza et al., 2007).

1.1.2.1.4 Staphylococcal Hemolysins

Hemolytic toxins are entitled due to their capacity to specifically cause hemolysis. These hemolytic toxins are subdivided into four types: alpha (α) hemolysin (*Hla*), beta (β) hemolysin (*Hlb*), gamma (γ) hemolysin (*Hlg*), and delta (δ) hemolysin (*Hld*) based on their structural characteristics. Hemolysins are mostly secreted by the general secretory pathway (*sec*) (Peng et al., 1988; Sibbald et al., 2006).

1.1.2.1.4.1 Alpha hemolysin

Alpha toxin is encoded by *hla* gene and is important for *S. aureus* sepsis, arthritis, pneumonia, sepsis, corneal infection and brain abscess (Bubeck Wardenburg et al., 2007; Callegan et al., 1994; Kielian et al., 2001; Nilsson et al., 1999). Hla is a 33kDa pore forming toxin and is secreted by most of *S. aureus* isolates (Bhakdi and Tranum-Jensen, 1991). Alpha toxin is secreted as monomer which represents cap domain and a β-strand. Monomers combine to form a heptamer where β-strands join to for stem which is inserted into the membrane (Figure 4).

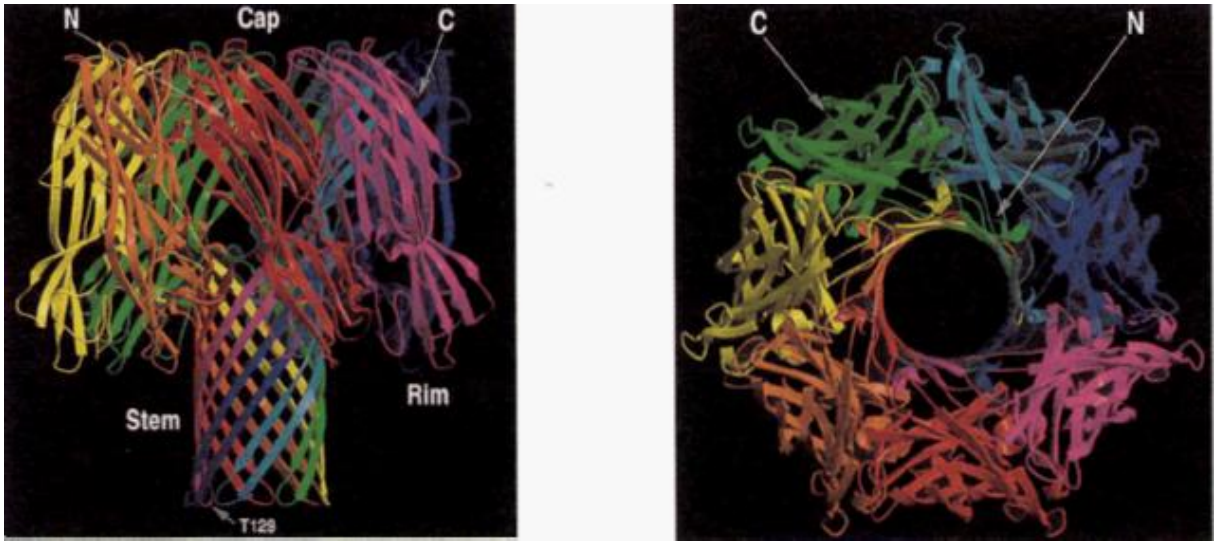

Figure 4: Three-dimensional presentation of alpha toxin.

View of alpha hemolysin a heptamer drawn in a ribbon representation which is approximately parallel to the membrane plane. Each protomer is presented in a different color. Different domains cap, rim, and stem are

labeled (left). View of the heptamer (right), the axis from the top of the Cap domain to bottom. (Gouaux et al., 1997)

This toxin is active against a vast variety of mammalian cells with a remarkable activity against rabbit erythrocytes (Bhakdi and Tranum-Jensen, 1991; Husmann et al., 2009). Moreover, it is capable of triggering cytokines and chemokines release such as IL-1a, IL-1b, IL-6, IL-8, TNF-α and MIP-2 from neutrophils (Bartlett et al., 2008; Bhakdi et al., 1989; Onogawa, 2002).

1.1.2.1.4.2 Beta hemolysin

Beta toxins are also known as sphingomyelinase, it is encoded by *hlb* gene. Beta hemolysin is a 35 kDa protein toxin that is hemolytic in nature (Huseby et al., 2007). In contrast to alpha hemolysin it is more active on sheep RBCs rather than rabbit erythrocytes (Dinges et al., 2000). Sometimes, β-toxin is depicted as "hot-cold" toxin due to their ability to become more active at temperature below 10°C (Dinges et al., 2000). The β-toxin has been isolated from cases of corneal and lung infections and is often associated with pneumonia due to its inhibitory effect on the ciliary movement of nasal epithelial cells (Kim et al., 2000; O'Callaghan et al., 1997).

1.1.2.1.4.3 Gamma hemolysins

Gamma hemolysins are the most important of hemolysin family members produced by *S. aureus*. They are encoded by *hlg* locus, in contrast to the other hemolysins, there are three open reading frames designated as *hlgA*, *hlgB* and *hlgC* (Cooney et al., 1988). Each of the encoded proteins is preceded by a putative signal translation sequence and mature forms are nominated as HlgA, HlgB and HlgC each of 31.9, 34.1 and 32.5 kDa respectively (Table IV) (Cooney et al., 1988). The sequence comparison demonstrates 70 % residual identity between HlgA and HlgC proteins, where each has approximately 30 % sequence similarity with HlgB (Cooney et al., 1993). Additionally, alpha toxins and HlgB have 27 % sequence identity there is almost no sequence identity between HlgA/C and alpha toxins (Cooney et al., 1993).

These toxins can act in combination with other γ-toxins components and sometimes even with the leucocidins. So, these are demarcated as bi-component toxins. HlgB and HlgA/C are nominated as class F and class S components respectively, due to their remarkable similarity with class F and class S components of PVL (98.5 and 99.1 % respectively) (Cooney et al., 1993). Biological activity usually results from the combination of one class S component with one class F component (Colin et al., 1994). Likewise, each toxin component in the gamma hemolysin assembly could in fact yield two biologically active

toxins. These are hemolytic (HlgA-HlgB) in nature, but complexity of their toxicity is more preeminent due to their ability of cross combine with other bi-component family members (Dalla Serra et al., 2005). Gamma hemolysins have leukotoxic effect in addition to hemolytic activity. HlgA-HlgB pair is more active on leukocytes in contrast to the HlgC - HlgB pair which is more active on erythrocytes (Colin et al., 1994; Prevost et al., 1995c). Recently, toxicity of gamma hemolysins has also been demonstrated towards neurons. In neuron cell cultures these can mediate rise in free intracellular Ca2+ via acidic stores and activation of store operated channels (Jover et al., 2012).

1.1.2.1.4.4 Delta hemolysin

Delta hemolysin is encoded by *hld* gene and the product is a 26 amino acid peptide capable of causing erythrolysis (Dinges et al., 2000; Peng et al., 1988; Wiseman, 1975). This toxin is more prevalent as compared to other hemolysin members, produced by about 97% of *S. aureus* isolates. These are also active for variety of mammalian erythrocytes, in addition can bind to sub-cellular structures that are mostly membrane bound (Bhakoo et al., 1982; Freer and Birkbeck, 1982). Delta toxin does not possess a cleavable signal sequence as present in alpha and beta toxins (Dinges et al., 2000).

1.1.2.1.5 Panton valentine leucocidin

Panton-Valentine leucocidin (PVL) is one of the most important bi-component toxins that are composed of one class S (LukS-PV) and one class F (LukF-PV) component (Finck-Barbancon et al., 1991; Panton and Valentine, 1932; Ward and Turner, 1980; Woodin, 1960). LukS-PV is 33 kDa and LukF-PV is 34 kDa, these toxins act synergistically on mammalian polymorphonuclear cells, monocytes, and macrophages (Prevost et al., 1995c). PVL has been described as a marker of virulence, due to its strong association with necrotizing cutaneous infections (Couppie et al., 1994; Prevost et al., 1995a; Prevost et al., 1995b). PVL is active against a variety of cells with varying activity but primarily target PMNs. They display different activities in different species such as these are highly dermonecrotic for rabbit skin as compared to other mammalians (Couppie et al., 1994). As previously described for gamma hemolysins (Dalla Serra et al., 2005), these can combine with other components too, including Luk-E, Luk-D, γhlg (A, B, C), Luk-S, LukF, Luk-M and Luk-F' (Fromageau et al., 2010). However, the heterologous pairs are not as much active as parent pairs (Prevost et al., 1995c). PVL, at sublytic amounts have been demonstrated to stimulate PMNs, and lead to the

release of Interleukins and leukotriene B4 of the neutrophils (Colin et al., 1994; Hensler et al., 1994).

Table IV: The bicomponent toxins produced by S. aureus.

Toxins	Components	Class	MW	*Gene*	Reference
γ-hemolysin	hlgA	S	32 kDa	*Hlga*	(Prevost et al., 1995c)
	hlgB	F	34 kDa	*hlgb*	(Prevost et al., 1995c)
	hlgC	S	32.5 kDa	*hlgc*	(Prevost et al., 1995c)
PVL	LukS-PV	S	33 kDa	*pvl*	(Prevost et al., 1995c)
	LukF-PV	F	34 kDa	*pvl*	(Prevost et al., 1995c)
LukE/D	LukE	S	33 kDa	*luke*	(Gravet et al., 1998)
	LukD	F	34 kDa	*lukd*	(Gravet et al., 1998)
LukEv/LukDv	LukEv	S	33 kDa	*lukev*	(Morinaga et al., 2003)
	LukDv	F	34 kDa	*lukdv*	(Morinaga et al., 2003)
LukM/F'-PV	LukM	S	33 kDa	*lukm*	(Kaneko and Kamio, 2004)
	LukF'-PV	F	34 kDa	*lukf'-pv*	(Kaneko and Kamio, 2004)
LukG/H	LukG	S	36 kDa		(Ventura et al., 2010)
	LukH	F	36 kDa		(Ventura et al., 2010)
Leucolysin	LukS	S	33 kDa	*luks*	(Said-Salim et al., 2003)
	LukF	F	34 kDa	*lukf*	(Said-Salim et al., 2003)

1.1.2.1.6 Leukotoxin LukE/D

These toxins were first identified in the Newman strain of *S. aureus* (NTCC 8178) (Gravet et al., 1998). Cytotoxic activity was detected in the supernatants of double mutants for PVL and hemolysins which later confirmed the production of leukotoxin LukE/D. Later on toxin family genes were discovered named as *lukED*, and the product is LukE and LukD. These are produced by about 30 % of the *Staphylococcal* strains prevalent in hospitals. Leukotoxin LukE/D have been isolated in more than 70 % of the *S. aureus* strains responsible for impetigo and also been simultaneously produced by epidermolysins A and B producing strains (Gravet et al., 2001). About 96 % of post-antibiotic diarrheal cases have been reported

to have leukotoxin LukE/D in association with enterotoxins (more than 80 %) concurrently (Gravet et al., 2001). These present similar activity as PVL for rabbit skin model but are non-hemolytics and week leukocytic in nature. Additionally, these have been demonstrated to target T lymphocytes. Recently leucocidin ED have been described to contribute to systemic infection by targeting neutrophils in murine and rabbit cells (Alonzo et al., 2012). Two variants of leukotoxin LukE/D (LukEv and LukDv) have also been characterized with 91 and 94% sequence similarities with LukE and LukD respectively (Morinaga et al., 2003). Interestingly, these variants have few regions of similarity with PVL components that is might responsible for a little enhanced leukocytic week hemolytic activity against rabbit PMNs and erythrocytes (Morinaga et al., 2003). Neither LukED nor LukEDv have been reported to have K/R-R/K-X-T/S motif, which is known to be phosphorylated by protein kinase and is essential for leukocytolytic activity but genome sequencing of few strains (N315, Mu50) have revealed presence of K/R-R/K-X-T/S motif, on pathogenicity islands in chromosome of these strains. (Kuroda et al., 2001). There is a conflict of studies about the prevalence of *lukED* genes in different strains but regardless of this, LukE/D are important for virulence of *S. aureus* as the deletion of *LukE* and *LukD* gene can result in attenuation of virulence and decrease in lethality in murine blood stream infection model (Alonzo et al., 2012). LukE/D also mediates specific toxicity towards T-lymphocytes by specifically binding to CCR5. Binding with the CCR5 recepter is meadiated by LukE compoenent but not the LukD of the leukotoxin (Alonzo et al., 2013).

1.1.2.1.7 Other Bi-component cytolytic toxins

Few other cytolytic toxins have also been characterized in different strains of *S. aureus* including Luk-S/Luk-F, Luk-M/Luk-F' and LukG/LukH, targeting different mammalian species. Luk-S (33 kDa) and Luk-F (34 kDa) pair is cytolytic in nature, mostly targeting human and rabbit PMNs and specifically rabbit erythrocytes but have no effect on human erythrocytes (Kaneko and Kamio, 2004). Luk-M and Luk-F' is mostly involved in mastitis cases with a very astonishing specificity towards bovine leukocytes (Fromageau et al., 2010).

1.1.2.2 Proteases of S. aureus

S. aureus expresses several kinds of proteases. SspA serine protease (V8 protease) plays an important role in the degradation of surface proteins of the bacterium (Karlsson et al., 2001; McGavin et al., 1997). So, it is responsible for degradation of protein A and most

fibrinogen binding proteins which are expressed at the end of exponential growth phase. Aureolysin is another major protease, which has a dual function: it acts similarly to SspA on the degradation of surface proteins and also it catalyzes the activation of many proteases cleaving precursors at specific sites (McAleese et al., 2001; Rice et al., 2001). There is interdependence in activation of different proteases. For example, SspA aureolysin lead to maturation of AspA and mature SspA turn SSPB cysteine protease (Rice et al., 2001). Proteases of *S. aureus* are also important to reshape bacterial membrane by modification. They are also actively involved in fight against the immune system. Some are especially capable of degrading immunoglobulin heavy chain and several protease inhibitors (Prokesova et al., 1995).Additionally, they protect bacterial cell from antimicrobial peptides such as aureolysin (Sieprawska-Lupa et al., 2004). Finally, proteases provide a source of energy since bacteria degrade all macromolecules present in extracellular medium after apoptosis of host cells (Shaw et al., 2004). Recently, several new serine protease encoded on the same operon were identified such as proteases sPL A-F (Reed et al., 2001). The function of these proteins is still unknown but crystallographic structure of sPLA shows strong similarities with SspA (Stec-Niemczyk et al., 2009). sPLA-F proteases are probably involved in virulence of the bacterium; especially expression of SPLC has already been confirmed in patients with endocarditis (Rieneck et al., 1997).

1.2 Host defences against S.aureus infection

S. aureus infection usually starts with a breach in outer physical barrier of body, including skin and different mucous surfaces. The organism is confronted by the host immune system, comprising both innate and acquired responses. *S. aureus* infection of skin triggers a strong inflammatory response, which involves the migration of first line defence cells (neutrophils and macrophages) to site of infection. A brief exhibition of events occurring during the initial development of infection and triggered inflammatory responses are presented in figure 5 (Foster, 2005). These inflammatory cells will attempt to engulf and dispose of entering pathogens with the help of available antibodies and through complement activation. This is where the first vital internal conflict between *S. aureus* and host defense occurs. Complement system is a family of proteins that have many roles in innate and acquired immunity, comprising killing of foreign cells and regulation of other effectors of immune response (Moore, 2004). During *S. aureus* infection the role of complement is to recruit effector molecules that are essential for tagging the cells and target them for

demolition by immune effector cells such as neutrophils. The process of complement fixation takes place by three different pathways as follows: alternative, lectin and classical pathway (Figure 7). Alternative and lectin pathways are carried out by components of innate immunity, whereas the classical pathway requires interaction of antigen bounded with antibodies on target cells (Moore, 2004). One of key purposes of complement fixation is opsonization which promotes phagocytosis of infected cells by macrophages and neutrophils.

During the complement activation several small peptides are released such as (C3a and C5a) which results in chemo-attraction of Phagocytes to site of infection, also by formylated peptides released by developing bacteria. These small molecules have specific receptor on the phagocytic cell membrane that enhances phagocytosis efficiency. Similarly neutrophils also have specific receptor that recognize Fc region of the immunoglobulin (Figure 5). By the help of complement system, invading microorganisms and their products are cleared by immune cells and transported to lymphnodes, where the resting B-lymphocytes are activated. These activated cells differentiate between host and pathogen components and secret specific antibodies that help to neutralize circulating toxins and promote efficient killing of remaining infectious agents (Foster, 2005).

Regardless of high immune response, *S. aureus* survive such dreadful conditions. Most of time antibodies to *S. aureus* antigens are present in humans and there is rapid rise in titre following infection (Dryla et al., 2005; Etz et al., 2002). However, these antibodies and immunological memory appear to be inadequate to prevent *S. aureus* subsequent infections. Moreover, several mechanisms have been evolved by *S. aureus* to disrupt immune defense. Previously it was regarded as a non-invasive pathogen, but recent studies have suggested that *S. aureus* can invade different type of host cells by a mechanism which involves formation of fibronectin bridge between host α5β1 integrin molecules and bacterial fibronectin binding proteins (Schwarz-Linek et al., 2004; Schwarz-Linek et al., 2003). After internalization into host cells, it can survive in a semi-dormant form denoted as small colony variants (von Eiff et al., 2000). In addition to this dormancy, various mechanisms are hired by *S. aureus* that let them to evade innate and acquired immunity.

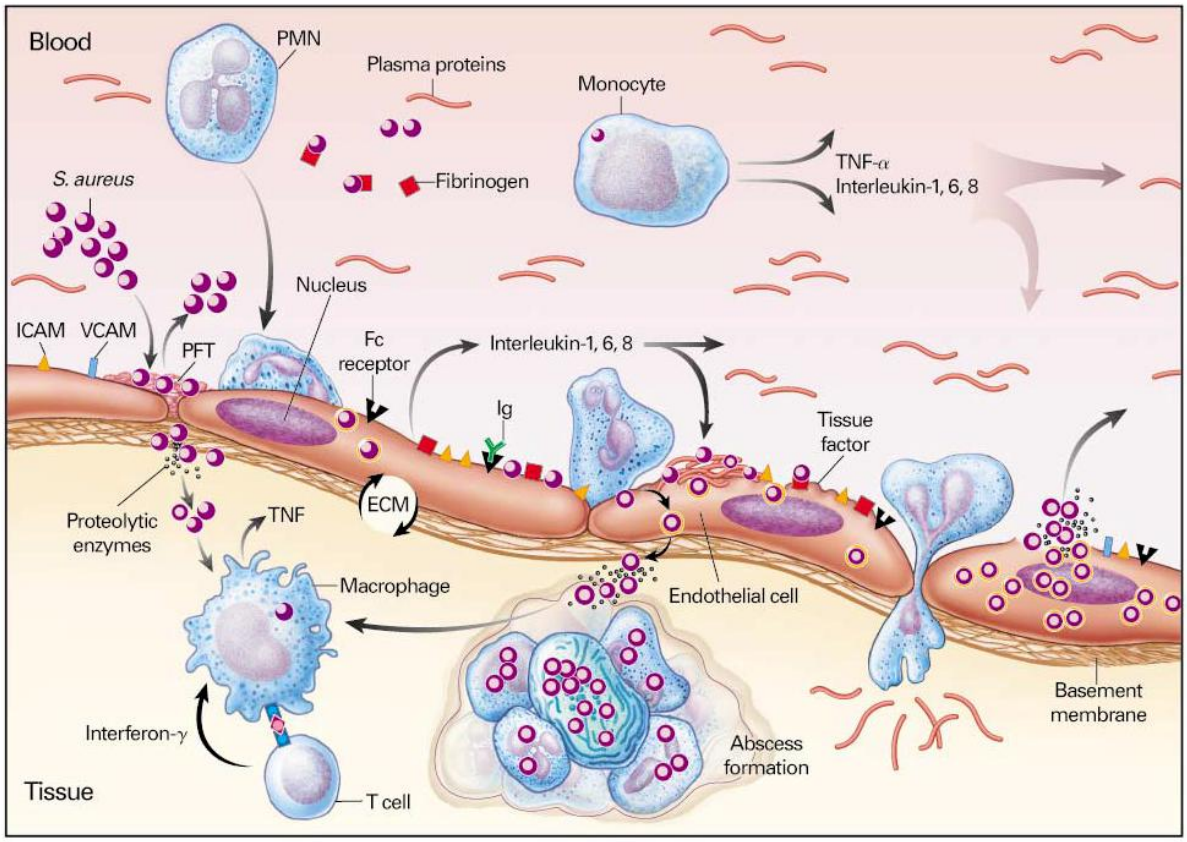

Figure 5: Immune activities against Staphylococcal infection following invasion of tissue.

The order of events progresses from left to right. Circulating *S. aureus* binds to damaged sites of endovascular where platelet–fibrin thrombi (PFT) have formed. The bacteria may attach through the development of MSCRAMM-mediated factors. Alternatively, they stick to to endothelial cells through adhesin–receptor interactions or through bridging ligands. Alterations in the extracellular matrix (ECM) can signal changes in cellular susceptibility to infection. After successful settling bacteria is phagocytosed by the endothelial cells, where it produce proteolytic enzymes to facilitate the spreading of infection. This process leads to release of staphylococci into the bloodstream. The pathogen elicits an inflammatory response which results in abscess formation. After phagocytosis, infected endothelial cells express Fc receptors and in addition certain adhesion molecules (vascular-cell adhesion molecules [VCAM] and intercellular adhesion molecules [ICAM]) and finaly the release of cytokines. As a result of inflammatory process stimulus, leukocytes adhere to endothelial cells and move to infected site by diapedesis. This whole process results in increased vascular permeability and seeping out of plasma proteins. Both tissue macrophages and circulating monocytes release IL-1a/b, IL-6, IL-8, and TNF-α after exposure to staphylococci. Cytokines are released into the blood from the activated macrophages or monocytes, as well as endothelial cells, which contribute to the appearances of the sepsis syndrome and vasculitis. Expression of Fc receptors by the endothelial cells may contribute to the vasculitis, by acting as a binding site for immunoglobulins. (Lowy, 1998)

1.3 Immune evasion strategies of S. aureus

S. aureus have evolved several cell surface associated and secreted virulence factors which promotes adhesion to host cell surface and to tissues, including blood proteins (Foster and Hook, 1998), all these factors help to evade immune response. Most of strains produce a polysaccharide capsule, in addition secret various proteases, hyaluronidase, nuclease and lipase that aid tissue destruction and facilitate infection spread. Moreover, several toxins are produced that cause cytolytic effect on host immune cells. Different strategies are employed by the *S. aureus* to modulate immune response (Figure 6), including interference with complement activation, resistance to phagocytosis, cloaking of opsonins, leukocytes lysis, resistance to oxidative burst and resistance to antimicrobial peptides (Nizet, 2007).

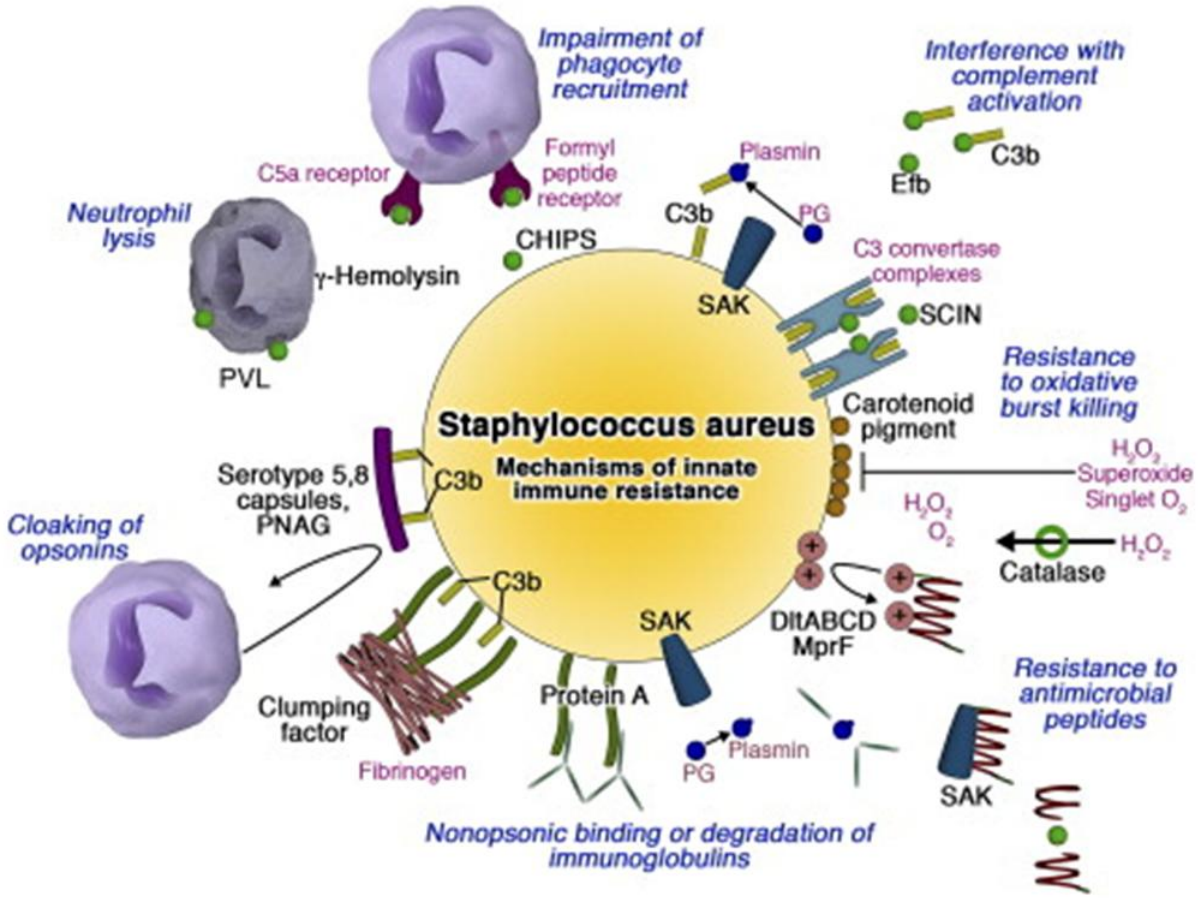

Figure 6: Mechanisms employed by S. aureus to resist immune response.

Various mechanisms by which *S. aureus* subverts host innate immune defense. CHIPS binding to chemokine recepters limit the phagocyte recruitment Phagocyte recruitment, additionaly complement activation is blocked. Golden carotenoid pigment helps to disable antioxidant shield whereas catalases detoxifies hydrogen peroxide. Resistance to AMPS is provided by charge modifications of cell wall contents and also by proteolytic

degradation by the proteases. Protein A binds to Fc domains of immunoglobulins in a nonspecific manner, whereas clumping factors and surface polysaccharide capsule and proteins and poly-N-acetylglucosamine (PNAG) act to cloak surface bound opsonins from phagocyte recognition. The pore-forming toxins of *S. aureus* target leukocyte membranes and ultimately cause cell lysis. The staphylokinase (SAK) activates the zymogen to active protease plasmin, which degrades immunoglobulin Fc domain and complement opsonin C3b. (Nizet, 2007)

1.3.1 Resistance to phagocytosis

S. aureus has the capability to resist phagocytosis by production of antiopsonin proteins, in addition polysaccharide capsule is produced which can hinder complement activation, either by classical pathway or alternative pathway. As the phagocytosis require bounded complement and antibodies which is prevented by capsule formation, regardless of high titer of antibodies against different cell surface components of *S. aureus*. Well known surface-protein of *S. aureus*, protein A is cell wall associated protein with four or five domains, which interact with the Fc region of IgG (Uhlen et al., 1984). Due to binding of IgG with protein A, bacterial cell surface is covered with immunoglobulins in an improper orientation that is unrecognizable by neutrophils Fc receptors.

Another protein produced by *S. aureus* is clumping factor A which is very dominant fibrinogen binding protein (Bischoff et al., 2004; O'Brien et al., 2002). Clf-A is predominantly produced during the exponential phase of growth and during stationary phase of growth unlike other surface proteins, it is 10 fold increased (O'Brien et al., 2002). When cells are densely packed *in vivo*, the γ-chain of Clf-A can bind to two different fibrinogen molecules (McDevitt et al., 1997). In this way, bacterial cell is covered with fibrinogens which inhibit deposition of opsonins.

Most of the *S. aureus* strains produce capsule especially MRSA, that is composed of various serotypes such as 5, 8 and 336 are more common (Roghmann et al., 2005). *In vitro*, phagocytosis assays revealed that presence of polysaccharide capsules reduced the uptake of cells by neutrophils which indicate the anti-opsonic property of capsule.

1.3.2 Complement inactivation

For the successful activation of complement, attachment of C3 convertases to bacterial surface is pre-requisite. C3 convertases (C4bC2a and C3bBb in classical/lecithin and alternative pathways respectively) lead to conversion of C3 protein to C3a (classical and lecithin pathways) and C3b (alternative pathway) (Figure 7). *S. aureus* secretes protein named

Staphylococcus complement inhibitor (SCIN), which binds to both C4bC2a and C3bBb, subsequently inhibiting further C3b formation (Rooijakkers et al., 2005a). As a result, SCIN can block phagocytosis and further killing of *S. aureus* cells by neutrophils. SCIN binding sites are indicated in black (figure 7).

In addition, extracellular fibrinogen binding protein (Efb) was demonstrated to bind C3 protein and block deposition to bacterial membrane and finally opsonization is blocked (Lee et al., 2004a). *S. aureus* also has the capability to disable complement factor C3b and IgG by staphylokinase, a plasminogen activator protein (Rooijakkers et al., 2005b). Moreover, serine protease of plasmin is triggered and chops surface-bound C3b and IgG, resulting in abridged phagocytosis by neutrophils (Rooijakkers et al., 2005b).

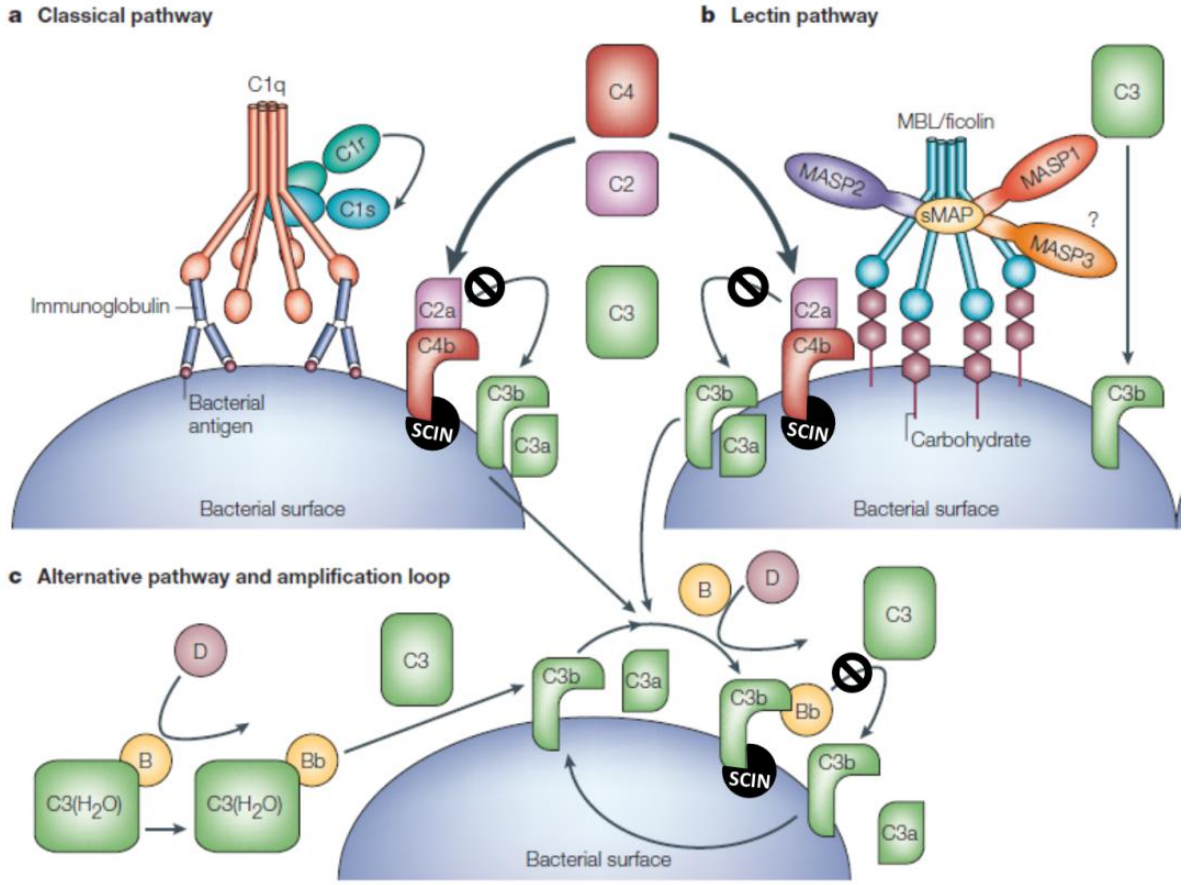

Figure 7: Schematic model of complement activation pathways.

(a) Classical pathway, (b) Lecithin pathway, (c) Alternative pathway. SCIN (black points) binding sites are indicated, which further blocks the activation pathways. (Foster, 2005)

1.3.3 Inhibition of neutrophil chemotaxis

Immediately, with the start of bacterial infection several chemo attractant are liberated, which are recognized by neutrophils. These chemo-attractants include C3a, C5a fragments of complement activation and several formylated petides (FPs) secreted by bacteria during growth. These factors results in strong chemotactic response, and are recognized by the transmembrane G-protein coupled receptors of neutrophils (Murdoch and Finn, 2000). With the recognition of these chemo attractants intracellular signaling cascade is activated, which result in migration of neutrophils from blood stream to site of infection. Approximately 60% of *S. aureus* strains produce CHIPS (chemotaxis inhibitory proteins) that can bind to C5a receptor and formyl peptide receptor (Figure 8) and finally block the recognition process (de Haas et al., 2004).

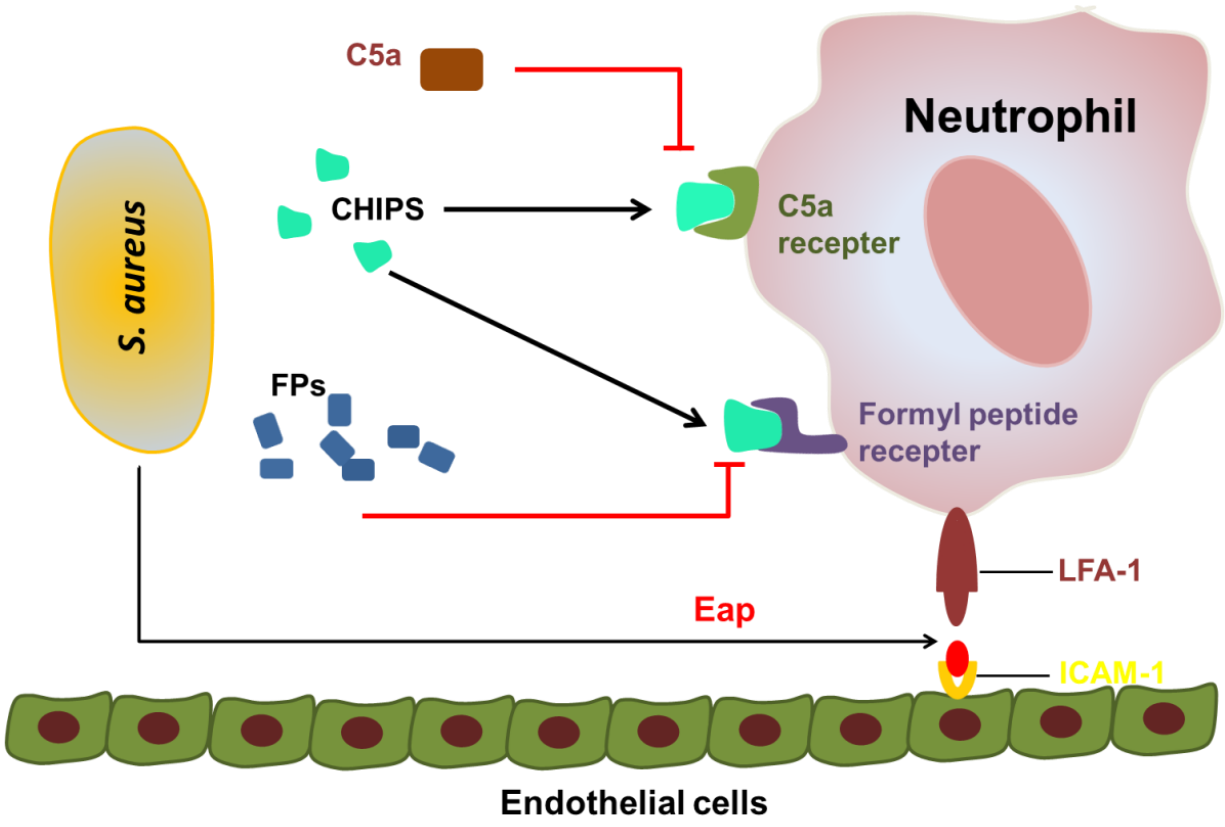

Figure 8: Schematic representation of methods employed by S. aureus to inhibit chemotaxis.

S. aureus produces CHIPS which occupy the C5a recepters and formyl receptor on the neutrophils cell surface. Additionally, Eap blocks neutrophil adhesion by occupying ICAM-1.

In addition to CHIPS, *S. aureus* also secretes extracellular adhesion protein (Eap) also called major histocompatibility class II analogue protein (MAP), which has a ligand on the endothelial cells surface called intercellular adhesion molecule-1 (ICAM-1) (Figure 8). Interaction between these two blocks the binding of LFA-1 (lymphocyte function associated antigen-1) of neutrophil surface (Chavakis et al., 2002). With blockade of LFA-1 neutrophil adhesion, extravasation and diapedesis is compromised, ultimately resulting in chemotaxis inhibition.

1.3.4 Leukocytes destruction by toxins

The expression of cytolytic toxins that lyse leukocytes is cardinal feature of *S. aureus*. Neutrophil lysis contributes to development of abscesses and diminishes their attempt to engulf bacteria. Most of the cytolytic toxins from *S. aureus* form beta-barrel pore in membrane, ultimately causing leakage and consequently cell death. Alpha-toxin is model of this class of virulence factors which is produced as monomers and these monomers join to form a heptamer in cell membrane. The β-strand from each monomer assemble into a β-barrel pore (Montoya and Gouaux, 2003).

The bi-component leukotoxins are secreted in two separate subunits (class S and class F component), those can assemble to form either hexamer or heptamer which have strong affinity for leukocytes. As previously described in the virulence factor section, *S. aureus* produces mainly four different type of bicomponent toxins including γ-toxins, PVL, leucocidin ED and Leucocidin M-F (PV like). The γ-hemolysin is more prevalent, it is produced by approximately more than 90% of the strains while PVL genes are found only in 1-2% strains (Peacock et al., 2002; Prevost et al., 1995c). PVL is more associated with the resistant strains (CA-MRSA), these community acquired methicillin resistant *S. aureus* strains has been widely isolated from cases of necrotizing pneumonia, and skin infections (Gillet et al., 2002; Said-Salim et al., 2003). These CA-MRSA strains have been demonstrated to carry staphylococcal cassette chromosome (type IV or V -SCCmec), which encode resistance to meticillin and other β-lactam antibiotics (Lina et al., 1999). Additionally, encoded by *pvl* genes located in a lysogenic bacteriophage (Narita et al., 2001).

1.3.5 Resistance to lysozyme

Lysozyme (muramidase) is produced by a vast array of host cell and is present almost all body fluids. Muramidase expression is enhanced by stimulated phagocytic cells. Muramidase cause cleavage of glycosidic link between *N*-acetylglucosamine and *N*-acetyl muramic acid, present in the bacterial cell wall (Bera et al., 2005). *S. aureus* resistance to lysozyme is attributed to a membrane-bound structure (*O*-acetyltransferase) which modifies C6 hydroxyl group of muramic acid (Bera et al., 2005). Ultimately, this leads to protection of glycosidic linkage from action of lysozyme.

1.3.6 Survival of S. aureus in neutrophil phagosomes

Neutrophils are most important in number and the first line of defense against pathogens especially *S. aureus,* as patient of granulomatous disease suffer recurrent infections. Recurrent staphylococcal infections are due to defective genes encoding of NADPH phagocyte oxidase subunits. Defective encoding for NADPH compromise production of superoxide radicals during respiratory burst (Heyworth et al., 2003). *S. aureus* has developed several mechanisms that resist the killing by phagocytes, including resistance to antimicrobial substances released and interference with endosome fusion (Gresham et al., 2000). Moreover, bacterium avoids the toxic effects of oxygen free radicals that are formed during respiratory burst. Carotenoid pigments are produced by *S. aureus* which give yellow coloration to colonies, helps to scavenge oxygen free radicals (Liu et al., 2005). In addition, *S. aureus* produces two superoxide dismutase enzymes that help to remove free oxygen radicals (Karavolos et al., 2003). Several other measure are employed to counter toxic effect of phagocyte environment including manganese homeostasis (Horsburgh et al., 2002), methionine sulphoxide reductases production (Singh and Moskovitz, 2003), high catalase production and several toxins are upregulated such as γ-hemolysin (Voyich et al., 2005).

1.3.7 Subversion of the humoral immune response

S. aureus heavily produce fibronectin binding protein (A and B) as surface adhesion proteins, which binds to platelets and finally lead to their activation. With the activation of platelets initiat thrombus starts to grow. *S. aureus* reside inside the thrombus where it is protected from neutrophils (Moreillon and Que, 2004). Moreover, fibronectin binding

proteins bind on the other end with immunoglobulins, which cover thrombus surface and finally pathogen saves itself from humural immune response.

1.3.8 Resistance to killing by antimicrobial peptides

Antimicrobial peptides are integral part of innate immune defense, mostly these are are attracted to wards bacteria due to negative charge, where they cause destabilization and rupture of bacterium. *S. aureus* execute few natural modifications to lipoteochoic acid, wall teichoic acid (WTA), and to membrane phospholipids. Dlt proteins expression result in d-alanine substitutions of lipoteichoic acid and ribitol teichoic acid that can partially neutralize the negative charge and moderate attraction of cationic force (Peschel et al., 1999). Similarly, MprF protein adds an L-lysine residue to phosphatidylglycerol (Staubitz et al., 2004). In both cases, cationic AMPs are repelled from the cytoplasmic membrane, in addition positively charges AMPs are also resisted such as lactoferrin and phospholipase A2. Mutants defective in MprF and Dlt are rapidly killed by cationic antimicrobial proteins and neutrophils *in vitro* and have reduced virulence in several animal infection models (Collins et al., 2002; Kristian et al., 2003).

In addition to neutralization of the charge, *S. aureus* also secretes several proteins that neutralize cationic peptides such as staphylokinase, a prothrombin activator. Staphylokinase degrade IgG and C3 factor of complement system, dissolve fibrin clot and moreover has high affinity for defensing peptides (Jin et al., 2004). Also, the extracellular proteases produce by *S. aureus* degrade antimicrobial peptides such as metalloprotease aureolysin cleaves and inactivates cathelicidin LL-37 and contributes considerably to resistance to antimicrobial peptide *in vitro* (Sieprawska-Lupa et al., 2004).

1.4 Resistance to chemotherapeutic agents

Chemotherapy is used against many bacterial infectious diseases including gram positive and gram negative pathogens. Chemotherapeutic agents such as aminoglycosides, β-lactams, and quinolones are used against *S. aureus*. The emergence of resistance to antibiotics makes it a huge problem to treat *S. aureus* infections. Initially, *S. aureus* became resistant to β-lactams antibiotics by acquiring the penicillinase (β-lactamase) gene by horizontal transfer (Chambers, 2001; Kirby, 1944; Thornsberry, 1988). To counter resistance to β-lactam antibiotics modified β-lactams resistant to β-lactamase, such as oxacillin and methicillin, were developed. Subsequently, through extensive use of these modified β-lactam antibiotics,

MRSA strains have emerged (Maple et al., 1989; Martin, 1994). Penicillin-binding-protein (PBP)-2 (2A) is responsible for this resistance against the modified β-lactams (Berger-Bachi, 2002; de Lencastre et al., 1994). *S. aureus* generally possesses four PBPs. In susceptible strains, they are inactivated in the presence of β-lactams, results in inhibition of cell wall biosynthesis and ultimately leading to cell death. MRSA can survive in presence of β-lactams due to an extra PBP2'. The difference between MRSA and methicillinsusceptible S. aureus (MSSA) is due to the presence or absence of the mec region including the mecA gene coding for PBP2'. The mec region is composed of mobile genetic elements that are called SCCmec cassettes. To date, five types of SCCmecs have been identified (Hiramatsu et al., 2001; Hiramatsu et al., 2002; Ma et al., 2002). These cassettes have a variable size of about 20–70 kbp and contain mecA, ccrA (recombinase A), ccrB (recombinase B), and other orf s of unknown function (Robinson and Enright, 2004).

The glycopeptides vancomycin and teicoplanin are powerful agents with significant activity in MRSA infections. Vancomycin binds to peptidoglycan precursor and then inhibits the incorporation of newly synthesized precursors in the cell wall peptidoglycan, thus causing inhibition of cell wall synthesis. Recently, vancomycin-intermediate susceptible *S. aureus* (VISA), or glycopeptide-intermediate susceptible *S. aureus* (GISA), emerged (Bobin-Dubreux et al., 2001; Cosgrove et al., 2004; Hiramatsu, 2001; Sieradzki et al., 1999), followed by a vancomycin-resistant *S. aureus* (VRSA) (Goldrick, 2002; Whitener et al., 2004). The mechanism of vancomycin resistance in the VRSA is due to expression of vanA gene, which modifies the structure of peptidoglycan, causing a loss of affinity of vancomycin for the modified peptidoglycan precursor (Clark et al., 2005).

Antibiotic resistance in MRSA has evolved to multiple drug resistances against chemotherapies. This implies that MRSA strains acquire extra factors at high frequency compared with the MSSA strains. This pattern has continued among the antibotics such as linezolid, which was introduced in 2000. Only a year later a resistance strain is reported named as linezolid-resistant MRSA (Quiles-Melero et al., 2012; Tsiodras et al., 2001). Similary fate was achieved daptomycin which was introduced in 2003 and MRSA was able to develop resistance against daptomycin within two years (Mangili et al., 2005; Marty et al., 2006). Therefore, it will become difficult in near future to cure MRSA infections because *S. aureus* adapts to new chemotherapeutics and new approaches to therapy will be required.

IMMUNE SYSTEM

2 IMMUNE SYSTEM

2.1 Introduction

The immune system evolved as a set of very well organized event that protect the host from the invading pathogens and cancer cells. Immune system helps to discriminate between self and non-self components. It acts as a mechanism of defense against the pathogens, such as bacteria, viruses, fungi, parasites and cancerous cells. Immune system usually functions in two steps first is the pathogen recognition and then response. Recognition is remarkably highly specific in nature, which carried out by various components of immune system. In recognition, subtle chemical differences are recognized by innate immune components, which distinguish invading agents from the host components. Later, effector cells come into play and clear the hazardous agents from the host body (Kindt et al., 2006).

On basis of time of play of the actors of immunity, it is broadly categorized in innate and adaptive immune systems. When the pathogen gets in, the components which first encounter are categorized as innate immune components; those build the first line of defense (Abbas and Lichtman, 2005). Innate immune components present the pathogen to adaptive immunity, which differentiate minor details of the pathogen and memorize the details for future. This form of immunity is not very rapid; it adapts itself according to situation, so named as adaptive immunity.

Adaptive immunity is able to recognize a large number of non-microbial and microbial substances. It possesses a great capacity to discriminate very finely different molecules and microbes, even in the case of high similarity. The adaptive immunity has further two main branches, the humoral and cellular immunity (Kindt et al., 2006). Humoral immunity is usually active against bacteria and viruses that invade into body fluids. Its major tools of action are immunoglobulins also known as antibodies (Burmester et al., 2003; Kindt et al., 2006). The cellular immunity targets infected cells by the action of T-lymphocytes and natural killer cells. Cellular immunity is very specific and potent in action, as it is able to clear infection reservoirs (Biron, 2010). With recent discoveries of various aspects of immune system, different categories of immune systems seem to be intermingled, these work in coordination, interdependent on each other. In addition to clear pathogens, immunity also maintains a homeostasis, by effectively clearing dead or dying cells from the body.

2.1.1 Innate immune system

Innate immune system is the first line of defense against the infections. It is composed of the components that recognize the pathogen well before the adaptive immunity comes into play. Innate immunity is one of the oldest defense mechanism that is evolved in multicellular organisms including insects and plants. While the adaptive system evolved in vertebrates as a more specific immune system (Danilova, 2006).

Innate immunity is very much important in defense mechanisms, as dysfunction of innate system remarkably increases the suceptibity to infection regardless of the persistence of adaptive immune system. A classic example is chronic granulomatous disease (CGD), which is characterized by the gene mutation encoding NADPH oxidase in neutrophils (Heyworth et al., 2003). Patients of CGD are more prone to recurrent infections due to inability of neutrophils to successfully lyse pathogens (Marodi and Notarangelo, 2007). Moreover, innate system provide base for the development of adaptive system, components of innate system such as phagocytes and complement system are used by the adaptive immune system. Additionally, innate immune system presents infection to adaptive system to trigger an effective immune response.

With the invasion of the infectious agents, innate immune response is triggered. First step is recognition of pathogens and declare it as self or non-self that is carried out by the pattern recognition receptors (PRRs) (Biron, 2010). After declaration of agent as non-self, cellular components comes into action and try to nullify the infection, meanwhile these develop chemical components of the immune system including complement system, cytokines and antimicrobial peptides (Biron, 2010).

2.1.1.1 Components of innate immune system

Innate immune system composed of following factors: physical/chemical barriers, pattern recognition receptors, cells of innate immune system, complement system, cytokines, and antimicrobial peptides.

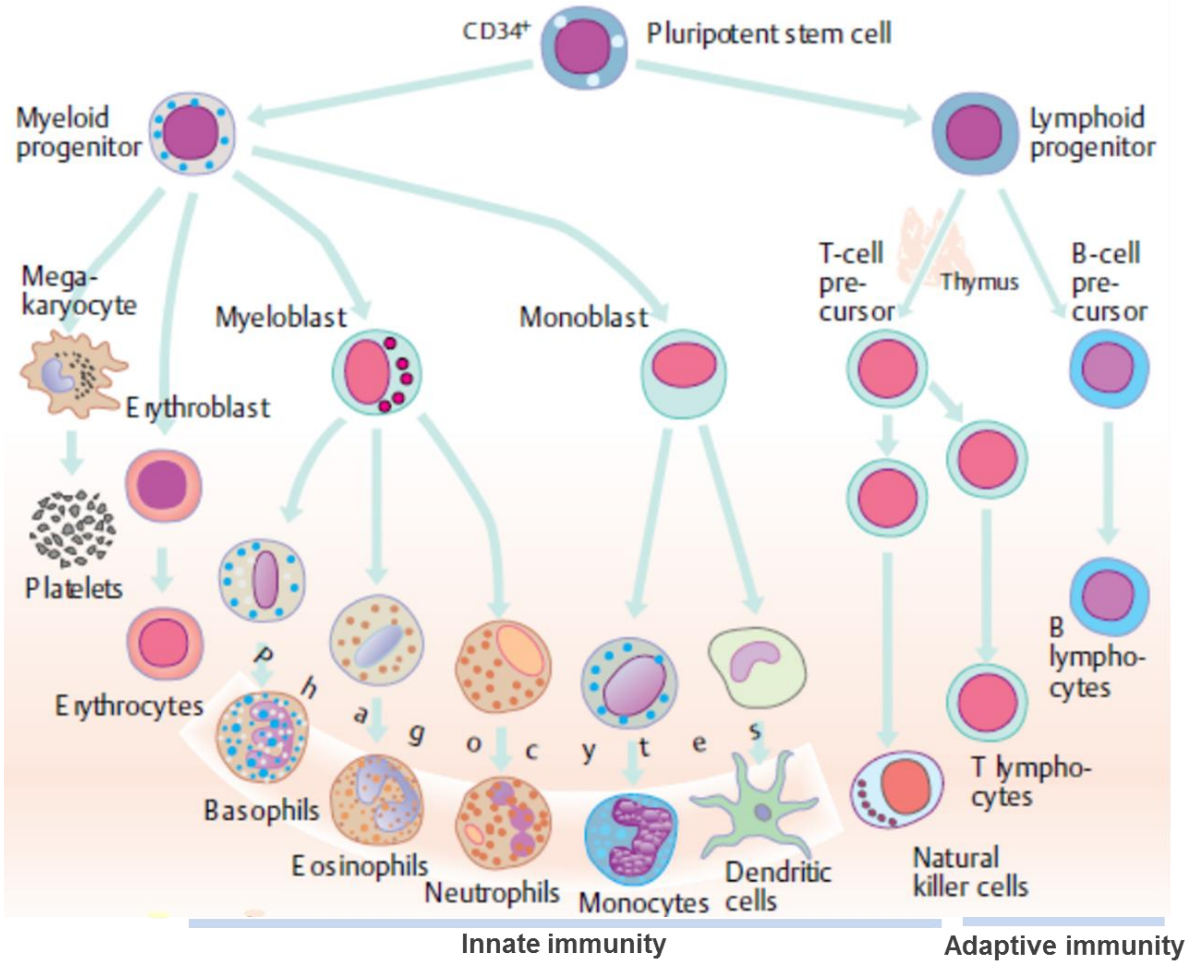

Figure 9: Presentation of different cell lineage involved in immunity.

Different cells of myeloid and lymphoid lineages that are involved in innate and adaptive immunity. Figure was modified from (Burmester et al., 2003).

2.1.1.1.1 Physical and chemical barriers

Epithelium is the first and more important barrier for the pathogens that oppose infectious agents both physically and chemically. This barrier protects the host from the outside environment (Biron, 2010) and is composed of skin (epithelium) and epithelium covered by mucous layer that is found in digestive, respiratory and urogenital tracts. Epithelium is a continuous protective layer separating body from outside environment. Any breach in this barrier can open the doors for the infection. This interfere the pathogens by producing various agents which include acidifying agents (Blanchette et al., 2009; Niyonsaba et al., 2007) and various antimicrobial agents such as lysozyme, defensins, LL-37, and chromogranin derived peptides (Metz-Boutigue et al., 2010; Niyonsaba et al., 2006;

Niyonsaba and Ogawa, 2005; Schroeder et al., 2011). Moreover, the chemical barrier provides an acidic pH at which it's too harsh for pathogens to survive (Niyonsaba et al., 2007). Inner tracts epithium carries various recognition receptors such as TLRs and PARs, that sense the infection and transmits signal (Biron, 2010; Kato et al., 2007). Later certain mediators are released, such as cytokines and chemokines in addition to AMPs. These chemokines and cytokines helps to attract and activate phagocytic cells (Maury et al., 2003).

2.1.1.1.2 Pattern recognition receptors (PRRs)

PRRs are the proteins expressed by various immune cells that help to recognize the pathogens with a remarkable high specificity. PRRs identify the pathogens by bind to much conserved molecules of pathogens called pathogen associated molecular patterns (PAMPs). Various PRRs are found in the host cells but few of them got remarkable importance including toll like receptors (TLRs), C-type lectin receptors (CLR), nucleotide binding oligomerization domain (NOD), lectins, collectin and ficolin. PRRs are broadly categorized into three groups:

- Endocytic receptors: they are expressed on the surface of phagocytes and are often involved in the ingestion and transport of microorganisms to the lysosomes. This is an example of scavenger receptors (Dommett et al., 2006).
- Secreted receptors: these bind to pathogen cell wall and mark the microbes for degradation by the system complement or phagocytosis such as mannose binding lectin (MBL) (Fraser et al., 1998).
- Signaling receptors: they are involved in the activation immune cells when they come in direct contact with the microorganism. These are mostly membrane bound (TLRs) and cytoplasmic (NOD like receptors) (Delneste et al., 2007; Franchi et al., 2006).

TLRs are very important in the pattern recognition; these are found on various cell surfaces, mostly, which are in direct contact with the external environment, such as lung epithelial cells, skin cells etc. These were first discovered in drosophila (Hoffmann et al., 1996). TLRs are transmembrane receptors with a rich leucine extracellular domain and a cysteine rich transmembrane domain. Till today 11 different TLRs have been identified, few are located in the plasma membrane (TLR-1, -2, -4, -5, and -6) and few are in the endosomes (TLR-3, -7, -8 and -9) (Kabelitz and Medzhitov, 2007). Upon recognition of the no-self factors these TLRs rapidly transmit signal to produce proinflammatory cytokines and interferons (Takeda and Akira, 2004, 2005).

2.1.1.1.3 Cells of innate immune system

These are blood cells actively implicated in innate immunity. After the recognition of non-self agents, these readily kill the microorganisms directly or indirectly and clear the cell debris. These cells include mast cells, natural killer cells, granulocytes (basophils and eosinophils) and phagocytes (macrophages/monocytes, neutrophils and dendritic cells) (Figure 9-10).

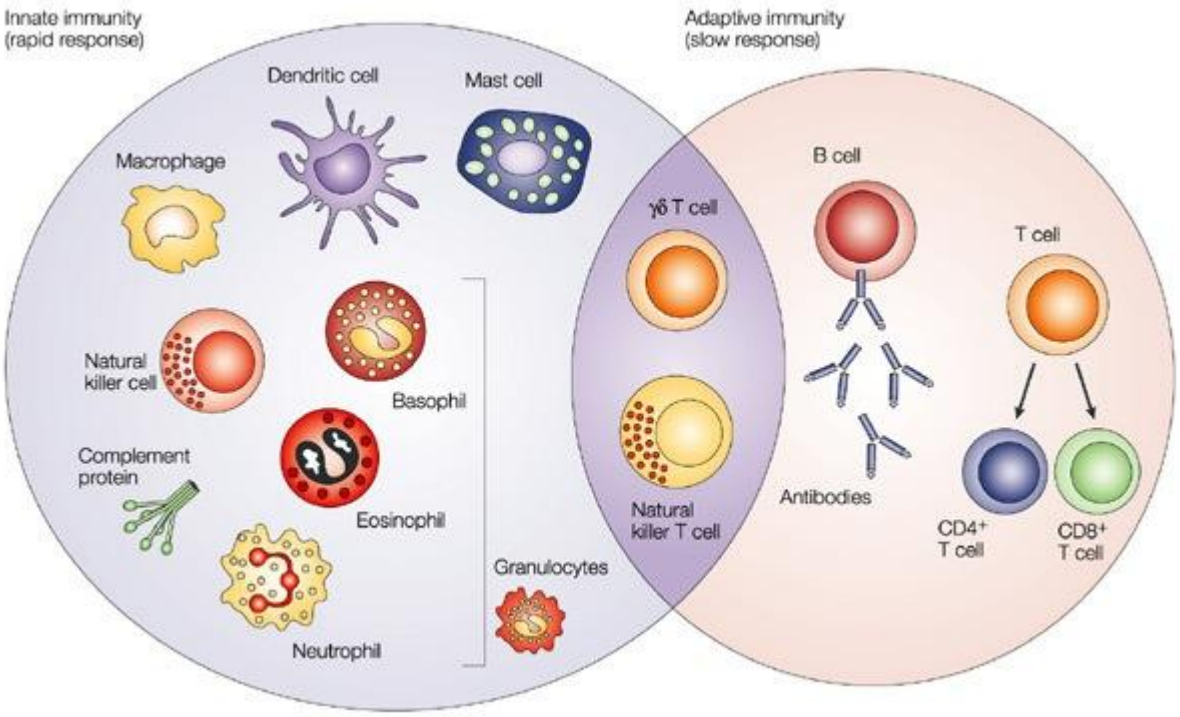

Figure 10: Different componets of the innate and adaptive immune system.

Different components of the immune system, classified according to their role in innate and adaptive immunity. The innate immune response functions as the first line of defense against infection. It consists of soluble complement proteins, and diverse cellular components including granulocytes (neutrophils, eosinophils, and basophils), dendritic cells, mast cells, NK cells, and macrophages. The adaptive immune response is slower in action, but with remarkable antigenic specificity and memory. It consists of antibodies, CD4+ and CD8+ T lymphocytes, and B cells. Natural killer T cells and T cytotoxic lymphocytes overlap the interface of innate and adaptive immunity (Dranoff, 2004).

2.1.1.1.3.1 Mast cells

Mast cells (mastocytes) are the residents of connective tissue and often found in mucous membranes. These cells are associated with the wound healing, more often to allergic

reaction and indirectly to the innate immune defense. When activated, mast cells rapidly produce large number of granules rich in histamine, chemokines, hormonal mediators and chemotactic cytokines (Prussin and Metcalfe, 2003). These cells produce characteristic signs of inflammation and help to recruit neutrophils and macrophages (Prussin and Metcalfe, 2003; Pulendran and Ono, 2008). Due to ability of secreted cytokine rich granules these cell develop a link between innate and adaptive immune system. Mast cells interrelation between innate ad adaptive immunity is briefly describe (Figure 11), indicating role of mast cells in tissue injury by recruiting T-lymphocytes through production of cytokines.

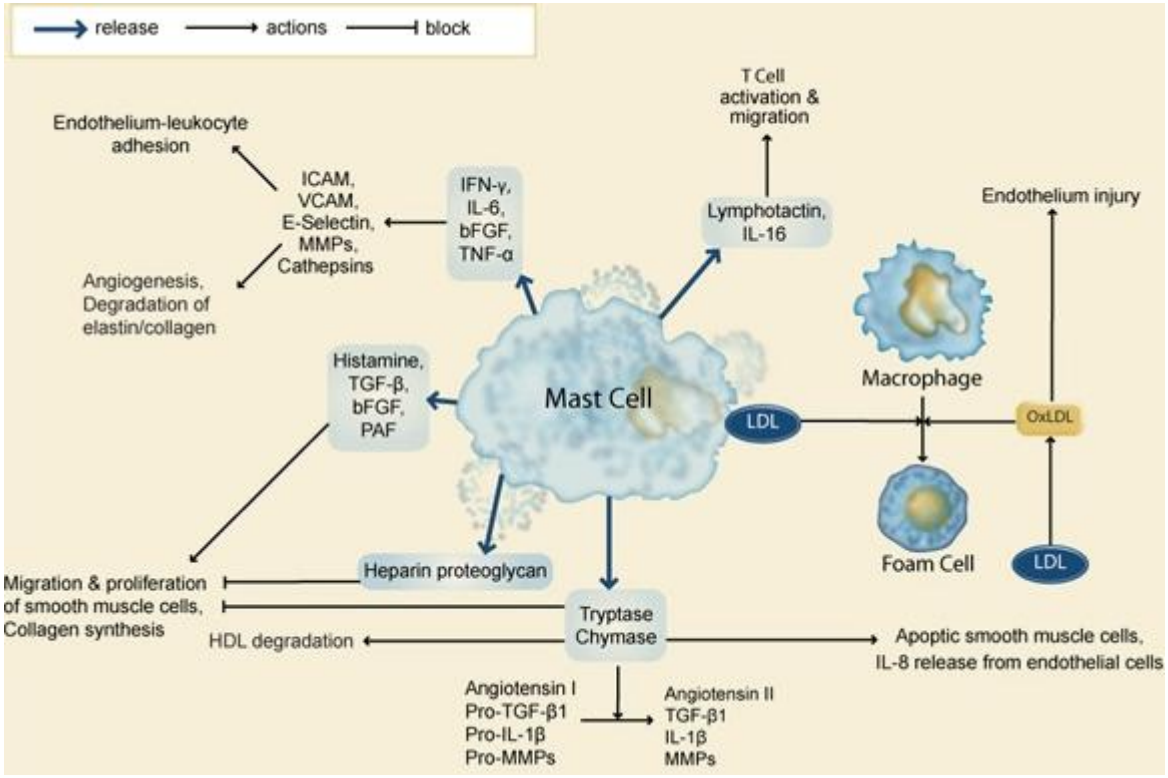

Figure 11: Role of mast cells in tissue injury.

Mast cells lead to activation and migration leukocytes and T cells by production of INF-γ, TNF-α and IL-6. These cells can also trigger proliferation, or can block of smooth muscle cells, in addition can also initiate angiogenesis (Libby and Shi, 2007; Sun et al., 2007).

2.1.1.1.3.2 Natural killer cells

Natural killer cells (NK cells) are the cytotoxic cells, vitally important in innate immune system modulation. Typical function of the NK cells is analogous to cytotoxic T cells (adaptive immune cells). These cells are associated with viral infection and response to tumor

formation. NK cells are unique in their function as recognize stressed cells even in the absence of MHC-1 (major histocompatibility complex-1) (Vivier et al., 2011). Unlike other innate immune cells, NK cells belong to the lymphoid lineage rather than myeloid lineage. NK cells can differentiate into natural killer T cells. Moreover, these have the ability to adjust promptly according to the environment, and to formulate antigen-specific immunological memory (Arina et al., 2007; O'Leary et al., 2006). Various functions are attributed to NK cells including cytolytic granule mediated apoptosis, antibody dependent cell mediated cytotoxicity (Smyth et al., 2002), cytokine induced activation by secreting TNF-α and INF-γ (Lodoen and Lanier, 2005), tumor surveillance (Smyth et al., 2002), and memory maintenance and recall (Lodoen and Lanier, 2005).

2.1.1.1.3.3 Granulocytes (basophils and eosinophils)

Basophils and eosinophils are the granulocytes of the innate immunity; basophils are least common blood cells (0.01-0.3%) (Grattan et al., 2003) with characteristic staining by basic dyes while eosinophils are harbored by acidic dyes. These cells release large amount of granules rich in chemokines, histamine that modulate different immune cells functions (Figure 12). Additionally highly toxic proteins and free radicals are also released to kill the bacteria and parasites. In addition to kill pathogens, these cells are also responsible for tissue damage that is liable for allergic reactions.

Secretions of basophils are rich in histamine, cytokines, leukotriene C4 (Karasuyama et al., 2011) and they often bear characteristic receptors that help to bind immunoglobulin (IgE) (Mukai et al., 2005; Mukai et al., 2009; Schroeder, 2009). While eosinophils secrete cationic proteins (eosinophil cationic protein), in addition reactive oxygen radicals (superoxide, peroxide etc.) are also produced that are associated with direct killing of microbes (Dyer et al., 2010; Saito et al., 2004; Trulson et al., 2007). Eosinophils present various Toll like receptors including TLR1, TLR4, TLR7, TLR9 and TLR10 (Nagase et al., 2003).

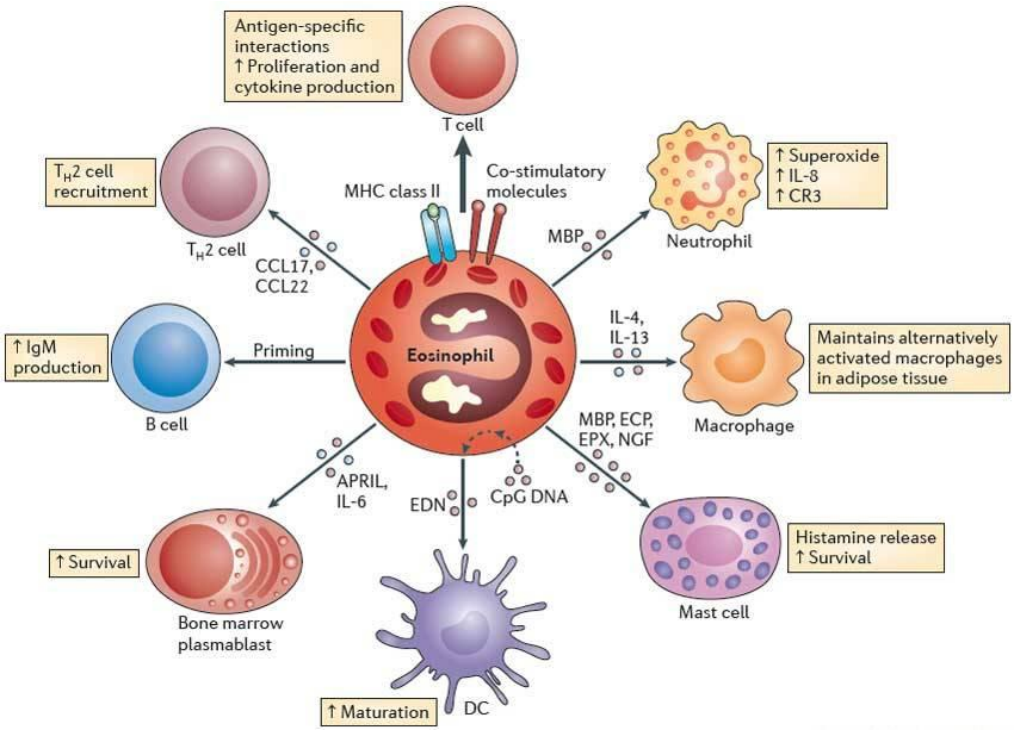

Figure 12: Modulation of immune cells by eosinophils.

Eosinophils can modulate activation and proliferation of various immune cells including dendritic cells, plasmablasts, B cells Th cells, T cells and phagocytes. Moreover, these express MHC class II and co-stimulatory molecules and also can process antigens (Rosenberg et al., 2013).

2.1.1.1.3.4 Phagocytic cells

Phagocytes are the protectors of body, characterized by the engulfing of the harmful foreign particles and the dead cells. In blood, mainly two types of phagocytes are present: neutrophils and the monocytes. Monocytes have the capability to differentiate into tissue macrophages when infiltrated into tissue and to dendritic cells. Mast cells also have phagocytic characteristics, but are more likely associated with allergic reactions. All phagocytes, and especially macrophages usually remain in a semi dormant state, mostly clear the non-infectious dead material present in the tissue and the blood. But, during an infection, upon receiving the chemical signal (proinflamatory cytokines), they increase the production of MHC II molecules, which prepare themselves for antigens. In activated state, these cells become good killer and antigen presenters, while if directly activated by the invader they become hyperactivated (Brechot et al., 2008; Sompayrac, 2008). Neutrophils resides in blood, but upon receiving signal by the macrophages, they escape from the blood vessels and infiltrate at the site of infection (Sompayrac, 2008).

2.1.1.1.3.4.1 Macrophages

Macrophage differentiation from the monocytes is mediated by the G-CSF (granulocyte-colony stimulating factor), GM-CSF (granulocyte-macrophage CSF) and IL-6 (Chomarat et al., 2000). Macrophages also present PRRs that help to recognize the invader and recruit neutrophils by cytokine production. Macrophages secrete IL-1, which stimulate the proliferation and differentiation of CD4 T cells and are thus involved in adaptive immunity (Galdiero et al., 2012; Mosser and Edwards, 2008). They contribute to homeostasis by eliminating cellular debris and other waste from the body. They also act as as antigen presenting cells expressing the major histocompatibility complex-II (MHC-II). There are two types of macrophages (M1 and M2): M1 macrophages are activated by classical pathway by use of LPS and INF-γ while M2 macrophages are activated by alternative pathway (IL-4 or IL-13) (Brechot et al., 2008; Mosser and Edwards, 2008).

2.1.1.1.3.4.2 Dendritic cells

Dendritic cells are also derived from the monocytes like macrophages. These are specialized antigen presenting cells with long outward growths which help to engulf the invaders (Guermonprez et al., 2002). Dendritic cells are present in various tissues that are in contact with the external environment, such as the skin (Langerhans cells) and the inner lining of the respiratory and digestive tracts. Once activated, they rapidly migrate to the lymph nodes where they intermingle with T cells and B cells to modulate the adaptive immune response. After stimulation, dendritic cells rapidly produce IL-12, which is a signal for CD4 T cell towards Th-1 cells (T helper cells) (Reis e Sousa et al., 1997; Sallusto and Lanzavecchia, 2002).

2.1.1.1.3.4.3 Polymorphonuclear neutrophils

Neutrophils are most abundantly found in the white blood cells constituting about 50% to 60% of the total circulating WBCs (Sompayrac, 2008). They are usually short lived only about five days (Sompayrac, 2008). Stimulated neutrophils rapidly leave blood and arrive at the site of infection in about thirty minutes. These are scavenger cells; they are ferocious eaters of invaders coated with complement or antibodies and cellular debris. These are poly-segmented nuclear cells usually have 2-5 segments connected with chromatin filaments.

Generally PMNs do not leave the bone marrow until maturity, but in case of severe infection neutrophil precursors (myelocytes and promyelocytes) are released (Linderkamp et al., 1998).

Neutrophils are highly enriched in granules containing bactericidal proteins, reactive oxygen compounds and protein destroying factors (Soehnlein et al., 2008b). In addition, they can secrete products that are helpful to stimulate monocytes and macrophages (Soehnlein et al., 2008b).

By the process of chemotaxis neutrophils migrate towards the infected or inflamed site which is under the influence of various chemical factors. Cell surface bears several receptors including cytokine receptors, complement receptors, receptors for chemokines, receptors to detect and adhere to endothelium, and Fc receptors for opsonin that allow neutrophils to detect the chemo-attractants such as interferon gamma (INF-γ), interleukin-8 (IL-8), complement factor C5a, and leukotriene B4 (Segal, 2005).

Neutrophils mainly produce three types of granules: azurophilic/primary granules, specific/secondary granules, gelatinase/tertiary granules (Borregaard and Cowland, 1997; Borregaard et al., 1993) (Figure 13). In addition to these granules PMNs also possesses several secretory vesicles (Figure 13) (Borregaard and Cowland, 1997; Borregaard et al., 1993; Lominadze et al., 2005).

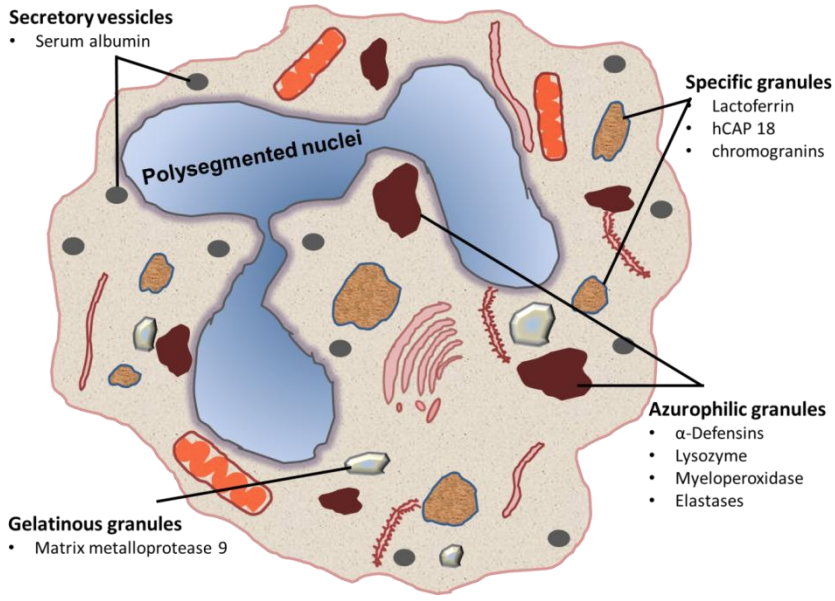

Figure 13: Human polymorphonuclear neutrophil.

Human polymorphonuclear neutrophil (PMNs), different granules with major characteristic proteins are demonstrated.

Secretory vesicles are produced by endocytosis having membranes rich in receptors, adhesion proteins and signaling proteins (Borregaard and Cowland, 1997). While the other three granules contain various host defense proteins (HDPs) and enzymes (Borregaard and Cowland, 1997; Lominadze et al., 2005). Secretions from the primary granules stimulate the phagocytosis of IgG-coated pathogens (Soehnlein et al., 2008a). Different granules are mobilized in a specific manner called graded exocytosis. Gelatinase granules are most easily mobilized followed by secondary and primary granules (Sengelov et al., 1995). These granules are highly enriched in antimicrobial peptides such as defensins, cathepsins, chromogranins, lactoferrins and lysozymes (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003; Lominadze et al., 2005).

In addition to direct killing by engulfing and degranulating, neutrophils are capable of constructing NETs (neutrophil extracellular traps) which is a hallmark of neutrophil function (Brinkmann et al., 2004). NETs comprise a web composed of chromatin fibers and serine proteases that trap and kill microbes. NETs provide localized high concentration of various antimicrobial components secreted by degranulation that facilitates adhesion and killing of microbes. NETs also serve to localize and prevent further spread of infection by providing a physical barrier (Clark et al., 2007). In addition NETs also exhibit pro-thrombotic effect (Borissoff and ten Cate, 2011; Fuchs et al., 2010).

2.1.1.1.4 Complement system

The complement system aids the antibodies and phagocytic cells to clear pathogens from an organism. It is part of the innate immune system; however it can be recruited by the adaptive immune system (Abbas and Lichtman, 2005). Components of the complement system include large number of small proteins, generally synthesized by the liver, and circulating in blood as inactive precursors. It is triggered by several factors, when stimulated proteases in the system cleave specific proteins to initiate an amplifying cascade of further cleavages. The end-result of this cleaving cascade is activation of the cell killing mechanism by formation of membrane attack complex (Sims and Wiedmer, 2000). Complement system is composed of more than 25 different proteins and their fragments. Several functions are attributed to complement system including opsonization, chemotaxis, cell lysis, and clumping of antigen bearing agents that are as a result of enhancement of phagocytosis, attracting phagocytic cells and rupturing membrane of foreign invaders respectively (Goldman and Prabhakar, 1996; Sims and Wiedmer, 2000).

Complement system is activated by three different pathways (Figure 14): classical complement pathway, alternative complement pathway, and lectin pathway (Goldman and Prabhakar, 1996). The classical pathway (Figure 14a) is initiated by activation of the C1-complex, which composed of C1q-r2-s2. This complex is usually triggered either by binding of C1q with the immunoglobulins (IgM/IgG) or by direct interaction of C1q with pathogen surface. Such binding leads to the activation of two molecules of C1r and C1s each (serine proteases). This complex of proteases cleaves C4 and C2 factors to C4a, C4b and C2a and C2b respectively (Mold et al., 1999; Sjoberg et al., 2006). C4b and C2a bind to form C4b2a complex (C3 convertase), which promotes cleavage of C3 into C3a and C3b. C3a bind to bacterial surface and promote phagocytosis, while C3b later binds to C3 convertase to make C5 convertase (C4b2a3b complex). This complex cleaves C5 to C5a and C5b, where former is a chemotactic protein which recriute inflammatory cells while the later forms a complex with C6, C7, C8, and C9. This final complex acts as membrane attacking complex (MAC) (Mold et al., 1999).

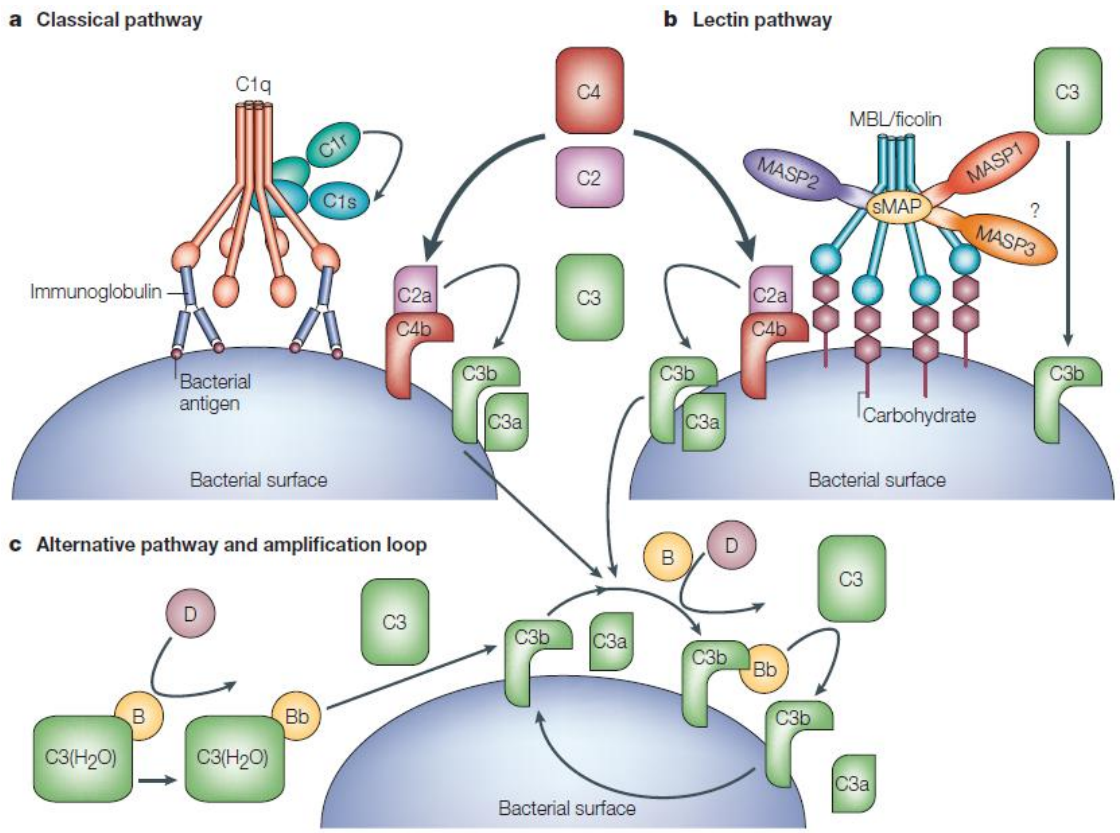

Figure 14: Complement activation pathways.

Complement system activation pathways: classical pathway (a), lectin pathway (b), alternative pathway (c) (Foster, 2004).

The lectin pathway (Figure 14b) is very much homologous to the classical pathway, that is triggered by mannose-binding lectin (MBL), opsonin, and ficolins, instead of C1q. Binding of MBL to mannose residues on the pathogen surface, stimulates the MBL-associated serine proteases (MASP-1, and MASP-2) (Degn et al., 2009; Wallis et al., 2010). These serine proteases can split C4 and C2 into C4a, C4b and C2a, C2b respectively (Sjoberg et al., 2006). Remaining pathway is same as described for the classical complement activation pathway (Mold et al., 1999).

The alternative pathway (Figure 14c) does not require binded antibodies and is continuously activated due to spontaneous C3 hydrolysis. The surface-bound C3b generated by cleavage of C3, binds to factor B to form C3bB (Conrad et al., 1978). Another factor D promotes the cleavage of B into Ba and Bb. C3b remain attached to Bb and this complex C3bBb (C3 convertase) complex is stabilized by binding of factor P. The complex C3bBbP can further intiate cleavage of C3 and this newly-bound C3b recruits more B, D and P factors and greatly amplifies the complement activation (McRae et al., 2005; Pangburn et al., 1977). Complement regulatory proteins such as CD35, CD46, CD55 and CD59 limit the alternative pathway activation. While pathogens do not have such complement regulatory proteins. Thus, the alternative complement pathway is able to distinguish self from non-self. Once the alternative C3 convertase enzyme is developed, it can bind to other C3b factor and form a complex C3bBbC3bP that act as C5 convertase.

2.1.1.1.5 Cytokines

Cytokines are soluble proteins that are modulators of immune response and inflammatory reaction. These develop a link between innate and adaptive immune cells. These are secreted by various immune cells, such as activated macrophages and neutrophils by presence of microbes. They are also produced by T lymphocytes, supplementing adaptive immunity (Lacy and Stow, 2011). An illustration of few cytokines with their role in innate immunity is given below (Table V).

Cytokines have been classed as interleukins (IL) chemokines, and lymphokines, based on their presumed function, cell of secretion, or target of action. Function based classification is more common now a days in research and clinical point of view which includes pro-inflammatory and anti-inflammatory cytokines (Abbas and Lichtman, 2005). Prior stimulates immune cells to secret more while the later works in inverse. This group of mediators

includes chemokines (chemoattractant cytokines), which act on the mobility of several cell types in coupling to G proteins, interferons (IFN α, β, γ), interleukins (IL-), the tumor necrosis factors (TNF-), the growth factors (G-CSF, GM-CSF) and growth factors transformation (TGF, Transforming Growth Factor) (Abbas and Lichtman, 2005).

Table V: Characteristics of few important cytokines implicated in immune system, with their principle source and targets. Table modified from (Abbas and Lichtman, 2005).

Cytokines	Cellular source	Principle cellular target and biological function
Tumor necrosis factor	Macrophages, T lymphocytes	Neutrophils and endothelial cells activation, pyrogenic (hypothalamus)
Interleukine-1	Macrophages, Dendritic cells, Epithelial cells	Endothelial cells activation, pyrogenic (hypothalamus)
Chemokines	Macrophages, Dendritic cells, T lymphocytes, Fibroblasts, Plateletts	Leukocytes activation and chemoataxis
Interleukine-12	Macrophages, Dendritic cells	Induce INF production by NK cells and T lymphocyte, increase cytolytic activity, differentiate T-cells to Th1 cells
Interferon-γ	NK cells, T lymphocytes	Increase immunoglobulin activity, activate macrophages.
Interferon-α	Macrophages	NK cells activation, increase MHC-1 expression
Interferon-β	Fibroblasts	NK cells activation, increase MHC-1 expression
Interleukine-10	Macrophages, T lymphocytes (Th2)	Inhibit IL-12 production by macrophages, reduce MHC-II expression
Interleukine-6	Macrophages, Endothelial cells, T lynphocytes	Proliferate Igs production by B-lymphocytes,
Interleukine-15	Macrophages	NK cells and T lymphocyte proliferation
Interleukine-18	Macrophages	Induces synthesis of IFN-γ by NK cells and T lymphocytes

2.1.1.1.6 Antimicrobial peptides

Antimicrobial peptides are the unique component of the innate immune response, that are highly conserved through evolutionary process (Ganz, 2003). AMPs are also called host defense peptides (Auvynet and Rosenstein, 2009). These are remarkably appreciable in their selection of target cells. These peptides are highly effective broad-spectrum antibiotics, which demonstrate their potential use as novel therapeutic agents. Antimicrobial peptides have been demonstrated to kill bacteria (including resistant strains), mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells (Auvynet and Rosenstein, 2009). In addition, they also have the ability to enhance immunity by functioning as immunomodulators (Auvynet and Rosenstein, 2009). A detailed description of structure, mode of action and different classes is provided in the relevant chapter.

2.1.2 Interrelation between immune, nervous and endocrine system.

The hypothalamic-pituitary-adrenal (HPA) axis helps to maintain homeostasis and participate to reproduction, growth and differentiation. Endocrine informations are controlled from the central nervous system. Adrenal cortex and adrenal medulla is predominantly composed of chromaffin cells. These secretory cells contain numerous granules rich in adrenaline, nor-adrenaline, serotonin, dopamine and simultaneously various proteins having diverse biological function such as chromogranins (Helle, 2004). Later, the discovery to TLRs on the adrenal paved the way to further establish the immune-nervous-endocrine triade (Sternberg, 2006). Stressful situations lead to secretion of AMPs from the endocrine and immune cells. These actions are well coordinated and regulated by the nervous system via the endocrine system. The relationship between nervous system and immune system via the endocrine system are not unidirectional but bidirectional. Several interrelations of neuro-endocrine immune system are demonstrated in figure 15. Brain controls immune system via endocrine system and directly by growth hormones and prolactin. Furthermore, several feedbacks involving of cytokines and autonomic system are also present. Several studies indicate the interrelation of nervous and immune system; for example an electric stimulus triggers the release of antibodies by lymphocytes (Liburdy, 1992). Conversely, interlukine-2 lead to changes in bioelectric pulse (Bindoni et al., 1988).

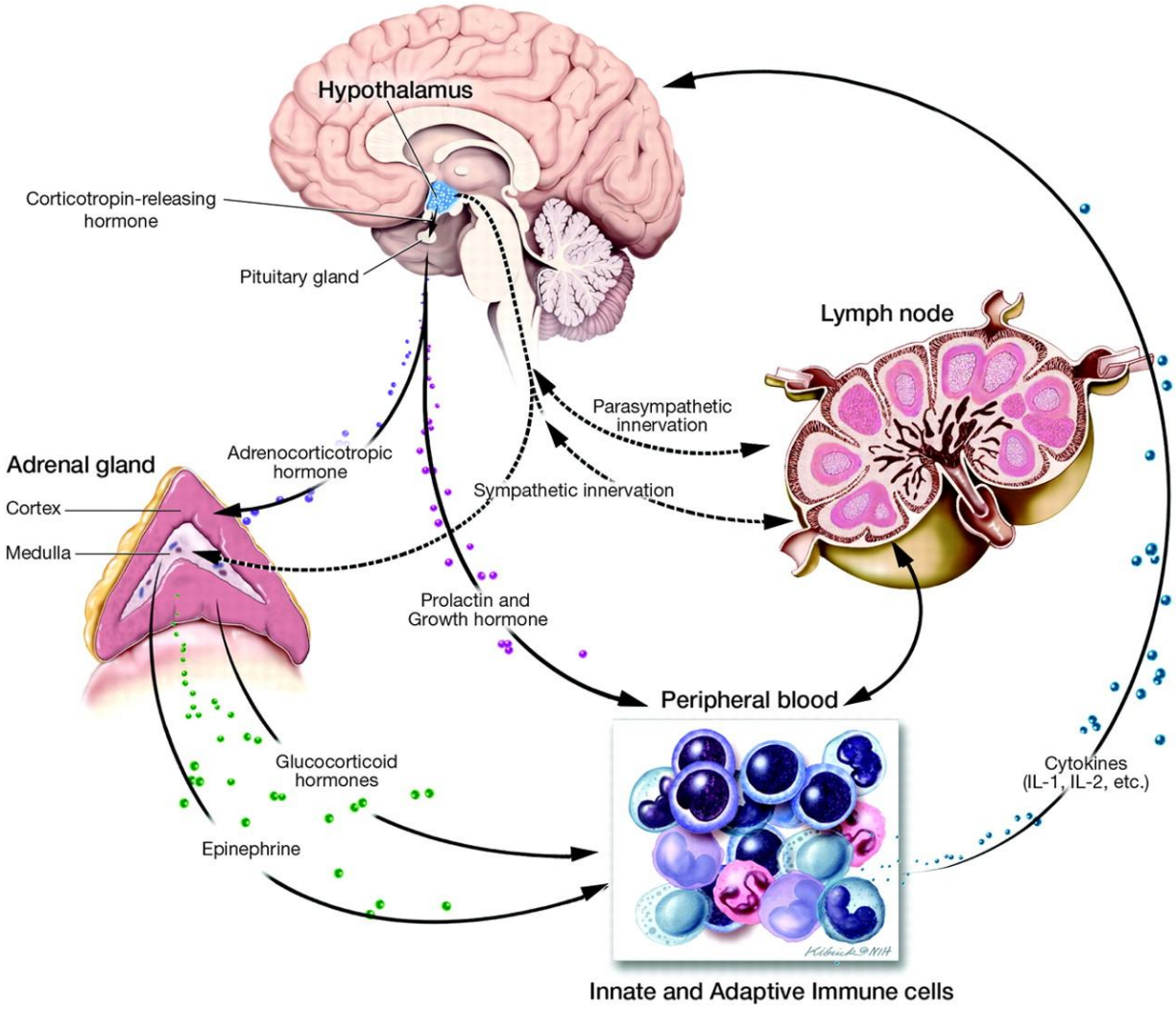

Figure 15: Immune, nervous and endocrine system interaction.

Brain regulates the immune system via the neuroendocrine and autonomic. The sympathetic innervation of the adrenal medulla, which secretes epinephrine and euroendocrine influences (hypothalamic pituitary axis, growth hormone and prolactin), together with epinephrine, influence innate and adaptive immune cells that secrete cytokines and AMPs. In addition to autonomic feedback, several different cytokine feedbacks also control brain function (Glaser and Kiecolt-Glaser, 2005).

Likewise, neuroendocrine control of the immune response at the site of inflammation is mediated by the release of neurotransmitters from nerve endings (Kawashima and Fujii, 2004). In addition, the autonomic nervous system, with its sympathetic and parasympathetic pathways influences innate immunity. Moreover, co-localization of adrenaline and antimicrobial chromogranin derived peptides indicate the active relationship between neuroendocrine and immune systems (Portela-Gomes et al., 1997). Injection of adrenaline actively recruite the natural killer cells, monocytes and T lymphocytes in patients with

rheumatoid arthritis (Kittner et al., 2002). Similarly, norepinephrine plays a role in the central neural regulation of the immune system (Besedovsky and del Rey, 1996; Elenkov et al., 2000; Sanders and Straub, 2002). Likewise, acetylcholine binds macrophages and endothelial cells through the α7 nicotinic receptor, reducing their activation and secretion of pro-inflammatory cytokines (Saeed et al., 2005). Other hormones such as CRH (corticotrophin releasing hormone), the substance P, CGRP (calcitonin gene-regulated peptides) are vigorously involved in the defense by controlling the pro-inflammatory activity of the innate immune cells. For example, stress may stimulate CRH production via cytokines secretion such as TNF-α, IL-1 or IL-6 secretion and finally increases the adrenocorticotropic hormone (ACTH) and cortisol. Conversely, cortisol inhibits pro-inflammatory cytokine secretion by macrophages. Substance P favors the release of vasoactive mediators, such as serotonin and histamine by mast cells (Guhl et al., 2005). Similarly, the β-endorphin and enkephalin inhibit the production of ACTH. In addition, sex hormones and glucocorticoids are also well implicated in immunity (Silva et al., 2010). Furthermore, stress and anxiety lead to weakening of the immune system due to release of adrenaline and prostaglandins that decrease NK cells activity (Inbar et al., 2011).

Similar to control of immune system by the endocrine system, components of immune system also regulates it in vice versa. Such as, AMPs modulate immune cell secretions. For example, the peptides derived from chromogranins such as catestatine has a chemotactic effect and thus contributes to mobilization of polymorphonuclear the site of inflammation (Shooshtarizadeh et al., 2010), further detail about the chromogranin derived peptides is provided in the relevant chapter. Similarly, catestatin and chromofungin trigger neutrophil secretion via SOC channels (Channels Store Operated) by inducing intracellular calcium influx (Zhang et al., 2009b).

ANTIMICROBIAL PEPTIDES

3 ANTIMICROBIAL PEPTIDES

3.1 Introduction

Antimicrobial peptides (AMPs) are small molecules that are integral part of innate immune defense (Yount et al., 2006; Zasloff, 1992), found in all living organisms including bacteria, archaea, protists, fungi, plants, animals and humans. These are evolutionary conserved components, existing through the history of life on earth (Yount et al., 2006). AMPs are generally 12-50 amino acids long peptides, although several large proteins are also discovered having antimicrobial properties such as lysozymes, lactoferrin and chromogranin (Strub et al., 1996a). AMPs are usually positively charged which can vary from +2 to +9 depending on the number of positively charged amino acids (arginine and lysine) (Hancock and Chapple, 1999). In addition to this positive charge a large hydrophobic portion is also present (>50%) (Hancock and Chapple, 1999; Yeaman and Yount, 2003; Yount et al., 2006). Few AMPs are negatively charged having large number of anionic residues such as aspartic acid or glutamic acid (Hancock and Chapple, 1999; Yeaman and Yount, 2003). Most of the AMPs are non-structured in solutions but they immediately attain their specific configuration in membranes or hydrophobic solutions (Hancock and Rozek, 2002). AMPs display a broad spectrum of activity against viruses, fungi, bacteria, and parasites (Hancock and Sahl, 2006; Izadpanah and Gallo, 2005). In addition to antimicrobial activities, AMPs also display various immuno-modulatory activities, such as anticancer activities and immune boosting properties (chemotactic peptides) (Izadpanah and Gallo, 2005).

AMPs could not get the research focus until late twenth century, which was further accelerated in the last two decades. The first AMP, lysozyme was described by the Alexander Flemming in 1922 (Fleming and Allison, 1922), he isolated lysozymes from the secretions produced against infectious agents. Few early reports are also present about AMPs described in 1902-1921, but a well-established report about the existence of AMPs was published in 1928 by Ridley (Ridley, 1928). Lysozymes were later used in medication as bacteriolytic agents in 1964 (Corone, 1964), which initiated the research to discover new AMPs and their use as potential antimicrobial agents. Later in the year 1930, the franco-american microbiologist, Rene Dubos discovered gramicidin D (Dubos and Cattaneo, 1939). This AMP was isolated from *Bacillus brevis* and is composed of 15 amino acids. It increases cationic permeability of plasma membrane by pore forming.

Another milestone in the discovery of AMPs is colicin, which is produced by certain strains of *Escherichia coli*. André Gratia observed inhibition of a strain of *Escherichia coli* by a substance (Gratia, 1925). Later in 1950, it was described as colicin (Gardner, 1950). Since then, multiple AMPs were isolated and identified. There are several databases that collect all the information about the AMPs discovered; one of them is APD (http://aps.unmc.edu/AP/links.php) (Wang et al., 2009; Wang and Wang, 2004). Till today (Feb. 2013) 2172 AMPs have been identified including 135 antiviral, 773 antifungal, 1762 antibacterial, 48 antiparasitic and 145 anticancerous peptides. Few of the nominated representative AMPs each year by APD, are listed below (Table VI).

Table VI: AMPs timeline: representative AMPs selected by antimicrobial peptide database (Wang et al., 2009; Wang and Wang, 2004).

Year	Representative AMPs
1928	Nisin (bacteria)
1939	Gramicidin
1968	Viscotoxin A3 (plants)
1970	Bombinin (amphibians)
1972	Purothionin (plants)
1973	Kalata B1, Melittin A (insects)
1979	Mastoparan
1981	Cecropins
1985	Bombolitin; PGLa; Human alpha defensins (HNP1); Microcin
1986	Enterocin AS-48, Pardaxin (fish)
1987	Magainin
1988	Human histatin
1989	Bactenecin (cathelicidin), Tachyplesin, Polyphemusin, HNP4
1990	Duramycin, Gamma-thionin (i.e. plant defensin)
1991	PR-39, the first beta defensin TAP
1992	human HD5, Brevinin, Indolicidin, Microcin J25
1993	human HD6, Bovine beta defensins, Protegrin, Caerin, plant antifungal peptides
1994	Dermaseptin, Esculentin, Avian defensin, Amoebapore
1995	the only human cathelicidin LL-37, hBD-1
1996	Temporin, Thanatin, Buforin, Uperin, Mytilin
1997	Clavanin, Styelin, hBD-2

1998	Lactoferrin, Ranaturin, Maculatin, Lycotoxin, Granulysin
1999	Circulin, RTD-1 (Theta-defensin, circular), Cyclotides (Kalata B1, circular)
2000	Aurein, rCRAMP, Palustrin
2001	hBD-3, hBD-4, Ponericin, Dermcidin, Piscidin
2002	human RNase 7; Maximin, Hepcidin, Cupiennin, Retrocyclin, Halocidin
2003	human angiogenins, human chemokine, Alo-1, Circularin A, PhD1, Halocin C8
2004	Arenicin, Brazzein, Ascaphin, Caenacin (Gly-rich)
2005	human psoriasin (S100A7), Plectasin, Phylloseptin
2006	human RNase 8; Latarcin, Cycloviolacin, Lividin
2007	Dybowskin, Pleurain, Hyposin, Odorranain, AvBD, Longicin
2008	human RNase 3; BTD, Vibi
2009	cn-AMP, Kassinatuerin, Lasioglossin, Lichennicidin, Myxinidin
2010	Caenopore-5, Lucifensin, Temporin-SHf, Centrocin, Beta-amyloid peptide, Thuricin
2011	LCI, Glycocin F (class 4 bacteriocin is found), frog cathelicidin-AL, PAM1
2012	Geobacillin I (7 thioether rings), uncyclotide ChaC7 (i.e open chained), Microcin S (103AA), Slerocin, fungal Eurocin, fungal Micasin

3.2 Structure of antimicrobial peptides

AMPs structural classification and characteristics are typically based on the amino acid sequences. It has been demonstrated that alteration in the sequence and modification of the charged amino acids vastly alter activities of peptides. In addition to sequence and nature of amino acids, secondary and tertiary structures are equally important in physiological characteristics. Generally four characteristics play important role in AMPs activities: chemical properties, primary structure, secondary structure and posttranslational modifications (Izadpanah and Gallo, 2005; Jenssen et al., 2006; Strub et al., 1997).

3.2.1 Chemical properties of the peptides

AMPs are widely isolated from various species including animals, plants and microbes; such diverse nature gives AMPs a vast variety of physico-chemical characteristics. These characteristics include polarity and non-polarity. Generally, AMPs possesses a unique balance of hydrophobic and hydrophilic residues that gives amphiphilic nature to peptides important for their activity (Helle et al., 2007; Izadpanah and Gallo, 2005; Yount et al., 2006).

Polarity of AMPs: Polarity is the first important characteristic of AMPs: there are two types of AMPs: cationic and anionic AMPs. Cationic antimicrobial peptides are more common in nature than anionic peptides. Generally, charge varies from +2 to +9 for cationic AMPs, but can be up to +12 in some cases. Positive charge of the AMPs is important for interaction with negatively charged microbial membrane. Bacterial surface usually contains high concentration of LPS (lipopolysaccharides) and techoic acid which gives negative charge to surface in Gram-negative and Gram-positive bacteria respectively (Madigan and Martinko, 2005). In case of fungi, phosphomannans is important to provide negative charge to surface (Salzman et al., 2004). In addition to surface proteins, microbial membrane is also rich in phospholipids, such as phosphatidylcholines which facilitate AMPs insertion deep into membrane (Yount et al., 2006).

While anionic AMPs are rich in glutamic acid and aspartic acid residues that denotes negative charge. These are more abundantly found in respiratory system of mammals. Anionic peptides generally require zinc co-factors to present antibacterial activities (Fales-Williams et al., 2002). Studies on anionic AMPs dermicidin and enkelytin indicate that negative charge and amphiphilic characteristics are important for their antimicrobial activities (Goumon et al., 1998; Kieffer et al., 1998). In addition to electrostatic interaction between peptide and membrane, polarity also provides a unique orientation to peptide that facilitates penetration to bacterial membrane (Dennison et al., 2006; Lins and Brasseur, 2008).

Amphiphilic nature of AMPs: A unique balance of hydrophilic and lipophilic residues provides amphiphilic character to AMPs. Most of the AMPs are amphipathic or they became amphipathic, when came across anisotropic medium (Yount et al., 2006). It has been demonstrated that alteration in this balance can change the properties of AMPs. By increasing amphiphilic character of synthetic peptides their effectiveness can be increased (Jin et al., 2005). Generally, AMPs contain about 50% hydrophobic residues that facilitate insertion and rests are hydrophilic (Helle et al., 2007; Jin et al., 2005; Yount et al., 2006). This dual nature provides the capability to solubilize both in hydrophilic and lipophilic solvents. However, excessive increase in the hydrophobicity can rapidly decrease their activity but can increase toxicity to mammalian cells (Zelezetsky et al., 2005).

3.2.2 Primary structure of AMPs

Antimicrobial activity is determined by the amino acids composition of AMPs. Lysine, arginine, and to a lesser extent, histidine, are likely to provide a positive charge to peptide

(cationic), while aspartic acid and glutamic acid are responsible for negative charge. Additionally, tryptophan, tyrosine, phenylalanine, leucine, isoleucine and methionine are responsible for hydrophobicity of AMPs. Alteration in amino acids sequence can profoundly change their antimicrobial activity, such as two tryptophan residues are essential for the activity of lactoferrin (Strom et al., 2002). Similarly, addition of few amino acids can increase the activity of AMPs by facilitating insertion into bacterial membrane such as tryptophan end tags (Pasupuleti et al., 2009a; Pasupuleti et al., 2009b; Schmidtchen et al., 2009).

3.2.3 Secondary structure of AMPs

Secondary structures can widely alter activity of AMPs. General characteristics found in AMPs are α-helices, β-sheets, and extended coils (Figure 16). However, AMPs mostly possesses several mixed structures, which provide an extended spectrum of activity. In general, secondary structures are observed only when the peptide interacts with membranes (Jenssen et al., 2006; Yount et al., 2006). For example indolicidin is mostly unstructured in aqueous solutions but it adopts a boat like conformation in the presence of lipid membranes (Hsu and Yip, 2007). A cathelicidin derived peptide tritrpticin is unstructured but in organic solvents such as dodecylphosphocholine micelles it adopts turn-turn amphipathic structure (Schibli et al., 2006).

3.2.4 Posttranslational modifications

In addition to structural and physico-chemical characteristics, posttranslational modifications also play important role in activity spectrum of AMPs. These modifications include phosphorylation, glycosylation, and ubiquitination. As, in case of enkelytine (AMP derived from proenkephalin) phosphorylation is essential for antimicrobial activity of the peptide (Goumon et al., 2000). Similarly, phosphorylations and glycosylations are important for chromacin (chromogranin A derived peptide) (Strub et al., 1996a).

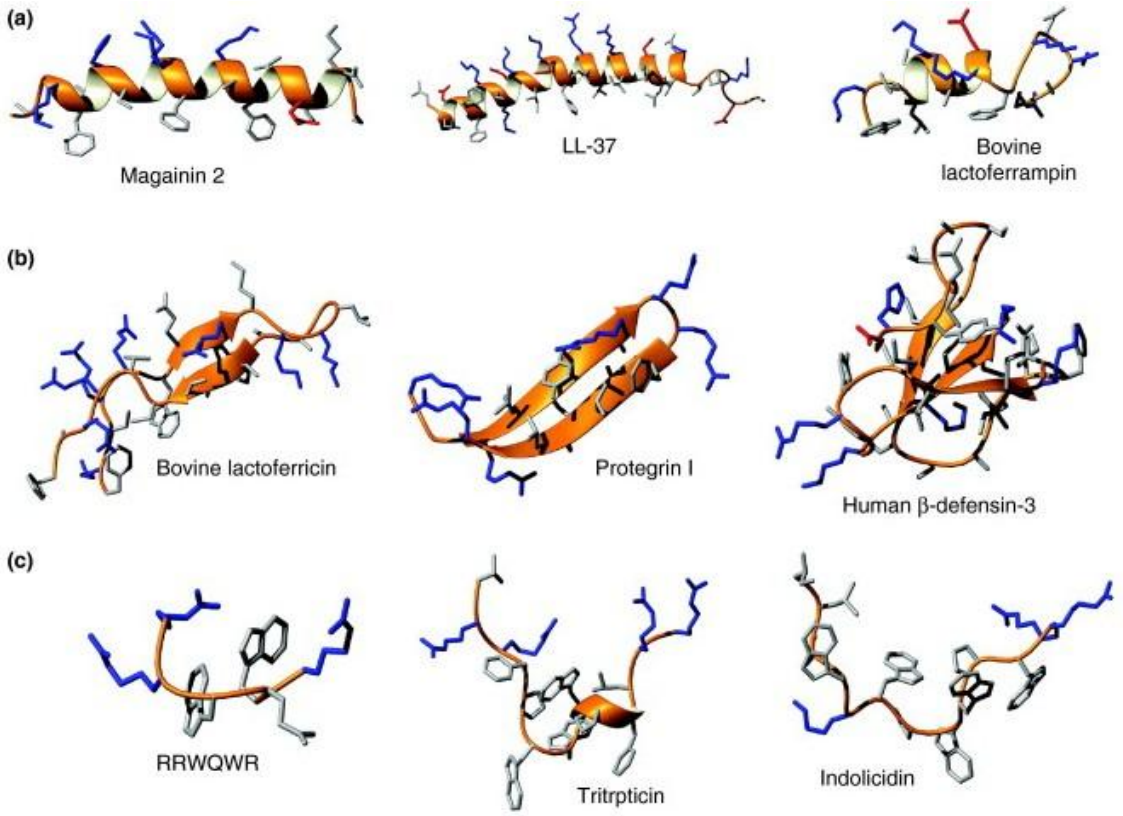

Figure 16: Structural classes of antimicrobial peptides (AMPs).

(a) α-Helical peptides, (b) β-sheet peptides and (c) extended peptides. Positively charged side chains are colored in blue, negatively charged side chains in red and remaining side chains in grey (Nguyen et al., 2011).

3.3 Classification of antimicrobial peptides

Regarding classification of AMPs, there are two methods based on functional characteristics, which is not more common due to dual properties of AMPs and secondly, based on structural characteristics which is more common now a days. AMPs are endogenous peptides synthesized by body under the influence of two processes either ribosomal oriented or non-ribosomal (Hancock and Chapple, 1999). Under the ribosomal synthesis, AMPs are synthesized in ribosomes and stored in secretory vesicles where they are modified to express active forms and secreted under stress conditions (Devine, 2003; Levy, 2004). These proteins obtained by ribosomal synthesis are usually of large sizes and serve precursors for synthesis of various biologically active peptides (e.g. chromogranins, lactoferrin). The non-ribosomal method is most commonly adopted by bacteria, fungi and streptomycetes for example polymixins, pencillins, colistin and bacitracin (Kleinkauf and von Dohren, 1988; Perlman and Bodanszky, 1971).

AMPs are posttranslationaly modified in secretory vesicles, which helps to protect peptides from degradation. These modifications include amidation, phosphorylation, glycosylation (Bulet et al., 1999; Goumon et al., 1998; Strub et al., 1997) that are helpful in ultimate structural modifications in their active form. Based on active form generation and structural characteristics AMPs are divided into following categories (Table VII). Generally, based on the physic-chemical characteristics AMPs are divided into five classes.

3.3.1 Cationic AMPs rich in specific amino acids

These types of AMPs are very vast in number having a single or multiple amino acids high in number. These amino acids include proline (abaecin), arginine (bactericin), phenylalanine (prophenin), glycine (hymenotaecin), histadin (histatin) and tryptophan (indolicidin). These can be linear in structure or can adopt different conformations. Generally these have less than 40 residues, out of which 40-60% are hydrophobic residues (Tossi et al., 2000). This category of AMPs include a large number of peptides rich in the amino acids such as proline (abacin), trptophan (indolicidin), glycine (hymenotaecin), histidin (histatin), arginine (bactenicin), phenylalanine (prophenin), or few other amino acids (Baumann et al., 2010; Brogden, 2005; Bulet et al., 1999; Kichler et al., 2006; Scocchi et al., 2011; Yu et al., 2010).

3.3.2 Cationic AMPs lacking cysteine

Cationic AMPs lacking cysteine are not very common in nature. These lack specifically cysteine amino acid, which limit their ability to form disulfide bond. Moreover, these strictly follow the conformation of α helices. These also possess 40-60% hydrophobic residues as previously described. Examples include cercopins, LL37, and magainin (Boman, 1991; Zaiou et al., 2003; Zasloff, 1987).

3.3.3 Anionic AMPs

These peptides are rich in aspartic acid or glutamic acid, which provide negative charge to peptides. These peptides are more commonly found in the respiratory system of the mammals, where they act as surfactants. Few examples include scygonadine, Maximin H5, and enkelytine (Goumon et al., 1998; Lai et al., 2002; Wang et al., 2007). The mode of action of these peptides is not clear few studies indicate their role as surfactants while others suggest that they act through a structure orientation by oblique helical shape (Dennison et al., 2006).

Table VII: Different types of antimicrobial peptides based on their structural characteristics with their characteristics and few prominent examples of the domain.

Cationic AMPs rich in specific amino acids	
Rich in proline, arginine, phenylalanine, glycine, tryptophan	abaecin, apidaecins, prophenin, indolicidin, drosocine, pyrrhocoricine, bactenicines, PR39, prophenine, hymenotaecine, histatin, coleoptericine, holotricine
Cationic AMPs lacking cysteine	
Lack in cysteine Linear α-helix in structure	Andropin, moricin, ceratotoxin cecropins, melittin, bombinin, magainin, dermaseptin, esculentins, brevinin-1, buforin II, CAP18, LL37, BMAP, SMAP
Anionic AMPs	
Rich in glutamic and aspartic acid	Maximin H5, scygonadine, dermcidin, enkelytin
Cationic/anionic AMPs having disulfide bond	
Having cysteine and 1-3 disulfide bonds	brevinins, protegrin, tachyplesins, defensins, drosomycin, SPAG11, cryptidine
Cationic/anionic peptides derived from the large proteins	
Shorter fragments derived from the large proteins	Lactoferricin, casocidin, lysozyme, catestatin antimicrobial domains of lactabumine, hemoglobin, ovalbumine

3.3.4 Cationic/anionic AMPs having disulfide bond

AMPs rich in cysteine residues are usually cyclic peptides with antiparallel β sheets that are stabilized by disulfide bridges. These cationic peptides may contain between 2 and 8 cysteine residues that can bind to form disulfide bonds and adopt a specific structures such as "hairpin". The main representative of this class of peptides is defensin (Dimarcq et al., 1998; Dimarcq et al., 1990; Lehrer and Ganz, 1996).

3.3.5 Cationic/anionic peptides derived from the large proteins

AMPs generated from large proteins are obtained by proteolysis carried out either by host proteases or bacterial proteases. Few examples of the AMPs belong to this category includes: peptides derived from granins (Briolat et al., 2005; Lugardon et al., 2001; Strub et al., 1995;

Strub et al., 1996b), lactoferrin (Tani et al., 1990), complement proteins (Cochrane and Muller-Eberhard, 1968; Hugli, 1975), pro-enkephalin (Goumon et al., 1998) and ubiquitin (Kieffer et al., 2003).

3.4 Mechanism of action of antimicrobial peptides

AMPs can be categorized in two subdivisions based on the mode of action that is either direct action or indirect activity (Diamond et al., 2009). Direct antimicrobial activity means their interaction with the membrane and indirect relates to their interaction with the intracellular components. However, in both case primary focus is to cross the cell membrane that is achieved by various methods.

3.4.1 Interaction with membranes

AMPs have the ability to interact with the bacterial membrane, which is favored by the cationic and hydrophobic nature of AMPs. Their mechanism of action is well described by amphipathic secondary structure of the peptides. Amphipathy is relative distribution of hydrophobic and hydrophilic residues along the sequence of protein/peptide, which favors their solubility in organic or inorganic solvents. Membrane interaction is achieved by three phases: attraction, attachment and then insertion (Diamond et al., 2009). Attraction phase is favored by cationic nature of the peptides and further attachment is also favored by this electrostatic interaction between peptides and lipopolysaccharide or teichoic acid residues of bacterial membrane (Nizet, 2006). Additionally, hydrophobic and aromatic residues also play a crucial role in the attachment phase. Third step of insertion is penetration by the amphipathic domain of peptide into membrane of pathogens. Then peptides enter the cell and can interact with multiple cytoplasmic targets. Three models have been proposed that demonstrate insertion of AMPs deep into membrane (Figure 17). In addition some distorted or mixed pores are also formed by few AMPs. Some of AMPs such as magainin-2 makes tetravalent and octavalent polymers which can increase pore forming ability by adopting mixed pore formation (Arnusch et al., 2007).

3.4.1.1 Carpet like mechanism

This model refers to demolition of membrane assembly by combined action of peptides (Shai, 2002). This model is achieved by the peptides that are mostly linear in structure. In this pore forming mechanism peptides self-associate and by electrostatic interaction stick to

bacterial membrane just like beta barrel antiparallel sheets. Peptide accumulation is specifically onto the acidic phospholipid-rich regions of lipid bilayers. This carpet structure continues to grow until a certain threshold level is reached which lead to permeation of membrane by destabilizing (Figure 17). AMPs rapidly permeate into the membrane after destabilization by mounting the bilayer positive potential (Li et al., 2006).

3.4.1.2 Barrel stave model

In this model, AMPs form clusters in hydrophobic regions of the membranes and creates pore by formation of transmembrane bundles (Yang et al., 2001). Initial steps are same as attraction and attachment which in followed by insertion of peptides perpendicular to the membrane axis. Peptides bind to the membrane and spread their hydrophilic heads towards phospholipids, and then they enter their pin hydrophobic heads by pushing the phospholipids. Peptides assemble themselves to carbon chains phospholipids leaving them to free their hydrophilic domains that form pores (Bechinger, 1999; Nguyen et al., 2011; Yang et al., 2001) (Figure 17). One typical example following this mode of action is alamethicin which was isolated from fungus *Trichoderma viride* (Bechinger, 1999; North et al., 1995).

3.4.1.3 Toroidal model

In this model, AMPs builds toroidal pores in lipid bilayers. The lipid polar head and helix bundles that orient vertically to the membrane to achieve pore construction. AMPs aggregate and induce the lipid monolayers to bend continuously through the pores so that both the inserted peptides and the lipid head groups line towards water core (Brogden, 2005). Toroidal pores act as channels for ions or metabolites, and eventually preventing bacteria from maintaining the homeostasis (Boland and Separovic, 2006) (Figure 17). This model of action is adopted mostly by α-helical peptides such as magainin (Matsuzaki et al., 1996), melittin (Lee et al., 2004b), LL-37 (Brogden, 2005; Henzler-Wildman et al., 2004), protegrin-1 (Yamaguchi et al., 2002), 2002).

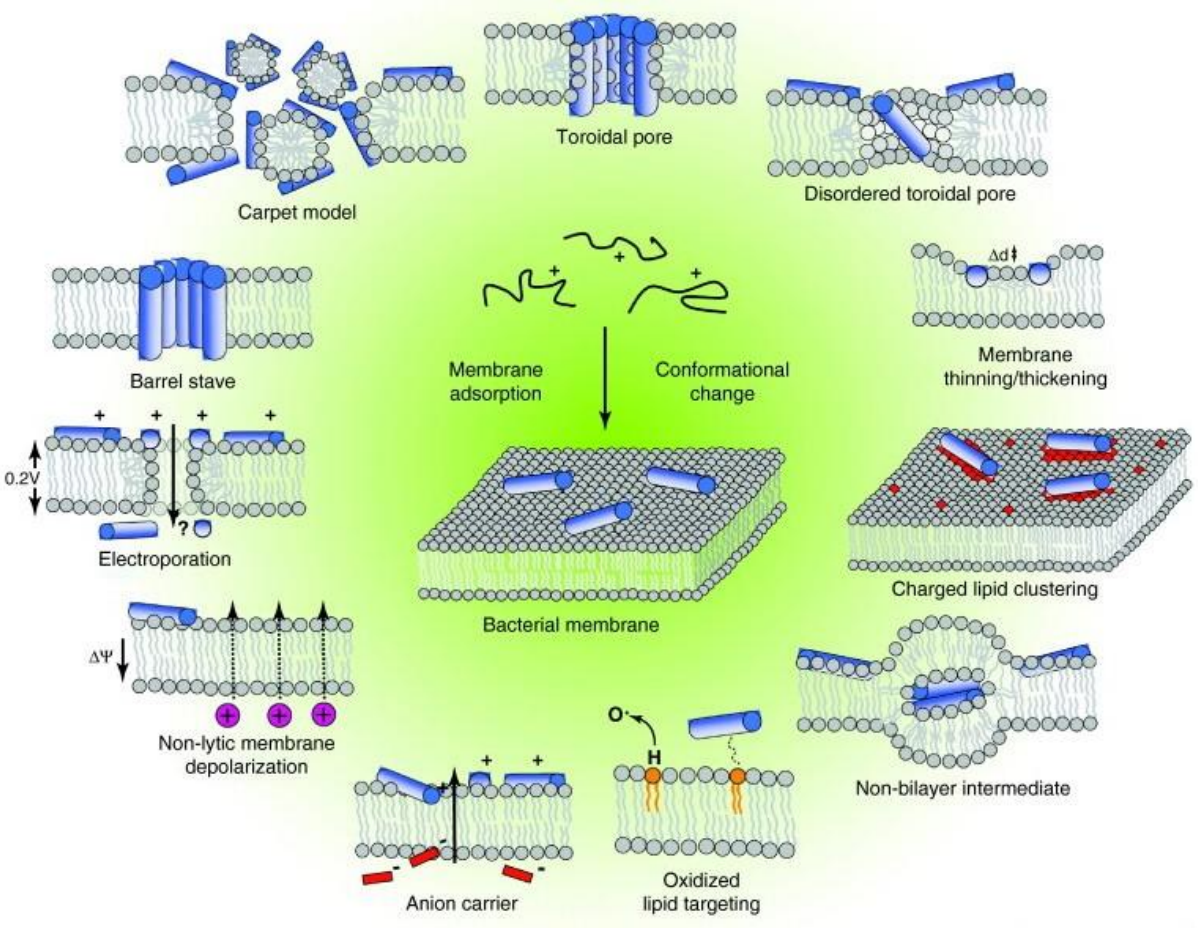

Figure 17: Different types of interaction between AMPs and microbial membrane.

Following AMPs adsorption to bacterial cytoplasmic membrane, different events that follows are demonstrated. AMPs after reaching threshold concentration insert themselves across the membrane to form different types of pores (Nguyen et al., 2011).

3.4.2 Interaction with intracellular components

Inhibition of DNA and interference with various physiological protein functions are the most popular intrcellular targets of AMPs. It has been postulated that the positive charges in AMPs boosts bacterial cell access and consequently, its entrance into the periplasmic space. Next step is to cross the microbial membrane that is achieved by either membrane disrupting or by receptor/transporter molecule binding and by the help of bacterial chaperone GroEL (Castle et al., 1999; Otvos et al., 2000). Ultimately, the peptide is displaced inside the cell where it can interact with multiple targets either ribosome leading to protein synthesis inhibition or DnaK leading to protein folding inhibition (Castle et al., 1999; Kragol et al., 2001; Otvos et al., 2000). Pyrrhocoricin a member of apidaecins family inhibits natural

ATPase activity in *E. coli* (Kragol et al., 2001). In contrast, PR-39 kills bacteria by interfering with DNA and ultimately blocking protein synthesis (Boman et al., 1993). Interestingly, PR-39 can hamper NADPH-dependent redox reactions by production of syndecans (Shi et al., 1996). In addition to ribosomal and DNA interference, AMPs have various other target sites including enzymatic activity and cell wall synthesis inhibition (Brogden et al., 2003; Jenssen et al., 2006).

3.5 Biological activities of antimicrobial peptides

AMPs are widely associated with host defense either by exerting direct antimicrobial activities or by modulating immune cell's function (Hancock and Sahl, 2006). Their spectrum of activity is so much diverse that they are often termed as host defense peptides (HDPs) rather than AMPs (Yeung et al., 2011; Yu et al., 2007).

3.5.1 Direct antimicrobial activity

AMPs can directly target and destroy microbes and infected cells. This direct action involves destabilization of microbial membrane as previously described in section 3.4 of this chapter. Based on this type of interaction these can be either antimicrobial or anticancerous. Antimicrobial nature of these peptides is attributed to negatively charged membranes of bacteria, fungi, viruses and parasites. Similar to this mode of action, AMPs can differentiate between healthy normal cells and cancer cells, due to selective presentation of negative charge on the surface. This selective presentation of negative charge on the surface is due to demonstration of some anionic molecules on the surface such as phosphatidylserine (Dobrzynska et al., 2004; Utsugi et al., 1991). Sometimes this recognition is potentiated by selective demonstration of O-glycosylated saccharide components (Burdick et al., 1997; Yoon et al., 1997). In addition to selective presentation, cancer cells grow larger than the normal cells with remarkably distorted orientation that renders them more prone to AMPs (Chan et al., 1998; Hoskin and Ramamoorthy, 2008; Schweizer, 2009).

AMPs are also effective against the DNA or RNA viruses by interacting to heparin sulfate that is required for viral entry into cells, for example lactoferricin and rabbit alpha defensin NP-1 (Jenssen et al., 2006; Sinha et al., 2003). Some peptides, such as NK-18 can simultaneously destabilize microbial membrane and interact with microbial genome, ultimately potentiating antimicrobial activity (Yan et al., 2013). AMPs can also interact with multiple targets to block various biological processes and ultimately inhibiting microbial

growth such as DNA, RNA, protein synthesis, and cell wall synthesis (Boman et al., 1993; Zasloff, 1992, 2002).

Chromogranin A derived peptides such as catestatin and chromofungin can destabilize membrane, and bind to plasmepsin and calceneurin in *Plasmodium falciparum* and fungal spp. respectively (Akaddar et al., 2010; Lugardon et al., 2001).

3.5.2 Immune modulation by AMPs

AMPs are usually very potent in exhibition of their direct antimicrobial activity, which is based on their direct contact to pathogen and effective concentration. However, in most cases this concentration not up to mark that can effectively kill overwhelming infectious agents. Moreover, non-physiological condition, such as high salt and saccharides concentration can decrease effectiveness of AMPs (Bowdish and Hancock, 2005; Diamond et al., 2009). Immunomodulatory activities of AMPs can help to overcome this issue. These activities include recruitment of innate immune cells, endotoxin binding, cellular proliferation and activation of adaptive immune cells (Figure 18).

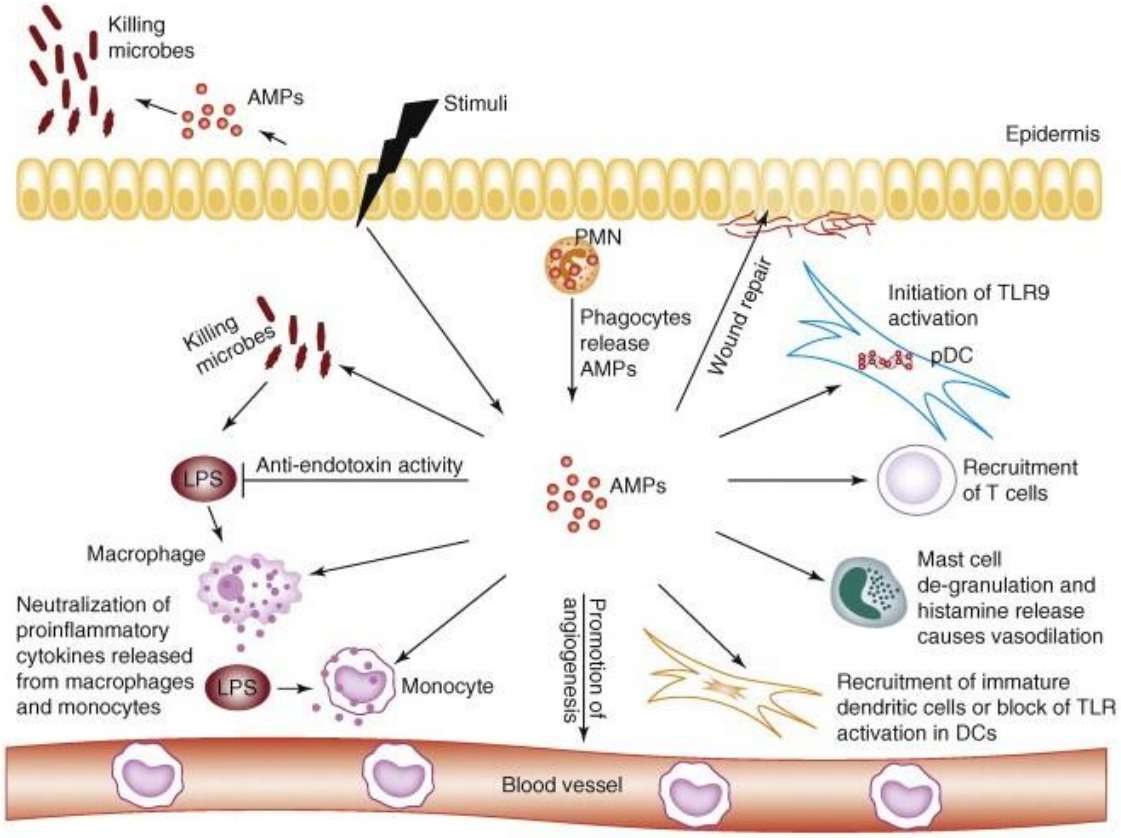

Figure 18: Multiple functions of AMPs in host defense. ***(Lai and Gallo, 2009)***

3.5.2.1 Recruitment of innate immune cells

AMPs are able to trigger selective production of pro-inflammatory and anti-inflammatory cytokines and ultimately leukocyte chemotaxis. For example, LL-37 attracts neutrophils, monocytes, T cells, and mast cells; by production of G-protein coupled receptors and N-formyl peptide like receptors that induce selective degranulation of phagocytes (De et al., 2000; Mookherjee and Hancock, 2007). LL-37 can also act synergistically with IL-1β, to enhance production of proinflammatory cytokines such as IL-6, IL-10, MCP-1, and MCP-3 (Yu et al., 2007). Catestatin, a chromogranin-A derived peptide has been demonstrated to attract neutrophils in vitro (Shooshtarizadeh et al., 2010). Defensins can also interact with MIP-3a receptor present on the inflammatory cells and regulate inflammatory response (Mookherjee and Hancock, 2007). Another AMP, IDR1002 (innate defense regulator) can modulate production of IL-1β by the inflammatory cells (Turner-Brannen et al., 2011).

3.5.2.2 Endotoxin binding

During infection a large amount of endotoxins are produced, which can initiate inflammatory response. Inflammation is beneficial as well as life threatening when exceeded by a certain limit (Mookherjee and Hancock, 2007). AMPs such as LL-37, indolicidin and cecropins, can modulate inflammatory response by binding to endotoxins and protect from endotoxemia (Bowdish and Hancock, 2005; Mookherjee et al., 2006). Furthermore, AMPs can inhibit expression of proinflammatory genes induced by endotoxin, such as LL-37 which can block TNF-alpha production mediated by endotoxins (Bowdish and Hancock, 2005; Mookherjee et al., 2006).

3.5.2.3 AMPs induce cellular proliferation and differentiation

AMPs can also modulate proliferation and differentiation of phagocytic cells, such as neutrophils by inhibiting apoptosis, activating mast cells, wound healing and promoting angiogenesis (Chen et al., 2006; Koczulla et al., 2003; Nagaoka et al., 2006). AMPs, such as LL-37, can also improve expression of CDs by innate immune cells and assist in antigen presentation (Mookherjee and Hancock, 2007; Tokumaru et al., 2005). Cathelicidin and neutrophil β-defensins can activate MAPK-p38 (Mitogen-Activated Protein Kinase) and ERK-1/2 (Extracellular signal-Regulated Kinase) pathways in mast cells, keratinocytes and monocytes (Bowdish et al., 2004; Chen et al., 2006; Niyonsaba et al., 2006). Furthermore,

AMPs can modulate transcription by regulation of STAT-3 (signal transducer and activator of transcription) (Tokumaru et al., 2005).

3.5.2.4 Adaptive immune cells activation

Besides the innate immune response modulation, AMPs can activate adaptive immune cells (Mookherjee and Hancock, 2007). Different AMPs such as human neutrophil defensins, β-defensins, cathelicidin (PR-39 and LL-37) can express CDs receptor and ultimately T and B lymphocyte activation (Chertov et al., 1996; Huang et al., 1997). Furthermore, LL-37 can also modulate dendritic cells differentiation and dendritic cells induced T-cells polarization (Davidson et al., 2004). Defensins can also increase proliferation of T cell induced by IFN-g, IL-10 and IL-6 (Lillard et al., 1999a; Lillard et al., 1999b).

3.6 Antimicrobial peptide avoidance mechanisms adopted by microorganisms

Microorganisms have adopted several mechanisms to disable antimicrobial host defense. Most of them are extra-cellular in origin including production of capsules, production of components that can bind and trap AMPs, degradation of AMPs, neutralization of charge, modification of cell surface proteins, change in membrane fluidity and expulsion of AMPs (Figure 19) (Gruenheid and Le Moual, 2012; Koprivnjak and Peschel, 2011).

Few resistant pathogens such as *S. aureus*, produces several proteins such as staphylokinase and SIC (Streptococcal inhibitor of complement) that can bind and trap the AMPs including α-defensin, CAMP (cathelicidin antimicrobial peptide) (Jin et al., 2004).

Neutralization of charge is carried out either production of capsules or various modifications of surface proteins including D-alanylation of teichoic acid. Most of bacteria produce cationic exopolymer polysaccharide intercellular adhesin (PIA) and anionic poly-c-glutamic acid (PGA), which can modulate surface charge and ultimately resist in expulsion of both cationic and anionic AMPs (Kocianova et al., 2005; Vuong et al., 2004a; Vuong et al., 2004b). Due to protection polysaccharide capsules, both cationic and anionic AMPs are expelled from bacterial surface by both electrostatic and mechanical interaction.

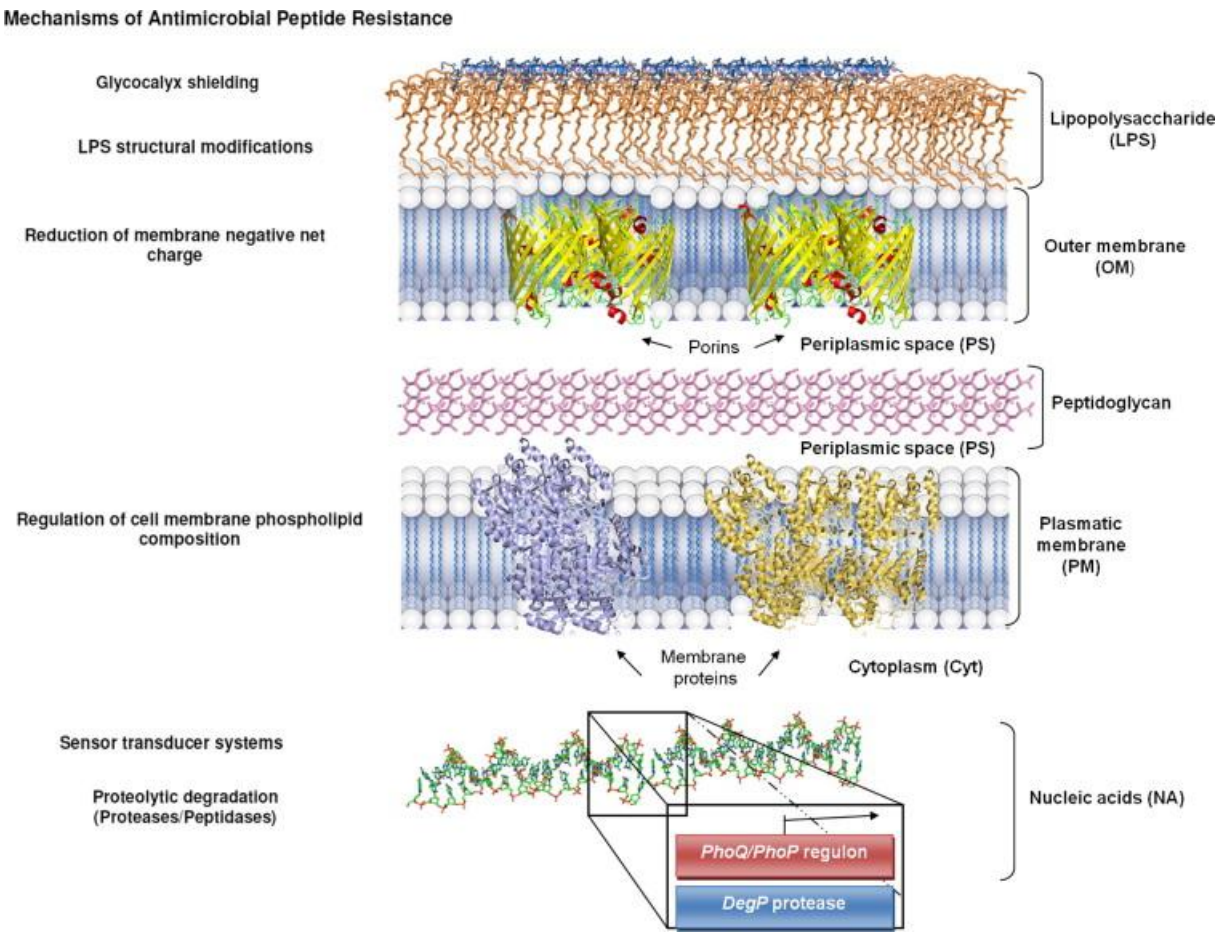

Figure 19: Different mechanism adopted by the pathogens to avoid antimicrobial peptides.

The most common mechanisms of resistance triggered by pathogens to decrease their susceptibility to AMPs are described (Teixeira et al., 2012).

In addition, bacteria such as *Salmonella typhimurium*, which can sense cationic AMPs and can alters Lipid A by acylation, carry out few modifications. This type of modification can ultimately reduce the membrane fluidity and permeability (Guina et al., 2000; Guo et al., 1997; Miller et al., 1990).

Furthermore, AMPs can be degraded by the bacterial proteases, such aureolysin and Glu-C in case of *S. aureus*. AMPs degradation ultimately renders them inactive or can decrease the effectiveness. For example, as previously demonstrated LL-37 is degraded by aureolysin, a metallo-protease produced by *S. aureus* (Sieprawska-Lupa et al., 2004).

GRANINS

4 GRANINS

Granin is a family of acidic proteins that are ubiquitous in various cells and tissues of body. Most common sites include chromaffin cells, endocrine glands, nervous cells, and immune cells (Eiden, 1987; Huttner et al., 1991a; Huttner et al., 1991b). These proteins are acid soluble and mostly heat stable in nature, have diverse biological functions. Members of granin family consist of single-polypeptide chains of about 180-700 residues, bearing a signal peptide at amino terminal. Granins are widely distributed in secretory granules of chromaffin cells (adrenal medulla), which is most abundant reservoir of chromogranins/secretogranins. Before to present different members of granin family and their physiological functions, let's have a look on the brief introduction of the adrenal glands and chromaffin cells.

4.1 Adrenal glands

Adrenal glands are one of the most important paired glands present in mammals. These are triangular in shape and are present on the upper edge of both kidneys. They are located at 12th thoracic vertebra and weigh about 4-10 g. Adrenal glands are composed of two parts: cortex and medulla.

Adrenal cortex is peripheral portion of adrenal gland that represents about 90% of the adrenal gland. Adrenal cortex usually mediates stress response via production of glucocorticoids (cortisol), mineralocorticoids (aldosterone) and androgens. Adrenal medulla is inner part of adrenal gland that is surrounded by adrenal cortex in parametric fashion. Adrenal medulla is originated from the embryonic neural crest and is innervated by the splanchnic nerve. It is reservoir of large number of secretory cells such as chromaffin cells. Chromaffin cell secretions are highly enriched in catecholamines (noradrenaline and adrenaline), dopamine, proteolytic enzymes (prohormone convertases like) (Seidah and Chretien, 1999), morphine and certain proteins like proenkephalin-1, chromogranins and many derived peptides (Goumon et al., 1996; Metz-Boutigue et al., 1998; Taupenot et al., 2003).

4.2 Chromaffin cells

Chromaffin cells are the most important secretory cells of adrenal medulla that are densely packed with secretory granules. These cells have high affinity towards chromium salts, which gives dark coloration to them that is why named as chromaffin cells (Ogata and Ogata, 1917). Preganglionic sympathetic nerves innervate chromaffin cells. These are rich in

secretory granules and provide a research model for analysis of molecular mechanisms of exocytosis (Aunis, 1998).

Secretory granules of chromaffin cells are storage vesicles of catecholamines (Blaschko and Welch, 1953; Hillarp, 1958; Hillarp and Hokfelt, 1953) and many other secretory proteins. Proteomic analysis of the secretory vesicles revealed different stress related components, including catecholamines, granins, cathelicidins (Wegrzyn et al., 2007). Chromaffin secretory granules highly enriched in different chromogranin/secretogranin family members. Recent proteomic analysis revealed eight out of nine members of granin family (Wegrzyn et al., 2007), which constitutes a major portion of chromaffin secretory granules. These secretory granules contain a vast variety of prohormone precursors such as chromogranins, cathepsin L, neurotransmitters and various other proteins categorized as enzymes/proteases (tyrosine-3-hydroxylase, prohormone convertase 1/2, and cystatin C), signal transduction proteins (RAS/Rho proteins and annexins), redox factors (cytochrome C) (Wegrzyn et al., 2007). The proteins present in the secretory granules usually act paracrine or autocrine upon secretion. Granule secretion is achieved by exocytosis or through a mechanism of Kiss and Run (Aunis, 1998; Henkel et al., 2001).

4.3 Granin family

Soluble proteins of secretory granules of chromaffin cells were found in 1958 (Hillarp, 1958). First member of granin family, CgA (chromogranin A) was identified in 1965 that is co-secreted with catecholamine (Banks and Helle, 1965; Blaschko et al., 1967). Later, other proteins have also been discovered and are integrated into granin family. Up-till now, nine members have been discovered (Bartolomucci et al., 2011; Chakraborty et al., 2006; Helle, 2004) that are divided into two groups: chromogranins (Cgs) and secretogranins (Sgs). The Cgs are acidic soluble proteins having multiple di- and tri-basic sites that are potential proteolytic cleavage sites, which can generate several biologicaly active short fragments (Metz-Boutigue et al., 1993). CgA, CgB/SgI and SgII constitute about 40%, 10%, and 2% respectively, of total protein constituents of bovine chromaffin cell secretions (Fischer-Colbrie and Schober, 1987; Wegrzyn et al., 2007). Relative proportion of different Cgs vary with stress condition and also with species: CgA is more dominant in bovine, sheep and horses but CgB is more in human and rat (Fischer-Colbrie and Schober, 1987). Different members of granin family of human origin, are presented in Table VIII with their number of residues, molecular weight and pI index.

Table VIII: Different members (human origin) of the granin family.

(MW: molecular weight, pI: isoelectric point)

Granins	MW	Residues	pI	Reference
Chromogranin A	49	439	4.5	(Helman et al., 1988)
Chromogranin B/Secretogranin I	76	657	5	(Benedum et al., 1987)
Secretogranin II	68	590	4.7	(Fischer-Colbrie et al., 1990)
Secretogranin III (1β1075)	51	449	4.9	(Dopazo et al., 1993)
Secretogranin IV (HISL-19)	35		5.6	(Huttner et al., 1991a)
Secretogranin V (7β2)	21	186	5.5	(Mbikay et al., 2001)
Secretogranin VI (Neuroendocrine secretory protein-55)	28	245	5.3	(Ischia et al., 1997)
Pro-SAAS	24	227	5.61	(Fricker et al., 2000)
Vaccina growth factor (VGF)	70	593	4.72	(Canu et al., 1997)

4.3.1 Biological activities of the granin derived peptides

Granins are produced as pre-proprotein, which include many cleavage sites for various endopeptidases such as serine proteases, plasmin, kallikrein, cathepsin L and prohormone convertases (Biswas et al., 2009; Dillen et al., 1993; Eskeland et al., 1996; Helle, 2004; Metz-Boutigue et al., 1993). These precursor proteins are posttranslationaly modified and short fragment are generated by proteolysis, during storage in secretory vesicles, exocytosis and blood stream. These posttranslational modifications include formation of disulfide bridge, N-glycosylation, O-glycosylation, phosphorylation, sulfation, oxidation, amidation, and C-terminal cyclization to N-terminal fragments. The peptides generated have autocrine effects, paracrine and endocrine depending on type of function (Eiden, 1987; Fischer-Colbrie et al., 1995; Taupenot et al., 2003). Granins derived peptides have been known for their vast array of biological activities in various tissues (Table IX).

These are involved in genesis and exocytosis of secretoty granules in various immune and endocrine cells (Aunis, 2009; Beuret et al., 2004; Huh et al., 2003; Kim et al., 2001). Granins are markers of various diseases such as neuroendocrine tumors (Bartolomucci et al., 2010) and sepsis e.g. SIRS (Systemic Inflammatory Response Syndrome and Sepsis) (Zhang et al., 2009a). Various studies describe expression of granins and their derived peptides in various neurological conditions (Alzheimer's disease, Parkinson's disease and multiple sclerosis) (Willis et al., 2011).

Table IX: Different granin derived peptides and their associated functions.

(Angelone et al., 2010; Helle, 2004, 2010c; Koshimizu et al., 2011; Metz-Boutigue et al., 2003; Metz-Boutigue et al., 2010; Shimizu et al., 2002; Taupenot et al., 2002).

	Peptides	Peptide function
CgA	Vasostatin I, II	Antifungal, Inhibits parathormone secretion, Vasodilatory, Cell adhesion promoter
	bCgA1-40	Inhibit parathyroid secretions and vasoconstriction, increase calcitonin expression
	CgA_{4-16}	Pain modulator, inhibit intestinal motility
	Chromofungin	Antifungal, pain modulator
	$rCgA_{1-64}$	Negative inotropic
	Prochromacin	Antibacterial and antifungal
	Chromacin I, II	Antibacterial and antifungal
	Pancreastatin	Inhibit insulin secretion, increase glycolysis, chemotaxis for monocytes, decrease gastric secretions
	Catestatin	Inhibit catecholamine, antibacterial, antifungal, antiplasmodial, attract monocytes
	Parastatin	Inhibition of parathyroid hormones
	Serpinine	Anti-apoptotic in neurons, regulate granules secretions
	Betagranin	----------
	WE-14	----------
CgB/SgII	CgB1–41	Inhibits bovine PTH secretion at low plasma Ca^{2+}
	BAM	----------
	GAWK	----------
	PE-11	----------
	CCB	----------
	Secretolytin	Antibacterial
	Chrombacin	Antibacterial
	$CgB_{564-626}$	Antibacterial
	$CgB_{614-626}$	----------
SgII	Secretoneurin	Stimulates release of neurotransmitters and dopamine, chemotaxis, migration of endothelial and monocytes.
SgIII	SgIII214–373	Associated with CgB in granule membrane, sorting partner for CgA to regulated pathway, binds to CgA48–111
SgIV	HISL-19	----------
SgV	7β2 C-terminal	Involved in maturation of proPC2 during intracellular transport, Inhibit PC2 activity.
SgVI	$SgIV_{159-162}$	5-HT1β receptor antagonist
SgVII	VGF	Energy homeostasis
	VGF20	
	VGF10	
ProSAAS	ProSAAS	Inhibit PC1

In addition to neurological conditions, granins are also involved in various infectious and metabolic conditions such as diabetic retinopathy, hepatocellular tumor, heart diseases (Ceconi et al., 2002; Corti et al., 2000; Fournier et al., 2010; Helle, 2010c; Massironi et al., 2009; Pieroni et al., 2007; Sciola et al., 2009; Tota et al., 2010). CgB is also involved in various conditions such as heart diseases, prion diseases, β-amyloid plaque and Creutzfeldt-Jacob disease (Rangon et al., 2003; Rosjo et al., 2010). A brief list of the various granin derived peptides and their biological functions have been described in table above (Table IX).

4.3.2 Chromogranin A

CgA is produced as a pre-proprotein by the various cells including chromaffin cells entero-chromaffin cells, certain neurons and neutrophils, having an isoelectric point of about 4.5. Human and bovine CgA contain 439 and 431 amino acid residues. The mature CgA has approximately 49 kDa molecular weight but apparently, it is of 70 kDa when estimated by SDS-PAGE. This retardation of SDS-gel is due to presence of certain posttranslational modification and large number of acidic residues (Gadroy et al., 1998; Metz-Boutigue et al., 1993).

Seven post-translational modification sites have been described for bovine CgA (Figure 20) including five phosphorylations and two O-glycosylations sites (Strub et al., 1997). Phosphorylation sites are located on Ser-81, Tyr-173, Ser-307, Ser-372, Ser-376 residues while O-glycosylation sites are located on Ser-186 and Thr-231(Figure 20) (Strub et al., 1997). In addition, a unique di-sulfide bridge is also present that is located betwen Cys-17 and Cys-38 residues, which is part of vasotatin I and vasostatin II fragments (Blois et al., 2006). Apart from these posttranslational modifications several dibasic residues are also present which are source for generation of several natural fragments (Figure 20) (Metz-Boutigue et al., 1993).

Similar to bCgA, human CgA also possesses several post-translational modifications (Figure 21). These modifications include three O-glycosylation sites (Thr-163, Thr-165 and Thr-223) (Gadroy et al., 1998). Phosphorylation sites vary with origin, as CgA isolated from urine of cancer patients have 3 phosphorylation sites (Ser-200, Ser-252 and Ser-315) (Gadroy et al., 1998), while CgA isolated from pituitary contains 7 phosphorylation sites that includes Ser-94, Ser-118, Ser-124, Ser-185, Ser-282, Ser-304 and Ser-384) (Beranova-Giorgianni et al., 2006; Giorgianni et al., 2004). In addition, two amidation sites (Ser-283 and Ser-420) and a di-sulfide bridge is also present that is located between Cys-17 and Cys-38 (Gadroy et al.,

1998; Metz-Boutigue et al., 1993). Furthermore, several dibasic cleavage sites are also located that lead to generation of natural fragments (Figure 21).

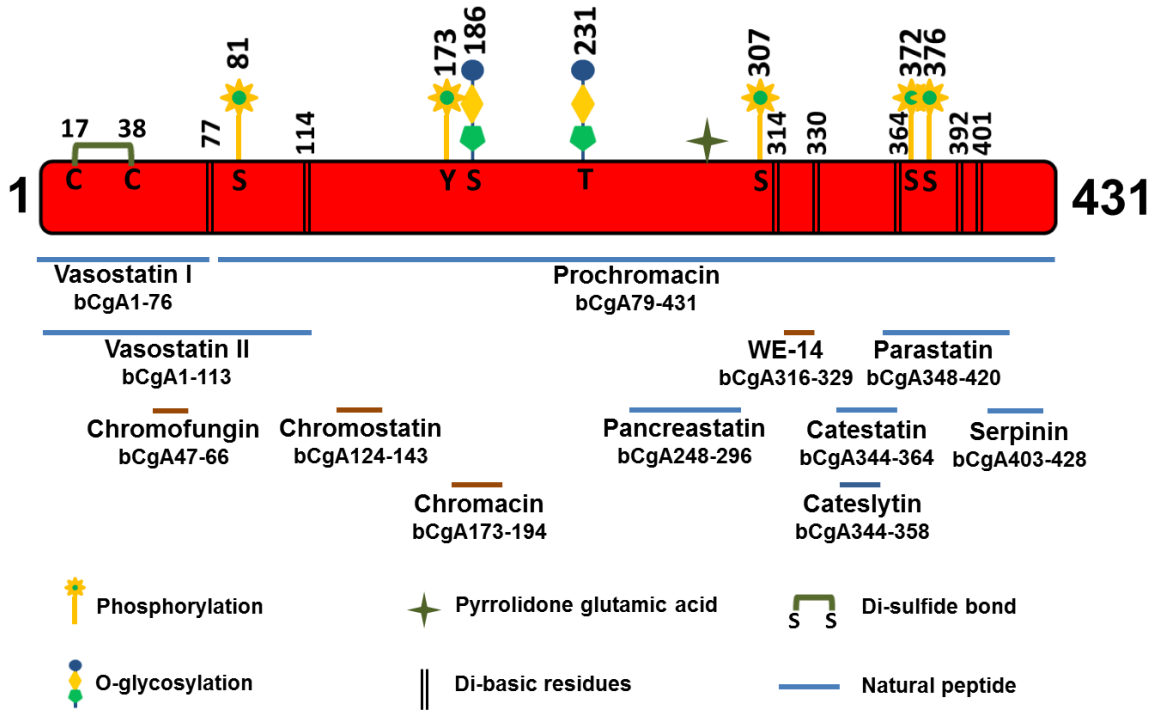

Figure 20: Presentation of mature bovine CgA.

Several dibasic cleavage sites and posttranslational modifications are indicated that include phosphorylation, O-glycosylation and di-sulfide bridge. In addition, several active fragments are also indicated.

4.3.2.1 Biological activities of the CgA derived peptides

Chromogranin A is the most important member of granin family that is widely studied in relation to various diseases. It is stored in secretory vesicles of endocrine, immune and nervous cells (Helle, 2004). Natural processing of CgA is well described in granules of endocrine cells and sympathoadrenal medullary chromaffin cells. In secretory granules of these cells, CgA is cleaved by several protease including prohormone convertases (PC1/3 and PC2), prohormone thiol proteases (PTP) and carboxypeptidase E (CPE), resulting peptides are cosecreted with catecholamines (Metz-Boutigue et al., 1993; Strub et al., 1997). In late 80's, the discovery of pancreastatin, a CgA-derived peptide inhibiting insulin secretion from pancreatic β-cells, initiated the concept of prohormone (Eiden, 1987; Tatemoto et al., 1986). In vivo studies reported numerous peptides produced by CgA, these peptides modulate homeostatic processes (Helle et al., 2007), but are also considered as defense molecules.

During last two decades, several new antimicrobial CgA-derived peptides have been characterized that participate actively against the combat with infectious agents (Helle et al., 2007; Lugardon et al., 2000; Metz-Boutigue et al., 1998; Strub et al., 1996a; Strub et al., 1996b) (Figure 21: Table IX).

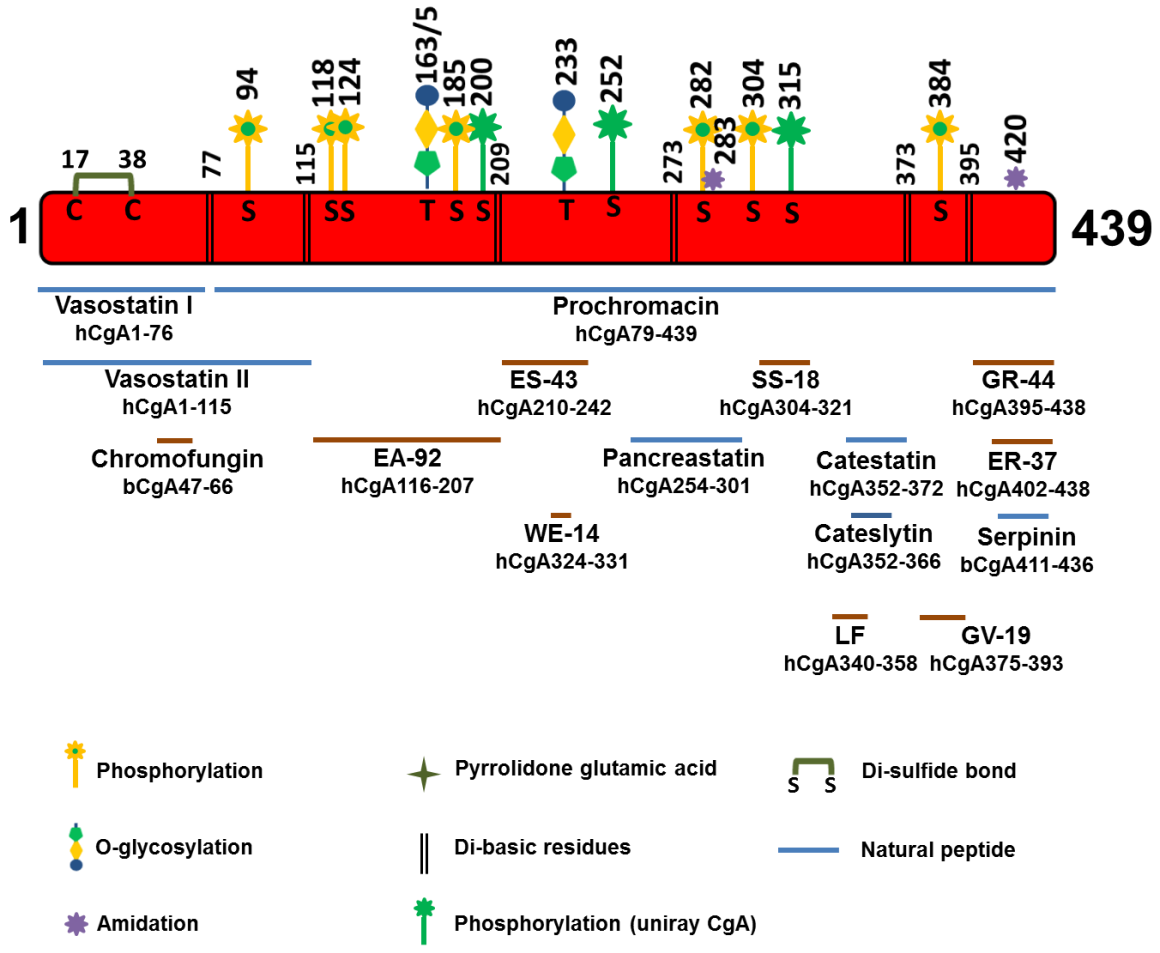

Figure 21: Presentation of mature human CgA.

Several dibasic cleavage sites and posttranslational modifications are indicated that include phosphorylation, O-glycosylation and di-sulfide bridge. In addition, several active fragments are also indicated.

Sequences of CgA-derived peptides are highly conserved during evolution. Interestingly, main cleavage site in bovine CgA (bCgA) at position 78-79 and subsequently removal of the two basic residues K77 and K78 by carboxypeptidase H (Metz-Boutigue et al., 1993), produces two major fragments: vasostatin-I (VS-I; bCgA1-76) (Lugardon et al., 2000) and prochromacin (ProChrom; bCgA79-431) (Strub et al., 1996a). In addition, another N-terminal fragment called vasostatin II (VS-II; bCgA1-113) is also generated that possesses similar activities as VS-1(Helle et al., 1993). Synthetic vasostatin and its shorter fragment

bCgA16-40 can actively inhibit PTH release from bovine gland (Angeletti et al., 1996). Moreover, these present antimicrobial activities against filamentous fungi, yeasts and Gram-positive bacteria in micromolar range from 0.1-2 µM (Lugardon et al., 2000) (Table X).

Table X: Antimicrobial peptides derived from CgA.

Peptides	Bacteria (G+)	Bacteria (G-)	Fungi	Yeast	Parasites	Reference
Vasostatine I (bCGA1−76)	X		X	X		(Lugardon et al., 2000)
Chromofungin (bCGA47-66)			X			(Lugardon et al., 2001)
Prochromacin (bCGA79−431)	X	X				(Strub et al., 1996a)
Chromacin (bCGA173−186)	X					(Strub et al., 1996a)
Catestatin (bCGA344−364)	X	X	X	X	X	(Briolat et al., 2005)

Furthermore, it has been previously demonstrated that the N-terminal bCgA1-40 fragment can interact with a model of phospholipid membranes (Blois et al., 2006; Maget-Dana et al., 2002; Shooshtarizadeh et al., 2010). Another important peptide derived from vasostatin region of CgA is chromofungin (CHR; CgA47-66) that is not naturally occurring but generated by bacterial proteases such as Glu-C from *S. aureus*. CHR belongs to CgA domain that is well conserved through evolution. CHR presents antifungal activity at 2-15 µM concentration against yeasts (*Candida albicans, Candida tropicalis,* and *Candida neoformans*) and filamentous fungi (*Fusarium culmorum, Fusarium oxysporum, Aspergillus fumigatus, Neurospora crassa, Alternaria brassicola,* and *Nectria haematococca*) (Lugardon et al., 2001).

Similarly, prochromacin also generates several fragments including chromacin (Chrom; bCgA173-194), catestatin (CAT; bCgA344-364), cateslytin (CTL; bCgA344-358), pancreastatin (bCgA248-296), parastatin (bCgA348-420), chromastatin (bCgA124-143), and WE-14 (bCgA216-229) (Helle, 2004, 2010a, b, c; Shooshtarizadeh et al., 2010; Strub et al., 1996b). These C-terminal derived peptides also display several biological activities; few of them are summarized in Table IX. Most important multifunctional C-terminal derived peptides are cateslytin and catestatin that are described in detail below.

4.3.2.1.1 Catestatin

Catestatin (CAT) is short CgA derived fragment, which is produced by cleavage of prochromacin region of CgA (bovine and human CAT CgA344-364 and CgA352-372 respectively) (Table XI). CAT was first identified as non-competitive nicotinic cholinergic antagonist in chromaffin cells in 1997 (Mahata et al., 1997). Later studies revealed that CAT is produced in chromaffin granules by action of prohormone thiol protease (PTP), which was demonstrated to act on D343-R344 and L364-R365 (Lee et al., 2003). This cleavage leads to liberation of CAT, a further cleavage is also been demonstrated on the motif R-G of CAT that liberate cateslytin (CTL), the short active form of CAT (Biswas et al., 2009). Three human variants were identified: Gly364Ser, Pro370Leu and Arg374Gln. These varients have same properties as wild type CAT but their activity varies and their order of activity to inhibit nicotinic process is as follows Pro370Leu > wild type > Gly364Ser > Arg374Gln (Mahata et al., 2004).

CAT represents various biological activities: it can regulate blood pressure, is sympatho-inhibitor and attenuates sympathetic barosensitivity, and serves as an endogenous sympathetic nervous system regulator (Gaede and Pilowsky, 2012; Rao et al., 2007; Schillaci et al., 2011). Plasma concentration of CAT in hypertension patients is increased (Meng et al., 2011), which underlines its role as vasodilator (Angelone et al., 2011; Kennedy et al., 1998; Liao et al., 2011). Few contradictory results are also published, which indicate reduced plasma expression in hypertension patient than healthy persons (O'Connor et al., 2002). Despites all, CAT serves as heart protector by inhibiting adrenergic release, regulating blood pressure and as vasodilator (Bassino et al., 2011; Liao et al., 2011). It also serves as marker for various diseases such as heart failure, myocardial infarction, and carcinoid tumors of intestine (Conlon, 2010; Ji et al., 2012; Meng et al., 2012; Prommegger et al., 2003). CAT is also involved in mobilization of fat from adipose tissue through regulation of adrenergic and leptin signaling (Bandyopadhyay et al., 2012). Furthermore, CAT also serves as angiogenic cytokine by promoting fibroblast growth via a basic fibroblast growth factor dependent mechanism (Theurl et al., 2010).

In addition, CAT is very potent AMPs against bacteria, fungi and yeasts (Akaddar et al., 2010; Briolat et al., 2005; Metz-Boutigue et al., 2010; Shooshtarizadeh et al., 2010). CAT can effectively inhibit various fungal, parasite and bacterial growth in micromolar range. It interacts with plasmepsin and ultimately inhibits growth of plasmodium species (Akaddar et al., 2010). Antimicrobial properties of CAT are due to asymmetric structure adopted in

hydrophobic solvents. It is mostly unstructured but readily adopts β-strand-loop-β-strand conformation when it is in contact with lipid membrane. A short loop is formed between residue 7 and 11 of CAT. Three arginine residues adopt a conformation of positive side chain that facilitates its interaction with membrane (Sugawara et al., 2010; Tsigelny et al., 1998). This model is well explained for catecholamine release inhibition and is assumed to interact similarly against pathogens.

In addition to direct antimicrobial activities, it also acts as immune modulator peptide. Catestatin acts as chemoattractant for the monocytes (Egger et al., 2008), and as angiogenic cytokine for fibroblast (Theurl et al., 2010). These studies suggest its activity in immune modulation by acting as inflammatory cytokine. Furthermore, it can trigger production of IL-8 by keratinocytes via activation of mitogen-activated protein kinases (Aung et al., 2011a) and stimulates mast cells degranulation to release histamine that further initiates inflammatory cascade (Aung et al., 2011b; Kruger et al., 2003). Catestatin induce influx of calcium in neutrophils by calmodulin-regulated calcium independent phospholipase A2 (Zhang et al., 2009b). It also contributes in wound healing by increasing migration and proliferation of keratinocytes at wound site (Hoq et al., 2011).

4.3.2.1.2 Cateslytin

Cateslytin (bCTL; bCgA 344-358) is the short active form of CAT (Table XI). Endogenous production of CAT is previously described, which is further processed by an additional cleavage R358-G359 of catestatin by cysteine protease cathepsin L (CTSL) in chromaffin secretory vesicles (Lee et al., 2003). Cateslytin interacts with fungal membrane, by adopting an aggregated antiparallel beta-sheet structure at membrane interfaces (Jean-Francois et al., 2009). This mechanism lead to separation of fluid and rigid membrane structures on ergosterol (fungal membrane) containing models and ultimately destabilization of membrane (Jean-Francois et al., 2009). *In vitro*, studies by using zwitterionic biomembranes, CTL was demonstrated to form beta-sheets and generates pores of 1 nm diameter (Jean-Francois et al., 2008b). By using NMR studies CTL was demonstrated to be converted into antiparallel beta-sheets that aggregate at negatively charged surface of bacterial membranes (Jean-Francois et al., 2008a; Jean-Francois et al., 2007). Arginine residues are known to be important in binding to negatively charged lipids. Following the interaction of CTL to membrane, several rigid and thicker membrane domains were observed (Jean-Francois et al., 2008a).

Table XI: Sequence presentation of Catestatin and cateslytin.

Sequence the catestatin domain is well conserved through evolution. Here in this table sequence of the catestatin and cateslytin from different species and their locations are presented. (b: bovine, h: human, hr: horse, r: rat, m: mouse, mac: macaque, d: dog.)

Peptide	Location	Sequence
Catstatin	bCgA 344-364	RSMRLSFRARGYGFRGPGLQL
	hCgA 352-372	SSMKLSFRARAYGFRGPGPQL
	hrCgA 343-363	RSMKLSFRARAYGFRGPGLQL
	rCgA 363-383	RSMKLSFRARAYGFRDPGPQL
	mCgA 364-384	RSMKLSFRTRAYGFRDPGPQL
	macCgA 353-373	RSMKLSFRARAYGFRGPGPQL
	dCgA 344-364	RSMKLSFRARAYDFRGPGLPL
Cateslytin	bCgA 344-358	RSMRLSFRARGYGFR
	hCgA 352-366	SSMKLSFRARAYGFR
	hrCgA 343-357	RSMKLSFRARAYGFR
	rCgA 363-377	RSMKLSFRARAYGFR
	mCgA 364-378	RSMKLSFRTRAYGFR
	macCgA 353-367	RSMKLSFRARAYGFR
	dCgA 344-358	RSMKLSFRARAYDFR

CTL is very potent AMP that is known to inhibit bacterial, fungal, and yeast growth at micromolar concentrations. Moreover, it is non-toxic to mammalian cells (Briolat et al., 2005). Enhanced activity of CTL as compared to other Cgs derived peptides is attributed due to its small size (15 residues) and net charge of +5, which provides better interaction with negatively charged bacterial membrane. Moreover, high arginine ratio can also account for deeper penetration into microbial membrane.

4.3.3 Chromogranin B

CgB is the second member of granin family that has 657 and 626 residues and 5.02 and 5.19 pI in human and bovine CgB respectively. The difference of theoretical molecular weight (76 kDa) and estimated molecular weight (100 kDa) by SDS-PAGE is due to the post-translational modifications and abundance of acidic residues (Gasnier et al., 2004). Post

translational modifications include two O-glycosylations (T93 and T170), one N-glycosylation (N168), two sulfatations (Y138 and Y295), three phosphorylations (S564, S573 and S578) and two pyrolidone glutamic acid residues (Q456 and Q547) (Figure 22) (Gasnier et al., 2004). In addition, a disulfide bridge is also present at the N-terminal of CgB that is essential for CgB interaction with membrane and also mediates membrane binding in the TGN (Trans-Golgi-Network) (Glombik et al., 1999).

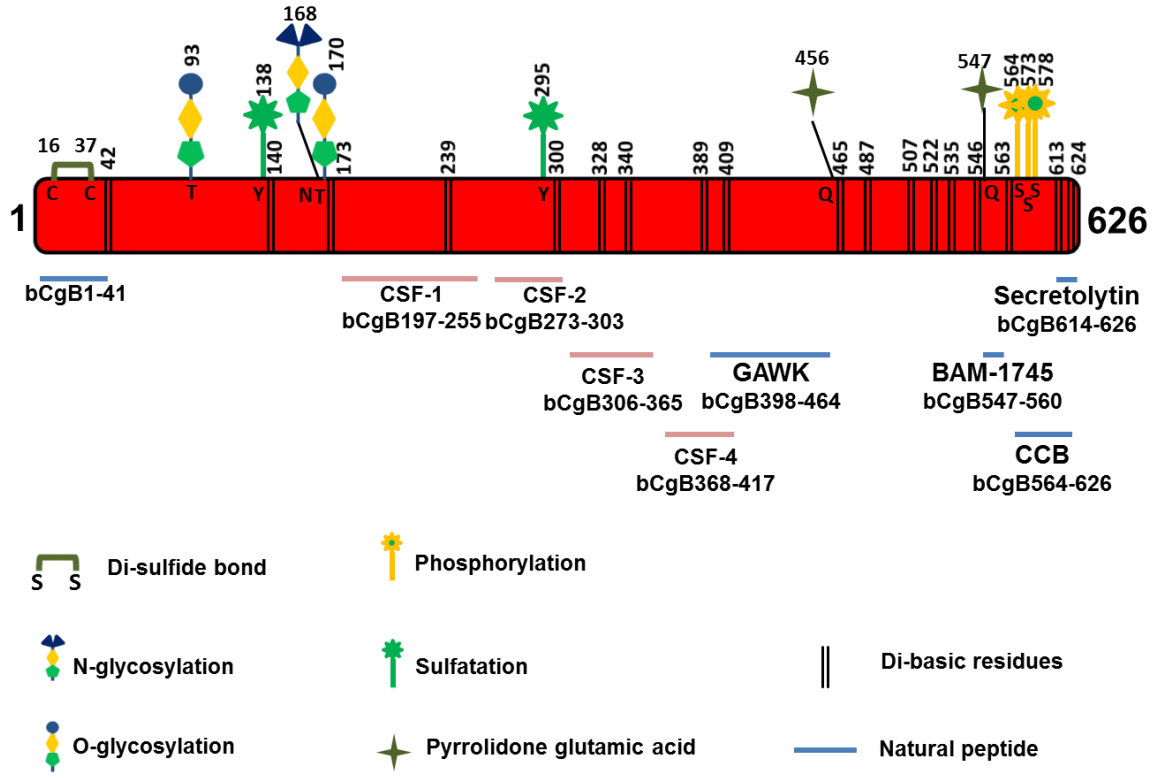

Figure 22: Presentation of mature bovine CgB.

Several dibasic cleavage sites and posttranslational modifications are indicated that include phosphorylation, O-glycosylation, N-glycosylation, sulfatation and disulfide bridge. In addition, several active fragments are also indicated.

CgB was first characterized from rat PC12 cells as a tyrosine-sulphated protein and later named chromogranin B when it was detected as a conspicuous component of bovine adrenal medulla (Eiden et al., 1987; Falkensammer et al., 1985; Lee and Huttner, 1983). Several CgB derived peptides have been identified in various tissues, including extracts of human pituitary glands, pheochromocytomas of human, brain extracts, splanchnic nerve in

pigs, and cephalo-rachidian fluid samples (Benjannet et al., 1985; Conlon et al., 1992; Depreitere et al., 2002; Depreitere et al., 2004; Marksteiner et al., 1999; Stark et al., 2001). Likewise, CgA derived peptides, CgB and its derived peptides also denote various biological activities, such as N-terminal peptide CgB1-41 that inhibits PTH secretion in bovine (Helle, 2004). Similarly, N-terminal domain (CgB1-49) has been implicated in interaction with membrane vesicles (Yoo and Kang, 1997).

C-terminal peptide (bCgB614-626) was identified in 1995 in secretions of stimulated chromaffin cells (Strub et al., 1996b). This C-terminal peptide is well known for its antibacterial activities, inhibiting growth of various bacteria at micro molar range (Strub et al., 1996b). Another C-terminal peptide CgB564-626, also presents antimicrobial activities (Strub et al., 1995), this peptide is homologous to N-terminal domain of cecropin, a well-known insect AMP. CgB derived AMPs are listed in the Table XII.

Table XII: Antimicrobial peptides derived from CgA.

Peptides	Bacteria (G+)	Bacteria (G-)	Fungi	Yeast	Reference
Secretolytin ($bCgB_{614–626}$)	**X**		**X**		(Strub et al., 1996b)
CCB ($bCgB_{564-626}$)	**X**				(Metz-Boutigue et al., 1998)

Several other CgB derived peptides have been isolated but still their biological activities are not very clear such as GAWK, BAM-1745, SR-17, HQ-34 and PE-11 (Benjannet et al., 1985; Flanagan et al., 1990; Laslop et al., 1998; Wang et al., 2001). CgB have also been detected in vagus nerves of human fetus, where may be playing important role in fetal developmental organization (Bitsche et al., 2006).

AMP CONJUGATED POLYELECTROLYTE FILMS

5 AMPS CONJUGATED POLYELECTROLYTE FILMS

5.1 Introduction to polyelectrolyte complexes

Polyelectrolytes have emerged as tools to study the complexity of the polymers and to develop a multilayer polyelectrolyte base. In this chapter: the evolution and the use of charged polymers to complex formation and development of multilayer by dipping or spraying methods will be discussed briefly. Finally, we focus on the comparison polyelectrolyte complex films and their potential applications in the field of biomaterials. Polyelectrolytes are polymers whose repeating unit is electrostatically charged. They may be synthetic, such as poly-allylamine hydrochloride (PAH) or poly-sodium styrene sulfonate (PSS) or natural polysaccharides such as DNA. The polyelectrolyte complex (PEC) is formed from the mixture of solutions of polyanion and polycation. They can be in the form of soluble complexes (Kabanov, 1994; Tsuchida and Abe, 1982), of liquid coacervates (Burke et al., 2007) or solid precipitates (Michaels and Miekka, 1961).

5.2 Application of polyelectrolyte complexes in the biomedical field

PECs are widely used in the field of biomedical, these present very diverse applications. These are widely used in various studies for example, transfection of DNA for which nanoparticles of chitosan are used (Duceppe and Tabrizian, 2009; Erbacher et al., 1998). The PECs are also used for the encapsulation such as targeted or non-targeted release of insulin for the oral treatment of diabetes, for asthma treatment, for targeted anticancer therapies (Damgé, 1991; He et al., 2009; Jain and Jain, 2008; Oyarzun-Ampuero et al., 2009; Yin et al., 2009) (Jain and Jain 2008; Choi et al., 2009; He et al. 2009).

5.3 Polyelectrolyte multilayered films

The material surface is the preferred interaction site between it and its environment. To control these interactions, it is necessary to control the properties of this interface. To control cell-material interaction is one of the most crucial aspects in the field of biomaterials. This is made possible by the deposition of thin films with controlled surface properties. The polyelectrolyte multilayers, thanks to their ease of implementation as well as the wide range of available compounds, constitute an appropriate method of functionalization of biomaterials by surface modification.

5.3.1 History of multilayered films

Two types of coating materials exist: inorganic and organic materials. Organic material films were initially started to construct in the early twentieth century by Langmuir and Blodgett, commonly known as "Langmuir-Blodgett films" (LB). These films are constructed by applying a monolayer of surfactants or amphiphilic molecules that are transferred from the air/water interface of a solution on the vessel substrate by dipping (Blodgett, 1934; Blodgett and Langmuir, 1937). The adsorption of the film is based on the hydrophilic / hydrophobic type of interactions with the substrate. A similar second immersion is carried out for the adsorption of a second layer. This method can be repeated several times varying species each deposition step. The thickness of the films ranges from a few angstroms to several nanometers. This method has many disadvantages such as instability of films, a limitation related to the shape of the substrate and the presence of parasitic reactions between the uncontrolled and the solvent molecules in the film.

Another method of preparation of multilayer films was proposed in 1966 by Iler (Iler, 1966). It is based on electrostatic interactions between the anionic and cationic colloidal particles for the construction of multilayer films. Later in 1980, Fromherz is the first to mention the application of this concept to the adsorption of proteins and oppositely charged polyelectrolytes (Fromherz, 1980; Gölander et al., 1982). Each deposit must be accompanied by a change of surface charge, by overcompensation of charge (Gölander et al., 1982).

Later in 1980s, other self-assembly methods have been developed to form organic films based on the systems of coordination chemistry of metal/phosphonate and covalent chemistry (Guang et al., 1992; Netzer and Sagiv, 1983). The first consists in the self-assembly of molecules, composed of a head and a tail phosphonate hydrophobic carbon, by fixing heads phosphonate on a metal substrate by coordination chemistry and hydrophobic interaction. These processes, however, are limited to a number of chemical systems and do not provide reproducibly homogeneous films.

In the early 1990s, with the adaptation of work to Iler polyelectrolytes, Decher have really exploited the capabilities of the method of layer-by-layer (LbL) assembly (Decher, 1997; Decher and Hong, 1991; Helm et al., 1992). This technique is based on the adsorption of polycations and polyanions alternatively, each deposition step being followed by a rinsing step with a buffer solution or water. Films made by this method are called PEMs (polyelectrolyte multilayers).

5.3.2 Methods to develop polyelectrolyte multilayer films

This method was well established in the 1990s, the layer by layer deposition is shown schematically in Figure 23. The successive deposition of polyelectrolytes are made on a substrate, usually negatively charged in contact with a solution of polycation followed by a rinsing step and then a solution of polyanion also followed by a rinsing step for a given time (5 to 20 min). Putting in contact with the polyelectrolyte solution allows the adsorption of the compounds in solution (Advincula et al., 1996; Hoogeveen et al., 1996; Ramsden et al., 1995), the surplus or unbound compounds are removed by rinsing step (Caruso et al., 1998; Lvov et al., 1999). At the end of the adsorption step and rinsing excessive polycations, the surface is positively charged: there is overcompensation of charge (Caruso et al., 1998; Ladam et al., 1999). This overcompensation load has been demonstrated by zeta potential measurements (Hoogeveen et al., 1996; Ladam et al., 1999). It is the driving force behind the construction of polyelectrolyte multilayer films.

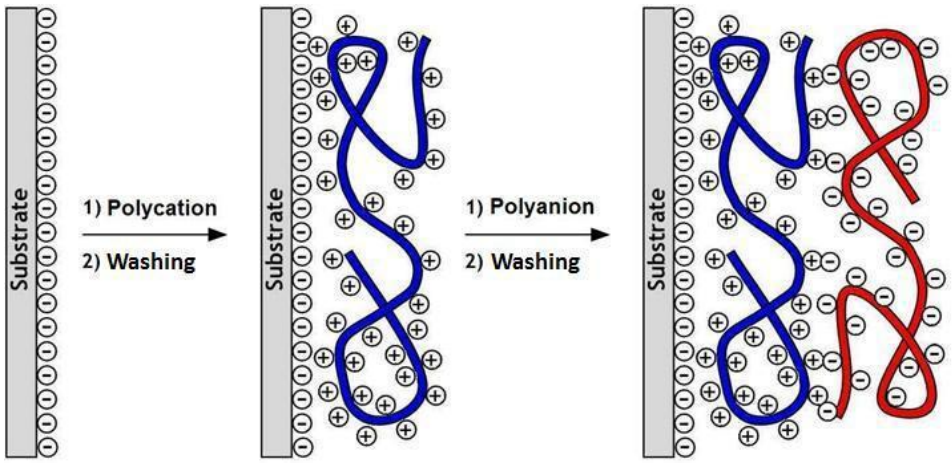

Figure 23: Schematic construction of multilayer polyelectrolyte adsorptions by successive polycations and polyanions. ***(Decher, 1997; Decher and Hong, 1991)***

Newly positively charged surface, can be then exposed to a solution of polyanions to be electrostatically adsorbed. Further rinsing allows the elimination of the surplus of polyanions. The surface is negatively charged. A pair of layers is formed finally. Consecutive cycles, alternating adsorption of polycations and polyanions, thus allow the growth stages of the multilayer film.

5.3.2.1 The soaking method (dipping method)

This simple technique allows coating a substrate, whatever its form. The substrate is soaked alternately in a solution of polycations and polyanions, each soaking being followed by a rinsing step (Decher, 1997; Decher and Hong, 1991) (Figure 24). The soak time in the polyelectrolyte solutions should permit adsorption of the compounds and is between five and twenty minutes. This is a slow depositing method, but consumes less product however since the polyelectrolyte solution can be used for the deposition of several layers. The characteristics of the resulting films such as thickness and roughness depend on parameters of construction: immersion time, concentration of polyelectrolytes, ionic strength, pH and temperature. A method derived from this technique is used in some devices such as the quartz crystal microbalance and optical waveguide spectroscopy.

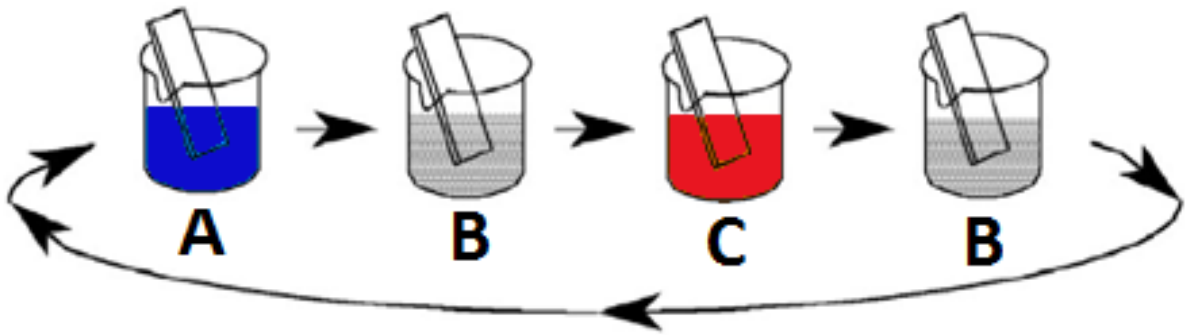

Figure 24: Representation of the soaking/dipping method.

A: polycation solution, B: flushing solution, C: polyanion solution polyanion. (Decher and Hong, 1991)

5.3.2.2 The centrifugation method (Spin coating method)

Later in the 2000s, a new method was adopted to overcome the time consuming previous method, called spin coating method. This technique was previously, widely used to deposit photoresistant resins in the development of integrated circuits in the semiconductor industry (Peter Bakos and John Rasile, 1977). Later, Lee and Hong modified this method to use in the field of polyelectrolyte multilayers (Lee et al., 2001). Under the effect of centrifugal force, the polyelectrolyte solutions are deposited on a rotating substrate. Excess solvent is then evaporated. The method continues with the solution of polyelectrolytes of opposite charge to form the second layer and so on (Figure 25). No rinsing step is required in this technique.

With a spin coating, the time required for the construction of a polyelectrolyte bilayer is more than about 80 seconds. The thickness and surface roughness of the films depends on the rotational speed, the viscosity of the solution and the volatility of the solvent in addition to the parameters already mentioned in the case of dipping. This method allows to obtain films with better mechanical strength than the films obtained by dipping (Jiang et al., 2004).

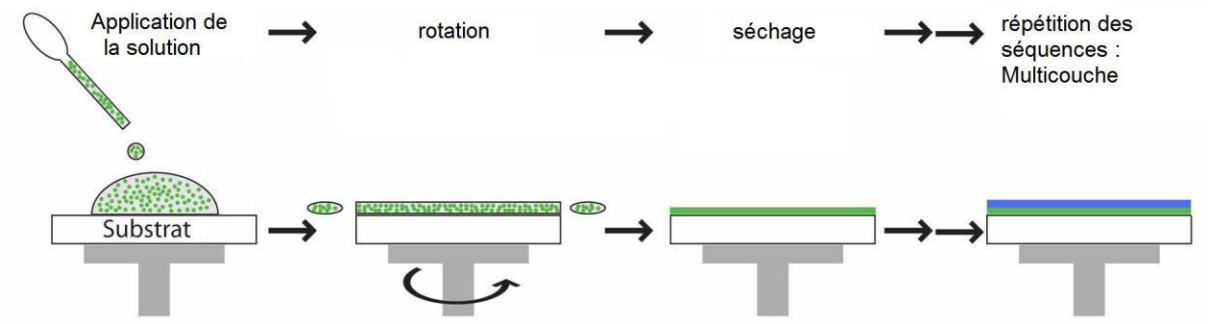

Figure 25: Representation of PEMs film construction by spin coating method.

5.3.2.3 Spray coating method

Another coating method was developed to build PEM films, known as spraying or assisted spray-coating method. The multilayer construction is done by spraying solutions of polyelectrolytes on a vertical or slightly inclined substrate, allowing the drainage of excess solution (Schlenoff et al., 2000). A step of spraying polyelectrolyte and then a rinsing step followed (Figure 26). When spraying, forming a thin film of liquid on the substrate is observed. The main disadvantage of this method is the high consumption solution. In fact, the majority of spray solutions is lost by drainage and is not reusable thereafter. Roughness and thickness of the films obtained are comparable to those of the films obtained by dipping. The flow of solutions sprayed, the spraying distance, solution concentrations, ionic strength, pH and temperature are the parameters that influence construction of the film. Sometimes rinsing step is deliberately removed to increase the thickness and roughness of the films (Izquierdo et al., 2005). Now a day, this technique is widely used for the surface treatment of contact lenses to make them hydrophilic.

Another modification of this technique is also used that uses simultaneous deposition of polyelectrolytes rather than alternatively spraying (Porcel et al., 2005).

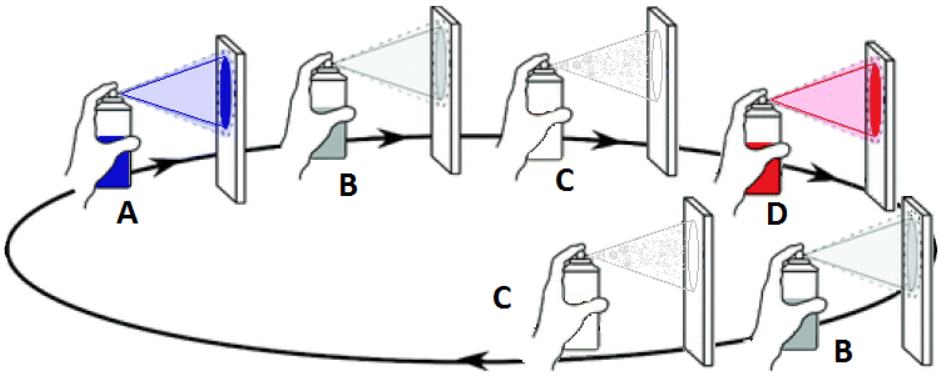

Figure 26: Representation of the spraying method.

A: polycations solution, B: rinsing solution, C: drying with air or nitrogen (2 bars), D: solution polyanions.

5.4 Application of polyelectrolyte multilayered films in the field of biomaterials

Biomaterial science has emerged as to overcome the health problems by replacing the missing organs or administrating body fluid. Most of the time, these equipment counter various problems including tissue rejection, toxicity, and infection. Research on biomaterials has been developed to preserve the integrity and comfort of life of people with functional impairment of an organ or tissue (Langer and Vacanti, 1993). Some of the applications of PEM films are briefly described here.

5.4.1 Biomaterials

Biomaterials are non-living materials used in a medical device, and designed to interact with biological systems, they participate in the formation of a device for diagnosis or to that of a replacement tissue or organ or to that of a replacement device (or assistance) function.

Biomaterials for medical devices are single-use prostheses. These biomaterials can be synthetic (metal alloy, ceramics, plastics) or of natural origin such as collagen or cellulose. Upon implantation of a biomaterial, recognition by proteins or the cells of the host is the first interaction that they encounter. It is therefore should be the first priority to control the adhesion between biomaterial and tissue components to avoid inflammatory or allergic reaction. To control the bacterial infections or a phenomenon of thrombosis during implantation of a foreign body should also be the priority in developing the biomaterial. Due

to these secondary infections, most of the time removal of implant becomes unavoidable. The new challenge in this area is the development of materials with mechanical and structural properties are adapted to their future application but also interact actively with the surrounding living environment. The control response of the material is sought to enable minimize reactions of the body to this foreign element and modulate the activation of immune cells (Ratner, 1996; Ratner and Bryant, 2004). These materials are called "bioactive" interact with cells and induce a specific response of the surrounding tissue.

To limit the discharge phenomena, biomaterials must be biocompatible. Biocompatibility is defined as the ability of the material to be accepted by the body. Recently, research has turned to the development of biodegradable compounds when their presence is required for a given time. Synthetic absorbable suture threads such as Vicryl® or Dexon®, bioresorbable stents, biodegradable membranes for wound healing, sponges to regenerate the bone tissue are some of classical examples that are extensively used (Freed et al., 1993; Ishaug et al., 1997; Jürgens et al., 2006; Zilberman and Eberhart, 2006).

5.4.2 Cell adhesion to the PEM films

Multilayer films can be used as coatings for biomaterials to control of the response of the medium surrounding the implanted biomaterial. In this perspective, understanding and studying the behavior of cells is extremely important. Indeed, depending on the desired application, the biomaterial must be attached or not to the cells. When cell migration should be encouraged to regenerate damaged tissue, the material is preferably non-stick. Conversely, to enable the integration of an implant in vivo following the removal of an organ, it may promote adhesion of healthy cells while disfavoring the tumor cells that may still be present in the surrounding tissue.

5.4.2.1 Adhesion to films based on synthetic polyelectrolytes

A means of controlling cell adhesion on film PAH/PAA (poly-allylamine hydrochloride/poly-acrylic acid), is pH change during the construction of the film. Mendelsohn studied the adhesion of mouse fibroblasts (NR6WT) on these two polyelectrolyte films, in function of pH to control cellular growth (Mendelsohn et al., 2003). Construction of the film at pH 6.5 allows good adhesion of fibroblasts, whereas the same film constructed at pH 2 is non-adherent. This change in pH control the hydration of the molecules, ultimately growth of cells via internal swelling capacity in relation to pH. At low pH molecules are

usually less hydrated and develop thin layer ultimately allowing cells to grow. Moreover, the amount of proteins adsorbed on the films is independent of the pH of construction.

PEM films (PAH / PSS) constructed on gels of polydimethylsiloxane (PDMS) by the microfluidics network technique, are non-adherent for the murine neural cells and becomes adherent with the passage of time. PEMs constructed by this method are also selective for growth pattern, as more elongated cell growth was observed (Reyes et al., 2004). Later, some *in-vivo* studies showed improved endothelial cell growth when implanted in arteries and human umbilical cord (Kerdjoudj et al., 2007).

5.4.2.2 Adhesion of the films based on biopolymers (polysaccharides and polypeptides)

Cell adhesion can also be modulated by the use of natural polymer based films instead of using synthetic polymers, such as CHI/HA (Chitosan/Hyaluronic acid) films. These films can regulate growth of many cells from different origin e.g. platelets and leukocytes (Thierry et al., 2003), chondrosacroma (Richert et al., 2004a; Richert et al., 2004b), MC3T3 preosteoblasts (Hillberg et al., 2009) or stem cells derived from bone marrow (Liu et al., 2010). Low cellular adhesion and proliferation on the films CHI/HA is mainly due to the mechanical properties of these films, which are not rigid enough. Another modification of these films is to crosslink, which can render these films more stiffer and resistant to enzymes such as hyaluronidases (Richert et al., 2004a).

5.4.3 Insertion of active molecules in a film

To make bioactive biomaterials to induce an appropriate response *in vivo*, it is necessary to add or graft bioactive molecules such as proteins, peptides or drugs.

5.4.3.1 Adsorption and absorption of molecules

The multilayer films are made by adsorption of polyanions and polycations. This property of the films can make it possible to incorporate a wide variety of charged molecules (particles, inorganic molecules, proteins). One method is to create reservoir films that may contain active molecules by use of adsorption. Berg in 2006, made a porous film by treatment at different pH after alternative building of PAH/PAA and PAH/PSS and include the ketoprofen and cytochalasin D (Berg et al., 2006). In this technique, exponential growth of the crosslinked films may serve as a reservoir for active molecules (Crouzier et al., 2010). The

amount of active molecules is modulated by varying the number of layers of the film and the release is affected by the modification of the medium or degradation of the film. A similar technique was adopted to construct a multilayered film made up of PLL/PGA (Poly-L-lysine/Poly-glutamic acid), with the insertion of Protein A from *S. aureus* (Jessel et al., 2003). Protein A has various biological activities, including antitumor, antifungal and antiparasitic. This film is gradually degraded when it is in contact with the cells, which enables them to liberate protein A.

5.4.3.2 Insertion of active molecules by coupling of the molecules

Another method of inserting active molecules to the film is the coupling with one of the components of the film. The α-MSH (α-melanocyte-stimulating hormone), anti-inflammatory peptide, has been successfully integrated into multilayer films of PLL/PGA while remaining active (Chluba et al., 2001). The α-MSH was grafted onto the PLL. Later, in 2005 this peptide is covalently coupled by thiol functionalization with the PGA to create prosthetic trachea (Fioretti et al., 2010; Schultz et al., 2005). The anti-inflammatory activity of α-MSH could be demonstrated *in-vivo* by detection of IL-10 in rats implanted with functionalized trachea.

5.4.4 Antibacterial PEM films

5.4.4.1 Passive antibacterial PEM films

Most of the pathogenic bacteria can form biofilms. Silverstein and Donatucci describe different stages of biofilm formation for various biomaterials such as catheters, heart valves (Silverstein and Donatucci, 2003). As previously described in the first chapter about *S. aureus*, infection usually starts with the adhesion of bacteria to host surface that is potentiated by use of MSCRAMs. Various studies have enabled the creation of multi-resistant bacterial adhesion films. Ultimately, reduced adhesion of the pathogen can indirectly reduce infection. PEMs films based on PLL/PGA can be potentiated by applying of last layer which is grafted with poly-ethylene glycol (PLL / PGA-g-PEG) (Boulmedais et al., 2004). PEG is indeed a hydrophilic molecule and its hydration prevents bacterial adhesion. Reisch et al. also proposed another approach to make non-adherent surfaces to inhibit fungal adhesion. The surface covering by deposition of polyelectrolyte multilayer films modified with oligoethylene glycol chains or phosphorylcholine (Reisch et al., 2010; Reisch et al., 2008).

Chitosan is extensively used for the development of PEM films. It is a hydrophilic polymer obtained industrially by N-deacetylation of chitin, also has antimicrobial properties against a wide range of fungi, algae and some bacteria. In addition, it can regulate growth of some viruses (Rabea et al., 2003).

5.4.4.2 Active antibacterial PEM films

Films can be made by inserting molecules having antibacterial properties. Initially, antibacterial films were developed by the insertion of silver nanoparticles in multilayers of PAH/PAAm (Poly-alanine hydrochloride/polyacrylamide) (Lee et al., 2005). Antibacterial activity can be controlled by the deposition of varying number of layers on the planner surface. The zone of inhibition increases as the film thickness increases, which can be described by a simple diffusion model. It was also demonstrated that the release of silver ions, responsible for antibacterial, can be controlled by an oxidation mechanism on the surface of nanoparticles. The deposition of these layers on magnetic microspheres allows release of the antimicrobial agent to specific sites. Similar experiments were carried out in 2008, by using the antibacterial encapsulated silver ions in liposomes inserts that are inserted in the films based on PLL/HA (Malcher et al., 2008). The bactericidal action was observed against *E. coli*, which is due to the diffusion of silver ions through the film under the effect of temperature.

Similarly, it is also possible to incorporate antibiotics or AMPs in multilayers such as gentamycin and gramicidin, which was coupled to HA of the film (Chuang et al., 2008; Guyomard et al., 2008; Guyomard et al., 2006). These bactericidal compounds inserted films can effectively kill gram-positive and gram-negative bacteria and these are non-toxic to host cells (murine MC3T3 osteoblasts). The integrated amount of peptide is modulated by changing the number of deposited layers. The antibacterial activity is due to two phenomena: the contact between the bacteria and the film on the one hand and the release of the peptide in the surrounding medium. It is therefore possible to integrate many molecules in multilayer films and release them to give them different properties.

PART: II

MATERIALS AND METHODS

1 PREPARATION AND PURIFICATION OF BIOLOGICAL MATERIALS

1.1 Preparation of Leucocidin E-D

Staphylococcal leucocidin E-D were purified from *S. aureus Newman* strain (NTCC 8178) according to previous studies (Finck-Barbancon et al., 1991; Gravet et al., 1998; Morinaga et al., 2003). Briefly, one colony was grown in 5mL of YCP medium (yeast extract-Casamino Acids-pyruvate) composed of 3% w/v yeast extract (Oxoid), 2% w/v bacto-casaminoacids (Difco), 2 % w/v sodium pyruvate (Merck), 0.25 % w/v Na_2HPO_4, 0.042 % w/v KH_2PO_4, pH 7.0 at 37°C for 8h with vigorous rotary shaking. Proteins were precipitated from the supernatant by the help of 80 % w/v ammonium sulfate and resuspended and subjected to a first chromatography on a SP sepharose fast flow column (Pharmacia, Uppsala, Sweden) (Finck-Barbancon et al., 1991). The further purification was achieved by subjecting the eluted fractions separately to a two steps chromatography including MonoS-cation-exchange FPLC (Fast performance liquid chromatography, Pharmacia, Uppsala, Sweden) and later to an Alkyl-Superose hydrophobic FPLC (Finck-Barbancon et al., 1993; Staali et al., 1998). Purified compounds were controlled by SDS-PAGE (12 %) on PHAST System (Pharmacia) and then solubilized in 50 mM Na-phosphate buffer (pH 7.0) supplemented with 150 mM NaCl, (Gravet et al., 1998; Prevost et al., 1995c) at OD of 1.0 at 280 nm. Leucocidin E-D were stored at -80° C until further use. LukE and LukD were further analysed by the Edman sequencing and gel-electrophoresis (12 %) to analyze purity and integrity. After Edman degradative analysis, for sequences identification a query was made in the database SWISS-prot (http://www.expasy.org/tools/Blast/). The protein characteristic parameters are given by the (http://www.uniport.org/). Uniport accession numbers are as follows: LukD A6QI08 and LukE A6QI09.

1.2 Gel electrophoresis and coomassie blue staining of the gel

- **Preparation of gel**

Western blot analysis was carried out by the use of the SDS-PAGE (sodium dodecyle sulphate polyacrylamide gel electrophoresis) by the protocol previously described (Laemmli, 1970). For electrophoresis 4-12 % polyacrylamide gel was prepared which composed of two gel portion separating gel and stacking or concentrating gel. First the 15 % gel was

polymerized between two glass plates fixed in jacket and followed by polymerization of 4 % polyacrylamide gel polymerization after insertion of the comb (10 wells; 1 mm)

- **Sample preparation for electrophoresis**

Leucocidin E-D were prepared for electrophoresis separately, by adding Laemmli buffer (4x) (Laemmli, 1970) (SDS 3 %, EDTA 1 mM, Tris HCl 10 mM, DDT 20 mM, glycerol 40 %, bromophenol blue 0.025 % at pH 6.8). Samples were denatured by heating at 95° C for 6 min.

- **Migration of the samples**

Samples after denaturing were poured to wells and migration was achieved at 45 V for 30 min and followed by 110 V till the migration was completed till the lower end of the gel. Migration buffer composed of glycine 192 mM, SDS 0.1 %, Tris HCl 0.025 M, pH 8.2. Mixture of known molecular markers was used to determine the mass of the proteins.

- **Coomassie blue staining**

After the migration of the proteins onto 12 % polyacrylamide gel, gel was stained by the coomassie blue solution (40 % methanol, 10 % acetic acid, 50 % water; one gram coomassie blue) for 1 h with shaking. After staining, destaining was done with decoloration solution (7.5 % methanol, 10 % acetic acid and 82.5 % water).

1.3 RP-HPLC of Leucocidins E-D

Leucocidins E-D were further analyzed for the purity by using RP-HPLC (Reverse phase-high performance liquid chromatography). DIONEX-Ultimate 3000 Dual Gradient System (Dionex, Sunnyvale USA) was used to achieve purification which comprises of dual solvent (A and B) system which was used at a flow rate of 700 µL/min. Solvent A comprises of 0.1 % (v/v) Trifluoroacetic acid and solvent B comprises of 0.09 % (v/v) Trifluoroacetic acid in 70 % (v/v) acetonitrile. Gradient used for analysis is presented on the relevant chromatograms. Absorbance was monitored at 214 nm and whole process of purification was carried out at room temperature.

1.4 Isolation of polymorphonuclear neutrophils

Human PMNs were prepared to about 98 % homogeneity as previously described, (Finck-Barbancon et al., 1993) from Buffy coat of healthy donors of either sex, obtained from the Centre de Transfusion Sanguine de Strasbourg (France). Briefly, twelve mL of J-Prep solution (TechGen) added added to 30 mL of buffy coats from healthy donors and centrifuged

at 200 x g for 20 min after centrifugation lower dark band was collected without disturbing the sediment and washed with EGTA buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM ethylene glycol-bis-(L-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 10 mM HEPES, 3 mM Tris-base, pH 7.3). PMNs were further processed by dextran sedimentation (Colin et al., 1994) for 30 min decantation and then followed by centrifugation at 1600 x g for 6 min. Remaining impurities of red blood cells were lysed by the hypotonic shock of distilled water (18 mL) for 45s and immediately suspended in 2 mL hypertonic NaCl (9 % w/v) solution to maintain a concentration of 0.9 % (w/v) (Barrio et al., 2006). This step of RBCs lysis can be repeated if necessary but for shorter time. Later two washings were performed by EGTA buffer and suspended at a concentration of 5×10^6 cells/mL in the same buffer.

1.5 Preparation of secretions after PMNs stimulation by Leucocidin E-D

PMNs were suspended in EGTA buffer solution (140 mM NaCl, 5 mM KCl, 10 mM Hepes, 0.1 mM EGTA and 10 mM glucose pH 7.3) at 10^8 cells per mL. A total of 10 mL (10^8) cell suspension was used. Two components of leucocidins of *S. aureus* Luk-D and Luk-E were used each at 10 nM. Before stimulating the cells $CaCl_2$ was used at a concentration approximate to the concentration of normal biological solutions (1.1 mM) (Moussa et al., 2006). For proper stimulation cell suspensions were kept at 37° C for 25 min. Centrifuged ($800 \times g$) for 10 min and supernatants were recovered and stored at -80° C.

1.5.1 Purification of PMNs secretions by RP-HPLC

PMNs secretion were purified by using RP-HPLC to separate in different fractions for identification by Lc-MS/MS. DIONEX-Ultimate 3000 Dual Gradient System (Dionex, Sunnyvale USA) was used with a Nucleosil RP300-5C18 column (Macherey-Nagel, Hoerdt, France) (0.4 x 25 cm: 5 µm particle size, 300-Å porosity). Dual solvent (A and B) system was used at a flow rate of 700 µL/min. Absorbance was monitored at 214 nm at room temperature. Gradient with reference to time is indicated on the chromatograms and fractions were collected. Fractions were collected according to one minute time interval.

1.5.2 Desalting of the PMNs secretions

Desalting method was used to eliminate the salts of the PMNs secretion medium. PMNs secretions were dispatched to tube each containing 1 mL, were concentrated by speed vacuum (concentrator 5301, Hamburg, Germany) to approximately one third of the volume

and 3-4 tubes were combined to one tube and desalting was performed with Sep-PAK C18 cartridge (Waters, Ireland). Protocol used is as follows: first cartridge was activated with 10 mL of absolute methanol, followed by 10 mL of acetonitrile (pure). After activation, cartridge was calibrated with 10 mL of 0.1 % TFA (trifluoroacetic acid). After those PMNs secretions were passed through the cartridge, followed by 10 mL of 0.1 % TFA to remove the salts. Proteins were eluted from the cartridge by 5-7 ml of acetonitrile. Eluted proteins were lyophilized to remove acetonitrile.

1.6 Synthesis of peptides

All the synthetic peptides were prepared on an Applied Biosystems 433A peptide synthesizer (Foster City, USA). Amino acids and the all the reagents used were purchased from Novabiochem (Novabiochem, Darmastadt, Germany). Stepwise solid-phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry was used for the synthesis of the peptides (Merrifield, 1965). Then, the synthetic peptides were purified by a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Macherey Nagel Nucleosil RP 300-5C18 column (10 × 250 mm; particle size 5µm and pore size 100 nm). Synthetic peptides were analysed by mass spectrometry (MALDI-TOF) and automated Edman sequencing on an Applied Sequencing System Procise (Applied Biosystems, Foster City, USA). MALDI-TOF mass measurements were carried out on an Ultraflex™ TOF/TOF (BrukerDaltonics, USA) to perform a rapid control of synthetic peptides (Metz-Boutigue et al., 1998; Sizova et al., 2007).

1.6.1 Purification of synthetic peptides by RP-HPLC

Peptides after synthesis were analyzed and purified by using RP-HPLC, DIONEX-Ultimate 3000 Dual Gradient System (Dionex, Sunnyvale USA) was used with a C18 Vydac Grace 218TP510 column (Grace Davidson, USA) (1 x 25 cm: particle size was 5 µm and pore size of 300 A). Dual solvent (A and B) system was used at a flow rate of 1500 µL/min. Absorbance was monitored at 214 nm at room temperature.

1.7 Isolation and characterization of staphylococcal strains

S. aureus strains used to demonstrate peptide degradation *S. aureus* ATCC-25923, ATCC-49775, S1 and S2 were provided by the Institute of Bacteriology, Strasbourg, France. S1 was isolated from the blood of an 83 y. o. patient and S2 was isolated from the *sputum* of a 12 days old neonate. After isolation and identification, *S. aureus* strains were assessed for

their susceptibility to various antibiotics, using the agar disc diffusion method. S1 was found resistant to Amoxicillin, Oxacillin, Amikacin, Tobramycin, Fluoroquinolones, Erythromycin and Clindamycin, but was susceptible to Gentamicin, Synercid, Co-trimoxazole, Rifampicin, Fusidic acid, Vancomycin, Teicoplanin and Linezolid. These tests indicate that S1 is a MRSA (Methicillin resistant *S. aureus*) strain. In contrast, S2 was MSSA (Methicillin susceptible *S. aureus*) as is sensitive to all the antibiotics tested.

2 PREPARATION AND CHARACTERIZATION OF MATERIAL FOR POLYMER DEVELOPMENT

2.1 Preparation of modified synthetic antimicrobial peptide (CTL-Cys) for insertion in biomaterials

The synthetic peptides corresponding to bovine CGA (Uniprot P05059), named CTL, ($CGA_{344-358}$: RSMRLSFRARGYGFR) was synthesized with a Cysteine at the C-terminal end (CTL-Cys) on an Applied Biosystems 433A peptide synthesizer using the stepwise solid-phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as previously described (Merrifield, 1965).

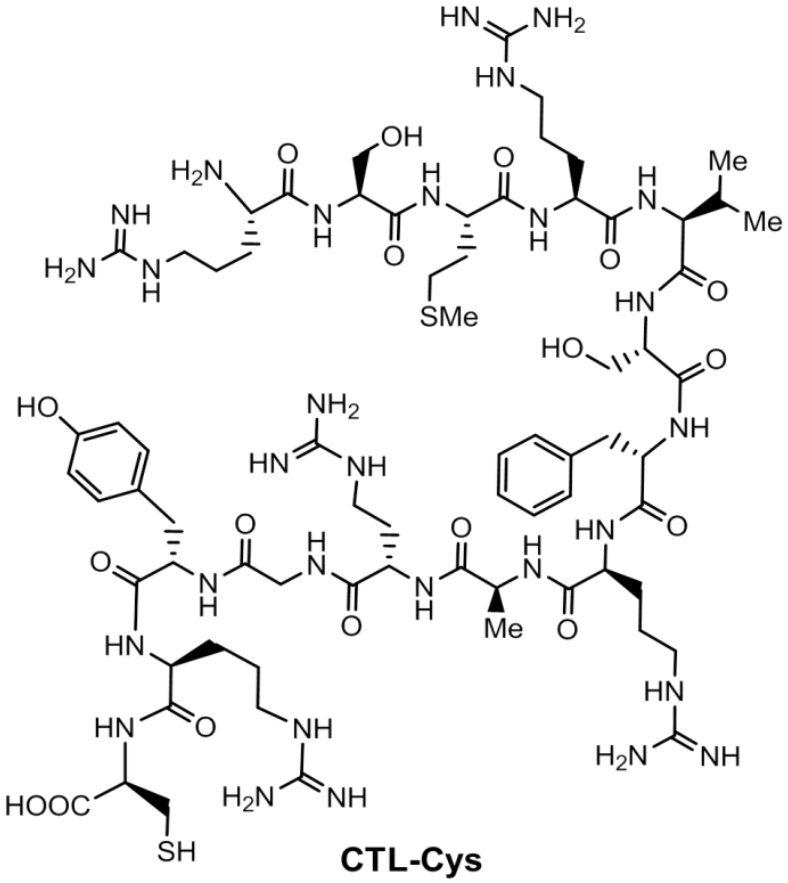

Figure 27: Cateslytin (CGA 344-358) + cysteine at C-terminal

2.1.1 Synthesis of rhodaminated peptides

For confocal microscopy peptides are rhodaminated by the fluorescent molecule, 5(6)-carboxytetramethylrhodamine (Interchim, Montlucon, France). Rhodamine was fixed to N-terminal of the peptide which was achieved by combining rhodamine to N-terminal when the sequence is finished before to remove resin.

2.2 Preparation of modified HA.

Peptide and FITC functionalized HA, *i.e.* HA-CTL, HA^{FITC} and HA^{FITC}-CTL, have been prepared according the following general synthetic pathway described below.

2.2.1 Preparation of HA-CTL-Cys

Hyaluronic acid (HA) 420 000 Da, was functionalized with maleimide group according to previously reported method (Wall et al., 2008). HA (100.9 mg) was dissolved in 15 mL of 0.01M HEPES buffer (pH 6). To this solution, EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) (35.2 mg) and sulfo-NHS (3-sulfo-N-hydroxysuccinimide ester) (10.2 mg) were added and the resulting mixture was stirred 2 hours at room temperature. Afterwards, N-(2-aminoethyl) maleimide trifluoroacetate salt (6.7 mg) was added to the reaction mixture and let stirred during 20 hours. The mixture was dialyzed (cut-off 12 000-14 000 Da) first against 0.5 M NaCl during one day and MilliQ water during 6 days. Water was changed every day. HA-Mal (70 mg) was obtained as a white solid after freeze-drying. By ^{1}H NMR (400MHz, D_2O), was determined the degree of substitution (DS) of HA-Mal, defined as the number of maleimide groups per 100 disaccharide units of HA.

All spectra were done in D_2O containing 5% of tBuOH: the singlet of the t-butyl group of tBuOH was calibrated at δ 1.24 ppm and thus used as internal reference. The singulet at δ 7.1 ppm was assigned to the two symmetric protons on the double bond of the maleimide group. By comparing the integration of this signal with the singlet at δ 2.0 ppm, assigned to the methyl protons of the acetamide group, the DS was determined equivalent to 5%.

^{1}H NMR (D_2O, 400MHz, δ ppm): δ 6.9 (s, maleimide), 4.5 (br d, HA), 3.5 (m), 2.0 (s, acetyl group of HA).

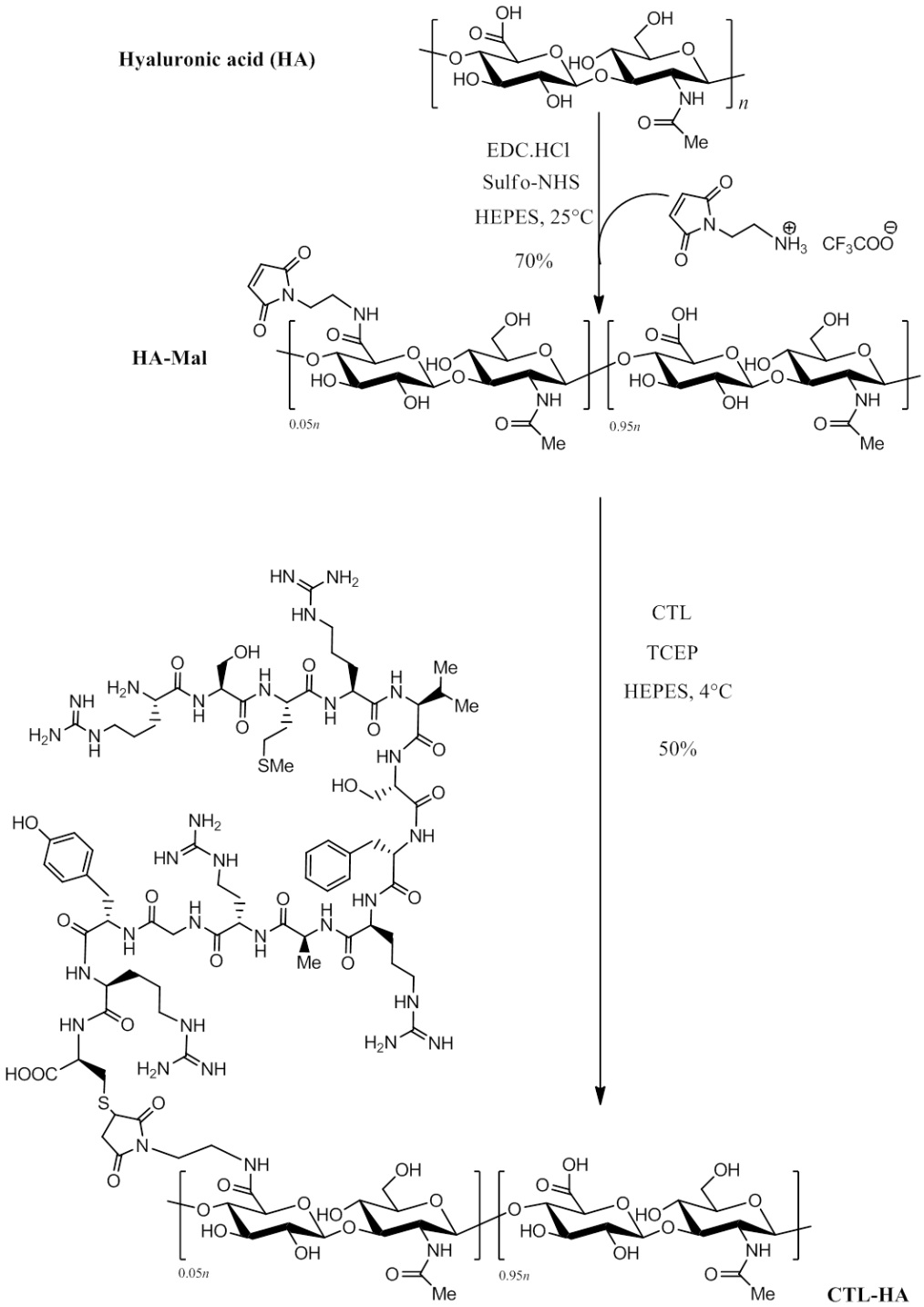

Figure 28: Coupling reaction of HA to maleimide and followed by conjugation of CTL (CGA344-358) to HA.

The coupling reaction between HA-Mal 5% (51mg) and CTL-Cys (7mg) was performed at 4°C during 20 h in 16mL of 0.01M HEPES buffer (pH 6) and 50 µM of TCEP. Then, the mixture was dialysed (cut-off 50 000Da) against 0.5 M NaCl during one day and MilliQ water during 6 days. Water was changed every day. Peptide-conjugated HA, named

HA-CTL, was freeze dried to provide a white solid with 80-85% of overall yield (~45 mg). The DS, defined as the number of CTL peptide per 100 disaccharide units of HA, was determined by ^{1}H NMR (D_2O, 400 MHz). All aromatic signals between δ 7.0 and 7.5 ppm corresponding to the protons of the aminoacids Phe and Tyr were compared to the singlet at δ 2.0 ppm (assigned as the methyl group of the acetamide). A DS of 5% is determined.

^{1}H NMR (D_2O, 400MHz, δ ppm): 7.35 (br s, Ar aminoacid of CTL), 7.25 (br s, Ar aminoacid of CTL), 7.10 (br s, Ar aminoacid of CTL), 6.85 (br s, Ar aminoacid of CTL), 4.50 (br s, HA), 4.40 (br s, CTL), 3.10 (br s, CTL), 2.50 (br s, CTL), 2.00 (s, methyl from acetyl group of HA), 1.75 (br s, CTL), 0.95 (br s, CTL).

2.2.2 Preparation of HA^{FITC}

Fluorescein Isothiocyanate (FITC) has been covalently attached to HA according to the following procedure: a solution of FITC (41 µmol of FITC in 2mL of DMSO) and a solution of HA (0.31 µmol of HA dissolved in 18 mL of deionized water) were mixed together and the pH was adjusted to 9 by using a 0.01M NaOH solution. The reaction mixture was stirred 12h at room temperature. Then, 40 mL of deionized water was added and this final mixture was dialyzed (cut-off: 12 000 – 14 000 Da) against deionized water until no absorbance is detected in the water (λ=494nm). The obtained compound is a yellowish solid and corresponds to HA functionalized with 1% of fluorescein. The 1H NMR (D_2O) spectra of HA^{FITC} is identical to the non-modified HA.

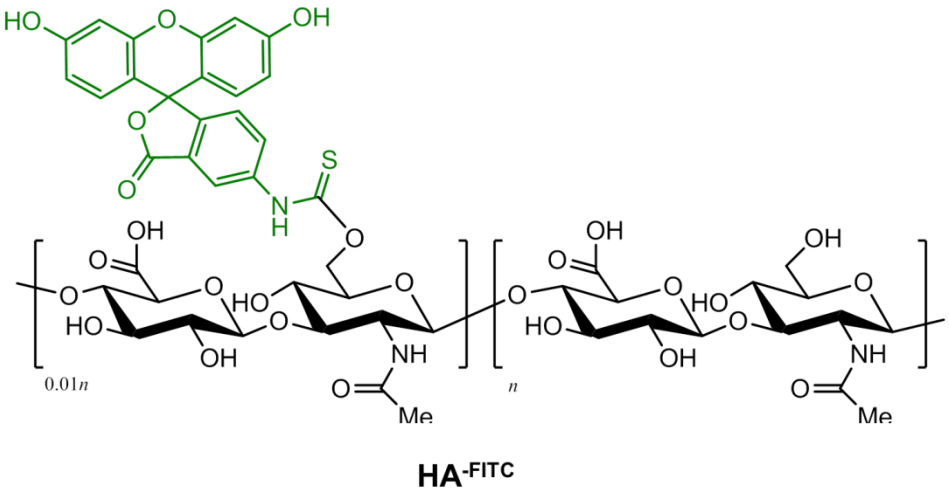

Figure 29: Preparation of HA^{FITC}

2.2.3 *Preparation of HA^{FITC}-CTL*

HA^{FITC}-CTL polymer has been prepared from HA-Mal. This polymer was labeled with 1% FITC has described above to provide HA^{FITC}-Mal. Then, CTL-Cys peptide reacted with free maleimide group to lead to HA^{FITC}-CTL as a yellow solid. The procedure used to get HA^{FITC}-CTL was identical to the one described above to prepare HA-CTL. ^{1}H NMR (D_2O) spectra of HA^{FITC}-CTL was identical to the spectra of HA-CTL.

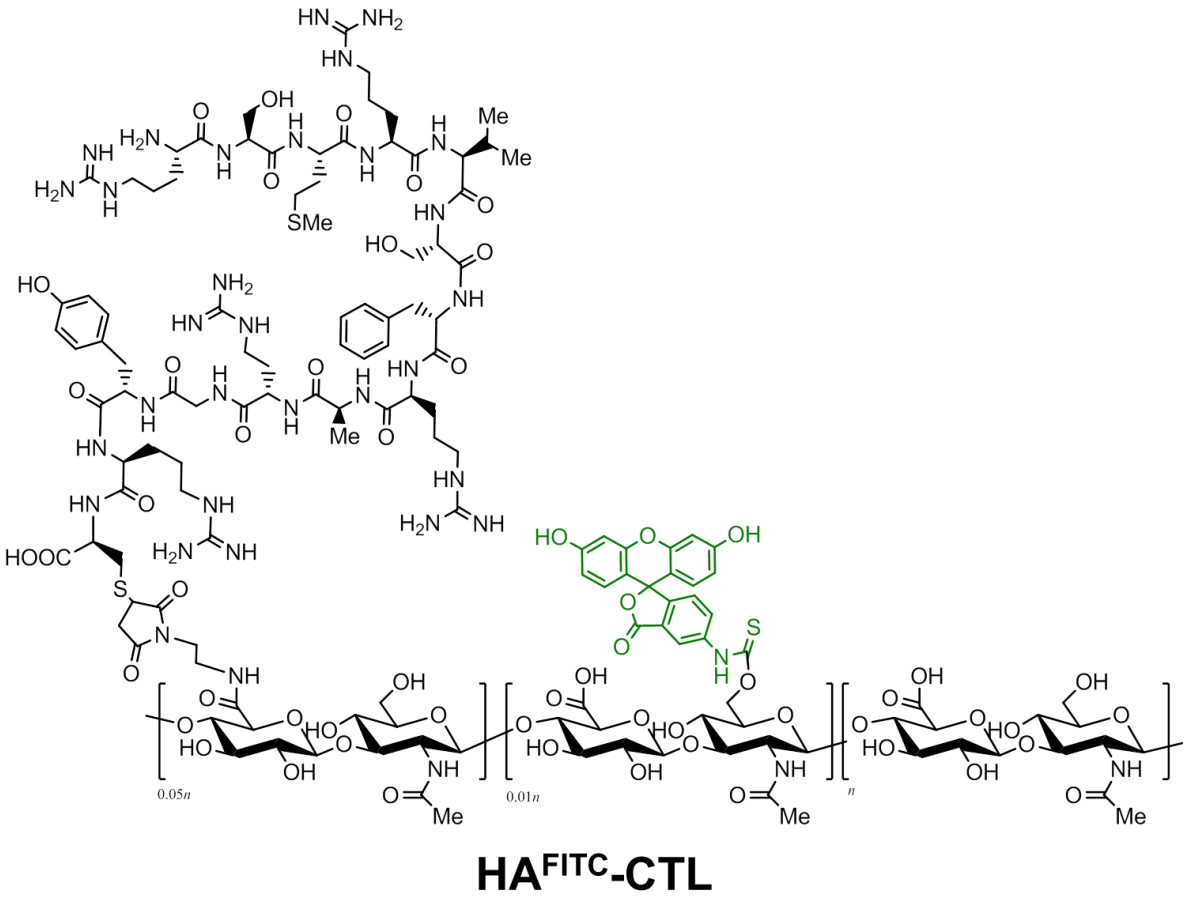

Figure 30: Preparation of HA^{FITC}-CTL

2.3 *Gel filtration chromatography*

Gel filtration chromatography was used to perform a rapid control of the HA and HA-CTL prepared. For chromatography HPLC DIONEX-Ultimate 3000 (Dionex, Sunnyvale USA) system was used, instead of using dual gradient only one solvent system was used which comprises of 100% H_2O. A constant flow rate of 1 mL was used and absorption was monitored at 214 nm. Gel filtration column used was TSK-GEL G3000PW$_{XL}$ (TOSOH

Bioscience; Zettachring, Stuttgart, Germany) (0.75 x 30 cm: 6 µm particle size). All the elutions were performed at room temperature.

2.4 PEM films buildup

2.4.1 Polysaccharide solutions

Chitosane (CHI, PROTASAN Ultrapure Chitosan CL213, 260 000 g/mol, DA 83 %) was purchased by Novamatrix (Sandvika, Norway). Dried Sodium Hyaluronate (HA, 420 000 g/mol) was purchased by Lifecore (Chaska, USA). Poly (ethylene imine) (PEI, 60 000 g/mol, 50% in water), sodium chloride, sodium dodecyl sulfate (SDS), HCl and NaOH were purchased by Sigma-Aldrich (Quentin-Fallavier, France). All products were used without further purification. Both solutions were prepared by dissolution of 0.3 mg/mL in 150 mM NaCl aqueous solution prepared using ultrapure Milli-Q® water having a resistivity of 18.2 MΩ.cm and were adjusted at pH 4 with NaOH or HCl solutions. PEI solution was prepared at 0.5 mg/mL in 150 mM NaCl solution and used without adjusting the pH.

2.4.2 Film buildup

Before PEM buildup, glass coverslips with a 14 mm in diameter (VWR, Strasbourg, France) were cleaned in a SDS solution at 10^{-2} M, ultrasonicated during 5 min and then submitted to a hot 0.1 M HCl solution during 10 min and finally rinsed with ultrapure Milli-Q® water. A precursor PEI layer was adsorbed with an adsorption time of 15 min followed by a rinsing step of 5 min. CHI/HA films were deposited by using an automated spraying device described in a previous work (Cado et al., 2012). CHI/HA-CTL and CHI/HA^{FITC}-CTL films have been prepared in 24 wells plates on glass coverslips. 300 µl of polycation solution has been deposited for 5 min on glass coverslips followed by three rinsing steps (1 mL) with ultra Milli Q water. Then, 300 µl of polyanionic solution has been added for 5 min followed by three rinsing steps (1 mL) with ultra Milli Q water. To test the antimicrobial activity of PEM films depending of the number of HA-CTL layers, PEI-$[HA/CHI]_{15-n}[HA\text{-}CTL/CHI]_n$ with n equal to 0, 5, 10 and 15 and PEI-$[HA\text{-}CTL/CHI]_{30}$ films were built.

2.5 Physico-chemical characterization of the polymer films

2.5.1 Quartz Crystal Microbalance

The construction of multilayer films was monitored in situ by quartz crystal microbalance (QCM) (Q-Sense E4, Goteborg, Sweden). The measurement methodology has been addressed in details elsewhere (Boulmedais et al., 2004). Briefly, after cleaning of the crystal with a 2% (v/v) Hellmanex solution during half an hour, rinse with Milli-Q water, further immersion in a 0.1M HCl solution and intensive rinse with Milli-Q water, 600 µL of NaCl solution were injected into the measurement cell. After stabilization of the signals, 600 µL of the polycation solution containing NaCl were injected, left in the cell for 5 min, and rinsed with the NaCl solution and left again for 5 min. The frequency shifts were recorded during the whole process. The same procedure was used for the deposition of the polyanion. A negative value of Δfn (or of the normalized shift Δfn/n) reveals a decrease of the resonance frequency during the deposition process and an increase of the mass adsorbed.

2.5.2 Atomic Force Microscopy (AFM)

Atomic force microscopy measurements (AFM) were realized with a Veeco Multimode Nanoscope IIIA (Digital Instrument) in the wet state and in PeakForce TappingR mode (Bruker) using cantilevers with a spring constant of 3 N/m.

2.5.3 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) observations were carried out with a Zeiss LSM-510 microscope using a 63x/1.4 oil immersion objective and a 0.43-mm z-section interval. FITC fluorescence was detected upon excitation at 488 nm, through a cut-off dichroic mirror and an emission band-pass filter of 505–530 nm (green).

3 METHODS OF PROTEOMIC ANALYSIS

3.1 Sequence characterization by Edman sequencing

Sequencing was used to perform a rapid control of the amino acid sequence of the leucotoxins purified, synthetic peptides and for the identification. Edman sequencing is recurrent process of detaching a single amino acid starting from the N-terminal of the proteins and comparing a satandard of amino acids. Degradation analysis is based on the reaction of Edman (Edman, 1949).

The N-terminal sequencing was carried out by the method of automatic Edman sequencing with a bi-sequencer cartridge (Procise 473A, Applied Biosystems, Foster city, USA). All the products for the peptide sequencing were purchased from Applied Biosystem (Applied Biosystem, Warrington, UK). The sensitivity of detection is in Picomoles (5-10pmol: 0.01-0.02mL). Samples were deposited on a fiber filter by repeating cycles of sample addition (10µl volume) and drying by argon gas. Fiber filters were previously treated with 10µL of polybrene, (BioBrène plus, Applied Biosystems) was used. Amino acids are identified as Phenylthiothiohydantoine-amino acids (PTH-aas) by HPLC on reverse phase column coated with C_{18} (PTH-C18, 2.1 x 200µm, Applied Biosystems, Warrington, UK) at a temperature of 55°C. Solvents used for elution consists of dual solvent system comprising: solvent A (Tetrahydrofurane 3.5% and Premix 2% in water) and solvent B (Acetonitrile 88% and Propanol 12%). Detection is performed by measuring the absorbance at 269 nm and the identification of PTH-ass is done by comparison with the chromatogram of a standard mixture of different PTH-aas.

For sequences identification a query was made in the database SWISS-prot (http://www.expasy.org/tools/Blast/). The protein characteristic parameters are given by the (http://www.uniport.org/)

3.2 Western blot analysis of the PMNs secretion

- **Gel electrophoresis**

Western blot analysis was carried out by the use of the SDS-PAGE (sodium dodecyle sulphate polyacrylamide gel electrophoresis) by the protocol previously described (Laemmli, 1970). For electrophoresis 4-15% polyacrylamide gel was prepared. Desalted PMNs secretion were used, which were prepared by adding Laemmli buffer (4x) (Laemmli, 1970) (SDS 3%,

EDTA 1mM, Tris HCl 10mM, DDT 20mM, glycerol 40%, bromophenol blue 0.025% at pH 6.8). Samples were denatured by heating at 95°C for 6 min. Migration was achieved at 45V for 30 min and followed by 110 V till the migration was completed till the lower end of the gel. Migration buffer composed of glycine 192 mM, SDS 0.1%, Tris HCl 0.025M, pH 8.2. Mixture of known molecular markers was used to determine the mass of the proteins identified and in case of PMNs secretions soluble protein rich granules of the chromaffin cells were used as positive control which is rich in chromogranins.

- **Electro-transfer onto PVDF membrane**

Transfer was achieved by subjecting to 75 V for 50 min. PVDF membrane (Amersham Biosciences, Little Chalfon, UK) with a pore size of 0.22 µm was used for transfer. Transfer buffer composed of 20% of the migration buffer, 10% methanol and 70% water.

- **Immuno detection**

For immunodetection Millipor Snap i.d protein detection system was used using 0.5 % BSA (bovine serum albumin) (Euromedex, Muldosheim, France) in PBS as blocking agent. Proteins were immunodetected by the antibodies specific to the proteins, two type of antibodies were used monoclonal anti-CGA (anti-$CgA_{47–68}$) and polyclonal anti-CgB (anti-$CGB_{547-560}$) for CGA and CGB respectively. ECL conjugated antibodies were used as secondary antibodies: anti-mouse IgG and anti-rabbit IgG ((GE Healthcare, Little Chaflond, UK) for anti-CgA and anti-CgB respectively. Finally, detection was achieved by the use of SuperSignal (SuperSignal West Femto Maximum Sensitivity Substrate, Thermoscientific, Rochford, USA) and visualized by the use of ChemiDOC (BioRad).

3.3 Matrix assisted laser desorption ionization time-of-flight Mass spectrometry (Maldi-TOF)

Mass determination was carried out on a Brucker BIFLEX™ matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF) equipped with the high resolution optics (SCOUT™) with X-Y multi-sample probe, a grid less reflector and with the HIMAS™ linear-detector. With a maximum accelerating potential of 30 kV, the system can be operated either in linear or the reflective mode. Ionization was carried out with a 148 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 250 MHz in linear mode and 500 MHz in reflector mode using a 1-GHz digital oscilloscope (Lecroy model). For the data processing and the instrumental control, software supplied by Bruker using a Sun sparcworkstation was accomplished (Pons et

al., 2002). These studies were realized using the matrix α-cyano-4-hydroxycinnamic acid, obtained from Sigma, and prepared as a saturated solution in acetone. A total of 1-2 Nl of the sample matrix aliquot solution was deposited on the probe and air dried by ambient air. A thin layer was obtained of matrix crystal after fast spreading and evaporation (Kussmann et al., 1997). A micro-molar analyte solution was applied to the matrix and allowed to dry under moderate vacuum. The whole preparation was washed by 1 µl of a 0.5% trifluoroacetic acid aqueous solution. This cleaning procedure often leads to an increase in sensitivity and mass accuracy by removing the remaining alkali cations

3.4 Nano LC-MS/MS Mass spectrometry analysis

Secretions were digested by the modified porcine trypsin (promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at 37°C for 14 hrs. The generated peptides were analyzed directly by NanoLC-MS/MS on an Agilent 1100 series HPLC-Chip/MS system (Agilent Technologies, Palo Alto, USA). The voltage applied to the capillary cap was optimized to -1850V. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides, performing doubly charged ions, were selected on each MS spectrum for further fragmentation. The MS/MS scanning was performed in the ultra-scan resolution mode at a scan rate of 26.000m/z per second. A total of six scans were averaged to obtain a MS/MS spectrum. The complete system was fully controlled by ChemStation (Agilent Technologies) and EsquireControl (BrukerDaltonics) software. Mass data collected during nanoLC-MS/MS analysis were processed before interpretation using a local Mascot™ (Matrix Science, London, UK) server. The searches were performed against the NCBI database (Delalande et al., 2005).

4 METHODS TO CHARACTERIZE BIOLOGICAL ACTIVITIES

4.1 Flow-cytometry measurements:

For the flow cytometry PMNs were suspended at a concentration of 5x 10^5 cells per mLin the EGTA buffer (pH 7.3 adjusted with Tris HCl) containing EGTA 0.1 mM, Hepes 10mM, NaCL 140mM, KCl 5mM, Glucose 10 mM. Flow cytometry measurements were carried out using a FacSort flow cytometer (Becton-Dickinson, Le pont de Claix, France) equipped with a 15mW argon laser tuned to 488 nm (Meunier et al., 1995).

Activity of leucotoxins (E-D) on the human PMNs was evaluated using flow-cytometry of cells previously incubated with Ethidium Bromide (25nM) (Moussa et al., 2006) for 10 min before addition of LukE/LukD. Pore formation by the toxins was revealed by the penetration of ethidium through the pores. Ethidium fluorescence was measured using Cell QuestPro^{TM} software (Becton, Dickinson and company, Franklin Lakes-USA) by using (FL3: λEm = 650nm) (Meunier et al., 1995). Each component constituting the leucotoxins was generally added at concentrations as follows: 2nM, 3nM, 6nM, 10nM, 20nM, 40nM and 60nM. First image was taken at the time of addition of toxins then after 5 min fluorescence was recorded till 30 min of time. Fluorescence is increased when the molecule entered the cells by the pores formed through the plasma membrane and combined with nucleic acids. As positive control, the fluorescence corresponding to the dead cells was considered with a value of 100. Fluorescence without toxins was subtracted from the data and the results are presented as percentile by take positive control as 100%. Kinetics of the intracellular calcium were measured by using flow-cytometry of the cells previously loaded with 5 µM of Fluo-3 (Molecular Probes, New Brunswick, USA) during 1 h at 37°C and the washed and resuspended in EGTA buffer supplemented with 1.1mM Ca2+ (Moussa et al., 2006). Each component constituting the leucotoxins was generally added at concentrations as follows: 2nM, 3nM, 6nM, 10nM, 20nM, 40nM and 60nM. Fluo-3 measurement were carried out from the fluorescence light 1(FL1: λEm = 530 nm) using cell Quest ProTM software (Becton-Dickinson, Le Pont de Claix, France)(Meunier et al., 1995) for every 30 seconds during 10 minutes. Data provided is the average of at-least three individual experiments.

4.2 Analysis of antimicrobial activity

Antimicrobial activities of the peptides and secretion of the PMNs were analyzed. For the demonstration of activity two types of assays were carried out: antibacterial activities and the antifungal activities. Bacterial strains used are as follows: *Micrococcus luteus* (A270), *Staphylococcus aureus* (ATCC25923, ATCC49775, S1, S2) and *E. coli* (ATCC 25922). Fungal and yeast strains used to demonstrate antifungal activities were: *Neurospora crassa* (CBS 327–54), *Aspergilus fumigatus, Trichophyton mentagrophytes, Candida albicans, Candida glabrata* and *Candida tropicalis.*

4.2.1 Antibacterial test

Bacterial strains were precultured aerobically at 37°C in a Mueller-95 Hinton-Broth medium (Difco Laboratories, Detroit, MI), pH 7.3. For which two isolated *S. aureus* strains were plated on the agar plates and cultivated for 24 h at 37°C. After incubation, one colony *per* isolate was transferred to 10 mL of the Mueller-Hinton-Broth medium and incubated at 37°C for about 18-22 h to the stationary growth phase.Bacteria were suspended in the Mueller-Hinton Broth medium, and antibacterial activity was tested by measuring the inhibition of bacterial growth. 10 µL final volumes of synthetic peptides or the polymer (HA-CTL-C) and 20µL in case of PMNs secretion fractions purified by RP-HPLC were incubated in microtitration plates (Falcon, Becton Dickinson, USA) with 90 µl of a mid-logarithmic phase culture of bacteria, with a starting absorbance of 0.001 at 620 nm. To test the antibacterial and properties of the multilayer films, PEI-(HA/CHI)15-n-(HA-CTL/CHI)n with n = 0,3, 5, 10, 15 and PEI-(HA-CTL/CHI)30 films were prepared. 400 µL of a mid-logarithmic-phase culture of bacteria or yeast with OD620 of 0.001 were placed in 24-well plate containing multilayer films. In the initial inoculums with absorbance of 0.001, bacteria were quantified by agar plate spreading method which was $5x10^5$ Colony forming unit (CFU)/mL (NCCLS, 2003). Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) were used as positive control. Microbial growth was assessed by the increase of absorbance after 16 h incubation at 37°C (Bulet et al., 1993; Wu and Hancock, 1999). The A620 nm value of control cultures growing in the absence of peptide and antibiotics was taken as 100% growth and when the A620 nm is zero with the antibiotics (Tetracycline and Cefotaxime) was taken as 100% inhibition. Absence of bacterial growth was verified by agar plate spreading. Each assay was verified three times at least.

4.2.1.1 Kinetics of bacterial growth inhibition

For the bacterial growth inhibition kinetics in respect to time, the initial inoculum was prepared at concentration of $5x10^5$ CFU/mL (NCCLS, 2003) (OD620nm = 0.001) as described above. Quantification was performed by converting OD to bacterial count by devising a conversion factor (Lee et al., 2009). Briefly, OD of fresh bacteria culture was measured at 620 nm and was plated to MH agar for 24 h at 37°C. Colony count was performed and plotted versus the OD620 and the gradient of the linear line was taken as conversion factor. Later on intermittent controls were performed by colony counting to verify the accuracy of conversion factor. Quantification was performed at different time intervals (0h, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, and 24h). For each tested film, 100 µL of the supernatant was taken to measure the OD620 of the supernatant by a microplate reader. The withdrawn volume was compensated with the appropriated fresh bacteria or yeast solution of the same measured OD620. Intermittent controls were performed by inoculating the culture medium on MH agar plates by spreading method and counting colonies for verification at different steps. Several controls were used: fresh medium without inoculation of pathogens was used to ensure sterility, mixture of Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) were used as positive control (90 µL of culture and 10 µL of antibiotics) and fresh inoculated culture medium without any addition was taken as negative control. Each assay was performed in triplicate and the experiments were repeated minimum three times.

4.2.2 Antifungal activity

To analyze the antifungal activities of the synthetic peptides, PMNs secretion or the polymer either in solution or the multilayered film, two types of fungi were used: Moulds (filamentous fungi) and yeasts. Filamentous fungi used were *Neurospora crassa* (CBS 327–54) and *Aspergilus fumigatus*, and three candida strains were used *C. albicans C. glabrata, C. tropicalis*.

- **Filamentous fungi:**

For the filamentous fungi, spores were suspended at a concentration 10^4 spores/mL, in a growth medium containing Potato Dextrose Broth (PDB) (Difco, Becton Dickson Microbiology system) in half-strength, supplemented with Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) to suppress bacterial growth (Broekaert et al., 1990). Synthetic peptides and the polymers in solution form (10 µL) and aliquots of RP-HPLC fractions (20 µL) of PMNs secretions were incubated in micro-titration plates (Falcon, Becton Dickinson,

USA) with 90 and 80µL respectively, of fungal spores already diluted. The suspension was incubated at 30 C° for 24-72 h without agitation (Broekaert et al., 1990; Wu and Hancock, 1999). Growth of fungi was monitored microscopically after 24 h in case of Neurospora and Aspergilus spp. and 72 h in case of Trichophyton spp. Inoculated media was used as negative control and was taken as 100% fungal growth and voriconazole (1µg/mL) was used as positive control which was taken as 100% growth inhibition.

- **Yeasts:**

For the yeast spp. method used was the same as previously described for the antibacterial assay with few exceptions. Broth medium used was sabouraud medium (BioMerieux S.A., Marcy l'Etoile, France) and the incubation temperature was modified to 30°C instead of 37° as in case of bacterial strains with agitation. Inoculated media (OD_{620nm} = 0.001) was used as negative control and was taken as 100% fungal growth and voriconazole (1µg/mL) was used as positive control which was taken as 100% growth inhibition.

4.2.2.1 Kinetics of fungal growth inhibition

For the quantification of fungal growth with respect to time *Candida albicans* was used. Protocol used was the same as previously described for the antibacterial assay with few exceptions. Broth medium used was sabouraud medium (BioMerieux S.A., Marcy l'Etoile, France) and the incubation temperature was modified to 30°C instead of 37° as in case of bacterial strains with agitation. For the colony counting sabouraud agar (BioMerieux S.A., Marcy l'Etoile, France) was used.

4.3 Confocal laser scanning microscopy(CLSM)

To follow the interaction of fluorescently labeled HA^{FITC}-CTL in solution and HA^{FITC}-CTL/CHI films with *C. albicans*, CLSM was used a protocol previously described with few modifications(Lugardon et al., 2001). Briefly for experiments done with HA^{FITC}-CTL in solution, Poly-L-Lysine coated microscopic slides were covered with fresh medium containing *C. albicans* (OD620 = 0.001) in SB culture medium and incubated during 24 h at 30°C without agitation. The medium was then removed and replaced by 50 µM (in peptide) of HA^{FITC}-CTL. After an incubation of 45 min, the slides were washed with fresh culture medium and subsequently treated for 30 min with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3.

After several rinsing with PBS, slides were covered with Moviol 4-88 (Aldrich, Steinheim, Germany). For experiments done with HA^{FITC}-CTL/CHI, fresh *C. albicans medium*, previously incubated at OD620 =0.001 in SB culture medium during 24 h at 30°C without agitation, was put in contact with PEI-(HA^{FITC}-CTL/CHI)15 films during 45 min at 30°C without agitation. HA^{FITC} in solution and HA^{FITC}/CHI films were used as control. *C. albicans* were subjected to optical serial sectioning (0.2-0.3 μm) to produce image in the x-y plane. Each optical section was scanned several times to obtain an average image. Images were recorded digitally in a 768 x 576 pixel format.

4.4 Degradation analysis of synthetic peptides by S. aureus strains

S. aureus strains (ATCC25923, ATCC49775, S1 and S2) were precultured in MH broth medium at 37°C for 18-20h. Preculture was diluted to OD_{620nm} 0.001, which contains $5x10^5$ CFU/mL. Synthetic peptides were incubated with the diluted preculture at ½ MICs as previously determined. Suspension of bacteria and peptides were incubated at 37°C with agitation for 18-20 h. Triplicate wells were treated for each concentration of peptide. Media was sampled at different time intervals (0h, 2h, 4h, 8h, and 20h). Samplings were centrifuged at *10000 x g* for 15 min and the supernatants were filtered using Millex®-GV 0.22 μM (Millipore, Carrigtwohill, Ireland) to eliminate bacteria. The supernatants were stored -20°C until further use. In order to check sterility, 1 mL of each supernatant was incubated at 37°C for 72 h. Absence of a colony was interpreted as lack of viable bacteria. In parallel, several controls were performed: i) without peptides for bacterial growth control and ii) without bacteria, with peptides in the MHB medium.

4.4.1 Purification of bacterial supernatants by RP-HPLC

Bacterial supernatants were analyzed by using RP-HPLC to separate in different fractions for comparison. RP-HPLC system used was the same as described for PMNs secretions except the change of gradient which is indicated on the relevant chromatograms. Fractions were collected according to one minute time interval.

4.5 PSG degradation by the V8 protease

Secreted proteins from stimulated chromaffin cells were isolated from bovine adrenal medulla as previously described (Metz-Boutigue et al., 1998). The soluble material (1 mg) was incubated for 18 h at 37°C with *S. aureus* V8 protease Glu-C (E.C. 3.4.21.19;

Boehringer, Mannheim, Germany) at a protein-to enzyme weight ratio of 50:1 in 200 µL of 50 mM Tris-HCl, pH 8.3. The reaction was stopped by addition of 0.1% (v/v) trifluoroacetic acid (TFA) and concentrated by evaporation, but not to dryness, using a speed-vac centrifuge.

4.6 Human gingival fibroblasts viability assays

The biocompatibility of films was tested using Human gingival fibroblasts (HGFs). HGFs were extracted from human gingival connective tissue of healthy individuals according to a protocol approved by the ethics committee for patient protection of CPP Strasbourg Hospitals. Cells were grown in DMEM containing, 1g/L of glucose and supplemented with 10% fetal bovine serum and 100 IU/mL penicillin-streptomycin (all from GibcoR). Cells were cultured at 37°C in 5% CO2 in 75 cm2 flasks. HGF were used between the 6th and the 9^{th} passage. Before cell seeding, glass coverslips (O = 14 mm) coated with different architectures were irradiated by UV for 15 min. Then HGFs were seeded at $3x10^4$ cells per cm^2 and cultivated at 37°C under a 5% CO2 humidified atmosphere for different culture times (Day 1, Day 2 and Day 7).

Cell viability was assessed by Alamar BlueTM assay (Biosource International). This assay is based on the reduction of the blue, non-fluorescent resazurin dye to the pink and fluorescent resorufin dye by living cells (Page et al., 1993). The overall conversion rate is proportional to the metabolic activity of living cells (Voytik-Harbin et al., 1998). Viability was assessed for different times 1, 2 and 7 days.

After rinsing, cells with PBS, 10% reagent in complete medium were incubated for 2 h. After incubation optical density (OD) at 570 nm and 630 nm were determined with a microplate reader. The percentage of reduction of Alamar BlueTM was calculated according to the procedure provided by the manufacturer. The experiments were performed in triplicate.

PART: III

RESULTS AND DISCUSSION

MANUSCRIPT: I

PROTEOMIC ANALYSIS OF ACTIVATED NEUTROPHILS BY LUKE/D

Activation of neutrophils by the two-component leukotoxin LukE/D from *Staphylococcus aureus*: proteomic analysis of the secretions

(Under revision in Journal of proteome research)

Rizwan Aslam [‡], Benoît-Joseph Laventie [≠†], Céline Marban [‡], Gilles Prévost [≠], Daniel Keller [≠], Jean-Marc Strub [§], Alain van Dorsselaer [§], Youssef Haikel[‼], Corinne Taddei[‼] and Marie-Hélène Metz-Boutigue [‡*]

[‡] Inserm UMR 1121, Université de Strasbourg, Strasbourg, F-67000, France

[≠] EA 7290 Virulence bactérienne précoce, Fédération de Médecine Translationnelle de Strasbourg, Université de Strasbourg - CHRU Strasbourg, Institut de Bactériologie, F-67000 Strasbourg, France

[§] CNRS UMR 7178, Université de Strasbourg, Strasbourg, F-67000, France

[‼] Faculté d'Odontologie, Université de Strasbourg, Strasbourg, F-67000, France

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ABSTRACT

Staphylococcus aureus is responsible for severe bacterial infections in hospitals and healthcare facilities. It produces single and bi-component toxins (leukotoxins and hemolysins) that hinder innate immune function. Leukotoxin subunits bind to leukocyte cell membrane thus inducing transmembrane pores and subsequently, cell lysis. Leukotoxin LukE/D is a member of the bi-component toxin family, but to date, no study concerning its involvement in host-pathogen interactions has been reported. In the present study, we performed the proteomic analysis of the secretions recovered after activation of human neutrophils by leukotoxin LukE/D. The neutrophil secretions were purified by RP-HPLC and different fractions were analyzed by Edman sequencing, LC-MS/MS, immunoblotted for chromogranin-derived peptides and further analyzed for antimicrobial properties. Proteomic analysis revealed that neutrophil secretions constitute a large number of proteins related with immune boosting mechanisms, proteolytic degradation, inflammatory process and antioxidant reactions.

INTRODUCTION

Staphylococcus aureus (*S. aureus*), a commensal bacterial strain present in about 20–30% of the general population, is responsible for numerous nosocomial infections.[1] It causes a wide range of illnesses from minor skin infections to some of life-threatening diseases, such as sepsis, osteomyelitis, pneumonia, endocarditis, and meningitis.[2] Mostly, *S. aureus* infection starts with bacterial adhesion to host tissues and continues with colonization.[3, 4] Colonization is associated with adhesion proteins that belong to the "Microbial Surface Components Recognizing Adhesive Matrix Molecules" (MSCRAMMs) family. These proteins are important for the attachment of bacteria to fibronectin[5], plasma clot, etc.[6] In addition, *S. aureus* produces several virulence factors, including toxins[7] such as staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins A and B (ETA and ETB), α-, β-, γ- and δ-hemolysins, the bicomponent Panton-Valentine Leucocidin (PVL)[8] and the LukE/D[9] leukotoxin. LukE/D consists of two subunits: LukE and LukD. The toxin subunits bind to leukocyte cell membranes, inducing Ca^{2+} activated trans-membrane pores and subsequent cell lysis.[9-11] Expression of LukE/D is associated with several common diseases. As an example, it has been isolated with both epidermolysin A and B from 78% of retrospective and prospective cases of impetigo.[10] In addition, LukE/D has been isolated from 93.6% of patients with *S. aureus* associated post-antibiotic diarrhea, in combination with the enterotoxins (80.9% of isolates).[12] However, no study regarding the involvement of LukE/D on the host-pathogen interactions have been reported so far.

Neutrophils (PMNs) are part of the first line of defense against pathogens, as they are involved in infection clearing and contribute to the development of adaptive immunity. Previous data indicate that in humans, chromogranins (CgA and CgB) are present in secretory granules of PMNs[13, 14] and are overexpressed in plasma of the patients with systemic inflammatory response syndrome (SIRS) or sepsis.[15] Several endogenous CgA- and CgB-

derived fragments have been described as antimicrobial peptides (AMPs).[16] In addition to their direct antimicrobial effect against pathogens, they activate PMNs,[17] inducing a calmodulin-mediated influx of calcium and therefore the secretion of innate immune components such as AMPs and cytokines. [18] A comprehensive understanding of the regulation of neutrophils responses could be obtained by proteomic analysis of the secretions from activated neutrophils.[19, 20] Here, by using proteomics and computational analysis, we identified the secreted proteins from LukE/D-activated neutrophils in order to better define the effects of the dynamic regulatory network of neutrophils.

MATERIALS AND METHODS

PREPARATION OF LEUKOTOXIN LUKE/D

LukE and LukD were purified from *S. aureus* Newman strain (NTCC 8178) according to previous studies.[9, 21, 22] Briefly, one colony was grown in 5 mL of YCP medium composed of 3% (w/v) yeast extract (Oxoid), 2% (w/v) Bacto-Casamino acids (Difco), 2% (w/v) sodium pyruvate (Merck), 0.25% (w/v) Na_2HPO4, 0.042% (w/v) KH_2PO_4, pH 7.0 at 37°C for 18 h with vigorous shaking. Proteins were precipitated from the supernatant with the help of 80% (w/v) ammonium sulfate, resuspended and subjected to a first chromatography on a 50 mL XK26 SP Sepharose fast flow column onto an AktaPurifyer-10® (GE-Healthcare, Uppsala, Sweden).[21] The further purification was achieved by subjecting the eluted fractions separately to a two-step chromatography including a 8 mL Mono S® cation exchange FPLC column (GE-Healthcare, Uppsala, Sweden) followed by a 8 mL ReSource-Iso® hydrophobic FPLC column (GE-Healthcare, Uppsala, Sweden) conditioned into a HR-10® column (GE-Healthcare, Uppsala, Sweden).[21, 22] Purified compounds were controlled by SDS-PAGE (12%) on PHAST System (GE-Healthcare, Uppsala, Sweden) and solubilized in 50 mM Na-phosphate buffer (pH 7.0) supplemented with 150 mM NaCl[8, 9] at $A_{280\ nm}$ of 1.0. LukE and LukD were stored at -80°C until use. Purity and integrity of LukE and LukD were analyzed by Edman sequencing and MALDI-TOF.

ISOLATION OF POLYMORPHONUCLEAR NEUTROPHILS

Human PMNs were prepared with a purity of 98% as previously described[23, 24] from Buffy Coat from healthy sex non-discriminated donors, obtained from the Centre de Transfusion Sanguine de Strasbourg (France). Briefly, 12 mL of lympho-Prep solution (EuroBio, Courtaboeuf Cedex B, France) was added to 30 mL of Buffy Coat from healthy donors and centrifuged at 200 *g* for 20 min. After centrifugation the lower dark band was collected without disturbing the sediment and washed with neutrophil buffer (EGTA buffer) (140 mM

NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM EGTA (ethylene glycol-bis-(2-aminoethylether) N,N,N`,N`-tetraacetic acid), 10 mM HEPES, 3 mM Tris-base, pH 7.3). PMNs were further processed by dextran sedimentation[25] for 30 min and then centrifugated at 1600 *g* for 6 min. The remaining impurities of red blood cells were lysed by hypotonic shock in distilled water (18 mL) for 45 s and immediately suspended in a 2 mL hypertonic NaCl (9% w/v) solution to a concentration of 0.9% (w/v).[26] Finally, cells were washed with mM EGTA buffer and suspended at a concentration of 5×10^6 cells/mL in the same buffer.

FLOW CYTOMETRY MEASUREMENTS

For the flow cytometry analysis, PMNs were cultured at a concentration of 5 x 10^5 cells/mL in EGTA buffer. Flow cytometry measurements were carried out using a FacSort flow cytometer (Becton-Dickinson, Le Pont de Claix, France) equipped with a 1 mW argon laser tuned to 488 nm.[27] Activity of LukE/D on PMNs was evaluated using flow cytometry of cells previously incubated with ethidium bromide (25 nM)[28] for 10 min prior to addition of LukE and LukD. Ethidium fluorescence was measured using the Cell QuestProTM software (Becton, Dickinson and company, Franklin Lakes, USA) by using (FL3: λ_{Em} = 650 nm).[27] Cells were supplemented with different concentrations of each leukotoxin component: 2, 3, 6, 10, 20, 40 and 60 nM. The first image was taken at the time of addition of the toxins. Subsequently, fluorescence was recorded from 5 to 30 min post-treatment. As a control, the fluorescence measuring for dead cells was considered as 100. Fluorescence measurements in the absence of toxin were subtracted from the data and the results are presented as percentiles. Intracellular calcium kinetics were measured by using flow cytometry with cells previously loaded with 5 µM of Fluo-3 (Molecular Probes, New Brunswick, USA) for 1 h at 37°C, washed and resuspended in 0.1 mM EGTA buffer supplemented with 1.1mM Ca^{2+}.[28] Fluo-3 measurement were carried out every 30 s during 10 min from the fluorescence light 1 (FL1:

λ_{Em} = 530 nm) using the Cell Quest Pro™ software (Becton-Dickinson, Le Pont de Claix, France)[27]. Data represent an average of at least three independent experiments.

PREPARATION OF SECRETIONS AFTER PMNs STIMULATION BY LEUKOTOXIN LUKE/D

PMNs were cultured in EGTA buffer solution at 10^7 cells/mL. The two components of leukotoxin (LukE and LukD) were used each at 10 nM. Cells were stimulated with 1.1 mM $CaCl_2$ for 25 min at 37°C and centrifuged at 800 *g* for 10 min.[28] The supernatants were recovered and stored at -80°C.

RP-HPLC OF NEUTROPHILS SECRETIONS

Total protein concentration was measured using a Bradford Assay,[29] and 1 mg of total proteins was purified by RP-HPLC using a DIONEX Dual Gradient System (Dionex, Sunnyvale USA) and a Nucleosil RP300-5C18 column (5 µm particle size, 300-Å porosity; Macherey-Nagel, Hoerdt, France) at 20°C. Absorbance was monitored at 214 nm, at room temperature (25° C). The solvent system consists of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile-water (solvent B) with a flow rate at 700 µL/min. A gradient with elution time reference is indicated on the chromatograms. The fractions were collected every 1 min interval and concentrated by speed-vac and stored at -20°C.

WESTERN BLOT ANALYSIS

Secretion samples were loaded on a 15% SDS-PAGE and transferred to PVDF membrane (Hybond™-P, Amersham Bioscience, Little Chalfont, Bucks, UK). During migration a voltage of 50 V was applied for the first 30 min and then 110 V till end and 75 V during transfer for 70 min. For immunodetection Millipore Snap i.d.® 2.0 protein detection system (Millipore, Billerica, MA, USA) was used, using ChemiBlocker™ as blocking agent. The antibodies used are as follows: mouse monoclonal anti-CgA (anti-$CgA_{53–57}$),[13, 30] rabbit

polyclonal anti-CgB (anti-$CgB_{547-560}$)[31]. Immunolabelled bands were visualized by autoradiography after addition of ECL reagent (Amersham Bio-science, Buckinghamshire, UK).

ANTIBACTERIAL ASSAY

Micrococcus luteus (A270) and *S. aureus* (ATCC 25923) strains were used to assess the antibacterial properties of the secreted material. Bacterial strains were precultured aerobically at 37°C in a Mueller Hinton Broth (MHB) medium (Merck KGaA, Darmstadt, Germany), pH 7.3. For each isolate, one colony was transferred to 10 mL of MHB medium and incubated at 37°C for 18-20 h. Bacteria were resuspended in MHB medium, and antibacterial activity was assessed by measuring the inhibition of bacterial growth. 10 to 20 µL of PMNs secretion fractions purified by RP-HPLC were incubated with 90 µL of a mid-logarithmic phase culture of bacteria, with a starting absorbance of 0.001 at 620 nm. In the initial inoculums, bacteria were quantified to 5 x 10^5 colony forming unit (CFU)/mL by agar plate spreading method.[32] Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) were used as a positive control. Microbial growth was assessed by the increase of absorbance after 20h incubation at 37°C.[33, 34] The A_{620nm} values of control cultures growing in the absence of secretions and antibiotics were considered as 100% growth. Moreover, the A_{620nm} value with the antibiotics (Tetracycline and Cefotaxime) was considered as 100% inhibition (absence of bacterial growth was confirmed by agar plate spreading). Each assay was performed at least three times.

ANTIFUNGAL ACTIVITY

Neurospora crassa, Aspergillus fumigatus, Candida albicans, and *Candida tropicalis* strains were tested to evaluate the antifungal properties for the secreted material.

For the filamentous fungi, spores were resuspended at a concentration of 10^4 spores/mL, in Potato Dextrose Broth (PDB) medium (Difco, Becton Dickson Microbiology system, Sparks,

MD, USA) in half-strength, supplemented with Tetracycline (10 μg/mL) and Cefotaxime (0.1 μg/mL) to suppress bacterial growth.[35] Aliquots of PMN secretions purified by RP-HPLC (20 μL) were incubated with 80 μL of fungal spores. The suspension was incubated at 30° C for 24 h without agitation.[34, 35] Fungal growth was monitored microscopically after 24 h. Media alone was used as negative control and corresponds to 100% fungal growth and voriconazole (1 μg/mL) was used as a positive control and corresponds to 100% growth inhibition.

Concerning yeast, the protocol described above to test the antibacterial properties of the secretions was used, with two exceptions: the culture medium was Sabouraud (BioMerieux S.A., Marcy l'Etoile, France) and the incubation temperature was modified to 30°C with agitation. Media alone was used as negative control and corresponds to 100% fungal growth and voriconazole (1 μg/mL) was used as a positive control and corresponds to 100% growth inhibition.

PROTEOMIC CHARACTERIZATION BY EDMAN SEQUENCING

The N-terminal sequencing was carried out by automatic Edman sequencing method, with a bi-sequencer cartridge (Procise 473A, Applied Biosystems, Foster City, USA).[13] For sequences identification a query was made in the SWISS-prot database http://www.expasy.org/tools/Blast/. The protein characteristic parameters were given by http://www.uniport.org/

TRYPTIC DIGESTION

After RP-HPLC, collected fractions were concentrated using a SpeedVac. Collected fractions were submitted to a reduction with 10 μl of 10 mM DTT in 25 mM NH4HCO3 (1 h at 57°C) and alkylation with 10 μl of 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature. For tryptic digestion, the trypsin (12.5 ng/μL; Promega V5111, Madison, WI, USA) was suspended in 2 ml of 25 mM NH_4HCO_3.The enzymatic digestion was performed at 37°C

during 4 h at an estimated ratio enzyme/substrate 1/50. Before nano LC-MS/MS analysis, 1 µl of H_2O/TFA 0.1% was added and fractions were concentrated using a SpeedVac.

MASS SPECTROMETRY ANALYSIS

NanoLC-MS/MS was performed using a nanoACQUITY ultra performance liquid chromatography (UPLC®) system (Waters, Milford, MA, USA) coupled to a maXis 4G Q-TOF mass spectrometer (BrukerDaltonics, Bremen, Germany). The system was fully controlled by HyStar 3.2 (BrukerDaltonics). The UPLC system was equipped with a Symmetry C18 precolumn (20 x 0.18 mm, 5 µm particle size, Waters, Milford, USA) and an ACQUITY UPLC® BEH130 C18 separation column (75 µm × 200 mm, 1.7 µm particle size, Waters). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). 3 µL of each sample was injected. Peptides were trapped during 1 min at 15 µL/min with 99% A and 1% B. Elution was performed at 60 °C at a flow rate of 450 nL/min, using a linear gradient of 6-35% B over 28 minutes. The mass spectrometer was operating in positive mode, with the following settings: source temperature was set to 160°C while dry gas flow was at 5 liter per minute. The nanoelectrospray voltage was optimized to −5000 V. External mass calibration of the TOF was achieved before each set of analyses using Tuning Mix (Agilent Technologies, Paolo Alto, USA) in the mass range of 322-2722 m/z. Mass correction was achieved by recalibration of acquired spectra to the applied lock masses (methylstearate ([M+H]+ 299.2945 m/z) and hexakis(2,2,3,3,-tetrafluoropropoxy)phosphazine ([M+H]+ 922.0098 m/z)). For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes in the range of 100-2500 m/z (MS acquisition time of 0.4 s, MS/MS acquisition time between 0.05 s (intensity > 250,000) and 1.25 s (intensity <5000)). The 6 most abundant ions (absolute intensity threshold of 1500), were selected from each MS spectrum for further isolation and CID fragmentation using nitrogen as collision gas. Ions were excluded after acquisition of one

MS/MS spectrum and the exclusion was released after 0.2 minutes. Peak lists in mascot generic format (.mgf) were generated using Data Analysis (version 4.0; Bruker Daltonics, Bremen, Germany).

MS/MS DATA INTERPRETATION

The peak list has been searched against a NCBInr-derived combined target-decoy database (created 2010-03-23, containing 219120 target sequences plus the same number of reversed decoy sequences) using Mascot (version 2.4.1, Matrix science, London, England). During the database search, up to one missed cleavage by trypsin and two variable modifications (oxidation of Methionine (+16 Da), carbamidomethylation of Cysteine (+57 Da), were considered. The search window was set to 15 ppm for precursor ions and 0.05 Da for fragment ions. Mascot result files (.dat) were imported into the Scaffold 3 software (version 3.00.03; Proteome Software Inc., Portland, OR, USA) used to validate MS/MS based peptide and protein identifications. Mascot identifications required at least ion minus identity scores greater than 0 and ion scores greater than 40. Protein identification was accepted if the protein contained at least 1 identified peptide. The false discovery rate (FDR) was calculated to be <1% based on the number of decoy hits.

RESULTS:

PURIFICATION OF LUKE/D

LukE/D were isolated from the *Staphylococcus aureus* Newman strain (NTCC 8178) as described in the Materials and Methods section. Once isolated, they were analyzed by RP-HPLC to determine their purity (Figure 1). HPLC peaks were collected and analyzed by SDS-PAGE after Coomassie Blue staining and later by Edman sequencing (Figure 1). Experimental masses obtained by SDS-PAGE and MALDI-TOF were in accordance with the theoretical molecular values reported in www.uniprot.org, (LukE: UniProt; A6QI09, LukD: UniProt; A6QI08). For each protein, the N-terminal sequence determined by Edman sequencing confirms the identification of LukE/D compared to UniProt sequences.

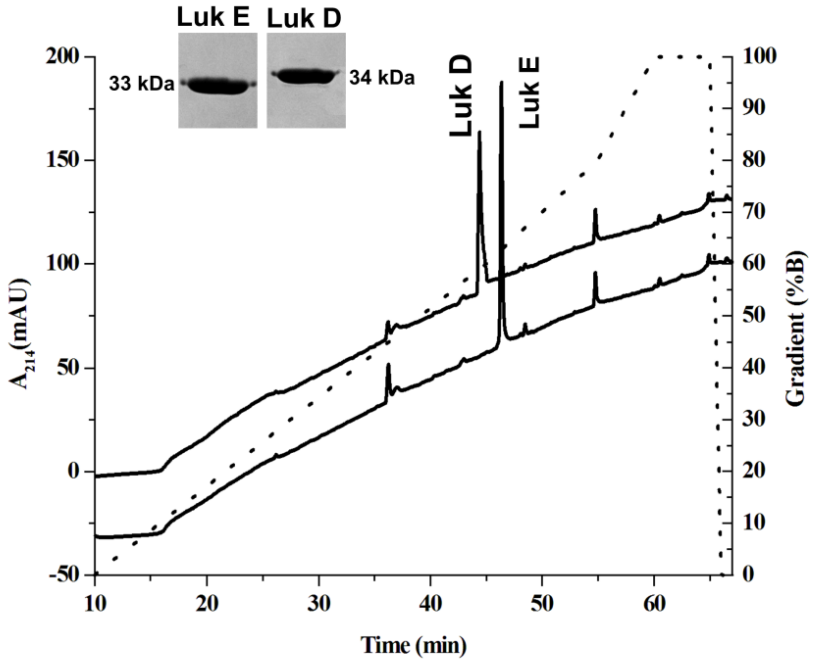

Figure 1: Reversed-phase HPLC of LukE and LukD and sequence analysis.

LukE and LukD (10 µg) were separately loaded on a reverse phase Nucleosil RP300-5C18 column. Elution was performed at a flow rate of 700 µL/min with a linear gradient as indicated by dotted line on the right-hand scale. Fractions with one min time interval were collected for mass spectrometry. LukE and LukD were further loaded on a 12% SDS-PAGE and stained by Coomassie Blue.

LUKE/D STIMULATE PMNs

To determine the effects of LukE/D on PMNs activation, we carried out flow cytometry. Two parameters were evaluated: firstly, the kinetics of the ethidium bromide entry into the cells (Figure 2A) and secondly the kinetics of Ca^{2+} influx into the cells (Figure 2B). LukE and LukD were used at a concentration ranging from 2 nM to 60 nM (Figure 2A, 2B). Ethidium

bromide uptake by PMNs is typically used as an indicator of pore formation.[23] We observed a rapid increase in ethidium bromide uptake, which continues during the first hour of the treatment (Figure 2A). These results are indicative of the toxicity of LukE/D towards PMNs. The presence of the toxins induced an increase of fluorescence and the time-course of ethidium bromide entry was dependant on the toxin concentration. Meanwhile an increase in calcium uptake by PMNs was also observed.

LukE/D at nanomolar concentrations stimulate PMNs by the entry of Ca^{2+} (Figure 2B), similarly to the effect observed for PVL,[17] which is mediated by the opening of the Ca^{2+} channels of the neutrophils plasma membrane.[24] With 10 nM of LukE/D, calcium uptake was rapid and later stabilized (Figure 2B). The observed increase in the intracellular calcium concentration is directly related to PMNs activation and production of cell secretions.[36] LukE/D leads to stimulation of PMNs, by triggering extracellular calcium influx and consequently production of secretory products. From the different concentrations tested, we selected 10 nM of LukE/D to induce cell secretion for further analyses, as 10 nM of LukE/D leads to a steady influx of calcium and abundant cell secretion.

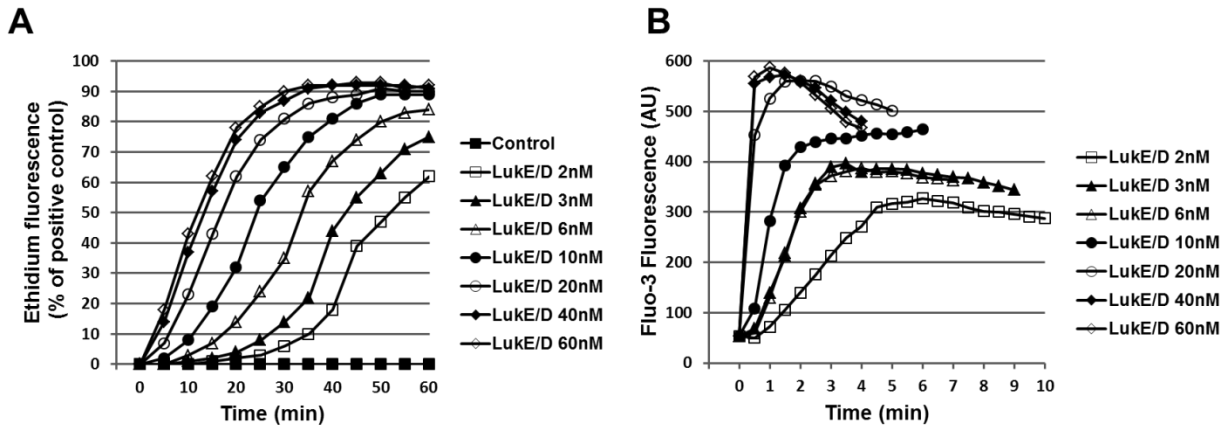

Figure 2: Time course of ethidium bromide and Ca2+ entry in human neutrophils.

A: incubation with various concentrations (2, 3, 6, 10, 20, 40 and 60 nM) of LukE/D. **B:** Time course of Ca^{2+}entry in human neutrophils incubated with various concentrations of LukE/D (2, 3, 6, 10, 20, 40 and 60 nM).

LEUKOTOXIN LUKE/D STIMULATES PRODUCTION OF AMPs AND OTHER IMMUNE RELATED PROTEINS

Secretions of PMNs were obtained after stimulation by 10 nM LukE/D, which leads to steady increase in ethidium positive cells (about 57% at 25 min incubation) (Supplementary Figure 1A, B). Moreover, cell debris was evaluated 6% of total cells (Supplementary Figure

1A, B). PMNs secretions were purified by RP-HPLC as described in the Materials and Methods section (Figure 3). One mg of PMN secretions was loaded on the HPLC column. Absorbance was monitored at 214 nm and elution was performed at a flow rate of 700 µL/min with a linear gradient, as indicated by the dotted line. Fractions were collected with 1 min time interval and marked according to the collection time. Collected fractions were concentrated using a speed-vac. Compared to the control of non-stimulated PMNs (PBS) (Figure 3: Chromatogram 1), LukE/D stimulated neutrophils (Figure 3: Chromatogram 2) and induced a rapid secretion of a large number of immune related factors. The 223 secreted proteins identified by mass spectrometry are listed according to the RP-HPLC fraction numbers (Supplementary data 1), together with their number of unique peptides, sequence coverage, Mascot scores and the peptides used to identify them (Supplementary data 2).

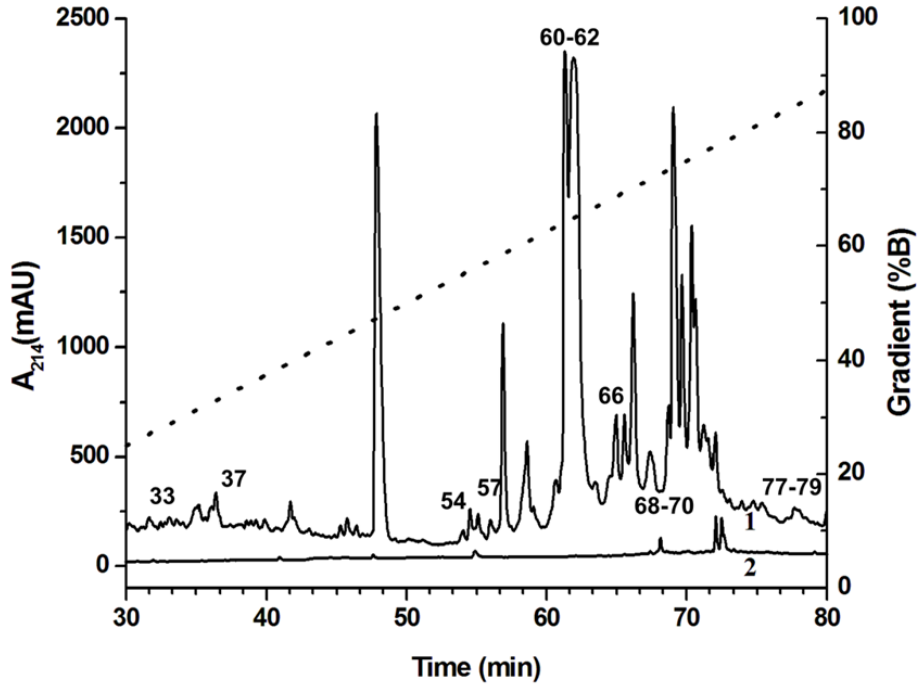

Figure 3: Reverse-phase HPLC of proteic material from LukE/D-induced PMNs secretions compared to the control.

(1) After stimulation with 10 nM of LukE/D and (2) as a control, after treatment with PBS.

Using gene ontology analysis software (GoTermMapper), all these factors were classified into different functional categories on the basis of their molecular functions, according to the Gene ontology database analysis (Figure 4). Based on the theme of innate immunity, we decided to classify the proteins into the following functional categories: (1) AMPs and other

immune related proteins, (2) binding proteins, (3) inflammatory proteins, (4) enzymatic proteins, (5) cell growth and differentiation proteins, (6) transport proteins, (7) cytoskeleton proteins, (8) antioxidant proteins, (9) proteins involved in proteolysis and (10) proteins related to protein synthesis (Figure 4).

The pie chart representing the identified proteins revealed a major secretion of AMPs and other immune related proteins (27% of total secreted products) (Figure 4; Table 1). HPLC fraction numbers containing the identified proteins were listed together with their NCBI accession numbers and their identification probability. In addition, the type of granule containing the proteins is also listed next to their position. Other abundant secreted proteins belong to the binding proteins (11%), inflammatory proteins (8%), enzymatic proteins (13%), cell growth and differentiation proteins (12%), transport proteins (11%), cytoskeleton proteins (10%), antioxidant proteins (2%), proteins involved in proteolysis (4%) and proteins related to protein synthesis (2%) (Figure 4). All these secreted factors well illustrate acute activation of the neutrophil cell machinery.

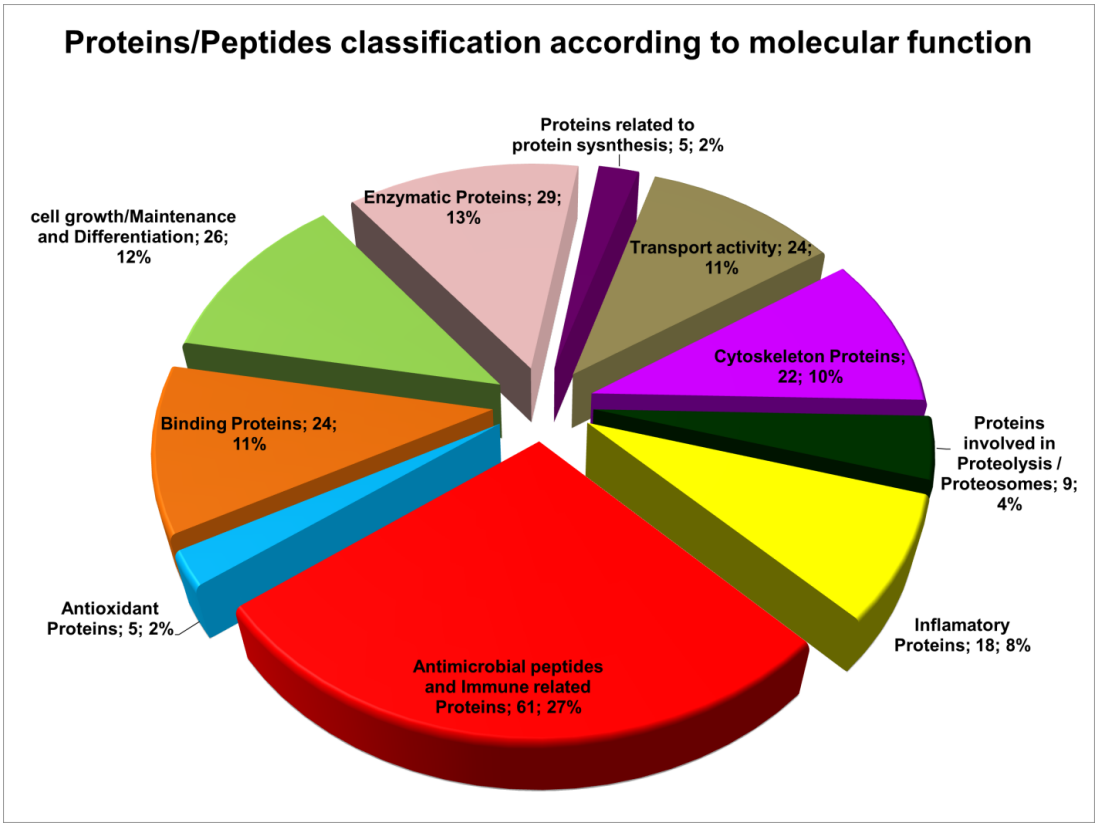

Figure 4: Pie chart representing the protein distribution according to their molecular function (GoTermMapper).

Table 1: Identification of proteins involved in innate immunity by LC-MS/MS analysis of RP-HPLC fractions corresponding to LukE/D-induced PMN secretions.

HPLC fraction number	Protein name	Protein accession numbers	Protein molecular weight (Da)	Total number of unique peptide	Percentage sequence coverage (%)	Granule type	Reference
28-31, 74-75, 80	alpha-1 antitrypsin variant	gi|110350939	46 607.8	4	12	AZ	[37]
29	Alpha-1-antichymotrypsin	gi|112874	47 653.0	1	3	AZ	[37]
39-42, 51-59, 68-69, 75-79	Alpha-enolase	gi|119339	47 170.2	12	41	GE	[14]
28-31	anti-SARS-CoV S protein immunoglobulin kappa light chain	gi|110626504	23 566.5	3	24	GE	[14]
74	Antithrombin-III	gi|113936	52 604.1	1	2	SP, GE	[14, 37]
62, 63	Calreticulin	gi|117501	48 142.9	1	7	GE, SP, AZ	[14]
56-57, 68	cathelicidin antimicrobial peptide	gi|119585258	19 591.6	5	30	GE, SP, AZ	[14, 38]
63	cathepsin C	gi|119579767	35 640.0	2	11	GE, SP, AZ	[14, 38]
37, 57-63	Cathepsin G	gi|115725	28 837.5	10	46	GE, SP, AZ	[14, 38]
37, 60-63	cationic antimicrobial protein CAP37	gi|227250	24 051.0	7	58	GE, AZ	[14, 38]
59	CD93 antigen	gi|119630576	68 549.1	1	2	AZ	[39]
68	Chitinase 3-like 1	gi|23512215	42 527.9	8	29	SP	[14, 38]
47-48	Chitotriosidase-1	gi|37999493	51 682.1	8	25	GE, SP, AZ	[14]
75	chromogranin A	gi|180529	50 929.5	1	1	SP	[14]
37, 61-62	Cofilin-1	gi|116848	18 503.2	8	57	GE	[14]

61	complement factor properdin	gi|119579721	49 664.4	1	3	GE, SP, SV	[14, 38]
59, 76	dermcidin isoform 2	gi|148271063	12 414.5	1	9	GE, SP	[14]
53-59	Eosinophil cationic protein	gi|147744558	18 385.3	5	37	GE, SP, AZ	[14]
78-80	Neuroleukin	gi|17380385	63 148.5	16	43	GE	[14]
28, 31, 34	hCG2029987	gi|119602340	43 900.2	7	28	GE	[14]
44	histocompatibility (minor) HA-1	gi|119589958	122 920.8	1	1	GE, SP	[14, 38]
28	Ig alpha-1 chain C region	gi|113584	37 653.8	3	20	GE, SP, AZ	[14]
28	Ig gamma 2 H chain	gi|243169	38 721.8	3	14	GE	[14]
56-60	IgG receptor IIIB	gi|11344591	26 189.0	3	15	GE, SP	[14]
57-61	IgG receptor precursor	gi|183037	26 299.0	3	14	GE, SP	[14]
28, 31	immunoglobulin heavy chain constant region	gi|10799664	35 919.3	4	19	GE, SP, AZ	[14]
37-38, 57-70, 72-79	lactoferrin	gi|34412	78 338.5	42	69	GE, SP, AZ	[14, 38]
61-63	lactoferrin precursor	gi|12083188	78 382.5	42	69	GE, SP, AZ	[14, 38]
77-79	Leukotriene A-4 hydrolase	gi|126353	69 287.1	15	36	SP	[14]
38-40, 42	Lymphocyte-specific protein 1; WP34	gi|462553	37 191.1	1	8	GE, SP	[14]
37-38, 60-63	lysozyme	gi|1335210	14 700.5	6	59	GE, SP, AZ	[14, 38]
44, 47, 70-72	matrix metallopeptidase 8 (neutrophil collagenase)	gi|119587431	53 426.3	10	31	GE, SP	[14]
69-79	myeloperoxidase	gi|119614877	88 763.7	17	28	GE, SP, AZ	[14]
40, 47, 50, 70-73, 79	Neutrophil collagenase	gi|116862	53 414.2	10	31	SP	[14]
54-55	Neutrophil defensin 4	gi|399352	10 504.1	1	18	SP, GE	[14, 38]

71, 73-79	Neutrophil elastase	gi|119292	28 517.4	3	27	SP, AZ	[14, 38]
63-68	Neutrophil gelatinase-associated lipocalin	gi|1171700	22 588.9	7	48	GE, SP	[14, 38]
46-53, 60-63	neutrophil granule peptide HP1	gi|228797	3 448.1	4	83		
57	non-specific cross reacting antigen	gi|189102	38 139.8	2	10		
37, 60-64	Peptidoglycan recognition protein 1	gi|18202143	21 730.6	5	49	GE, SP	[14]
63-66, 74, 77-79	Plastin-2	gi|1346733	70 292.1	25	49	GE, SP, AZ	[14]
34, 54-58	polyubiquitin	gi|11024714	68 492.8	5	8	GE, SP, AZ	[14]
78	PRO2783 (Mitogen-activated protein kinase kinase 1-interacting protein 1)	gi|11493534	13 589.0	1	13	SP	[40]
53, 57-63, 67, 76-77	Profilin-1	gi|130979	15 054.3	7	62	GE, SP, AZ	[14]
48	Proteasome activator complex subunit 1	gi|1170519	28 723.9	1	4		
79	proteasome activator subunit 2 (PA28 beta)	gi|119586505	21 752.7	1	12		
75	Proteasome subunit alpha type-6	gi|46397659	27 399.5	2	10		
76	proteasome subunit, alpha type, 3	gi|119601121	24 217.1	3	15		
79	proteasome subunit, alpha type 4	gi|119619571	25 047.6	3	18		
74	proteasome subunit, beta type 2	gi|119627813	20 208.6	1	9		
46, 67-74	Protein S100-A11; Calgizzarin	gi|1710818	11 741.1	3	34	SP	[14]
69-72, 77-78	Protein S100-A12;Calgranulin-C	gi|2507565	10 575.4	4	65	SP	[14]
73	Protein S100-A4; Metastasin	gi|115601	11 729.0	1	8	GE, SP, AZ	[14]
74, 76-77	Protein S100-A6; Calcyclin	gi|116509	10 180.4	4	48	GE, SP AZ	[14]
34, 63, 66-80	Protein S100-A8; Calgranulin-A	gi|115442	10 835.0	9	76	GE, SP, AZ	[14]

34, 36, 66-80	Protein S100-A9; Calgranulin-B	gi|115444	13 242.3	8	80	GE, SP AZ	[14]
76-79	Protein S100-P	gi|134142	10 400.4	4	72	GE, SP, AZ	[14]
28	serum amyloid A1	gi|119588814	13 562.6	1	12	GE, SP AZ	[14, 37]
60-61	SGP28 protein	gi|1587691	27 640.1	4	28	SP	[41]
47-49, 66-67	Vimentin	gi|55977767	53 652.7	14	34	GE, SP, AZ	[14]

GE: gelatinous granules, SP: specific granules, AZ: azurophilic granules

Table 2: Antibacterial assays on the RP-HPLC fractions.

Antibacterial assay on the RP-HPLC fractions (n=96), each containing 1 mg of LukE/D-induced PMNs secretions (Figure 3). Fractions with 100% of bacterial growth inhibition are indicated for each bacterial strain (*Micrococcus luteus* and *S. aureus*)

Antimicrobial HPLC Fraction no.	**Name of protein identified**	**Active (100% inhibition) against**	
		Micrococcus luteus	***Staphylococcus aureus***
54	Neutrophil defensin IV, Eosinophil cationic protein, Polyubiquitin	X	
57	Cathepsin G, Cathelicidin, Eosinophil cationic protein, Polyubiquitin, lactoferrin	X	X
60	Azurocidin, Eosinophil cationic protein, Lysozyme	X	
61	Cathepsin G, Lactoferrin, Lysozyme, Azurocidin	X	
62	Cathepsin G, Lactoferrin, Lysozyme, Azurocidin	X	
66	Lysozyme C, Lactotransferrin, Calgranulin A, Calgranulin B	X	
68	Calgranulin A, Calgranulin B, Calgizzarin, Cathelicidin antimicrobial peptide, Neutrophil gelatinase associated lipocalin, cathepsin G, lactoferrin	X	X
69	Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin,	X	X
70	Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin,	X	
77	Calcyclin, Lactoferrin, Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin, Protein S100-P,	X	X
79	Lactoferrin, Calgranulin A, Calgranulin B, Protein S100-P,	X	

Table 3: Antifungal assays on the RP-HPLC fractions.

Antifungal assay on the reverse phase HPLC fractions (n=96), each containing 1 mg of LukE/D-induced PMNs secretions (Figure 3). For each microorganism (*Neurospora crassa, Aspergillus fumigatus, Candida albicans* and *Candida tropicalis)* fractions displaying 100% of fungal growth inhibition are indicated.

Antifungal HPLC Fraction no.	**Protein identified**	**Active (100% inhibition) against**			
		Neurospora crassa	***Aspergillus fumugatus***	***Candida albicans***	***Candida tropicalis***
33	Calgranulin A, Calgranulin B, Polyubiquitin	X			
37	Cathepsin G, lactoferrin, Cationic antimicrobial peptide, Lysozyme	X	X	X	X
54	Neutrophil defensin IV, Eosinophil cationic protein, Polyubiquitin	X		X	
57	Cathepsin G, Cathelicidin, Eosinophil cationic protein, Polyubiquitin, lactoferrin	X	X	X	X
60	Azurocidin, Eosinophil cationic protein, Lysozyme	X	X		X
61	Cathepsin G, Lactoferrin, Lysozyme, Azurocidin	X			
62	Cathepsin G, Lactoferrin, Lysozyme, Azurocidin	X			
66	Lysozyme C, Lactotransferrin, Calgranulin A, Calgranulin B	X	X	X	
68	Calgranulin A, Calgranulin B, Calgizzarin, Cathelicidin antimicrobial peptide, Neutrophil gelatinase associated lipocalin, cathepsin G, lactoferrin	X	X	X	X
69	Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin,	X	X	X	X
70	Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin,	X	X	X	
77	Calcyclin, Lactoferrin, Calgranulin A, Calgranulin B, Calgranulin C, Calgizzarin, Protein S100-P,	X	X		
78	Calgranulin A, Calgranulin B, Calgranulin C, Lactoferrin, Protein S100-P,	X		X	X
79	Lactoferrin, Calgranulin A, Calgranulin B, Protein S100-P,	X	X		

ANTIMICROBIAL ACTIVITIES OF THE PMNs SECRETIONS

Antimicrobial assays were performed on RP-HPLC fractions against different bacterial, fungal and yeast strains. Two Gram-positive bacteria (*M. luteus* and *S. aureus*) were tested for their antibacterial activity and *N. crassa*, *A. fumigates, C. albicans* and *C. tropicalis* were challenged for their antifungal activity. Several fractions were active against the pathogenic strains used. Fractions expressing 100% bacteria and fungi growth inhibition are presented in Table 2 and Table 3, respectively.

CHROMOGRANIN A AND B-DERIVED PEPTIDES ARE SECRETED BY PMNs IN RESPONSE TO LUKE/D STIMULUS

In LukE/D-induced PMNs secretions, CgA and CgB were identified (Figure 5 A, B) by using two types of antibodies: a monoclonal anti-CgA ($CgA_{53–57}$) and a polyclonal anti-CgB ($CgB_{547-560}$). The control used in both cases is the granular proteic material of chromaffin cells (PSG), which is highly rich in Cgs and their derived peptides.[42] In the present experiment, we identified several fragments of both CgA and CgB: a major band of 75 kDa (apparent molecular weight) was recovered and corresponds to full-lenght CgA (CgA_{1-439}) (Figure 5A). In addition, according to previous studies, several fragments were also recovered including CgA_{1-291} (51 kDa), CgA_{1-249} (34 kDa), CgA_{1-209} (28 kDa) and CgA_{1-115} (24 kDa)[43, 44] (Figure 5A). Similarly, regarding CgB, a band of 100 kDa (apparent molecular weight), which corresponds to full-length CgB (CgB_{1-657}) (Figure 5B) as well as several shorter fragments were identified: $CgB_{156-657}$ (70 kDa), $CgB_{439-657}$ (37 kDa), $CgB_{498-657}$ (30 kDa), $CgB_{520-657}$ (25 kDa) and $CgB_{568-657}$ (20 kDa)[45] (Figure 5B).

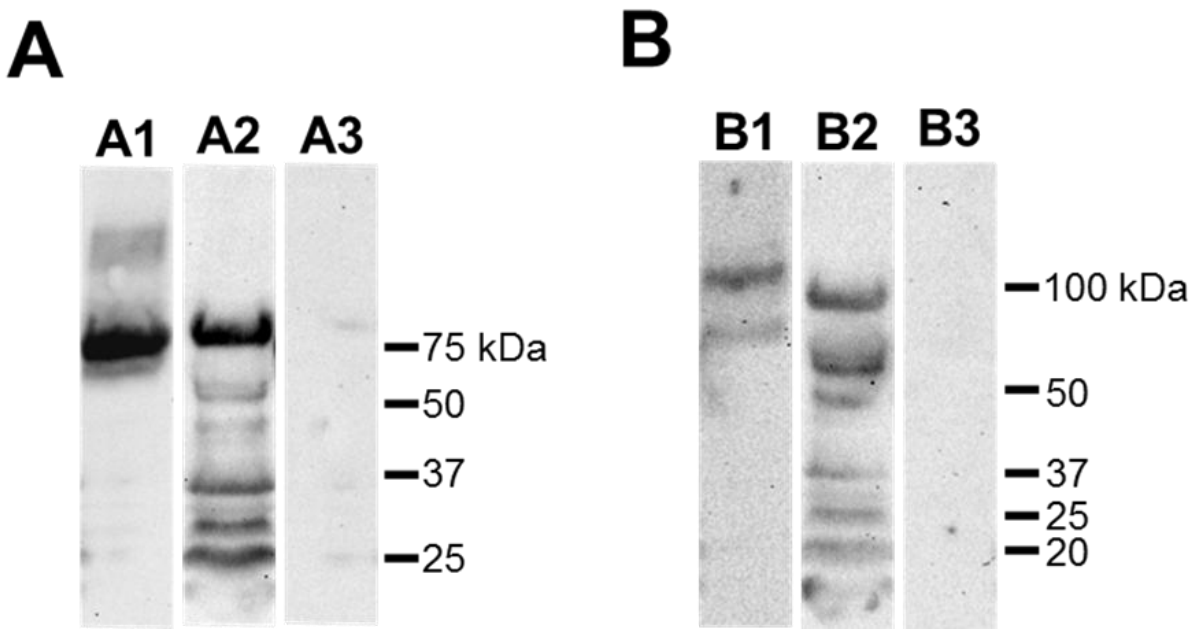

Figure 5: Immunodetection of CgA and CgB in the secretions of LukE/D-treated PMNs. ***A:*** Western blot analysis (12%, SDS-PAGE) with monoclonal antibody (anti- $CgA_{47–68}$). **A1,** Soluble material from chromaffin granules used as a positive control. A**2,** PMNs secretions obtained after stimulation with LukE/D. A**3,** Extra-cellular medium after PBS treatment. ***B:*** Western blot analysis (12%, SDS-PAGE) with polyclonal antibody anti-$CgB_{547-560}$. **B1,** Soluble material from chromaffin granules used as a positive control. B**2,** PMNs secretions obtained after stimulation with leukotoxin LukE/D. **B3,** Extra-cellular medium after PBS treatment.

DISCUSSION

LukE/D, a pore-forming leukotoxin produced by *S. aureus* strains infecting human, is specific against host defense cells and T-lymphocytes.[46-48] Several recent studies have underlined the crucial role of LukE/D in *S. aureus* virulence. LukE/D gene expression was significantly higher in community-associated methicillin-resistant *S. aureus* (*CA-MRSA*) compared with methicillin sensitive *S. aureus* (*MSSA*), suggesting that they contribute to bacterial virulence.[49] Analysis of the most prevalent genes associated with *MRSA* strains with type III *SCCmec* points out LukE/D in 72.9% of the isolates.[50] Furthermore, it was reported that in addition to PVL, a high prevalence of LukE/D was recorded in HIV (+) furuncles patients.[51] The role of LukE/D to trigger inflammation by acting synergistically with PVL to amplify IL-1b release in human macrophages has been demonstrated.[52] A biological mechanism explaining the toxic activity of LukE/D was proposed in a recent study.[48] Indeed, the authors demonstrate that LukE/D is specifically cytotoxic on human T-cell line expressing CCR5. Moreover, LukE, but not LukD binds to CCR5 in a time-dependent manner.[48]

In the present study, we characterized the proteins secreted by PMNs upon *S. aureus* LukE/D activation. PMNs are potent actors of innate immunity and serve as an important interface between innate and adaptive immunity by producing reactive oxygen species, antimicrobial peptides, chemokines and cytokines.[53, 54]

The 223 proteins identified in the neutrophil secretions after proteomic analysis were classified according to their molecular function by using go.princeton.edu/cgi-bh/GoTermMapper (Figure 4). Major proteins correspond to AMPs and immune related proteins (27%). PMNs display a potent microbicidal activity including oxygen-dependent and oxygen-independent strategies to destroy invading pathogens.[54] The production of ROS derived from NADPH oxidase is critical for host defense mechanisms against invading microorganisms.[55] In relation with oxidative mechanisms, the proteomic analysis indicates that 2% of antioxidant proteins and oxygen-independent microbicidal agents include AMPs[38] such as defensins, cathelicidins, azurocidin, cathepsins, lactoferrin, chromogranin A and lysozyme (Table 1). Enzymatic proteins, such as cathepsin, lysozyme, pyruvate kinase, neutrophil collagenase, eosinophil peroxidase and matrix metallo peptidase correspond to 13% and are important for proteolysis of AMPs and to manage the neutrophil turnover. Proteins for cell growth and development, such as epidermal growth factor (EGF), elongation factors, olfactomedin 4 (OLFM4) and neuroleukin represent 12% of the total released proteins and they participate to the rapid turnover of PMNs (Figure 4). All these proteins are crucial

for the dynamics of host-pathogen interactions with the multistep progression into mature PMNs.[56] Binding proteins correspond to 11% of the total proteins and include DNA binding proteins (histones, high mobility group protein B2), calcium binding proteins (calmodulin and its isoforms), adenylate cyclase and fatty acid binding proteins. The proteomic analysis also includes proteins involved in transport activity (hemoglobin, lactoferrin, and neutrophil gelatinase associated lipocalin), which play an important role in boosting innate defense at the site of infection. PMNs are the most efficient vertebrate motile defense cells. Their directional movement is governed by the binding of chemoattractants and chemokines to G-proteins coupled receptors. These motility responses start with the polymerization of F-actin and the rearrangement of cytoskeleton and cell crawling. The stimulation of neutrophils by LukE/D induces the release of cytoskeleton proteins (10%) such as actin, actinin, gelsolin, profilin and vimentin. The proteins involved in the modulation of inflammation represent 8% of the total proteins and play a key role in the early pro-inflammatory signals, as they release proinflammatory molecules to promote efficient pathogen recognition and removal.[54] Numerous secreted proteins are pro-inflammatory molecules such as cathepsin, chitotriosidase, cofilin, complement C3, cysteine rich secretory protein, eosinophil cationic protein, eosinophil peroxidase, neutrophil elastase, defensin-4, proteasome proteins, S100 A12, calgranulin, vimentin. In contrast, others such as alpha-1 antitrypsin, serpin, anti-thrombin III, neuroleukin, lactoferrin, lysozyme, MMP8, peptidoglycan recognition protein 1 and polyubiquitin are anti-inflammatory. Collectively, these observations suggest that LukE/D can enhance rather than hinder the host innate immune response to *S. aureus* infection. Nevertheless, the ability of LukE/D to enhance neutrophils bactericidal activity *in vivo* needs further investigation. For the first time Brinkmann *et al.* reported that activated PMNs release their DNA that is laden with antimicrobial molecules and form PMNs extracellular traps (NETs).[57] Later, in 2007 with a very detailed series of imaging experiments it was demonstrated that the formation of NETs in response to *S. aureus* is the last step in a process of active PMN death.[58] It was also reported that over the period of 3h, the PMNs underwent important morphological changes. The granular membranes disappeared and the nuclear, cytoplasmic and granular components were mixed together, allowing the extrusion of the NETs and therefore the antimicrobial role of PMNs even after cell death. PVL was described to be the major NET inducer, after inhibition of NET production by treatment with antiserum against PVL.[59] Our proteomic analysis of the PMNs secretions induced by LukE/D shows the presence of 6.25% cytoskeleton proteins (Figure 4) also suggesting the involvement of severe morphological changes.

Calcium is a universal secondary messenger involved in many cellular signal transduction pathways, regulating crucial functions such as secretion, cell motility, proliferation and cell death. Increase in intracellular Ca^{2+} derives mainly from two sources: internal stores releasing Ca^{2+} into the cytosol and Ca^{2+} channels in the plasma membrane that open to allow external Ca^{2+} to flow into the cell. In PMNs, calcium signaling has been reported to be involved in oxidase activation, cell degranulation and priming response to a wide range of proinflammatory molecules.[60] In the present study, we demonstrated that calcium binding proteins represent ~11% of the PMNs secreted proteins highlighting the importance of calcium in the stimulation of PMNs by LukE/D. For instance, vesicular amine transport (VAT-I) plays a crucial role in calcium regulated cell activation.[61]

Our previous studies have established the presence of CgA in neutrophils and its role as an immune activator.[17] Immunodetection of CgA (Figure 5) showed that LukE/D induces the predominant release of full-length CgA (75 kDa) but also of minor fragments with apparent molecular masses of 50, 35, 30 and 25 kDa that might correspond to fragments 1-298, 1-210, 1-182 and 1-115. Moreover, immunodetection of CgB (Figure 5) shows the release of 2 major fragments corresponding to full-length CgB (100 kDa) and the 143-657 fragment (70 kDa). In addition, several minor fragments 259-657 (50kDa), 306-657 (37 kDa), 555-657 (20 kDa) and 564-657 (10 kDa) were identified.

CONCLUSION

We previously described that an increase in intracellular free Ca^{2+} can induce secretion in PMNs.[62] These secretions can result from cell activation by the two LukE and LukD components of the LukE/D toxin of *S. aureus*. A similar effect was observed previously by using PVL of *S. aureus*.[23] Complement C3 factor (CD35) was recovered upon PVL treatment, which indicates neutrophil activation.[63] Here, we demonstrated that in addition to toxicity, LukE/D can also stimulate PMNs. Identification of trademark proteins from different PMNs granules underlines the ability of this leukotoxin to degranulate all type of compartments. PMNs secrete various proteins belonging to different functional categories, such as immune related proteins, antioxidant proteins, proteases and inflammatory components. Beyond these functional categories, AMPs and immune activation proteins represent a significant proportion of the secretions in this specific inflammatory stimulus. The release of all these factors is crucial for innate immune functions. As an example, we recovered Cgs-derived

fragments, which are active participants of the immune system. These findings underlie the importance to further study the role of leukotoxin LukE/D as immune activating components.

ASSOCIATED CONTENTS

Extended information of all the proteins/peptides identified by LC-MS/MS in PMNs secretions induced by *S. aureus* leukotoxin LukE/D, are listed in the supporting information. Data are presented according to RP-HPLC fraction numbers, which correspond to collection time (Supplementary data 1). Identified proteins are provided with their NCBI database accession numbers, molecular weight, percent sequence coverage, and corresponding peptide sequences (Supplementary data 1). Proteomic analysis was supplemented with Mascot Ion score, Mascot identity score and ion score-identity score (Supplementary data 2). Proteins/peptides identified by only one unique peptide and with Ion score less than 40 are presented with MS/MS spectra (Supplementary data 3).This information is available free of charge via the Internet at http://pubs.acs.org

* CORRESPONDING AUTHOR

Marie-Hélène METZ-BOUTIGUE,

Inserm UMR1121, Bâtiment Leriche, Faculté de Chirurgie Dentaire, Hôpital civil, Porte de l'Hôpital, F-67000, Strasbourg, France.

E-mail: marie-helene.metz@inserm.fr; Tel. (+33)3 68 85 54 71; Fax (+33)3 68 85 54 72.

PRESENT ADDRESS

† Division of Molecular Microbiology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of all authors. Rizwan Aslam has performed the experiments and prepared the manuscript and figures. Benoît-Josef Laventie

and Daniel Keller have purified and characterized LukE/D. Gilles Prévost supervised the study and with Céline Marban participated in preparation of the manuscript. Jean-Marc Strub and Alain van Dorsselaer brought their expertise in proteomic studies; Youssef Haikel and Corinne Taddei participated to interesting discussions and Marie-Hélène Metz-Boutigue supervised the experiments and the preparation of the manuscript. All authors have given approval to the final version of the manuscript.

NOTES

The authors declare no competing financial interest.

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MANUSCRIPT: II

CHROMOGRANIN A DERIVED PEPTIDE AND *S. AUREUS* INTERACTION

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Cateslytin, a chromogranin A derived peptide is active against *Staphylococcus aureus* and resistant to degradation by its proteases

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Rizwan Aslam[1], Céline Marban[1], Christian Corazzol[2], François Jehl[2], François Delalande[3], Alain Van Dorsselaer[3], Gilles Prévost[2], Youssef Haïkel[1,4], Corinne Taddei[4], Francis Schneider[5], Marie-Hélène Metz-Boutigue[1#]

[1] Inserm UMR-1121, Université de Strasbourg, Strasbourg, France

[2] EA-7290, Virulence bactérienne précoce, Fédération de Médecine Translationnelle de Strasbourg, Institut de Bactériologie, Université de Strasbourg - CHRU Strasbourg, Strasbourg, France

[3] CNRS UMR-7178, Institut Pluridisciplinaire Hubert Curien, Université de Strasbourg, Strasbourg, France

[4] Faculté de chirurgie dentaire, Université de Strasbourg, Strasbourg, France

[5] Service de Réanimation Médicale, Hôpital de Hautepierre, Université de Strasbourg, Strasbourg, France

#, Corresponding author: Marie-Hélène Metz-Boutigue, Inserm UMR 1121, Bâtiment Leriche, Faculté d'Odontologie, Hôpital Civil, Porte de l'Hôpital, Strasbourg, F-67000, France.

E-mail : marie-helene.metz@inserm.fr; Tel. +33 3 68 85 54 71; Fax +33 3 68 85 54 72.

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ABSTRACT

Innate immunity involving antimicrobial peptides represents an integrated and highly effective system of molecular and cellular mechanisms that protects host against infections. One of the most frequent hospital-acquired pathogens, *Staphylococcus aureus*, capable of producing proteolytic enzymes, which can degrade the host defence agents and tissue components. Numerous antimicrobial peptides derived from chromogranins, are secreted by nervous, endocrine and immune cells during stress conditions. These kill microorganisms by their lytic effect at micromolar range, using a pore-forming mechanism against Gram-positive bacteria, filamentous fungi and yeasts. In this study, we tested antimicrobial activity of chromogranin A-derived peptides (catestatin and cateslytin) against *S. aureus* and analysed *S. aureus*-mediated proteolysis of these peptides using HPLC, sequencing and MALDI-TOF mass spectrometry. Interestingly, this study is the first to demonstrate that cateslytin, the active domain of catestatin, is active against *S. aureus* and is interestingly resistant to degradation by *S. aureus* proteases.

INTRODUCTION

Chromogranins (Cgs) constitute the predominant family of proteins enclosed in secretory vesicles of chromaffin cells.[1] They are naturally processed to produce numerous peptides with various biological activities.[2-4] During the past decade, our group characterized several new antimicrobial peptides (AMPs) derived from chromogranin A (CgA) [5-8] and chromogranin B (CgB).[3,9] These peptides are released by stimulated chromaffin cells of adrenal medulla and also by activated polymorphonuclear neutrophils (PMNs).[5,7] Sequences of these peptides are highly conserved during evolution, suggesting that they are well-integrated in innate immune system.[10] Among these AMPs: chromofungin (CHR, CgA_{47-66}) and catestatin (CAT, $CgA_{344-364}$), derived from bovine CgA, activate PMNs and induce a calcium influx into immune cells.[11]

Staphylococcus aureus is the most frequently isolated pathogen in Gram-positive sepsis, often involved in blood clotting disorders and destruction of endocardial tissue.[12] *S. aureus* has developed several mechanisms to avoid immune response including resistance to AMPs,[13] impairment of phagocyte recruitment,[14] escape from neutrophil extracellular traps,[15] interference with complement,[16] neutrophil lysis, resistance to oxidative burst[17] and non-specific binding and degradation of immunoglobulins.[18] The AMPs evasion mechanisms deployed by *S. aureus* include proteolytic degradation by extracellular proteases of three major catalytic classes, namely metallo-, serine- and papain-like cysteine proteases.[19] The expression of proteolytic enzymes is controlled directly by global regulators of virulence factors such as *agr*, *sar* [20,21] and indirectly by RsbU that controls Sigma(B) activity.[22] Moreover, *SarA* is also a regulator of methicillin resistance factor (fmtA).[23] It has been previously reported that *S. aureus* metallo-protease aureolysin can cleave and inactivate human cathelicidin LL-37, thereby contributing to bacterial escape from the innate immune system.[13]

As staphylococci easily colonize skin and epithelia, regardless of the expression of antimicrobial Cgs-derived peptides,[24] we aimed to investigate the antimicrobial effects of CAT and its shorter fragment cateslytin (CTL, $CgA_{344-358}$) against *S. aureus*. Taking into account the difference in the activity of these peptides, using HPLC and proteomic analysis (sequencing and MALDI-TOF mass spectrometry), we examined the degradation of these CgA-derived peptides by staphylococcal proteases released into bacterial supernatants. Here, we report the relationship between peptide sequences and their sensitivity to bacterial proteases, as well as the possibility to use them as new antimicrobial agents in combination with antibiotics.

MATERIALS AND METHODS

Preparation and analysis of synthetic antimicrobial peptides

Synthetic peptides were prepared on an Applied Biosystems 433A peptide synthesizer (Foster City, USA), using the stepwise solid-phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Sequences of the synthetic peptides are as follows: bCAT ($bCgA_{344-364}$: RSMRLSFRARGYGFRGPGLQL), hCAT ($hCgA_{352-372}$: SSMKLSFRARGYGFRGPGPQL), bCTL (bCgA344-358: RSMRLSFRARGYGFR) and LL-37 ($hCAP_{134-170}$ LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES). Then, the synthetic peptides were purified by a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Macherey Nagel Nucleosil RP 300-5C18 column (10 × 250 mm; particle size 5 µm and pore size 100 nm). Synthetic peptides were analysed by an automated Edman sequencing on an Applied Sequencing System Procise (Applied Biosystems, Foster City, USA).[9] Finally, MALDI-TOF mass measurements were carried out on an Ultraflex™ TOF/TOF (BrukerDaltonics, USA), as previously described.[25]

Isolation and characterization of Staphylococcus aureus strains

Different *S. aureus* strains were used to demonstrate the peptide antimicrobial activity and subsequently, the peptide degradation: strains ATCC 25923, ATCC49775, S1 and S2, were provided by the Institute of Bacteriology, Strasbourg, France. S1 was isolated from the blood of an 83 y. o. patient and S2 was isolated from the *sputum* of a 12 days old neonate. After isolation and identification, *S. aureus* strains were assessed for their susceptibility to various antibiotics, using the agar disc diffusion method.[26] S1 was found Methicillin resistant (MRSA) and is also resistant to Amoxicillin, Oxacillin, Amikacin, Tobramycin, Fluoroquinolones, Erythromycin and Clindamycin. However, it was susceptible to Gentamicin, Synercid, Co-trimoxazole, Rifampicin, Fusidic acid, Vancomycin, Teicoplanin and Linezolid. In contrast, S2 was found to be Methicillin susceptible (MSSA) and is sensitive to all the antibiotics tested.

Antibacterial activity against S. aureus

Different *S. aureus* strains described above were first pre-cultured aerobically at 37°C for 20 h in a Mueller-Hinton-Broth (MHB) medium, pH 7.3 (Difco Laboratories, Detroit, MI). Bacteria were suspended at absorbance of 0.001 at 620nm in the MHB medium. Antibacterial activity was tested for 24 h incubation at 37°C with shaking by measuring the inhibition of bacterial growth. Ten µl final volumes (10-200 µg/mL) of synthetic peptides (LL-37, bCAT, hCAT and bCTL) were incubated in microtitration plates with 90 µl of a mid-logarithmic phase culture of bacteria, with a starting absorbance of 0.001 at 620 nm. In the initial inoculi,

bacteria were quantified by the agar plate spreading method which was 5 x 10^5 colony forming units (CFU)/mL.[27] Tetracycline (10 mg/L) and Cefotaxime (0.1 mg/L) were used as positive controls. Microbial growth was assessed by the increase of absorbance after 24 h incubation at 37°C.[28,29] The $A_{620\ nm}$ value of control cultures growing in the absence of peptide and antibiotics was defined as 100% growth. $A_{620\ nm}$ zero with the antibiotics (Tetracycline and Cefotaxime) was taken as 100% inhibition. Absence of bacterial growth was verified by agar plate spreading. Each assay was performed in triplicates.

Killing kinetics

Bacterial strains were first grown in MHB medium as described above. Bacteria killing kinetic activity was measured according to the previously described method, after making few modifications.[30] Initial inoculum was prepared at a concentration of 5 x 10^5 CFU/mL with absorbance of 0.001 at 620 nm, which was calculated by agar plate spreading method.[27] Bacteria were incubated with different concentrations of peptides (MIC and 2 x MIC), determined by MIC assay *via* microdilution method.[27] Viable bacterial count was then assessed at different time intervals up to 24 h. Briefly, aliquots were taken at different time intervals and were diluted in phosphate buffer saline (pH 7.4). After appropriate shaking 100 µL of the dilutions were plated on MH agar plates. Plates were incubated at 37°C and colony count was performed to determine CFU/mL after 24 h.

Preparation of S. aureus supernatants

S. aureus strains were precultured and plated on the agar plates and cultivated for 24 h at 37°C. After incubation, one colony *per* isolate was transferred to 10 mL of the MHB medium and incubated at 37°C for about 30 h to late stationary growth phase. After incubation, cultures were centrifuged at 10,000 x *g* for 15 min and supernatants were filtered using a 0.22 µM Millex®-GV (Millipore, Carrigtwohill, Ireland) to eliminate bacteria. The supernatants were stored at -20°C until further use. In order to check sterility, 1 mL of each supernatant was incubated at 37°C for 48 h. Absence of a colony was interpreted as lack of viable bacteria.

Degradation analysis of the synthetic antimicrobial peptides by S. aureus supernatants

Synthetic peptides catestatin (bovine and human) and cateslytin (bovine) were incubated at 37°C for 24 h, with the different strain supernatants, at a concentration corresponding to the MIC values. Triplicate wells were treated for each concentration of peptide. In parallel, several controls were performed: with and without peptides in MHB medium.

In order to prevent degradation, the above experiment was repeated. For which, protease inhibitor cocktail (complete protease inhibitors except metalloproteases) (Roche Diagnostic GmbH, Mannheim, Germany) supplemented with 100 µM of 1-10 phenanthroline (metalloprotease inhibitor) (Sigma Aldrich GmbH, Steinheim, Germany) was added to bacterial supernatants before the addition of synthetic peptides. The whole suspension was incubated at 37°C for 24 h prior to being analyzed by RP-HPLC.

RP-HPLC purification of CgA-derived peptides after incubation with S. aureus supernatants

The synthetic peptides treated with bacterial supernatants and different controls including culture medium, bacterial supernatants, and synthetic peptides were separated using a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Nucleosil reverse-phase 300-5C18-column (4×250 mm; particle size, 5 µm; porosity, 300 Å) (Macherey Nagel Hoerdt, France). Absorbance was monitored at 214 nm, and the solvent system consisted of 0.1% (v/v) TFA in water (solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile-water (solvent B). Elution was performed at a flow rate of 700 µL/min, with the gradient as indicated on chromatograms. Fractions were collected with one min time interval. They were subsequently concentrated by evaporation by using a speed-vac, however not allowing them to dry completely.

Automatic Edman sequencing of CgA-derived peptides

The N-terminal sequence of purified peptides was determined by automatic Edman degradation analysis on a Procise microsequencer (Applied Biosystems, Courtaboeuf, France). Samples purified by HPLC were loaded on polybrene-treated glass-fiber filters. Phenylthiohydantoin-amino acids (Pth-Xaa) were identified by chromatography on a C_{18} column (PTH C-18, 2.1 mm × 200 mm).[2]

Proteomic analysis

Mass determination was carried out on a Brucker BIFLEX™ Matrix-Assisted Laser Desorption Ionization - Time-Of-Flight mass spectrometer (MALDI-TOF) (equipped with the high resolution optics (SCOUT™) with X-Y multi-sample probe, a gridless reflector and with the HIMAS™ linear detector). With a maximum accelerating potential of 30 kV, the system can be operated either in linear or the reflective mode. Ionization was carried out with a 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 250 MHz in linear mode and 500 MHz in reflector mode using a 1 GHz digital oscilloscope (Lecroy model). Brucker supplied software hosted in Sun sparcworkstation was used for data processing and the instrumental control.[31] These

studies were realized using the matrix α-cyano-4-hydroxycinnamic acid, obtained from Sigma, and prepared as a saturated solution in acetone. A total of 1-2 µl of the sample matrix aliquot solution was deposited on the probe and dried by ambient air. A thin layer matrix crystal was obtained after rapid spreading and evaporation.[32] A micromolar analyte solution was applied to the matrix and allowed to dry under moderate vacuum. The whole preparation was washed by 1 µl of trifluoroacetic acid (0.5%) aqueous solution. This cleaning procedure helps remove remaining alkaline cations and often leads to an increase in sensitivity.

Statistical analysis

The MIC values (µg/mL) are reported as means of three independent experiments. To determine significance, between different peptides (rows) and bacterial strains (columns), one way ANOVA was used. Overall significance and correlation was evaluated by 4x4 factorial ANOVA for independent samples. Significance was accepted at $p \leq 0.05$.

RESULTS

Antibacterial activity of CgA-derived peptides against S. aureus

The aim of present study was to examine antibacterial activity of bovine and human CAT ($bCgA_{344-364}$ and $hCgA_{352-372}$) and bovine CTL ($bCgA_{344-358}$), against *S. aureus*. In comparison with the other peptides tested, CTL was found to be most active against all *S. aureus* strains tested (ATCC49775, ATCC25923, S1, and S2). Activity of these CgA derived peptides was compared to the well-known C-terminal peptide (LL-37) of hCAP-18 (Table I). Activity of bCTL was comparable to that of LL-37 ($p > 0.05$) against S1 and S2, but significantly different ($p < 0.05$) for ATCC49775 and ATCC25923. However, MIC (presenting 100 % growth inhibition) values for bCAT and hCAT were highly significantly different ($p < 0.01$) that were about 2-4 fold higher than short fragment CTL (Table I). MIC value for CTL varies between different *MRSA* and *MSSA* strains ($p < 0.05$) and can effectively kill bacteria at 45 µg/mL. While both human and bovine CAT have more than 100 µg/mL MIC values.

Table I: Antibacterial assays of catestatin (bovine, bCAT and human, hCAT) and cateslytin (bovine, bCTL) against different *S. aureus* strains, compared to the cathelicidin antimicrobial peptide-18 (LL-37). Results are presented as MIC (µg/mL) of each peptide against four *S. aureus* strains. Values represent the means of the triplicate (n=3) wells. Means with same letters are not significantly different ($p < 0.05$). Small letters (a, b, c) represents significance between different *S. aureus* strains, while capital letters (A, B, C) represent significantly difference between peptides.

Antimicrobial peptides	MIC (µg/mL)			
	S. aureus **ATCC49775**	***S. aureus*** **ATCC25923**	***S. aureus*** **S1 (MRSA)**	***S. aureus*** **S2 (MSSA)**
bCAT	100 ^{aA}	100 ^{aA}	100 ^{aA}	115 ^{bA}
hCAT	125 ^{aB}	135 ^{aB}	130 ^{aB}	150 ^{bB}
bCTL	37 ^{aC}	40 ^{aC}	37 ^{aC}	45 ^{bC}
LL-37	30 ^{aD}	30 ^{aD}	30 ^{aC}	35 ^{bC}

In order to detail CTL antimicrobial activity, we further determined the killing rate of CTL. Time-killing kinetic assays were performed against *S. aureus* (ATCC 25923). As shown in Figure 1, CTL acts very rapidly against *S. aureus*. AT concentration 2 x MIC CTL present 4 logs bacterial killing at within 20 min of treatment and 100% within 40 min (Figure 1A).

Moreover, at MIC, CTL reaches 100% bacterial killing within 60 min of treatment. This killing assay was continued till 24 h (Figure 1B) and bacteria were unable to grow further during 24 h. As CTL is the most active, we hypothesized this fragment is more resistant to proteases produced by *S. aureus*. In order to demonstrate the stability of CTL compared to human and bovine CAT, we incubated the three peptides with the supernatants of four different *S. aureus* strains.

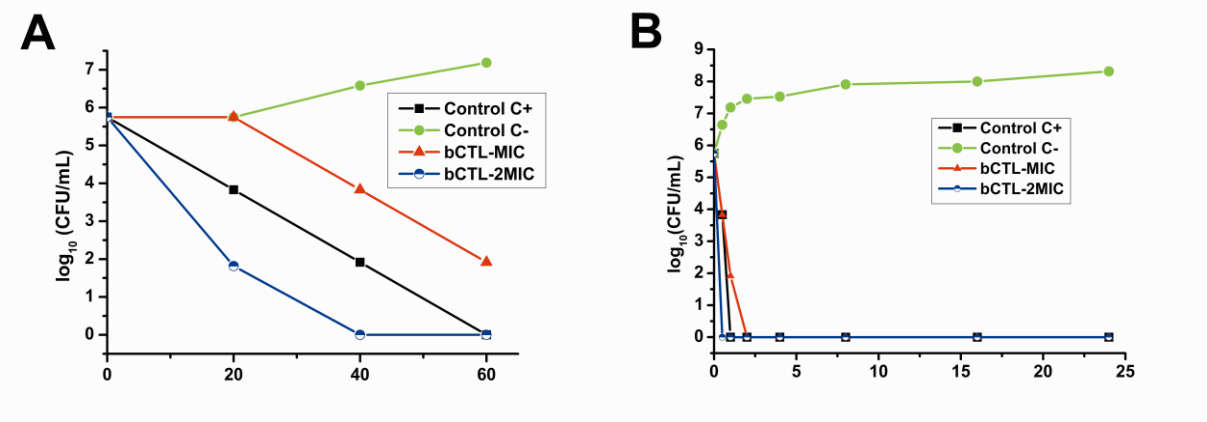

Figure 1: **The bacterial killing kinetics of the bCTL against the *S. aureus* ATCC 25923.** Different concentrations (MIC 40 µg/mL and 2x MIC 80 µg/mL) of bCTL were used. C+, represents antibiotic control (Cefotaxime 0.1 µg/mL + Tetracycline 10 µg/mL) and C-, represents phosphate buffer saline control. (A): *S. aureus* killing kinetics at time zero to 60 min. (B): *S. aureus* killing kinetics over 24 h time period.

Analysis of the proteolytic cleavage of catestatin (h/bCAT) and cateslytin (CTL) in the presence of S. aureus strain supernatants

S. aureus agr and *sar* regulate expression of proteases, which often are modulated by *SarA* regulator during biofilm formation.[33] These proteases are mostly expressed in the late growth phase and secreted in the extracellular environment to facilitate the bacterial spread.[34] As described above, *S. aureus* supernatants were prepared by the extended incubation period and are rich in proteases. Four *S. aureus* strains were tested for these experiments: two of them are referenced strains (ATCC 49775 and ATCC 25923), while two strains (S1 and S2) were isolated from the patients of Strasbourg hospital. S1 is a *MRSA* (methicillin resistant *S. aureus*) and S2 is a *MSSA* (methicillin sensitive *S. aureus*). The synthetic peptides were incubated with *S. aureus* supernatants and were separated by RP-HPLC (Figure 2 A, C, E). The HPLC profiles of CAT and CTL are compared with HPLC profiles of MHB medium and *S. aureus* supernatants. Peaks resulting from the bacterial

degradation were analyzed by automatic Edman sequencing and MALDI-TOF. In contrast to bovine and human CAT (Figure 2A, 2C), CTL is not degraded (Figure 2E).

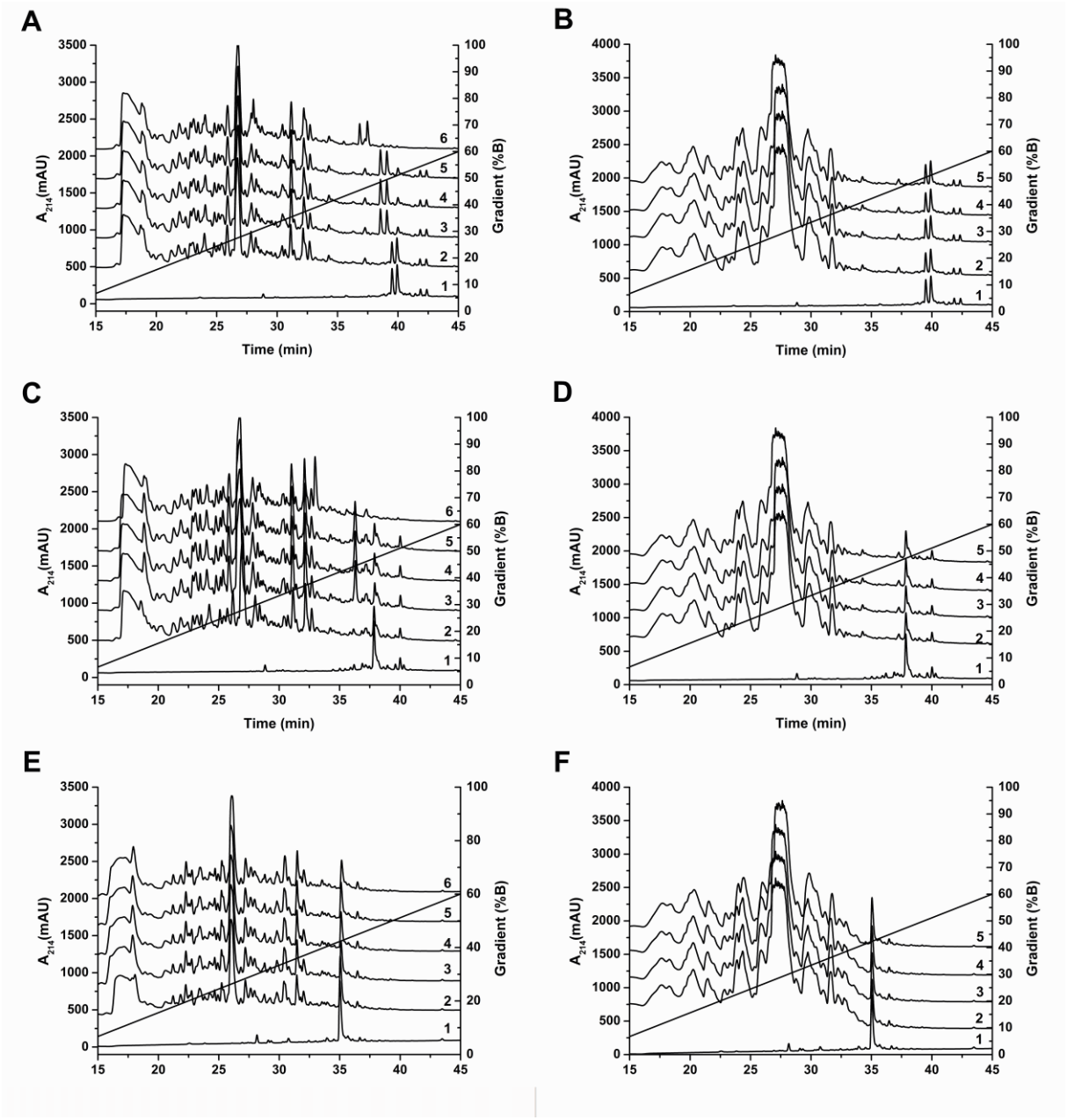

Figure 2: HPLC chromatograms of bCAT, hCAT and CTL alone or with different bacterial strain supernatants, with or without protease inhibitors. (A): Alignment of the HPLC chromatograms corresponding to: (1) bCAT, (2) bCAT+MHB, (3) bCAT+S49775, (4) bCAT+S25923 (5) bCAT+S1 (6) bCAT+S2. (B): Alignment of the HPLC chromatograms corresponding to: (1) bCAT, (2) bCAT+Pi+S49775, (3) bCAT+Pi+S25923 (4) bCAT+Pi+S1 (5) bCAT+Pi+S2. (C): Alignment of the HPLC chromatograms corresponding to: (1) hCAT,

(2) hCAT+MHB, (3) hCAT+S49775, (4) hCAT+S25923 (5) hCAT+S1 (6) hCAT+S2. (D): Alignment of the HPLC chromatograms corresponding to: (1) hCAT, (2) hCAT+Pi+S49775, (3) hCAT+Pi+S25923 (4) hCAT+Pi+S1 (5) hCAT+Pi+S2. (E): Alignment of the HPLC chromatograms corresponding to: (1) bCTL, (2) bCTL+MHB, (3) bCTL+S49775, (4) bCTL+S25923 (5) bCTL+S1 (6) bCTL+S2. (F): Alignment of the HPLC chromatograms corresponding to: (1) bCTL, (2) bCTL+Pi+S49775, (3) bCTL+Pi+S25923 (4) bCTL+Pi+S1 (5) bCTL+Pi+S2.

HPLC of bCAT (Figure 2A) indicates 2 major peaks, eluted at 38.7 and 40 min, corresponding to full-length peptide according to sequencing and MALDI-TOF mass spectrometry (2426 Da) (Figure 3). Presence of these 2 isoforms may be related to beta-turn conformation induced by proline residue in position 360 and its isomeric state (cis/trans).[35] In MHB medium, bCAT was not processed. Whereas, in presence of culture supernatants (S49775, S25923 and S1), bCAT was processed to generate fragments eluted at 38.3 and 38.6 min (Figure 2A). Sequencing and MALDI-TOF analysis indicate that these 2 fragments correspond to the isoforms of $bCgA_{349-364}$ (1782 Da) (Figure 3). In presence of culture supernatant of S2, bCAT was largely cleaved to generate fragments eluted at 28.0, 36.6 and 37.5 min (Figure 2A). Sequencing and MALDI-TOF analysis indicate that these fragments correspond to $bCgA_{350-356}$ (826 Da) and the 2 isoforms of $bCgA_{357-364}$ (887 Da) (Figure 3).

Moreover, the HPLC profile of hCAT (Figure 2C) showed one major peak at 38 min that corresponds to the full-length peptide according to sequencing and MALDI-TOF mass spectrometry (2327 Da) (Figure 3). In presence of MHB medium, hCAT was not cleaved. However, after incubation with S49775, S25923 and S1, hCAT was partially processed to generate a fragment eluted at 36 min. Sequencing and MALDI-TOF analysis indicated that this fragment corresponds to $hCgA_{357-372}$ (1780 Da) (Figure 3). In presence of supernatant S2, hCAT was completely cleaved to generate fragments eluted at 28.5 and 33 min (Figure 2C). Sequencing and MALDI-TOF analysis indicated that these fragments correspond to $hCgA_{358-364}$ (840 Da) and $hCgA_{365-372}$ (871 Da) (Figure 3).

Finally, the shortest peptide bCTL (bCTL, $CgA_{344-358}$), is eluted as a single peak (Figure 2E) and is not degraded by any of the staphylococcal strains tested.

Interestingly, incubation of synthetic peptides with bacterial supernatants supplemented with protease inhibitors, prevents the peptide degradation (Figure 2B, D, F). HPLC profiles of bCAT (Figure 2B: chromatogram 2-5) indicate that it is not cleaved by any of the four strain

supernatants tested in presence of protease inhibitors. Similar results were obtained for hCAT (Figure 2D: chromatogram 2-5). In addition, CTL remains unaffected by *S. aureus* supernatants with or without protease inhibitors (Figure 2E, F).

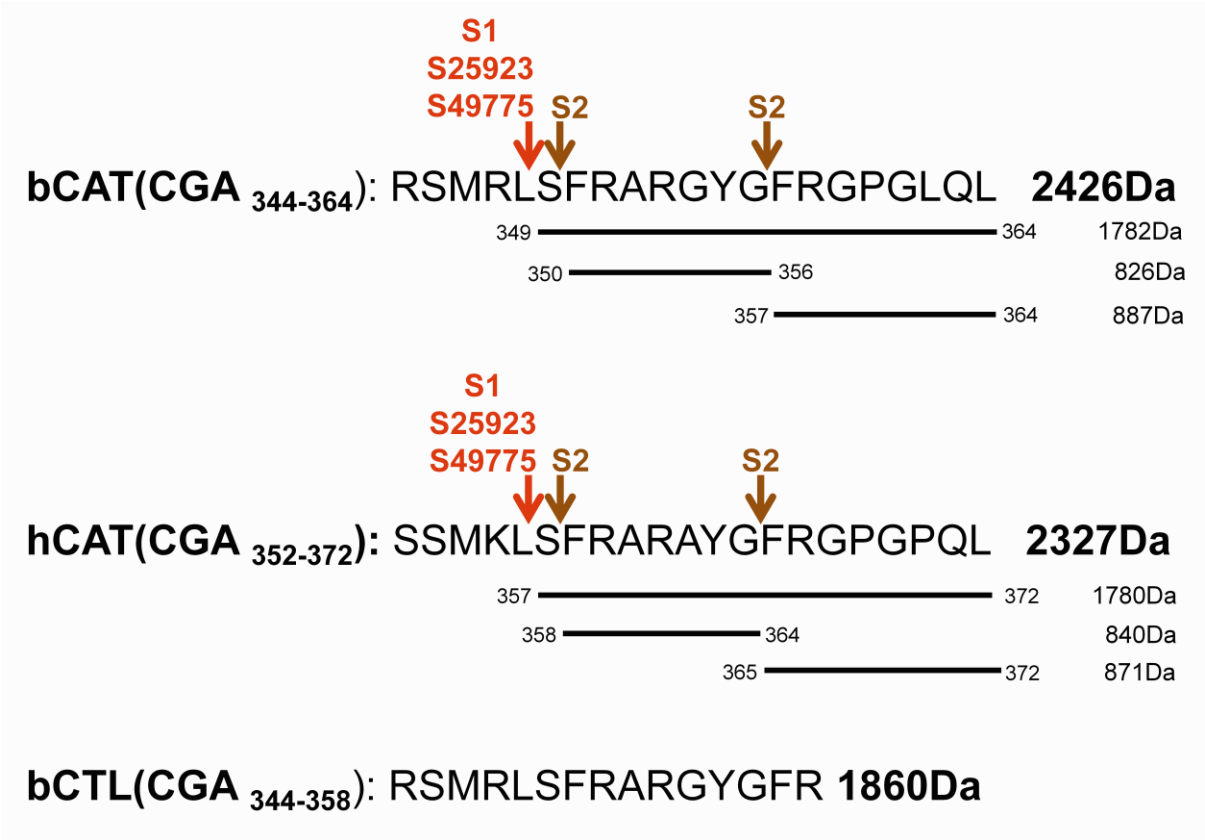

Figure 3: The proteolytic cleavage sites induced after treatment of bCAT, hCAT and CTL with different *S. aureus* strains (ATCC25923, ATCC49775, S1 and S2). The experimental molecular masses obtained after MALDI-TOF analysis, are indicated.

The proteolytic cleavage sites of bCAT and hCAT obtained after incubation with S49775, S25923 and S1 were identical (Figure 3). They corresponded to the peptide bonds L_{348}-S_{349} and L_{356}-S_{357} for bCAT and hCAT, respectively. With S2, they correspond to the peptide bonds S_{349}-F_{350}+G_{356}-F_{357} and S_{357}-F_{358}+G_{364}-F_{365} for bCAT and hCAT, respectively. It is important to point out that the short peptide bCTL ($bCgA_{344-358}$) derived from CAT was not cleaved by *S. aureus* proteases (Figure 2E, F) demonstrating that CTL resists to the proteolytic cocktail produced by *S. aureus*.

DISCUSSION

Due to continuous resistance development by *S. aureus*, the interest for natural AMPs is increasing because some of them display potent antibacterial activity. However, secondary reactions such as hemolytic activity,[36] limit their use as systemic anti-infective drugs. Until recently, no such reaction was reported for the antimicrobial Cgs-derived peptides in the literature. Several CgA-derived peptides were previously characterized to display antimicrobial properties,[9] however, data concerning killing of *S. aureus* is not reported. CAT was previously reported, to be active against different bacterial strains at micromolar range.[24] CTL is well characterized for antimicrobial activity against *Micrococcus luteus*, few yeasts and fungal strains. Moreover, it is not hemolytic for human erythrocytes.[5]. In the present study, for the first time, we brought to light the antibacterial activity of CTL against different strains of *S. aureus* with a MIC value of 37-45 µg/mL (Table I).

In vivo, a cross talk is established *via* dynamics of the interactions between host and bacteria. Many bacterial strains express a variety of proteases, ranging from non-specific and powerful enzymes that degrade many proteins involved in innate immunity, to proteases that are extremely specific in their mode of action.[37] Here, we questioned whether *S. aureus* proteases can cleave the three CgA-derived peptides studied. This proteolytic degradation could explain the 3-4 fold difference observed in antibacterial activity. To address this issue, we hypothesized that the human and bovine CAT peptides are degraded by the staphylococcal proteases and that CTL resists to the degradation and maintains its activity. Therefore, we tested the effect of four staphylococcal supernatants and demonstrated that bovine and human CAT are similarly degraded to generate inactive fragments, while CTL is not degraded. For bovine and human CAT, the cleavage sites induced by the supernatants are different. For S2 these correspond to the cleavage of S-F/G-F. Whereas, for ATCC49775, ATCC25923, and S1, it corresponds to cleavage of L-S (Figure 3). Upon cleavage by *S. aureus* proteases, the CAT peptides are no longer active against *S. aureus.* Previous data showed that bovine $CgA_{344-351}$ and $CgA_{348-358}$ display an antimicrobial activity against *Micrococcus luteus* at 20 µM, suggesting that L348/356 is important for its antibacterial activity.[5] Furthermore, in the protease inhibition assay, we demonstrated that degradation of human and bovine CAT can be prevented by the use of protease inhibitors. CTL maintains its position either with or without protease inhibitors indicating that proteases have no effect on the CTL.

The biosynthesis of CTL results by the action of prohormone convertases PC1/2 present in the granular matrix of chromaffin cells.[2,38] The prohormone thiol protease (PTP) was also reported to be essential for CAT synthesis ($bCgA_{344-364}$) by cleaving D-R and L-R.[38] In

addition, in chromaffin secretory vesicles, the cysteine protease cathepsin L (CTSL)[39] generates CTL by the additional cleavage R-G of CAT. CAT fragment is known to activate the neutrophils by inducing extra-cellular calcium influx in human neutrophils *via* calmodulin-regulated calcium independent phospholipase A2.[11] Here, we demonstrate that CTL is resistant to the degradation by staphylococcal proteases, which strengthens the involvement of this CgA-derived domain in innate immunity.[10,40]

The primary structures of CAT and CTL are highly conserved during evolution (Figure 4). Arginine ratio of these peptide sequences is important, since it modulates the interaction with negatively charges of the microorganism's membranes. In addition, it was previously shown that arginine residues have a high tendency to interact with the lipids as suggested for other peptides, such as HIV-1 transcriptional activator Tat protein.[41,42] For hCAT, bCAT, and bCTL the arginine ratios are 15%, 23%, and 33% respectively. High arginine ratio of bCTL supports a strong interaction with the negatively charged lipid bilayer as compared to the both CAT. Structure activity relationship of CTL with bacterial membrane is also demonstrated by recent experiments concerning the CTL derived peptide with a hydrophobic N-terminal end FLE-CTL (FLE-RSMRLSFRARGYGFR) (5R). With the help of HPLC and antimicrobial assays, we have shown that this synthetic peptide is inactive against *S. aureus* at 400 µg/mL and interacts strongly with bacterial membrane. In contrast, the shorter peptide that is lacking the C-terminal end (YGFR) of CTL: FLE-RSMRLSFRARG, displays antibacterial activities at 200 µg/mL (data not shown). Thus, CTL sequence possesses the cationic amphipathic features to exhibit potent antimicrobial activities.

Organism	Code	Sequence (5 10 15 20)	%
Bovine	P05059	RSMRL SFRAR GYGFR GPGLQ L	100
Human	P10645	SSMKL SFRGR GYGFR GPGPQ L	85
Human G364S	P10645	SSMKL SFRGR GYSFR GPGPQ L	80
Human P370L	P10645	SSMKL SFRGR GYGFR GPGLQ L	80
Macacafascicularis	Q4R4V1	RSMKL SFRAR AYGFR GPGPQ L	90
Macacamulata	12CTA3	RSMKL SFRAR AYGFR GPGPQ L	90
Pan troglodytes	H2Q8T7	SSMKL SFRAR AYGFR GPGPQ L	85
Gorillagorillagorilla	G3SJY7	SSMKL SFRAR AYGFR GPGPQ L	85
Nomascusleucogenys	G1S5U4	SSMKL SFRAR AYGFR GPGPQ L	85
Pongo abelii	H2NM30	WSMKL PFRAR AYGFR GPGPQ L	75
Otolemurgarnettii	H0WYW2	RSMKL SLQTR AYDFR GPGPQ L	76
Horse	Q9XS63	RSMKL SFRAR AYGFR GPGLQ L	95
Sus scrofa	P04404	RSMKL SFRAP AYGFR GPGLQ L	90
Caviaporcellus	H0VR23	RSMKL SFRAQ AYGFP GPEPQ L	80
Ailuropodamelanoleuca	GIM4X7	RSMKL SFRAR AYDFR GPGLQ L	90
Canislupus	C5NM83	RSMKL SFRAR AYDFR GPGLP L	85
Rattusnorvegicus	P10354	RSMKL SFRAR AYGFR DPGPQ L	85
Heterocephalusglaber	G5B2Y1	RSMKL SFQAP AYDFR GSGPQ L	76
Mus musculus	P26339	RSMKL SFRTR AYGFR DPGPQ L	80
Oryctolaguscuniculus	g1TSN0	RSMKL SFQPR AYGFR GPGPQ L	85
Cricetulusgriseus	G3HU91	RSMKL SFRAR AYGFR DPRPQ L	85
Callithrixjacchus	F3T376	HSMKL SFQAR AYDFR GPGPQ L	80
Loxodontaafricana	G3TBQ8	RSMKL SFRAQ AYGFP GPEPQ L	80
Consensus		+SM+L SF+a+ aYaF+ aPaPQ L	

Figure 4: Sequence alignment of bovine catestatin ($CgA_{344-364}$) with corresponding fragments from several species. For each position predominant identical residues are indicated in bold letters. Homology sequence is indicated (%).The data base used is UniProtKB. (+, basic residue; a, for A/G/T/P).

Concerning its secondary structure, an aggregated antiparallel beta sheet structure was previously reported for CTL [43] by CD (Circular Dichroism) and ATR (Attenuated total reflectance) experiments, whereas hCAT and bCAT form short helical structures (residue 7 to residue 11) in the presence of high concentrations of DPC, as confirmed by CD and NMR (Nuclear magnetic resonance) experiments.[44] CTL adopts a major β-sheet character only on negatively charged membranes, whereas it is essentially unstructured in water. These β-sheet formations give a much more stability to peptide than the helical formation reported for CAT.[44] In addition, the (HR-MAS) ^{1}H NMR analysis of CTL indicates that the arginine and hydrophobic residues are in close proximity, thus creating a deep penetration of charged

residues into the membrane.[42] Therefore, the electrostatic interaction between positively charged arginine residues and negatively charged lipids appears to be responsible for binding of CTL to the lipid bilayer and the aromatic residues stabilize the lipid-peptide interaction.

In our group, we recently evaluated the synergistic effect of three CgA-derived peptides (CAT, CTL and amidated CTL) with antibiotics and demonstrated that this co-treatment induce a reduction of the antibiotics concentration used and can potentiate their activities. Antimicrobial assays were carried out by combining the AMPs at concentration below the MIC. The comparison was made with antibiotic or peptide separately at the same doses. For all these experiments, we evaluated the Fractional Inhibitory Concentration (FIC) of CgA-derived peptides combined with Minocyclin or Voriconazole. FIC corresponds to a synergistic effect in the range of ≤0.5, an additive effect when it is between >0.5 and <2, and an antagonistic effect when it is >2.[45] For the combination of amidated CTL and Minocyclin, we obtained a FIC of 0.37 against *S. aureus,* and for CTL and Voriconazole, we obtained a FIC of 0.25, and 0.5 against *Candida albicans* and *Candida tropicalis* respectively.[10] Regarding these data, one could imagine a mechanism in which, the peptides could favor destabilization of bacterial membrane, thus allowing the antibiotics to rapidly penetrate inside bacterial cells to reaching their site of action. These *in vitro* results are likely to occur *in vivo*, especially during systemic inflammation. Indeed, sepsis triggers numerous changes in proteases and inhibitors activity.[46] These regulations have been strongly related with sepsis severity.[47] Under such influences, CAT and CTL could even be difficult to release, owing to structural modifications of CgA,[48] due to sepsis-related oxidative stress. These modifications include oxidation of methionine, aromatic residues, glycanes, phosphorylation and also aggregation of the complete protein that might prevent the processing of CgA to produce active antimicrobial peptides. The structure-function relationship of AMPs is also very important to unravel the regular pattern of antibacterial activity. It has been previously demonstrated, that selective end tagging or short hydrophobic stretches can be, added to increase AMPs activity.[49,50] Recently, we studied the properties of a modified CTL by inserting cysteine at C-terminal. This modified CTL was used to develop antimicrobial polymer coatings *via* alternative deposition of polyelectrolytes conjugated to CTL-C.[51] Activity of CTL is not altered, even with this modification or when inserted into PEM (Polyelectrolyte multilayer) coatings. Moreover, it is non-toxic for human gingival fibroblast.[51]

To conclude, for the first time we present the involvement of staphylococcal proteases on the cleavage of antimicrobial CgA-derived peptides. As the CTL domain is resistant to

staphylococcal proteases, it constitutes a promising natural antibacterial peptide for further studies.

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Transparency Declarations

None to declare.

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MANUSCRIPT: III

CHROMOGRANINS AND GLU-C PROTEASE

MANUSCRIPT: IV

BIODEGRADEABLE ANTIMICROBIAL POLYELECTROLYTE MULTILAYER COATING

Self-Defensive Biomaterial Coating Against Bacteria and Yeasts: Polysaccharide Multilayer Film with Embedded Antimicrobial Peptide

*G. Cado, R. Aslam, L. Séon, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J.-C. Voegel, B. Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf, H. Kerdjoudj, M.-H. Metz-Boutigue, and F. Boulmedais**

Prevention of pathogen colonization of medical implants is a major medical and financial issue since infection by microorganisms constitutes one of the most serious complications after surgery or critical care. Immobilization of antimicrobial molecules on biomaterials surfaces is an efficient approach to prevent biofilm formation. Herein, the first self-defensive coating against both bacteria and yeasts is reported where the release of the antimicrobial peptide is triggered by enzymatic degradation of the film due to the pathogens themselves. Biocompatible and biodegradable polysaccharide multilayer films based on functionalized hyaluronic acid by cateslytin (CTL), an endogenous host-defensive antimicrobial peptide, and chitosan (HA-CTL-C/CHI) were deposited on a planar surface with the aim of designing both antibacterial and antifungal coating. After 24 h of incubation, HA-CTL-C/CHI films fully inhibit the development of Gram-positive *Staphylococcus aureus* bacteria and *Candida albicans* yeasts, which are common and virulent pathogens agents encountered in care-associated diseases. Hyaluronidase, secreted by the pathogens, leads to the film degradation and the antimicrobial action of the peptide. Furthermore, the limited fibroblasts adhesion, without cytotoxicity, on HA-CTL-C/CHI films highlights a medically relevant application to prevent infections on catheters or tracheal tubes where fibrous tissue encapsulation is undesirable.

1. Introduction

Implantable medical devices are widely used in surgery not only to replace altered or lost tissues but also in critical care for fluid or gas administration using catheters or tracheal tube, respectively. These devices constitute an open gate for pathogens invasion.[1] Prevention of pathogen colonization of medical implants constitutes a major medical and financial issue since nosocomial infection represents one of the most serious complications after surgery or critical care. Indeed each year in Europe, 5% of patients admitted to hospitals suffer from hospital-acquired infections leading to a mortality of 10%.[2] *Staphylococcus aureus*, a Gram-positive bacterium, is responsible for hospital-acquired infections especially in immunocompromized patients. It is one of the most virulent bacteria leading to high rates of device-related systemic infections and mortality.[3] A recent study has genetically characterized the strains of *S. aureus*

Dr. G. Cado, L. Séon, Dr. T. Garnier, Dr. A. Parat,
Dr. Y. Frère, Dr. L. Jierry, Prof. P. Schaaf, Dr. F. Boulmedais
Centre National de la Recherche Scientifique
Institut Charles Sadron
UPR 22, 23 rue du Loess,67034 Strasbourg Cedex 2, France
E-mail: fouzia.boulmedais@ics-cnrs.unistra.fr
R. Aslam, Dr. R. Fabre, A. Chassepot, Dr. J.-C. Voegel,
Dr. B. Senger, Prof. P. Schaaf, Dr M.-H. Metz-Boutigue
Institut National de la Santé et de la Recherche Médicale
INSERM UMR 1121, 11 rue Humann, 67085 Strasbourg Cedex, France
Université de Strasbourg
Faculté de Chirurgie Dentaire
1 place de l'Hôpital, 67000 Strasbourg, France
Prof. F. Schneider
Service de Réanimation Médicale
Hôpital de Hautepierre
Hôpitaux Universitaires de Strasbourg
1 avenue Molière, 67098 Strasbourg Cedex, France
Dr. H. Kerdjoudj
EA 4691, Université de Reims Champagne Ardenne
SFR-CapSanté, 1 avenue du Maréchal Juin
51100 Reims, France
Dr. L. Jierry, Prof. P. Schaaf
Université de Strasbourg
Ecole Européenne de Chimie
Polymères et Matériaux
25 rue Becquerel, 67087 Strasbourg Cedex 2, France
Dr. L. Jierry, Prof. P. Schaaf, Dr. F. Boulmedais
International Center for Frontier Research in Chemistry
8 allée Gaspard Monge, 67083 Strasbourg, France
Prof. P. Schaaf
Institut Universitaire de France
103 boulevard Saint-Michel, 75005 Paris, France

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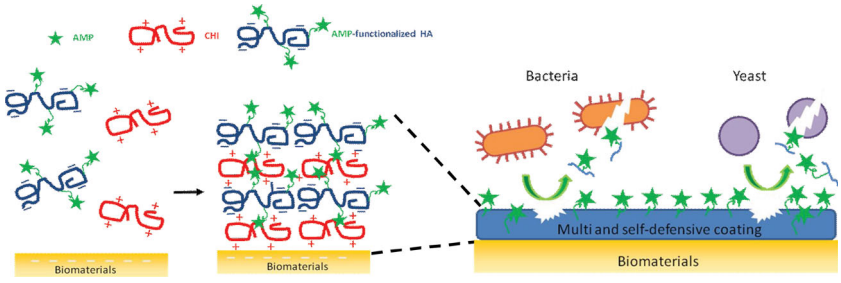
responsible of catheter-related infections and demonstrated that 82% of these strains are methicillin resistant and contain numerous genes involved in biofilm formation and bacterial dispersion.[4] *Candida albicans*, the most common human yeast pathogen, possesses the ability to form biofilms that are sources of local and systemic infection. Moreover *C. albicans* biofilms allow the formation of *S. aureus* microcolonies on their surface and even enhanced *S. aureus* resistance to antibiotics.[5] When associated with bacterial infections, fungal proliferation induces an increased frequency or severity of diseases.[6–8] The recent resistance of *C. albicans* to antifungal therapies[9–10] and of *S. aureus* to antibiotics points out the need of multifunctional coatings that prevent infections of both yeast and bacteria.

Various approaches based on immobilization or release of bactericidal substances, using self-assembled monolayers or grafting of polymers have been explored and extensively reviewed.[11,12] Polyelectrolyte multilayer (PEM) films, based on an alternated deposition of polycations and polyanions onto a solid surface, emerged as a simple and efficient approach to functionalize surfaces in a controlled way.[13,14] The first antibacterial PEM films were designed by insertion of silver nanoparticles.[15–20] Later on, hydrophobic bactericide using dendritic block copolymer[21] and liposomes containing silver ion[22] were inserted in PEM films to obtain efficient bactericidal coatings. Chitosan based PEM films were demonstrated to be antibacterial against *Escherichia coli* and *Enterococcus faecalis*.[16,23,24] Antibiotics, like gentamicin, have been inserted in hydrolysable[25] or crosslinked PEM films[26] to be later released. Despite an improvement of the antibacterial activity of the films, the use of antibiotics or silver particles have serious drawbacks because of their limited efficiency, their toxicity or their role in the emergence of multi-resistant pathogens.[27,28]

Natural antimicrobial peptides (AMPs), secreted by numerous living organisms against pathogens, gain increased attention due to their broad spectrum of antimicrobial activity and their low cytotoxicity.[12] They predominantly cause disruption of the membrane integrity of pathogen agents and thus unlikely initiate the development of resistance.[12] Positively charged AMPs were already used as a part of the PEM architecture to obtain antibacterial effect by contact[29] or by release.[26,30] Guyomard et al. succeeded in embedding in PEM films a complex of poor water soluble AMPs and amphiphilic polyelectrolytes. They obtained antibacterial activity against Gram-positive bacteria.[31] Active PEM films with embedded antifungal peptides were also reported.[32,33] Yet, it would be of high interest to design coatings bearing both antibacterial and antifungal properties. Up to now, only few systems share both properties. They are mainly based on silver coating[34] or quaternary ammonium cationic molecules as surfactant,[35] synthetic polymer,[36] or silane.[37]

To our knowledge, no coating based on AMP peptides possessing both properties has been reported so far. To achieve this goal, we used bovine cateslytin (CTL), a Chromogranin A (CGA) derived peptide, an endogenous protein, secreted with its numerous natural derived peptides by nervous, endocrine and immune cells during infection[38] acting in the innate immunity system.[39] CTL, an AMP corresponding to CGA344-358, acts in the micromolar range with a wide spectrum of antimicrobial activities against Gram-positive bacteria, yeasts and filamentous fungi, without cytotoxic effect on mammalian cells.[40,41] Moreover, it is very stable against bacterial proteases.[42] We used polysaccharide multilayer films based on CTL-C-functionalized hyaluronic acid as polyanion and chitosan as polycation, (HA-CTL-C/CHI), that were deposited on a planar surface with the aim of designing a self-defensive coating against both bacteria and yeasts (**Scheme 1**). A cysteine residue (C) was added at the C-terminal end of the CTL sequence to allow its grafting to HA. HA and CHI are biodegradable by enzymatic hydrolysis with hyaluronidase[43] and chitosanase[44] respectively. Both polysaccharides are already widely used in biomedical applications due to their interesting intrinsic properties.[45,46] The ability of *Staphylococcus*,[47] *Candida* species[48] and *M. luteus* to degrade HA, by producing hyaluronidase, allows the CTL–C to be released from PEM films only in the presence of the pathogens. Release of antibacterial compounds (AMPs or classical antibiotics) are usually obtained by passive diffusion from the films at physiological pH[31,32] or by pH-induced degradation of the films.[25,30] Pavlukhina et al. reported the release of antimicrobial agents using pH variations associated with growth of bacteria as an internal trigger to release.[26] This coating can thus be named as self-defensive as it is related to a local change of the environment of the coating due to the pathogens themselves. This was the first system developed based on this idea. We developed herein a new self-defensive coating where the release of the antimicrobial peptide is triggered by enzymatic degradation of the film due to the pathogens themselves. Polysaccharides adsorbed mass was determined by Surface Plasmon Resonance (SPR). The buildup and the topography of the films were characterized by Atomic Force Microscopy (AFM). Antibacterial and antifungal activities of HA-CTL-C in solution and HA-CTL-C/CHI films were tested against two strains Gram-positive bacteria, i.e., *S. aureus* and *M. luteus* and one strain of yeast strain *C. albicans*, respectively, by using microdilution assays.[49] Confocal laser scanning microscopy (CLSM) allowed following the penetration of the fluorescently labeled HA^{FITC}-CTL-C, diluted in solution or embedded in a PEM film, into the cell membrane of *C. albicans*. Finally, the cytotoxicity of HA-CTL-C/CHI films was tested through human gingival fibroblasts (HGFs) viability.



Scheme 1. Schematic representation of CHI/HA multilayers functionalized by an antimicrobial peptide (AMP) and its activity towards bacteria and yeasts based on the degradation of the film.

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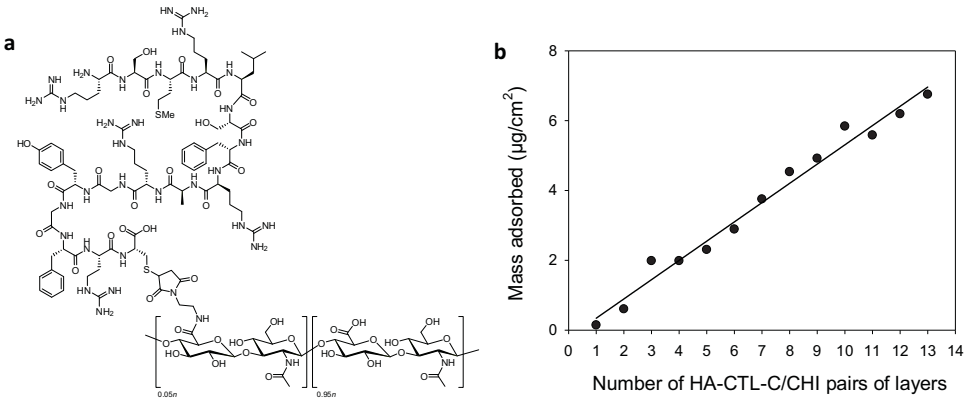

Figure 1. a) Formula of CTL-C functionalized hyaluronic acid, HA-CTL-C, at 5% in grafting ratio. b) Evolution of the mass adsorbed, measured by SPR, (black disks) for the buildup of PEI-(HA-CTL-C/CHI) multilayer film as a function of the number of deposited pairs of layers. The straight line serves to guide the eye.

2. Results and Discussion

2.1. Physical-Chemical Characterization of HA-CTL-C/CHI Films

CTL-C peptide was covalently coupled to HA in two steps using successively the carbodiimide chemistry to graft maleimide functions on HA and the thiol-maleimide coupling reaction to graft CTL-C on the modified HA. Synthetic procedures of CTL-C peptide and its conjugation to HA are described in Supporting Information (SI). After dialysis and freeze-drying, a coupling ratio of 5% was determined by ^{1}H-NMR, corresponding to the grafting of approximately 5 CTL-C peptides for 100 HA dimer units (**Figure 1**a). The antimicrobial and antifungal activities of CTL and modified CTL were tested in solution against two bacterial strains *M. luteus* and *S. aureus* (ATCC 25923) and a yeast *C. albicans*. Minimal inhibitory concentration (MIC) in peptide of CTL, CTL-C and HA-CTL-C were determined using bacterial and fungal assays described in SI. CTL-C remains antimicrobial at micromolar concentration (<100 µM) and displays an even better activity against *M. luteus* compared to the cysteine free CTL peptide. In comparison with the non-grafted CTL-C peptide, MIC value of HA-CTL-C increases from 35 to 45 µM for *S. aureus* and from 20 to 25 µM for *C. albicans* (**Table 1**). In the case of *M. luteus,* the MIC of HA-CTL-C (5 µM) is five times higher than that of free CTL-C (1 µM). In spite of this decrease in efficiency, the CTL-C peptide covalently linked to HA polymer can still be considered as antimicrobial, acting in the micromolar range. The buildup of HA-CTL-C/CHI film was monitored by SPR. A linear increase of the mass adsorbed is observed at each deposited layer of polysaccharide indicating the buildup of the film (Figure 1b). We investigated the topography and the roughness of HA-CTL-C/CHI films at different numbers of deposition steps by means of AFM in dry state (Supporting Information Figure S1). It was difficult to obtain good quality AFM images in the wet state due to the viscoelasticity of the films as we found for HA/CHI films in our previous work.[50] AFM imaging allows measuring film thickness after scratching. Supporting Information Table S1 summarizes the thicknesses and roughnesses measured by AFM. With 5 bilayers, the surface is already entirely covered with a 5-nm thick film with a roughness of 1.6 nm. As the buildup process goes on, the film thickness increases up to 52 nm at 30 bilayers. The film grows linearly up to 30 bilayers (Supporting Information Figure S2) and the film roughness increases up to 16.5 nm (for 30 pairs of layers).

Table 1. Minimal inhibitory concentration (MIC_{100}) of CTL, CTL-C and HA-CTL-C measured in solution leading to 100% of pathogens inhibition. In the case of HA-CTL-C, the MIC values given correspond to the concentration in CTL-C.

Pathogens	MIC_{100} [µM in peptide]		
	CTL	CTL-C	HA-CTL-C
S. aureus	30	35	45
M. luteus	5	1	5
C. albicans	20	20	25

2.2. Bacterial and Fungal Assays of HA-CTL-C/CHI Films

After characterization of HA-CTL-C/CHI multilayers buildup, the antibacterial and antifungal activities of the functionalized films were evaluated against two strains of bacteria *M. luteus* and *S. aureus* and one of yeast strain *C. albicans,* respectively. The influence of the number of embedded functionalized layers was studied by monitoring pathogen growth for different films with increasing number of HA-CTL-C/CHI bilayers. To this aim, PEI-$(HA/CHI)_{15-n}$-$(HA\text{-}CTL\text{-}C/CHI)_n$ films, with n = 0, 5, 10 and 15, and PEI-$(HA\text{-}CTL\text{-}C/CHI)_{30}$ films were built. Pathogens were incubated for 24 h in contact with HA-CTL-C/CHI films at 37°C for bacteria strains and at 30°C for *C. albicans*. For each pathogen, the microbial growth was measured at different times (1, 4, 6, and 24 h) by determination of the optical density at 620 nm (OD_{620}) of the bacterial suspension in contact with the film. The data were normalized to OD_{620} value obtained in similar conditions in the absence of films and are expressed as a percentage of growth (**Figure 2**a–c). For each tested pathogen,

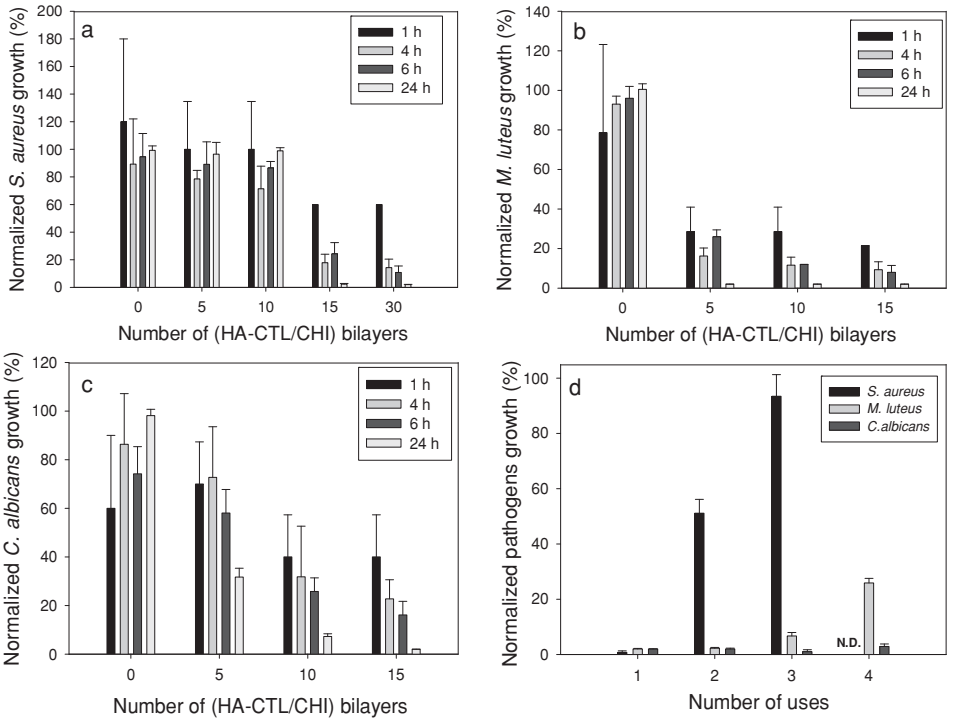

Figure 2. Normalized growth of a) *S. aureus*, b) *M. luteus* and c) *C. albicans* incubated for 1 to 24 h in contact with PEI-$(HA/CHI)_{15-n}$-$(HA\text{-}CTL\text{-}C/CHI)_n$ with $n = 0$ to 15 and $(HA\text{-}CTL\text{-}C/CHI)_{30}$ multilayer films. d) Normalized growth of *S. aureus* incubated on PEI-$(HA\text{-}CTL\text{-}C/CHI)_{30}$ and *M. luteus* and *C. albicans* incubated on PEI-$(HA\text{-}CTL\text{-}C/CHI)_{15}$ films, as a function of the number of uses. The film was brought in contact with a fresh pathogen suspension for 24 h. Every 24 h, the supernatant is removed and replaced by a fresh suspension and its OD_{620} is measured. The films were built in 150 mM NaCl at pH 4. The normalization was performed with respect to OD_{620} measured in the absence of film and antibiotics taken as 100% growth and in the presence of antibiotic taken as 0% growth. ND means not determined.

we observed that by increasing the number of HA-CTL-C/CHI bilayers the normalized microbial growth decreases. An important decrease of microbial growth is obtained for at least 5 bilayers for *M. luteus* and *C. albicans* and 15 bilayers for *S. aureus*. After 6 h of incubation, at least 70% of inhibition is obtained with 15 bilayers for all tested pathogens. After 24 h of incubation, the growth of *M. luteus*, *C. albicans* and *S. aureus* are fully inhibited with 5, 15 and 30 HA-CTL-C/CHI bilayers, respectively. These results are in agreement with the MIC values of HA-CTL-C in solution (Table 1) that follow the same trend towards the different pathogens. In order to check if the films can be reused several times as antimicrobial coatings, the growth of the three pathogens was monitored when incubated for 24 h in contact with reused PEI-$(HA\text{-}CTL\text{-}C/CHI)_{15}$ films for *M. luteus* and *C. albicans* and PEI-$(HA\text{-}CTL\text{-}C/CHI)_{30}$ film for *S. aureus*. To this aim, a fresh pathogen suspension was brought in contact with the functionalized film. After an incubation of 24 h, the supernatant was withdrawn and replaced by a fresh pathogen suspension. After each withdrawal, the OD_{620} of the supernatant was measured to determine pathogen growth after 24 h of incubation. When *M. luteus* and *C. albicans* suspensions in contact with $(HA\text{-}CTL\text{-}C/CHI)_{15}$ film are renewed every 24 h, a complete inhibition was observed at least for two and three cycles of use respectively. A significant decrease in efficiency of $(HA\text{-}CTL\text{-}C/CHI)_{15}$ film is detectable after the following renewal of pathogens suspension (Figure 2d). *S. aureus* growth is only inhibited by 40% for the second use of the $(HA\text{-}CTL\text{-}C/CHI)_{30}$ film and becomes fully inefficient in the third cycle. In parallel, the supernatants withdrawn were incubated with fresh pathogen suspensions for 24 h and the OD_{620} was also measured (Supporting Information Figure S3). No inhibition of growth was observed showing that there is no or a weak amount (quite less than the MIC) of CTL-C released in solution.

2.3. Mechanism of Pathogen Growth Inhibition of HA-CTL-C/CHI

To clarify the mechanism of HA-CTL-C inhibition, we synthesized fluorescently labeled HA^{FITC}-CTL-C and HA^{FITC} to analyze the films after contact with the pathogens. *S. aureus* and *C. albicans* are known to secrete hyaluronidase, a class of enzymes able to hydrolyze HA.[47,48] The cutting of one constitutive partner of the film should lead to its degradation as it was observed by Etienne et al.[51] We were then interested in imaging PEI-$(HA^{FITC}/CHI)_{15}$ films, by CLSM, before and after 24 h of contact with the pathogens. The three pathogens induce the degradation of HA/CHI films with, however, some differences in the resulting film morphologies (**Figure 3**). After 24 h of incubation with *S. aureus*, HA^{FITC}/CHI films were almost totally degraded (Figure 3a). The film appears inhomogeneous with fluorescent dots after incubation with *M. luteus* (Figure 3b). *C. albicans* induces the formation of honeycombs in the film

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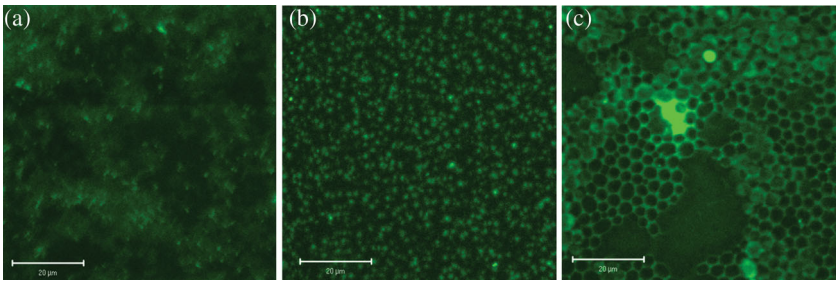

Figure 3. CLSM images of PEI-$(HA^{FITC}/CHI)_{15}$ after 24 h of incubation with a) *S. aureus*, b) *M. luteus* and c) *C. albicans*. All the films underwent a PFA treatment. The scale bars represent 20 µm.

due to the degradation of HA (Figure 3c). Degradation of HA should release CTL-C in the supernatant and promote the contact between CTL-C peptides and the pathogens. To check this hypothesis, we built hyaluronidase resistant films functionalized by CTL-C. CTL-C grafted on poly(allylamine hydrochloride) was thus synthesized and used to build poly(acrylic acid)/CTL-C functionalized poly(allylamine hydrochloride) (PAA/PAH-CTL-C) film. After 24 h of incubation, $(PAA/PAH\text{-}CTL\text{-}C)_{15}$ films show no inhibition against *C. albicans* (data not shown). This emphasizes the fact that the antimicrobial activity of the HA-CTL-C/CHI film is due to its degradation by the pathogens. This property renders the film specifically active in the presence of hyaluronidase secreted by the pathogens. Pathogens thus initiate their own death when brought in contact with the HA-CTL-C/CHI film. Even though the film is degraded with time in the presence of pathogens, it can be reused at least two and three times without losing its activity against *M. luteus* and *C. albicans*.

Using fluorescently labeled CTL-C, a previous study showed that the peptides penetrate into cell membranes and accumulate inside yeasts.[40,41] The interactions of HA^{FITC}-CTL-C and HA^{FITC} with *C. albicans* was thus studied when solubilized in solution or embedded in multilayer films. After 45 min of incubation at 30 °C with HA^{FITC} or HA^{FITC}-CTL-C in solution, *C. albicans* were observed by CLSM. The fluorescent HA^{FITC}-CTL-C was detectable in cytoplasm without inducing cell lysis (**Figure 4**a). On the contrary, HA^{FITC} is clearly

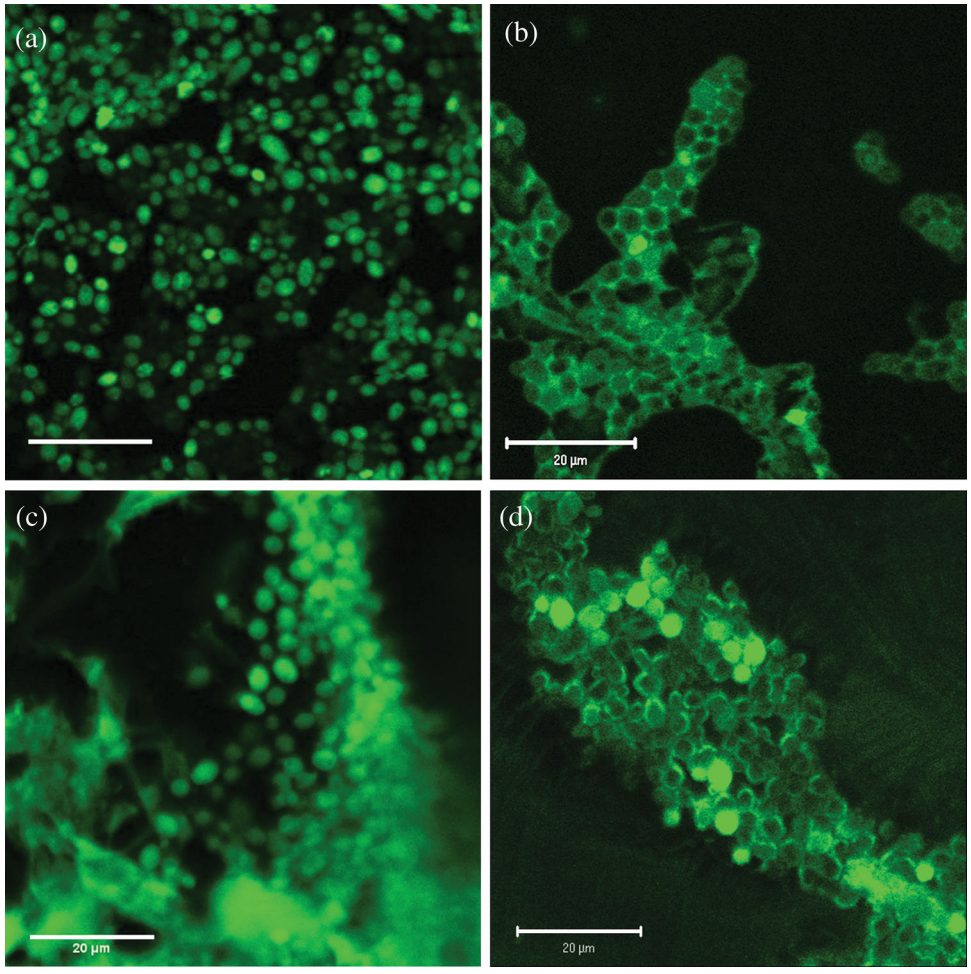

Figure 4. CLSM images of *C. albicans,* after 45 min of incubation a) in the presence of HA^{FITC}-CTL-C in solution (50 µM in peptide), b) in the presence of HA^{FITC} in solution, c) in contact with PEI-$(HA^{FITC}$-CTL-C/CHI$)_{15}$, and d) in contact with PEI-$(HA^{FITC}/CHI)_{15}$ multilayer films. The scale bars represent 20 µm

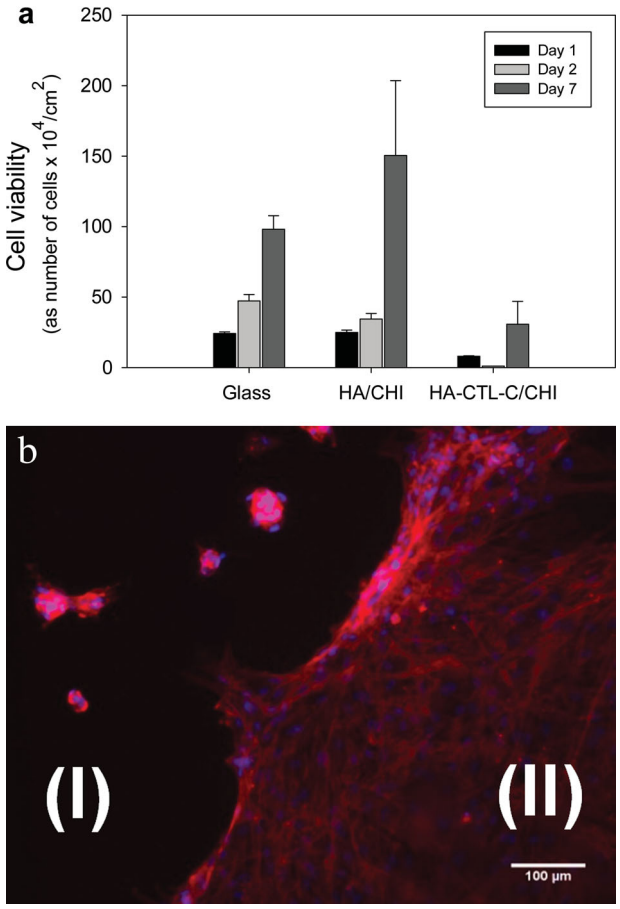

Figure 5. a) Viability of HGFs, evaluated by metabolic activity of cells, cultured on PEI-$(HA/CHI)_{15}$, named HA/CHI and PEI-$(HA\text{-}CTL\text{-}C/CHI)_{15}$, named HA-CTL-C/CHI, films built on glass substrates. Cell viability was determined by Alamar Blue assays after 1, 2, and 7 days of culture. The values represent the mean and the standard deviation of three films. b)Cytoskeleton visualization by actin filament immunofluorescent staining with phalloidin (red labeling) and DAPI nuclei counterstaining (blue labeling) of HGFs after 24 h of culture on half coated glass substrate by PEI-$(HA\text{-}CTL\text{-}C/CHI)_{15}$: the areas represent (I) the HA-CTL-C/CHI film and (II) the bare glass substrate.

observed all around the yeast cells, stacking probably on the membranes and leading to a honeycomb structure (Figure 4b). This suggests that CTL–C can cross the cell membrane, even when it is coupled to HA, leading to an accumulation of HA inside the cytoplasm. To image them by CLSM, the films were treated by paraformaldehyde (PFA) to fix the pathogen in contact. This treatment was first applied on the films to evaluate its effect. PFA treatment induces no change in the case of HA^{FITC}/CHI films in contrary to HA^{FITC}-CTL-C/CHI films (Supporting Information Figure S4) where heterogeneities appear. *C. albicans* were incubated for 45 min at 30 °C in contact with PEI-$(HA^{FITC}\text{-}CTL\text{-}C/CHI)_{15}$ film and then observed by CLSM. Among the heterogeneities due to PFA treatment, a strong green fluorescence is observed mainly inside the yeast (Figure 4c). In the case of HA^{FITC}/CHI films, a little fluorescence is localized inside the yeasts and only few of them are strongly fluorescent (Figure 4d). In spite of its insertion into the PEM films, CTL-C allows the penetration of HA^{FITC}-CTL-C inside the yeasts explaining the activity of the films.

2.4. Biocompatibility Tests of HA-CTL-C/CHI Films

Finally, it is important to ensure that the film is not cytotoxic to healthy wound healing cells. Fibroblasts are one of the first anchorage-dependent cells to come at an implant surface during the wound healing process. The viability of HGFs cultivated on PEI-$(HA\text{-}CTL\text{-}C/CHI)_{15}$ films, compared to PEI-$(HA/CHI)_{15}$ films and glass substrate, has been evaluated through their mitochondrial activity, monitored by Alamar blue assays. Already after one day, the metabolic cell activity measured on HA-CTL-C/CHI films is lower compared to HA/CHI films and non-coated glass substrate (**Figure 5**a). The good biocompatibility of HA/CHI films towards HGFs, mediated via CD44 receptor, was already reported in our previous work.[50] After 7 days of culture, the number of viable HGFs on HA-CTL-C/CHI films represents 25% of viable HGFs on HA/CHI films. The functionalization of HA by CTL-C peptide induces a lower adhesion of HGFs which seems to slow down the proliferation of cells. Knowing that HA-CTL-C in solution at 100 µM did not show any cytotoxicity (data not shown), such behavior would suggest that the multilayers are not cytotoxic. To discriminate between cytotoxic or low initial adhesion properties of HA-CTL-C/CHI, we performed two experiments. First after 24 h of contact, suspended cells harvested from HA-CTL-C/CHI film supernatant were passed to fresh culture plates. After 24 h of culture, many cells readily attached and spread as fresh cells. Second, we analyzed the biocompatibility of films by a complementary assay based on cell spreading via cytoskeleton arrangement when the cells are seeded on a glass substrate half coated by $(HA\text{-}CTL\text{-}C/CHI)_{15}$. After 24 h, HGFs adhere on the glass substrate (Figure 5b, zone II), but to a lesser degree on $(HA\text{-}CTL\text{-}C/CHI)_{15}$ film (Figure 5b, zone I). After 24 h of culture, a confluent layer with a typical fibroblastic cell shape and with polymerized F-actin fibers is observed on the glass substrate (Supporting Information Figure S5a). On the HA-CTL-C/CHI film, cells appear less elongated and have a peripheral actin distribution (Supporting Information Figure S5b). This indicates that $(HA\text{-}CTL\text{-}C/CHI)_{15}$ films are not cytotoxic but seem rather anti-adherent towards HGFs. It is know that persistent excessive functions of fibroblasts have been linked to detrimental fibrous tissue formation which may cause implant failure. The present results of decreased fibroblast adhesion on functionalized substrate with HA-CTL-C/CHI films shows promise for implant applications.

3. Conclusions

In conclusion, we designed a new surface coating based on polysaccharide multilayer films containing a functionalized HA with 5% of CTL-C, a peptide possessing both antibacterial and antifungal properties. Antimicrobial properties of CTL-C were preserved when grafted on HA either in solution or when embedded into PEM films. After 24 h of incubation,

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HA-CTL-C/CHI films fully inhibit the development of *S. aureus* and *C. albicans,* which are common and virulent pathogens agents encountered in care-associated diseases. The presence of CTL-C peptides on HA allows the penetration of the modified polysaccharide inside *C. albicans* after 45 min of contact. The secretion of hyaluronidase by all tested pathogens seems to be responsible for HA-CTL-C release from the film and for its activity. The film can keep its activity during 3 cycles of use against fresh incubated *C. albicans* suspension. Furthermore, the limited fibroblasts adhesion, without cytotoxicity, on HA-CTL-C/CHI films highlights a medically relevant application to prevent infections on catheters or tracheal tubes where fibrous tissue encapsulation is undesirable.

4. Experimental Section

Polysaccharide Solutions: Chitosan (CHI, PROTASAN Ultrapure Chitosan CL213, 260 000 g/mol, DA 83%) was purchased by Novamatrix (Sandvika, Norway). Dried Sodium Hyaluronate (HA, 420 000 g/mol) was purchased by Lifecore (Chaska, USA). Poly(ethylene imine) (PEI, 60 000 g/mol, 50% in water), sodium chloride, sodium dodecyl sulfate (SDS), HCl and NaOH were purchased by Sigma-Aldrich (Quentin-Fallavier, France). All products were used without further purification. 150 mM NaCl solution was prepared with Ultrapure Milli-Q water having a resistivity of 18.2 MΩ cm. CHI and HA solutions were prepared at 0.3 mg/mL in 150 mM NaCl adjusted at pH 4 with NaOH or HCl solutions. HA-CTL-C and HA^{FITC}-CTL-C solutions were prepared at 0.2 mg/mL and 0.1 mg/mL, respectively. PEI solution was prepared at 0.5 mg/mL in 150 mM NaCl solution adjusted at pH 7.5 with NaOH or HCl solutions.

Film Buildup: Before PEM buildup, glass coverslips (Ø = 14 mm; VWR, Strasbourg, France) were cleaned in a SDS solution at 0.01 M, ultrasonicated for 5 min and then submitted to a hot 0.1 M HCl solution for 10 min and finally rinsed with ultrapure Milli-Q water. The precursor PEI layer and CHI/HA films were deposited using an automated spraying device described in a previous work.[50] CHI/HA-CTL-C and CHI/HA^{FITC}-CTL-C films have been prepared in 24-well plates on PEI precoated glass substrates. 300 µL of the polycation solution were deposited for 5 min on glass coverslips followed by a rinsing step (500 µL) with ultrapure Milli-Q water. Then, 300 µL of polyanion solution were deposited for 5 min followed by a rinsing step (500 µL) with ultrapure Milli-Q water. To test the antimicrobial activity of PEM films versus the number of HA-CTL-C layers, PEI-$[HA/CHI]_{15-n}[HA\text{-}CTL\text{-}C/CHI]_n$ with n equal to 0, 5, 10, and 15 and PEI-$[HA\text{-}CTL\text{-}C/CHI]_{30}$ films were built.

Surface Plasmon Resonance: Surface plasmon resonance (SPR)[52,53] as some other detection techniques (optical waveguide lightmode spectroscopy, quartz crystal microbalance), provides a label-free, in situ method to monitor the buildup of e.g., a polymer film on a solid surface. When a beam of light passes from a material of refractive index, n_1, into a material with a lower refractive index, n_2, some light is reflected from the interface. When the angle of incidence of the light, θ, on the interface is greater than $\theta_{TIR} = \arcsin(n_2/n_1)$, the light is completely reflected (total internal reflection). In the case of the SPR instrument used here (SPR Navi 200, Bionavis, Finland), the most refractive medium is a BK7-glass prism. Because the bottom surface of the prism is coated with a thin film of a noble metal (gold), this reflection is not total; some of the light is 'lost' into the metallic film. There then exists a second angle, $\theta_{SPR} > \theta_{TIR}$, at which the intensity of the reflected light goes through a minimum. In addition, an evanescent electrical field travels for a short distance into the medium (e.g., film) from the metallic film. The probing distance reaches generally a few hundreds of nm depending on the refractive index of the probed medium and θ_{SPR} is sensitive to the optical characteristics of the deposited film. The reflection curves, i.e., the variation of the intensity of the reflected light with θ, corresponding to the successive polymer deposition steps have been analyzed with a home-made software based on Maxwell's equations. The parameters involved are the refractive indices of the prism, the chromium and gold layers, the polymer film (n_{film}) and the buffer solution (n_{sol}), as well as the thickness of the chromium and gold layers and of the film (d_{film}). From n_{film}, n_{sol} and d_{film}, we derive the mass of film per unit area:

$$m = \frac{(n_{film} - n_{sol})\, d_{film}}{dn/dc} \quad (1)$$

where dn/dc is the change in refractive index with concentration and is equal to 0.197 cm^3/g for polyelectrolytes.[54] The SPR experiment was performed under a continuous flow rate of 100 µL/min of 150 mM NaCl solution adjusted at pH 4. The time of deposition and of rinsing of polyelectrolytes was fixed at 5 min.

Atomic Force Microscopy (AFM): Atomic force microscopy (AFM) images were obtained in contact mode in dry state with the Nanoscope IV from Veeco (Santa Barbara, CA). The images were carried out with silicon nitride cantilevers, spring constant 0.03 N/m (model MSCTAUHW, Veeco, CA). Several scans were performed over a given surface area. These scans had to give reproducible images to ascertain that there is no sample damage induced by the tip. Deflection and height mode images are scanned simultaneously at a fixed scan rate (2 Hz) with a resolution of 512 × 512 pixels. Data evaluation was performed with the NanoScope software version 5.31r1 (Digital Instruments, Veeco). The buildup of the film was made on glass substrate and the film thickness was measured by using the "scratch" method. Profilometric section analyses of a scratched film allowed us to determine precisely the quality of the film and its thickness over the scanned area. The scratches were achieved with a plastic cone tip and were always imaged perpendicular to the fast scan axis. The profiles correspond to a cross section along this axis. The mean thickness of the scratched film was determined by measuring the thickness at least on three areas.

Confocal Laser Scanning Microscopy (CLSM): Confocal laser scanning microscopy (CLSM) observations were carried out with a Zeiss LSM-510 microscope using a 40×/1.31 oil immersion objective and a 0.43 µm z-section interval. FITC fluorescence was detected upon excitation at 488 nm, through a cut-off dichroic mirror and an emission bandpass filter of 505–530 nm (green).

Bacterial and Fungal Growth: To determine the antibacterial activity, microdilution assay was used on two bacterial strains *M. luteus* (A270) and *S. aureus* (ATCC25923). The antifungal activity was tested against one yeast strain *C. albicans*. Bacteria were precultured aerobically in Mueller-Hinton (MH) broth (Merck, Darmstadt, Germany) with agitation at 37 °C for 18 h. *C. albicans* preculture was carried out on a Sabouraud broth (SB) medium (BioMérieux S.A., Marcy l'Etoile, France) with agitation at 30 °C for 24 h. The antimicrobial activity was tested using a mid-logarithmic-phase culture of bacteria or yeast with an initial optical density at 620 nm (OD_{620}) of 0.001. For the tests performed in solution, aqueous solutions of CTL, CTL-C and HA-CTL-C were prepared in bacterial or yeast medium. Aliquots of CTL-C or HA-CTL-C (10 µL) were incubated in 96-well microplates (Falcon, Becton Dickinson, USA) with 90 µL of bacteria or yeast at final concentrations of 100 µM to 2 µM of peptide. After 24 h of incubation at 37 °C under gentle stirring, the OD_{620} of the 96-well plate was measured by a microplate reader.[55–56] MIC_{100}, the minimal inhibitory concentration, is the lowest concentration that is able to completely inhibit the growth of bacterial or fungi after 24 h of contact. To test the antibacterial and antifungal properties of the multilayer films, PEI-$(HA/CHI)_{15-n}$-$(HA\text{-}CTL\text{-}C/CHI)_n$ with n = 0, 5, 10 and 15 and PEI-$(HA\text{-}CTL\text{-}C/CHI)_{30}$ films were prepared. 400 µL of a mid-logarithmic-phase culture of bacteria or yeast with OD_{620} of 0.001 were placed in 24-well plate containing multilayer films. For each tested film, 100 µL of the supernatant was taken to measure its OD_{620} by a microplate reader. The withdrawn volume was compensated with the appropriated fresh bacteria or yeast solution of the same measured OD_{620}. Intermittent controls were performed by inoculating the culture medium on MH (resp. SB) agar plates by spreading method and counting colonies for verification at different steps. Several controls were used: a fresh medium without inoculation of pathogens was used

to ensure sterility, a mixture of Tetracycline (10 µg/mL) and Cefotaxime (0.1 µg/mL) was used as positive control (90 µL of culture and 10 µL of antibiotics) and a fresh inoculated culture medium without any addition was taken as negative control. For the bacterial and yeast quantification, a conversion factor was devised to convert OD into bacterial and yeast counting, respectively. Briefly, OD of fresh bacteria (respectively yeast) culture was measured at 620 nm and was plated to MH (respectively. SB) agar for 24 h at 37 °C (respectively 30 °C). Colony count was performed and plotted versus the OD_{620} and the slope of the linear curve was taken as conversion factor. Pathogen quantification (colony forming unit (CFU) per mL) was performed at time zero and then at each hour for 6 h and then finally at 24 h. For quantification, conversion factors were determined for each strain used separately.[57] Each assay was performed in triplicate and the experiments were repeated at least three times. The normalized growth of pathogens (%) was estimated by comparing the OD_{620} values in the presence of multilayer films and the positive and the negative controls. The OD_{620} value of control cultures growing in the absence of films and antibiotics was taken as 100% growth (negative control) and the OD_{620} value of cultures growing in the presence of antibiotics (Tetracycline and Cefotaxime) was taken as 0% growth (positive control). The following equation definition was used:

$$\textit{Normalized pathogen growth}(\%) = \frac{\left(OD_{620,sample} - OD_{620,positive\ control}\right)}{\left(OD_{620,negative\ control} - OD_{620,positive\ control}\right)} \times 100$$

To follow the interaction of fluorescently labeled HA^{FITC}-CTL-C in solution and HA^{FITC}-CTL-C/CHI films with *C. albicans,* CLSM was used based on a protocol previously described with few modifications.[58] Briefly, for experiments done with HA^{FITC}-CTL-C in solution, poly-L-Lysine coated microscopic slides were covered with fresh medium containing *C. albicans* (OD_{620} = 0.001) in SB culture medium and incubated for 24 h at 30 °C without agitation. The medium was then removed and replaced by 50 µM (in peptide) of HA^{FITC}-CTL-C. After an incubation period of 45 min, the slides were washed with fresh culture medium and subsequently treated for 30 min with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3. After several rinsing steps with PBS, slides were covered with Moviol 4-88 (Aldrich, Steinheim, Germany). For experiments performed with HA^{FITC}-CTL-C/CHI, fresh *C. albicans* medium, previously incubated at OD_{620} = 0.001 in SB culture medium for 24 h at 30 °C without agitation, was put in contact with PEI-$(HA^{FITC}$-CTL-C/CHI$)_{15}$ films for 45 min at 30 °C without agitation. HA^{FITC} in solution and HA^{FITC}/CHI films were used as control. *C. albicans* were subjected to optical serial sectioning (0.2–0.3 µm) to produce images in the x–y plane. Each optical section was scanned several times to obtain an average image. Images were recorded digitally in a 768 × 576 pixel format.

Human Gingival Fibroblasts Viability Assays: The biocompatibility of films was tested using human gingival fibroblasts (HGFs). HGFs were extracted from human gingival connective tissue of healthy individuals according to a protocol approved by the ethics committee for patient protection of CPP Strasbourg Hospitals. Cells were grown in DMEM containing 1 g/L of glucose and supplemented with 10% fetal bovine serum and 100 IU/mL penicillin-streptomycin (all from Gibco). Cells were cultured at 37 °C in 5% CO_2 in 75 cm^2 flasks. HGFs were used between the 6th and the 9th passage. Before cell seeding, glass coverslips (Ø = 14 mm) coated with different architectures were irradiated by UV for 15 min. Then HGFs were seeded at 3×10^4 cells per cm^2 and cultivated at 37 °C under a 5% CO_2 humidified atmosphere for different culture times (Day 1, Day 2 and Day 7). Cell viability was assessed by Alamar Blue assay (Biosource International). This assay is based on the reduction of the blue, non-fluorescent resazurin dye to the pink and fluorescent resorufin dye by living cells.[59] The overall conversion rate is proportional to the metabolic activity of living cells.[60] Viability was assessed for different times 1, 2, and 7 days. After rinsing with PBS, cells were incubated with 10% reagent in complete medium for 2 h. After incubation optical density (OD) at 570 nm and 630 nm were determined with a microplate reader. The percentage of reduction of Alamar Blue was calculated according to the procedure provided by the manufacturer. The experiments were performed in triplicate. Actin filament staining with tetramethylrhodamine B isothiocyanate coupled phalloidin (Sigma-Aldrich) and DAPI (Invitrogen) nuclei counterstaining were performed as follows: cells were fixed with 3.7% paraformaldehyde for 10 min at 4 °C, permeabilized in 0.25% Triton X-100 in PBS for 10 min, and blocked in 1% BSA-PBS for 30 min. Thereafter, cells were incubated for 30 min at room temperature with 5×10^{-5} mg/mL phalloidin followed by nuclear counterstaining with DAPI (50 ng/mL) incubated for 2 min at room temperature. Washed slides were mounted on blades with DAKO fluorescent mounting medium and fluorescence distribution was examined by means of an inverse fluorescence microscope (Axiovert, Zeiss).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Self-Defensive Biomaterial Coating Against Bacteria and Yeasts: Polysaccharide Multilayer Film with Embedded Antimicrobial Peptide

*G. Cado, R. Aslam, L. Séon, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J.-C. Voegel, B. Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf, H. Kerdjoudj, M.-H. Metz-Boutigue, and F. Boulmedais**

Supporting Information

Self-defensive biomaterial coating against bacteria and yeasts: polysaccharide multilayer film with embedded antimicrobial peptide

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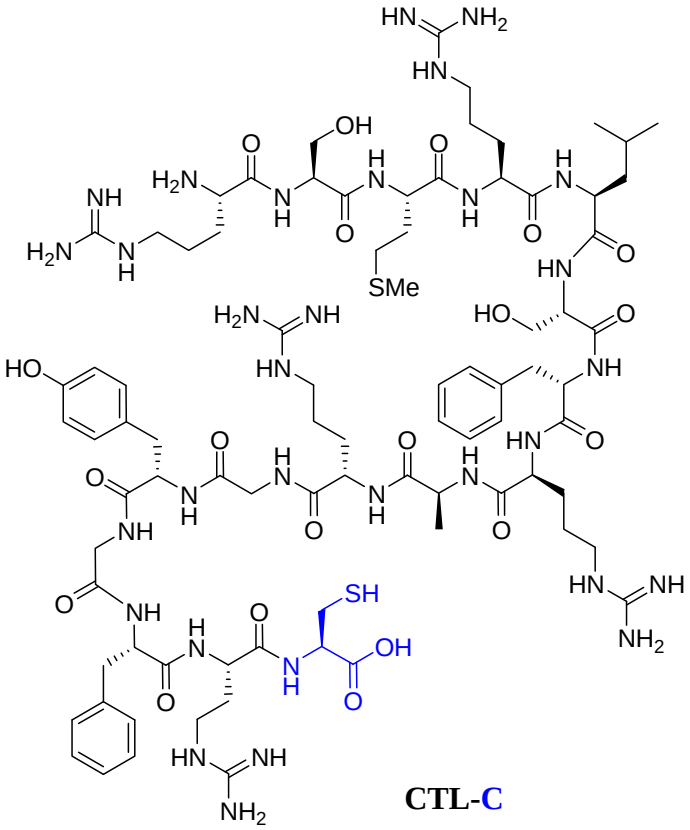
Abbreviations: AMP = antimicrobial peptides, $CGA_{344-358}$ = chromogranin A 344-358; CTL = cateslytin; CHI = chitosan; CTL-C = cateslytin with a cystein in C terminal; HA = hyaluronic acid; EDC = N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; sulfo-NHS = N-Hydroxysulfosuccinimide sodium salt; HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; TCEP = tris(2-carboxyethyl)phosphine hydrochloride; Da = Dalton; tBuOH = tertio-butanol; DS = degree of substitution; NMR = nuclear magnetic resonance; MHz = megahertz; ppm = parts per million ; br = broad; s = singlet; d = doublet; m = multiplet.

1. Preparation and analysis of synthetic antimicrobial peptides.



CTL-C

The synthetic peptide corresponding to bovine CGA (Uniprot P05059), named CTL, ($CGA_{344-358}$: RSMRLSFRARGYGFR) was synthesized with a cystein at the C-terminal end (CTL-C) on a phase approach with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.[1] After purification by a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Macherey Nagel Nucleosil RP 300-5C18 column (10 × 250 mm; particle size 5 µm and pore size 100 nm), the peptide was analyzed by mass spectrometry (MALDI-TOF) and automated Edman sequencing on an Applied Sequencing System Procise (Applied Biosystems, Foster City, USA).[2] MALDI mass measurements were carried out on an Ultraflex™ TOF/TOF (BrukerDaltonics, USA) to perform a rapid control of synthetic peptides according to the procedure previously reported.[3]

2. Preparation of modified HA and PAH.

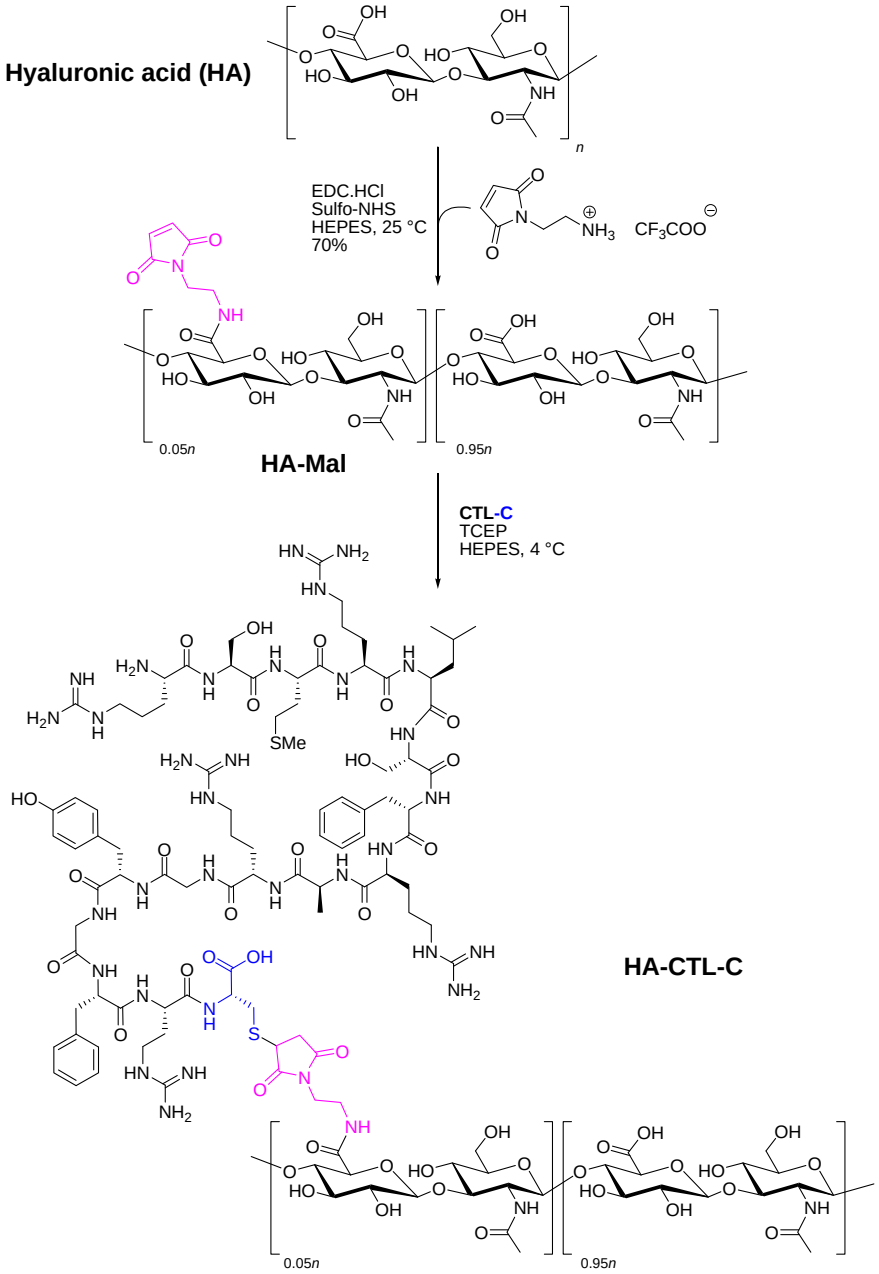
Peptide and FITC functionalized HA, *i.e.* HA-CTL-C, HA^{FITC} and HA^{FITC}-CTL-C, and peptide modified PAH have been prepared according to the general synthetic pathway described below.

2.1. Preparation of HA-CTL-C

Hyaluronic acid (HA) 420 000 Da, was functionalized with maleimide group according to previously reported method.[4] HA (100.9 mg) was dissolved in 15 mL of 0.01M HEPES buffer (pH 6). EDC (35.2 mg) and sulfo-NHS (10.2 mg) were added to this solution and the resulting mixture was stirred 2 h at room temperature. Afterwards, N-(2-aminoethyl) maleimide trifluoroacetate salt (6.7 mg) was added to the reaction mixture and let stirred for 20 h. The mixture was dialyzed (cut-off 12 000-14 000 Da) first against 0.5 M NaCl for one day and MilliQ water for 6 days. Water was changed every day. HA-Mal (86 mg) was obtained as a white solid after freeze-drying. The degree of substitution (DS) of HA-Mal, defined as the number of maleimide groups per 100 disaccharide unit of HA, was determined by ^{1}H NMR (D_2O, 400 MHz). All spectra were realized in D_2O containing 5% of *t*BuOH: the singlet of the *t*-butyl group of *t*BuOH was calibrated at δ 1.24 ppm and thus used as internal reference. The singulet at δ 7.1 ppm was assigned to the two symmetric protons on the double bond of the maleimide group. By comparing the integration of this signal with the singlet at δ 2.0 ppm, assigned to the methyl protons of the acetamide group, the DS was determined equivalent to 5%.

^{1}H NMR (D_2O, 400MHz, δ ppm): δ 6.9 (s, maleimide), 4.5 (br d, HA), 3.5 (m), 2.0 (s, acetyl group of HA).

The coupling reaction between HA-Mal 5% (80 mg) and CTL-C (10.1 mg) was performed at 4°C for 20°h in 16mL of 0.01M HEPES buffer (pH 6) and 50 µM of TCEP. Then, the mixture was dialyzed (cut-off 50 000Da) against 0.5 M NaCl for one day and MilliQ water for 6 days. Water was changed every day. Peptide-conjugated HA, named HA-CTL-C, was freeze-dried to provide a white solid with 80-85% of overall yield (≈ 79 mg). The DS, defined as the number of CTL-C peptide per 100 disaccharide units of HA, was determined by ^{1}H NMR (D_2O, 400 MHz). All aromatic signals between δ 7.0 and 7.5 ppm corresponding to the protons of the aminoacids Phe and Tyr were compared to the singlet at δ 2.0 ppm (assigned as the methyl group of the acetamide). A DS of 5% is determined.

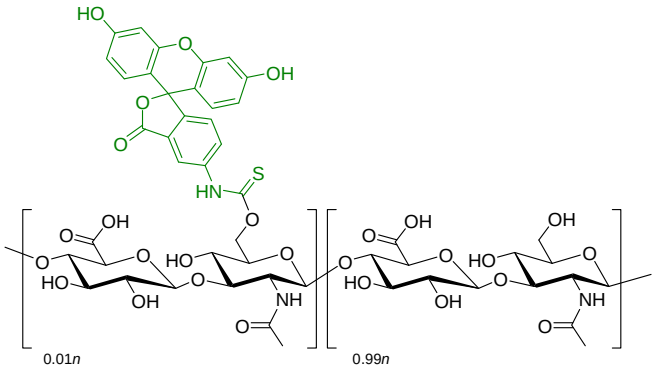


^{1}H NMR (D_2O, 400MHz, δ ppm): 7.35 (br s, Ar aminoacid of CTL-C), 7.25 (br s, Ar aminoacid of CTL-C), 7.10 (br s, Ar aminoacid of CTL-C), 6.85 (br s, Ar aminoacid of CTL-C), 4.50 (br s, HA), 4.40 (br s, CTL-C), 3.10 (br s, CTL-C), 2.50 (br s, CTL-C), 2.00 (s, methyl from acetyl group of HA), 1.75 (br s, CTL-C), 0.95 (br s, CTL-C).

2.2. Preparation of HA^{FITC}

Fluorescein Isothiocyanate (FITC) has been covalently attached to HA according to the following procedure: a solution of FITC (41 µmol dissolved in 2 mL of DMSO) and a solution of HA (0.31 µmol dissolved in 18 mL of deionized water) were mixed. The resulting

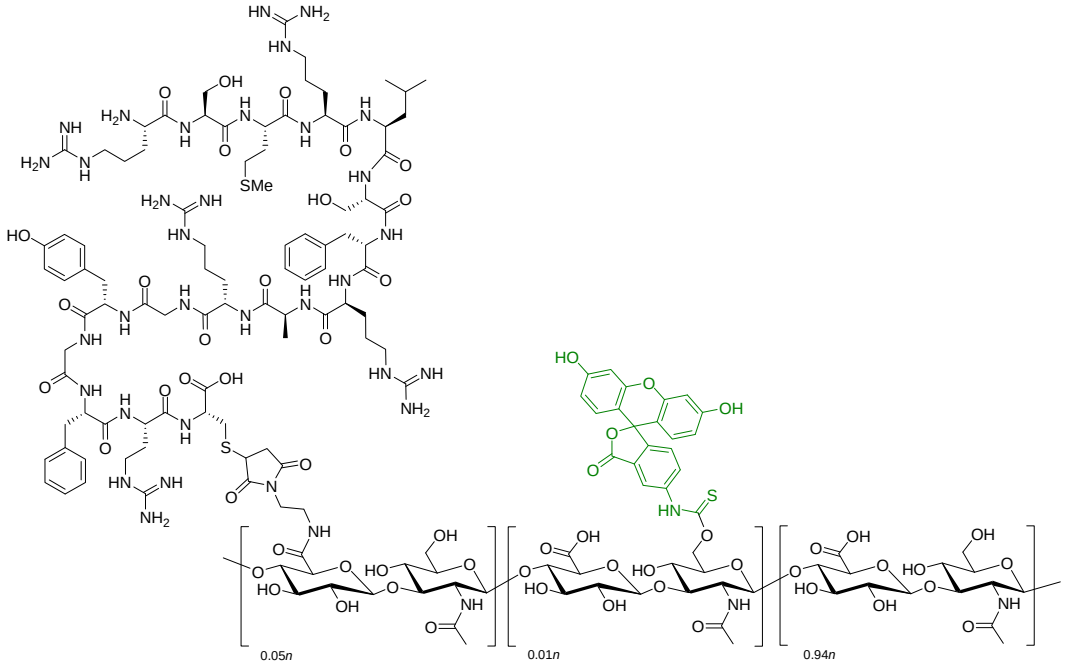
solution was then adjusted at pH 9 by using a 0.01M NaOH solution. The reaction mixture was stirred for 12 h at room temperature. Then, 40 mL of deionized water was added and this final mixture was dialyzed (cut-off: 12 000 – 14 000 Da) against deionized water until no absorbance was detected in the water (λ = 494 nm). The obtained compound is a yellowish solid and corresponds to functionalized HA by 1% of fluorescein. The 1H NMR (D_2O) spectra of HA^{FITC} is identical to the non-modified HA. Because of the very low loading of fluorescein on HA, the ^1H NMR (D_2O) spectra of HA^{FITC} is identical to the non-modified HA.



HA^{FITC}

The fluorescein moiety may also be linked through the secondary alcohol groups.

2.3. Preparation of HA^{FITC}-CTL-C



HA^{FITC}-CTL

The fluorescein moiety may also be linked through the secondary alcohol groups.

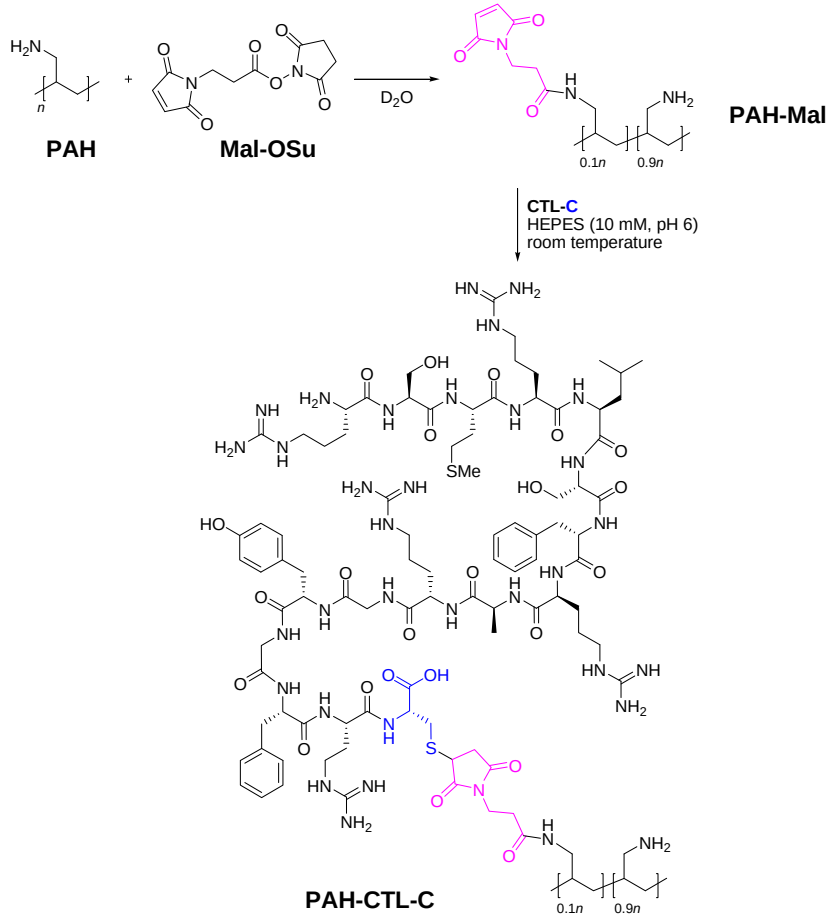
HA^{FITC}-CTL-C polymer has been prepared from HA-Mal. This polymer was labeled with 1% FITC as described above to provide HA^{FITC}-Mal. Then, CTL-C peptide reacted with free maleimide group to lead to HA^{FITC}-CTL-C as a yellow solid. The procedure used to get

HA^{FITC}-CTL-C was identical to the one described above to prepare HA-CTL-C. ^1H NMR (D_2O) spectra of HA^{FITC}-CTL-C was identical to the spectra of HA-CTL-C.

2.4. Preparation of PAH-CTL-C

All the following reactions were carried out in NMR tube and monitored by ^1H NMR (D_2O). To a solution of PAH (22.16 mg) in D_2O (0.6 mL) was added Mal-OSu (15 mg). The resulting mixture was stirred mechanically at room temperature until RMN monitoring indicated total consumption of the reagent (48 h). Evaporation of solvent provided the desired PAH-Mal (27 mg) as a white solid which was used without further purification. The DS of PAH-Mal was determined by ^1H NMR (400MHz, D_2O) and defined as the number of maleimide groups per 100 allylamine units of PAH. NMR Spectrum was realized in D_2O and calibrated at δ 4.79 ppm (residual water). The singulet at δ 6.9 ppm was assigned to the two symmetric protons on the double bond of the maleimide group. By comparing the integration of this signal with the singlet at δ 3.1 ppm, assigned to the $C\underline{H_2}$-NH protons of the allylamine group, the DS was determined equivalent to 10%.

^1H NMR (D_2O, 400MHz, δ ppm): δ 6.9 (s, maleimide), 3.8 (t, Mal-$C\underline{H_2}$-CH_2-CO), 3.1 (br s, CH_2 of PAH), 2.7 (t, Mal-CH_2-$C\underline{H_2}$-CO), 2.1 (br s, CH of PAH), 1.6 (br s, CH_2 of PAH).



The coupling reaction between PAH-Mal 10% (1.5 mg) and CTL-C (15.6 mg) was performed overnight at room temperature in 0.6 mL of 10 mM HEPES buffer solution in D_2O (pH 6). Then, the mixture was dialyzed (cut-off: 12 000 – 14 000 Da) against MilliQ water for 6 days (water was changed every day). Peptide-conjugated PAH, named PAH-CTL-C, was freeze dried to provide a white solid (17 mg). This quantitative yield and the ^{1}H NMR described below allowed us to define a 10% DS (number of CTL-C peptide per 100 allylamine units of PAH).

^{1}H NMR (D_2O, 400MHz, δ ppm): 7.35 (br s, Ar aminoacid of CTL-C), 7.25 (br s, Ar aminoacid of CTL-C), 7.13 (br s, Ar aminoacid of CTL-C), 6.86 (br s, Ar aminoacid of CTL-C), 3.67-4.68 (m, CTL-C), 3.10 (br m, PAH and CTL-C), 2.50-2.68 (br m, CTL-C), 1.34-2.18 (br m, PAH and CTL-C), 0.96 (br s, CTL-C).

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4. Supplementary Figures

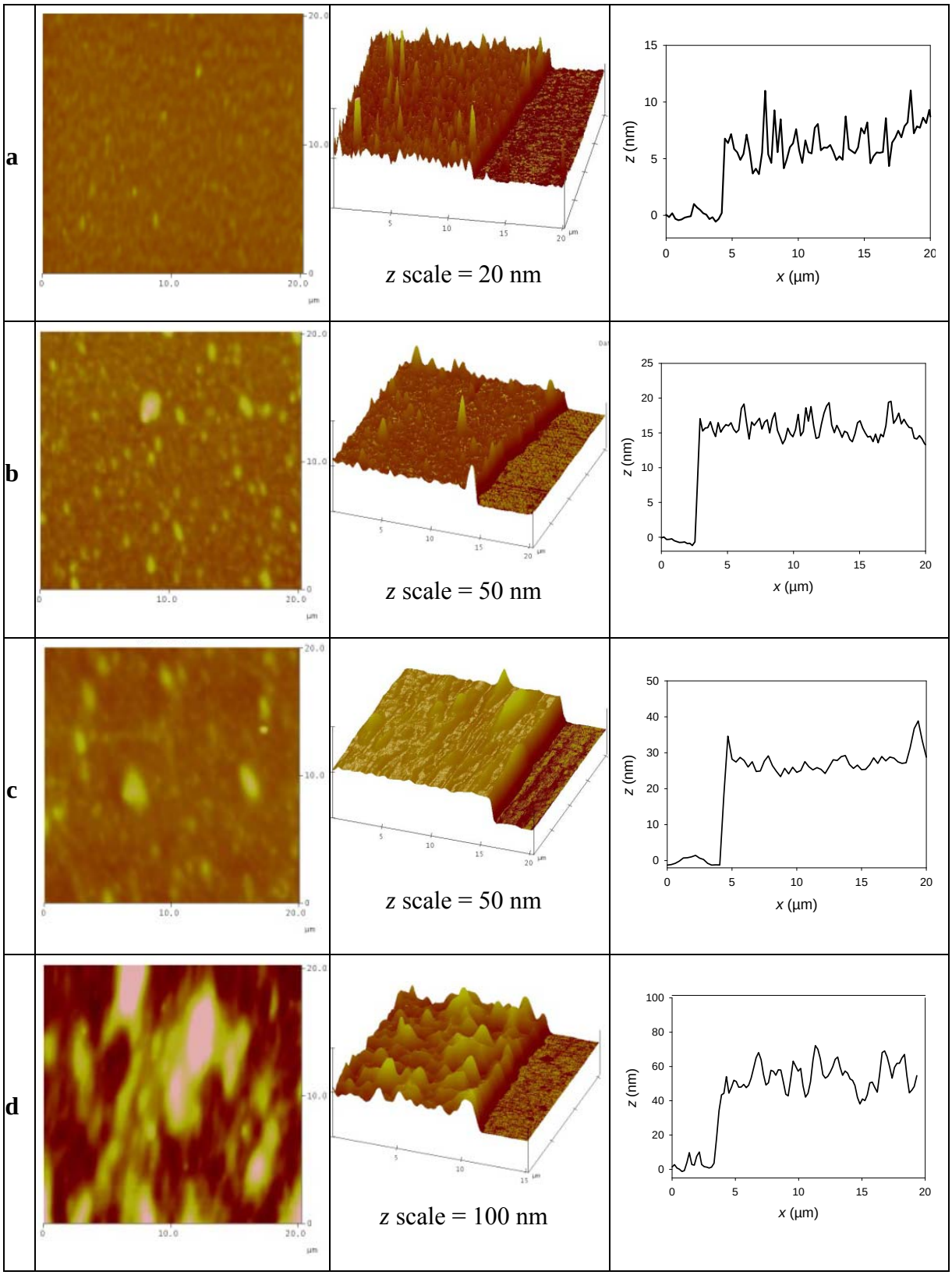

Figure S-1: AFM images of non-scratched and 3D images and their profilometric sections of scratched PEI-(HA-CTL-C/CHI)$_n$ films built at (a) 5, (b) 10, (c) 15 and (d) 30 bilayers obtained in height mode in dry state.

Table S-1. Thicknesses and roughnesses of PEI-(HA-CTL-C/CHI)$_n$ films built at different number of deposited pair of layers, *n*. The errors on the thickness values measured by AFM were obtained from the measurement of three different areas.

n	d_{AFM} **(nm)**	**RMS (nm)**
5	5 ± 1	1.6 ± 0.1
10	17 ± 2	4.2 ± 0.8
15	28 ± 3	4.6 ± 0.9
30	52 ± 9	16.5 ± 2.8

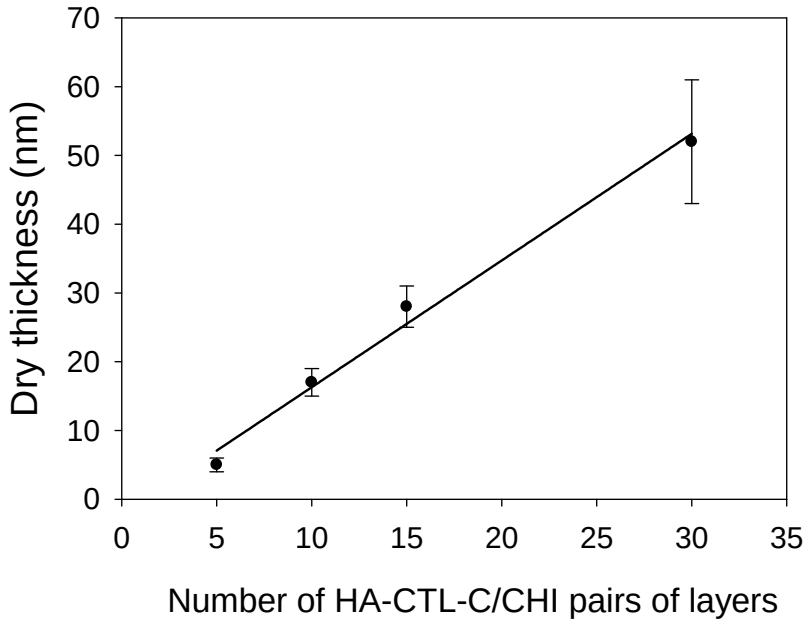

Figure S-2: Evolution of the thickness of (HA-CTL-C/CHI) films, measured by AFM in dry state after scratching, at different number *n* of pairs of layers.

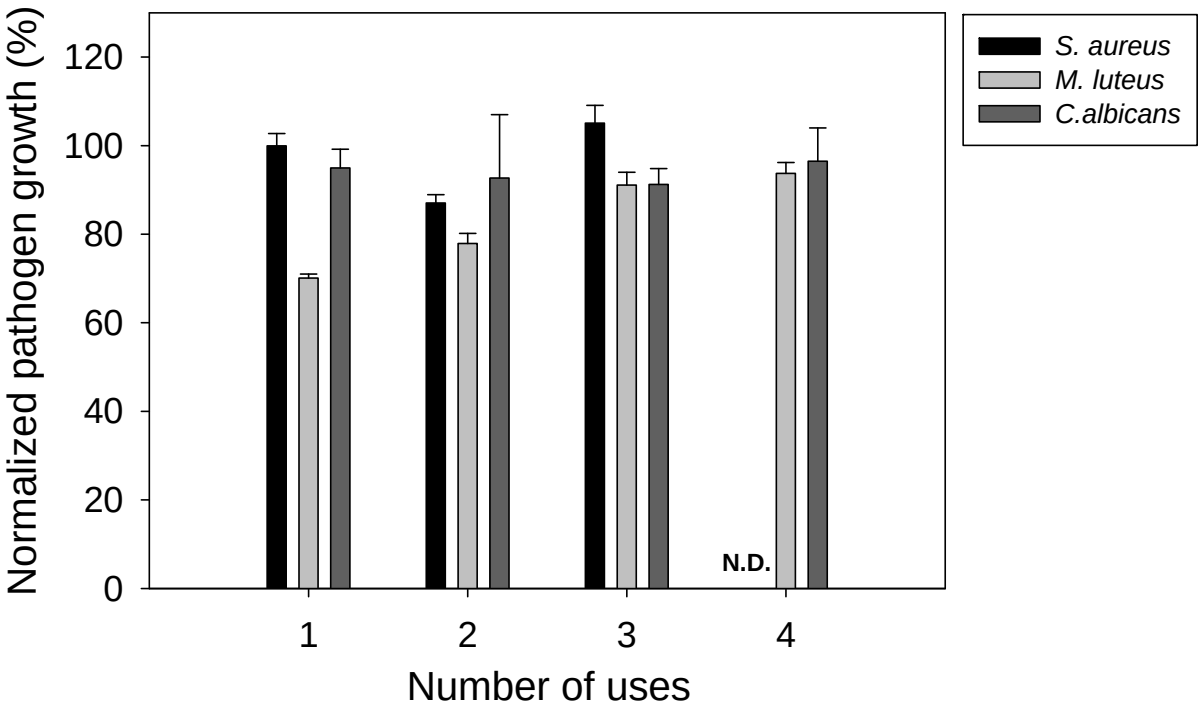

Figure S-3: Normalized growth of *S. aureus*, *M. luteus* and *C. albicans,* incubated for 24 h in contact with the supernatant withdrawn from HA-CTL-C/CHI films used in Figure 2d. Every 24 h, the film was put in contact with a fresh pathogen suspension. After 24 h of incubation with the film, the supernatant was withdrawn and incubated for 24 h with a fresh pathogen suspension. The normalization was performed with respect to OD_{620} measured in the absence of film and antibiotics taken as 100% growth and in the presence of antibiotic taken as 0% growth. ND means not determined.

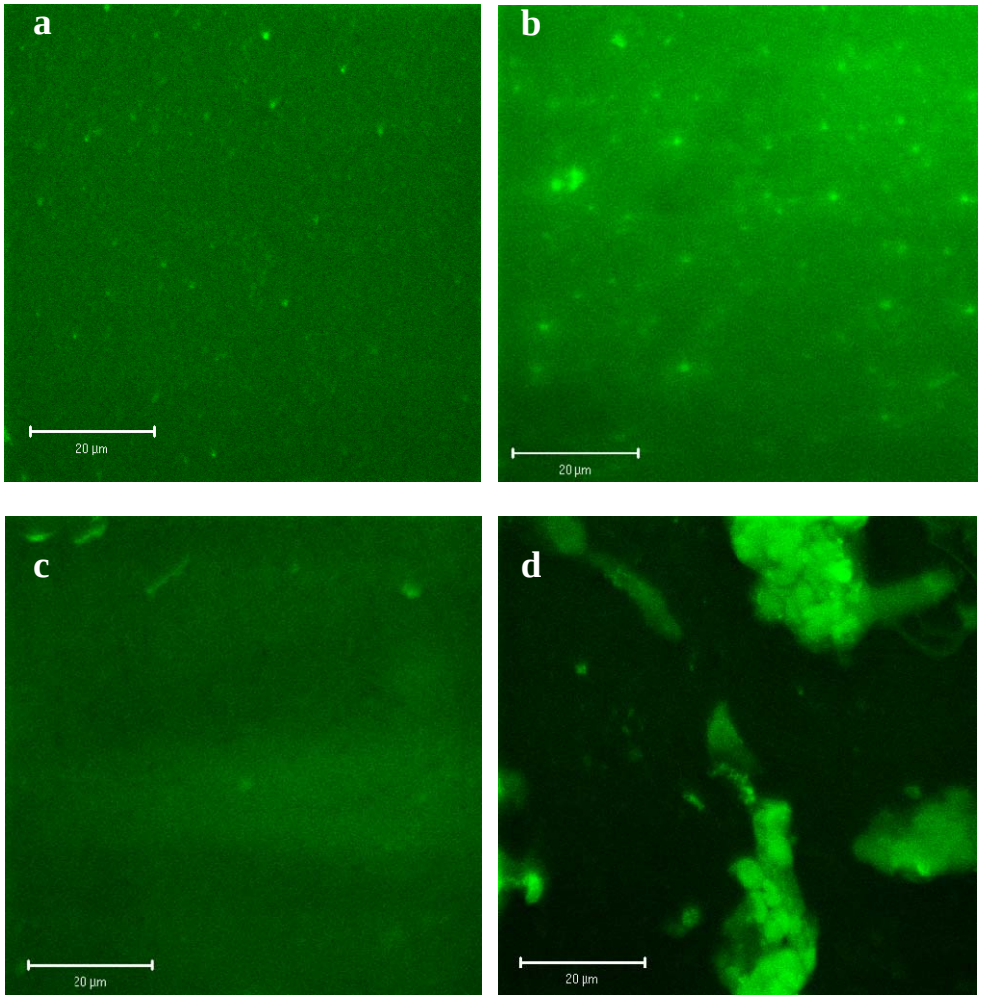

Figure S-4: CLSM images of PEI-$(HA^{FITC}/CHI)_{15}$ (a) before and (b) after paraformaldehyde (PFA) treatment and of PEI-$(HA^{FITC}$-CTL-C/$CHI)_{15}$ (c) before and (d) after PFA treatment. PFA treatment was performed to allow the observation of polysaccharide multilayer films after contact with pathogens. The films are put into contact for 30 min with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.3 and, after several rinsing with PBS, were covered with Moviol 4-88 (Aldrich, Steinheim, Germany). The scale bars represent 20 µm.

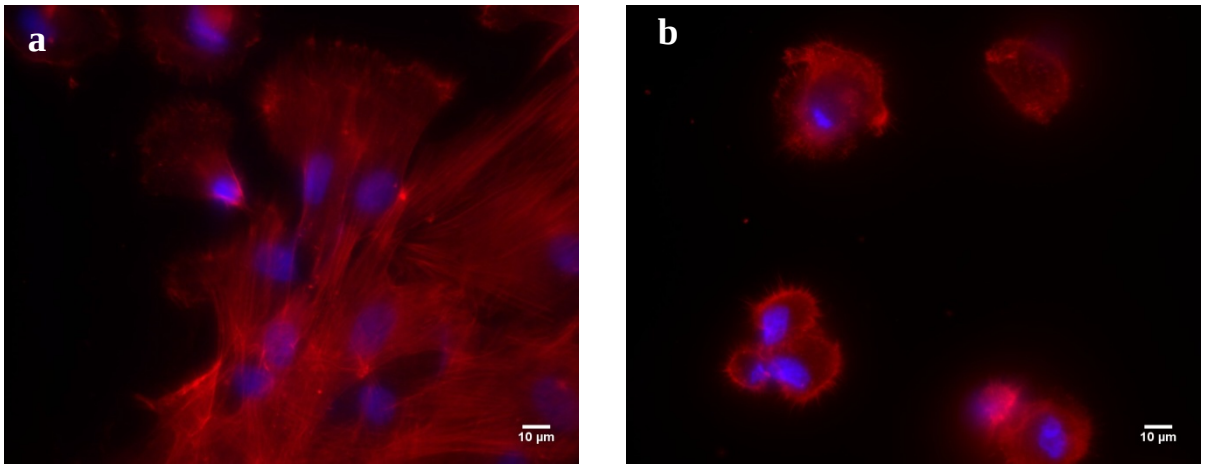

Figure S-5: Immunolabeling of HGFs after 24 h of culture on (a) a glass substrate and (b) on PEI-(HA-CTL-C/CHI)$_{15}$ film. The cytoskeleton was visualized by actin filament immunochemistry staining with phalloidin® (red labeling) and the nuclei with DAPI counterstaining (blue labeling) of HGFs. The scale bars represent 10 µm.

CONCLUSIONS

CONCLUSIONS

My PhD thesis report data of basic and applied research with the development of new antimicrobial materials. The basic studies correspond to the analysis of new cellular and molecular connections in the dynamic of Host-Pathogens interactions. *S. aureus* is a dangerous Gram-positive bacterium responsible of numerous severe diseases that release more than 60 virulence factors. During my PhD I have studied *S. aureus* leukotoxins LukE/D which are characterized to cause lysis of PMNs and other immune cells. By using a proteomic approach we demonstrated that they induce the secretion of granular contents. Since, it has been shown that LukE/D triggered a rise in intracellular free Ca2+ in PMNs which ultimately incited cell secretion. These secretions are results of the cell activation generated by the two LukE and LukD components, a similar effect was observed previously by using PVL of S. *aureus.* Complement C3 factor (CD35) was recovered with neutrophils secretions, which showing the cellular activation of neutrophils and the identification of trademark proteins of different types of granules underlines the ability of leukotoxins LukE/D to degranulate all vesicles. PMNs secrete numerous proteins (243) belonging to different functional categories, such as immune related proteins, antioxidants proteins, proteases and inflammatory components. Out of these functional categories AMPs and immune activation proteins present major share in this specific inflammatory stimulus. Interestingly, we recovered Cgs-derived fragments from CgA and CgB in the secretions of neutrophils, as active participants of the immune system. Cgs play a bi-directional role as secretagogue (immunomodulators) and antimicrobial agents. Because of these important properties with innate immunity, we decided to analyze the antibacterial activity against *S. aureus* of several synthetic Cgs-derived peptides included in the large pro-chromacin C-terminal fragment.

Chromogranins (Cgs) and their derived peptides are highly expressed during various stress and infectious conditions including SIRS and severe sepsis. On the other hand, *S. aureus* is well known for different immune evasion strategies, such as degradation of AMPs. As demonstrated in manuscript II, CTL (CgA344-358) was found to be the only CgA derived peptide that can efficiently effective against different *S. aureus* strains. Moreover, in comparison to the other CgA derived AMPs, it can effectively resist degradation by proteases. Furthermore, we investigated the processing of Cgs by the Glu-C protease of *S. aureus* and observed that Glu-C can effectively process Cgs and render them inactive against different bacterial spp. but interestingly, several new antifungal peptides are generated. These new antifungal peptides are very active and can inhibit growth of different fungal and candida

strains at micromolar concentration. Moreover, it is important to point out that the CTL containing domain of CgA is not processed by Glu-C protease. Additionally, CTL also present promising ability of synergism in combination with other antibiotics. These can be promising features for further studies and to use as potent antibacterial drugs in combination with other antimicrobial agents.

Regarding the Cgs derived peptides production in response to S. aureus LukE/D, and ultimate effectiveness of CTL to kill *S. aureus* coupled with its ability to resist bacterial proteolytic degradation, underlines the necessity to use CTL as antimicrobial agent. Keeping in view these features, we designed a new surface coating based on polysaccharide multilayer films containing functionalized HA with 5% of CTL-C. Antimicrobial properties of CTL-C were preserved when grafted on HA either in solution or when embedded into PEM films. After 24 h of incubation, HA-CTL-C/CHI films fully inhibit the development of *S. aureus* and *C. albicans*, which are common and virulent pathogens agents encountered in care-associated diseases. The presence of CTL-C peptides on HA allows the penetration of the modified polysaccharide inside *C. albicans* after 45 min of contact. The secretion of hyaluronidase by all tested pathogens seems to be responsible for HA-CTL-C release from the film and for its activity. The film can keep its activity during 3 cycles of use against fresh incubated *C. albicans* suspension. Furthermore, the limited fibroblasts adhesion, without cytotoxicity, on HA-CTL-C/CHI films highlights a medically relevant application to prevent infections on catheters or tracheal tubes where fibrous tissue encapsulation is undesirable.

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ANNEXES

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CHROMOGRANIN A-DERIVED PEPTIDES ARE INVOLVED IN INNATE IMMUNITY.

ASLAM R, ATINDEHOU M, LAVAUX T, HAÏKEL Y, SCHNEIDER F, METZ-BOUTIGUE MH.

Chromogranin A-Derived Peptides Are Involved in Innate Immunity

R. Aslam[1], M. Atindehou[1], T. Lavaux[1,2], Y. Haïkel[1,3], F. Schneider[1,2], M.-H. Metz-Boutigue*,[1]

[1]*University of Strasbourg, Biomatériaux et ingénierie Tissulaire, Inserm U977, Strasbourg, France;* [2]*University of Strasbourg, Hôpital de Hautepierre, Service de Réanimation médicale, Strasbourg, France;* [3]*University of Strasbourg, Faculty of Odontology, Strasbourg, France*

Abstract: New endogenous antimicrobial peptides (AMPs) derived from chromogranin A (CgA) are secreted by nervous, endocrine and immune cells during stress. They display antimicrobial activities by lytic effects at micromolar range using a pore-forming mechanism against Gram-positive bacteria, filamentous fungi and yeasts. These AMPs can also penetrate quickly into neutrophils (without lytic effects), where, similarly to "cell penetrating peptides", they interact with cytoplasmic calmodulin, and induce calcium influx *via* Store Operated Channels therefore triggering neutrophils activation. *Staphylococcus aureus* and *Salmonella enteritis* are bacteria responsible for severe infections. We investigated here the effects of *S. aureus* and *S. enteritis* bacterial proteases on CgA-derived peptides and evaluated their antimicrobial activities. We showed that the Glu-C protease produced by *S. aureus* V8 induces the loss of the AMPs antibacterial activities and produces new antifungal peptides. In addition, four antimicrobial CGA-derived peptides (chromofungin, pro-catestatin, human/bovine catestatin) are degraded when treated with bacterial supernatants from *S. aureus* and *S. enteritis,* whereas, cateslytin, the short active form of catestatin, resists to this degradation. Finally, we demonstrate that several antimicrobial CgA-derived peptides are able to act synergistically with antibiotics against bacteria and fungi indicating their roles in innate defense.

Keywords: Innate immunity, antimicrobial peptides, antibiotics, chromogranins, catestatin, chromofungin, *Staphylococcus aureus*, *Salmonella enteritis*, neutrophils, bacterial proteases, synergy, neuroendocrine system, immune system.

INTRODUCTION

The innate immune system, since 2 billion years, is the primary defense mechanism in most living organisms. Antimicrobial peptides (AMPs) are fundamental components of this system in both animals and plants [1-3]. Multidrug antibiotic resistance is a worldwide health problem, therefore the need to produce new efficient antibiotics, acting alone or in combination, is pressing. A major factor in the emergence of antibiotic resistant organisms is the overuse of antibiotics in hospitals and communities. To overcome this abuse, numerous efforts are undertaken to reduce antibiotics prescription and/or to promote their synergistic effects with others molecules. In this context, AMPs represent a promising way considering their direct lytic effect against pathogens in combination with their immunomodulatory properties,

The antimicrobial peptides (AMPs) are highly conserved throughout evolution ensuring organism's defense against a large number of pathogens in various conditions. In addition to their direct antimicrobial activity, they also have a wide range of endogenous functions, notably the modulation of both innate and adaptive immunity. To date more than 1414 antibacterial, 560 antifungal and 107 antiviral peptides have been identified, (antimicrobial peptides database http://aps.unmc.edu/AP/main.php). Among them, cytokines, chemokines, several neuropeptides and fragments derived from proteins exhibit antimicrobial activity. The folding of many AMPs adopts an amphipathic profile important for their antimicrobial activity: their cationic properties induce an electrostatic attraction to the negatively-charged phospholipids of microbial membranes while their hydrophobicity favors their integration into the microbial cell membrane, leading to membrane disruption. Furthermore, the amphipathic structure also allows the peptides to be soluble in both aqueous environments and lipid membranes [4]. In mammals, the most well-studied AMPs are human defensins and cathelicidins [5, 6]. Several proteins such as lysozyme, caseins, hemoglobin, lactalbumin, secretory phospholipase A2 and lactoferrin also display antimicrobial activity against multiple microorganisms.

*Address correspondence to this author at the University of Strasbourg, Biomatériaux et Ingénierie Tissulaire, Inserm U977, Faculté de chirurgie dentaire, Bâtiment Leriche, Hôpital Civil, Porte de l'Hôpital, 67000 Strasbourg, France; Tel: +33368 85 54 71; Fax: +33368 85 54 72; E-mail: marie-helene.metz@inserm.fr

STRUCTURAL AND BIOLOGICAL PROPERTIES OF THE ANTIMICROBIAL PEPTIDES DERIVED FROM CHROMOGRANINS/SECRETOGRANINS

Chromogranins/secretogranins (CGs/SGs) of the granin family are acidic proteins present in secretory vesicles of nervous, endocrine and immune cells [7]. The natural processing of bovine CGs is well described in granules of particular endocrine cells, the sympathoadrenal medullary chromaffin cells (referred as chromaffin cells). In these cells, CGs are cleaved in the chromaffin granules by prohormone convertases (PC1/3 and PC2) and carboxypeptidase E (CPE) and the resulting peptides are co-secreted with the catecholamines [8-10]. Moreover, secretogranin II (SGII), the third member of the chromogranin family, is also processed to generate several natural fragments [11-13]. In the late 80's, the discovery of pancreastatin, a chromogranin A (CGA)-derived peptide inhibiting insulin secretion from pancreatic beta-cells, initiated the concept of prohormone [14, 15].

In vivo studies reported numerous peptides produced by CGA cleavage, some of which displaying dual biological activities. These peptides modulate homeostatic processes [16], but are also considered as defense molecules of an infected host. During the past decade, our laboratory has characterized new antimicrobial CGs-derived peptides [16, 17-20] Fig. (**1**) and has also reported host defense agents derived from CGA during infections [21].

The AMPs derived from CGA act at micromolar range against bacteria, fungi, yeasts and are non-toxic for mammalian cells. They are found in biological fluids involved in defense mechanisms (serum, saliva) and in supernatants of stimulated human neutrophils [19, 20].

These new AMPs are well integrated in the innate immune system, highlighting the key role of the adrenal medulla which releases large amount of IL-6 and TNF-alpha in response to pro-inflammatory stimuli, such as LPS, IL-1 alpha and beta [22]. The discovery of the AMPs together with the presence of TLRs on adrenal cortex cells indicate that the adrenal gland is involved in innate immunity by the activation of the innate immune response and by the clearing of infectious agents [22].

Sequences of CGA-derived AMPs are highly conserved during evolution. Interestingly, the main cleavage site in bovine CGA (bCGA) at position 78-79 and the subsequent removal of the two basic residues K77 and K78 by the carboxypeptidase H [8]

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Peptide	Location	Sequence	Net charge
CGA			
VS-I	1-76	LPVNSPMNKGDTEVMKC*IVEVISDTLSKPSPMPVSKEC*FETLRGDERILSILRHQNLLKELQDLALQGAKERTHQQ	+3
NCA	4-40	NSPMNKGDTEVMKC*IVEVISDTLSKPSPMPVSKEC*FE	-1
CHR	47-66	RILSILRHQNLLKELQDLAL	+1.5
Chrom	173-194	YPGPQAKEDSEGPSQGPASREK	-1
CAT	344-364	RSMRLSFRARGYGFRGPGLQL	+5
CCA	418-427	LEKVAHQLEE	-2
ProChrom	79-431	HSSYEDELSEVLEK.....	-37
CGB			
Chromb	564-626	SAEFPDFYDSEEQMSPQHTAENEEEKAGQGVLTEEEEKELENLAAMDLELQKIAEKFSGTRRG	-12
SEC	614-626	QKIAEKFSGTRRG	+3
CGC			
Rrf	131-138	RKLKHMRF	+4.5
Kvk	430-443	KVLSRLPYGPGRSK	+4

Fig. (1). The antimicrobial bovine CGs-derived peptides according to the sequences reported in the Protein Knowledge Base UniprotKB for CGA (P05059), CGB (P23389) and CGC (P20616). For each AMP, the sequence, the location and net charge are indicated. *, cysteine residues of the disulfide bridge; phosphorylated residue are underlined and the glycosylated residue is in bold. This figure was originally published in Regul. Peptides, Shooshtarizadeh P *et al.*, The antimicrobial peptides derived from chromogranin/secretogranin family, new actors of innate immunity. Regul. Peptides, 2010 165(1), 102-110.

produces two antimicrobial fragments: vasostatin-I (VS-I; bCGA1-76) [19] and prochromacin (ProChrom; bCGA79-431) [23]. These large N- and C-terminal antimicrobial domains contain shorter active fragments: bCGA4-40 (NCA) [24, 25], bCGA47-66 (chromofungin; CHR) in VS-I and bCGA173-194 (chromacin; Chrom) and bCGA344-364 (catestatin; CAT) in ProChrom [25]. VS-I and NCA contain the unique disulfide bridge and two post-translational modifications are important for the antibacterial activity of Chrom: the phosphorylation of Y173 and the O-glycosylation of S186 [23].

Vasostatin-I

Vasostatin-I (VS-I) displays antimicrobial activity against (i) Gram-positive bacteria (*Micrococcus luteus* and *Bacillus megaterium*) with a minimal inhibitory concentration (MIC) of 0.1-1 μM; (ii) filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Alternaria brassicola, Nectria haematococca, Fusarium culmorum, Fusarium oxysporum*) with a MIC of 0.5-3 μM and (iii) yeast cells (*Saccharomyces cerevisiae, Candida albicans*) with a MIC of 2 μM [19]. However VS-I is unable to inhibit the growth of *Escherichia coli* SBS363. VS-I presents AMPs-specific structural features, such as a global positive charge (+3), an equilibrated ratio of polar and hydrophobic residues (20:23) and the presence of a helical region CGA40-65 corresponding to a calmodulin-binding sequence [26, 27] Fig. (**1**). Furthermore, we have demonstrated that the N-terminal bCGA1-40 fragment interacts with a model of phospholipid membrane in a concentration range relevant for biological activity in mammalian tissue [28].

Chromofungin

Treatment of VS-I by endoprotease Glu-C from *Staphylococcus aureus* generates the evolutionary conserved and shortest VS-I peptide, chromofungin (CHR), suggesting that this peptide may be released during infections by *S. aureus*. CHR presents antifungal activity at 2-15 μM against filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Alternaria brassicola, Nectria haemato-cocca, Fusarium culmorum, Fusarium oxysporum*) and yeast cells (*Candida albicans, Candida tropicalis, Candida neoformans*) [26].

Catestatin

Two CGA-derived fragments bCGA333-364 and bCGA343-362 were characterized after the extensive processing of bCGA by prohormone convertases (PC 1/3 or 2) in chromaffin granules [29]. More recently, it has been shown that cathepsin L co-localizes with CGA in chromaffin granules. Digestion of recombinant human CGA (hCGA) *in vitro* generates a catestatin (CAT)–derived fragment hCGA360-373 [30]. In addition to the inhibitory effect of bovine catestatin (bCAT) on catecholamine release from chromaffin cells [31], we have shown that this peptide, highly conserved through evolution [20] and its shorter active sequence bCGA344-358 (cateslytin, CTL), Fig. (**1**) present a potent antimicrobial activity with a MIC in the low-micromolar range against Gram-positive bacteria (*Micrococcus luteus, Bacillus megaterium* at concentration of 0.8 μM), Gram-negative bacteria (*Escherichia coli D22* at concentration of 8 μM), filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Nectria haematococca* at concentration of 0.2-10 μM) and yeasts (*Candida albicans, Candida tropicalis, Candida glabrata, Candida neoformans* at concentration of 1.2-8 μM). The two human variants of CAT, P370L and G364S, present antibacterial activity against *M. luteus* with a MIC of 2 and 1 μM, respectively, and against *E. coli* with a MIC of 20 and 10 μM, respectively [20]. The shorter peptide, CTL, from bovine origin, is the most active. Bovine CTL contains a cationic sequence with a global net charge of +5 (R344, R347, R351, R353, R358) and five hydrophobic residues (M346, L348, F360, Y355, F357) Fig. (**1**), and is able to completely kill bacteria at concentration lower than 10 μM even in the presence of NaCl (0-150 mM) [20].

C-Terminal CGA-Derived Fragment

CCA, the C-terminal CGA-derived fragment bCGA418-427 Fig. (**1**), with a remarkable net charge of -2, displays antifungal activity and belongs to the less abundant anionic AMPs family. CCA is conserved through evolution and is homologous to both the C-terminal sequence of CGB and the antibacterial peptide SEC (secretolytin) Fig. (**1**) [17]. *In vitro*, this peptide was generated from chromaffin secretory vesicles after digestion, by the *S. aureus*

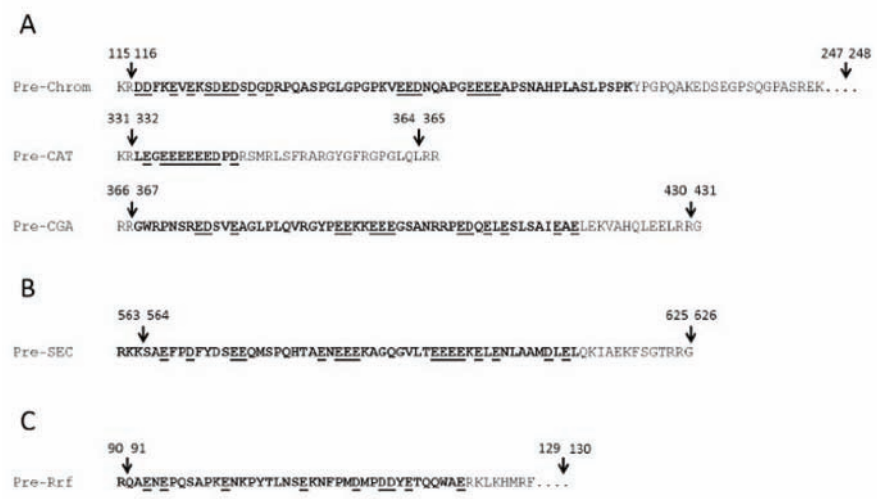

Fig. (2). Anionic pro-peptides sequences found in bovine CGs-derived peptides. The sequence of each antimicrobial peptide is indicated in grey. The acidic residues present in the propeptide sequence are underlined. (A), CGA-derived fragments (Chrom, CAT, CCA); (B), CGB-derived fragment (SEC); (C), CGC/SGII-derived fragment (Rrf). Arrows represent natural cleavage sites occurring during the storage in chromaffin granules. This figure was originally published in Regul. Peptides, Shooshtarizadeh P *et al.*, The antimicrobial peptides derived from chromogranin/secretogranin family, new actors of innate immunity. Regul. Peptides, 2010 165(1), 102-110.

protease Glu-C. As previously hypothesized for CHR, CCA could be generated during infections induced by *S. aureus*.

Antimicrobial Peptides Derived from Bovine Chromogranin B

To date, the natural C-terminal fragment of bovine CGB (CCB; bCGB564-626), isolated from chromaffin granules of the adrenal medulla, presents antibacterial activity against both *M. luteus* and *E. coli*. The complete inhibition of bacterial growth was observed at a concentration of 1.8 µM [17]. This large fragment contains the natural short antibacterial peptide secretolytin (SEC, bCGB614-626) with a net positive charge (+3) Fig. (**1**). A structure-activity analysis suggests that an alpha-helical amphipathic structure, common to SEC and cecropins, may account for the antibacterial activity [17].

Antimicrobial Peptides Derived from Bovine Secretogranin II

The weak expression of bSGII in the intragranular matrix of chromaffin secretory vesicles (2% of total proteins) prevents the detection of its endogenous AMPs by classical methods. However, *in-silico* analysis allowed the identification and preparation of two synthetic peptides with cationic amphipathic sequences: Rrf and Kvk, corresponding respectively to the sequences bSGII131-138 (net charge +4.5) and bSGII430-443(net charge + 4) Fig. (**1**). Rrf completely inhibits the bacterial growth of *M. luteus* and *B. megaterium* with a MIC of 5 and 15 µM, respectively, while Kvk displays antifungal properties at 19 µM against *N. crassa* [25]. Like numerous AMPs, antimicrobial CGs/SGs derived peptides are expressed as pro-peptides which undergo subsequent proteolytic processing to release active molecules.

THE CGA-DERIVED PEPTIDES: PRODUCTION AND MOLECULAR MECHANISM OF THE ANTIMICROBIAL ACTIVITIES

Previously, we have identified a number of AMPs derived from chromogranins (CGs), proenkephalin-A and ubiquitin which are co-secreted with catecholamines upon stimulation of chromaffin cells from the adrenal medulla [20, 25]. It should be noted that the pro-sequences of several active peptides derived from CGA (Chrom, CAT, CCA), CGB (SEC) and SGII (Rrf) are composed of anionic amino acids similar to those observed in pre-prodefensins [32] Fig. (**2**).

When polymorphonuclear neutrophils, known to accumulate at sites of infection, are stimulated by bacterial agents, such as Panton-Valentine leucocidin (PVL), they produce and secrete complete and processed forms of CGA, notably vasostatin-I (residues 1-76), vasostatin-II (residues 1-113) [19] and cateslytin (residues 344-358) [20] Fig. (**3A**). Others experiments have detected catestatin in infected skin [21], consistent with the presence of CGA in keratinocytes. Catestatin present an antimicrobial activity against skin microbes, including Gram-positive and Gram-negative bacteria, yeast, and fungi. In human skin, CGA was found to be proteolytically processed to produce catestatin. Interestingly, catestatin expression in murine skin is increased in response to injury and infections, potentially providing protection against infection Fig. (**3B**). These results demonstrate that a neuroendocrine peptide with antimicrobial function is upregulated upon injury, suggesting a direct link between the neuroendocrine and cutaneous immune systems [21].

The AMPs have two modes of action on microorganisms [33]. They can either target and permeabilize cell membranes following several described mechanisms [34] or alternatively they can act on intracellular targets to induce DNA binding, enzyme inactivation and inhibition of cell wall synthesis. These two mechanisms can be illustrated by the antimicrobial action of chromofungin (CHR) and catestatin (CAT), two AMPs derived from CGA. The NMR structure of CHR showed a helical amphipathic character in the presence of trifluoroethanol [26]. This property allows the peptide to bind and to cross fungal membranes (*Aspergillus fumigatus*) [26] Fig. (**4B**). CHR penetrates into microorganisms creating holes, through which the inactive rhodaminated CGB602–626 peptide is able to pass Fig. (**4C** and **D**) inducing cell wall and membrane destabilization. In order to kill the microorganism, the membrane destabilization is completed by the interaction of CHR with calmodulin, an intracellular target [26]. CAT acts similarly to CHR [20].

BIOLOGICAL ROLES OF CHR AND CAT WITH NEUTROPHILS

On mammalian cells, CHR and CAT act following different ways: (i) membrane crossing and stimulation of innate immunity

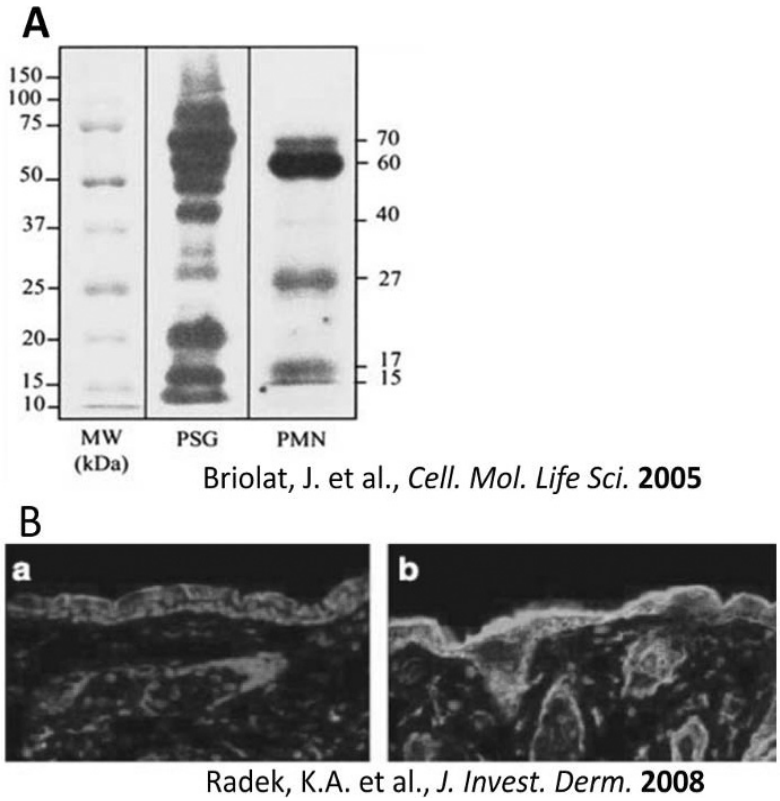

Fig. (3). Production of catestatin by neutrophils and keratinocytes. (A), Western blot analysis (12%, SDS-PAGE) with polyclonal anti-catestatin antibody. 1st lane, Molecular weight standards (MW); 2nd lane, soluble proteins from chromaffin granular matrix (PSG); 3rd lane, secretions from PMNs activated by Panton-Valentine leucocidin. (B), CAT staining is shown at (a) baseline, (b) 3 hours post-tape stripping. This work was originally published in Cell. Mol. Life Science, Briolat J. *et al*., New antimicrobial activity for the catecholamine release-inhibitory peptides from chromogranin A, 2005, (21), 377-385 and in J. Invest. Dermatol., Radek, K.A. *et al.*, J. Invest. Dermatol. 2008, 128(6), 1525-1534.

[35], (ii) binding to AchR and/or surrounding lipids, acting as an anti-stress hormone [36] and (iii) modulating myocardial cells by a yet unknown mechanism [37].

In 2009, our group has investigated the effects of CHR and CAT on PMNs activation using a broad range of techniques (biochemistry, confocal microscopy, flow cytometry, calcium imaging, surface plasmon resonance and proteomic analysis) [35]. By confocal microscopy, we first demonstrated the uptake of rhodaminated-labeled CHR and CAT into isolated PMNs Fig. (**5**). We showed that these CGA-derived peptides stimulate exocytosis from PMNs by triggering a transient Ca^{2+} influx, independent from intracellular vesicles release. For the two peptides, the molecular mechanisms involve calmodulin-binding, subsequent activation of the calcium-independent phospholipase A2 and opening of the store-operated channels which induce the secretion of numerous factors involved in innate immunity. The complete mechanism is reported in Fig. (**6**). Stress and infection lead to two different pathways stimulating PMNs secretion: release of CGA and CGA-derived peptides from the adrenal medulla, as indicated by 1a–6a (black), and PVL leucocidin stimulation by *S. aureus* infections, as indicated by 1b–3b (grey), respectively. The stress-stimulated pathway leads to the penetration of P into the cytoplasm (2a), resulting in the removal of inhibitory CaM, the activation of iPLA2 to produce LysoPL (3a) and the activation of Ca2+ influx through SOC (4a), finally converging on the activated docking of secretory granules (5a) and subsequent release of proteins relevant for innate immunity (6a). The negative feedback induced by P' on nicotinic cholinergic receptor in chromaffin cell is also indicated (7a). The infective route leads to the activation of the putative PVL receptor triggering the opening of Ca2+-channels and subsequent increase in intracellular Ca2+ (3b) leading to the docking of secretory granules and secretion of proteins relevant for innate immunity (6a) [35].

HOST-PATHOGEN INTERACTIONS: THE DEGRADATION OF ANTIMICROBIAL CGA-DERIVED PEPTIDES BY BACTERIAL PROTEASES

The AMPs avoidance mechanisms deployed by bacteria include the proteolytic degradation of the active forms by the bacterial proteases. To examine the effects of bacterial proteases, we have tested *Staphylococcus aureus* V8 protease Glu-C and supernatants S1 and S2 from *S. aureus* on isolated AMPs derived from CGs using biochemical methods. In addition, supernatants from *Salmonella enteretica*, *Klebsiella oxytoca*, *Shigella sonnei* and *Vibrio cholera* were also tested.

Interaction of Antimicrobial CGs-Derived Peptides with Proteases from Staphylococcus Aureus

After incubation of intragranular chromaffin cell contents with *S. aureus* V8 protease Glu-C, 21 new peptides located along the CGA sequence Fig. (**8**), were isolated by HPLC Fig. (**7**) and analyzed by sequencing and MALDI-TOF mass spectrometry. These peptides were tested against Gram positive bacteria (*Micrococcus luteus* and *S. aureus*), Gram negative bacteria (*Escherichia coli*), fungi (*Neurospora crassa*) and yeast (*Candida albicans*). Surprisingly, they do not present any antibacterial activity; however 5 peptides corresponding to CGA47-60, CGA418-426 and CGB279-291, CGB450-464 and CGB470-486 showed antifungal activity at the micromolar range against *N. crassa*. Thus, *S. aureus* subverts innate immunity by degrading the

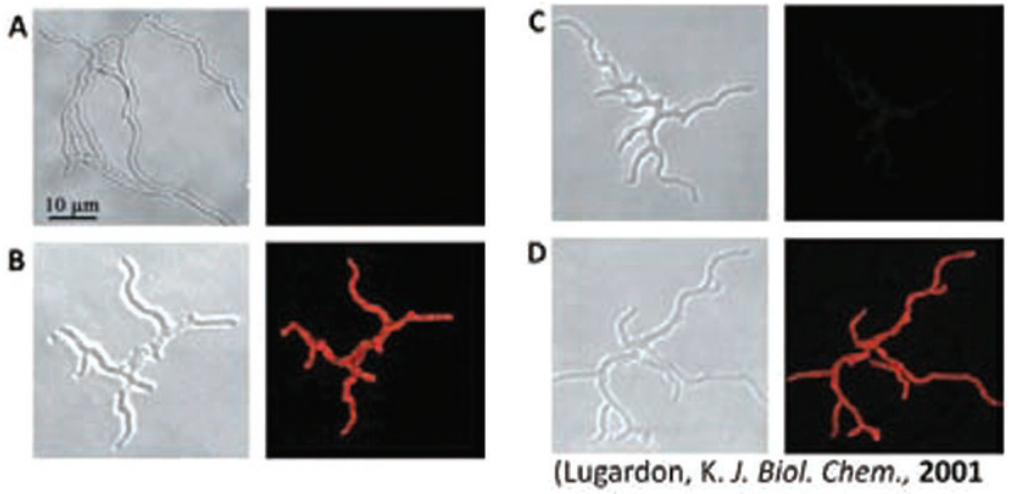

Fig. (4). Phase-contrast (*left*) and fluorescence confocal laser (*right*) micrographs of *Aspergillus fumigatus* after incubation with rhodamine-labeled synthetic peptides (CHR and CGB602–626, as a control). (A), in the absence of rhodamine-labeled synthetic peptide; (B), after incubation with 10 µM rhodaminated CGA47–66 during 1 h; (C), after incubation with 10 µM rhodaminated CGB602–626 for 1 h. (D), *A. fumigatus* was examined after 24 h in cultured medium (30 °C) and preincubated during 1 h at 30 °C with 100 µM unlabeled CGA47–66 and 10 µM rhodaminated CGB602–626. An intense staining was observed, indicating that CHR induced cell wall and membrane destabilization with formation of holes through which rhodaminated CGB602–626 peptide is able to pass. This research was originally published in J. Biol. Chem. Lugardon K. *et al.*, Structural and biological characterization of chromofungin, the anti-fungal chromogranin A-(47-66)-derived peptide. 2001, J. Biol. Chem. 276(38), 35875-35882.

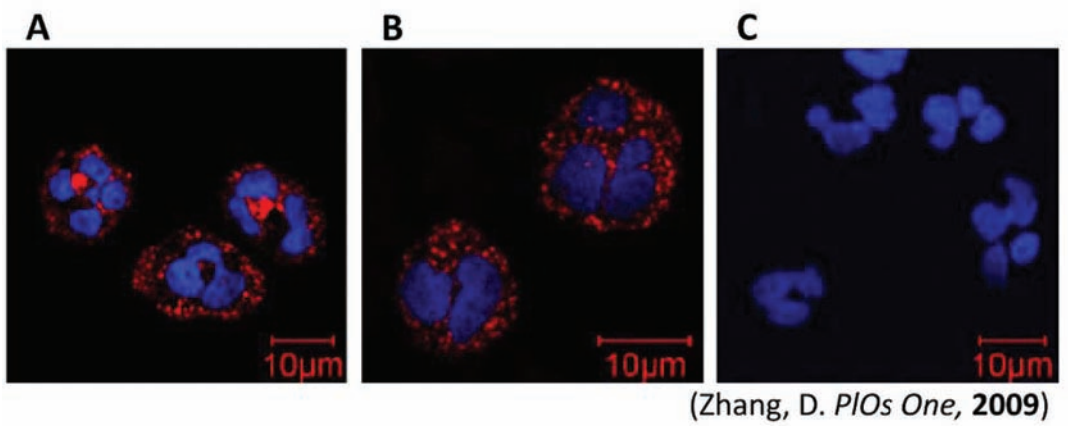

Fig. (5). Fluorescence confocal microscopy of PMNs after incubation with 20 µM rhodaminated labeled CHR (A) and CAT (B). Unactive rhodaminated HCNP (Hippocampal Cholinergic Neurostimulating Peptide) was used as control (C). This research was originally published in PlOsOne. Zhang, D. *et al.*, Two Chromogranin A-derived peptides induce calcium entry in human neutrophils by calmodulin regulated calcium independent phospholipase A2. PLoS One., 2009, *4*(2):e4501.

antibacterial CGs-derived peptides to produce new antifungal molecules. Finally, *S. aureus* protects itself by degrading endogenous antibacterial peptides and generating new products presenting antifungal properties.

Degradation of the natural antimicrobial peptide Vasostatin-I [19] generates the peptides 1-13, 14-20 linked to 38-40 by a disulfide bridge, 21-37, 47-60 and 61-71 Fig. (**8**). Prochromacin, the antimicrobial C-terminal domain [23] was also degraded to form 14 peptides (79-85, 89-101, 89-104, 148-154, 157-180, 200-207, 228-244, 245-254/264, 274-283, 284-303, 334-378, 379-391, 387-394 and 418-426) Fig. (**8**). Chromacin ($CGA_{173-194}$), [23] was proteolysed after the residue E_{180} and pro-catestatin ($CGA_{334-378}$) was resistant to the proteolysis by *S. aureus* V8 protease Glu-C in our experimental conditions Fig. (**8**).

In a second series of experiments three antimicrobial CGA-derived peptides (bCAT, hCAT and CTL) were incubated in the presence of staphylococcal supernatants S1 (a Methicillin resistant strain, MRSA) and S2 (a non-resistant strain). Bacteria were isolated from patients of the Strasbourg Civil Hospital and the supernatants were prepared by the Bacteriology Institute at University of Strasbourg, (EA-4438). CTL (CGA344-358), the active domain of CAT, is able to kill *S. aureus* (strain ATCC 49775) at 30 µM, but the two others peptides (bCAT and hCAT) are inactive. By proteomic analysis (HPLC, sequencing and mass spectrometry), we demonstrated that the two fragments bCGA344-364 (bCAT) and hCGA352-372 (hCAT) were cleaved at the L-S bond by S1 and at the S-F/G-F bonds by S2. In contrast, CTL was very weakly degraded by both S1 and S2 supernatants Fig. (**9**). These data suggest that the C-terminal sequence of CAT (GPGP/LQL) induces a conformation of the total peptide that might be cleaved by *S. aureus* proteases, whereas CTL remains stable. Using the GOR secondary structure prediction method [38], it appears that the CTL domain with extended strand prediction corresponds to 60.3% of the total sequence compared with 38% for the 2 CAT sequences. Consistently, previous reports indicated the

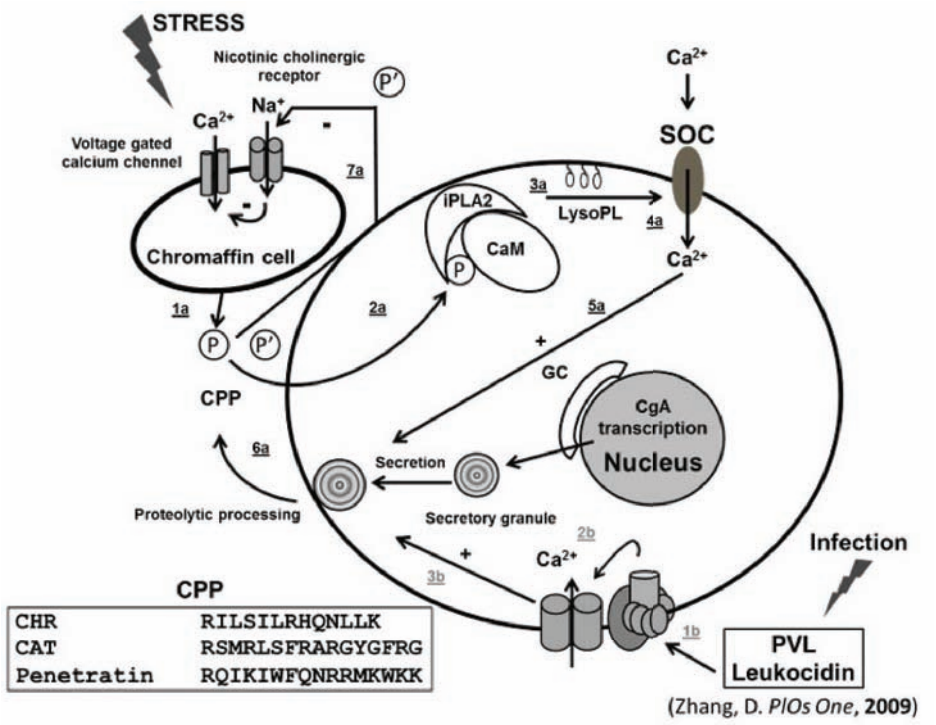

Fig. (6). Model for the activation of PMNs by CHR and CAT. P (CHR), P' (CAT), CPP (cell penetrating peptide, CaM (calmodulin), iPLA2 (calcium independent phospholipase A2), LysoPL (lysophospholipids), GC, Golgi complex, PVL, Panton-Valentine leucocidin, R (receptor). Stress and infection lead to two different pathways for stimulation of PMN secretion, by release of CgA and CgA-derived peptides from the adrenal medulla, as indicated by 1a–6a (black), and by PVL leucocidin stimulation by S. aureus infections, as indicated by 1b–3b (grey), respectively. Abbreviated symbols: P (CHR, CAT), P' (CAT), CPP (Cell Penetrating Peptide), CaM (calmodulin), iPLA2 (calcium independent phospholipase A2) LysoPL (lysophospholipids); GC (Golgi complex); PVL (Panton-Valentine leucocidin), R (receptor). The stress-stimulated pathway leads to penetration of P into the cytoplasm (2a), resulting in removal of inhibitory CaM, activation of iPLA2 to produce LysoPL (3a) and activation of Ca^{2+} influx through SOC (4a), converging on activated docking of secretory granules (5a) and subsequent release of proteins of relevance for innate immunity (6a). The negative feedback induced by P' on nicotinic cholinergic receptor of chromaffin cell is also indicated (7a). The infective route leads to activation of the putative PVL receptor coupled to opening of Ca^{2+}-channels and a rise in intracellular Ca2+ (3b) that converges on docking of secretory granules and subsequent secretion of proteins of relevance for innate immunity (6a). This research was originally published in PlOs One. Zhang, D. *et al.*, Two Chromogranin A-derived peptides induce calcium entry in human neutrophils by calmodulin regulated calcium independent phospholipase A2. PLoS One., 2009, *4*(2):e4501.

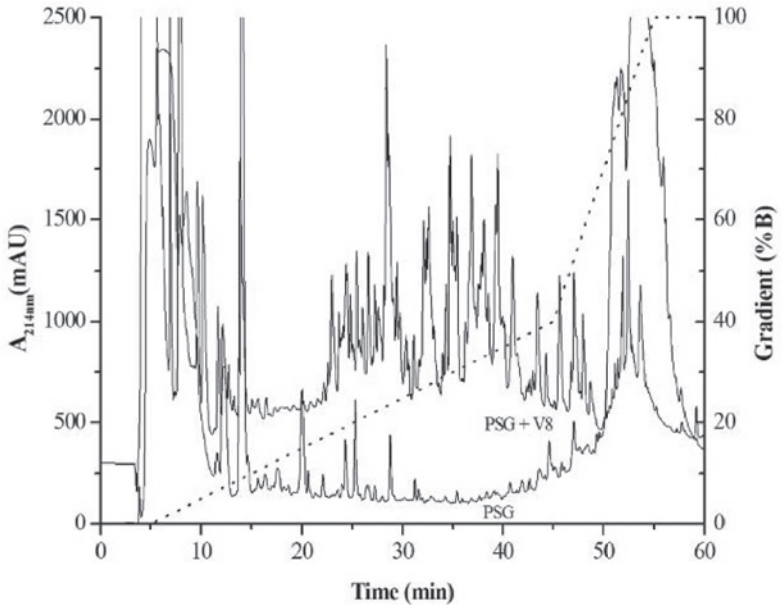

Fig. (7). Separation by HPLC of bovine chromaffin intragranular matrix (grey curve) and of V8 protease Glu-C digest of bovine chromaffin intragranular matrix (1 mg, black curve). HPLC was performed on a Macherey Nagel Lichrospher 100-5-RP-C18 column (4 x 250 mm; particle size 5 µm and pore size 100 nm). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile and 30% (v/v) water (solvent B). Absorbance was monitored at 214 nm and elution was performed with a linear gradient as indicated on the right-hand scale.

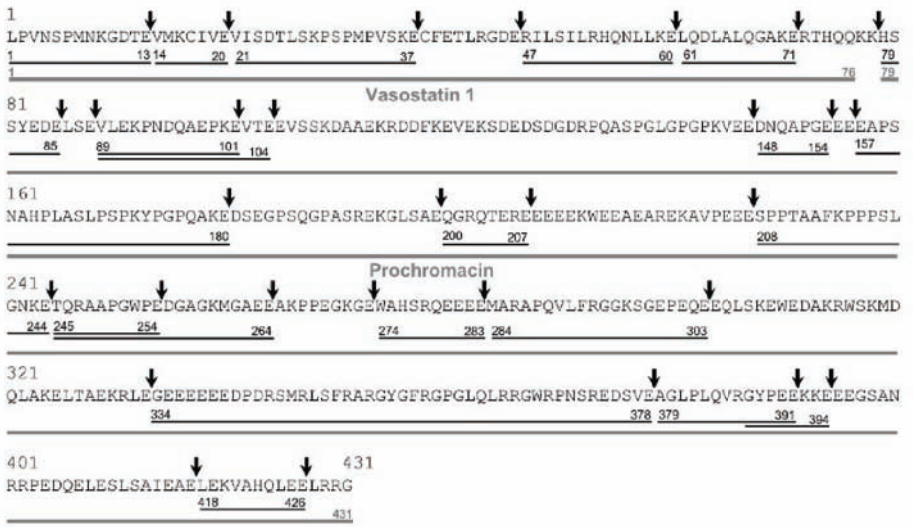

Fig. (8). Sequence alignment of the identified peptides produced after incubation of the stimulated chromaffin cell secretions with *S. aureus* V8 protease Glu-C. Antimicrobial regions Vasostatin and Prochromacin are marked by grey lines and the new generated peptides are in black. Arrows indicate cleavage site by Glu-C protease.

importance of an extended strand for several classes of protease inhibitors [39]. Our observation indicates that the structure of CTL might present a protease inhibitor activity.

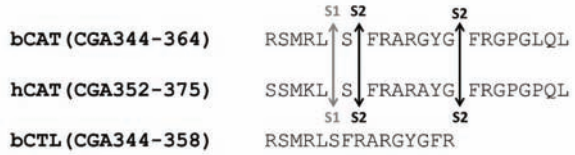

Fig. (9). Degradation analysis of bCAT, hCAT and CTL with S1 and S2 two *Staphylococcus aureus* supernatants. Arrows indicate cleavage sites produced by proteases from S1 and S2. CTL resists to the proteolytic degradation.

Interaction of Antimicrobial CGs-Derived Peptides with Proteases from Diarrheogenic Bacteria

Bacteria were isolated from patients of the Strasbourg Civil Hospital by the Bacteriology Institute, University of Strasbourg, (EA-4438). *Klebsiella oxytoca, Salmonella enterica, Shigella sonnei, and Vibrio cholera* infections have a clinical interest as they cause other symptoms beside diarrhea. *Klebsiella oxytoca* may be commensal [42] and is involved in post-antibiotic diarrheas [40], as well as nosocomial infections in newborns and adults [41]. *K. oxytoca* has also been associated with hemorrhagic colitis [43] and intercurrent colitis in Crohn's disease [44]. *Salmonella enterica* destroys infected cells and causes gastrointestinal infections which propagates through blood (sepsis) or through lymphatic vessel (typhoid fever). *Shigella sonnei* and *Vibrio cholera* non O1 produce Shiga toxin which causes inflammation of the intestinal mucosa. *Klebsiella oxytoca, Salmonella enterica, Shigella sonnei, and Vibrio cholera* all develop an antibiotic resistance. *Salmonella* was reported to be resistant to the action of Ciprofloxacin [45] and Ceftriaxone [46].

We tested the antimicrobial activity of bovine (bCGA344-364), rat (rCGA344-364) and human (hCGA352-372) CAT, bovine CTL (bCGA344-358), two short fragments hCGA360-372 and the conserved tetrapeptide LSFR (bCGA348-351) against the four bacterial strains mentioned above. In addition, we tested a scrambled peptide relative to the sequence of bovine CAT and the procatestatin fragment bCGA332-364. We found antimicrobial activities only for bovine CAT and CTL, indicating that CTL, the shorter fragment, corresponds to the active domain of CAT. Procatestatin was inactive in similar experimental conditions. Bovine CAT and CTL were active against *Klebsiella oxytoca*, *Salmonella enterica* and *Vibrio cholera* at 100 µM and 30 µM respectively and against *Shigella Sonnei* at 50 µM and 25 µM. In addition, CHR and the C-terminal fragment (CGA387-431) were inactive for concentration below 100 µM. We then compared the profiles of the peptides alone and with the inoculated medium, using HPLC.

These experiments showed that all the peptides were completely degraded by the bacteria, except for CTL. The degradation by bacterial proteases was observed by the HPLC profiles of proCAT (CGA332-364) after incubation with *Salmonella enterica* Fig. (**10**). The complete peptide and the processed forms were analyzed by sequencing and mass spectrometry (MALDI-TOF) Fig. (**10**).

SYNERGY OF THE COMBINATION OF ANTIMICROBIAL CGA-DERIVED PEPTIDES WITH ANTIBIOTICS

The high toxic effects of antibiotics have shifted the research focus towards the discovery of new combination of antimicrobial agents with broad spectrum of activity and less toxicity. Synergy is the combined activity of two agents which is higher than the added activity of each agent separately [47]. Considering the direct interaction of AMPs with membranes, they represent good candidates to potentiate antibiotics activity through synergistic actions. To demonstrate that antimicrobial peptides are able to reduce the doses of antibiotics used and to potentiate their activity, we evaluated the synergistic effects of three CGA-derived peptides (CAT, CTL and amidated CTL) together with Minocyclin and Voriconazole. Antimicrobial tests were carried out using the antimicrobial peptides (at concentration below MIC), the antibiotics or a combination of both, evaluating the fractional inhibitory concentration (FIC): FIC $\leq$0.5 corresponds to a synergistic effect, between 0.5 and 2 to an additive effect, and > 2 to an antagonistic effect [47]. Importantly, the combination of amidated CTL and Minocyclin led to a FIC of 0.37 against *S. aureus*. In addition, for CTL and Voriconazole, we obtained a FIC of 0.25 and 0.5 against *Candida albicans* and *Candida tropicalis,* respectively.

One could imagine a mechanism by which the AMPs could favor the destabilization of the membrane allowing the antibiotics to rapidly penetrate inside the bacterial cells and more efficiently

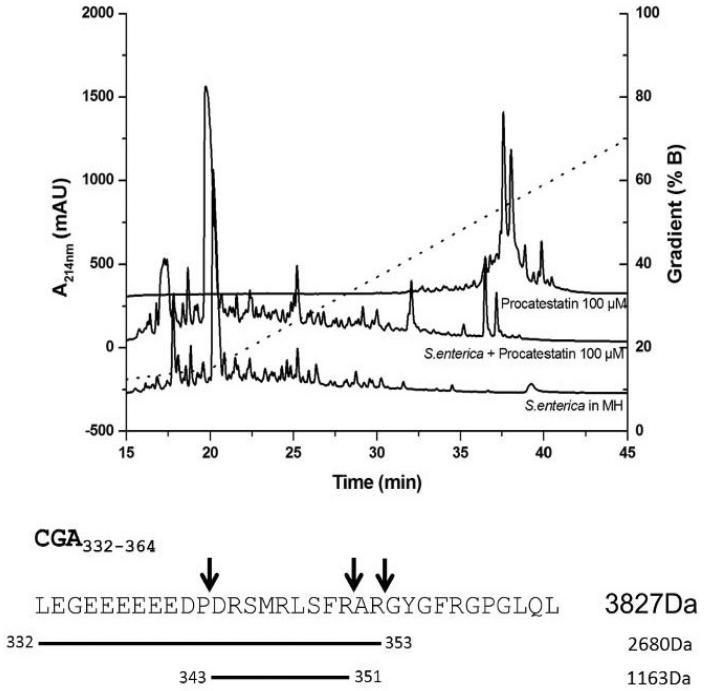

Fig. (10). Analysis by HPLC of the procatestatin (proCAT) and degradation by *Salmonella enterica*. The HPLC system is composed by a Dionex chromatogram, (Germerong, Germany), using a Nucleosil 300-5 C18 column (4×250 mm, particle size 5 µm, porosity 300 Å; Macherey Nagel, Düren, Germany). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in milliQ water (solvent B). Elutions were performed at a flow rate of 700 µL min^{-1} using the gradient indicated on the chromatogram. Arrows correspond to the cleavage sites induced by *S. enteretica*.

reach its site of action. Together our results suggest that CGA-derived peptides could potentiate the effects of antibiotics by favoring membrane destabilization.

CONCLUSIONS

The natural AMPs derived from CGA are important components in innate immunity. These peptides act rapidly to destroy microorganisms after destabilization of their outer membrane. AMPs are non-toxic for the host cells and stimulate neutrophils *via* a calcium influx and store operated channels.

Together with recent data reporting that numerous antifungal peptides correspond to short sequences (<25 residues), we showed that *S.aureus* is able to degrade antibacterial peptides producing smaller fragments with antifungal activities [48]. Thus, a new concept might be proposed in which the proteolytic degradation of long natural and antibacterial peptides by host pathogens might produce new antifungal fragments. Our studies show that, unlike larger CAT peptides, the short CTL peptide resists to bacterial supernatants. Its secondary structure prediction corresponds to a larger domain of extended strand, characterized by proteolytic inhibitory properties. Finally CAT and CTL peptides might be used with antibiotics to synergistically destabilize the pathogen's membrane allowing the drug to rapidly penetrate inside the microorganisms.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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The Natural Antimicrobial Chromogranins/Secretogranins-Derived Peptides – Production, Lytic Activity and Processing by Bacterial Proteases

Ménonvè Atindehou*, Rizwan Aslam*, Jean-François Chich, Youssef Haïkel, Francis Schneider and Marie-Hélène Metz-Boutigue
Biomatériaux et Ingénierie Tissulaire, University of Strasbourg, Strasbourg France

1. Introduction

1.1 Multidrug antibiotic resistance and innate immunity

Multidrug-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant enterococci (VRE) have important infection control implications in all healthcare stings. Multidrug antibiotic resistance is a worldwide crucial health problem and the production of new potent antibiotics, acting alone or in combination is urgent. In addition, a major factor in the emergence of antibiotic resistant organisms is the overuse of antibiotics in the hospital or the community. To overcome this abuse, numerous efforts are undertaken to reduce antibiotics prescription and/or promote synergistic effects by others molecules.

Indeed, stimulating organism defense is a promising way to struggle against pathogens. The innate immune system is, since 2 billion years, the primary defense in most living organisms and antimicrobial peptides (AMPs) are fundamental components of the innate immune defense of multicellular organisms, either animal or vegetal (Bulet et al., 2004; Aerts et al. 2008; Manners, 2007).

1.2 The antimicrobial peptides

The antimicrobial peptides (AMPs) have been well conserved throughout the evolution and they ensure the organism's defense against a large number of pathogens. They serve as endogenous antibiotics that are able to rapidly kill bacteria, fungi and viruses. Interestingly, they are not toxic for the host cells. Taking into consideration the diversity of the living beings, it is presumed that a large number of specific antibiotic peptides have been developed during evolution, allowing a protection of each organism in various conditions and the last years it has clearly appeared that many of these peptides, in addition to their direct antimicrobial activity, also have a wide range of functions in modulating both innate and adaptive immunity. Most of these are small molecules (less than 40 aminoacids) but some can be proteins. To date more than 1414 antibacterial, antifungal and 107 antiviral peptides have been

* These authors contributed equally

identified, (antimicrobial peptides database http://aps.unmc.edu/AP/main.php), including peptides from several tissues and cell types from invertebrates, plants and mammals (Wang Z. & Wang G. 2004). Among them are found cytokines and chemokines, several neuropeptides and fragments derived from proteins exhibiting antimicrobial activity. They carry an average of 40-50 percent hydrophobic residues in such a structure that the folded peptide adopts an amphipathic profile. These properties are important for their microbial killing mechanism: the cationic character of AMPs induces an electrostatic attraction to the negatively-charged phospholipids of microbial membranes and their hydrophobicity aids the integration into the microbial cell membrane, leading to membrane disruption. Furthermore, the amphipathic structure also allows the peptides to be soluble both in aqueous environments and in lipid membranes (Yeaman & Yount 2003).

In mammals, the most well studied AMPs are human defensins and cathelicidins (Zanetti, 2004; Yang et al., 2002). Furthermore, some large proteins such as lysozyme, caseins, hemoglobin, lactalbumin, secretory phospholipase A2 and lactoferrin display antimicrobial activity against numerous microorganisms. Several of them, such as lysozyme and phospholipase A2 are ubiquitous and secreted by a large number of cells (*i.e.* epithelial cells, leukocytes and Paneth cells in the small intestine) (Keshav, 2006).

Because a large number of AMPs were identified in gut and skin, in the first part of this chapter we report a review of the well-studied AMPs expressed in these tissues and in the second part we present recent data relative to the new active CGs-derived peptides in relation with pathogens involved with intestine diseases, skin infections and sepsis.

1.3 Gut and antimicrobial peptides

Gastrointestinal mucosa is a large host-environmental interface, showing a remarkable organization (Figure 1) and operating several functions including the digestive absorptive processes and the nutrients peristaltism, but also a physical and immunological protection of the body against microbes and a reconnaissance between commensal and pathogenic microorganisms.

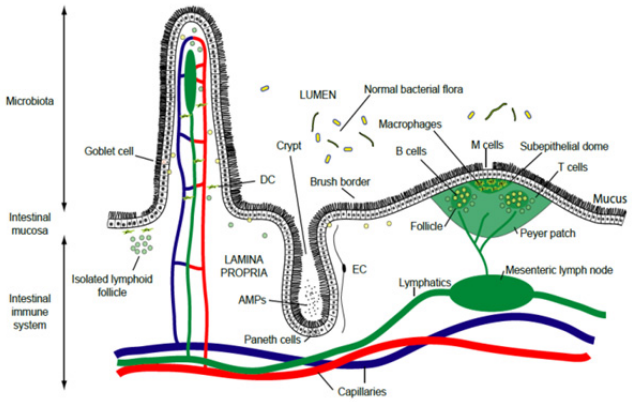

Fig. 1. Schematic representation of the gut epithelium. The different cellular actors involved in innate and/or adaptative immunity are represented. (DC: dendritic cells ; EC: enterochromaffin cells.)(According to Metz-Boutigue, M.H. et al., Curr Pharm Des.2010;16(9):1024-39).

Lamina propria is a conjunctive tissue composed of fibroblasts, immune cells and collagen. It also contains capillaries and lymphatic vessels. Epithelial cells, or enterocytes, are disposed on a single layer separating the *lumen* from the *lamina propria*. These cells are tightly bound by tight junctions forming an impermeable barrier to commensal flora and to pathogens. Brush-border *microvilli* are present on the apical surface of absorptive enterocytes, representing a large absorbing surface and allowing of microorganisms to the gut. In contrast, microfold cells are not present in *microvill*i (Figure 1); these cells express cathepsin E (a proteolytic enzyme) and Toll-like receptors able to secrete proinflammatory cytokines and chemokines. The main function of microfold cells is the transport of antigens from the lumen to the subepithelial lymphoid tissue and thus to the adaptive immune system.

Several anatomical structures are present along the gastrointestinal tractus. Peyer's patches are lymphoid structures containing B and T cells, macrophages and dendritic cells (Figure 1). Lieberkühn crypts are found in the small intestine and they constitute the basis of the intestinal *villi* (Figure 1). They contain multipotent stem cells and cells, involved in gastrointestinal immunity. Two other cellular types are also present in intestine: i) goblet cells synthesize and secrete large quantities of mucin, ii) enterochromaffin cells that originate from neural crest synthesize serotonin (5-HT) and numerous neuropeptides (Figure 1).

In addition to humoral and cellular immunity, non-immunological defense mechanisms represent an important line of intestinal defenses. Some of these protective factors have been amply documented: pancreatic and gastric juices, intestinal motility and intestinal flora (Sarker, 1992).

Mucosal epithelial cells and Paneth cells produce a variety of AMPs (defensins, cathelicidins, cryptdin related peptides, bactericidal/permeability increasing protein (BPI), chemokine CCL20 and bacteriolytic enzymes such as lysozyme and group IIA phospholipase A2 (Müller et al., 2005). In addition to their direct role in killing pathogenic microorganisms, AMPs are involved in attraction of leukocytes, alarming the adaptive immune system and neutralizing the proinflammatory bacterial molecules (Müller et al., 2005).

Lysozyme

Lysozyme is synthesized and secreted by Paneth cells, macrophages, neutrophils and epithelial cells (Mason & Taylor, 1975; Satoh et al., 1988). Its role and selectivity towards microbes are the same as in skin.

Lactoferrin

Lactoferrin (LF) exhibits a wide spectrum of antimicrobial and immunotropic properties (Artym et al., 2005). In contrast to caseins, LF is particularly resistant to proteolytic degradation in alimentary tract. LF is absorbed from the intestine by means of specific receptors located on brush border cells. Orally administered LF stimulates both local and systemic immune responses. It suppresses the growth of pathogenic bacteria, while promoting the multiplication of non-pathogenic *Lactobacillus sp.* and *Bifidobacterium sp.* (Artym et al., 2005).

Studies on mice showed LF to be protective against bacteremia and endotoxemia. LF inhibits the activity of proinflammatory cytokines, nitric oxide and reactive forms of oxygen. Furthermore, LF promotes the differentiation of T and B cells from their immature precursors and increases the activity of NK and LAK (lymphokine activated killer) cells (Artym et al., 2005).

Phospholipase A2

The hydrophobic layer of phosphatidylcholine (PC) overlies and protects the surface of the gastrointestinal (GI) tract, contributing to barrier integrity. In addition, phospholipase A2 is synthesized by Paneth cells and this enzyme hydrolyses bacterial membrane phospholipids to generate both free fatty acids and lysophospholipids. An important prerequisite for the action of phospholipase A2 is the successful binding to the phospholipids surface. *In vitro* studies utilizing recombinant enzymes and artificial phospholipids substrates have shown that phospholipases act on anionic phospholipids (phosphatidylglycerol, phosphatidylserine and phosphatidylethanolamine), but are inactive with phosphatidylcholine due to the lack of high affinity binding (Wu et al., 2010). During critical illness such as sepsis, gut barrier integrity may be compromised, which could be related to degradation of PC. Pretreatment with an orally active sPLA(2) inhibitor blocks the LPS-induced increase in GI permeability, and may suggest a new approach to reinforce the GI mucosal barrier and prevent complications from endotoxin in trauma in other septic conditions (Zayat et al., 2008).

Cathelicidins

Cathelicidin LL-37/hCAP18 is synthesized by neutrophils, where it was first identified (Romeo et al., 1988) and epithelial cells of the colon (Hase et al., 2002). *In vitro*, it has chemotactic properties for monocytes, macrophages and T cells (Koczulla et al., 2003). LL-37 is found in sites of inflammation where it modifies dendritic cells (DCs) differentiation, relying innate and adaptive immunity. *In vitro*, modified DCs had, among others characteristics induced by the peptide, enhanced secretion of Th-1 inducing cytokines and promoted Th1 responses (Davidson et al., 2004). LL-37 acts synergistically with IL-1-beta to increase the production of suppressive cytokines (IL-6 and IL-10) and chemokines MCP-1 and MCP-3 by macrophages (Yu et al., 2007). It acts *via* the transcription factor CREB and the activation of phosphorylation of the kinase Akt. In LPS-stimulated monocytes, LL-37 inhibits the release of TNF-alpha modulating inflammatory response induced by LPS, endotoxins and other agonists of TLRs (Mookherjee et al., 2006).

RNAses

Angiogenin-4 (Ang-4) is a member of the ribonucleases family. This protein is synthesized by Paneth cells and is similar to RNase 7 found in skin. Its secretion is stimulated by exposure to LPS. Ang-4 kills *E. faecalis* and *L. monocytogenes* at concentrations as low as 1 μM, whereas its concentration in crypts can be 1000 times greater (Hooper et al., 2003). Similarly to defensins, it is sensitive to salt concentration and is potentially cytotoxic to eukaryotic cells (Saxena et al., 1992).

C-type lectins

C-type lectins HIP/PAP are synthesized in human by enterocytes and Paneth cells. The same protein exists in mouse and is named RegIII gamma. These lectins bind Gram-positive peptidoglycan and act by direct killing. Several members of this family are found in gastrointestinal tissues (Dieckgraefe et al., 2002).

Defensins

As in skin, defensins have a direct antimicrobial role as well as immunomodulatory function. Alpha-defensins are synthesized by Paneth cell in the gastrointestinal tractus (Porter et al., 2002). Alpha-defensin expression does not require microbe induction since

they are synthesized in germ-free conditions (Putsep et al., 2000) and/or prenatally (Mallow et al., 1996). In transfected mouse, it was shown that the alpha-defensin hBD-5 protects efficiently against *Salmonella typhimurium*, demonstrating the direct antimicrobial effect of this peptide. In mouse, alpha-defensins are named cryptdins and several families of peptides related to cryptdins are regrouped under the term CRS (Cryptdin Related Sequences). Interestingly, these CRS can form homo- or heterodimers, thus allowing a combinatorial diversity to struggle against pathogens (Hornef et al., 2004).

Beta-defensins are expressed in enterocytes of the small and large intestines. 28 beta-defensin encoding genes have been identified in human genome, but only 8 were found to be expressed. hBD-1 is constitutively expressed in absence of stimulus or bacterial infection (O'Neil et al., 1999), while some nutrients can stimulate its production in cell lines (Sherman et al., 2006). In mouse, an infection by the *Cryptosporidium* parasite resulted in a down-regulation of mBD-1 (Zaalouk et al., 2004), while *in vitro*, sporozoites are killed by this defensin. Some authors conclude on an unique and important regulation of hBD-1, during small intestine infections (Dann et Eckmann, 2007). hBD-2 is not constitutively expressed, but is induced by an infection or by proinflammatory stimuli (O'Neil et al., 1999). hBD-3 and -4 are inducible and particularly expressed in crypt regions (Fahlgren et al., 2004). Defensins can also act as chemotactic agents for immune cells in a similar way to that described for the skin.

Bactericidal Permeability Increasing protein

Bactericidal/permeability-increasing protein (BPI), a constituent of primary neutrophil granules, is a potent natural antibiotic and anti-BPI antibodies are detected during infectious enteritis. In addition, BPI is a target antigen for anti-neutrophil cytoplasmic antoantibodies in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Walmsley et al., 1997).

Neuropeptides

Enterochromaffin cells (EC) (Siddique et al., 2009) (Figure 1) are enteroendocrine cells present in the intestine, especially colon (Kuramoto et al., 2007) and containing large amounts of serotonin (5-HT). These cells can sense luminal content before its basolaterally release, and activate afferent neuron endings within *lamina propria*, allowing information exchange between gut and central nervous system (Hansen & Witte et al., 2008). Besides this important role, EC secrete also numerous other products, among which VIP (Zanner et al., 2004), Substance P (Heitz et al., 1976), CgA, CgB and secretogranin II/CgC (Cetin & Grube, 1991) and melatonin (Raikhlin et Kvetnoy, 1976).

Despite the crucial role of these cells, their sparse repartition and their low number did not allowed their extensive study. However, the BON cells were proposed as a model (Kim et al., 2001), that will enhance further research. When EC were stimulated by odors, they released serotonin, showing that these cells can also be stimulated by spices and fragrances (Braun et al., 2007). Moreover, a new method was proposed allowing isolating and purifying EC from biopsies (Modlin et al., 2006).

1.4 Skin and antimicrobial peptides

Mammal skin is an essential defense barrier against external aggressions, such as microbial pathogens, oxidant stress, chemical aggressions, mechanical insults, burns etc. For a long time,

skin was considered as a simple physical barrier, but it is in a process of continual regeneration and has its own immunological, histological and nervous responses to environment.

Skin is composed of three layers, from inside to outside (Figure 2): i) *hypodermis* or subcutaneous tissue, ii) *dermis*, or *corium*, with a 3 to 5 mm thickness, iii) *epidermis*, with a thickness varying from 0.06 to 0.8 mm. *Epidermis* can be subdivided itself into four layers : *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Figure 2). The deeper layers are composed of keratinocytes, melanocytes, Langerhans cells, Merkel cells and malpighian cells (Figure 2). Epidermis is composed as a gradient of differentiated keratinocytes, synthesizing keratine in *stratum granulosum*, and losing nuclei and organelles.

Skin, and more specifically *stratum corneum*, acts as a barrier in several ways (Elias, 2007). Corneocytes and extracellular matrix represent a physical barrier ("brick wall" model) (Figure 2).

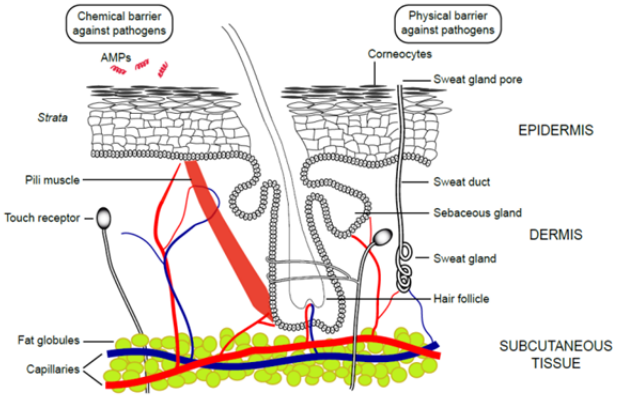

Fig. 2. Schematic representation of the different skin layers, adapted from different sources. A: Epidermis, dermis and subcutaneous tissues are shown with the different structures composing them. (according to Metz-Boutigue, M.H. et al., Curr Pharm Des. 2010;16(9):1024-39).x

The slightly acidic surface (pH~ 5.0), as well as the low hydration level of the skin represents a hostile area for pathogens, such as *Staphylococcus aureus*. Lipids (ceramides, cholesterol, free fatty acids) and their metabolic products present in *stratum corneum* act also as antimicrobial defense. Last, constitutive (and/or inducible) expression of antimicrobial peptides and proteins helps to maintain skin integrity and to prevent pathogen colonization. On the contrary, the surface of healthy skin is ideal for the growth of the normal cutaneous microflora (*Micrococcacea*, i.e. *Staphylococcus epidermidis* and *Corynebacteriacea*) that colonizes skin, competes with pathogens for nutrients and synthesizes antimicrobial compounds. These evolutionary conserved components of the innate immune system can act as direct antimicrobial agents and exert a role as immunomodulatory molecules in normal skin and during skin diseases, such as atopic dermatitis or psoriasis.

Lysozyme

Lysozyme is the first antimicrobial protein found in human skin. It was located in cytoplasm of epidermal cells in granular layers and in malpighian cells present in the *stratum spinosum* layer (Ogawa et al., 1971; Papini et al., 1982). Lysozyme is mainly active against Gram-

positive bacteria (*S. aureus*), but is also active against Gram-negative bacteria, acting probably as a control of bacterial growth. Still recently, the contribution of lysozyme to cutaneaous defense was subjected to debate since it was not detected in *stratum corneum* as well as in washing fluid. However, it was recently detected in skin wash of adults, and lysozyme concentration was 5 times higher in newborn skin than in adult (Walker et al., 2008), confirming its status of antimicrobial molecule, as well as giving it an important role in preventing infections in newborn children.

Lactoferricin

Lactoferricin is an antimicrobial peptide originally produced by pepsin digestion of lactoferrin. It is active against Gram-positive, Gram-negative bacteria and also against *Candida albicans* (Bellamy et al., 1993). This molecule was also detected in skin wash of adult and newborn children (Walker et al., 2008). Synthesized by melanocytes, cutaneous lactoferrin is an iron-binding protein with antibacterial properties due to its ability to sequester iron in biological fluids or to destabilize bacterial membranes, limiting microorganism proliferation and adhesion. It has also immunomodulatory properties by up and down regulating immune cells involved in inflammatory processes (Legrand et al., 2005). The protective anti-inflammatory role of lactoferrin is due to its ability to bind free ferric ion acting as an anti-oxidant (Walker et al., 2008). It can bind to LPS and their receptors during an infection as well (Legrand et al., 2005). Expression of virulence factors of *S. aureus* is modulated by transferrin and lactoferrin (Kansal et al., 2005), demonstrating that these iron-binding proteins play an important role in the host-pathogen interaction in skin and in mucosal tissue probably by LPS or its receptors binding.

Dermcidin and its derived peptides

Dermcidin, is constituvely and specifically expressed in the eccrine sweat glands within the dermis of human skin, secreted into the sweat and transported *via* sweat to the epidermal surface (Schittek et al., 2001). It is a 47 amino acids peptide produced from hydrolysis of a 9.3 kDa precursor by cathepsin D (Baechle et al., 2006). It possesses antibacterial properties at low concentration against *S. aureus*, *E. faecalis*, *E. coli* and *C. albicans*. The *in vivo* importance of DCD in prevention of infections has been demonstrated by its low expression in patients with atopic dermatitis. It was shown that dermcidin induces the production by SepA of *S. aureus*, a proteolytic virulence factor that cleaves and inactivates dermcidin (Lai et al., 2007). In the eccrine sweat, several proteolytically generated DCD fragments (DCD-1, DCD-1L) have been identified. DCD-1L is the most abundant antimicrobial peptide present in sweat, but other peptides derived from dermcidin by proteolysis are also found (Baechle et al., 2006; Rieg et al., 2006). The distribution of these peptides was found to be different according to the individuals. Most of them have 2 to 4 of the major DCD-derived peptides with the constant presence of at least one of the following peptides: DCD-1L (63-110), LEK-45 (66-110) and SSL-29 (63-91). The authors also showed that the distribution of these peptides is dependent on the body sites, which correlates with the presence of eccrine sweat glands and not with apocrine glands. Body parts in contact with pathogens (arms, face etc.) produce high levels of DCD-derived peptides. The molecular analysis of the antimicrobial activity of dermcidin-derived peptides showed that peptides like DCD-1L or SSL-23 do not disrupt bacterial membranes, but kill bacteria by still unknown mechanisms (Steffen et al., 2006).

Recently, by using a proteomic approach, a dermcidin precursor was found in human cervico-vaginal fluid (Shaw et al., 2007), together with haptoglobin, neutrophil defensin,

lysozyme and lactoferrin. Dermcidin precursor was also found in human gestational tissue (Lee Motoyama et al., 2007), where it is proposed to play a role in pregnancy by regulating trophoblastic functions.

Cathelicidin, LL-37, hCAP18

Cathelicidin is found in eccrine gland cells, but also released into circulation. The CAP18 precursor is produced in skin by keratinocytes and is processed within neutrophils, keratinocytes and mast cells by inflammation or injury. In circulation, the mature form is LL-37, after processing of CAP18 by neutrophil-derived elastase and proteinase-3, but other proteases can also produce KS-30 and RK-21, two peptides active against pathogenic bacteria. Cathelicidin expression is also regulated at the transcriptional level by bacterial LPS, cutaneous injury and pro-inflammatory mediators (IL-6, retinoic acid). The LL-37 is intensively studied and besides its wide antibacterial spectrum, it is considered as a mediator between innate and adaptive immunity (Kai-Larsen & Agerberth, 2008) and cell differentiation can also regulate its activity.

RNase A superfamily

Eight known functional RNase A ribonucleases genes are encoding small polypeptides of 15 kDa (Dyer & Rosenberg, 2006). Besides their well-documented ribonuclease activity, some of these proteins display unexpected antimicrobial activities unrelated to their primary function. Eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic peptide (ECP/RNase 3) are proteins secreted by eosinophilic leukocytes and were primarily tested for their toxic role against parasites. *In vitro*, ECP has also an activity against Gram-positive and Gram-negative bacteria (Lehrer et al., 1989).

RNase A7 was identified as a major agent of the innate immune response of the skin acting on Gram-positive and Gram-negative bacteria and also on *C. albicans* (Harder & Schröder, 2002). RNase 7 transcripts were induced in keratinocyte culture by addition of TNF-alpha, interferon-gamma, interleukin-1 beta and in the presence of bacteria (Harder & Schröder, 2002). More recently, RNase 5 was added to the list of antimicrobial molecules present in skin (*stratum corneum*), and the same authors showed that skin proteases are involved in inhibition of RNases 5 and 7 (Abtin et al., 2009).

Psoriasin (S100A7)

Psoriasin belongs to the S100 family of calcium-binding proteins. This family is composed of 21 genes and 11 proteins that have been found to be expressed in human epidermis or in cultured keratinocytes. Langerhans cells and melanocytes (Boni et al., 1997; Broome et al., 2003) express S100B and Meissner's corpuscules (sensorial receptors localized in the upper part of dermis) express S100P (Del Valle et al., 1994). These proteins possess two EF hands (helix-loop-helix calcium binding domains) and they act probably as calcium sensors. Several functions have been proposed for S100 proteins in keratinocytes, the main role being an implication in skin inflammatory processes (Jinquan et al., 1996). Another role could be keratinocytes membrane remodeling, that occurs during differentiation: psoriasin and another member of the S100 family, calgranulin-A (S100A9), have been shown to have their expression correlated with the degree of keratinocyte differentiation, suggesting that they are involved in this process (Martinsson et al., 2005]. A third role could be an involvement in the formation of calcium channels, in conjunction with annexins. Other postulated roles

concern S100 proteins as substrate for transglutaminase, resulting in an incorporation of S100 in the cornified envelope; a last role could be a response to exogenous agents that modulate S100 proteins distribution and consequently their function (Eckert et al., 2004). Psoriasin has been found to be overexpressed in psoriasis. It is produced in *stratum corneum* by keratinocytes (Martinsson et al., 2005) and its basal expression is influenced by extracellular calcium level. Its expression in normal adult tissue is low, but high expression levels were detected in fetal skin, as for transferrin, suggesting a protective role in innate immunity. Psoriasin was found to be the main *E. coli*-cidal agent in the skin. It is a chemotactic agent for neutrophils and $CD4^+$ T cells (Jinquan et al., 1996). Moreover, psoriasin mediates the production of several inflammatory cytokines and chemokines from neutrophils *via* MAPK p38 and ERK activation. It also induces reactive oxygen species production and the exocytosis of alpha-defensins from neutrophils (Zheng et al., 2008).

Defensins

To date 4 defensins (hBD-1 to -4) in neutrophils and 2 defensins (hBD-5 and hBD-6) produced by Paneth cells were identified. The first inducible human defensin, hBD-2, was identified in psoriatic lesions as the most abundant AMP. It was found to be expressed in terminally differentiated keratinocytes, in a structure located in *stratum corneum*, lamellar bodies that contain lipid-rich secretory granules. It is probably released with lipid-like content of these lamellar bodies (Oren et al., 2003). hBD-2 is also up-regulated locally by infections (Radek & Gallo, 2007) or wounds (Butmarc et al., 2004). It has preferential bactericidal properties against Gram-negative bacteria (Harder et al., 1997) and like LL-37, its effect is sensitive to the concentration of NaCl. hBD-2 derived from neutrophils, promotes prostaglandins production and histamine release from mast cells, playing a role in allergic response (Bals et al., 1998). hBD-2 has also chemotactic properties for immature dendritic cells and memory T cells; it was described to bind to CCR-6, the receptor for macrophage inflammatory protein 3 alpha. In monocytes, hBD-2 expression is stimulated by several cytokines (Ganz, 2003; Kanda & Watanabe, 2008) and Il-1 seems to be the major inducer of hBD-2 production. Bacteria can also stimulate the expression of hBD-2 by epithelial cells, in a cytokine-independent pathway. *P. aeruginosa* is a powerful inducer of hBD-2 by primary keratinocytes (Schroeder & Harder, 2006).

hBD-1 was considered as a constitutively expressed antimicrobial peptide and in particularly not induced by proinflammatory cytokines. However, its production can be induced by peptidoglycan or LPS exposure (Sorensen et al., 2005). It is expressed in malpighian layer and in *stratum corneum* (Ali et al., 2001) and this expression is induced by increasing concentration of calcium (Harder et al., 2004), condition that provokes keratinocyte differentiation *in vitro* (Lichti et al., 2008).

hBD-3 has its expression induced by EGF that provokes keratinocytes proliferation in skin wounds (Sorensen et al., 2006).

It has chemotactic properties for monocytes (Garcia et al., 2001). While its expression is not induced by infection, hBD-3 displays a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, as well as against fungi (Harder et al., 2001). Regarding the adaptive immune system, hBD-2, 3 and 4 stimulate expression of proinflammatory cytokines, IL-10 and MCP-1 (Niyonsaba et al., 2007). They also stimulate the phosphorylation of STAT-1 and STAT-3 that induce keratinocytes migration and proliferation.

Neuropeptides in skin immunity

It was reported that neuropeptides display antimicrobial activities, linking together nervous and immune system (Radek & Gallo, 2007; Sternberg, 2006). Both systems can influence each other: brain and peripheral nervous system directly influence the activity of innate and adaptive immune system. Immune system can relay signals to the nervous system *via* the production of growth factors and cytokines. For example, stress can induce alterations in the immune response (Webster et al. 2002), or can be elicited by infection or injury with release of neuropeptides (Brogden et al., 2005).

Exchange between both systems can occur at systemic, as well as at regional or local levels (Sternberg, 2006). The first, global level gathers sympathetic nervous system, the hypothalamic-pituitary-adrenal axis and circulating AMPs. The second, local level, is composed of nervous endings, neuropeptide-releasing cells and receptors-exhibiting cells.

At the skin level, important structures, such as Merkel cells (Lucarz & Brand, 2007) localized at the basement membrane, separating epidermis from dermis, are neuropeptide-producing cells, cutaneous nervous cells and target cells. Merkel cells have characteristics of both epidermal and neuroendocrine cells. They are connected to nervous system with terminal sensory synapses and dense-core granules contain CGRP (Calcitonin Gene Related Peptide), VIP (VasoIntestinal Peptide), and CgA (Chromogranin A)-derived peptides (Hartschuh et al., 1989a; Hartschuh et al., 1989b).

Alpha-melanocyte-stimulating hormone (alpha-MSH), a 13 amino-acid peptide, is synthesized by keratinocytes, melanocytes, monocytes and astrocytes (Wikberg et al., 2000). This peptide derives from the pro-opiomelanocortin (POMC) after a processing by a proteolytic cascade (Pritchard & White, 2007), producing also five other peptides. Alpha-MSH acts as an AMP by inhibiting *S. aureus* and *C. albicans* growth at picomolar concentration (Cutuli et al., 2000). Interestingly, the tripeptide KPV (alpha-MSH 11-13) exhibited similar antimicrobial properties (Hiltz & Lipton, 1990; Mandrika et al., 2001; Mugridge et al., 1991), without effect on melanocytes (Sawyer et al. 1990). Alpha-MSH acts in two ways; it has a direct antimicrobial effect at very low concentration and reduces inflammatory responses associated with UV induced epithelial injury (Radek & Gallo, 2007).

2. Structural and biological properties of the antimicrobial peptides derived from chromogranins/secretogranins

2.1 Introduction

Chromogranins/secretogranins (CGs/SGs) constitute the granin family of genetically distinct acidic proteins present in secretory vesicles of nervous, endocrine and immune cells (Helle, 2004). The natural processing of bovine CGs is well described in granules of sympathoadrenal medullary chromaffin cells, where the resulting peptides are co-secreted with the catecholamines (Metz-Boutigue et al., 1993). The numerous cleavage sites are consistent with the specificity of prohormone convertases (PC1/3 and PC2) and carboxypeptidase E (CPE), that reside within chromaffin granules (Metz-Boutigue et al., 1993; Seidah & Chretien 1999). Secretogranin II (SgII), the third member of the chromogranin family is also processed to generate several natural fragments (Metz-Boutigue et al., 1993; Anouar et al., 1998; Marksteiner et al., 1993; Yajima et al., 2004). The discovery

that pancreastatin, a chromogranin A (CGA)-derived peptide inhibits insulin secretion from pancreatic beta-cells, initiated the concept of prohormone (Eiden, 1987; tatemoto et al., 1986). The release of these CGs-derived peptides from chromaffin cells results from the nicotinic cholinergic stimulation and regulates several neuroendocrine functions (Helle & Serck-Hanssen, 1975).

Numerous cleavage products of the granins have been characterized, among which some display biological activities (Tatemoto et al., 1986; Aardal et al., 1993; Curry et al., 1992; Fasciotto et al., 1993; Lugardon et al., 2001; Mahata et al., 1997; Strub et al., 1996a,b). Neuroendocrine activities are reported from *in vivo* studies, with modulations of homeostatic processes, such as calcium regulation and glucose metabolism (Helle et al., 2007), cardiovascular functions (Brekke et al., 2002; Corti et al., 2004), gastrointestinal motility (Amato et al., 2005; Ghia et al., 2004a), nociception (Ghia et al., 2004b) tissue repair (Gasparri et al., 1997; Ratti et al., 2000), inflammatory responses (Ceconi et al., 2002; Corti et al., 2000) and as host defense agents during infections (Radek et al., 2008). During the past decade, our laboratory has characterized new antimicrobial CGs-derived peptides (Strub et al., 1996a,b; Metz-Boutigue et al., 1998; Lugardon et al., 2000, 2001; Briolat et al., 2005; Helle et al., 2007) (Figure 3).

Peptide	Location	Sequence	Net charge
CGA			
VS-I	1-76	LPVNSPMNKGDTEVMKC*IVEVISDTLSKPSPMPVSKEC*FETLRGDERILSILRHQNLLKELQDLALQGAKERTHQQ	+3
NCA	4-40	NSPMNKGDTEVMKC*IVEVISDTLSKPSPMPVSKEC*FE	-1
CHR	47-66	RILSILRHQNLLKELQDLAL	+1.5
Chrom	173-194	YPGPQAKEDSEGP**S**QGPASREK	-1
CAT	344-364	RSMRLSFRARGYGFRGPGLQL	+5
CCA	418-427	LEKVAHQLEE	-2
ProChrom	79-431	HSSYEDELSEVLEK.....	-37
CGB			
Chromb	564-626	SAEFPDFYDSEEQMSPQHTAENEEEKAGQGVLTEEEEKELENLAAMDLELQKIAEKFSGTRRG	-12
SEC	614-626	QKIAEKFSGTRRG	+3
CGC			
Rrf	131-138	RKLKHMRF	+4.5
Kvk	430-443	KVLSRLPYGPGRSK	+4

Fig. 3. The antimicrobial bovine CGs-derived peptides according to the sequence of CGA (P05059), CGB (P23389) and CGC ((P20616) For each antimicrobial peptide the sequence, the location and net charge are indicated. *, cysteine residues of the disulfide bridge; phosphorylated residue are underlined and the glycosylated residue is in bold.

They act at micromolar range against bacteria, fungi, yeasts and are non-toxic for mammalian cells. They are recovered in biological fluids involved in defense mechanisms (serum, saliva) and in secretions of stimulated human neutrophils (Briolat et al., 2005; Lugardon et al., 2000).

These new AMPs are integrated in the concept that highlights the key role of the adrenal medulla in the immunity (Sternberg, 2006) as previously reported for adrenaline and neuropeptide Y that regulate immunity systemically once released from the adrenal medulla. Furthermore, the adrenal medulla contains and releases large amounts of IL-6 and TNF-alpha in response to pro-inflammatory stimuli such as LPS, IL-1 alpha and IL-1 beta (Metz-Boutigue et al., 1998). The discovery of the presence of TLRs on the adrenal cortex cells raises the interesting possibility that the adrenal gland might have a direct role in the response to pathogens, activation of innate immune response and clearing of infectious agents (Sternberg, 2006).

2.2 Antimicrobial peptides derived from chromogranin A

Several new antimicrobial peptides isolated from the granules of chromaffin cells of the bovine adrenal medulla correspond to CGA-derived peptides (Figure 3). The corresponding sequences are highly conserved in human. Interestingly, the main cleavage site in position 78-79 of bCGA and the subsequent remove of the two basic residues K77 and K78 by the carboxipeptidase H (Metz-Boutigue et al., 1993) produces two antimicrobial fragments: vasostatin-I (VS-I; bCGA1-76) (Lugardon et al., 2000) and prochromacin (Prochrom; bCGA79-431) (Strub et al.,1996b). For these N- and C-terminal domains with antimicrobial activities several shorter active fragments were identified: for VS-I, bCGA1-40 (N CgA; NCA) (Shooshtarizadeh et al., 2010), bCGA47-66 (chromofungin; CHR) and for ProChrom, bCGA173-194 (Chromacin; Chrom) (Strub et al., 1996b) and bCGA344-364 (Catestatin; CAT) (Shooshtarizadeh et al., 2010). The unique disulfide bridge of bCGA is present in VS-I and NCA sequences. Two post-translational modifications are important for the expression of the antibacterial activity of Chrom: the phosphorylation of Y173 and the O-glycosylation of S186 [130] (Strub et al., 1996a). Furthermore, it is important to point out that a dimerization motif GXXXG similar to that reported for Glycophorin A (Brosig & Langosch, 1998) is present in the Chrom sequence (G184-G188).

Vasostatin-I

Vasostatin-I (VS-I) displays antimicrobial activity against (i) Gram-positive bacteria (*Micrococcus luteus* and *Bacillus megaterium*) with a minimal inhibitory concentration (MIC) in the range 0.1-1 µM; (ii) against filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Alternaria brassicola, Nectria haematococca, Fusarium culmorum, Fusarium oxysporum*) with a MIC of 0.5-3 µM and (iii) against yeast cells (*Saccharomyces cerevisiae, Candida albicans*) with a MIC of 2 µM (Lugardon et al., 2000). However VS-I is unable to inhibit the growth of *Escherichia coli* SBS363 and *Escherichia coli* D22. VS-I (Figure 3) possesses structural features specific for antimicrobial peptides, such as a global positive charge (+3), an equilibrated number of polar and hydrophobic residues (20:23) and the presence of a helical region CGA40-65 characterized to be a calmodulin-binding sequence (Lugardon et al., 2001; Yoo, 1992). The loss of the antibacterial activity of CGA7-57 suggests that the N- and C-terminal sequences are essential, nevertheless CGA7-57 is less efficient than VS-I against fungi. Besides, the disulfide bridge is essential for the antibacterial, but not the antifungal property. Altogether, these data suggest that antibacterial and antifungal activities of VS-I have different structural requirements (Lugardon et al., 2001). Interestingly, two helix-helix dimerization motifs important for the interaction with membranes such as LXXXXXXL,

present in DAT and dopamine transporter sequences (Torres et al., 2003) are present in the bovine and human VS-I sequences (L42-L49; L57-L64).

Surface interaction of rhodamine-labelled bCGA1-40 was demonstrated using confocal microscopy after incubation of the labeled peptide with *Aspergillus fumigatus*, *Alternaria brassicola* and *Neurospora crassa* (Blois et al., 2006). In addition, the interaction of bCGA1-40 with monolayers of phospholipids and sterols, as models for the interaction with mammalian and fungal membranes was investigated by the surface tension technique (Blois et al., 2006; Maget-Dana et al., 1999). These studies demonstrated that the N-terminal bCGA1-40 fragment interacts with model membrane phospholipids in a manner consistent with an amphiphilic penetration into membranes in a concentration range relevant for biological activity in mammalian tissue (Blois et al., 2006).

Chromofungin

When VS-I was treated with the endoprotease Glu-C from *Staphylococcus aureus*, one of the generated peptide, chromofungin (CHR), is the shortest active VS-I-derived peptide with antimicrobial activities (Figure 3). It is well conserved during evolution and displays antifungal activity at 2-15 µM against filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Alternaria brassicola, Nectria haematococca, Fusarium culmorum, Fusarium oxysporum*) and yeast cells (*Candida albicans, Candida tropicalis, Candida neoformans*) (Lugardon et al., 2001). Since this peptide was generated after digestion of the material present in chromaffin secretory vesicles by the endoprotease Glu-C from *S. aureus*, it may be hypothesized that it is produced during infections by this class of pathogens.

The 3-D structure of CHR has been determined in water-trifluoroethanol (50:50) by using ^{1}H-NMR spectroscopy. This analysis revealed the amphipathic helical structure of the sequence 53-56, whereas the segment 48-52 confers hydrophobic character (Lugardon et al., 2001).The importance of the amphipathic sequence for antifungal activity was demonstrated by the loss of such activity against *N. crassa* when two proline residues were substituted for L61 and L64, disrupting the helical structure, the amphipathic character and the dimerization motif helix-helix L57-L64 (Lugardon et al., 2001).

Catestatin

Two CGA-derived fragments bCGA333-364 and bCGA343-362 were characterized after the extensive processing of bCGA by prohormone convertases (PC 1/3 or 2) in chromaffin granules (Taylor et al., 2000). More recently, it was shown that cathepsin L colocalizes with CGA in chromaffin granules. *In vitro* it is able to generate after digestion of recombinant hCGA, a catestatin (CAT)–derived fragment hCGA360-373 (Biswas et al., 2009). In addition to the inhibitory effect of CAT on catecholamine release from chromaffin cells (Mahata et al., 1997), we have shown for this peptide and its shorter active sequence bCGA344-358 (cateslytin, CTL), (Figure 3) a potent antimicrobial activity with a MIC in the low-micromolar range against Gram-positive bacteria (*Micrococcus luteus, Bacillus megaterium* at concentration of 0.8 µM), Gram-negative bacteria (*Escherichia coli D22* at concentration of 8 µM), filamentous fungi (*Neurospora crassa, Aspergillus fumigatus, Nectria haematococca* at concentration of 0.2-10 µM) and yeasts (*Candida albicans, Candida tropicalis, Candida glabrata, Candida neoformans* at concentration of 1.2-8 µM). The sequence of CAT (Figure 3) has been highly conserved during evolution (Briolat et al., 2005). The two human variants P370L and

G364S display antibacterial activity against *M. luteus* with a MIC of 2 and 1 μM, respectively, and against *E. coli* with a MIC of 20 and 10 μM, respectively (Briolat et al., 2005). However, the most active peptide corresponds to the bovine sequence. Bovine CTL, a cationic sequence with a global net charge of +5 (R344, R347, R351, R353, R358) and five hydrophobic residues (M346, L348, F360, Y355, F357) (Figure 3), is able to completely kill bacteria at concentration lower than 10 μM even in the presence of NaCl (0-150 mM) (Briolat et al., 2005). The C-terminal sequence bCGA352-358 is inactive, whereas the N-terminal sequences bCGA344-351 and bCGA 348-358 are antibacterial at 20 μM.

C-terminal CGA-derived fragment

CCA, the C-terminal CGA-derived fragment bCGA418-427 (Figure 3), with a remarkable net charge of -2, displays antifungal activity and belongs to the less abundant anionic AMPs family. It is well conserved during evolution and is homologous to the C-terminal sequence of CGB and the antibacterial peptide SEC (secretolytin) [(Strub et al., 1996b). This peptide was generated *in vitro* after digestion, by the protease Glu-C from *S. aureus*, of the material present in chromaffin secretory vesicles. As previously postulated for CHR, CCA could be generated during infections induced by this pathogen.

2.3 Antimicrobial peptides derived from bovine chromogranin B

To date, the natural C-terminal fragment of bovine CGB (CCB; bCGB 564-626), isolated from chromaffin granules of the adrenal medulla, was found to display antibacterial activity against both *M. luteus* and *E. coli*. The complete inhibition of bacterial growth was observed at a concentration around 1.8 μM (Strub et al., 1996b). This large fragment contains the natural short antibacterial peptide secretolytin (SEC, bCGB614-626) with a net positive charge (+3) (Figure 3). We observed the natural formation of a pyrrolidone glutamic acid at the N-terminal end of SEC and both forms displayed antibacterial activity against *M. luteus*, reaching 100% of growth inhibition at 2 μM (Strub et al., 1996ab). A structure-activity analysis suggests that an alpha-helical amphipathic structure common to SEC and cecropins may account for the antibacterial activity (Strub et al., 1996b).

2.4 Antimicrobial peptides derived from bovine secretogranin II

Because bSGII is weakly expressed in the intragranular matrix of chromaffin secretory vesicles (2% of total proteins), the detection of endogenous AMPs by classical methods was unsuccessful. After *in-silico* analysis, two synthetic peptides with cationic amphipathic sequences were prepared: Rrf and Kvk, which correspond to the sequences bSGII131-138 and bSGII430-443 with respective net charges of +4.5 and 4 (Figure 3). Rrf, completely inhibits the bacterial growth of *M. luteus* and *B. megaterium* with a MIC of 5 and 15 μM, respectively, and Kvk displays antifungal properties at 19 μM against *N. crassa* (Shooshtarizadeh et al., 2010).

3. Interaction of antimicrobial chromogranins-derived peptides with bacterial proteases

The AMPs avoidance mechanisms deployed by bacteria include the proteolytic degradation of the active forms by the bacterial proteases. In order to examine the effects

of bacterial proteases on the isolated AMPs derived from CGs, we have tested the effects of *Staphylococcus aureus* V8 protease Glu-C and several supernatants from *S aureus*, *Salmonella enteretica*, *Klebsiella oxytoca*, *Shigella sonnei* and *Vibrio cholera*. By using biochemical methods we have analyzed the degradation of the peptide in presence of bacteria.

Interaction of antimicrobial CGs-derived peptides with proteases from diarrheogenic bacteria
Bacteria were isolated from patients of the Strasbourg Civil Hospital by the Bacteriology Institute, University of Strasbourg, (EA-4438). The four strains have a clinical interest because apart from inducing diarrhea, they may cause other infections.

Thus, *Klebsiella* was involved in the occurrence of post-antibiotic diarrheas (Gorkiewicz 2009). Many studies show that *Klebsiella oxytoca* is also involved in nosocomial infections for newborns or adults (Biran et al., 2010) *Klebsiella* infections may also be commensal (Tsakris et al., 2011). *Klebsiella oxytoca* has also been associated with hemorrhagic colitis (Hoffmann et al., 2010) and intercurrent colitis in Crohn's disease (Plessier et al. 2002). *Salmonella* destroys infected cells and the infection continues through blood (sepsis) or through lymphatic vessel (typhoid fever). Salmonella cause also gastrointestinal infections. *Shigella sonnei* and *Vibrio cholera* non O1 cause inflammation of the intestinal mucosa by producing the Shiga toxin.

Klebsiella oxytoca, Salmonella enterica, Shigella sonnei, and Vibrio cholera develop phenomena of antibiotic resistance. Thus, *Salmonella* was reported to be resistant for the action of Ciprofloxacine (Medalla et al., 2011) and Ceftriaxone (Su et al., 2011).

Concerning CgA, we have tested bovine, rat and human CAT corresponding to the sequences bCgA344-364, rCgA6344-364 and hCgA352-372, bovine CTL located at bCgA344-358, two short fragments hCgA360-372 and the conserved tetrapeptide LSFR (bCgA348-351). In addition, we have tested a scrambled peptide relative to the sequence of bovine CAT and the procatestatin fragment bCgA332-364.

We have found antimicrobial activities only for the bovine CAT and CTL, showing that CTL is the shorter active fragment and that it corresponds to the active domain of CAT. Procatestatin was inactive in similar experimental conditions. Bovine CAT and CTL were active against *Klebsiella oxytoca*, *Salmonella enterica* and *Vibrio cholera* at 100 μM and 50 μM respectively and against *Shigella Sonnei* at 50 μM and 25 μM. In addition, CHR and the C-terminal fragment (CgA387-431) were inactive for concentration up to 100 μM. In contrast, CTL is active at 30 μM against the four pathogens.

Three CgB-derived peptides (CgB58-62, CgB279-291, and CgB547-560) and secretoneurin corresponding to SgII189-254 were examined against the four strains in order to analyse their degradation by bacterial proteases. By using HPLC we have compared the profiles of the peptide alone and the peptide with the inoculated medium.

These experiments show that except CTL all the peptides are completely degraded by the bacteria. To illustrate these data, we present on Figure 4, the profiles relative to CAT and CTL in presence of buffer with *Salmonella enterica*. The complete peptide and the processed form are analysed by sequencing and mass spectrometry (MALDI-TOF).

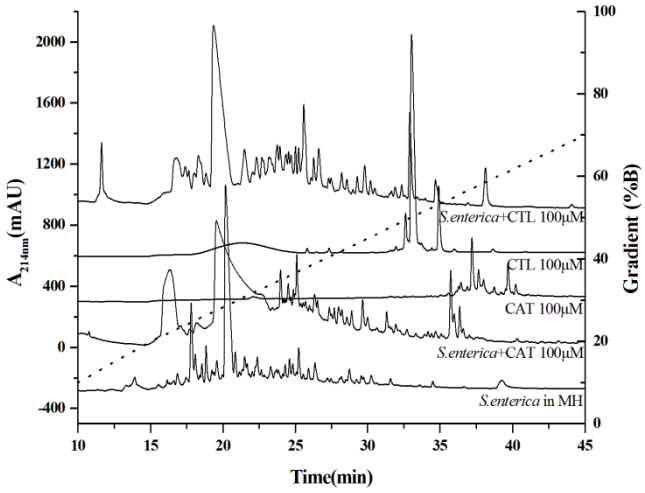

Fig. 4. Analysis by HPLC of the catestatin (CAT) and cateslytin (CTL) degradation by *Salmonella enterica.* The HPLC system is composed by a Dionex chromatogram, Germerong, Germany), using a Nucleosil 300-5 C18 column (4×250 mm, particle size 5 µm, porosity 300 Å; Macherey Nagel, Düren, Germany). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in milliQ water (solvent B). Elutions were performed at a flow rate of 700 µL min−1 using the gradient indicated on the chromatogram.

3.1 Interaction of antimicrobial Cgs-derived peptides with proteases from *Staphylococcus aureus*

After incubation with *S. aureus* V8 protease Glu-C of the proteic intragranular material of chromaffin cells present in the adrenal medulla, 21 new peptides were isolated by HPLC and analysed by sequencing and mass spectrometry. These peptides were tested against Gram positive bacteria (*Micrococcus luteus* and *S. aureus*), Gram negative bacteria (*Escherichia coli*), fungi (*Neurospora crassa*) and yeast (*Candida albicans*). They are not antibacterial but 5 peptides corresponding to CgA47-60, CgA418-426 and CgB 279-291, CgB 450-464 and CgB470-486 display antifungal activity at the micromolar range against *N. crassa.* Thus, *S. aureus* subverts innate immunity to degrade the antibacterial Cgs/Sgs-derived peptides and produce new antifungal peptides (manuscript in preparation).

Four antimicrobial CgA derived peptides (CHR CgA47-66, bovine CAT CgA344-364, human CgA352-372 and CTL CgA344-358) were incubated in presence of staphylococcal supernatants from S1 (a Methicillin resistant strain) and S2 (a non-resistant strain). CTL, the active domain of CAT, is able to completely kill *S. aureus* at 30 µM, but the two others peptides are inactive. By using a proteomic analysis (HPLC, sequencing and mass spectrometry) we demonstrated that CHR and CTL were not degraded by supernatants, whereas bovine and human CAT are processed to produce several fragments (Figure 5).

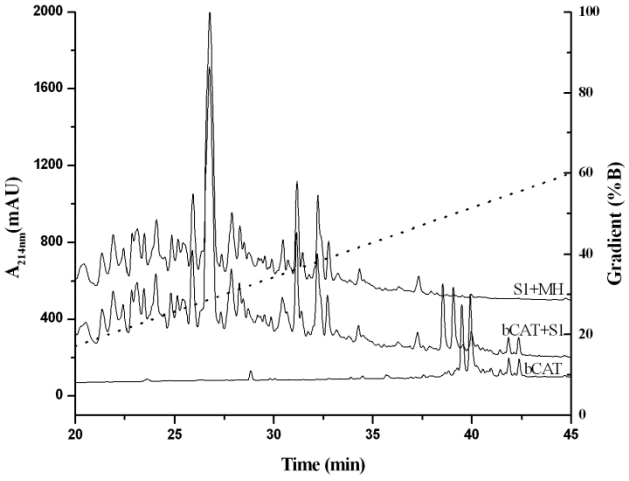

Fig. 5. Analysis by HPLC of the catestatin (CAT) degradation by a methicillin resistant *Staphylococcus aureus* (MRSA). The HPLC system is composed by a Dionex chromatograph, (Germerong, Germany), using a Nucleosil 300-5 C18 column (4×250 mm, particle size 5 µm, porosity 300 Å; Macherey Nagel, Düren, Germany). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile in milliQ water (solvent B). Elutions were performed at a flow rate of 700 µL min−1 using the gradient indicated on the chromatogram.

4. Synergy of the combination of antimicrobial peptides with antibiotics

The emergence of multi-drug resistant bacteria (MDR), with therapeutic failure against *Staphylococcus aureus* (MRSA), *Klebsiella pneumonia, Acinebacter baumannii and Pseudomonas aeruginosa* have paved the way to develop new therapeutic agents by the help of the synergism. In addition the highly toxic effects of antibiotics have shifted the research focus to discover new peptides with broad spectrum of activity and less toxicity. Synergy is the combined activity of two antimicrobial agents that can never be attained by any one of them singly (Serra et al., 1977). Numerous AMPs demonstrate broad spectrum of activity against pathogens, interacting directly with membranes or acting with a specific mode. They represent interesting candidates to synergistically act with antibiotics.

4.1 For *Staphylococcus aureus* MRSA

Most of the patients can prone to serious bacterial infections caused mainly by the multi-resistant microorganisms, *Staphylococcus aureus* coagulase negative spp are one of them. These coagulase negative strains (MRSA) have got approximately 90% of methicillin resistance due to β-lactam resistance (Silva et al., 2011). Story just not stopped here, but still it continues, some of the staphylococcal strains got resistance to the other drugs such as Vancomycin, which was previously widely used against the MRSA infections, and to treat

infections of central nervous system, bone infections and sometimes for the pulmonary infections which require a more concentrations to get treated (Dehority, 2010). *S. aureus* have also developed resistance to the Vancomycin due to the use at low level concentrations and recently, *S. aureus* was isolated that had got the *VanA* gene from the *Enterococcus spp* (Sievert et al., 2008) which leads to drug resistance.

In our group, we have examined the synergically effects of three CGA-derived peptides (CAT, CTL and CHR) with Minocyclin, Amoxicillin and Linezolide. To demonstrate that antimicrobial peptides are able to reduce the doses of antibiotics used and to potentiate the activity of antibiotics, antimicrobial tests were carried out by combining the antibiotic peptides at doses below the MIC. The comparison was made with the antibiotic or peptide separately at the same doses.

Minocyclin has a MIC of 2 µg/ml alone against the *S. aureus* ATCC 49775, but when it was combined with CTL at a concentration corresponding to 75% of the MIC, the, concentration of Minocycline was lowered to 0.5 µg/ml. Similar data were obtained by the use of the two others peptides (Figure 6). Thus we demonstrate that amidated bCTL acts synergistically with Minocycline against *S. aureus*. In addition CTL acts synergistically with Voriconazole against *Candida albicans* and *Candida tropicalis.*

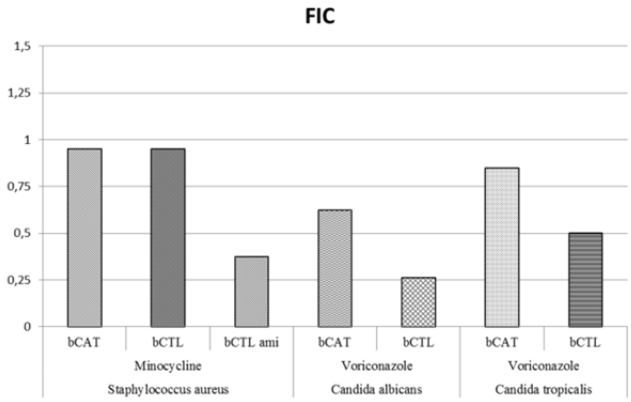

Fig. 6. Fractional inhibitory concentration (FIC) of the chromogranin derived peptides combined with the antimicrobials (Minocycline against the *Staphylococcus aureus* and Voriconazole against *Candida albicans* and *Candida tropicalis*). FIC in range of ≤0.5 gives a synergistic effect, ≤0.5 - <2 is an additive effect but if more than 2 have an antagonistic effect.

4.2 From *Shigella*

Some of the strains of *Shigella* got resistance to antibiotics. A 9-year study of shigellosis in Malaysia, show that 58.4% of the studied strains were resistant to tetracyclin and 53.8% to trimetropin-sulfamethoxasol (Banga Singh et al., 2011). In China, another study establish for *Shigella* the resistance to aztrenam (30,8%), ampicillin (92,3%), piperacilline (61,5%), ceftazidime (30,8%), cefotaxime (30,8%), gentamicine (53,8%) (Zhang et al., 2011). Furthermore, *Vibrio cholera* was also described to develop several resistances against antibiotics (Lamrani et al., 2010).

In conclusion these studies show that CGA-derived AMPs potentiate the effects of antibiotic drugs. One could imagine a mechanism in which the peptide would favor the destabilization of the membrane allowing the antibiotic to rapidly penetrate inside the bacterial cells and thus to reach its site of action.

5. Chromogranin A, a new marker of severity

In clinical practice, CGA has been used as a marker of pheochromocytomas (O'Connor et al., 1984), carcinoid tumors (O'Connor & Deftos, 1986; Syversen et al., 1993), neuroblastomas (Hsiao et al., 1990), neuroendocrine tumors (Berruti et al., 2005), and neurodegenerative diseases (Rangon et al., 2003). Recent data have shown CGA to be a useful prognostic indicator in patients with chronic heart failure (Omland et al., 2003, suggesting that CGA may have some association with cardiovascular diseases. Furthermore, a pilot study has shown CGA to be a predictor of mortality in patients with acute myocardial infarction (Estensen et al., 2006). Characterization of the severity of organ failures and prediction of patient outcome are of major importance for physicians who care for critically ill patients. Multiple organ failure (MOF) remains the main problem in intensive care because of its impact on morbidity, mortality, and resources (Baue et al., 1998). MOF can develop as a consequence of multiple causes, such as infection, systemic inflammatory response syndrome (SIRS), myocardial infarction, septic shock, leading to the activation of various endogenous cascades, cellular dysfunction and death (Baue et al., 1998).

In a recent study we have evaluated whether unselected critically ill patients at ICU (Hautepierre Hospital, Strasbourg, France) admission demonstrate increased plasma CGA concentrations and whether CGA can be of any interest in the care of patients at high risk of death. Patients older than 18 years were recruited consecutively over 3 months during 2007. Exclusion criteria included: duration of stay >24 h and conditions known to increase CGA concentrations independently of acute stress [i.e., a history of documented neuroendocrine tumors (O'Connor & Deftos, 1986) or chronic treatment with proton pump inhibitors before admission (Giusti et al., 2004). Patients who required surgical interventions were also excluded. Of the 120 participants included in the study, 70 patients had a primary diagnosis severe infection, and 50 had a SIRS. Serum CGA concentrations were measured with a commercial sandwich RIA kit (a gift of Cisbio Bioassays, Marcoule, France. In the central 95% of the healthy population, serum CGA concentrations range from 19μg/L to 98μg/L. In neuroendocrine system tumors, the CGA serum concentration varies from the typical range up to 1200 μg/L, depending on the biological and structural characteristics of the tumor, as well as on the extent of tumor spread (Degorce et al., 1999). As a control Procalcitonin (PCT) concentrations were measured on the Kryptor system (Brahms Diagnostic) with the time-resolved amplified cryptate emission methodology in accordance with the manufacturer's recommendations. The Simplified Acute Physiological Score II (SAPS II) and the Logistic Organ Dysfunction System (LODS) score were calculated at admission according to published standards (Levy et al., 2003; Le Gall et al., 1993). Our data show that CGA concentration was positively but weakly correlated with age, PCT concentration, creatinine concentration, SAPS II, and LODS score (P <0.001 for all variables) and was correlated with CRP concentration (Zhang et al., 2008). Thirty-three deaths occurred during the median follow- up time of 23 days. The death rates for CGA and PCT are shown by quartiles in Figure 7. Statistical analysis revealed a significant difference in death rates between CGA quartile 4 and CGA quartiles 1, 2, and 3 (P < 0.001, log-rank test). The death rate for CGA quartile 3 was also significantly different from that of CGA quartile 1 (P= 0.033).

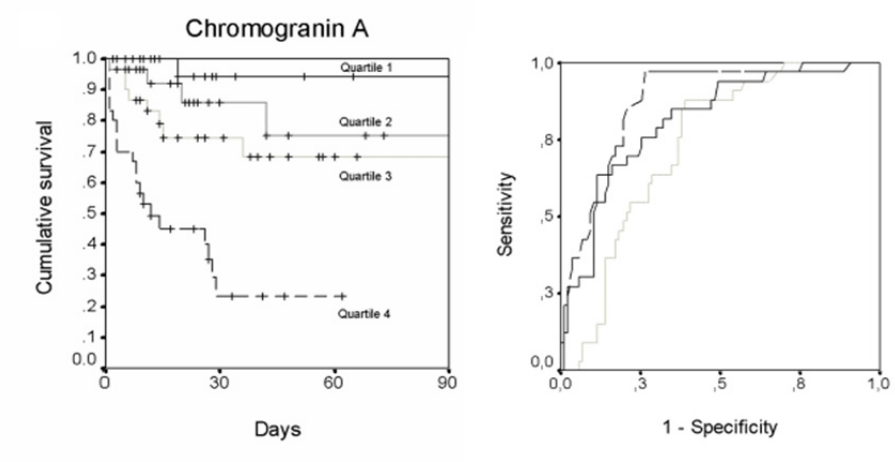

Fig. 7. Kaplan–Meier analysis: cumulative incidence of death by CGA and PCT quartiles. (A), Median (interquartile range) for CGA concentration data: quartile 1, 35 _g/L (30–53 _g/L); quartile 2, 84 _g/L (77–94 _g/L); quartile 3, 174 _g/L (151–197 _g/L); quartile 4, 563 _g/L (355–974 _g/L). Each quartile includes 30 patients. (B), ROC curve to test the ability of CGA (black line), SAPS II (black dashed line), and PCT (gray dashed line) to predict outcome.

ROC curves for CGA, PCT, and SAPS II are shown in Figure 7. To assess the best positive likelihood ratio, we chose the cutoff value that was associated with the best specificity. For CGA, we chose a cutoff value of 255µg/L, which produced a sensitivity of 0.63 and a specificity of 0.89 (positive likelihood ratio, 5.73; negative likelihood ratio, 0.42; AUC, 0.82). A cutoff value of 65 for SAPS II produced a sensitivity of 0.61 and a specificity of 0.85 (positive likelihood ratio, 4.07; negative likelihood ratio, 0.46; AUC, 0.87). For a PCT cutoff value of 4.82 µg/L, sensitivity and specificity were 0.60 and 0.71, respectively (positive likelihood ratio, 2.07; negative likelihood ratio, 0.56; AUC, 0.73). To conclude, in this clinical study of critically ill nonsurgical patients, we demonstrate that plasma CGA is a strong and independent prognostic in consecutive critically ill nonsurgical patients. The over expression of complete CGA suggests that for these patients the processing machinery to procuce antimicrobial peptides in not correct.

6. Insertion of synthetic antimicrobial chromogranins-derived peptides in biomaterials

The surface of medical devices is a common site of bacterial and fungal adhesion, first step to the constitution of a resistant biofilm leading frequently to chronic infections. In order to prevent such complications, several physical and chemical modifications of the device surface have been proposed. In a previous study, we experimented a new type of topical antifungal coating using the layer-by-layer technique. The nanometric multilayer film obtained by this technique is functionalized by the insertion of a CgA-derived antifungal peptide (CGA 47-66, Chromofungin). We show that the embedded peptide keeps its

antifungal activity by interacting with the fungal membrane and penetrating into the cell. *In vitro* studies demonstrated that such an antifungal coating is able to inhibit the growth of yeast *Candida albicans* by 65% and completely stop the proliferation of filamentous fungus *N.crassa*. The cytotoxicity of such a coating was also assessed by growing human gingival fibroblasts at its surface. Finally, the antifungal coating of poly (methylmethacrylate), a widely used material for biomedical devices, is successfully tested in an *in vivo* oral candidiasis rat model (Etienne et al., 2005).

7. Conclusions

CGs family emerges as prohormones able to modulate homeostatic processes in response to excessive stimulations such as microbial infections. The studies concerning the expression of CGs and their antimicrobial peptides in patients with inflammatory diseases and the correlation with the proteolytic processes occurring in these pathologies vs. controls are crucial to understand the involvement of these prohormones and their derived peptides in innate and adaptive immunities. Calcium is a universal secondary messenger involved in many cellular signal transduction pathways, regulating crucial functions such as secretion, cell motility, proliferation and cell death. The calcium-dependent immunomodulatory properties of CHR and CAT are important for the understanding of their involvement in inflammatory mechanisms. In sum, these linear peptides may represent prototypic lead molecules useful for the development of new therapeutic agents and also biomaterials.

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PRESENTATION OF PROTEOMIC ANALYSIS OF ACTIVATED PMNS SECRETION, BY *STAPHYLOCOCCUS AUREUS* LEUKOTOXINS LUKE/D.

Fraction Number	Protein name	Protein accession numbers	Protein molecular weight (Da)	Protein identification probability	Number of unique peptides	Percentage sequence coverage
25	PREDICTED: hypothetical protein	gi|113420837	12 055,40	87,30%	1	11,80%
25	Serum albumin	gi|113576	69 348,90	87,30%	1	2,46%
25	Stathmin; AltName:Phosphoprotein pp19; AltName: Prosolin	gi|134973	17 285,00	87,30%	1	8,05%
25	unnamed protein product	gi|189067580	39 941,50	87,30%	1	7,20%
25	unnamed protein product	gi|31092	50 123,20	87,30%	1	3,25%
25	putative	gi|553734	2 195,70	87,30%	1	38,10%
26	Serum albumin	gi|113576	69 348,90	100,00%	4	5,09%
26	unnamed protein product	gi|189067580	39 941,50	93,60%	1	7,20%
27	PREDICTED: hypothetical protein	gi|113420837	12 055,40	93,40%	1	11,80%
27	hCG1979072, isoform CRA_b	gi|119583466	10 641,10	99,80%	2	16,00%
27	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	93,40%	1	1,65%
27	unnamed protein product	gi|189067580	39 941,50	93,40%	1	7,20%
27	putative	gi|553734	2 195,70	93,40%	1	38,10%
28	immunoglobulin heavy chain constant region	gi|10799664	35 901,30	100,00%	3	29,10%
28	alpha-1 antitrypsin variant	gi|110350939	46 719,90	99,90%	3	10,30%
28	anti-SARS-CoV S protein immunoglobulin kappa light chain	gi|110626504	28 708,20	100,00%	3	19,00%
28	Alpha-1-acid glycoprotein 1; Short=AGP 1	gi|112877	23 522,20	100,00%	4	23,40%
28	Alpha-2-HS-glycoprotein	gi|112910	39 305,40	80,50%	1	7,90%
28	Serum albumin	gi|113576	69 348,90	100,00%	46	74,50%
28	Ig alpha-1 chain C region	gi|113584	53 205,20	100,00%	3	14,40%
28	Apolipoprotein A-I	gi|113992	30 760,50	100,00%	4	17,20%
28	Apolipoprotein A-II	gi|114000	11 166,20	80,50%	2	29,00%
28	Complement C3	gi|119370332	187 147,20	99,80%	2	2,47%
28	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	99,80%	2	6,16%
28	Complement component C1q receptor	gi|88758613	68 541,20	99,60%	1	1,69%
28	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	gi|119585668	103 368,30	80,50%	1	2,80%
28	serum amyloid A1, isoform CRA_a	gi|119588814	13 490,60	80,50%	1	11,50%
28	hemopexin, isoform CRA_d	gi|119589127	51 658,50	100,00%	4	15,60%
28	hCG2029987	gi|119602340	36 133,10	100,00%	4	35,50%
28	hCG2001591	gi|119623974	192 754,80	80,50%	1	1,49%
28	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	80,50%	1	5,73%
28	Serotransferrin	gi|136191	77 032,20	100,00%	15	33,50%
28	Vitamin D-binding protein	gi|139641	52 933,50	80,50%	1	4,64%
28	unnamed protein product	gi|16554039	64 751,30	100,00%	8	18,70%
28	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	80,50%	1	3,03%
28	alpha-2-macroglobulin precursor	gi|177870	160 785,70	80,50%	1	1,38%
28	Ig gamma 2 H chain	gi|243169	38 703,80	80,50%	1	23,30%
29	alpha-1 antitrypsin variant	gi|110350939	46 719,90	99,80%	2	6,46%
29	Alpha-1-antichymotrypsin	gi|112874	48 621,00	99,10%	2	7,16%
29	Serum albumin	gi|113576	69 348,90	100,00%	14	28,10%
29	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	86,30%	1	2,96%

29	unnamed protein product	gi|16554039	64 751,30	99,80%	2	6,50%
29	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	86,30%	1	3,03%
29	unnamed protein product	gi|189067580	39 941,50	86,30%	1	7,20%
29	unnamed protein product	gi|31092	50 123,20	86,30%	1	3,25%
30	Serum albumin	gi|113576	69 348,90	100,00%	4	10,70%
30	desmoplakin I	gi|1147813,gi|115502381	331 765,20	50,00%	1	1,04%
30	RCSD domain containing 1, isoform CRA_a	gi|119611202	47 131,10	50,00%	1	3,62%
30	JUP protein	gi|15080189	81 727,90	50,00%	1	2,42%
30	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	50,00%	1	3,03%
30	TALDO1 protein	gi|48257056	37 523,70	50,00%	1	2,97%
30	Complement factor D	gi|158515408	27 014,40	99,60%	1	10,30%
31	immunoglobulin heavy chain constant region	gi|10799664	35 901,30	88,60%	1	7,36%
31	alpha-1 antitrypsin variant	gi|110350939	46 719,90	88,60%	1	2,39%
31	anti-SARS-CoV S protein immunoglobulin kappa light chain	gi|110626504	28 708,20	88,60%	1	5,95%
31	Serum albumin	gi|113576	69 348,90	100,00%	27	50,10%
31	hCG2029987	gi|119602340	36 133,10	88,60%	1	16,70%
31	Serotransferrin	gi|136191	77 032,20	99,80%	2	4,73%
31	unnamed protein product	gi|16554039	64 751,30	100,00%	3	7,50%
31	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	5,79%
31	myristoylated alanine-rich C-kinase substrate	gi|187387	31 526,20	88,60%	1	4,52%
31	unnamed protein product	gi|189067580	39 941,50	88,60%	1	7,20%
31	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	88,60%	1	7,08%
31	protein Len,Bence-Jones	gi|229528	24 203,90	81,70%	1	15,50%
31	Gox, cytosolic GTP-binding protein	gi|459639	881,8	88,60%	1	100,00%
32	Serum albumin	gi|113576	69 348,90	100,00%	5	11,70%
32	hCG1979072, isoform CRA_b	gi|119583466	10 641,10	93,70%	1	13,00%
32	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	93,70%	1	5,73%
32	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	93,70%	1	3,03%
32	unnamed protein product	gi|193787391	32 417,00	93,70%	1	4,07%
32	unnamed protein product	gi|194382178	63 522,00	93,70%	1	1,52%
32	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	93,70%	1	7,08%
33	Serum albumin	gi|113576	69 348,90	91,30%	1	2,13%
33	vasodilator-stimulated phosphoprotein	gi|11414808	39 592,80	99,80%	2	3,97%
33	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	5,79%
33	unnamed protein product	gi|194382178	63 522,00	91,30%	1	1,52%
34	polyubiquitin	gi|11024714	68 474,80	83,10%	2	2,63%
34	HIST1H4H protein	gi|111309351	11 349,70	83,10%	1	7,77%
34	TMSB4X protein	gi|112180539	5 034,90	99,20%	2	43,20%
34	Serum albumin	gi|113576	69 348,90	100,00%	4	11,80%
34	Annexin A1	gi|113944	38 697,90	83,10%	1	3,76%
34	vasodilator-stimulated phosphoprotein	gi|11414808	39 592,80	83,10%	1	3,17%
34	HIST1H2BC protein	gi|114205460	13 918,60	83,10%	1	7,14%
34	Protein S100-A8, AltName:Calgranulin-A	gi|115442	10 816,90	83,10%	1	11,80%
34	Protein S100-A9; AltName:Calgranulin-B	gi|115444	13 224,30	83,10%	1	13,20%

34	Elafin; AltName:Elastase-specific inhibitor	gi|119262	12 251,10	83,10%	1	13,70%
34	hCG1743774	gi|119573727	7 786,50	83,10%	1	12,50%
34	ras-related C3 botulinum toxin substrate 1	gi|119575445	21 432,60	83,10%	1	5,73%
34	hCG2029987	gi|119602340	36 133,10	83,10%	1	16,40%
34	psoriasin	gi|12053626	11 453,70	99,80%	2	30,70%
34	Galectin-7	gi|1346431	15 056,70	83,10%	1	11,80%
34	60S ribosomal protein L6	gi|1350762	32 711,30	83,10%	1	5,21%
34	Heat shock 27kDa protein 1	gi|15126735	22 764,60	99,80%	2	13,20%
34	unnamed protein product	gi|158258082	26 770,50	83,10%	1	7,50%
34	unnamed protein product	gi|16554039	64 751,30	99,80%	2	6,33%
34	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	4	20,70%
34	epithelial cell marker protein 1	gi|187302	27 756,80	99,80%	2	13,70%
34	squamous cell carcinoma antigen-1 isoform SCCA-PD	gi|193792580	44 517,90	100,00%	6	19,00%
34	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	83,10%	1	4,87%
34	Calmodulin-like protein 5	gi|215273944	15 874,90	83,10%	1	7,53%
34	Fatty acid-binding protein,	gi|232081	15 146,40	99,80%	2	25,90%
34	Protein disulfide-isomerase;	gi|2507460	57 100,10	83,10%	1	5,91%
34	unnamed protein product	gi|31092	50 123,20	83,10%	1	6,28%
34	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	99,80%	2	8,66%
34	Myosin-9; AltName:Myosin heavy chain 9	gi|6166599	226 519,50	83,10%	1	0,61%
35	TMSB4X protein	gi|112180539	5 034,90	99,80%	2	43,20%
35	Serum albumin	gi|113576	69 348,90	98,70%	2	6,57%
35	ras-related C3 botulinum toxin substrate 1	gi|119575445	21 432,60	85,60%	1	5,73%
35	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	99,80%	2	19,80%
35	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	85,60%	1	3,03%
35	unnamed protein product	gi|31092	50 123,20	85,60%	1	6,28%
36	glucose-6-phosphate dehydrogenase isoform a	gi|109389365	59 249,10	92,70%	1	1,94%
36	TMSB4X protein	gi|112180539	5 034,90	99,80%	2	43,20%
36	Serum albumin	gi|113576	69 348,90	99,70%	2	4,93%
36	Protein S100-A9; AltName:Calgranulin-B	gi|115444	13 224,30	92,70%	1	18,40%
36	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	100,00%	3	12,80%
36	High mobility group protein B2	gi|123374	24 016,60	92,70%	1	7,18%
36	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	99,80%	2	19,80%
36	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	6,06%
36	unnamed protein product	gi|189053774	45 819,00	99,30%	2	1,97%
36	unnamed protein product	gi|194382178	63 522,00	92,70%	1	2,19%
36	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	92,70%	1	4,87%
37	glucose-6-phosphate dehydrogenase isoform a	gi|109389365	59 249,10	81,50%	1	1,94%
37	TMSB4X protein	gi|112180539	5 034,90	99,80%	2	43,20%
37	Serum albumin	gi|113576	69 348,90	99,60%	2	3,94%
37	Cathepsin G	gi|115725	28 819,50	81,50%	1	5,10%
37	Cofilin-1	gi|116848	18 485,10	81,50%	1	16,90%
37	calmodulin 3	gi|119577833	17 145,40	81,50%	1	10,50%
37	lactoferrin	gi|12083188	78 364,50	81,50%	1	57,00%
37	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	99,80%	2	5,50%
37	High mobility group protein B2	gi|123374	24 016,60	81,50%	1	6,22%

37	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	99,80%	2	22,90%
37	Neutrophil cytosolic factor 2	gi|12804409	59 744,20	81,50%	1	2,47%
37	lysozyme	gi|1335210	14 682,50	100,00%	8	68,50%
37	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	5,79%
37	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	99,80%	2	15,80%
37	lactoferrin	gi|186833	78 378,50	100,00%	34	57,00%
37	unnamed protein product	gi|194382178	63 522,00	99,80%	2	8,60%
37	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	99,80%	2	5,31%
37	cationic antimicrobial protein CAP37	gi|227250	26 635,30	100,00%	3	18,10%
37	unnamed protein product	gi|34412	78 061,10	81,50%	1	57,10%
37	gelsolin isoform b	gi|38044288	80 622,80	81,50%	1	2,46%
37	TALDO1 protein	gi|48257056	37 523,70	81,50%	1	2,97%
37	Vimentin	gi|55977767	53 634,60	81,50%	1	2,15%
38	TMSB4X protein	gi|112180539	5 034,90	93,90%	1	27,30%
38	Serum albumin	gi|113576	69 348,90	93,90%	1	1,48%
38	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	93,90%	1	3,85%
38	pyruvate kinase, muscle, isoform CRA_c	gi|119598292	57 919,50	93,90%	1	1,88%
38	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	99,80%	2	5,50%
38	lysozyme	gi|1335210	14 682,50	100,00%	3	40,00%
38	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	5,79%
38	lactoferrin	gi|186833	78 378,50	100,00%	3	6,05%
38	unnamed protein product	gi|194382178	63 522,00	93,90%	1	6,41%
38	Lymphocyte-specific protein 1, WP34	gi|462553	37 173,10	93,90%	1	7,96%
38	TALDO1 protein	gi|48257056	37 523,70	93,90%	1	3,26%
39	Serum albumin	gi|113576	69 348,90	89,30%	1	1,48%
39	Leucine-rich repeat flightless-interacting protein 1	gi|114149995	86 419,20	89,30%	1	1,40%
39	zyxin, isoform CRA_b	gi|119572234	61 258,00	99,80%	2	6,12%
39	hCG26831	gi|119584724	24 975,90	99,50%	2	15,30%
39	capping protein (actin filament) muscle Z-line, beta	gi|119615294	30 611,70	89,30%	1	3,31%
39	psoriasin	gi|12053626	11 453,70	89,30%	1	19,80%
39	Histone H1.0; AltName:Histone H1(0)	gi|121897	20 846,10	89,30%	1	5,15%
39	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	89,30%	1	5,05%
39	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	4	7,99%
39	Rho GDP-dissociation inhibitor 2	gi|1707893	22 970,40	99,80%	2	12,90%
39	unnamed protein product	gi|189053774	45 819,00	99,70%	2	1,97%
39	unnamed protein product	gi|194382178	63 522,00	99,80%	2	8,26%
39	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	99,80%	2	10,20%
39	Lymphocyte-specific protein 1, WP34	gi|462553	37 173,10	89,30%	1	5,60%
39	Vimentin	gi|55977767	53 634,60	89,30%	1	1,72%
40	TMSB4X protein	gi|112180539	5 034,90	100,00%	3	54,50%
40	Serum albumin	gi|113576	69 348,90	99,80%	2	2,63%
40	Neutrophil collagenase; AltName:Matrix metalloproteinase-8	gi|116862	53 396,20	94,10%	1	1,71%
40	bridging integrator 2, isoform CRA_c	gi|119578592	61 729,30	94,10%	1	1,95%
40	hCG26831	gi|119584724	24 201,20	94,10%	1	7,11%
40	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	94,10%	1	5,50%
40	Heat shock cognate 71 kDa protein	gi|123648	53 500,80	94,10%	1	3,25%
40	Neutrophil cytosolic factor 2	gi|12804409	59 744,20	99,80%	2	6,84%

40	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	3,31%
40	Rho GDP-dissociation inhibitor 2	gi|1707893	22 970,40	94,10%	1	8,46%
40	unnamed protein product	gi|194382178	63 522,00	94,10%	1	5,73%
40	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	94,10%	1	5,31%
40	Lymphocyte-specific protein 1, WP34	gi|462553	37 173,10	94,10%	1	7,96%
41	TMSB4X protein	gi|112180539	5 034,90	95,00%	1	27,30%
41	Serum albumin	gi|113576	69 348,90	99,80%	2	3,94%
41	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	99,80%	2	5,50%
41	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	3,31%
41	small GTP binding protein RhoG	gi|20379122	21 290,10	95,00%	1	9,95%
41	neutrophil granule peptide HP1	gi|228797	3 430,10	95,00%	1	30,00%
41	Vimentin	gi|55977767	53 634,60	95,00%	1	2,15%
42	TMSB4X protein	gi|112180539	5 034,90	99,80%	2	43,20%
42	Serum albumin	gi|113576	69 348,90	94,30%	1	1,48%
42	septin-9 isoform c	gi|116256489	47 451,70	94,30%	1	3,55%
42	formin-like 1, isoform CRA_a	gi|119571919	121 814,20	94,30%	1	1,18%
42	zyxin, isoform CRA_b	gi|119572234	61 258,00	94,30%	1	2,10%
42	Eosinophil peroxidase	gi|182146	81 025,90	99,60%	3	6,99%
42	talin 1, isoform CRA_b	gi|119578757	269 747,10	94,30%	1	0,43%
42	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	94,30%	1	5,05%
42	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	3	6,06%
42	unnamed protein product	gi|194382178	63 522,00	99,80%	2	2,70%
42	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	94,30%	1	4,87%
42	neutrophil granule peptide HP1	gi|228797	3 430,10	94,30%	1	30,00%
42	Lymphocyte-specific protein 1; WP34	gi|462553	37 173,10	99,80%	2	13,30%
42	Vimentin	gi|55977767	53 634,60	99,80%	2	3,86%
43	unnamed protein product	gi|10439230	38 361,30	93,90%	1	2,92%
43	Heterogeneous nuclear ribonucleoproteins C1/C2	gi|108935845	25 213,00	93,90%	1	3,98%
43	TMSB4X protein	gi|112180539	5 034,90	93,90%	1	27,30%
43	Serum albumin	gi|113576	69 348,90	93,90%	1	1,48%
43	neutrophil cytosol factor 1	gi|115298672	44 665,80	93,90%	1	3,59%
43	integrin, beta 2	gi|119629788	83 863,70	93,90%	1	1,45%
43	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	93,90%	1	5,05%
43	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	4	8,26%
43	L-plastin polypeptide	gi|190030	63 863,50	93,90%	1	1,93%
43	unnamed protein product	gi|194382178	63 522,00	93,90%	1	2,36%
43	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	93,90%	1	4,87%
43	EF-hand domain-containing protein D2	gi|20140139	26 680,40	93,90%	1	5,00%
43	YWHAZ protein	gi|30354619	27 727,90	93,90%	1	5,71%
43	Myosin-9	gi|6166599	226 519,50	99,80%	2	1,22%
44	TMSB4X protein	gi|112180539	5 034,90	93,50%	1	27,30%
44	Serum albumin	gi|113576	69 348,90	93,50%	1	1,48%
44	vasodilator-stimulated phosphoprotein	gi|11414808	39 592,80	93,50%	1	3,44%
44	histocompatibility (minor) HA-1, isoform CRA_b	gi|119589958	124 597,00	93,50%	1	1,23%
44	pyruvate kinase, muscle, isoform CRA_c	gi|119598292	57 919,50	93,50%	1	3,39%
44	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	93,50%	1	5,05%
44	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	6	12,10%

44	Rho GDP-dissociation inhibitor 1	gi|1707892	23 175,40	93,50%	1	7,84%
44	unnamed protein product	gi|194382178	63 522,00	93,50%	1	2,36%
44	small GTP binding protein RhoG	gi|20379122	21 290,10	93,50%	1	9,95%
44	neutrophil granule peptide HP1	gi|228797	3 430,10	93,50%	1	30,00%
44	YWHAZ protein	gi|30354619	27 727,90	99,80%	2	10,60%
44	Phosphoglycerate kinase 1	gi|52788229	44 597,30	93,50%	1	2,16%
44	filamin A	gi|53791219	277 478,30	93,50%	1	0,54%
45	Serum albumin	gi|113576	69 348,90	91,90%	1	1,48%
45	apolipoprotein B-100 receptor	gi|119220598	114 813,50	91,90%	1	1,29%
45	formin-like 1, isoform CRA_a	gi|119571919	121 814,20	91,90%	1	1,18%
45	Protein kinase C inhibitor protein 1	gi|119596301	28 065,10	91,90%	1	4,88%
45	pyruvate kinase, isoform CRA_c	gi|119598292	57 919,50	91,90%	1	3,39%
45	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	6	14,00%
45	Rho GDP-dissociation inhibitor 1	gi|1707892	23 175,40	91,90%	1	7,84%
45	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
45	Phosphoglycerate kinase 1	gi|52788229	44 597,30	91,90%	1	3,60%
46	Serum albumin	gi|113576	69 348,90	93,50%	1	1,48%
46	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	93,50%	1	3,30%
46	heterogeneous nuclear ribonucleoprotein M	gi|119589327	77 499,30	93,50%	1	2,05%
46	Protein kinase C inhibitor protein 1	gi|119596301	28 065,10	93,50%	1	4,88%
46	pyruvate kinase, isoform CRA_c	gi|119598292	57 919,50	93,50%	1	3,39%
46	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	93,50%	1	5,05%
46	Stathmin; AltName:Phosphoprotein pp19	gi|134973	17 285,00	96,90%	2	9,40%
46	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	6	11,30%
46	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	93,50%	1	8,57%
46	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
46	YWHAZ protein	gi|30354619	27 727,90	93,50%	1	5,71%
47	Serum albumin	gi|113576	69 348,90	100,00%	6	8,21%
47	Neutrophil collagenase; Matrix metalloproteinase-8	gi|116862	53 396,20	76,20%	1	1,71%
47	pyruvate kinase, isoform CRA_c	gi|119598292	57 919,50	76,20%	1	3,39%
47	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	76,20%	1	3,03%
47	Rho GDP-dissociation inhibitor 2	gi|1707893	22 970,40	99,80%	2	12,90%
47	unnamed protein product	gi|194382178	63 522,00	76,20%	1	2,70%
47	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
47	YWHAZ protein	gi|30354619	27 727,90	76,20%	1	5,71%
47	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	76,20%	1	4,48%
47	Chitotriosidase-1; AltName:Chitinase-1	gi|37999493	51 664,10	76,20%	1	3,65%
47	Vimentin	gi|55977767	53 634,60	76,20%	1	2,15%
48	Proteasome activator complex subunit 1	gi|1170519	28 705,80	88,50%	1	4,42%
48	pyruvate kinase, isoform CRA_c	gi|119598292	57 919,50	88,50%	1	2,07%
48	hCG1729650, isoform CRA_a	gi|119612629	67 561,50	88,50%	1	1,78%
48	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	4	11,60%
48	neutrophil granule peptide HP1	gi|228797	3 430,10	100,00%	6	100,00%
48	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	88,50%	1	5,07%
48	Chitotriosidase-1; AltName:Chitinase-1	gi|37999493	51 664,10	88,50%	1	3,65%
48	Vimentin	gi|55977767	53 634,60	88,50%	1	2,15%
49	Serum albumin	gi|113576	69 348,90	86,40%	1	1,48%

49	pyruvate kinase, isoform CRA_c	gi|119598292	57 919,50	86,40%	1	2,07%
49	reticulon 4, isoform CRA_h	gi|119620534	129 926,60	86,40%	1	1,09%
49	moesin, isoform CRA_b	gi|119625804	67 803,80	86,40%	1	1,73%
49	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	86,40%	1	1,25%
49	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	3	10,70%
49	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	86,40%	1	4,87%
49	neutrophil granule peptide HP1	gi|228797	3 430,10	99,90%	3	80,00%
49	Vimentin	gi|55977767	53 634,60	86,40%	1	2,15%
50	glucose-6-phosphate dehydrogenase isoform a	gi|109389365	59 249,10	92,50%	1	2,14%
50	Serum albumin	gi|113576	69 348,90	99,60%	2	3,45%
50	Neutrophil collagenase	gi|116862	53 396,20	92,50%	1	1,71%
50	reticulon 4, isoform CRA_h	gi|119620534	129 926,60	92,50%	1	1,09%
50	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	92,50%	1	5,05%
50	High mobility group protein B2	gi|123374	24 016,60	92,50%	1	7,18%
50	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	92,50%	1	3,03%
50	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	92,50%	1	4,87%
50	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
51	Serum albumin	gi|113576	69 348,90	100,00%	4	6,73%
51	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	93,80%	1	3,30%
51	Alpha-enolase	gi|119339	47 152,20	93,80%	1	1,84%
51	Rho GDP-dissociation inhibitor 1	gi|1707892	23 175,40	93,80%	1	7,84%
51	Peroxiredoxin-6; Antioxidant protein 2	gi|1718024	25 018,10	93,80%	1	9,38%
51	Histone H1.5; AltName:Histone H1a	gi|19856407	22 563,70	93,80%	1	4,87%
51	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
51	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	93,80%	1	5,07%
52	PKP1 protein	gi|109730327	80 480,80	50,00%	1	1,52%
52	Serum albumin	gi|113576	69 348,90	100,00%	5	10,00%
52	desmoplakin I	gi|1147813	331 765,20	100,00%	14	7,38%
52	Alpha-enolase	gi|119339	47 152,20	50,00%	1	3,46%
52	hCG1743774	gi|119573727	7 786,50	50,00%	1	12,50%
52	small proline-rich protein 1A, isoform CRA_b	gi|119573742	9 868,60	50,00%	1	8,99%
52	thioredoxin, isoform CRA_b	gi|119579464	11 719,30	50,00%	1	12,40%
52	peroxiredoxin 1, isoform CRA_b	gi|119627382	22 092,90	99,80%	2	10,60%
52	desmoglein-1 preproprotein	gi|119703744	113 698,80	100,00%	5	8,77%
52	JUP protein	gi|15080189	81 727,90	100,00%	8	16,00%
52	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	50,00%	1	4,96%
52	Desmocollin-1	gi|223590198	93 818,30	99,80%	2	3,69%
52	neutrophil granule peptide HP1	gi|228797	3 430,10	50,00%	1	30,00%
52	Fatty acid-binding protein	gi|232081	15 146,40	100,00%	6	51,90%
52	suprabasin isoform 1 precursor	gi|260436922	60 519,20	100,00%	5	13,90%
52	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	4	17,90%
52	hornerin precursor	gi|40795897	282 354,70	100,00%	5	3,40%
52	Filaggrin-2; Short=FLG-2	gi|74755309	248 034,30	98,40%	2	1,51%
53	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	85,60%	1	3,30%
53	LIM and SH3 protein 1, isoform CRA_a	gi|119580947	29 698,80	99,80%	2	6,51%
53	Profilin-1	gi|130979	15 036,30	85,60%	1	10,00%
53	Non-secretory ribonuclease	gi|133168	18 335,70	85,60%	1	8,70%

53	Eosinophil cationic protein; Ribonuclease 3	gi|147744558	18 422,40	99,80%	2	17,50%
53	ARP2 actin-related protein 2 homolog	gi|15778930	44 743,70	100,00%	3	5,08%
53	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	85,60%	3	7,99%
53	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
53	TALDO1 protein	gi|48257056	37 523,70	85,60%	1	2,97%
53	filamin A	gi|53791219	277 478,30	100,00%	4	2,49%
54	polyubiquitin	gi|11024714	68 474,80	82,10%	2	2,63%
54	Serum albumin	gi|113576	69 348,90	100,00%	3	5,91%
54	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	82,10%	1	3,30%
54	Cystatin-A	gi|118177	10 988,60	99,60%	2	30,60%
54	solute carrier family 9	gi|119609595	38 850,30	82,10%	1	3,91%
54	moesin, isoform CRA_b	gi|119625804	67 803,80	100,00%	4	4,85%
54	SMT3 suppressor of mif two 3 homolog 3	gi|119629798	10 853,20	82,10%	1	12,60%
54	Histone H1.4; AltName:Histone H1b	gi|121919	21 717,70	82,10%	1	5,05%
54	High mobility group protein B2	gi|123374	24 016,60	82,10%	1	7,18%
54	Non-secretory ribonuclease	gi|133168	18 335,70	100,00%	3	19,90%
54	Eosinophil cationic protein; Ribonuclease 3	gi|147744558	18 422,40	100,00%	6	38,70%
54	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,90%	3	5,79%
54	Calmodulin-like protein 5	gi|215273944	15 874,90	82,10%	1	15,80%
54	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	82,10%	1	4,48%
54	Neutrophil defensin 4	gi|399352	10 486,10	99,50%	2	22,70%
54	Actin-related protein 3	gi|47117647	47 353,80	99,80%	2	8,13%
54	TALDO1 protein	gi|48257056	37 523,70	82,10%	1	2,97%
54	Phosphoglycerate kinase 1	gi|52788229	44 597,30	99,80%	2	6,71%
55	coronin-like protein	gi|1002923	51 008,00	80,50%	1	6,29%
55	polyubiquitin	gi|11024714	14 710,90	100,00%	6	41,40%
55	Serum albumin	gi|113576	69 348,90	80,50%	1	1,48%
55	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	80,50%	1	3,30%
55	moesin, isoform CRA_b	gi|119625804	67 803,80	100,00%	3	3,29%
55	Brain abundant, membrane attached signal protein 1	gi|12653493	22 675,30	100,00%	4	33,90%
55	Non-secretory ribonuclease	gi|133168	18 335,70	100,00%	3	19,90%
55	Eosinophil cationic protein; Ribonuclease 3	gi|147744558	18 422,40	100,00%	5	32,50%
55	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
55	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	80,50%	1	4,48%
55	Neutrophil defensin 4	gi|399352	10 486,10	80,50%	1	17,50%
55	filamin A	gi|53791219	277 478,30	100,00%	5	2,76%
55	hypothetical protein	gi|5912028	26 856,20	0,00%	1	24,70%
56	coronin-like protein	gi|1002923	51 008,00	79,70%	1	6,29%
56	polyubiquitin	gi|11024714	14 710,90	100,00%	3	21,10%
56	IgG receptor IIIB	gi|11344591	26 171,00	79,70%	1	6,44%
56	Serum albumin	gi|113576	69 348,90	99,80%	2	3,94%
56	Alpha-enolase	gi|119339	47 152,20	79,70%	1	3,00%
56	arginase, isoform CRA_b	gi|119568439	34 718,00	79,70%	1	3,42%
56	cathelicidin antimicrobial peptide	gi|119585258	19 284,30	100,00%	4	23,50%
56	Non-secretory ribonuclease	gi|133168	18 335,70	79,70%	1	8,70%
56	Eosinophil cationic protein	gi|147744558	18 422,40	100,00%	3	26,90%
56	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
57	polyubiquitin	gi|11024714	14 710,90	99,80%	2	19,50%

57	Alpha-1-acid glycoprotein 1	gi|112877	23 522,20	89,60%	1	4,48%
57	IgG receptor IIIB	gi|11344591	26 171,00	100,00%	3	13,30%
57	Serum albumin	gi|113576	69 348,90	100,00%	5	8,21%
57	Cathepsin G	gi|115725	28 819,50	99,80%	2	10,60%
57	Chromobox protein homolog 3	gi|116241284	20 794,00	89,60%	1	15,80%
57	Cystatin-A	gi|118177	10 988,60	99,60%	2	30,60%
57	Alpha-enolase	gi|119339	47 152,20	89,60%	1	3,00%
57	talin 1, isoform CRA_b	gi|119578757	269 747,10	89,60%	1	0,47%
57	cathelicidin antimicrobial peptide	gi|119585258	19 284,30	100,00%	5	30,60%
57	Profilin-1	gi|130979	15 036,30	89,60%	1	11,40%
57	Non-secretory ribonuclease	gi|133168	18 335,70	89,60%	1	8,70%
57	Eosinophil cationic protein; Ribonuclease 3	gi|147744558	18 422,40	100,00%	3	26,90%
57	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	99,80%	2	5,79%
57	92 kDa type IV collagenase	gi|177205	78 410,70	99,80%	2	2,97%
57	IgG receptor precursor	gi|183037	26 281,00	89,60%	1	9,01%
57	lactoferrin	gi|186833	78 378,50	89,60%	1	1,83%
57	non-specific cross reacting antigen	gi|189102	38 135,90	99,80%	2	9,74%
57	unnamed protein product	gi|194385108	53 123,90	89,60%	1	2,28%
57	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
57	Protein disulfide-isomerase	gi|2507460	57 100,10	89,60%	1	1,97%
57	Actin-related protein 2/3 complex subunit 1B	gi|3121763	40 931,80	89,60%	1	4,84%
57	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	89,60%	1	4,48%
57	filamin A	gi|53791219	277 478,30	99,80%	2	2,11%
58	coronin-like protein	gi|1002923	59 594,60	80,90%	1	5,20%
58	polyubiquitin	gi|11024714	68 474,80	80,90%	2	2,63%
58	Alpha-1-acid glycoprotein 1	gi|112877	23 522,20	100,00%	3	19,40%
58	IgG receptor IIIB	gi|11344591	26 171,00	100,00%	5	23,60%
58	Serum albumin	gi|113576	69 348,90	100,00%	3	3,78%
58	beta-2-microglobulin precursor	gi|114319011	12 781,80	100,00%	3	31,80%
58	Cytochrome b-245 heavy chain	gi|115211	53 587,60	99,80%	2	5,98%
58	Cathepsin G	gi|115725	28 819,50	100,00%	14	54,10%
58	Niemann-Pick disease, type C2, isoform CRA_b	gi|119601584	16 552,00	80,90%	1	15,20%
58	hypothetical protein	gi|12052955	12 783,70	100,00%	6	69,30%
58	High mobility group protein B2	gi|123374	24 016,60	80,90%	1	6,22%
58	Cu/Zn-superoxide dismutase	gi|1237406	15 778,10	80,90%	1	7,84%
58	Profilin-1	gi|130979	15 036,30	80,90%	1	11,40%
58	Phosphatidylethanolamine-binding protein 1	gi|1352726	21 038,90	100,00%	5	40,10%
58	92 kDa type IV collagenase	gi|177205	78 410,70	99,80%	2	3,54%
58	IgG receptor precursor	gi|183037	26 281,00	80,90%	1	21,90%
58	lactoferrin	gi|186833	78 378,50	100,00%	7	16,70%
58	neutrophil granule peptide HP1	gi|228797	3 430,10	99,70%	2	60,00%
58	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	80,90%	1	4,18%
58	gelsolin isoform b	gi|38044288	80 622,80	99,80%	2	3,42%
58	FKBP1A	gi|47115163	11 932,70	80,90%	1	12,00%
58	filamin A	gi|53791219	277 478,30	100,00%	7	5,21%
59	IgG receptor IIIB	gi|11344591	26 171,00	100,00%	3	15,00%
59	Serum albumin	gi|113576	69 348,90	100,00%	4	7,06%
59	Cytochrome b-245 heavy chain	gi|115211	53 587,60	82,40%	1	2,35%
59	Cathepsin G	gi|115725	28 819,50	100,00%	11	48,20%

59	Elongation factor 2	gi|119172	95 322,10	99,80%	2	2,56%
59	Alpha-enolase	gi|119339	47 152,20	82,40%	1	3,00%
59	calmodulin 3	gi|119577833	17 145,40	82,40%	1	10,50%
59	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	100,00%	4	8,87%
59	Niemann-Pick disease, type C2, isoform CRA_b	gi|119601584	16 552,00	98,20%	2	21,90%
59	CD93 antigen	gi|119630576	68 541,20	82,40%	1	2,45%
59	Profilin-1;	gi|130979	15 036,30	100,00%	6	56,40%
59	Eosinophil cationic protein	gi|147744558	18 422,40	82,40%	1	7,50%
59	dermcidin isoform 2	gi|148271063, gi|20141302	11 266,10	82,40%	1	10,00%
59	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	82,40%	1	4,96%
59	92 kDa type IV collagenase	gi|177205	78 410,70	99,80%	2	2,97%
59	IgG receptor precursor	gi|183037	26 281,00	99,80%	2	17,60%
59	lactoferrin	gi|186833	78 378,50	100,00%	8	15,00%
59	unnamed protein product	gi|189053774	45 819,00	99,80%	2	5,16%
59	unnamed protein product	gi|194373815	38 499,90	99,80%	2	8,05%
59	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
59	suprabasin isoform 1 precursor	gi|260436922	60 519,20	82,40%	1	3,05%
59	TIMP-2, CSC-21K=tissue inhibitor of metalloproteinase	gi|262883	21 630,00	99,80%	2	18,20%
59	gelsolin isoform b	gi|38044288	80 622,80	99,80%	2	4,79%
59	FKBP1A	gi|47115163	11 932,70	82,40%	1	12,00%
59	filamin A	gi|53791219	277 478,30	100,00%	12	7,54%
60	IgG receptor IIIB	gi|11344591	26 171,00	99,80%	2	11,60%
60	Serum albumin	gi|113576	69 348,90	100,00%	5	8,21%
60	Cathepsin G	gi|115725	28 819,50	100,00%	6	30,60%
60	ADP-ribosyl cyclase 2	gi|116241273	35 721,40	99,80%	2	8,81%
60	Elongation factor 2;	gi|119172	95 322,10	79,90%	1	1,52%
60	calmodulin 3, isoform CRA_e	gi|119577833	17 145,40	99,80%	2	10,50%
60	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	100,00%	7	16,70%
60	phosphohistidine phosphatase 1, isoform CRA_b	gi|119608696	13 804,60	79,90%	1	9,60%
60	Coronin-1C	gi|12643898	53 232,30	79,90%	1	2,53%
60	Profilin-1	gi|130979	15 036,30	79,90%	1	11,40%
60	Heterogeneous nuclear ribonucleoproteins A2/B1	gi|133257	37 412,30	79,90%	1	2,55%
60	lysozyme	gi|1335210	14 682,50	100,00%	6	58,50%
60	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	79,90%	1	6,24%
60	SGP28 protein	gi|1587691	27 622,10	100,00%	5	27,30%
60	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	99,80%	2	15,80%
60	IgG receptor precursor	gi|183037	26 281,00	79,90%	1	9,87%
60	lactoferrin	gi|186833	78 378,50	100,00%	13	27,10%
60	unnamed protein product	gi|189053774	45 819,00	99,70%	2	4,42%
60	unnamed protein product	gi|194388072	42 725,80	79,90%	1	3,17%
60	cationic antimicrobial protein CAP37	gi|227250	26 635,30	99,80%	2	9,24%
60	neutrophil granule peptide HP1	gi|228797	3 430,10	79,90%	1	30,00%
60	Fatty acid-binding protein,	gi|232081	15 146,40	79,90%	1	15,60%
60	gelsolin isoform b	gi|38044288	80 622,80	79,90%	1	3,01%
60	filamin A	gi|53791219	277 478,30	100,00%	10	6,01%
61	Serum albumin	gi|113576	69 348,90	100,00%	5	8,21%
61	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	74,00%	1	3,85%

61	ribonuclease T2 precursor	gi|114319041	29 463,20	74,00%	1	5,86%
61	Cathepsin G	gi|115725	28 819,50	100,00%	4	16,50%
61	Cofilin-1	gi|116848	18 485,10	100,00%	8	57,20%
61	calmodulin 3	gi|119577833	17 145,40	74,00%	1	10,50%
61	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	99,80%	2	3,69%
61	complement factor properdin, isoform CRA_a	gi|119579721	51 256,10	74,00%	1	2,77%
61	WD repeat domain 1, isoform CRA_b	gi|119613093	66 175,20	74,00%	1	3,47%
61	lactoferrin precursor	gi|12083188	78 364,50	74,00%	1	73,40%
61	Profilin-1;	gi|130979	15 036,30	74,00%	1	10,00%
61	Heterogeneous nuclear ribonucleoproteins A2/B1	gi|133257	37 412,30	99,80%	2	6,80%
61	lysozyme	gi|1335210	14 682,50	100,00%	11	80,00%
61	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	100,00%	3	4,37%
61	SGP28 protein	gi|1587691	27 622,10	100,00%	5	30,20%
61	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	100,00%	5	43,40%
61	IgG receptor precursor	gi|183037	26 281,00	74,00%	1	4,72%
61	lactoferrin	gi|186833	78 378,50	100,00%	50	73,40%
61	cationic antimicrobial protein CAP37	gi|227250	26 635,30	100,00%	6	29,30%
61	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
61	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	99,70%	2	8,66%
61	unnamed protein product	gi|34412	78 061,10	99,80%	2	73,60%
61	Guanine nucleotide-binding protein G	gi|52783599	7 300,30	74,00%	1	13,20%
61	filamin A	gi|53791219	277 478,30	100,00%	4	2,37%
62	coronin-like protein	gi|1002923	51 008,00	100,00%	3	4,12%
62	valosin-containing protein	gi|11095436	89 306,80	73,80%	1	1,74%
62	Serum albumin	gi|113576	69 348,90	100,00%	4	5,09%
62	Cathepsin G	gi|115725	28 819,50	99,80%	2	12,20%
62	Cofilin-1	gi|116848	18 485,10	100,00%	5	42,20%
62	Calreticulin	gi|117501	48 124,90	73,80%	1	6,95%
62	calmodulin 3	gi|119577833	17 145,40	73,80%	1	10,50%
62	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	99,70%	2	6,16%
62	WD repeat domain 1, isoform CRA_b	gi|119613093	66 175,20	73,80%	1	3,47%
62	hCG1789360	gi|119628861	17 844,30	99,80%	2	26,00%
62	lactoferrin precursor	gi|12083188	78 364,50	73,80%	1	73,80%
62	Profilin-1	gi|130979	15 036,30	100,00%	7	62,10%
62	lysozyme	gi|1335210	14 682,50	100,00%	3	36,20%
62	Serotransferrin	gi|136191	77 032,20	73,80%	2	4,87%
62	unnamed protein product	gi|158255138	17 994,90	99,10%	2	17,60%
62	DJ-1	gi|16751471	19 873,10	73,80%	1	10,10%
62	Rho GDP-dissociation inhibitor 2	gi|1707893	22 970,40	99,40%	3	27,40%
62	92 kDa type IV collagenase	gi|177205	78 410,70	73,80%	1	2,12%
62	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	100,00%	6	53,10%
62	lactoferrin	gi|186833	78 378,50	100,00%	45	73,80%
62	cationic antimicrobial protein CAP37	gi|227250	26 635,30	100,00%	8	51,80%
62	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	99,80%	2	8,66%
62	small nuclear ribonucleoprotein Sm D1	gi|32959908	13 263,90	73,80%	1	16,80%
62	unnamed protein product	gi|34412	78 061,10	73,80%	1	74,00%
62	lactoferrin	gi|38154680	77 963,00	73,80%	1	70,10%
63	coronin-like protein	gi|1002923	51 008,00	100,00%	4	7,59%
63	Serum albumin	gi|113576	69 348,90	100,00%	5	8,21%

63	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	74,80%	1	11,80%
63	Cathepsin G	gi|115725	28 819,50	74,80%	1	7,06%
63	Spectrin beta chain, brain 1	gi|116242799	274 617,40	74,80%	1	0,51%
63	Neutrophil gelatinase-associated lipocalin;	gi|1171700	22 570,90	100,00%	8	49,00%
63	Calreticulin	gi|117501	48 124,90	74,80%	1	6,95%
63	calmodulin 3	gi|119577833	17 145,40	74,80%	1	10,50%
63	haptoglobin, isoform CRA_a	gi|119579598	45 186,90	100,00%	4	10,80%
63	cathepsin C, isoform CRA_a	gi|119579767	51 824,20	99,80%	2	7,56%
63	lactoferrin precursor	gi|12083188	78 364,50	74,80%	1	74,40%
63	Profilin-1	gi|130979	15 036,30	100,00%	5	50,00%
63	lysozyme	gi|1335210	14 682,50	74,80%	1	21,50%
63	Plastin-2	gi|1346733	70 274,10	74,80%	1	3,03%
63	unnamed protein product	gi|158255138	17 994,90	100,00%	10	66,70%
63	Rho GDP-dissociation inhibitor 2	gi|1707893	22 970,40	100,00%	4	32,80%
63	92 kDa type IV collagenase	gi|177205	78 410,70	99,80%	2	3,54%
63	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	100,00%	4	31,60%
63	lactoferrin	gi|186833	78 378,50	100,00%	42	74,40%
63	unnamed protein product	gi|194388742	30 942,00	74,80%	1	6,23%
63	cationic antimicrobial protein CAP37	gi|227250	26 635,30	99,90%	3	21,70%
63	neutrophil granule peptide HP1	gi|228797	3 430,10	99,80%	2	60,00%
63	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	3	12,80%
63	unnamed protein product	gi|34412	78 061,10	74,80%	1	74,60%
63	filamin A	gi|53791219	277 478,30	74,80%	1	0,54%
63	Vimentin	gi|55977767	53 634,60	74,80%	1	1,93%
63	Thioredoxin domain-containing protein 17	gi|74732856	13 922,40	99,80%	2	19,50%
64	Serum albumin	gi|113576	69 348,90	100,00%	5	6,24%
64	Neutrophil gelatinase-associated lipocalin	gi|1171700	22 570,90	100,00%	4	26,80%
64	Plastin-2	gi|1346733	70 274,10	99,60%	2	4,63%
64	Peptidoglycan recognition protein 1	gi|18202143	21 712,60	100,00%	3	29,60%
64	lactoferrin	gi|186833	78 378,50	100,00%	7	14,80%
64	serum albumin	gi|62113341	69 066,60	85,90%	1	6,24%
65	Serum albumin	gi|113576	69 348,90	100,00%	11	14,90%
65	Neutrophil gelatinase-associated lipocalin	gi|1171700	22 570,90	100,00%	6	42,40%
65	serum albumin	gi|62113341	69 066,60	83,80%	1	14,40%
66	Serum albumin	gi|113576	69 348,90	100,00%	8	13,10%
66	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	100,00%	6	30,20%
66	HIST1H2BC protein	gi|114205460	13 918,60	98,60%	2	20,60%
66	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	76,60%	1	11,80%
66	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	99,80%	2	24,60%
66	Neutrophil gelatinase-associated lipocalin;	gi|1171700	22 570,90	100,00%	6	42,40%
66	thioredoxin, isoform CRA_b	gi|119579464	11 719,30	76,60%	1	12,40%
66	SH3 domain binding glutamic acid-rich protein like 3, isoform CRA_a	gi|119628236	10 419,80	99,80%	2	46,20%
66	Plastin-2;	gi|1346733	70 274,10	100,00%	3	5,90%
66	Vitamin D-binding protein	gi|139641	52 933,50	76,60%	1	4,64%
66	unnamed protein product	gi|158260331	32 905,10	99,80%	2	12,20%
66	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	11	22,50%
66	lactoferrin	gi|186833	78 378,50	99,80%	2	4,36%
66	unnamed protein product	gi|194373815	38 499,90	100,00%	4	27,00%
66	OLFM4 protein	gi|29126831	57 263,00	76,60%	1	2,94%

66	gelsolin isoform b	gi|38044288	80 622,80	100,00%	4	7,66%
66	Vimentin	gi|55977767	53 634,60	76,60%	1	2,79%
67	coronin-like protein	gi|1002923	51 008,00	100,00%	5	17,40%
67	APEX nuclease	gi|1090503	35 537,70	99,80%	2	14,20%
67	Serum albumin	gi|113576	69 348,90	100,00%	5	8,21%
67	Fructose-bisphosphate aldolase A	gi|113606	39 402,60	100,00%	10	34,60%
67	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	99,90%	3	32,30%
67	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	6	63,20%
67	Neutrophil gelatinase-associated lipocalin	gi|1171700	22 570,90	100,00%	4	30,30%
67	calmodulin 3	gi|119577833	17 145,40	57,40%	1	11,20%
67	Profilin-1	gi|130979	15 036,30	99,80%	2	21,40%
67	proteinase 3	gi|1335280	27 586,20	99,80%	2	6,69%
67	Glutaredoxin-1;	gi|1346143	11 757,80	99,80%	2	31,10%
67	unnamed protein product	gi|158260331	32 905,10	99,80%	2	12,20%
67	Cytidine deaminase;	gi|1705718	16 166,70	99,90%	3	40,40%
67	Rho GDP-dissociation inhibitor 1;	gi|1707892	23 175,40	57,40%	1	7,35%
67	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	99,50%	2	23,80%
67	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	14	28,00%
67	lactoferrin	gi|186833	78 378,50	100,00%	5	11,40%
67	OLFM4 protein	gi|29126831	57 263,00	100,00%	4	12,40%
67	Actin-related protein 2/3 complex subunit 2	gi|3121764	34 315,70	100,00%	7	32,30%
67	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	7	27,80%
67	gelsolin isoform b	gi|38044288	80 622,80	100,00%	6	11,90%
67	Phosphoglycerate kinase 1	gi|52788229	44 597,30	100,00%	3	8,63%
67	Vimentin	gi|55977767	53 634,60	100,00%	5	12,70%
68	HIST1H4H protein	gi|111309351	11 349,70	99,80%	2	17,50%
68	Serum albumin	gi|113576	69 348,90	100,00%	5	6,24%
68	vasodilator-stimulated phosphoprotein	gi|11414808	39 592,80	64,80%	1	3,70%
68	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	12	84,90%
68	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	8	81,60%
68	Neutrophil gelatinase-associated lipocalin	gi|1171700	22 570,90	64,80%	1	5,56%
68	Alpha-enolase	gi|119339	47 152,20	100,00%	14	49,10%
68	calmodulin 3	gi|119577833	17 145,40	64,80%	1	11,20%
68	cathelicidin antimicrobial peptide	gi|119585258	19 284,30	100,00%	3	19,40%
68	coactosin-like 1	gi|119615881	15 927,20	64,80%	1	11,30%
68	moesin, isoform CRA_b	gi|119625804	67 803,80	99,80%	2	4,51%
68	proteinase 3	gi|1335280	27 586,20	100,00%	6	37,80%
68	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	100,00%	3	34,30%
68	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	6	11,90%
68	lactoferrin	gi|186833	78 378,50	100,00%	4	7,74%
68	EF-hand domain-containing protein D2	gi|20140139	26 680,40	99,50%	2	7,92%
68	Chitinase 3-like 1	gi|23512215	42 609,00	100,00%	8	29,20%
68	OLFM4 protein	gi|29126831	57 263,00	64,80%	1	2,94%
68	Actin-related protein 2/3 complex subunit 5	gi|3121767	16 302,60	100,00%	3	29,10%
68	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	8	47,80%
68	Chitotriosidase-1	gi|37999493	51 664,10	100,00%	9	31,30%
68	gelsolin isoform b	gi|38044288	80 622,80	100,00%	15	31,90%
68	Phosphoglycerate kinase 1	gi|52788229	44 597,30	99,80%	2	6,71%
69	HIST1H4H protein	gi|111309351	11 349,70	100,00%	3	27,20%

69	Serum albumin	gi|113576	69 348,90	100,00%	6	8,21%
69	MYL6 protein	gi|113812151	16 942,80	100,00%	4	25,80%
69	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	7	71,00%
69	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	10	86,80%
69	Alpha-enolase	gi|119339	47 152,20	100,00%	4	13,60%
69	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	5	8,32%
69	moesin, isoform CRA_b	gi|119625804	67 803,80	100,00%	6	10,70%
69	Nucleoside diphosphate kinase A	gi|127981	15 511,20	62,40%	1	12,40%
69	proteinase 3	gi|1335280	27 586,20	100,00%	8	51,20%
69	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	99,80%	2	23,80%
69	Transketolase	gi|1729976	67 861,40	62,40%	1	2,89%
69	beta-hemoglobin	gi|183945	15 947,70	100,00%	10	76,90%
69	Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	100,00%	4	65,20%
69	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	5	24,80%
69	Chitotriosidase-1	gi|37999493	51 664,10	62,40%	1	4,51%
69	gelsolin isoform b	gi|38044288	80 622,80	100,00%	6	11,60%
70	unnamed protein product	gi|10439139	58 277,60	100,00%	7	23,70%
70	T200 leukocyte common antigen precursor	gi|10999057	147 237,90	56,90%	1	0,84%
70	Serum albumin	gi|113576	69 348,90	100,00%	4	7,06%
70	MYL6 protein	gi|113812151	16 942,80	100,00%	6	41,70%
70	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	5	55,90%
70	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	9	81,60%
70	Neutrophil collagenase	gi|116862	53 396,20	100,00%	8	26,80%
70	Alpha-enolase	gi|119339	47 152,20	98,60%	2	7,83%
70	matrix metallopeptidase 8	gi|119587431	53 409,20	56,90%	1	28,50%
70	calponin 2, isoform CRA_e	gi|119589975	33 679,60	56,90%	1	5,50%
70	spectrin, alpha, non-erythrocytic 1	gi|119608212	282 267,10	100,00%	5	3,43%
70	myeloperoxidase,	gi|119614877	83 839,00	100,00%	18	30,50%
70	actin related protein 2/3 complex, subunit 3	gi|119618319	20 529,80	99,80%	2	10,10%
70	proteinase 3	gi|1335280	27 586,20	100,00%	6	37,80%
70	Serpin B10	gi|1345616	45 386,90	99,50%	2	13,40%
70	hemoglobin alpha-1 globin chain	gi|13650074	15 239,60	56,90%	1	10,60%
70	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	99,80%	2	23,80%
70	Transketolase	gi|1729976	67 861,40	56,90%	1	1,93%
70	Resistin; Cysteine-rich secreted protein	gi|18202962	11 401,00	100,00%	4	51,90%
70	beta-hemoglobin	gi|183945	15 980,00	98,20%	3	23,10%
70	lactoferrin	gi|186833	78 378,50	56,90%	1	1,55%
70	Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	100,00%	5	67,40%
70	glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36 036,30	100,00%	5	26,30%
71	Serum albumin	gi|113576	69 348,90	100,00%	4	6,24%
71	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	3	46,20%
71	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	8	63,20%
71	Adenylyl cyclase-associated protein 1	gi|116241280	51 655,20	100,00%	7	20,80%
71	Neutrophil collagenase	gi|116862	53 396,20	100,00%	10	30,80%
71	RecName: Full=Grancalcin	gi|1170014	23 992,60	50,00%	1	3,23%
71	Neutrophil elastase	gi|119292	28 499,40	100,00%	3	27,00%
71	Alpha-enolase	gi|119339	47 152,20	86,80%	2	8,53%
71	vinculin, isoform CRA_a	gi|119574932	116 706,30	50,00%	1	1,03%
71	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	20	33,30%
71	proteinase 3	gi|1335280	27 586,20	50,00%	1	12,60%

71	hemoglobin alpha-1 globin chain	gi|13650074	15 239,60	100,00%	5	50,70%
71	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	100,00%	3	34,30%
71	Transketolase	gi|1729976	67 861,40	100,00%	7	16,90%
71	Resistin	gi|18202962	11 401,00	50,00%	1	10,20%
71	Fatty acid-binding protein	gi|232081	15 146,40	50,00%	1	6,67%
71	Full=Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	100,00%	3	58,70%
72	fructose-1,6-biphosphatase	gi|1000078	36 825,20	78,50%	1	4,73%
72	Serum albumin	gi|113576	69 348,90	100,00%	5	6,73%
72	Annexin A1	gi|113944	38 697,90	100,00%	8	32,40%
72	Annexin A6	gi|113962	75 859,50	78,50%	1	2,38%
72	neutrophil cytosol factor 1	gi|115298672	44 665,80	78,50%	1	5,90%
72	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	3	46,20%
72	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	7	57,00%
72	Adenylyl cyclase-associated protein 1	gi|116241280	51 655,20	100,00%	6	16,60%
72	Neutrophil collagenase; Matrix metalloproteinase-8	gi|116862	53 396,20	100,00%	5	16,90%
72	Grancalcin	gi|1170014	23 992,60	78,50%	1	5,07%
72	small GTP binding protein Rab7	gi|1174149	23 472,00	98,80%	2	13,00%
72	Alpha-enolase	gi|119339	47 152,20	78,50%	1	3,46%
72	arginase, isoform CRA_b	gi|119568439	34 718,00	100,00%	3	14,90%
72	vinculin, isoform CRA_a	gi|119574932	116 706,30	99,80%	2	3,10%
72	heat shock protein 90kDa alpha	gi|119602173	84 645,20	78,50%	1	2,05%
72	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	8	14,20%
72	proteinase 3	gi|1335280	27 586,20	78,50%	1	12,60%
72	hemoglobin alpha-1 globin chain	gi|13650074	15 239,60	78,50%	1	10,60%
72	unnamed protein product	gi|158255120	66 843,30	100,00%	3	11,40%
72	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	78,50%	1	10,50%
72	Transketolase	gi|1729976	67 861,40	100,00%	6	19,70%
72	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	3	4,95%
72	lactoferrin	gi|186833	78 378,50	100,00%	3	7,17%
72	Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	78,50%	1	34,80%
72	CAP, adenylate cyclase-associated protein 1	gi|55859737	51 812,50	78,50%	1	16,50%
73	unnamed protein product	gi|10432682	22 872,30	75,20%	1	7,32%
73	alpha-1 antitrypsin variant	gi|110350939	46 719,90	100,00%	4	14,10%
73	Serum albumin	gi|113576	69 348,90	100,00%	4	4,76%
73	Annexin A1	gi|113944	38 697,90	100,00%	14	48,80%
73	Annexin A6	gi|113962	75 859,50	99,80%	2	3,86%
73	neutrophil cytosol factor 1	gi|115298672	44 665,80	99,70%	2	8,21%
73	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	3	46,20%
73	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	7	57,00%
73	Protein S100-A4; Calvasculin	gi|115601	11 711,00	75,20%	1	7,92%
73	Neutrophil collagenase	gi|116862	53 396,20	100,00%	6	18,80%
73	Neutrophil elastase	gi|119292	28 499,40	75,20%	1	3,75%
73	transgelin 2, isoform CRA_e	gi|119573148	22 373,90	100,00%	3	22,10%
73	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	7	11,30%
73	glutaminyl-peptide cyclotransferase	gi|119620798	28 513,70	99,80%	2	9,96%
73	Protein-arginine deiminase type-4	gi|12230488	74 122,10	99,80%	2	5,28%
73	L-lactate dehydrogenase A chain	gi|126047	36 671,20	75,20%	1	3,01%
73	Chloride intracellular channel protein 1	gi|12643390	23 525,50	100,00%	7	51,90%
73	proteinase 3	gi|1335280	27 586,20	75,20%	1	12,60%
73	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	100,00%	10	22,90%

73	78 kDa glucose-regulated protein	gi|14916999	72 316,70	75,20%	1	1,68%
73	unnamed protein product	gi|158255120	66 843,30	100,00%	3	7,95%
73	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	100,00%	5	37,10%
73	Transketolase	gi|1729976	67 861,40	99,80%	2	5,30%
73	Triosephosphate isomerase 1	gi|17389815	26 651,10	100,00%	10	53,80%
73	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	3	5,94%
73	Resistin	gi|18202962	11 401,00	99,80%	2	24,10%
73	lactoferrin	gi|186833	78 378,50	100,00%	3	8,02%
73	unnamed protein product	gi|193785841	28 285,10	100,00%	3	17,40%
73	unnamed protein product	gi|194388072	42 725,80	100,00%	9	28,00%
74	alpha-1 antitrypsin variant	gi|110350939	46 719,90	100,00%	6	19,90%
74	valosin-containing protein	gi|11095436	89 306,80	70,70%	2	4,22%
74	Serum albumin	gi|113576	69 348,90	100,00%	4	6,73%
74	Antithrombin-III	gi|113936	52 675,10	70,70%	2	3,44%
74	Annexin A1	gi|113944	38 697,90	100,00%	3	12,40%
74	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	99,80%	2	34,40%
74	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	4	43,00%
74	PSMA7 protein	gi|116283481	27 869,10	100,00%	4	20,60%
74	Protein S100-A6; Calcyclin	gi|116509	10 162,40	70,70%	1	8,89%
74	Ras GTPase-activating-like protein IQGAP1	gi|1170586	189 240,60	100,00%	8	7,66%
74	Neutrophil elastase	gi|119292	28 499,40	100,00%	5	19,90%
74	Alpha-enolase	gi|119339	47 152,20	99,80%	2	8,53%
74	transgelin 2, isoform CRA_e	gi|119573148	22 373,90	99,80%	2	27,10%
74	actinin, alpha 4, isoform CRA_c	gi|119577215	102 253,60	100,00%	12	27,80%
74	bridging integrator 2, isoform CRA_c	gi|119578592	61 729,30	100,00%	3	8,69%
74	GDP dissociation inhibitor 2, isoform CRA_a	gi|119606836	50 647,50	100,00%	18	48,10%
74	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	8	13,80%
74	glutaminyl-peptide cyclotransferase	gi|119620798	28 513,70	99,80%	2	12,40%
74	proteasome subunit, isoform CRA_b	gi|119627813	22 819,50	70,70%	1	7,46%
74	hCG401289, isoform CRA_d	gi|119629883	35 083,70	70,70%	1	6,73%
74	phosphorylase, glycogen; brain, isoform CRA_b	gi|119630497	96 680,30	70,70%	1	1,42%
74	L-lactate dehydrogenase A chain	gi|126047	36 671,20	100,00%	4	14,80%
74	Chloride intracellular channel protein 1	gi|12643390	23 525,50	99,80%	2	14,30%
74	proteinase 3	gi|1335280	27 586,20	70,70%	1	12,60%
74	Plastin-2	gi|1346733	70 274,10	70,70%	1	3,99%
74	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	100,00%	5	9,83%
74	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	70,70%	1	4,96%
74	Annexin A4	gi|1703319	36 068,60	100,00%	4	15,60%
74	Annexin A11	gi|1703322	54 373,70	99,80%	2	4,75%
74	Protein S100-A11; Calgizzarin	gi|1710818	11 723,10	100,00%	3	34,30%
74	Transketolase	gi|1729976	67 861,40	99,80%	2	5,78%
74	Triosephosphate isomerase 1	gi|17389815	26 651,10	100,00%	13	71,90%
74	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	6	12,90%
74	lactoferrin	gi|186833	78 378,50	100,00%	4	7,03%
74	unnamed protein product	gi|189067522	53 910,80	100,00%	6	15,60%
74	unnamed protein product	gi|194376354	20 806,90	99,80%	2	13,60%
74	unnamed protein product	gi|194388072	42 725,80	100,00%	18	51,70%
74	unnamed protein product	gi|28334	103 043,00	100,00%	6	8,07%
74	YWHAZ protein	gi|30354619	27 727,90	70,70%	1	5,71%
74	VAT1 protein	gi|32425722	41 901,80	70,70%	1	2,80%

75	unnamed protein product	gi|10439139	58 277,60	80,80%	1	1,98%
75	alpha-1 antitrypsin variant	gi|110350939	46 719,90	100,00%	4	12,40%
75	Serum albumin	gi|113576	69 348,90	100,00%	4	6,24%
75	Annexin A3	gi|113954	36 359,40	100,00%	12	39,90%
75	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	3	43,00%
75	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	99,90%	3	36,00%
75	Adenylyl cyclase-associated protein 1	gi|116241280	51 655,20	80,80%	1	3,79%
75	Ras GTPase-activating-like protein IQGAP1	gi|1170586	189 240,60	99,80%	3	3,14%
75	Neutrophil elastase	gi|119292	28 499,40	100,00%	5	25,80%
75	Alpha-enolase	gi|119339	47 152,20	80,80%	1	5,07%
75	actinin, alpha 4, isoform CRA_c	gi|119577215	102 253,60	99,90%	3	12,90%
75	protein phosphatase 1, catalytic subunit, alpha isoform, isoform CRA_b	gi|119595014	37 495,90	100,00%	3	13,60%
75	GDP dissociation inhibitor 2, isoform CRA_a	gi|119606836	50 647,50	80,80%	1	4,27%
75	family with sequence similarity 49, member B, isoform CRA_b	gi|119612532	36 730,70	80,80%	1	4,63%
75	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	4	7,65%
75	hCG401289, isoform CRA_d	gi|119629883	35 083,70	80,80%	1	6,73%
75	Ferritin light chain	gi|120523	19 979,70	80,80%	1	8,62%
75	PYD and CARD domain containing	gi|13325316	21 609,80	80,80%	1	8,72%
75	proteinase 3	gi|1335280	27 586,20	80,80%	1	12,60%
75	Tryptophanyl-tRNA synthetase, cytoplasmic	gi|135191	53 149,60	80,80%	1	3,40%
75	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	99,80%	2	4,84%
75	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	3	12,40%
75	Annexin A11	gi|1703322	54 373,70	100,00%	7	16,60%
75	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	5	9,05%
75	chromogranin A	gi|180529	50 911,50	99,50%	2	2,63%
75	unnamed protein product	gi|189067522	53 910,80	99,80%	2	7,39%
75	unnamed protein product	gi|194388072	42 725,80	100,00%	13	37,70%
75	unnamed protein product	gi|28334	103 043,00	100,00%	7	11,20%
75	YWHAZ protein	gi|30354619	27 727,90	80,80%	1	5,71%
75	VAT1 protein	gi|32425722	41 901,80	99,80%	2	8,65%
75	Proteasome subunit alpha type-6	gi|46397659	27 381,50	100,00%	3	13,80%
75	filamin A	gi|53791219	277 478,30	100,00%	12	6,93%
75	hypothetical protein	gi|5912028	26 856,20	80,80%	1	2,51%
76	Purine nucleoside phosphorylase	gi|108935929	32 533,50	100,00%	11	50,90%
76	alpha-1 antitrypsin variant	gi|110350939	46 719,90	100,00%	5	14,40%
76	Serum albumin	gi|113576	69 348,90	100,00%	4	6,24%
76	Annexin A3	gi|113954	36 359,40	99,80%	2	9,60%
76	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	99,80%	2	34,40%
76	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	3	36,00%
76	Protein S100-A6; Calcyclin	gi|116509	10 162,40	100,00%	4	47,80%
76	Neutrophil elastase	gi|119292	28 499,40	100,00%	3	19,50%
76	Alpha-enolase	gi|119339	47 152,20	99,80%	2	8,53%
76	glutamic-oxaloacetic transaminase 1	gi|119570253	46 230,10	68,90%	1	4,84%
76	serpin peptidase inhibitor, clade B	gi|119575505	42 572,70	68,90%	1	3,99%
76	talin 1, isoform CRA_b	gi|119578757	269 747,10	68,90%	1	1,18%
76	protein phosphatase 1, catalytic subunit, alpha isoform, isoform CRA_b	gi|119595014	37 495,90	68,90%	1	5,15%
76	proteasome subunit, alpha type, 3, isoform	gi|119601121	28 415,70	100,00%	3	12,90%

	CRA_a					
76	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	5	7,92%
76	Neutrophil cytosolic factor 2	gi|12804409	59 744,20	98,30%	2	4,94%
76	Phosphoglycerate mutase 1	gi|130348	28 786,80	100,00%	10	65,70%
76	Profilin-1	gi|130979	15 036,30	99,50%	2	21,40%
76	PYD and CARD domain containing	gi|13325316	21 609,80	68,90%	1	8,72%
76	Protein S100-P; S100 calcium-binding protein P	gi|134142	10 382,30	68,90%	1	29,50%
76	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	68,90%	1	2,34%
76	dermcidin isoform 2	gi|148271063	11 266,10	68,90%	1	10,00%
76	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	18	66,70%
76	Transketolase	gi|1729976	67 861,40	68,90%	1	3,05%
76	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	10	18,10%
76	unknown	gi|18027726	66 422,70	68,90%	1	3,37%
76	lactoferrin	gi|186833	78 378,50	100,00%	4	7,17%
76	unnamed protein product	gi|194384098	50 079,80	99,70%	2	7,02%
76	unnamed protein product	gi|194388072	42 725,80	100,00%	8	24,50%
76	unnamed protein product	gi|194391276	50 419,90	68,90%	1	2,73%
76	unnamed protein product	gi|28334	103 043,00	100,00%	30	41,60%
76	OLFM4 protein	gi|29126831	57 263,00	68,90%	1	2,94%
76	TALDO1 protein	gi|48257056	37 523,70	100,00%	5	16,60%
76	Phosphoglycerate kinase 1	gi|52788229	44 597,30	100,00%	10	31,90%
76	filamin A	gi|53791219	277 478,30	100,00%	5	2,57%
76	6-phosphogluconolactonase	gi|7387511	27 529,50	99,80%	2	14,30%
77	coatomer protein	gi|1002369	138 331,30	100,00%	3	3,59%
77	Purine nucleoside phosphorylase	gi|108935929	32 533,50	66,00%	1	7,51%
77	alpha-1 antitrypsin variant	gi|110350939	46 719,90	66,00%	1	3,35%
77	Serum albumin	gi|113576	69 348,90	100,00%	4	6,24%
77	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	99,90%	3	46,20%
77	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	4	49,10%
77	Protein S100-A6; Calcyclin	gi|116509	10 162,40	99,90%	3	46,70%
77	Neutrophil elastase	gi|119292	28 499,40	100,00%	4	22,80%
77	Alpha-enolase	gi|119339	47 152,20	99,10%	2	5,76%
77	ras-related C3 botulinum toxin substrate 1	gi|119575445	21 432,60	66,00%	1	5,21%
77	hCG1995701, isoform CRA_g	gi|119584408	19 649,40	100,00%	4	23,20%
77	translin-associated factor X, isoform CRA_a	gi|119590367	33 095,10	66,00%	1	6,90%
77	protein phosphatase 1	gi|119595014	37 495,90	99,80%	2	10,60%
77	family with sequence similarity 49, member B, isoform CRA_b	gi|119612532	36 730,70	66,00%	1	4,01%
77	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	5	8,86%
77	L-lactate dehydrogenase B chain	gi|126041	36 620,60	99,80%	2	9,88%
77	L-lactate dehydrogenase A chain	gi|126047	36 671,20	66,00%	1	5,12%
77	Leukotriene A-4 hydrolase	gi|126353	69 269,10	100,00%	12	25,00%
77	Phosphoglycerate mutase 1	gi|130348	28 786,80	100,00%	6	40,20%
77	Profilin-1	gi|130979	15 036,30	99,80%	2	20,00%
77	Protein S100-P; S100 calcium-binding protein P	gi|134142	10 382,30	100,00%	4	71,60%
77	Plastin-2	gi|1346733	70 274,10	66,00%	1	3,99%
77	Transcobalamin-1	gi|146345530	48 177,60	66,00%	1	4,62%
77	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	15	59,00%
77	Transketolase	gi|1729976	67 861,40	99,80%	2	4,65%
77	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	11	21,60%

77	lactoferrin	gi|186833	78 378,50	99,80%	3	5,49%
77	unnamed protein product	gi|194388072	42 725,80	100,00%	4	16,40%
77	Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	66,00%	1	34,80%
77	unnamed protein product	gi|28334	103 043,00	100,00%	13	20,40%
77	OLFM4 protein	gi|29126831	57 263,00	66,00%	1	2,94%
77	Actin-related protein 3	gi|47117647	47 353,80	100,00%	12	40,20%
77	TALDO1 protein	gi|48257056	37 523,70	100,00%	15	39,20%
77	Phosphoglycerate kinase 1	gi|52788229	44 597,30	100,00%	13	44,40%
77	filamin A	gi|53791219	277 478,30	66,00%	1	0,42%
77	6-phosphogluconolactonase	gi|7387511	27 529,50	100,00%	3	19,00%
78	ubiquitin-conjugating enzyme	gi|1096569	16 718,00	99,80%	2	23,80%
78	Serum albumin	gi|113576	69 348,90	100,00%	5	6,24%
78	Annexin A6	gi|113962	75 859,50	100,00%	5	9,51%
78	PRO2783	gi|11493534	13 605,00	63,80%	1	12,90%
78	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	100,00%	3	46,20%
78	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	3	36,00%
78	Neutrophil elastase	gi|119292	28 499,40	100,00%	5	25,80%
78	actinin, alpha 4, isoform CRA_c [Homo sapiens]	gi|119577215	102 253,60	100,00%	6	15,40%
78	N-acylsphingosine amidohydrolase 1	gi|119584197	44 633,10	63,80%	1	2,78%
78	hCG1995701, isoform CRA_g	gi|119584408	19 649,40	100,00%	5	34,50%
78	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	63,80%	1	1,34%
78	2-deoxyribose-5-phosphate aldolase homolog	gi|119616763	35 213,80	63,80%	1	5,03%
78	Beta-hexosaminidase subunit alpha	gi|123079	60 671,80	98,40%	2	5,29%
78	L-lactate dehydrogenase B chain	gi|126041	36 620,60	63,80%	1	8,68%
78	L-lactate dehydrogenase A chain	gi|126047	36 671,20	100,00%	5	16,60%
78	Leukotriene A-4 hydrolase	gi|126353	69 269,10	100,00%	22	43,20%
78	Protein S100-P; S100 calcium-binding protein P	gi|134142	10 382,30	99,80%	2	43,20%
78	Plastin-2	gi|1346733	70 274,10	100,00%	4	9,41%
78	Heat shock 70 kDa protein 1A/1B	gi|147744565	70 036,00	63,80%	1	2,34%
78	ARP2 actin-related protein 2 homolog	gi|15778930	44 743,70	100,00%	10	37,80%
78	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	100,00%	10	36,90%
78	Transketolase	gi|1729976	67 861,40	99,80%	2	5,94%
78	Neuroleukin	gi|17380385	63 130,40	100,00%	3	8,24%
78	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	11	20,70%
78	lactoferrin	gi|186833	78 378,50	99,80%	2	3,66%
78	unnamed protein product	gi|189067522	53 910,80	63,80%	1	2,26%
78	unnamed protein product	gi|194385108	53 123,90	100,00%	5	12,80%
78	unnamed protein product	gi|194388072	42 725,80	100,00%	6	20,10%
78	Protein S100-A12; Calgranulin-C	gi|2507565	10 557,40	63,80%	1	34,80%
78	unnamed protein product	gi|28334	103 043,00	100,00%	9	9,98%
78	Actin-related protein 3	gi|47117647	47 353,80	100,00%	13	48,80%
78	TALDO1 protein	gi|48257056	37 523,70	100,00%	7	21,40%
78	Phosphoglycerate kinase 1	gi|52788229	44 597,30	100,00%	5	21,10%
78	filamin A	gi|53791219	277 478,30	100,00%	3	1,68%
79	Serum albumin	gi|113576	69 348,90	100,00%	6	8,21%
79	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	99,90%	3	43,00%
79	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	100,00%	3	36,00%
79	Phosphoglucomutase-2	gi|116242708	68 253,60	100,00%	4	8,82%
79	Neutrophil collagenase	gi|116862	53 396,20	60,30%	1	3,43%

79	Neutrophil elastase	gi|119292	28 499,40	100,00%	4	9,74%
79	Alpha-enolase	gi|119339	47 152,20	60,30%	1	3,46%
79	actinin, alpha 4, isoform CRA_c	gi|119577215	102 253,60	60,30%	1	6,22%
79	EGF-like-domain, multiple 4, isoform CRA_a	gi|119577536	254 547,10	60,30%	1	0,25%
79	hCG1995701, isoform CRA_g	gi|119584408	19 649,40	60,30%	1	11,30%
79	proteasome activator subunit 2	gi|119586505	27 344,30	60,30%	1	9,21%
79	myeloperoxidase, isoform CRA_b	gi|119614877	83 839,00	100,00%	4	7,38%
79	proteasome subunit, alpha type, 4, isoform CRA_b	gi|119619571	29 466,80	100,00%	4	18,80%
79	DKFZP564J102 protein, isoform CRA_a	gi|119625030	53 232,10	60,30%	1	1,87%
79	L-lactate dehydrogenase B chain	gi|126041	36 620,60	60,30%	1	8,38%
79	L-lactate dehydrogenase A chain	gi|126047	36 671,20	100,00%	13	43,10%
79	Leukotriene A-4 hydrolase	gi|126353	69 269,10	100,00%	5	11,50%
79	Phosphoglucomutase 1	gi|127801388	61 433,00	99,80%	2	7,47%
79	proteinase 3	gi|1335280	27 586,20	60,30%	1	4,72%
79	Protein S100-P; S100 calcium-binding protein P	gi|134142	10 382,30	60,30%	1	29,50%
79	Plastin-2	gi|1346733	70 274,10	100,00%	27	56,00%
79	Transcobalamin-1	gi|146345530	48 177,60	60,30%	1	4,62%
79	ARP2 actin-related protein 2 homolog	gi|15778930	44 743,70	100,00%	3	15,50%
79	Unknown (protein for IMAGE:3538275)	gi|16924319	40 486,40	60,30%	2	7,99%
79	Transketolase	gi|1729976	67 861,40	100,00%	3	9,47%
79	Neuroleukin	gi|17380385	63 130,40	100,00%	22	49,60%
79	92 kDa type IV collagenase	gi|177205	78 410,70	100,00%	8	14,40%
79	lactoferrin	gi|186833	78 378,50	99,80%	2	3,52%
79	unnamed protein product	gi|194385108	53 123,90	100,00%	5	14,50%
79	unnamed protein product	gi|194388072	42 725,80	100,00%	5	17,20%
79	unnamed protein product	gi|28334	103 043,00	100,00%	3	4,26%
79	Actin-related protein 3	gi|47117647	47 353,80	100,00%	5	26,80%
79	TALDO1 protein	gi|48257056	37 523,70	60,30%	1	3,26%
79	Phosphoglycerate kinase 1	gi|52788229	44 597,30	100,00%	4	14,40%
80	unnamed protein product	gi|10434865	24 415,80	93,60%	1	4,25%
80	Protein S100-A8; Calgranulin-A	gi|115442	10 816,90	93,60%	1	22,60%
80	Protein S100-A9; Calgranulin-B	gi|115444	13 224,30	93,60%	1	11,40%
80	L-lactate dehydrogenase A chain	gi|126047	36 671,20	93,60%	1	5,12%
80	Neuroleukin	gi|17380385	63 130,40	97,80%	2	5,38%

UNIVERSITÉ DE STRASBOURG

Rizwan ASLAM

Inserm
BioMaterials
BioEngineering
UMR-S 1121
Strasbourg

Les peptides antimicrobiens dérivés de la chromogranine A et *Staphylococcus aureus*: de l'analyse de l'interaction hôte-pathogène au développement de revêtement de polymère antimicrobien

Résumé

Les chromogranines (Cgs) sont une famille de protéines acides exprimées dans les granules des cellules neuroendocrines et immunitaires. Plusieurs peptides dérivés des Cgs présentent des activités antimicrobiennes. L'objectif de ma thèse est d'évaluer l'interaction hôte-pathogène et ensuite de développer un polymère antimicrobien avec insertion du peptide antimicrobien cateslytin (CTL).

Dans une première partie, nous avons évalué l'aptitude de la leukotoxine LukE/D à induire la sécrétion des neutrophiles et rôle des protéases bactériennes à dégrader les peptides dérivés de la CgA. Les neutrophiles activés sécrètent de nombreux composés que nous avons identifiés. De plus, la dégradation des PAMs dérivés de la CgA par les protéases de *S. aureus* a été déterminée. Sur tous les PAMs testés, CTL est le seul qui tue *S. aureus* et résister à dégradation. Par ailleurs, CgA et CgB sont dégradés par la protéase Glu-C pour produire de nouveaux fragments sans activité antibactérienne, mais d'activité antifongique.

Dans une deuxième partie, nous avons décidé de préparer un revêtement conjugué à CTL. CTL-C est utilisé pour préparer des films avec le dépôt alterné de CHI et HA-CTL-C. Par la suite nous avons synthétisé HAFITC-CTL-C and HAFITC pour analyser leur interaction. HAFITC-CTL-C est rapidement détectable dans le cytoplasme sans provoquer la lyse cellulaire. De plus, les films contenant CTL-C ne sont pas toxiques pour les fibroblastes gingivaux humains.

En conclusion, CTL est le seul peptide antimicrobien dérivé de la CgA qui peut tuer *S. aureus* et résiste à la dégradation protéolytique, ce qui est de bon augure pour de nouvelles études visant à développer des biomatériaux antimcrobiens.

Mots clés: *Staphylococcus aureus*, leucotoxine, LukE/D, l'immunité innée, les peptides antimicrobiens, chromogranines, cateslytin, revêtement de polymère antimicrobien, biomatériaux antimcrobiens

Abstract

Chromogranins (Cgs) are a family of acidic proteins, expressed in secretory granules of neuro-endocrine and immune cells. Several Cgs derived peptides express antimicrobial activity. Current study was aimed to evaluate host-pathogen interaction and ultimately to develop antimicrobial polymer with insertion of cateslytine (CTL).

In first part, stimulatory ability of leukotoxin LukE/D to induce neutrophils secretions and role of bacterial proteases to degrade CgA-derived AMPs was evaluated. Activated neutrophils secrete various components which were identified. Later by using antimicrobial assays, several fractions were found active and later discussed with respect to proteomic analysis. Additionally, degradation of CgA derived AMPs by *S. aureus* proteases was demonstrated. Out of various AMPs tested, CTL was only that can kill *S. aureus* and resist protease degradation. Furthermore, CgA and CgB are processed by Glu-C protease to produce new fragments lacking antibacterial activity but presenting antifungal activity.

Secondly, we aimed to prepare CTL conjugated biomaterial coating. CTL-C was used to prepare PEM films with alternative deposition of CHI and HA-CTL-C and evaluated for antimicrobial activities. Later on, we synthesized HA^{FITC}-CTL-C and HA^{FITC} to analyze their interaction. HA^{FITC}-CTL-C was readily detectable in cytoplasm without provoking cell lysis. Moreover CTL-C inserted PEM films are non-toxic to human gingival fibroblast cells.

In conclusion, CTL is the only CgA-derived AMP that can kill *S. aureus* and resistant to proteolytic degradation, which is a promising feature for further studies in order to develop antimicrobial biomaterials.

Key words: *Staphylococcus aureus*, leukotoxin, LukE/D, innate immunity, antimicrobial peptides, chromogranins, cateslytin, antimicrobial polymer coating, antimicrobial biomaterial.

UNIVERSITÉ DE STRASBOURG

Rizwan ASLAM

Inserm
BioMaterials
BioEngineering
UMR-S 1121
Strasbourg

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