Développement de l'immunothérapie anti-tumorale médiée par vecteur bactérien vivant basé sur le système de sécrétion de type III de Pseudomonas aeruginosa

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Abbreviations

ACT	adoptive cell transfer
ADPRT	ADP-ribosyltransferase
APC	antigen presenting cell
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
bp	base pair
CCR	C-C chemokine receptor
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CPP	cell-penetrating peptide
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte–associated antigen 4
COX2	cytochrome c oxidase subunit II
CRPC	castrate resistant prostate cancer
DNA	deoxyribonucleic acid
DC	dendritic cell
DLN	draining lymph node
DMEM	Dulbecco's modified Eagle's medium
EBV	Epstein-Barr virus
EGF	epidermal growth factor
EGTA	ethylene glycol bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FOXP 3	forkhead box P3
GM-CSF	granulocyte–macrophage colony-stimulating factor
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
HPV	human papillomavirus
HSP	heat shock protein
HSV	herpes simplex virus
HTLV I	human T-cell lymphotropic virus type 1
ICAM	intracellular adhesion molecule
IDO	indoleamine dioxygenase
IFN-γ	interferon γ
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-*β*-D-thiogalactopyranoside
kb	kilo-base
kDa	kilo-Dalton
LAK	lymphokine-activated killer cell
LB	Luria Broth medium
LDH	lactate dehydrogenase
LFA	lymphocyte function-associated antigen
LLO	listeriolysin O
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
MOI	multiplicity of infection
NK	natural killer cell
NKT	natural killer T cell
OD	optical density
PAMP	pathogen-associated molecular pattern
PAP	prostatic acid phosphatase

PCR	polymerase chain reaction
PIA	*Pseudomonas* isolation agar
PBS	phosphate-buffered saline
PGE2	prostaglandin E2
RBS	ribosome binding site
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-PolyAcrylamide Gel Electrophoresis
SPI-1	*Salmonella* Pathogenicity Island-1
SPI-2	*Salmonella* Pathogenicity Island-2
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAP	transporter for Antigen Presentation
TCM	central memory T cell
TCR	T-cell receptor
TEM	effector memory T cell
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	transforming growth factor β
Th	T helper
TIL	tumor infiltrating lymphocyte
T3SS	type III secretion system
TLR	Toll-liked receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
iTreg	induced regulatory T cell
nTreg	natural regulatory T cell
Tris	Tris-(hydroxymethyl)aminomethane
TRP	tyrosinase related protein

TSA	tumor specific antigen
UV	ultraviolet
VEGF	vascular endothelial growth factor
YopE	*Yersinia* outer protein

INTRODUCTION

(Résumé en français)

Malgré les grands efforts réalisés pour développer des traitements efficaces, le cancer reste une des causes importantes de la mortalité dans le monde. En plus des stratégies de lutte classiques contre cette pathologie comme la chirurgie, la radiothérapie et la chimiothérapie, l'immunothérapie anti-tumorale est devenue une approche prometteuse. En effet, la spécificité du système immunitaire permettrait de cibler les cellules cancéreuses sans endommager les cellules normales. Dans ce domaine, la vaccination thérapeutique contre des antigènes associés aux tumeurs est devenue très attractive. La réponse immune stimulée par cette voie est plus spécifique et plus efficace pour empêcher la croissance de la tumeur ou pour éliminer les tumeurs résiduelles.

A L'immunologie du cancer et l'immunothérapie anti-tumorale

A-I *Cancer immunoediting* – de l'immunosurveillance à l'échappement tumoral

Le débat concernant le rôle du système immunitaire au cours de la progression tumorale a duré plus d'un siècle. C'est récemment qu'on a expliqué de façon appropriée comment le système immunitaire interagit avec les cellules tumorales d'une part et comment les tumeurs échappent à l'éradication immunitaire d'autre part.

L'hypothèse selon laquelle le système immunitaire réprime la croissance des carcinomes a été évoquée pour la première fois par Paul Ehrlish en 1909. Burnet et Thomas ont proposé la théorie de l'immunosurveillance du cancer en 1957. Selon ce concept, les lymphocytes pourraient, en permanence, reconnaître et éliminer les cellules cancéreuses (14). Néanmoins, cette théorie a été quelque peu battue en brèche par la suite. En effet, Shankaran a démontré en 2001 que le système immunitaire est capable de favoriser la formation des tumeurs primaires parce que les cellules tumorales ont une immunogénicité réduite. Ces cellules sont capables d'échapper à la reconnaissance et la destruction par le système immunitaire. Par conséquent, il a été reconnu que le système immunitaire possède une double fonction au cours de la progression tumorale. D'une part, le système immunitaire empêche la formation et le développement des tumeurs, c'est la fonction de «*immunosurveillance*» du cancer.

D'autre part, le système immunitaire pourrait modifier la nature intrinsèque de la tumeur. Donc, l'immunogénicité de cette dernière change et elle échappe aux actions du système immunitaire. Ceci correspond à la fonction appelée «*tumor sculpting*». En se basant sur ces découvertes, une nouvelle théorie décrivant de façon appropriée la fonction du système immunitaire lors du développement tumoral a été établie. Elle correspond à la théorie de «*immunoediting*» (18, 22, 23). Selon cette théorie, l'interaction entre le système immunitaire et les tumeurs est un processus dynamique comprenant trois phases : l'élimination, l'équilibre et l'évasion (Fig. 1) (18).

A-II le système immunitaire et le cancer

Il existe plusieurs voies par lesquelles le système immunitaire peut inhiber la croissance des tumeurs. Il est bien connu que la voie la plus efficace, *in vivo*, consiste à activer les cellules de la réponse immunitaire. En particulier, les cellules T CD8 + sécrétant l'interféron-γ (IFN-γ) et les cellules T-helper 1 (Th1) CD4+ (60).

Au cours de la formation d’un cancer, la transformation en cellules malignes et la perturbation du microenvironnement qui les entoure peut générer des signaux néfastes pour les cellules tumorales. Ces signaux induisent fréquemment une inflammation. D'une part, cette inflammation active des cellules innées effectrices, telles que les NKTs, NKs et les lymphocytes T γδ. Ces cellules peuvent avoir un impact direct sur la croissance tumorale. D’autre part, l’inflammation est à l’origine d’une stimulation des cellules présentatrices d'antigènes (CPAs), en particulier les cellules dendritiques (CDs). Ces dernières peuvent engloutir les antigènes dérivés des tumeurs et activer une réponse immunitaire adaptative médiée par les lymphocytes T et B. Cette réponse est spécifique des antigènes des tumeurs.

En absence de la présentation des antigènes, les lymphocytes T ne les reconnaissent pas (sauf pour les super-antigènes). Une présentation efficace des antigènes par les CPA est cruciale pour le développement d’une réponse spécifique médiée par les lymphocytes T. Il existe différents types des CPAs, telles que les lymphocytes B, les CDs et les macrophages. Les CDs sont les CPAs les plus

puissantes car les molécules du complexe majeur d'histocompatibilité (CMH) et des complexes CMH-peptide sont en concentration 10 à 100 fois plus élevées sur les CDs que sur les autres CPAs (62). Les lymphocytes T ne reconnaissent que des peptides antigéniques situés dans le sillon de liaison peptidique des molécules de MHC. Il existe deux classes majeures des molécules du CMH (CMH I et CMH II). Ces molécules assurent la présentation des antigènes via différentes voies afin d'activer les cellules T CD8 + et les cellules T CD4 + respectivement (63). Outre les voies classiques, les CDs peuvent également présenter les antigènes extracellulaires aux cellules T CD8+. Ces antigènes proviennent des particules phagocytées ou des complexes immunitaires renfermant les molécules du CMH I. Ce phénomène est appelé « *cross-presentation* » (64). Ce processus est nécessaire pour la défense immunitaire contre la plupart des tumeurs et les virus qui n'infectent pas les cellules présentatrices d'antigènes. Il est également nécessaire pour l'induction de l'immunité cytotoxique par la vaccination anti-tumorale (65-67).

Afin d'expliquer le mécanisme d'activation des lymphocytes T, un modèle appelé le modèle des deux signaux « *Two-signals* » a été établi (Fig. 2) (68). Le premier signal est médié par la réorganisation entre les récepteurs des cellules T (TCR) exprimés par les lymphocytes T et les CDs avec les peptides présentés dans le contexte des molécules du CMH. Le deuxième signal est généré par l'interaction entre des membres de la famille B7 (CD80/86) exprimés par les CDs et la molécule CD28 exprimée par les lymphocytes T; le CD40 exprimé par les CDs et le CD40L exprimé par les lymphocytes. Le second signal est aussi appelé co-stimulateur. Il est nécessaire pour la division et la différenciation des lymphocytes T en cellules effectrices. Les deux signaux sont indispensables pour une réponse immunitaire efficace. Récemment, l'existence d'un troisième signal a également été démontrée. La sécrétion ou non de facteurs par les CDs, en particulier l'IL-12, est impliquée dans la différenciation terminale des cellules T CD4 + en cellules Th1 ou Th2 respectivement (73).

Le développement et la différenciation de la réponse médiée par les cellules T

contre des agents pathogènes infectieux a été considérée comme un processus dynamique se composant généralement de quatre phases (89-90) : 1) la phase d'expansion : au cours de laquelle les cellules T naïves sont induites et se différencient en lymphocytes T effecteurs (CD4 + Th ou CD8 + CTL); 2) la phase de contraction : au cours de laquelle la majorité des cellules T effecteurs meurent, laissant 5% -10% des cellules de la phase d'expansion comme des cellules mémoires de long terme, 3) la phase mémoire : au cours de laquelle le nombre des cellules T mémoires se stabilise. Ces cellules sont maintenues pendant des longues périodes de temps, tout au long de la vie de la souris et pendant de nombreuses années chez l'homme; 4) Une réponse de rappel : rapide et médiée par des cellules T mémoires après une réexposition à l'agent pathogène.

Les cellules T activées de façon adaptative peuvent être divisées en deux catégories : les cellules T CD8 + cytotoxiques et les cellules T CD4 + helper. Les lymphocytes T CD8+ cytotoxiques (CTLs) sont impliqués dans l'immunité contre les virus et la surveillance de la transformation néoplasique. Il a été démontré que les CTL détruisent leurs cellules cibles par au moins deux différents mécanismes. L'un est médié par la voie perforine / granzyme (81) et l'autre est médié par la voie Fas (82). Ces deux mécanismes peuvent spécifiquement et directement induire l'apoptose de cellules cibles.

De nombreuses études ont montré que les CD4 + Th jouent un rôle central dans l'amplification et la régulation de la réponse immunitaire. D'une part, à travers leur capacité à aider les lymphocytes B à produire des anticorps, d'induire les macrophages à développer une forte activité microbicide, de recruter des neutrophiles, des éosinophiles et des basophiles au niveau du site d'infection et d'inflammation. D'autre part, les les CD4 + Th produisent des cytokines et des chemokines pour orchestrer toute la panoplie des réponses immunitaires. Selon leur profil de cytokines, les facteurs de transcription caractéristiques et les fonctions, les cellules Th peuvent être principalement classées en quatre types : Th1, Th2, Th17 et T régulatrices (Treg).

(83-87)

Les cellules B sont des lymphocytes jouant un rôle important dans la réponse immunitaire humorale. Les principales fonctions de ces cellules sont la production des anticorps dirigés contre les antigènes. Elles agissent également comme des cellules présentatrices d'antigène et éventuellement, elles peuvent se développer en cellules B mémoires après activation par l'interaction avec l'antigène. (93)

Outre la fonction de protection contre les tumeurs qui est assurée par le système immunitaire, les interactions entre les cellules tumorales et les cellules immunitaires dans le microenvironnement de la tumeur peuvent également créer un réseau immunosuppressif. Cette immunosuppression protège les tumeurs de l'attaque immunitaire et favorise leur croissance et leur progression. Plusieurs mécanismes ont été décrits, par lesquels les tumeurs peuvent supprimer l'action du système immunitaire. Parmi ces mécanismes : 1) le profil anormal de cytokines; 2) l'altération des molécules co-stimulantes et/ou co-inhibiteurs; 3) des anomalies des sous-ensembles des cellules présentatrices d'antigène; 4) la modification du ratio entre les Tregs et les lymphocytes T effecteurs, etc. (94-96)

A-III l'Immunothérapie anti-tumorale (97-103)

L'immunothérapie anti-tumorale est une classe de traitement contre le cancer. Elle a pour le but de moduler le système immunitaire de l'hôte, et/ou utiliser les composantes de ce système afin d'induire ou d'augmenter la réponse anti-tumorale. L'apparition de ce concept peut remonter à plus de 100 ans. Au cours du siècle dernier, des progrès impressionnants ont été réalisés dans le domaine de la biologie et de l'immunologie du cancer. La plupart des aspects de la réactivité immunitaire ont été clairement énoncés. Les résultats obtenus ont démontré que les attaques anti-tumorales déclenchées par le système immunitaire sont plus spécifiques et caractérisées par une moindre nocivité pour l'hôte. Par conséquent, l'immunothérapie est devenue un traitement de plus en plus prometteur contre le cancer. Pour atteindre cet objectif, différentes stratégies ont été testées. Elles peuvent généralement être

classées en deux catégories, chacune d'elles consiste en des approches différentes : l'immunothérapie anti-tumorale active et l'immunothérapie anti-tumorale passive (ou appelée adoptive).

L'immunothérapie anti-tumorale active comprend les approches qui consistent à stimuler la réponse immunitaire intrinsèque de l'hôte contre les tumeurs. Elle peut être subdivisée en immunothérapie active non-spécifique et l'immunothérapie active spécifique. Le rôle de l'immunothérapie anti-tumorale active non-spécifique est l'activation d'une réponse immunitaire générale par l'administration de cytokines ou de médiateurs qui pourraient induire la libération des cytokines. L'immunothérapie anti-tumorale active et spécifique consiste à activer les réponses immunitaires médiées par des cellules et/ou des anticorps ciblant les antigènes spécifiques exprimés par les cellules tumorales.

L'immunothérapie anti-tumorale passive correspond à des approches apportant une immunité active extrinsèque plutôt que l'activation de l'immunité intrinsèque. Dans ce cas, la réponse immunitaire peut être médiée par des cellules ou des anticorps.

B - Les vaccins anti-cancers

Le développement des vaccins anti-cancers visant à améliorer la réponse immunitaire anti-tumorale est un domaine prometteur de recherche. De nombreux résultats encourageants ont été obtenus à partir d'animaux et de nombreux essais cliniques de plusieurs vaccins sont en cours. Une vaccination anti-tumorale efficace doit être réalisée en se basant sur : l'identification des antigènes spécifiques ou associés aux tumeurs, le système de délivrance des antigènes doit être efficace et stimuler une réponse immunitaire appropriée et spécifique, et la définition d'une formulation optimale du vaccin et les stratégies d'immunisation (110).

B-I Les antigènes tumoraux

L'identification de l'existence des antigènes tumoraux est une découverte cruciale pour l'immunothérapie anti-tumorale basée sur l'antigène. Au cours des dernières décennies, de nombreux antigènes tumoraux ont été successivement identifiés. En général, les antigènes tumoraux peuvent être répartis en deux classes : les néo-antigènes (les antigènes spécifiques des tumeurs) et les antigènes de soi (les antigènes associés aux tumeurs) (112, 113). Les antigènes spécifiques des tumeurs sont exprimés exclusivement par les tumeurs mais pas par les tissus normaux. Les antigènes associés aux tumeurs sont exprimés non seulement par les cellules tumorales (avec des quantités ou des localisations anormales), mais aussi par les tissus normaux.

B-II Les modalités des vaccins anti-tumoraux

En général, les vaccins anti-tumoraux peuvent être classés en trois catégories : les vaccins basés sur les cellules, les vaccins basés sur les protéines et les vaccins basés sur des vecteurs.

B-II-a Les vaccins anti-tumoraux basés sur les cellules

Les vaccins anti-tumoraux basés sur les cellules comprennent généralement des vaccins basés sur des cellules tumorales entières et des vaccins basés sur les cellules dendritiques. Cette classe de vaccins a été largement explorée dans des essais précliniques et cliniques.

Les vaccins basés sur les cellules tumorales entières peuvent être divisés en deux sous-ensembles : 1) les vaccins autologues dans lesquels les cellules tumorales utilisées proviennent de la même personne à vacciner; 2) les vaccins allogéniques dans lesquels les cellules tumorales proviennent d'autres patients, généralement de lignées cellulaires tumorales préexistantes (150). Les cellules tumorales, toutes seules, sont généralement faiblement immunogènes, donc elles sont habituellement administrées avec des adjuvants ou des cytokines (150). En outre, les lysats des cellules tumorales peuvent être utilisés comme source d'antigènes et pas uniquement les cellules tumorales entières. La méthode usuelle de préparation de lysats des

cellules tumorales consiste à faire des cycles répétitifs de congélation-décongélation afin d'induire une nécrose des cellules tumorales (154). Récemment, des efforts importants ont été effectués pour produire des cellules tumorales autologues ou allogéniques génétiquement modifiées. Ces cellules peuvent sécréter des cytokines par exemple. En effet, l'administration systémique des cytokines est souvent associée à une toxicité profonde pour le patient, surtout lorsque les doses utilisées sont fortes. Parmi les cytokines, la transduction des cellules tumorales faiblement immunogènes pour qu'elles expriment le GM-CSF ou l'IL2 a été activement étudiée (163-172).

Les CDs sont considérées comme les cellules présentatrices d'antigène les plus puissantes. Elles sont également les stimulateurs les plus forts des cellules T naïves. Grâce à cette propriété, la vaccination basée sur les CDs représente une stratégie potentiellement puissante pour l'immunothérapie anti-tumorale. Cette stratégie est devenue le centre d'intérêt de nombreuses études scientifiques et cliniques. Jusqu'à présent, les CDs chargées avec des antigènes associés aux tumeurs et les CDs génétiquement modifiées et exprimant des antigènes tumoraux entiers ou des cytokines ont été largement testées dans des modèles animaux ainsi que dans des essais cliniques (182-186).

B-II-b Les vaccins anti-tumoraux basés sur les protéines

Les vaccins anti-tumoraux basés sur les protéines consistent généralement en un simple antigène tumoral ou des antigènes tumoraux combinés. Ce genre de vaccins a été étudié dans des modèles animaux et des essais cliniques ont été également réalisés. Ils sont généralement administrés avec des adjuvants, tels que le GM-CSF, l'IL 2, l'IFN-α, le CpG, etc., afin de générer une réponse immunitaire forte et durable (217-219). En plus de l'approche basée sur l'antigène tumoral, une famille de protéines de choc thermique (HSP) a également été utilisée dans l'immunothérapie anti-tumorale actuelle. D'une part, des peptides antigéniques de faible masse moléculaire ont été ajoutés à une préparation d'HSPs des cellules tumorales. Ceci indique que les interactions peptidiques de certaines HSP sont similaires à celles des

protéines du CMH (221-225). D'autre part, et indépendamment des propriétés peptidiques, les HSPs ont une propriété d'immunomodulation. Elles induisent une réponse immunitaire innée (229). Grâce à ces propriétés d'immunostimulation de l'immunité innée et adaptative les HSPs sont maintenant utilisées dans le développement de différents vaccins anti-tumoraux.

Comme les lymphocytes T reçoivent un message antigénique sous forme de peptide, l'utilisation de peptides comme agents thérapeutiques a été adoptée dans le traitement du cancer. Cependant, l'immunisation avec des séquences natives de peptides était souvent insuffisante pour générer une réponse médiée par des lymphocytes T au cours d'essais chez les animaux ou d'essais cliniques. Plusieurs stratégies ont été développées afin d'améliorer l'immunogénicité et de diriger le système immunitaire vers le type de réponses souhaitées. Parmi ces stratégies : la modification de la séquence de l'épitope, l'incorporation de cytokines, de chimiokines, de molécules costimulantes ou d'immunomodulateurs avec le vaccin peptidique, ou encore charger les peptides sur les CDs, etc. (240-253).

Récemment, la vaccination avec des anticorps anti-idiotype (Id) est apparue comme une approche intéressante pour l'immunothérapie anti-tumorale. Cette approche est basée sur la théorie du réseau idiotypique de Niels Jerne. Selon ce concept (254), un antigène induit la production d'anticorps (Ab1) dont la structure sérologique unique (idiotype) peut induire la production des Abs anti-Id (Ab2). Certaines de ces molécules Ab2 (Ab2β) sont spécifiques de la région variable d'Ab1 dédiée à la liaison avec l'antigène. Elles peuvent fonctionner comme une "image interne" de l'antigène cible en mimant sa structure tridimensionnelle. La vaccination avec ces anticorps Ab2β devrait stimuler la production d'anticorps anti-anti-Id (AB3 aussi appelé Ab1') qui reconnaissent l'antigène original. Cette nature cyclique de la complémentarité des sites de liaison et des idiotypes représente la base des vaccins idiotypiques.

B-II-c Les vaccins anti-tumoraux basés sur des vecteurs

Une expression de l'antigène et un système de délivrance appropriés sont importants pour l'efficacité d'un vaccin. Les différents systèmes de délivrance d'antigènes peuvent être classés en deux catégories. Les vecteurs non pathogéniques comprenant l'ADN plasmidique, les liposomes, les peptides cationiques pénétrants (*Cell Penetrating Peptides, CPPs*) et les vecteurs dérivés de microorganismes. La deuxième catégorie correspond aux vecteurs pathogéniques comprenant des vecteurs viraux et des vecteurs bactériens.

Les vaccins correspondant à l'ADN plasmidique sont basés sur des plasmides bactériens modifiés afin d'exprimer l'antigène cible. Ce dernier est sous le contrôle de promoteurs actifs dans les cellules de l'hôte. Ils contiennent également un terminateur transcriptionnel pour mettre fin à la transcription dans les cellules mammifères et un marqueur de sélection pour faciliter la production des plasmides dans des cellules bactériennes transformées pour la production du vaccin (261). Les moyens courants d'administration de l'ADN plasmidique comprennent : 1) des technologies de délivrance physique : l'injection intramusculaire par une aiguille; les ADN plasmidiques enrobées avec des particules d'or; l'électroporation après l'injection par l'aiguille, etc.; 2) l'application d'adjuvants.

Les liposomes sont des vésicules sphériques composées d'une ou plusieurs bicouches de phospholipide et un centre aqueux interne. Ils sont capables d'encapsuler les Ag et les agents immunomodulateurs. Les agents d'intérêt peuvent être véhiculés par des liposomes de différentes façons notamment : 1) une encapsulation dans le noyau, 2) ancrés dans la bicouche ou 3) adsorbés ou greffés à la surface extérieure (271). Les liposomes injectés localement peuvent être capturés avidement par les CPAs infiltrant le site d'injection ou ceci dans les vaisseaux lymphatiques (272). En outre, les liposomes permettraient de protéger leur contenu d'ADN de l'attaque de désoxyribonucléase (273). Ainsi, les liposomes sont considérés comme des vecteurs appropriés de vaccin pour délivrer des antigènes ou des ADNs

Diverse CPPs naturels et/ou synthétiques ont été aussi largement appliqués dans

les vaccins. Les CPPs sont des peptides cationiques et/ou amphipathiques de 9-35 acides aminés qui sont capables d'internaliser et traverser des membranes cellulaires (291). Cette capacité de translocation membranaire est possédée non seulement par les CPPs seuls, mais aussi par les CPPs attachés avec des cargaisons différentes, comme des peptides, des protéines, des oligonucléotides, des ADNs/ARNs, des nanoparticules, des liposomes, des bactériophages et des colorants fluorescents (291). Deux applications des CPPs déjà validées par des études de vaccins sont les vecteurs délivrant des antigènes associés aux tumeurs dans les CPAs et les véhicules non-viraux appliqués dans les vaccins ADN (291).

Les levures ont également été utilisées comme vecteur de délivrance d'antigène dans des vaccins anti-tumoraux. Ces organismes peuvent être avidement phagocytés par les CPAs et induire par la suite des réponses immunitaires chez les mammifères (300, 301). Il a été démontré que les cellules recombinantes de levure sont capables de stimuler le système immunitaire pour déclencher des réponses cellulaires très spécifiques et puissantes contre les antigènes cibles et ce avec une faible toxicité (302, 303).

Les virus sont des petits agents infectieux pouvant se répliquer uniquement à l'intérieur des cellules vivantes des organismes. Les virus peuvent infecter tous les types d'organismes, animaux, plantes jusqu'aux bactéries (309). Ils ont évolué pour avoir des structures et des mécanismes efficaces afin d'infecter les cellules et produire des protéines virales en utilisant la machinerie cellulaire (310). Ainsi, les vecteurs viraux représentent des véhicules attractifs pour délivrer des antigènes dans un but d'immunothérapie anti-tumorale. Ils peuvent mimer une infection naturelle et exprimer des signaux de danger. Ici, nous évoquons plusieurs vecteurs viraux largement employés dans l'immunothérapie contre le cancer, y compris : des virus recombinants de *vaccinia*, des vecteurs basés sur les adénovirus, des rétrovirus recombinants et des virus du type herpès simplex.

Par ailleurs, plusieurs caractéristiques intrinsèques des bactéries pathogènes,

vivantes et atténuées ont permis leur utilisation comme vecteurs de délivrance de l'ADN ou d'antigènes protéiques. Ces caractéristiques comprennent : 1) un tropisme positif pour les CPAs et 2) la bactérie elle-même est un adjuvant naturel pour le vaccin parce que l'infection bactérienne est connue pour induire la libération de plusieurs cytokines pro-inflammatoires qui pourraient améliorer les réponses immunitaires (318). Nous évoquons en détail plusieurs vecteurs bactériens bien caractérisés et largement appliqués dans des vaccins anti-tumoraux, tels que *Listeria monocytogenes*, le genre *Salmonella* et la délivrance d'antigènes médiée par le système de sécrétion de type 3 des bactéries à Gram négative.

B-III Les récentes stratégies pour l'amélioration des vaccins contre le cancer

Un défi majeur dans le développement des vaccins anti-tumoraux est la tolérance immunitaire vis-à-vis de la tumeur. En fait, beaucoup de TAAs sont des protéines du soi et donc souvent n'arrivent pas à induire des réponses antitumorales efficaces médiées par des lymphocytes T. En effet, une clef pour développer des vaccins anti-tumoraux efficaces consiste à surmonter les mécanismes potentiels de suppression immunitaire contre des tumeurs antigéniques mais faiblement immunogéniques (409). Pour atteindre cet objectif, plusieurs stratégies ont été appliquées afin d'améliorer les performances des vaccins anti-tumoraux. Parmi ces stratégies : le ciblage des voies co-inhibitrices, le ciblage des cellules T régulatrices, des stratégies utilisant différents protocoles de vaccination par prime-boost et celles combinant les vaccins anti-tumoraux et d'autres thérapies, etc.

C Le système de sécrétion de type III de Pseudomonas aeruginosa

C I La bactérie *P. aeruginosa*

P. aeruginosa est un bacille Gram négatif de l'environnement. Il est non fermentaire. C'est un pathogène opportuniste pour l'homme. Il infecte les personnes atteintes de la mucoviscidose, les immunodéprimés (en particulier les iatrogènes), les personnes avec une ventilation assistée invasive ou atteintes de bronchopathies chroniques, les personnes avec des effractions cutanées nosocomiales (chirurgie, …)

et les patients porteurs de diverses prothèses en contact avec des surfaces muqueuses telle que les lentilles de contact ou les sondes urinaires. En dehors de ces situations, *P. aeruginosa* est incapable de développer une infection à cause des barrières physiques (muqueuses, peau, clairance mucociliaire …etc.), de l'immunité innée (macrophages pulmonaires …etc.) et de l'immunité acquise.

C-II Le Système de sécrétion de type 3 (SSTT)

Le SSTT a été décrit initialement chez *Yersinia* et *Salmonella*. Par la suite, il a été retrouvé chez la plupart des bacilles à Gram négatif. Le SSTT semble être un facteur de virulence majeur jouant un rôle prépondérant au cours de la phase aiguë de l'infection par *P. aeruginosa*. Il peut induire la nécrose ou l'apoptose des différentes populations cellulaires et ainsi provoque l'altération de la fonction des phagocytes. Les dommages causés par le SSTT sont plus marqués dans l'infection pulmonaire.

L'appareil de sécrétion du SSTT de *P. aeruginosa* se compose d'une vingtaine de protéines remarquablement conservées. Ces protéines s'assemblent pour former une structure qui ressemble à une aiguille. Cette dernière peut traverser les deux membranes bactériennes et s'insère dans la membrane des cellules eucaryotes. Cette structure constitue un canal continu à travers lequel les toxines bactériennes sont directement délivrées dans le cytoplasme des cellules eucaryotes selon un mécanisme actif et spécifique. (446-448)

Quatre toxines sont sécrétées par le SSTT de *P. aeruginosa* : l'exotoxine (Exo) S, ExoT, ExoU et ExoY. En plus de ces toxines, PopB et PopD peuvent également être considérées comme des toxines car le pore qu'elles forment au niveau de la membrane des cellules eucaryotes peut induire en lui-même une certaine cytotoxicité selon le type de cellules. En générale, les exotoxines ont la capacité de modifier les fonctions des cellules de l'hôte pour le profit des bactéries. Parmi ces effets, il y a les transformations du cytosquelette et par conséquent la modification de la phagocytose et le transport des organelles, les altérations de la signalisation intracellulaire induisant ainsi l'apoptose des cellules, la diminution de la production des médiateurs,

l'induction de la nécrose, etc.

Le SSTT de *P. aeruginosa* est régulé au niveau transcriptionnel et post-transcriptionnel de façon sophistiquée. Tous les gènes codant pour les protéines du SSTT sont régulés par ExsA, un activateur transcriptionnel de type AraC. Il se lie à une séquence consensus au niveau du promoteur des gènes (449). Outre la régulation dépendante d'ExsA qui est un mécanisme majeur, plusieurs facteurs tels que : les facteurs métaboliques, le système du quorum sensing (QS), l'AMP cyclique, Vfr, PtrA, etc. peuvent également avoir un impact sur l'activation du SSTT (450).

C-III Travaux précédemment menés au laboratoire

Pendant plusieurs années, nous avons étudié le SSTT de *P. aeruginosa.* En utilisant ce système, nous avons essayé de construire des souches bactériennes qui délivrent des antigènes. La translocation intracytoplasmique de ces antigènes a été réalisée par la fusion des antigènes d'intérêt avec la toxine ExoS du SSTT. Le développement et l'utilisation des vecteurs bactériens sont encore en cours. Les travaux déjà réalisés concernent la détermination de la séquence minimale d'ExoS qui est nécessaire pour la sécrétion d'une protéine de fusion ExoS-antigène (3), le développement de différentes souches atténuées (3, 496), le développement d'une construction plasmidique dans laquelle ExsA a été inséré sous le contrôle d'un promoteur inductible et l'application des vecteurs bactériens atténués dans des modèles d'immunothérapie anti-tumorales chez la souris (3, 6, 10).

D Objectifs du travail

Dans le but de générer et maintenir une réponse immunitaire acquise efficace, la stimulation de l'immunité innée et la délivrance adéquate de l'antigène sont deux facteurs cruciaux. Au cours des dernières décennies, l'utilisation de la machinerie de sécrétion de type III des bactéries à Gram-negatif telle que *Salmonella, Shigella, Yersinia* et *Pseudomonas* a séduit énormément grâce à leurs potentiels pour développer des vaccins anti-tumoraux. En fait, ces bactéries utilisent cette machinerie

pour délivrer les effecteurs bactériens dans les cellules de l'hôte.

Grâce à une manipulation génétique facile, différentes souches atténuées de *P. aeruginosa* ont été préparées dans notre laboratoire. Leurs potentiels à délivrer des vaccins anti-tumorales a été vérifié.

L'objectif de cette étude est de continuer à développer et améliorer la performance de nos souches atténuées utilisées comme vecteur vaccinal pour des futures applications cliniques.

INTRODUCTION

(In English)

In spite of great efforts to develop better treatments, cancer remains an important cause of mortality in the world. Each year, there are millions of people worldwide died from cancer. According to the statistical report published by World Health Organization in 2010, cancer is actually the leading cause of death in economically developed countries and the second leading cause of death in developing countries (1).

The main cancer treatments include surgery, chemotherapy, radiotherapy, immunotherapy and other methods. However, surgery needs to be realized in the early stage before the tumor spreads to distant area and required the removal of a wide surgical margin; chemotherapy and radiotherapy can not affect tumor cells without adverse affects on normal cells, which results in unpleasant side effects. Since several decades, the increase in knowledge of the immune system and its regulation have led to a great interest in immunologic approaches to target and eliminate cancer, because the exquisite specificity of the immune system could be marshaled to precisely target cancer cells without harming normal cells.

Cancer immunotherapy can be either active immunotherapy in which case the patient receive a cancer vaccine and his own immune system is trained to recognize tumor cells as targets to be destroyed, or passive immunotherapy in which case the patient receive either therapeutic antibodies or *ex vivo* manipulated lymphocytes as drugs and his immune system is recruited to destroy tumor cells by the transferred effectors. In active immunotherapy, therapeutic vaccination against defined tumor-associated antigens (TAAs) for preventing tumor growth or eliminating residual tumors is becoming an intense area for medical research, because the immune response stimulated by this way is more specific and more efficient. In order to generate and maintain efficient acquired cellular immune response, stimulation of the innate immune system and adequate antigen delivery are both crucial. In the last decades, the use of Gram-negative bacteria, such as *Salmonella, Shigella, Yersinia* and *Pseudomonas* which use their powerful secretion machinery - type III secretion

system (T3SS) to deliver bacterial effectors into the host cell cytoplasm, has attracted more and more attention for their potential use for cancer vaccine development (2-5). Until now, T3SS based bacteria has been proved as a carrier for cancer vaccines which provide the protection against several tumor models like glioma, prostate cancer, breast cancer and fibrosarcoma in mice (6-9).

Due to the ease of genetic manipulation, several attenuated *Pseudomonas aeruginosa* strains have been established in our laboratory and their potential use as the carriers for anti-tumor vaccination purpose has also been reported (3, 10, 11). Due to previous achievements, I have the honor, during my thesis, to continue developing and improving the performance of our attenuated *P. aeruginosa* strains as vaccine vectors for future clinical amplifications purpose.

A - Cancer Immunology and Cancer Immunotherapy

It has been well documented that cancer cell formation and growth is initiated by multiple factors. Genetic mutations and physiological changes within normal cells as well as within body's normal defense mechanisms play a crucial role in tumor formation and development (12, 13). Mutations in normal cells, such as gain of function mutation in oncogenes and loss of function mutation in tumor suppressor genes, can induce relative cellular immortality, proliferation, and carcinogenesis. During malignant transformation, cells acquire numerous molecular and biochemical changes which could be recognized by immune system and provoke interactions between the immune system and cancer cells. This last situation is emphasized when tumor are induced upon viral infection.

A-I Cancer Immunoediting – from Immunosurveillance to Tumor Escaping

Understanding how the immune system affects cancer development and progression has long been the biggest challenge question in cancer immunology. The exact ways in which the immune system interacts with tumor cells and how tumors are able to escape immunological eradication have only recently started to be appropriately explained.

A-I-a Cancer Immunosurveillance and Cancer Immunoediting

The debate about the role of the immune system during tumor progression has undergone for over a century. The hypothesis that the immune system represses the growth of carcinomas was first speculated by Paul Ehrlish in 1909. However, during the following 50 years, little progress about this idea has been achieved because the composition and function of immune system was mainly unknown. With an enhanced understanding about transplantation and the demonstration of tumor antigens existence, Burnet and Thomas proposed cancer immunosurveillance theory in 1957. According to this concept, lymphocytes could continuously recognize and eliminate arising, nascent transformed cells (14). Nevertheless, this theory has suffered the challenge after that. Studies by Stutman and by Rygaard & Povlsen demonstrated that

the susceptibility of immune-competent mice to both spontaneous and carcinogen-induced tumors was similar to that of nude mice which had major immunodeficiency (15-17). After these experiments, the cancer immunosurveillance theory was greatly abandoned. Until the 1990s, interest in cancer immunosurveillance was re-incited by two important findings: 1) interferon γ (IFN-γ) can promote immunologically induced rejection of transplanted tumor cells and prevent the

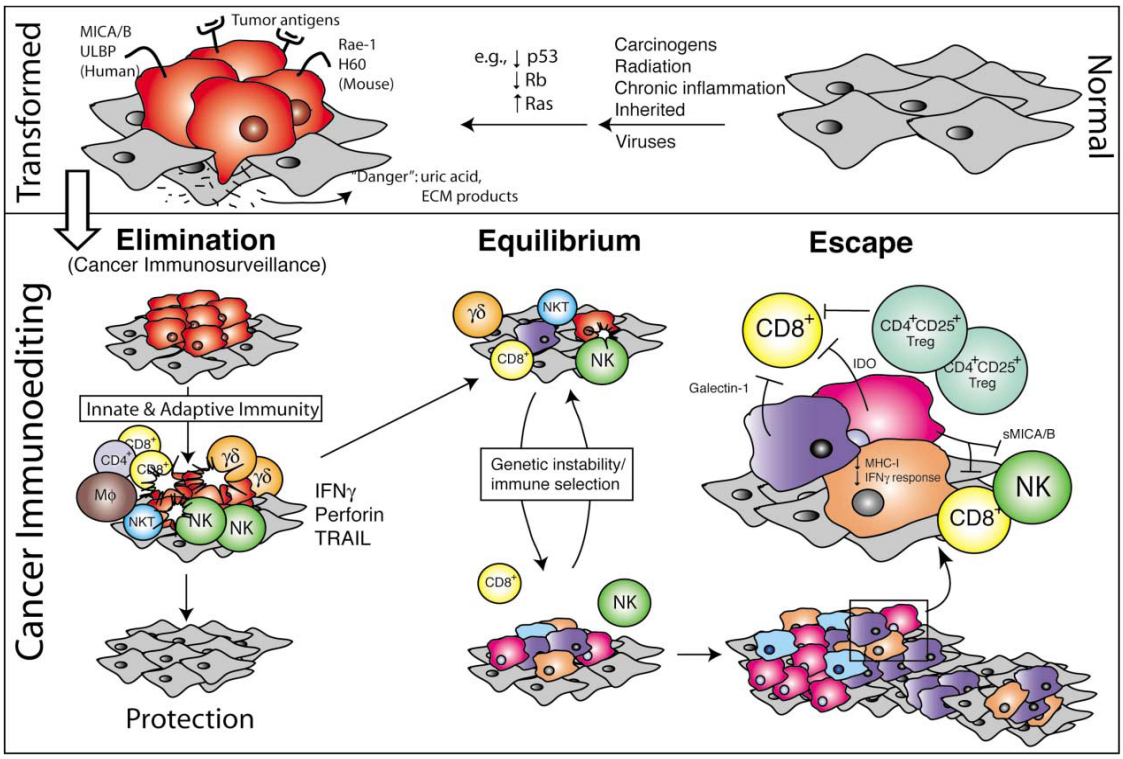

Figure 1: The Three Phases of the Cancer Immunoediting Process.

Normal cells (gray) subject to common oncogenic stimuli ultimately undergo transformation and become tumor cells (red) (top). Even at early stages of tumorigenesis, these cells may express distinct tumor-specific markers and generate pro-inflammatory "danger" signals that initiate the cancer immunoediting process (bottom). In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immunosurveillance network, may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculpted by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase. Reprinted from Dunn GP *et al*. 2004 (18).

formation of chemical induced and spontaneous tumors (19); 2) mice lacking either IFN-γ responsive ability or adaptive immunity were more susceptible to carcinogen induced and spontaneous tumor formation (20, 21). In the same period, other experiments demonstrated the similar results and thus the extrinsic tumor suppressor function of immune system was proved to indeed exist. However, one growing evidence that tumor protection represents only one dimension of the complex relationship between the immune system and cancer has emerged (20, 22). The work of Shankaran in 2001 revealed that the immune system can also promote the formation of primary tumors because the tumor cells have a reduced immunogenicity and are capable to escape immune recognition and destruction. Thereby, it is now accepted that immune system has dual function during tumor progression. On the one hand, immune system prevents tumor formation and development, the function of cancer immunosurveillance. On the other hand, the immune system can modify the intrinsic nature of tumor which can change the tumor immunogenicity and lead to tumor escape, the function of tumor sculpting. Basing on these findings, a new theory which can describe more broadly and appropriately the function of immune system throughout tumor development was established – immunoediting (18, 22, 23). According to immunoediting theory, the interaction between immune system and tumors is a dynamic process comprising three phases: elimination, equilibrium and escape (Fig. 1, (18)).

A-I-b Cancer Immunoediting – Elimination

The elimination phase represents the original concept of cancer immunosurveillance. During this phase, both innate and adaptive immune system can detect the presence of tumor cells and destroy them so as to prevent tumor development. The mechanisms of immunosurveillance initiation have not been fully understood. It can be generally considered that accumulated transformed cells can express the danger signals to recruit the cells of innate immune system, such as NKT (natural killer T) cells, NK (natural killer) cells and γδ T cells, and stimulate them to secret IFN-γ (24-26). The early produced IFN-γ can start a cascade of innate immune

reactions which result in some tumor death by several mechanisms, such as anti-proliferative mechanism (27), apoptotic mechanism (28), chemokines induced angiostatic mechanism (29-31). The chemokines produced in this stage can help to recruit more NK and macrophages to kill more tumor cells through the mechanisms involving tumor necrosis factor-related apoptosis-inducing ligand, perforin and reactive oxygen and nitrogen intermediates (32-36). Then, dead tumor cells or tumor cell debris can be captured by dendritic cells (DCs) which will migrate to draining lymph node to activate tumor antigen specific CD4+ and CD8+ T cells (37-40). After that, tumor specific CD4+ and CD8+ T cells can migrate to tumor site to eliminate remaining antigen expressing tumor cells (20). If tumor cells are not successfully deleted by the elimination process, they may enter the subsequent phase – the equilibrium phase.

A-I-c Cancer Immunoediting – Equilibrium

Although cancer elimination process can clear away a significant quantity of tumor cells, there are still some malignant cells variants that can stand up with the pressure exerted by immunosurveillance and enter with immune system into the equilibrium stage. During this phase, the host immune system and tumor cells maintain a dynamic balance. The analysis in immunodeficient mice model revealed that adaptive immunity, but not innate immunity, was responsible for maintaining the equilibrium (41). The adaptive immunity could on the one hand restrict tumor outgrowth and maintain residual tumor cells in a functional dormant state (42, 43), on the other hand shape the intrinsic nature of tumor cells. By reason of genetic instabilities in tumor cells, such as nucleotide-excision repair instability, microsatellite instability and chromosomal instability (44) , this sculpting effect of immune system to tumor cells can results in a large quantity of mutations. These mutations may endue tumor cells with the new phenotypes with reduced immunogenicity, which enable tumor cells evade the immune system and enter into the escape phase.

A-I-d Cancer Immunoediting – Escape

During this phase, surviving tumor cells have acquired enough ability to escape the recognition and the destruction by immune system, which might result in either recurrent primary tumor or distant metastases. Recent studies have documented the potential mechanisms of tumor escaping. First of all, the inherent genetic instability in tumor cells and selected pressure exerted by immune system can yield numbers of mutation in tumor cells. These mutations can lead tumor cells to be poorly immunogenic and invisible for immune system through several ways: 1) inducing tumor cells lacking rejection antigen expression (45, 46); 2) inducing tumor cells lacking responsible molecules for antigen presentation, such as major histocompatibility complex (MHC) molecules, β2m (45, 47) and 3) inducing tumor cells lacking intra-cellular antigen processing function (48). Moreover, even the rejection antigens are correctly expressed and tumor cells can still be detected by immune system, they can also develop the means to evade the immune destruction. These last mechanism can consist of: 1) the up-regulation of anti-apoptotic molecules, such as FLIP (49) and BCL-XL (50); 2) the expression of mutated inactive death receptors, such as TRAIL receptor, DR5 and Fas (51, 52); 3) the secretion of cytokines which can suppress the function of immune effector cells, such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), indoleamine dioxygenase (IDO), IL10, etc. (53-56); 4) the secretion of cytokines which can recruit the regulatory cells, such as regulatory T cells (Treg cells) and myeloid derived suppressor cells (MDSCs) (57-59).

A-II The Immune System and Cancer

Advances in cellular and molecular immunology lead to the brilliant insights into the nature of the interaction between immune system and tumors. The immune system can restrain tumor growth in several ways. However, it is well accepted that the most effective way to eradicate a tumor mass *in vivo*, as well as for the other infectious diseases, is to activate the cell mediated immune response, specially the combined action of CD8+ and IFN-γ-secreting T-helper 1 (Th1) CD4+ T cells (60). Reflecting

current consensus and our own research emphasis, the discussion will focus on the cellular branch of anti-tumor immunity.

During the course of cancer formation, the malignant cell transformation and the disruption of surrounding microenvironment can bring out the expression of danger signals on tumor cells. These danger signals frequently induce inflammations which can on one hand activate innate effector cells, such as NKT, NK and γδ T cells, to directly impact tumor growth; on the other hand, stimulate professional antigen-presenting cells (APCs), particularly DCs, to engulf tumor-derived antigens and to activate antigen-specific, T and B lymphocytes mediated adaptive immune response.

A-II-a Dendritic Cells

Dendritic cells (DCs) are the most potent APCs that were discovered in 1973 by Ralph Steinman. Numerous documented results prove that DCs can present antigens to both CD4+ and CD8+ naive T lymphocytes and prime antigen-specific immune responses. On the other hand, DCs also participate in the regulation of T cell-mediated immune responses including T type 1 helper (Th1) cell- and Th2 cell-responses. Thus, DCs are clearly central to the maturation, maintenance the regulation of a cellular immune response to cancer.

Both in mice and humans, DCs consist of distinct subsets (61). In mice, early used classification which distinguished DCs as myeloid or lymphoid subset is now thought to be inappropriate, whereas these terms are still frequently used in actual scientific experiments. Multiple DC subsets have now been defined in mouse lymphoid organs on the basis of cell surface marker expression. The expression of CD11c and MHC class II (MHC II) in combination with CD4, CD8α, CD11b, and CD205 has been used to separate five subsets which are diversely represented in spleen, thymus and lymph node. These subsets are myeloid or lymphoid origin and differ in the secretion level of certain cytokines and the capacity of migrating to secondary lymphoid organ to interact with T lymphocytes. In humans, DCs can be

classified in three subsets: Langerhans DCs, interstitial DCs and plasmacytoid DCs. Similar to mouse DCs, human DCs are defined in terms of the expression of a range of cell surface markers, such as CD4 and MHC II combined with CD1a, CD11c, CD14 and BDCA, but these might reflect differences in the maturation status rather than separate sub-lineage. However, for both species, even certain phenotypic differences have been observed among these different DC subsets, cells' origins, maturation stages, and functional differences have not been clearly established.

DCs residing in peripheral tissues are immature and act as sentinels. Immature DCs are characterized by: 1) an enhanced sensibility to chemo-attractors by their chemokine receptors (CCR1, CCR2, CCR5 and CXCR1); 2) a high Ag capture capacity through endocytosis and phagocytosis; 3) the low expression of MHC II molecules and co-stimulatory molecules on their surface. These characters permit the recruitment DC precursors, in response to the production of chemokines upon local inflammation induced by microbial infections and tissue damages. Then, immature DCs can efficiently capture Ag through several pathways: 1) macro-pinocytosis; 2) receptor-mediated endocytosis via C-type lectin receptors (mannose receptor, DEC-205) or Fcc receptor types I (CD64) and II (CD32) for the uptake of immune complexes or opsonized particles; and 3) phagocytosis of particles such as latex beads, apoptotic and necrotic cell fragments, viruses, bacteria and intracellular parasites.

During the recruitment, antigen capturing and immigration processes, immature DCs gain the maturation signals which are composed of Toll-liked receptors (TLRs) and various agents, such as the inflammatory compounds (TNF-α and IL-1), Pathogen-derived compounds (dsRNA, LPS, etc.) and cell-derived compounds (HSP, CRT, uric acid, etc.). The DCs maturation process is associated with several characters: 1) loss of endocytic and phagocytic receptors; 2) high-level expression of MHC II molecules at the cell surface and increased production of co-stimulatory molecules including CD40, CD80, and CD86; 3) changes in morphology; 4) re-programming of chemokine receptor expression and responsiveness and 5)

activation of the Ag-processing machinery (61). The maturation process can consequently trigger the migration of DCs from inflamed tissues to T cell-enriched draining lymph nodes (DLNs) in response to certain chemokines such as CCR7. Then, DCs select rare Ag-specific naive T cells and induce their activation and differentiation into effector cells, which thereby initiates primary immune responses.

A-II-b DCs Mediated Antigen Presentation

Unlike B cells, T cells fail to recognize antigens (except for super-antigens) in the absence of antigen presentation. The effective presentation of antigens by APCs is crucial for the development of specific T cell response. Although APCs contain different types of cells, such as B cells, DCs and macrophages, DCs are the most potent APCs because the MHC molecules (the indispensable components for antigen presentation) and MHC-peptide complexes are 10-100 times higher on DCs than on the other APCs (62). The T cell is restricted to recognizing antigenic peptides in the peptide-binding groove of MHCs molecules. There are two major classes of MHC molecules (MHC I and MHC II) that execute the antigen presentation function through the different pathways for activating CD8+ and CD4+ T cells, respectively.

In general, through MHC I pathway, the antigens from self and intracellular pathogens (such as viral infection, the presence of intracellular bacteria or cellular transformation) can be reported to CD8+ cytotoxic T cells (CTL). First of all, antigenic particles will undergo the ubiquitinylation and then the digestion by a proteasome complex so as to be transformed into small peptides of 8 to 10 amino acids. Then, peptides will be moved by Transporter for Antigen Presentation (TAP) molecules to the endoplasmic reticulum (ER). MHC I molecules can be bridged to TAP by the transmembrane protein tapasin. By this mean, the antigenic peptides could be coupled with MHC Class I molecules and be transferred to the cell surface to encounter with CD8+ cytotoxic T cells. (63)

Through MHC II pathway, the antigens from the extracellular milieu can be presented to CD4+ T cells. In contrast to the widespread expression of MHC I

molecules, MHC II molecules are restrictedly expressed by professional APCs, particularly by DCs. Peripherally resided DCs are immature and express low level of MHC II molecules. However, immature DCs have the efficient antigen capturing ability. Exogenous pathogens such as bacteria, parasites, and toxins in the tissues can be phagocytosed by immature DCs and targeted to MHC II compartments (endosomes) where the pathogen-associated proteins can be digested into smaller peptides by lysosome-associated enzymes. Meanwhile, immature DCs can migrate to T cell-enriched lymph nodes in response to certain chemokines. During migration and antigen capturing, immature DCs undergo the maturation process during which stage they obtain several new characters including enhanced expression of MHC II molecules in MHC II compartments. In result, peptide can be loaded to MHC II molecules and then transported to the cell surface where they engage antigen-specific CD4+ T cells. (63)

Besides the standard pathways, dendritic cells can also process and present extracellular antigens originated from phagocytosed particulates or immune complexes with MHC I molecules to CD8+ T cells. This phenomenon is termed *cross-presentation* (64). This process is necessary for immunity against most tumors and against viruses that do not infect antigen-presenting cells (65, 66). It is also required for induction of cytotoxic immunity by anti-tumor vaccination (67).

A-II-c Interaction between DCs and T Cells

DCs play a central role in T lymphocyte activation and differentiation into Th1, Th2 and CTL effectors. After acquiring antigen in the periphery, the DCs migrate to the DLNs to encounter and activate both CD4+ and CD8+ T cells. To explain the mechanism of T cell activation, “Two-signal” model has been well accepted (Fig. 2) (68). Signal 1 is mediated by the reorganization between T-cell receptor (TCR) expressed by T cells and DCs with peptide presented in the context of MHC molecules. Signal 2 is generated by the interaction between members of the B7 family (CD80/86) on DCs and CD28 on T cells; CD40 on DCs and CD40L on lymphocytes.

The second signal is also called co-stimulatory which is required to ensure that T cells will divide and differentiate into effector cells. Both signals are indispensable for production of an effective immune response. Recently, there are also the other additional co-stimulatory signals, such as signals mediated through 4-1BBL–4-1BB (69, 70), OX40L–OX40 (71, 72) interactions, are documented to be required for promoting the survival and proliferative capacity of activated T cells and the generation of memory T cells. Recently, the existence of "Signal 3" has also been documented. Secretion or non-secretion of factors by DCs, particularly IL-12, are proved to be instrumental in the final differentiation of CD4+ T cells into Th1 or Th2 cells, respectively (73).

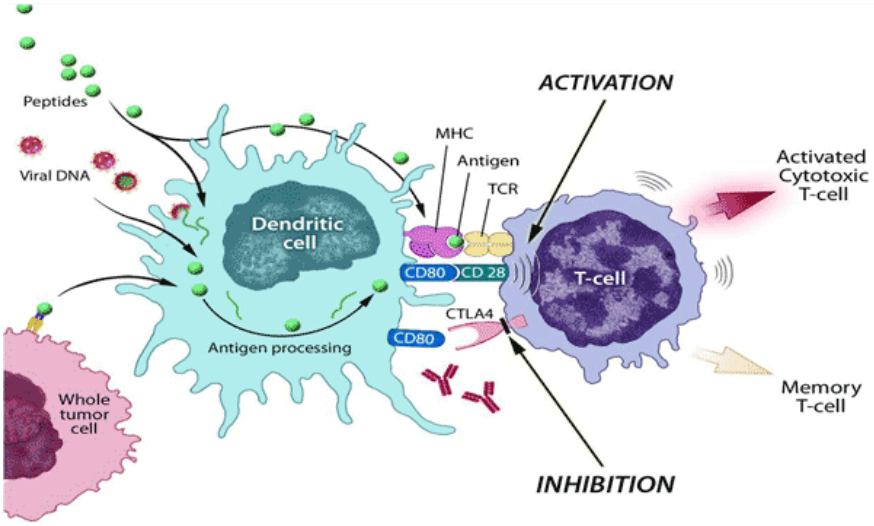

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Figure 2: Two Signals Model for T Cells Activation.

Two signals are indispensable for T cell activation mediated by dendritic cells. Signal 1: reorganization of TCR expressed on T cells by DCs with the appropriate peptide in the context of MHC molecules. Signal 2: co-stimulatory mediated by the reorganization between the B7 proteins (CD80/CD86) expressed by DCs and their lymphoid ligand CD28.

Furthermore, it has also been revealed that co-stimulation pathway is quite complex, it involves not only activating signals but also inhibitory signals issued from the interaction between CD28 and cytotoxic T lymphocyte–associated antigen 4

(CTLA-4) molecules (74). CTLA-4 is a co-stimulatory receptor which is only expressed by activated T cells. CTLA-4 is structurally similar to CD28 and can bind to B7 molecules with an affinity 500 – 2000 folds higher than CD28 (75). However, in contrast to CD28, CTLA-4 down-regulates T-cell responses and promotes the tolerance.

On the other hand, while the DC-T cell interaction has been traditionally viewed as a one way interaction, recent data suggested that T cells may play an important role in activating DCs thus further enhancing the T cell stimulatory capacity of the DCs (76, 77).

A-II-d CD8+ Cytotoxic T Cells Mediated Response (78-80)

The CTLs mediate immunity against viruses and surveillance against neoplastic transformation. CTLs can circulate in organs with various specificities. When CTLs encounter their target cells, which means the TCR molecules expressed by CTLs recognize their appropriate peptides expressed by target cells in MHC context, CTLs can eliminate the target cells through different pathway.

It has been proved that CTLs destroy their target cells through at least two different mechanisms. Both mechanisms can specifically and directionally induce the apoptosis of target cells, whereas these processes are totally different. One is perforin/granzyme-mediated pathway and the other is Fas-mediated pathway.

Perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to eliminate virus-infected or transformed cells. When CTLs encounter infected/dysfunctional somatic cells, they can release the cytotoxins (perforin, granzymes) through exocytosis to the contact region between the killer and target cells. Perforin can form via polymerization the aqueous channels in the membrane of target cell which can further allow the entry of perforin itself and granzymes. Granzymes are serine proteases that cleave at aspartate residues in the substrates and can be activated by perforin action in intracellular space. Granzyme B is one serine

protease and it can activate numbers of caspases which eventually lead to apoptosis (programmed cell death). Granzyme A is the other well-expressed granzyme in CTLs and it does not directly process pro-caspases like granzyme B. However, the effection of granzyme A has not been clearly defined, some research revealed that granzyme A can possibly trigger a novel form of apoptosis associated with DNA single-strand breaks (81).

The second pathway to induce apoptosis is via cell-surface interactions between the CTLs and the target cells. Activated CTLs express the surface protein FAS ligand (FasL) which can bind to Fas molecules expressed on target cells. Fas molecules receptors belong to the TNF receptor family. Engagement of Fas with FasL allows for recruitment of the death-induced signaling complex (DISC). Then, the Fas-associated death domain (FADD) translocates with the DISC which can activate numbers of caspases and result in classical caspase-dependent apoptosis. The main function of the FAS–FASL pathway is to eliminate self-reactive lymphoid cells (82).

.A-II-e CD4+ T Cells Mediated Response (83-87)

Extensive studies have demonstrated that Th cells play a central role in the amplification and regulation of immune response. They do so through their capacity to help B cells make antibodies, to induce macrophages to develop enhanced microbicidal activity, to recruit neutrophils, eosinophils, and basophiles to sites of infection and inflammation, and, through their production of cytokines and chemokines, to orchestrate the full panoply of immune responses. Actually, stimulation of a CD4+ Th response combining with long lasting CD8+ CTL response is appearing more and more important for cancer immunotherapy. According to their functions, their unique products, their characteristic transcription factors, and cytokines critical for their fate determination, Th cells can be principally classified in four types: Th1, Th2, Th17 and regulatory Treg cells (Fig. 3).

Th 1 cells are mainly generated with the help of IL-12 and are important for the eradication of intracellular pathogens, including bacteria, parasites, yeast, and viruses.

T-bet and Stat4 are the important transcription factors for Th1 response amplification. More and more proofs demonstrated that modulating the Th1 cell response against a tumor antigen may lead to effective immune-based therapies. Th1 cells can mainly secret IFN-γ, IL-2 and TNF-β. IFN-γ can: 1) sensitize tumor cells to CD8+ T cells by upregulating MHC I and other components of the antigen-processing machinery; 2) promoting the recruitment of NK cells; 3) activate macrophages, enhancing their microbicidal action and 4) providing help to B cells to stimulate the production of immunoglobulin (Ig-) G antibodies (IgG1 in human and IgG2 in mice) that are involved in opsonization and phagocytosis. TNF-*β* can stimulate innate and T-cell immune responses. IFN-γ and IL-2 can also promote the differentiation of CD8+ lymphocytes into cytotoxic cells.

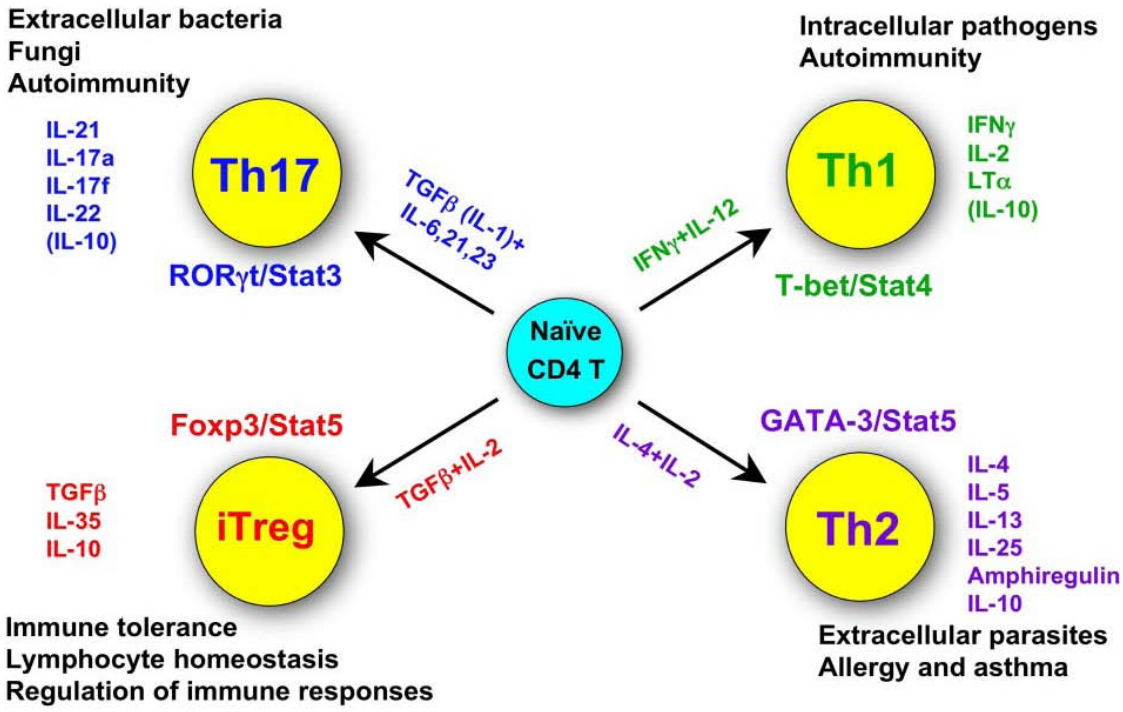

Figure 3: Classification of CD4+ Th Cells.

Th cells can be classified in four types according to their functions, their unique products, their characteristic transcription factors, and cytokines critical for their fate determination. Reprinted from Zhu JF *et al.* 2008 (85).

Th2 differentiation is promoted by IL-4 and GATA-3 and signal transducer and activator of transcription (STAT) 5 are two important transcription factors for Th2 differentiation. The Th2 response is often associated with a humoral response and mediate host defense against extracellular parasites, such as helminthes. Besides, Th2 cells are also important in the induction and persistence of asthma and other allergic diseases. Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin. IL-4 is the inhibitor cytokine for Th2 cell differentiation and is the major mediator of IgE class switching in B cells. IL-5 plays a critical role in recruiting eosinophils. IL-9 can affect mast cells and lymphocytes and also induce mucin production in epithelial cells during allergic reactions. IL-10 can suppresses Th1 cell proliferation and also suppress dendritic cell function. IL-13 is the effector cytokine in the expulsion of helminthes and in the induction of airway hypersensitivity. Amphiregulin is a member of the epidermal growth factor (EGF) family. It induces epithelial cell proliferation.

Th17 cells were recently identified as a distinct Th subset because they exhibit a cytokine profile distinct from Th1 and Th2 cells, which was characterized by the expression of IL-17A, IL-17F, IL21 and IL-22. The biological activities of IL-17A and F are very similar. Both molecules induce multiple pro-inflammatory mediators, including IL-6, IL-8, keratinocyte chemo-attractant (KC), granulocyte colony stimulating factor (G-CSF), etc. Thus, IL-17A and F play an important role in inducing inflammatory responses. IL-21 made by Th17 cells is a stimulatory factor for Th17 differentiation and serves as the positive feedback amplifier. The development of Th17 subset is depended on IL-6, IL-21, IL-23 and TGF-β. RORγt and Stat3 are important in T17 differentiation. Th17 cells can mediate immune responses against extracellular bacteria and fungi, but also participate in the amplification of autoimmune diseases.

Treg cells are a highly specialized subset of immune cells capable of suppressing auto-reactive cells and thereby maintaining tolerance to self-antigens and preventing autoimmunity. On the other hand, Tregs also play an important role in the suppression

of immune response to tumors and infection agents and are thought to be the main obstacle of successful immunotherapy. Forkhead box P3 (FOXP3) and Stat5 are two important transcription factors required for Treg development. Some categories of Tregs have been characterized and two subsets are best known: natural Tregs (nTregs) and induced Tregs (iTregs). nTregs develop and mature in the thymus and are characterized as $CD4^{+}CD25^{+}FOXP3^{+}$ T cells. They are essential for the induction and maintenance of self-tolerance and control of autoimmunity. Various mechanisms responding to the suppressive action of nTregs have been identified. nTreg can act through (Fig. 4): 1) the secretion of inhibitory cytokines, such as IL-10, TGF-β; 2) the down-regulation of DCs function; 3) killing of APCs or responder T cells by means of granzyme and perforin; 4) the constitutive expression of high amounts of CTLA4 molecules which can bind to B7 molecules expressed by DCs and activate indoleamine 2,3-IDO pathway. In contrast to nTregs, iTregs do not develop in the

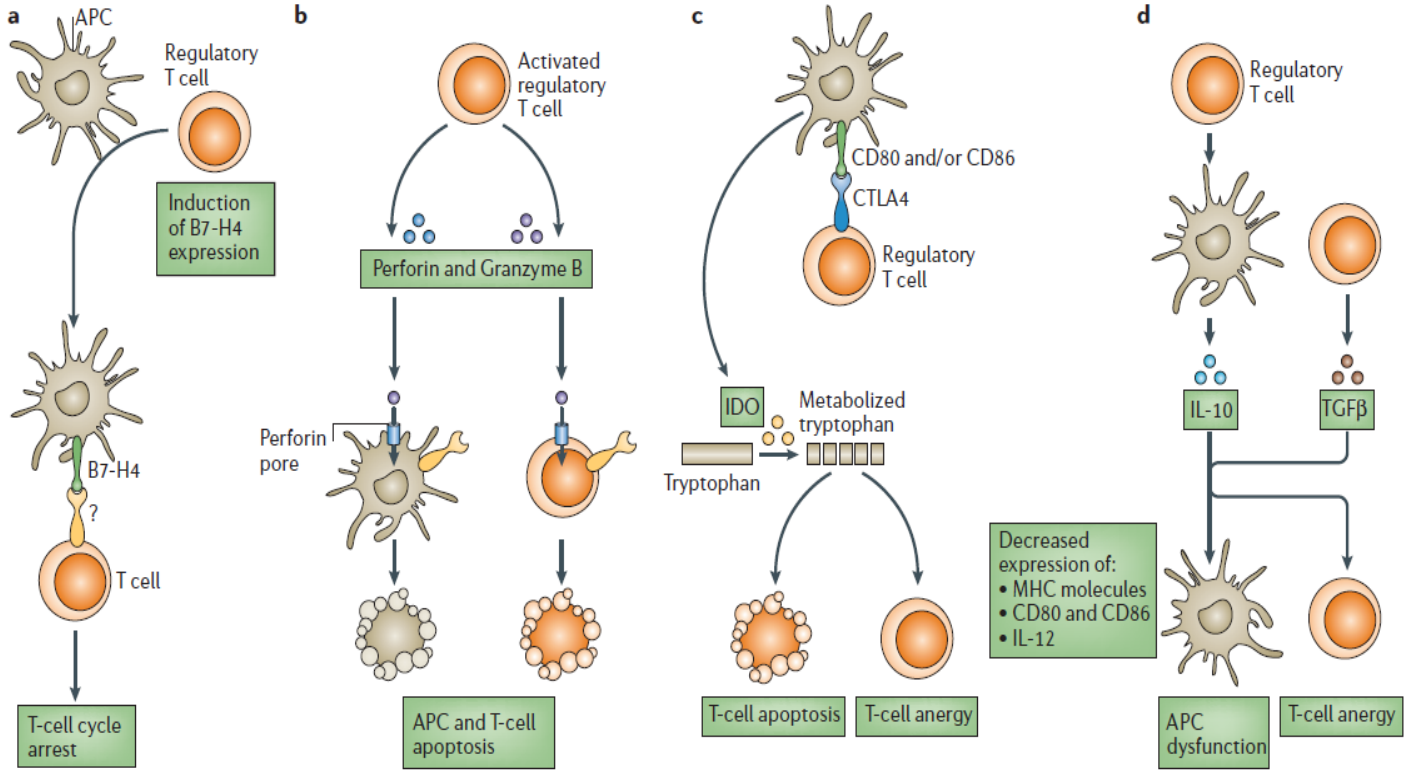

Figure 4: Possible Suppressive Mechanisms of Tumor Environmental Regulatory T Cells.

Reprinted from Zou W *et al* 2006 (87).

thymus but from peripheral T cells. iTregs can be either FOXP3-positive or negative. This cell population is characterized by their cytokine pattern rather than by the surface markers. Among Treg cell populations, "T-regulatory 1 cells (T_R1 cells)" is the term used to represent $CD4^+IL\text{-}10^+FOXP3^-$ regulatory T cells that develop under the influence of the immunosuppressive cytokine IL-10 and whose suppression function is partly mediated by IL-10, this type of iTregs can be induced in response to exogenous antigen challenge. "T_H3 cells" is the term used to represent the $CD4^+TGF\beta^+$ T cells that are induced in the context of oral tolerance and can synthesize TGF-β. In addition, $CD8^+$ Tregs that play an important role in the regulation of autoimmune disease have been recently identified. This kind of Treg cells only target at the activated T cells and can down-regulate target T cell or APC function by cell–cell contact mechanisms or through secreting immunosuppressive cytokines, for example, IL10 (87, 88).

A-II-f T Lymphocytes Differentiation (89, 90) and Memory T Cells (91, 92)

The development and differentiation of T cell response to infectious pathogen has been well studied. It has been identified as a dynamic process and can be generally divided into 4 phases (Fig. 5): 1) Expansion phase: T cell immune response initiated in the lymphoid tissues. After encountering DCs with antigen in appropriate MHC context, naive T cells can be induced to expand and differentiate into effector T cells (CD4+ Th cells or CD8+ CTLs) and travel to infection loci. During this stage, numerous inflammatory cytokines can be secreted through the combined action of CD4+ and CD8+ effector T cells to kill infected cells and a typical acute viral infection can be resolved within several days. 2) Contraction phase: Over the weeks that follow pathogen clearance, the majority of effector T cells die, leaving behind 5%–10% of the original burst size as long-lived memory cells. 3) Memory phase: The surviving T cells enter the third stage in which the number of memory T cells stabilizes, and these cells are maintained for long periods of time, throughout the life of the mouse and for many years in humans. 4) The rapid recall response of memory T cells following re-exposure to the pathogen.

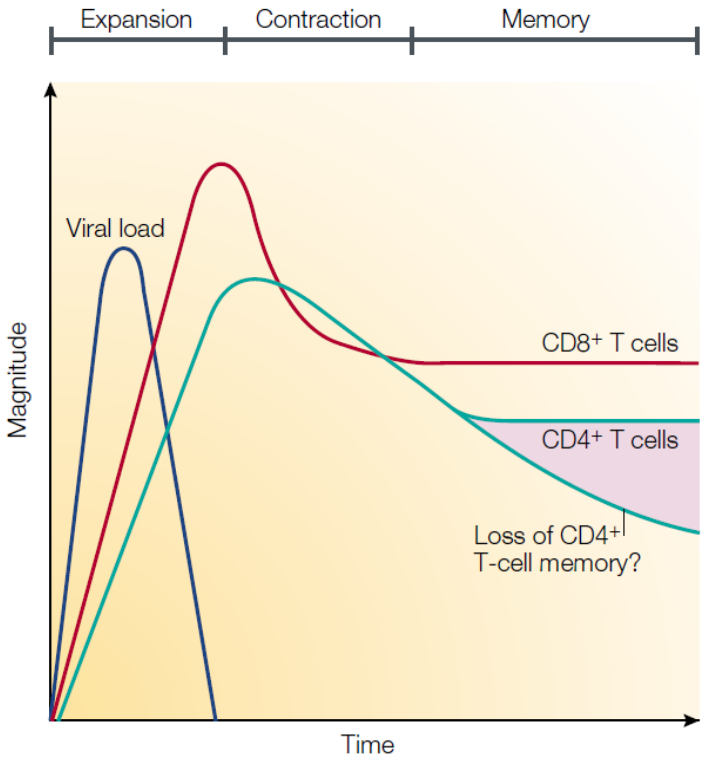

Figure 5: Differentiation of the T Cells Mediated Immune Response. Reprinted from Kaech SM *et al*. 2002 (89)

It is well accepted that memory T cells play an important role in cancer immunotherapy, because these cells have an enhanced ability to control secondary exposure to antigen and are very crucial for preventing tumor recurrence. Recently, a model of "central memory and effector memory T cells" has been proposed. These two memory T populations are characterized by distinct migration capacity and effector function. Central memory T cells (TCMs) mediate the reactive memory. This T memory subset maintains the similar homing and chemokine receptors and recirculation routes as naïve T cells and has little or no effector function. However, TCMs have a higher sensitivity to antigenic stimulation and can rapidly proliferate and differentiate to effector cells following secondary encounter with antigen. The other memory T subset — Effector memory T cells (TEMs) mediate the protective memory. Differing from TCM, effector memory T cells (TEM) no longer routinely enter lymph nodes and more tend to migrate to inflamed peripheral tissues and display

immediate and local effector function. Numerous surface molecules have been identified to interpret the distinct homing properties between TCMs and TEMs, among which L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7) are two lymph-node-homing receptors and have been best defined to discriminate the two memory T subsets. CD62L can interact with peripheral-node addressin (PNAd, the high endothelial venule (HEV)–specific ligand for L-selectin) and mediate the attachment and rolling of lymphocytes to peripheral lymph nodes. CCR7 can bind the chemokines CCL19 and CCL21 that are presented on the luminal surface of endothelial cells in the lymph nodes, which causes firm arrest and the initiation of extravasation. The two molecules are highly expressed by TCMs ($CD62L^{hi}CCR7^{+}$), which lead to their efficient migration to peripheral lymph nodes. For the same reason, TEMs ($CD62L^{lo}CCR7^{-}$) demonstrate a reduced potential for homing to lymph nodes because of the decreased expression of CD62L and CCR7 by this subset. However, TEMs have a greater capacity to migrate to inflamed tissues, which is owned to increased expression of chemokine receptors such as CCR5 and CCR2. Besides, the different functional proprieties between TCMs and TEMs have also been laid out. TCMs can secrete interleukin-2 (IL-2), but little interferon-γ, IL-4 or IL-5. By contrast, TEMs rapidly produced these effector cytokines, but produced less IL-2. And, only the $CD62L^{lo}CCR7^{-}$ subpopulation of CD8+ T cells was found to contain intracellular perforin.

A-II-g B Cells Mediated Response (93)

B cells are lymphocytes that play an important role in the humoral immune response. The principal functions of B cells are to produce antibodies against antigens, act as APCs and eventually develop into memory B cells after activation by antigen interaction.

A critical difference between B cells and T cells is how each lymphocyte recognizes its antigen. B cells recognize their cognate antigen in its native form. Each B cell has a unique receptor protein (B cell receptor, BCR) on its surface that will

bind to one particular antigen. In contrast to T cells which recognize their cognate antigen in a processed form, which means a peptide fragment presented in MHC context by APCs to the T-cell receptor (TCR), B cells recognize free (soluble) antigen in the blood or lymph using their BCR without MHC mediation, thereby for which the APCs are not necessary.

B cells don't produce antibodies until they become fully activated. The activation of B cells is frequently T cell-dependent, for which both the signal come from the recognition of antigen by BCR and the signal come from co-stimulation provided by a CD4+ Th2 cell are indispensable. In this case, B-cell recognition of foreign protein via the BCR results in internalization, processing and presentation of peptide to cognate CD4+ T-cell which has been primed by the same peptide presented by APCs. This interaction can lead Th2 cell to secrete cytokines (IL-4, 5, 6) that activate B cell and trigger B cell proliferation and differentiation into plasma cells.

B-cells are directly implicated in various infectious and autoimmune diseases, but seldom participated in anti-tumor therapy.

A-II-h Tumor Immunosuppressive Environment (94-96)

The immune system plays a crucial role in detecting and eliminating tumor cells. However, the protective function of the immune system against tumors is not always efficient, the interactions between tumor cells and the immune cells in tumor microenvironment can also create an immunosuppressive network that protects the tumors from immune attack and promotes tumors growth and progression.

Several mechanisms have been described by which tumors can suppress the immune system, including: 1) Secretion of cytokines: Series of studies have revealed that tumor microenvironment possesses an anomalous cytokine pattern. For a range of tumors, cytokines or substances which can suppress DC maturation and function can be abundantly expressed in tumor environment, such as VEGF, IL-6, macrophage colony-stimulating factor (M-CSF), TGF-β, IL-10, cytochrome c oxidase subunit II

(COX2), Prostaglandin E2 (PGE2), gangliosides. However, cytokines which induce DC differentiation and maturation, such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4, as well as the Th1-type cytokines IL-12 and IFN-γ, are rarely found. As a result, DCs present in the tumor environment are either immature or partially differentiated. 2) Co-stimulatory and co-inhibitory molecule alterations: Tumor factors present in the local environment can also affect the functional capacity of DCs by altering co-stimulatory and co-inhibitory molecules expression. In the environment of numerous tumors, B7-H1 and B7-H4, co-inhibitory molecules shown to be the negative regulators of T-cell responses *in vitro* by inhibiting T-cell proliferation, cell-cycle progression and cytokine production, might be up-regulated; B7-1 and B7-2, co-stimulatory molecules for T cell activation and differentiation might be down-regulated. Thereby, DCs present in the tumor environment induce either T-cell anergy or suppressive Treg cells. 3) Alterations in antigen-presenting cell subsets: Beside the immaturation and dysfunction of DCs, the balance between the DCs subsets could also be broken in tumor environment. Tumor environments seem to lack angiogenesis-inhibitory myeloid DCs, but present abundant angiogenesis-stimulatory DCs, such as plasmacytoid DCs and vascular DCs, which could promote tumor vascularization. 4) Altered ratios of Tregs to effector T cells. Treg cells are suppressor T cell that inhibits an immune response by influencing the activity of another cell type. We have discussed before about the suppressive mechanism of Treg. IL-10 and TGF-β produced by tumors favor the induction and differentiation of Treg cells. Dysfunctional DCs and tumor-conditioned plasmacytoid DCs would directly contribute to Treg cell induction in the tumor microenvironment.

It is well demonstrated that these mechanisms of immunosuppression can impair tumor specific immune responses.

A-III Cancer Immunotherapy (97-103)

Cancer immunotherapy is one class of anti-cancer treatment that attempts to modulate the host immune system, and/or use the components of immune system in

order to induce or augment anticancer response. The appearance of this concept can be traced back to more than 100 years ago. In 1890s, William B. Coley observed the regression and even the disappearance of tumors following the injection of bacterial products in and around tumors. His findings aroused the great enthusiasm for exploiting immune reactivity to treat cancer. Over the last century, impressive progress has been achieved in tumor biology and tumor immunology, most aspects of immune reactivity have been clearly laid out. Results obtained in related trials demonstrated that attracts triggered by immune system against tumors are more specific and with lower harmfulness to the host. Thereby, immunotherapy is becoming the more and more promising approach for cancer treatment today. For this goal, many ways have been performed and they can be generally classified into two classes, active anti-tumor immunotherapy and passive (or called adoptive) anti-tumor immunotherapy. Each of them comprises specific and non specific approaches (Fig. 6).

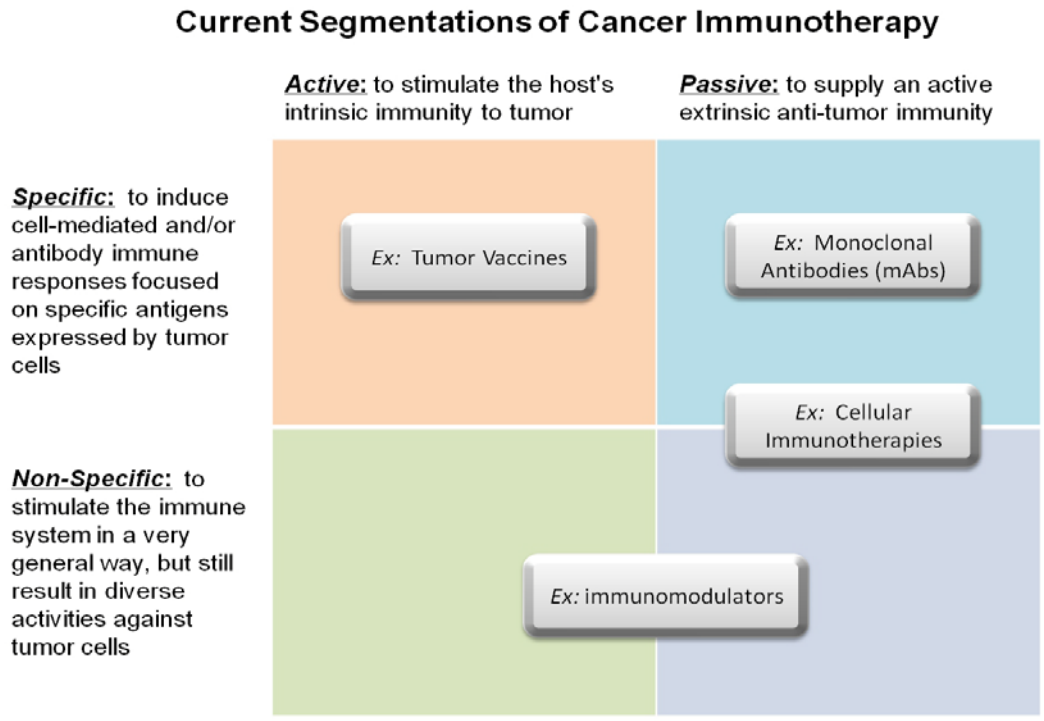

Figure 6: Current Segmentations of Cancer Immunotherapy.

Cancer immunotherapy can be either active or passive. Each subset consists of specific and non-specific approaches.

A-III-a Active Anti-tumor Immunotherapy

Active anti-tumor immunotherapy comprises the approaches that attempt to stimulate the host's intrinsic immune response to tumors. It can be sub-divided into non-specific active immunotherapy and specific active immunotherapy

Non-specific active anti-tumor immunotherapy refers to the activation of a general immune response through the administration of cytokines or the mediators which could induce the release of cytokines. The performance of this strategy has been first brought out by Coley's studies in which local injection of bacteria could induce tumor remission. The principle mechanism can be explained as following: Immune cells expressed Toll-like receptors can recognize Pathogen-associated molecular patterns (PAMPs). Thus the local administration of bacteria can provoke the recruitment and the activation of innate immune cells, such as macrophage, monocytes, and natural killer cells, which can further trigger the release of TNF and other pro-inflammatory cytokines to induce tumor shrinkage. Many subsequent reports have advocated this approach. For example, BCG (Bacillus Calmette-Guerin), a live attenuated form of *Mycobacterium tuberculosis*, has been applied to boost innate immunity to control tumor and now BCG represents the standard treatment for certain stages of superficial bladder cancer. However, the limited efficacy and significant toxicity of this approach has led to the exploration of the immunostimulatory potential of bacterial components or genetically modified bacteria. On the other hand, with the discovery of interferons in the mid 1950s, the vast research field of inflammatory cytokines has been opened. IFNs, TNFs, ILs and the other cytokines were studied with great expectations. Local cytokine administration was considered as one possible approach. TNF can arrest proliferation of tumor cells and prevent the angiogenesis necessary for tumor growth. IFN can demonstrate direct cytotoxic and possibly anti-proliferative properties. It can also enhance MHC expression and thereby potentially increase the efficiency of antigen processing and recognition. IL-2 is a T-cell growth factor and can stimulate lymphocyte proliferation, enhance cytolytic-T-cell activity, induce natural killer cell activity, and induce IFN-γ

and TNF. However, in spite of enormous efforts, beneficial responses in clinical trials were rare. With the exception of a small subset of patients with advanced renal cell carcinoma and malignant melanoma responding to IFN-α or IL-2 treatment, most solid tumors, high-risk leukemia and lymphomas are largely resistant to administration of cytokines.

Specific active immunotherapy attempts to induce cell-mediated and/or antibody immune responses focused on specific antigens expressed by tumor cells. Even the immune system can impact on tumor growth in several ways, it is well accepted that the most effective way of eradicating a tumor mass *in vivo* is through the combined action of CD8+ and interferon-γ (IFN-γ)-secreting T-helper 1 (Th1) CD4+ T cells. For this purpose, vaccination using either whole cells, proteins, peptides or a wide variety of immunizing vectors is the most common and efficient approach applied in specific active immunotherapy. However, the performance of anti-tumor vaccines in clinical trials is not satisfying; these vaccines have only succeeded in bringing about sporadic regressions and temporary stabilization. Effective T cell activation involves two sets of signals. One is mediated by MHC molecules performed antigenic peptides presentation to TCR; the other is mediated by the interaction of co-stimulators expressed on APCs with counter-receptors expressed on T cells. Therefore, the efficacy of a cancer vaccine would be improved by identifying highly immunogenic TAAs, the effective vaccine adjuvant and the optimal vaccine formulation and immunization strategies. We will discuss more in detail in the next chapter about the history, the actuality and the perspective of anti-tumor vaccine development.

A-III-b Passive (or adoptive) Anti-tumor Immunotherapy

Passive anti-tumor immunotherapy refers to the approaches that supply an active extrinsic immunity rather than activating the intrinsic immunity. The transferred immune response could be cell mediated or antibody-mediated.

Adoptive cell transfer (ACT) is a form of immunotherapy by which antitumor lymphocytes are induced and expanded *ex vivo* and then infused to the host. In

practice, because of MHC restriction, the lymphocytes manipulated *ex vivo* are normally autologous. Lymphocytes are collected from host, stimulated *in vitro* and finally re-infused to host. This approach has been investigated both in infectious (e.g., Epstein-Barr virus (EBV), Human immunodeficiency virus (HIV)) and malignant disease (metastatic melanoma). The clinical application of this approach was initially performed in 1980s as the activation of lymphocytes of cancer patients through non-tumor-specific signals. In several trials, the lymphocytes isolated from patients with advanced tumors were stimulated *in vitro* by IL-2 (lymphokine-activated killer cells, LAKs) and then re-infused along with high dose of IL-2. Objective responses have been obtained, whereas it was not sure that the responses were due to the effection of LAKs or IL-2. So far, some progress has been achieved to improve the efficacy of transferred lymphocytes. First, *in vitro* tumor infiltrating lymphocytes (TILs) preparation has been directed to the selection, stimulation and expansion of antigen-specific T cells. In general, TILs were screened by cytokine secretion assay for recognition of autologous tumor cells or allogeneic tumor cell lines. The TIL clones that exhibited specific tumor cell recognition were then expanded with irradiated allogeneic feeder cells, T-cell-stimulating antibody OKT3 (anti-CD3) and IL-2. Despite the high *in vivo* frequencies of tumor reactive effector cells achieved, only a fraction of patients responded because of the short persistence of these transferred cells *in vivo*. Later experiments revealed that preconditioning (lympho-depletion) of patients with cyclophosphamide and fludarabin was necessary for the survival and expansion of the infused TILs. Single-arm Phase II studies from separate institutions have shown objective response rates of 50% in patients with metastatic melanoma who are receiving this treatment. Approximately 10% of patients obtained a complete response, which may be durable. In the latest clinical trials, individualized and cumbersome micro-culture prepared tumor-reactive T cells are being superseded by genetically engineered TCR – transduced T cells for ACT, in which genes encoding TCRs with defined antitumor properties are transduced into short-term cultured peripheral blood lymphocytes. This therapy is potentially accessible to any patient whose tumor possesses the cognate human leukocyte antigen

(HLA) allele and expresses the target antigen recognized by the TCR. The first studies performed by the national cancer institute (NCI) using HLA-A2–restricted TCRs against melanoma antigen MART-1/Melan-A or gp100 leaded to objective responses in 20–30% of patients, while objective responses occurred in only 13-19% patients received ACT using native TCRs. However, the clinical use of highly avid TCRs has been associated with significant collateral destruction of normal tissues sharing the target antigen. Thereby, current efforts attempt to optimize gene transfer efficiencies, design TCR structural modifications, and identify target antigens, the expression of which is highly selective in tumor rather than non-transformed cells. Currently, although ACT clinical protocols with altered techniques are still being defined, ACT is the most effective treatment for patients with metastatic melanoma.

The discovery of monoclonal antibodies (mAbs) by Köhler and Milstein was the major breakthrough that leaded scientists to exploit the potential approach for tumor immunotherapy. mAb can induce tumor cell lysis through the different mechanisms, including antibody-depended cellular cytotoxicity, complement-mediated cytotoxicity, and enhancement of the adaptive immune response. Rituximab was the first mAb applied in clinical trial and approved by the US Food and Drug Administration (FDA). It can recognize the B cell antigen CD20 and can mediated complement-depended cytotoxicity and antibody-depended cellular cytotoxicity, which can induce the apoptosis in CD20+ B cell lymphomas. After that, remarkable progress has been achieved in this domain. Until now, a number of mAbs have been approved by FDA (98). They can be generally classified into two categories: Naked mAbs and Conjugated mAbs. For the second type, radio-labeling or conjugation with toxins further improved efficacy of monoclonal antibodies. Furthermore, More than 100 new therapeutic antibodies targeting different epitopes and multiple modifications of the antibody structure are now in different phases of their preclinical or clinical testing, which predict a great prospect for improving antitumor efficacy.

B - Cancer Vaccines

The development of cancer vaccines attempting to enhance the immune response against a tumor is a promising area of research. The term of cancer vaccine refers to a vaccine that could prevent either infections with cancer causing pathogen or the development of cancer in high risk individuals and treat existing cancer (104). Advances in molecular immunology and cancer biology have brought out impressive progress in this field. Several examples present the great promise of vaccination approach in terms of cancer, such as, following a nation-wide hepatitis B vaccination program in Taiwan, the incidence of hepatocellular carcinoma substantially declined in children (105); also, human papillomavirus-positive cervical cancer can be clinically prevented through the vaccination with a recombinant viral capsid protein and the Human papillomavirus (HPV) subunit vaccines have been already developed (106); moreover, there are also some experimental vaccines which could be hopeful in minimizing cancers associated with virus infection, including vaccines against the hepatitis C virus (hepatomas) (107), EBV (Burkitt lymphoma, nasopharyngeal carcinoma) (108), human T-cell lymphotropic virus type 1 (HTLVI, adult T cell leukemia) (109).

However, despite ongoing efforts to develop effective therapeutic vaccines against tumors, little progress has been obtained in clinical applications. It is possibly because that the vaccines have not yet adequately simulated critical aspects of a curative immune response. The simplest model of immune cell mediated antigen specific tumor rejection consists of three elements: appropriate antigen, efficient antigen presentation and the generation of potent effector cells. At this level, there are many hurdles which limit the development of cancer vaccine, such as: identification of antigens that could exploit the exquisite specificity of the immune system against cancer cells without harming normal cells; development of methods to induce an immune response sufficient to eradicate the tumor, in the face of self-tolerance to many tumor antigens; and overcoming the mechanisms by which tumors evade the

host immune response. Therefore, the efficacy of a cancer vaccine should be improved by identifying highly immunogenic tumor specific or associated antigens, using efficient antigen delivery system that stimulate the appropriate specific immune response and defining the optimal vaccine formulation and immunization strategies. So far, many aggressive strategies have been devised in many laboratories for the purpose of producing an effective vaccine (110). A number of therapeutic cancer vaccines have been already tested in animals and subsequently in humans (111).

In this part, we will discuss the progress made in developing cancer vaccines, including: 1) the discovery and characterization of tumor antigens used as targets for vaccination; 2) cancer vaccine modalities; and 3) recent strategies for cancer vaccines improvement.

B-I Tumor Antigens

The identification of tumor rejection antigens existence is the crucial breakthrough for antigen-based cancer immunotherapy. In the last decades, many tumor antigens have been successively identified (112, 113). In general, tumors antigens can be divided into two classes: neo-antigens (tumor specific antigens, TSAs) and self antigens (TAAs). TSAs are derived from mutated self-proteins (such as mutated p53 protein and mutated RAS protein) or oncogenic virus (such as HPV-16 for cervical cancer or EBV latent phase proteins for nasopharynx cancer) (112). This class of antigens is expressed exclusively by tumors but not in normal tissue. Thereby, they are foreign and not expressed in the thymus, TSAs activated lymphocyte precursors can escape thymic deletion and have a high affinity for the antigens so that antigen-specific immune response is strong.

TAAs are expressed not only by tumor cells with abnormal quantities or locations, but also in the normal tissues including the thymus. For these reasons, TAAs activated lymphocyte population could either be deleted in the thymus or have a low affinity for the antigens which lead to a weak antigen-specific immune response. TAAs can be sub-classified in three categories (37, 112): 1) tissue-specific

differentiation antigens, such as MART1/MelanA, gp100 and Tyrosinase Related Proteins (TRP) for melanoma are melanocyte specific; 2) the proteins that are over-expressed in tumor cells and promote cell growth by anti-apoptotic (such as survivin) or growth factor (such as HER2/neu) activities; 3) cancer/testis (C/T) antigen: the proteins that are more or less specifically expressed in various cancers, but almost totally absent in normal tissues, such as the MAGE, GAGE and BAGE protein families which are only normally expressed in testis in the adult, but can also be expressed in melanoma, mammary adenocarcinomas, the lung carcinoma, bladder carcinoma ...

B-II Cancer Vaccine Modalities

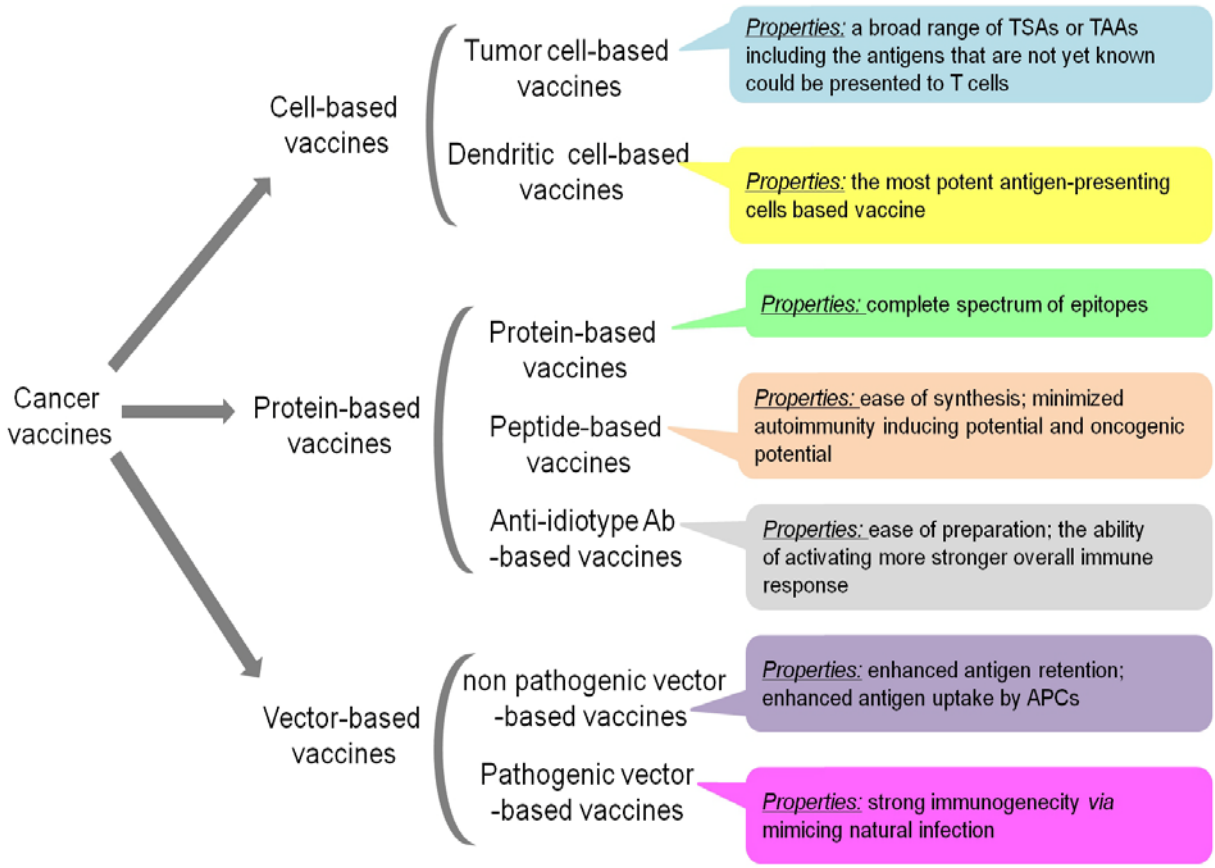

Figure 7: General Cancer Vaccine Modalities.

Various forms of vaccines can be essentially classified in three types: cell-based vaccine, protein-based vaccine and vector-based vaccine.

Actually, various forms of vaccines are being implicated for prevention or treatment of cancer. Essentially, they can be classified in three types: cell-based vaccines, protein-based vaccines and vector based vaccines (Fig. 7). Each of them presents the attractive properties as well as the limitations.

B-II-a Cell-based Vaccines

Cell-based vaccines generally consist of whole tumor cell-based vaccines and dendritic cell-based vaccines. This class of vaccines has been extensively explored with different tumor models in both preclinical and clinical trials. Some recent clinical applications of cell-based cancer vaccines are summarized in table 1.

Table 1: Some Cell-based Vaccines Applied in Recent Clinical Trials.

Cell-based vaccines	Tumor model	Ref.
Autologous tumor cell based vaccine	Melanoma	(114-116)
	Glioma	(117, 118)
	Leukemia	(119)
	Colorectal cancer	(120-122)
	Renal cell carcinomas	(123, 124)
	Non small cell lung cancer	(125)
Allogeneic tumor cell based vaccine	Melanoma	(126, 127)
	Renal cancer	(128, 129)
	Prostate cancer	(130, 131)
	Non small cell lung cancer	(132)
DCs based vaccine	Melanoma	(133-135)
	Glioma	(136-138)
	Breast cancer	(139, 140)
	Renal cell cancer	(140-142)
	Rectal cancer	(143)
	Prostate cancer	(144-146)
	Non small cell lung cancer	(147-149)

Whole Tumor Cell Vaccines

In recent decades, even series of TSAs or TAAs have been identified, characterized and further applied as targets in anti-tumor immunotherapy, there are undoubtedly a large number of antigens remaining undiscovered. Thereby, the appeal for using irradiation inactivated whole tumor cells as antigen sources has immerged, because a broad range of TSAs or TAAs including the antigens that are not yet known could be presented to T cells.

Whole tumor cell vaccines can be divided into two subsets (150): 1) autologous vaccines in which tumor cells used as a vaccine for a patient derive from the same person; 2) allogeneic vaccines in which tumor cells derive from other patients, usually from pre-existing tumor cell lines.

For preparing autologous tumor cell vaccines, autologous tumor tissues are generally obtained during the surgery and then cultured and processed *in vitro*. The consistency between the antigen source of a vaccine and those expressed by patient's tumor cells is very crucial for vaccine performance. Use of patient's own tumor cells as antigen source can ensure the optimal HLA matching and maximize the tumor-specific antigen matching, which is the obvious advantage of this type of vaccines. However, with the requirement for *in vitro* processing or culture, there may be a significant delay between surgical treatment and vaccine administration. Moreover, individualized manipulation, high cost of production and quality control are also required for the preparation of this type of vaccines. These challenges led to the limit application of autologous tumor vaccines. (150)

Allogeneic tumor cell vaccines express a similar load of antigens as autologous tumor cell vaccine. As allogeneic tumor cells do not show the perfect HLA matching and tumor antigen matching as autologous tumor cells do, allogeneic tumor cell vaccines are normally composed of a mixture of several selected allogeneic tumor cell lines that express high levels of tumor antigen and present a partial HLA matching to

most of the potential patient population. Such pattern can be hoped to give a better antigenic spread and generate an effective immunity. Presently, unmodified allogeneic cells have been used in clinical trials for melanoma and prostate cancer. The most advanced use of unmodified allogeneic cells in clinical trials comes from Donald Morton's group (151-153). A panel of 150 cell lines was tested and three cell lines (M10VACC, M24VACC and M101VACC) were selected for the use as vaccine (Canvaxin, Cancer Vax Corporation, Carlsbad, CA, USA) that expressed relatively high level of melanoma antigens and a spectrum of HLA types with a spatial match for approximately 95% of melanoma patients. Canvaxin is now the most thoroughly tested allogeneic live whole cell vaccine. Phase II studies demonstrated that immunization with Canvaxin resulted in prolonged survival in the treatment group compared with a control group receiving no vaccine. In the adjuvant treatment setting, the vaccine has also shown promising results. However, the results of phase III trials were disappointed, there was an excellent survival for the entire study population and no significant difference was found between the treatment group and the BCG control group. Beside of this, another vaccine composed of three irradiated prostate cancer cell lines (OnyCap23, LnCaP, and P4E6) has also been tested and its efficacy has been demonstrated against hormone-resistant prostate cancer in clinical trials (131). Compared with autologous tumor cell vaccines, allogeneic tumor cell demonstrate several advantages, including: 1) vaccines do not need to be individually prepared; 2) vaccines can be industrially prepared in large quantities and can be administrated immediately; 3) the quality control of vaccines can be more easily realized.

Tumor cells alone are generally poorly immunogenic, presumably because of low immunogenicity of TAAs, down-regulation of MHC molecules, the lack of adequate co-stimulatory molecule expression, secretion of immuno-inhibitory cytokines, etc. Thereby, whole tumor cells are usually administered together with adjuvants or cytokines. Adjuvants, such as aluminum salts, oil and water emulsions and bacterial (BCG, Detox) or viral compounds, can non-specifically stimulate the immune system and strengthen the immune responses to the target antigen. Besides, a

number of cytokines, such as GM-CSF, IL-2 and IFNs are administered with tumor cells to create a pro-inflammatory environment in which antitumor responses will arise. Such adjuvants and cytokines can be administrated at the site of vaccination or systemically (150).

Moreover, not only the whole tumor cells, but also the lysates of tumor cells can be used as an antigen source. The popular method of preparing tumor cell lysates is by repetitive freeze-thaw cycles to induce tumor cell necrosis. Lysates on one hand retain the most of the antigen diversity of whole cells; on the other hand, represent several advantages compared with whole tumor cells, including: 1) tumor cell's replication incompetence is assured; 2) lysates don't need the significant care to maintain the viability of cells and can be lyophilized or frozen. However, compared to whole tumor cells, lysates represent some limits, some epitopes are possibly lost through separation of the nuclear fraction and degradation of mRNA, and also more labor will be required (154). It has been proved that the antigens contained in tumor cell lysates can be taken up by DCs and subsequently be presented in MHC II context to CD4+ T cells; and, the antigens can be presented by DCs to CD8+ T cells through cross-presentation pathway (155, 156). As the cross-presentation process require certain conditions which is more easier to be achieved *in vitro*, current lysates based vaccine strategies generally apply lysates to DCs first *in vitro* and then administer the loaded cells as vaccine. Until now, lysates-based vaccines have been investigated in patients with different types of cancer, such as hepatocellular carcinoma (157), colon carcinoma (158), breast cancer (159), renal cell carcinoma (160), thyroid carcinoma (161), leukemia (162), etc.

Genetically Modified Tumor Cell Vaccines

Although systemically administered cytokines have been used to treat advanced cancers with some success, treatment with cytokines is often associated with profound toxicity for the patient, especially when used in high doses. Thereby, great efforts have been performed to generate genetically modified autologous or allogeneic tumor

cells which can secret the cytokines themselves. Among the cytokines, transduction of weakly immunogenic tumor cells to express GM-CSF or IL-2 has been actively investigated. GM-CSF can lead to the recruitment and differentiation of DCs. In animal studies, it has been shown that immunization with tumor cells expressing GM-CSF led to a long lasting systemic antitumor immunity (163, 164). In clinical trials, it has been tested in different cancer models, such as prostate cancer (165), metastatic malignant melanoma (166) and non-small cell lung cancer (167). IL-2 is a growth factor for lymphocytes and has been shown to restore responsiveness in anergic or unresponsive T cells. In animal studies, immunization with the engineered tumor cells secreting IL-2 resulted in a reduced tumorigenicity and a long lasting protective immunity (168, 169). Tumor cell vaccines transferred with IL-2 gene have also been investigated in clinical trials, largely in melanoma model (170-172). Besides, there are also other cytokines that have been investigated using this strategy, such as IL-4, IL-6, IL-7, IL-12, IFN-γ, etc.

Moreover, as the poor immunogenicity of tumor cells is partly due to the absence of co-stimulation, some studies have also focused genetically induced co-stimulator expression on tumor cells. Co-stimulation is normally provided by APCs that take up and present antigens to T cells. Engineered tumor cells expressing co-stimulator molecules could behave more like the APCs and probably prime efficient antitumor immune responses. B7-1 and B7-2 molecules have been largely studied in both animal and clinical trials. In murine model, it has been shown that B7-transfected tumor cells can elicit CTL responses (173) and can protect against tumor challenge in curative models (174, 175). B7-1 gene modified tumor cell vaccine has also been tested in clinical trials against different cancer models (176-178). Similarly, there are also some other co-stimulatory molecules, such as CD40L (179, 180), intracellular adhesion molecule (ICAM-1), lymphocyte function-associated antigen (LFA)-3 (181), etc. have been transduced to tumor cells to prime and strengthen antigen tumor response.

DCs Vaccines

DCs are considered as the most potent antigen presenting cells and the most powerful stimulators of naïve T cells. Due to this endowed property, DC-based vaccination represents a potentially powerful strategy for cancer immunotherapy and has become the focus of a vast array of scientific and clinical studies. Beside the classic MHC I and II antigen presentation pathway that DCs profit to present antigen to CD8+ and CD4+ T lymphocytes, respectively, DCs are also capable of cross-presenting exogenous Ags in MHC I context to generate antigen specific CTLs. This specialized capacity made DCs become one of the focal points of anti-tumor immunotherapy.

DCs can be exogenously loaded with TAAs through numerous approaches, including: 1) pulsed with synthetic peptides; 2) pulsed with protein; 3) pulsed with tumor lysates or apoptotic tumor cells; 4) fused to tumor cells; 5) transfected with cDNA or RNA encoding known TAAs; 6) infected with a viral vector. Up to now, TAAs loaded DCs vaccines have been widely tested in animal models as well as in clinical trials without any dose-limiting toxicity (182-186). In particular, one of autologous-DCs based therapeutic cancer vaccines for the treatment of advanced prostate cancer – *Provenge* (APC8015, also called Sipuleucel-T) has been recently approved by the USA Food and Drug Administration (FDA). *Provenge* is manufactured by Dendreon Corporation and is the only FDA-approved autologous cellular immunotherapy indicated for the treatment of asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer (CRPC) (187). *Provenge* is designed to train patient's immune system to seek and attack prostate cancer cells. One course of *Provenge* treatment consists of three basic steps (Fig. 8). Firstly, patient’s autologous peripheral blood mononuclear cells are extracted in a leukapheresis procedure. Then, the blood product is sent to the factory for the preparation of *Provenge* which involves enrichment for antigen-presenting cells from the leukapheresis product and activation ex vivo through incubation with a fusion protein (PA2024). PA2024 consists of two parts: one is the antigen prostatic acid

phosphatase (PAP) which is present in 95% of prostate cancer cells; the other one is GM-CSF that helps the APCs to mature. Finally, the activated blood product is re-infused into the patient to cause an immune response against cancer cells carrying the PAP antigen. Until now, Provenge showed overall survival (OS) benefit to patients in three double-blind randomized phase III clinical trials, D9901 (144), D9902 (145) and IMPACT (146). Presently, Dendreon Corporation continues to investigate this individual approach to treat cancer in other tumor types.

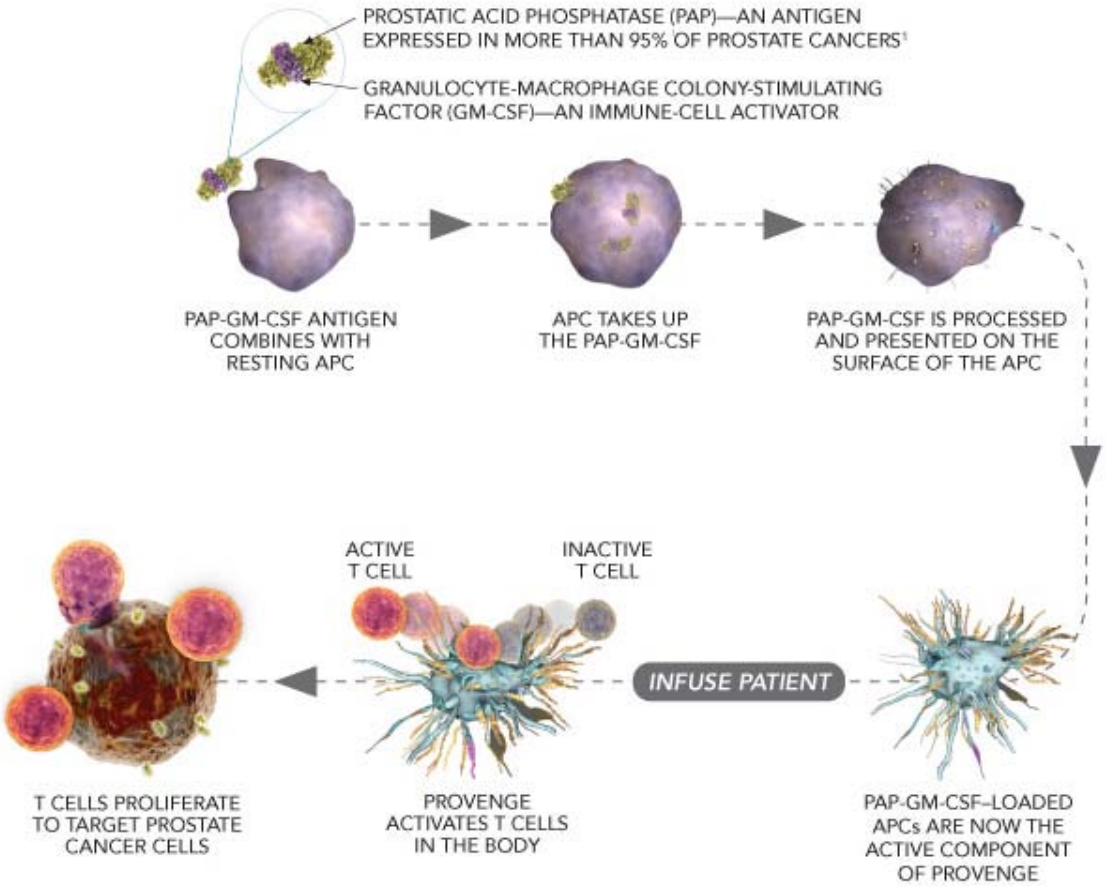

Figure 8: Diagram Explanation of PROVENGE Action Mechanism. Reprinted from www.provenge.com.

Although clinical trials have demonstrated immunological responses after vaccination with DCs loaded with tumor specific peptides, peptide-based vaccines are limited in their application because the use of specific TAAs depends on the

identification of relevant TAAs, but not all tumor cells have well defined TAAs. Moreover, both MHC I and MHC II epitopes are required for efficient T cell priming, while a number of MHC-I restricted peptides have been identified, fewer MHC II epitopes are known. However, loading DCs with tumor lysates or apoptotic tumor cells and fusing DCs with whole tumor cells could overcome these limits. Through these approaches, a broad spectrum of tumor-associated antigens, including those known and unidentified, are processed and presented on MHC I and MHC II molecules in the context of DCs-derived co-stimulatory signals to stimulate CD8+ and CD4+ T-cells. In animal models, tumor cell pulsed or tumor cell fused DCs could promote significant protective immunity against parental tumors (188-192). In the clinical trials, clinical and immunological responses were observed in patients with advanced stage of malignant tumors after being vaccinated with tumor cell-pulsed DCs or DC/tumor fusion cells, although the antitumor effect is not as vigorous as in the animal tumor models (135, 193-196).

Besides pulsing approaches, genetic modifications of DCs with genes encoding whole tumor antigens, widely mediated by adenoviral vectors (AdVs), recombinant poxviruses (rPs), and mRNA electroporation, have been investigated against a broad range of tumor models (197). In animal experiments, TAA-transduced DCs vaccinations have elicited stronger immune responses and led to obvious tumor protection (198-201). In clinical trials, this kind of vaccines has primed effective TAA specific T cell responses and contributed to promising clinical outcome (202-207). Compared to peptides or proteins loading methods, this pattern represents several advantages, including: 1) be capable of enhancing T cell priming potential via antigen presentation; 2) can avoid limitations associated with pulsing of DCs with defined TAAs, such as TAA identification requirement: modification of DCs with entire cancer cell-derived genetic material may enable them to present defined and uncharacterized TAAs to immune cells and to stimulate polyclonal cancer specific CTLs; 3) allow long lasting expression of introduced genes; 4) matching of MHC molecules is not required, the same gene construct may be inserted into DCs derived

from different patients. (97, 208)

In addition to loading DCs with TAAs, genetic approaches have also been used to introduce cytokine production (such as GM-CSF, IL-12, IL-2, IL-10, IFN-γ, etc.) or co-stimulatory molecules expression (such as CD40L, B7-1, etc.) on DCs to enhance their abilities. For example, GM-CSF is a key cytokine for the generation and stimulation of DCs, DCs transfected with GM-CSF demonstrated increased antigen presentation, better migration capacity and a prolonged lifespan, which contributed to an enhanced anti-tumor immune response *in vitro* and *in vivo* (209, 210). In a similar way, CD40L is usually expressed by CD4+ T cells, recent studies suggested that stimuli from CD4+ T-helper cells via the CD40/CD40L interaction could increase DC immune-stimulation with up-regulation of CD80/CD86- and interleukin-12 (IL-12) expression, which is the essential state of DCs for triggering antigen-specific T-cell responses (211-213), therefore numerous studies have focused on genetically expressing of CD40L on DCs to enhance the efficacy of DCs based the cancer vaccines (214-216).

B-II-b Protein-based Vaccines

Protein Vaccines

Protein vaccines generally consist of single or combined tumor antigens. This kind of vaccines has been investigated in both animal and clinical trials and is usually administered with adjuvant, such as GM-CSF, IL-2, IFN-α, CpG, etc., to produce a strong and long-lasting immune response (217-219).

In addition to tumor antigen based approach, a family of heat shock proteins (HSPs) has also been intensively studied and applied in actual cancer immunotherapy. HSPs are molecular chaperones that exist in almost all the sub-cellular compartments of eukaryotes and prokaryotes. Under normal condition, many of HSPs are chaperons that help protein folding and intracellular transport of peptides. HSPs can be unregulated by a variety of environmental stress, such as heat shock, fever, oxidative

stress, inflammation, etc., as well as by internal stresses, like malignant cell transformation. In 1980s, Srivastava *et al.* first proposed that certain HSPs purified from a particular tumor can elicit specific immune responses against that tumor. Afterwards, great amount of work has focused on the characterization of HSPs and their application in cancer immunotherapy (220).

Biochemical studies reported that the low molecular weight antigenic peptides were associated with HSPs preparation from tumor cells (221, 222), which proposed that certain HSPs perform the peptide-binding properties similar to that of MHC proteins. To confirm this theory, a number of studies revealed the existence of peptide-binding pocket frame in HSPs molecules, which provided the structural support (223-225). Then, immunological studies demonstrated that the HSP-peptide complexes can elicit a potent antigen specific adaptive cellular response. HSP-peptide complexes can be untaken and endocytosed by APCs through specialized receptors, such as CD91 and LOX-1. Once be internalized, the antigenic peptides carried by HSPs can be channeled into the endogenous presentation pathway and presented in MHC I context to activate CD8+ T cells (called cross-priming) (226, 227). In a variety of murine cancer models, vaccinations with certain HSPs induced anti-tumor activity via the generation of tumor reactive CTLs (228).

In the other hand, some studies also demonstrated that independent of their bound peptides, HSPs are endowed also the immunomodulator property to prime an innate immune responses. HSPs released by dead or dying cells can be regarded by immune system as danger signal and be acquired by APCs through specialized receptor. HSP–APC interaction leads to up-regulation of co-stimulatory molecules on APCs and secretion of pro-inflammatory cytokines or chemokines. TLR 2/4 and the downstream MyD88/NF-kB pathway have been proposed to mediate the HSP-triggered APCs activation (229). Therefore, HSPs are also considered as natural adjuvants.

These specialized immune-stimulatory properties of innate and adaptive

immunity lead HSPs to be widely utilized in various cancer vaccines design. Up to now, different types of HSPs based cancer vaccines have been developed, including: 1) autologous tumor-derived HSP vaccine; 2) reconstituted HSP–peptide complex, in which multiple peptides from different tumor antigens can be selected to create a polyvalent vaccine to generate a more vigorous immune response; 3) HSP–antigen fusion gene, which uses HSPs in a form of chimerical DNA through linkage of a known antigen gene to the HSP gene; 4) recombinant HSP–antigen fusion proteins; 5) *ex vivo* expanded HSP pulsed DC vaccine; 6) genetically modified HSP over-expressing tumor cells vaccines (230). The efficacy of HSPs based vaccines has been evaluated in animal models (231-234) as well as in clinical trials against different tumor models; promising results have been obtained in human tests (235-237).

Peptide Vaccines

Peptides derived from tumor-associated antigens and recognized by patient T cells have been firstly defined in the early of 1990's. The observation that short peptide segments (8–10 amino acids) fit into a groove in MHC molecules, combined with knowledge of the amino acid sequences of tumor epitopes, prompted the use of peptides as therapeutic agents in the treatment of cancer.

The use of peptides as immunogens demonstrates several advantages, such as: 1) this approach has been shown to facilitate the breaking of tolerance to self, for example in the case of Her-2/neu (238); 2) it can minimize the potential for inducing autoimmunity when compared to the use of a whole protein, which might share fragments with normal cellular proteins; 3) peptides can be modified to increase their immunogenicity and the affinity for the relevant MHC molecules; 4) synthetic peptides are chemically stable and free of bacterial/viral contaminating substances and are devoid of oncogenic potential; 5) the preparation of peptides is relatively easy and inexpensive (239).

It has been widely found that immunization with native peptide sequences was often insufficient to generate reactive T cell response in both animal and clinical trials, which is probably because of the poor immunogenicity of peptides, the lack of CD4+ helper-T-cell activation and the escape of tumor cells. Several strategies have been developed both to improve immunogenicity and to steer the immune system toward desired types of responses. First, the epitopes applied into the peptides vaccines can be improved by sequence modification, a strategy called epitope enhancement. This strategy can be used: 1) to increase the binding ability of peptides to MHC molecules and the binding stability between peptides and MHC molecules, which has been applied to tumor antigens like p53, gp100, PSA, MUC 1 (240-243); 2) to increase the affinity of peptide-MHC complex to TCRs, which has been applied to, for example, carcinoembryonic antigen (CEA) and p53 (244, 245); 3) to inhibit the proteolysis of peptides by serum peptidases (246). Second, incorporation of cytokines, chemokines, co-stimulatory molecules or other immunomodulators with peptide vaccines can enhance the immune response. In this category, a series of adjuvants have been applied alone or in combination, such as GM-CSF which can provoke the recruitment and differentiation of DC, CD40L or agonistic anti-CD40 which can induce DCs maturation, IL12 and IL15 which are Th1/CTL-supporting cytokines, CpG-oligodeoxynucleotide which are shown to elicit a broad range of immune responses (247-250). Moreover, as CD4+ Th cells activation is necessary for inducing an efficient CTL response, inclusion of helper T cell epitopes has also been tried to enhance the efficacy of peptides vaccines. Some papers reported that using peptides with broad MHC II binding, such as pan-HLA-DR-binding peptide (PADRE), endogenous helper epitopes or enhanced helper epitopes, to stimulate CD4+ Th responses also resulted in increased CD8+ CTL responses (251-253). Also, peptides can be loaded onto DCs or other APCs, which will be used as autologous cellular vaccines. The significant advantages of this approach are that DCs are the most potent APCs that could effectively activate naïve T cells and thereby can serve as the natural adjuvant; the loading of peptides and the induction of DCs maturation *in vitro* can be easily achieved.

Despite impressive results in animal models, the application of peptide vaccines in humans has met with only limited success, their efficacy remains to be ultimately proven. Moreover, individual peptides will be useful only in patients with appropriate HLA molecules capable of presenting that peptide, which might be also a potential restriction for this vaccine model.

Anti-idiotype Antibodies

Immunization with anti-idiotype (Id) antibodies (Abs) represents an attractive approach for tumor immunotherapy. This approach is based on Niels Jerne's idiotypic network theory. According to the network concept (254), an antigen generates antibodies (Ab1) whose serologically unique structure (idiotype) can induce the production of anti-Id Abs (Ab2). Some of these Ab2 molecules (Ab2β) are specific for the variable antigen-binding region of Ab1, and can function as an “internal image” of the target antigen by mimicking its three-dimensional structure. Immunization using these Ab2β antibodies should stimulate the production of anti-anti-Id antibodies (Ab3 also called Ab1’) that recognize the original Ag. This cyclic nature of complementary binding sites and idiotype is the basis for the approach to idiotype vaccines.

The administration of Ab2β as surrogate tumor-associated antigens represents a potential application of the idiotype vaccine concept and exhibit several advantages. The anti-idiotype hybridoma route of making surrogate antigens could alleviate the problem of preparing large amounts of purified antigens associated with a given tumor. Also, the internal antigens which are expressed in a different molecular environment may be able to overcome the immunosuppression and break the tolerance in the host by stimulating “silent” clones, or by activating T cell help, making the overall immune response stronger than the nominal antigen is capable to do. In addition, by virtue of being proteins, anti-idiotype antibodies can be easily manipulated and coupled to potential immunogenic carrier molecules to make them T cell dependent antigens which can receive full T help. Furthermore, idiotype-based tumor antigens used for immunotherapy are free of the potential danger of transmitting tumor viruses.

(255)

This strategy has been applied in different cancer models, such as B cell lymphoma, melanoma, colorectal cancer, ovarian cancers, and breast cancer (reviewed in Ref. (256)). Active immunization with tumor-specific anti-Id vaccines has been shown to inhibit the growth of tumors and cure mice with established tumors in animal models (257). Results obtained in human trials were promising, using, some experience using anti-Id to stimulate immunity against tumors has shown that immunization with true anti-Id reagents (Ab2β) can induce both cellular and humoral immunity (258-260).

The main problem for this strategy is that the Id is not only tumor-specific but also patient-specific. And also, large-scale feasibility remains still questionable for this moment.

B-II-c Vector-based Vaccines

The effectiveness of a vaccine depends upon optimal therapeutic gene expression, an appropriate DNA delivery method and the presentation of antigen to lymphocytes. Actually, the design of efficient vectors for cancer vaccine development is an area of intensive research. They should be able to deliver *in vivo* heterologous antigens into the antigen-processing pathways to stimulate MHC class I-restricted CTL responses as well as Th response. The different antigen delivery systems can be essentially classified in two categories, non pathogenic based vectors, such as DNA plasmid, liposome, cationic peptides (cell-penetrating peptides, CPPs) and yeast vectors; and pathogenic based vectors including viral and bacterial vectors.

Plasmid DNA

Plasmid DNAs are simple antigen delivery vehicles that can be easily designed and produced *in vitro*. Plasmid DNA vaccines are based on bacterial plasmids that have been engineered to express the target antigen using promoter elements that are active in mammalian cells. They also contain a transcriptional terminator to terminate

transcription in mammalian cells and a selectable marker to facilitate production of the plasmids in transformed bacterial cells. After administration, the role of plasmid DNA vaccines is duplex. On one hand, the antigen encoded by the plasmid is produced in host cells, either in professional antigen presenting cells (APCs) leading to direct priming of immune responses or in non-professional cells from where the antigen can be transferred to APCs leading to cross-priming. On the other hand, because DNA plasmids are derived from bacteria DNA, they stimulate the innate immune system by interacting with specific Toll-like receptors. This non-specific immune response increases the antigen-specific immune response (261).

Initial studies focused on the application of naked plasmid DNA vaccines. In general, naked plasmid DNA vaccines were administered trough intramuscular way. The action of DNA vaccines relies on the ability of myocytes to engulf the plasmid. Plasmid secreted by the myocytes or as such can also be taken up by APCs infiltrating the injected site. Meanwhile, some of the DNA might also be endocytosed by APCs infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics (262). Studies in animal models have demonstrated the feasibility of utilizing plasmid DNAs to induce both specific humoral and cell-mediated immune responses and elicit protective antitumor immune responses. (263, 264). Besides of this, the use of plasmid DNA demonstrated also some advantages, including that: they are easy and inexpensive to generate and can be tested rapidly; they do not require special handling or storage conditions; multiple or multi-gene vectors encoding several antigens and immune-modulatory molecules can be delivered as single administration; plasmid DNA vaccination will not generate anti-vector immune responses, thereby there is no preexisting immune responses that might interfere with the ability of the plasmid to be used or reused (265).

However, plasmid DNA vaccines were less efficient when tested in clinical trials. The inability of plasmid DNA vaccines to induce potent immune responses in humans may be mainly related to their low immunogenicity and associated with the fact that

high doses of plasmid DNA were needed to induce a sufficient cell-mediated immunity (CMI) (reviewed in ref (266)).

A variety of strategies are being followed to increase the immunologic potency of DNA based genetic immunization. In general, they could be classified in to 3 types: 1) physical delivery technologies: forcing the plasmid DNAs into cells of either the skin or muscle through intramuscular needle injection (267); application by bombardment using DNA-coated gold particles (268), electroporation following needle injection (269), jet injection (270) or topical exposure to mucosal sites; 2) application of adjuvants; 3) formation with different delivery system to target APCs. Most application methods were shown to induce more or less strong protective immune responses in a series of experimental animal models

Liposomes

In recent years, the use of liposomes as potential antigen carriers for cancer vaccines has been extensively explored.

Liposomes are spherical vesicles composed of one or more phospholipid bi-layers and an internal aqueous center and they are capable of encapsulating both Ag and immunomodulator agents (271). The target agents can be carried by liposome in different patterns, including: 1) encapsulated in the core, 2) embedded in the bi-layer or 3) adsorbed or engrafted to the outer surface.

It has been reported that locally injected liposomes can be taken up avidly by APCs infiltrating the site of injection or in the lymphatics, an event that has been implicated in their immune-adjuvant activity (272). And also, liposomes would protect their DNA content from deoxyribonuclease attack (273). Thus, liposomes are regarded as suitable antigen or DNA delivery structures for the development of potent cancer vaccines.

Many variations of liposomes have been studied with respect to their vaccine

potential. It has been revealed that vaccination with liposome based vaccine can elicit cell mediated immune response as well as humoral immune response (274). For this attractive property, some studies suggested that small liposomes can be delivered to the cytoplasm of cells by the process of endocytosis or pinocytosis. These different routes of uptake could result in either the partial or complete degradation of the antigen and consequently the antigen has the potential to enter different processing compartments such as the endosomes, the lysosomes or the cytosol (275). Thereby, liposome mediated antigen delivery could allow antigen to gain access to both MHC I and MHC II pathway in APCs. In addition to antigenic stimuli, liposomes can also be designed to carry adjuvants which will enhance anti-tumor immunity (276-278).

Besides their capacity of inducing a large spectrum of immune responses, the use of liposome as vaccine vector demonstrated also several advantages (279). For example, there were no granulomas at the site of injection (280); no hypersensitivity reactions were observed in pre-immunized animals when the antigen was given in the entrapped form (281); also, liposomes composed of the appropriate phospholipid do not develop antibodies against their phospholipid component (282) nor have they produced any side effects in repeatedly injected patients (283).

One well-recognized limitation of liposomes is their low entrapment efficiencies for water-soluble antigens. In such cases, in order to improve entrapment efficiencies, attentions were generally focused on optimizing the manufacturing method and/or altering the antigen properties, without affecting antigenicity (274).

In many animal experiments, tumor protection was achieved by immunization with a relevant antigen entrapped in liposome. And currently, liposome based vaccines have been carried to clinical trials, in which the delivery of tumor antigens using liposomes holds great promise as a safe and non-immunogenic approach to cancer immunotherapy (284, 285)

Cell-penetrating Peptides

Presently, various natural and/or synthetic cell-penetrating peptides (CPPs) have been widely used in vaccine design.

CPPs are cationic and/or amphipathic peptides containing 9-35 amino acids that are capable of internalizing across cell membranes. In fact, the cell membrane is impermeable and possesses an effective barrier for most hydrophilic substances. It was first discovered in 1980s that a natural poly-cationic protein, the trans-acting activator of transcription of the human immunodeficiency virus (HIV-TAT), contains the domain capable of cell membrane translocation (286, 287). Following this discovery, additional poly-cationic peptides of natural origin, such as herpes simplex virus (HSV-1) protein VP22 (288) and penetratin (Antp) derived from the homeodomain of *Antennapedia* (289); or of synthetic origin, like transportan (290), have also been identified to facilitate cellular uptake and translocation. The mechanism of cellular uptake and subsequent processing still remains controversial. It has been suggested that CPP mediate intracellular delivery via both endocytic and non-endocytic pathways (291).

This cell membrane translocation ability is processed not only by the CPPs alone but also by the CPPs attached with cargos. Different cargos, such as drugs, peptide/protein, oligonucleotide/DNA/RNA, nanoparticles, liposomes, bacteriophages, fluorescent dyes and quantum dots, have been linked to CPPs for intracellular delivery with possible use in future vaccine design (291). Cellular delivery using CPPs has several advantages over conventional techniques because it is efficient for a range of cell types, can be applied to cells *en masse*, and has a potential therapeutic application (292).

Two applications of CPPs already validated in vaccine studies are the delivery of tumor-associated antigens into APCs and the use as a non-viral gene delivery vehicle in DNA vaccines (291). The major drawback with CPPs is their lack of specific delivery, which is a highly desirable property for cancer treatment. This limitation can be overcome by addition of a receptor-targeting domain. Through this way,

polypeptides will recognize and bind to cell surface receptors that are unique to target cells and deliver cargos into the cells through receptor mediated endocytosis (293). Until now, the ability of CPPs to induce a superior broad anti-tumor immune response via enhanced delivery and presentation has been demonstrated in different murine tumor models, like cervical cancer (294-296), colorectal cancer (297), breast cancer (298), lymphoma (299). Research is now being extended to *in vitro* human systems.

Yeast Vectors

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism mainly used in the making of beer and bread.

Although be nonpathogenic, *S. cerevisiae* can be avidly phagocytosed by APCs and subsequently induce immunologic responses in mammals (300, 301). The phagocytosis of yeast by DCs is driven by the immunogenicity of yeast cell-wall components, such as β-1, 3-D-glucan and mannan, which can transmit 'danger signals' normally associated with microbial infection. These components have strong adjuvant properties and can be detected by Toll-like receptors and mannan receptors on DCs (302). Once inside in APCs, yeast can be degraded in proteasomes, antigens contained by yeast can be presented in MHC I context to be recognized by CD8+ CTLs. On the other hand, yeast can also be degraded in endosomes and antigens will be presented to MHC II molecules and be recognized by CD4+ T helper cells. Thereby, the use of yeast-based vaccines can lead to the recruitment and specific activation of both CD4+ and CD8+ T cells (303).

Due to this particular property, yeast has also been exploited as cancer vaccine vector for antigen delivery. It has been shown that recombinant yeast cells are capable of stimulating the immune system to produce highly specific and potent cellular responses against target protein antigens with little toxicity. Stubbs *et al.* demonstrated that whole recombinant *S. cerevisiae* expressing foreign antigens can activate DCs, elicit robust antigen-specific CTL responses, and confer protective immunity against

tumor challenge in mice (300). Lu *et al.* reported that whole recombinant yeast vaccines expressing mammalian mutant Ras proteins contributed complete regression of established Ras mutation-bearing lung tumors in mice in a dose-dependent, antigen-specific manner (304). It has been demonstrated that recombinant yeast expressing a tumor antigen could induce the activation and maturation of DCs *in vitro* and elicit immune and antitumor responses in different mice models, such as melanoma, pancreatic carcinoma and lung carcinoma (305-308). Following these encouraging pre-clinical experiments, recombinant yeast vaccines are currently investigated in clinical trials.

Viral Vectors

Viruses are small infectious agents that can replicate only inside the living cells of organisms. Viruses can infect all types of organisms, from animals and plants to bacteria (309). They have evolved highly efficient structures and mechanisms for infecting cells and utilizing the cellular machinery for production of virally encoded proteins (310). Thereby, viral vectors represent the attractive antigen delivery vehicles for cancer immunotherapy since they can mimic a natural infection and express potent danger signals.

A large number of viruses have been engineered as attenuated replication deficient vaccines. The first recombinant vaccinia viruses were constructed in 1982, since then several other viruses have also been developed as recombinant viral vectors. In the field of cancer immunotherapy, a variety of recombinant viruses has been used to deliver immune modulators (such as cytokine and co-stimulatory molecules) or tumor antigens. Generally, these virus vectors can be applied in two ways: 1) be directly administered for either the local administration of cytokines, co-stimulatory molecules and therapeutic genes, or systemic administration of viral vectors serving for delivery carriers of tumor antigen; 2) be used to modify cells *ex vivo* for the development of different cell mediated vaccines.

Within the last decade, many such recombinant vectors have made the transition from pre-clinical research to clinical development. Each viral vector system demonstrates some attractive properties that make it suitable for cancer vaccine application. Meanwhile, each system shows also some instinctive shortcomings. Next, we talk about several widely investigated viral vectors in cancer immunotherapy.

Recombinant vaccinia viruses were among the first vectors developed for vaccination purpose and have been widely utilized in clinical trials. Advantage of using vaccinia as a vector include the ease with which recombinant vectors can be formed and isolated; the large capacity of Pox viruses for foreign DNA; the relatively high level of transgene expression, and the wide host range. However, their efficacy for immunization has been shown to be limited by pre-existing anti-vaccinia immunity in the general population. Given the vaccination history of many individual, its application in the clinic is likely to be limited (311).

Adenovirus based vectors, are generally considered to be one of most potent vectors for vaccine development. Adenoviruses process several attractive features for vaccine use, for example, infection of a large variety of cells, expression of heterologous protein in both dividing and non-dividing cells, high levels of transgene expression, ability to grow to high titers *in vitro*, lack of integration in the host genome, and physical and genetic stability (312). Particularly, it has been reported adenoviruses can infect dendritic cells, up-regulate co-stimulatory molecules, and elicit cytokine and chemokine responses, thus effectively elicit immune responses and provide potent tumor protection (313). However, the use of adenoviruses is limited by several obstacles. They do not integrate into the host chromosomes, which results in a short-term expression of the transduced gene. There exist a widespread pre-existing anti-vector immunity in targeted populations and there is the novel generation of immunodominant immune responses against adenovirus Ags following vector delivery; and immunological responses to adenoviruses may also reduce the duration of expression therapeutic gene expression (311). Thereby, the clinical application of

recombinant adenoviral vectors for the treatment of tumor is shown with limited progress.

Cancer immunotherapy has also profited from the development of recombinant retroviruses. Because retroviruses are capable of integrating into the host genome and this property permits the long-term expression of the foreign gene (314). Also, this type of virus is not able to replicate in non-dividing cells, which displays a high degree of safety, whereas demonstrates also a low transfection efficacy (315). However, little clinical data support the development of recombinant retrovirus vectors in cancer immunotherapy. The main concerns in using recombinant retroviruses are accidentally random integration into the host chromosome resulting in deleterious effects such as the activation of certain proto-oncogenes by insertional mutagenesis, or suppression of other tumor suppressor genes. Furthermore, the requirement of cell division for the provirus integration limited their application. One solution to this problem is using lentivirus because this subset of adenovirus is capable of integrating into non-dividing cells (311).

HSV is also a promising vector for the delivery of tumor antigens due to its ability to infect a broad range of cells including both dividing and non-dividing host cells, its ability to accept large DNA inserts and its good safety profile (316). This vector has been shown to have a good clinical bio-safety profile due to the fact that following infection of the target cells there is no further replication (317). Major problems associated with the use of HSV vectors are the difficulty in completely eliminating lytic viral gene expression; vector induced cytotoxicity, and the transient nature of gene expression (311).

Bacterial Vectors

Due to several intrinsic features, live, attenuated pathogenic bacteria are being explored as vectors for protein antigen and DNA delivery, including: 1) they have a positive tropism for professional APCs; and 2) the bacteria itself is a natural adjuvant

for the vaccine because bacterial infection has been well known to induce the release of several pro-inflammatory cytokines which could enhance the immune responses.

Numerous preclinical and clinical studies have investigated the potential for bacterial delivery of therapies for cancer. The major problems of using bacteria as cancer vaccine vector are their toxicity at the dose required for therapeutic efficacy and the diminished efficacy at reduced dose.

As this work has been achieved in dependence of live, attenuated bacterial vectors, we will discuss next in more details the application of this type of delivery system for cancer immunotherapy.

B-III Bacteria-based Antigen Delivery Vehicles in Cancer Immunotherapy

Originally, bacteria were developed as live vaccines against bacterial infections. Induction of humoral and/or cellular immune response can be achieved by administration of attenuated strains derived from pathogenic bacteria, which leads to protection of the host against a subsequent challenge with the respective wild-type strain. In addition to be used to confer protection against self-antigens, live attenuated bacteria are also thought as promising carriers to deliver antigenic message *in vivo* for induction of a broad immune response against heterologous pathogens (reviewed in (318, 319)).

Presently, attenuated bacterial strains have been exploited as potential vaccine vectors for cancer immunotherapy. For this purpose, bacteria based vaccines function majorly through two mechanisms: bacteria mediated DNA delivery and bacteria mediated antigen delivery.

Numerous bacterial strains, majorly belonging to *Salmonella* genus, has been explored as DNA delivery vectors for inducing protective responses against a variety of tumor antigens (table 2). In this pattern, tumor antigen encoding plasmids carried

Table 2: Live Attenuated Bacteria-mediated DNA Delivery Applied for Anti-tumor Immunotherapy in Animal Models.

Cancer model	Vaccine antigen	Animal model	Vaccination strain	Ref
Melanoma	GP100	C57 BL/6 mice	*S. typhimurium* SL7207	(320)
	flk1	C57 BL/6 mice	*S. typhimurium* SL7207	(321)
	hTERT/ TRAIL	BALB/c & C57BL/6 mice	*S. typhimurium* SL3261	(322)
	TRP2/GP100	C57BL/6J	*S. typhimurium* SL7207	(323)
Breast carcinoma	MTDH/AEG-1	BALB/c mice	*S. typhimurium* RE88	(324)
	Endoglin (CD105)	BALB/c mice	*S. typhimurium* RE88	(325)
	Legumain	BALB/c mice	*S. typhimurium* RE88	(326)
	flk1	BALB/c mice	*S. typhimurium* RE88	(8)
Lung carcinoma	flk1	C57 BL/6 mice	*S. typhimurium* SL3261	(327)
	hTERT/ TRAIL	BALB/c & C57BL/6 mice	*S. typhimurium* SL3261	(322)
	CEA	C57 BL/6 mice	*S. typhimurium* SL7207	(328)
Colon carcinoma	flk1	BALB/c mice	*S. typhimurium* SL3261	(329)
	CEA	C57 BL/6 mice	*S. typhimurium* SL7207	(330)
Neuroblastoma	Survivin	A/J mice	*S. typhimurium* SL7207	(331)
	tyrosine hydroxylase (TH)	A/J mice	*S. typhimurium* SL7207	(332)
Prostate carcinoma	PSA	C57 BL/6 mice	*S. typhimurium* SL7207	(333)
Gastric carcinoma	MG7-Ag	C57BL/6J	*S. typhimurium* SL3261	(334)
Cervical carcinoma	HPV 16-E7	C57BL/6J	*L. monocytogenes*-LLO	(335)

by bacterial vectors will be expressed in the eukaryotic cells after transfection. With a few exceptions, most of the bacterial strains utilized *in vivo* for the delivery of vaccine plasmids are intestinal pathogens of humans and some other mammalian. The use of live attenuated enteric bacteria based vaccines permit colonic mucosa infections following oral administration. After crossing the intestinal mucosal barrier, mainly via M cells, a large fraction of bacterial carriers can be taken up by APCs, either in local lymphoid tissues or after systemic spread, in spleen, liver and lymph nodes (336). After being phagocytosed by the APCs, these bacteria either enter the cytosol of these cells, like *Listeria*, *shigella*; or remain in the phagosomal compartment, such as *Salmonella*, through which antigen could be expressed, processed and further presented on MHC class I and II molecules to T cells, respectively.

Besides, bacteria can also be used as vaccine vectors for antigen delivery. This pattern refers to the mechanism by which antigens are not produced by host cells but by the bacteria *in situ* and in addition bacteria act as adjuvants. More examples will be presented in following sections for individual bacteria species. In this model, heterologous antigens can be expressed either through chromosomal integration of antigen coding cassettes or by plasmid-based antigen expression systems. Chromosomal expression of antigens demonstrates several advantages, including genetic stability and the possibility of integrating and expressing multiple antigen genes. Meanwhile, this strategy shows an inherent drawback that generally one copy of the antigen gene will be expressed per bacterial cell, which might result in insufficient antigen expression and thereby the inefficient protection. However, expressing the antigen from a plasmid can circumvent this limitation. The quantity and location of antigen expression can be regulated by using a plasmid-based system. The amount of antigen expressed can be controlled by using either high-copy or low-copy plasmid backbones as well as inducible systems that produce large quantities of antigen upon addition of the induction agent. Furthermore, the location of antigen expression can be controlled *in vivo* to give the maximal antigen dosage depending on sub-cellular localization (337). Constant antigen synthesis can result in

decreased bacterial vector fitness and decreased immunogenicity; therefore, using *in vivo* inducible promoters to control antigen expression in a plasmid can improve immune responses (337). For bacteria-mediated antigen delivery, besides endocytosis /phagocytosis pathway, the *in situ* translocation of heterologous antigens that are locally produced by bacteria and engaged by their type III secretion system (T3SS) has been widely investigated. This will be discussed below.

Bacteria based protein/DNA delivery vectors offer multiple advantages: (1) there exist a variety of well-characterized virulence attenuating mutations; (2) the quantity and *in vivo* location of antigen expression can be regulated; (3) multiple vaccine delivery routes are possible; (4) they are potent innate and adaptive immune system stimulators; and 5) the others, like low batch preparation costs, facilitated technology transfer following development of the prototype, increased shelf-life and stability in the field respect to other formulations (e.g. subunit vaccines), easy administration and low delivery costs (337).

Bacterial vaccine delivery has demonstrated *in vivo* efficacy in several experimental animal models of infectious diseases and tumors and has been further evaluated in humans. However, despite these promising results, the development of bacterial vaccines for cancer treatment faces a number of technical and biological challenges. Bacteria are unable to carry out a variety of post-translational protein modifications observed in mammalian cells and do not readily fold proteins with great molecular weight. In addition, the necessity to secrete protein antigens expressed by bacteria limits the number of proteins that can be targeted by this approach. Also, high levels of antigen expression can sometimes lead to toxicity in host cells and consequently to the loss of its normal delivery function (338). Moreover, some other factors, including the reversion to virulent state, stability of the recombinant phenotype, pre-existing immunity, etc., should also be taken into consideration (319).

Furthermore, to be applied as vaccine *in vivo*, wild-type bacteria should be first attenuated. This process is in order to generate strains that can be well tolerated by the

immune system and reduce vector induced inflammatory responses. With a greater understanding of bacterial virulence and recombinant DNA technology, numerous genetically defined attenuated bacterial strains have been developed. One of the most widely studied classes of attenuated bacteria is auxotroph. Generally, this type of mutants will undergo limited replication and can be eliminated from host tissues within a short time (339). *Salmonella* strains with a deletion in the *aroA* gene of the aromatic amino acid (aro) biosynthetic pathway are immunogenic and less virulent in mice and can protect against a subsequent challenge with homologous *Salmonella* (340). Similarly, *aroA* mutants of *S. flexneri* (341), *Y. enterocolitica* (342), *L. Monocytogenes* (343), etc., have also been constitued. In our laboratory, we have also estabilished one mutant of *P. aeruginosa*-CHA-OAL in which the *aroA* gene is deleted and of which the lethal dose is significant enhanced than wild type strain (10).

Next, we will talk about several well characterized and widely applied bacterial vectors for cancer vaccine design.

Listeria Monocytogenes

L. monocytogenes is a gram-positive facultative intracellular bacterium that can cause listeriosis in both animals and humans. Its natural route of infection is oral, and translocation occurs across the gut epithelium prior to systemic dissemination.

L. monocytogenes is able to infect both phagocytic and non-phagocytic cells. *L. monocytogenes* invades cells either through direct phagocytosis or by binding to host cells via virulence factors called internalins. Once inside the phagosome, the majority of the bacteria could be killed and degraded lysozomal compartment. Meanwhile, there is still a small fraction of bacteria that can escape degradation and enter the cell cytosol. This phagosomal escape is mainly mediated by the action of one virulence factors - listeriolysin O (LLO). This enzyme can degrade the phagosomal membrane and allow the release of bacteria into the cytoplasm where they undergo cell division. At the same times, in the cytoplasm, the expression of ActA, an actin polymerase,

allows *L. monocytogenes* to polymerize host cell actin monomers to become motile. Ultimately, motile bacteria move to the periphery of the cell where they form pseudopod-like structures that are recognized and internalized by neighboring adjacent cells where the cycle is subsequently repeated. Through the direct cell-to-cell transfer mechanism, *L. monocytogenes* can spread without leaving the intracellular compartment. Due to the dual intracellular location, antigens secreted by *L. monocytogenes* can be targeted for both MHC class I and II presentation on the cell surface (344).

In addition to heterologous antigen delivery, *L. monocytogenes*, as the other bacterial vector, is endowed the ability to stimulate robust innate immune responses. *L. monocytogenes* can efficiently induce the functional maturation of DCs, and *L. monocytogenes* infection can trigger cascades of pro-inflammatory cytokine and chemokine production and promote the cellular recruitment to infectious loci, which are crucial for priming an antigen-specific CTL responses. The inherent immunostimulatory properties of *L. monocytogenes* vectors stimulate optimal antigenpresenting DCs *in vivo* (345).

Moreover, *L. monocytogenes* demonstrated several particular biological and immunological characteristics that make it an ideal vaccine vector, including: 1) it is capable of infecting a wide variety of different cell types inside the host (346); 2) as a Gram-positive bacterium, it does not express LPS and thereby is less likely to induce an LPS mediated septic shock in contrast to Gram-negative bacteria (347); 3) the complete genome sequences of two *L. monocytogenes* strains have been determined (348), which makes it possible to obtain virulence-attenuated strains through genetic modification; 4) the virulence attenuation or pre-existing anti-*listeria* immunity will not affect its ability of eliciting effective immune responses against heterologous antigens, which has been reported by several groups in a murine model (349-351); 4) it can be easily cultivated to large numbers in simple growth media, which renders the feasibility of producing a larger quantities of vaccine strains in an industrial setting.

In the past decades, the unique biological and immunological characteristics of *L. monocytogenes* prompted ones to examine the ability of these bacteria to act as vaccine vectors. It has been first reported in 1992 that *L. monocytogenes* could deliver heterologous antigens to the immune system with the induction of CTL responses (352). Since then, *L. monocytogenes* has been successfully explored as a vaccine vector for the delivery of antigens and antigen encoding plasmid (353) in diverse models (for recent reviews see ref (354-356). Nearly almost pre-clinical studies using *L. monocytogenes* to deliver tumor antigens as proteins in mouse models of cancer can be found in recent reviews (357, 358). Currently, the applications of *L. monocytogenes* as cancer vaccine carriers for human disease have entered phase I clinical trials. For example, a *L. monocytogenes* vaccine that secreted an LLO-E7 fusion product was the first bacteria vaccine evaluated in clinical trials. The results of this study showed that that a live-attenuated *L. monocytogenes* immunotherapeutic is safe to be administered to late stage invasive cancer patients (359).

During constructing and testing *L. monocytogenes* based immunotherapeutics, some interesting advantages have been revealed. For example, the delivery of the whole proteins can induce the immune responses against immuno-subdominant epitopes not previously identified (360); also expression of a chimerical product composed of multiple immunodominant regions can avoid the weak immune responses caused by the loss of CTL epitopes in tumors (361). However, some obstacles have also been observed, such as the difficulty of secreting large and hydrophobic molecules (362).

Some applications of *L. monocytogenes* as antigen delivery carrier for anti-tumor immunotherapy in animal models are summarized in Table 3.

Salmonella Genus

Salmonella is a gram-negative bacterium and the causal agent for salmonellosis. Based on antigenic determinants of LPS, flagellar and capsular antigens, over 2500

Table 3: Live Attenuated *Listeria*-mediated Antigen Delivery Applied for Anti-tumor Immunotherapy in Animal Models.

Cancer model	Vaccine antigen	Animal model	Ref
Cervical carcinoma	HPV16-E7	C57BL/6 mice	(363-366)
	HPV16-L1 $_{1–258}$	C57BL/6 mice	(367)
	Her2/neu	C57BL/6 & FVB/N mice	(362, 368, 369)
Breast carcinoma	VEGFR2	FVB/N mice	(370)
	Endoglin	FVB/N & Balb/c mice	(371)
	MAGE-b	Balb/c mice	(372)
	ISG15	FVB/N & Balb/c mice	(373)
Melanoma	TRP2	C57Bl/6 mice	(374)
	HMW-MAA	C57BL/6, BALB/c & FVB/N mice	(359)
Colon carcinoma	GP70, AH1epitope	C57Bl/6 mice	(375)
Prostate cancer	PSA	C57BL/6 mice	(376)

serotypes of *Salmonella* are defined (377). And, around 50 serovars are recognized as human or animal pathogens. Some of them, such as *S. typhi*, are host-restricted; and the others, like *S. typhimurium*, can infect a wide range of hosts (378) Generally, *Salmonella* infects humans and animals by the oral-faecal route.

Once inside the host, *Salmonella* can invade the intestinal mucosa and subsequently be captured by phagocytes. In contrast to *L. monocytogenes*, *Salmonella* lacks a phagosomal escape mechanism. After invasion into mammalian cells, it remains and divides within phagosomes (379). In order to survive in compartment, *Salmonella* can express several virulence factors encoded in the *phoP* locus and these molecules can induce the formation of spacious vacuoles from phagosomes that allow bacterial persistence and growth in these organelles (380). Proteins secreted by

Salmonella are degraded in the phagosome and are treated as exogenous antigens and presented by MHC II molecules resulting predominantly in Th2 type immune responses. Meanwhile, it has also been reported that the vaccination of mice with one attenuated *S. typhimurium* vaccine induced protective T cell response. It remains unclear how such antigens are released by *Salmonella* and target the MHC I antigen processing pathway. One hypothesis is that infection with *Salmonella* possibly causes leakage of the phago-lysosome (381).

Recently, vast progress has been acquired in *Salmonella* studies. First, the high level of homology in the genome between *Salmonella* and *Escherichia* (382) allows the extension of the enormous knowledge on the genetics of *E. coli* to the study of *Salmonella* and the genetic manipulation of the bacteria, which leads to the construction of a large number of attenuated mutants as well as chromosomal insertions of heterologous antigen-coding genes (383). And, a wide variety of plasmid-based strategies are available to realize the expression of heterologous antigens in *Salmonella* during infection of a host (384). Also, due to de expression of PAMPs and LPS, *Salmonella* can trigger a wide range of innate immune responses (344). Moreover, the growth of this bacterium can be easily achieved in laboratory conditions; it can be administered either systemically (intraperitoneally (i.p.) or intravenously (i.v.)) or orally and stimulate local and systemic immune responses; its biology and the interaction mechanisms with the host has been well studied. All these futures have turned *Salmonella* an attractive delivery carrier for tumor antigens.

Two *Salmonell*a serovars, *S. Typhi* and *S. Typhimurium*, have been extensively assessed as live vectors for antigen delivery for the design and development of new vaccines for human diseases (reviewed in ref (384)). One major limitation in the use of *Salmonella* as a vaccine platform has been its inefficiency at stimulating MHC I-restricted CD8+ T-cell responses. This limitation is most likely due to the intrinsic properties of intracellular lifestyle of *Salmonella* (385). In order to overcome this limitation and strengthen the performances of *Salmonella* as cancer vaccine,

numerous efforts have been consumed to promote antigen secretion by the bacterium. For example, one modified *Salmonella* was created to carry a construct encoding for listeriolysin (hly) from *L. monocytogenes,* which was shown to facilitate escape from phagosomes (381); the hemolysin (HlyA) secretion system of *E. coli*, the prototype of type I secretion systems, was employed in *Salmonella* to ensure secretion of the tumor antigen (386); Heterologous antigen was fused to cholera toxin subunit B (a potent mucosal adjuvant), which was shown to enhance antigen secretion and uptake by APCs and consequently induce a protective cell-mediated immunity (7). Moreover, one more abstract strategy, exploiting the T3SS of *Salmonella,* has been applied to improve antigen delivery and tumor specificity of *Salmonella*-based vaccines, about which we will talk in following section.

As cancer vaccine carriers, *Salmonella* genus has been widely used for DNA vaccine plasmids delivery (table 2) and tumor antigen delivery (majorly through T3SS, table 4). Furthermore, due to its tumor targeting capacity inherent antitumor activity, *Salmonella* genus, itself, has great potential as cancer therapeutics. Some cases have been reported in clinical trials. The clinical Phase I applications of an attenuated *S. Typhimurium* strain administered i.v. or intra-tumorally to an heterogeneous group of cancer patients resulted in tumor colonization and mild side effects (387-389).

T3SS Mediated Antigen Delivery

This secretion machinery is used by a broad spectrum of Gram negative pathogenic bacteria (*Salmonella, Shigella, Yersinia and Pseudomonas,* etc.) to deliver effector proteins to membrane or into cytoplasm of host cells, which alter the function of the host cell to promote the survival of the bacterium (390). The activation of T3SS can be induced *in vivo* via bacteria - host cells contact. Before that moment, most of the effector proteins are expressed and stored within bacteria (391). As the protection against tumor is highly dependent on the triggering of MHC class I-restricted cellular immune response, this distinctive intracellular protein-translocation machinery of T3SS has being widely applied for improving the performance of bacteria based

cancer vaccines (table 4).

Table 4: T3SS Mediated Antigen Delivery Applied for Anti-tumor Immunotherapy in Animal Models.

Cancer model	Vaccine antigen	Animal model	bacterial carrier strain	Ref.
Melanoma B16F10	TRP2153-417+HSP70	C57BL／6J mice	*S. typhimurium* (SL3261)	(392)
Colon carcinoma	Survivin	BALB/c mice	*S. typhimurium, MvP728 (ΔpurD/ΔhtrA)*	(393)
Hepatocellular carcinoma	Hepatitis B virus x (HBx)	C57BL／6J mice	*S. typhimurium* (SL3261)	(394)
Fibrosarcoma	p60	BALB/c mice	*S. typhimurium* (SB824)	(395)
	$p60_{130-477}$	BALB/c mice	*S. typhimurium* (SB824)	(396)
Sarcoma	NY-ESO-1	BALB/c mice	*S. typhimurium(ΔphoP ΔphoQ)*	(397)
Glioblastoma	survivn	BALB/c mice	*S. typhimurium, MvP728 (ΔpurD/ΔhtrA)*	(393)
	$TRP2L_{125-376}$	C57BL／6J mice	*P. aeruginosa(ΔexoS ΔexoT*	(6)

As mentioned before, attenuated *Salmonella* bacterium is considered as a promising candidate for antigen delivery. However, after invading host cells, *Salmonella* is confined within vacuole compartment, which prevents the delivery of heterologous antigens to the host cell cytosol. Several strategies have been established to overcome this problem, among which an innovative approach using the T3SS of *Salmonella* has been investigated with the aim to promote the cytosolic delivery of Ag and their subsequent engagement by MHC I pathway for the generation of CTL response. In *Salmonella*, there are two types of T3SSs that are encoded by *Salmonella* Pathogenicity Island-1 (SPI-1) and *Salmonella* Pathogenicity Island-2 (SPI-2),

respectively. In early stages of infections, the T3SS encoded by SPI1 mediates bacterial invasion into the host cell. In contrast, the T3SS encoded by SPI2 is activated under intracellular conditions and is required for the intracellular survival and proliferation of the bacterium (398).

It has been demonstrated in several infectious models that when peptide fragments from pathogenic viruses or bacteria were fused to *Salmonella* T3SS effector proteins, such as SopE and SptP of SPI-1 T3SS (385, 399, 400); SspH2 and SseF of SPI-2 T3SS (401, 402), they could be engaged by the T3SS and delivered to the host cell cytosol, resulting in the priming of a protective class I-restricted immune response. Similarly, *Salmonella* has also been engineered deliver tumor antigens to the cytosol of antigen-presenting cells via T3SS. Through this way, significant T-cell priming and anti-tumor protection have been observed (396-398).

Besides, T3SS mediated antigen delivery has also been applied in other live attenuated bacterial strains. In our laboratory, we have developed cancer vaccines on profiting the T3SS of *P. aeruginosa.* Different to the other bacteria vectors being investigated, *P. aeruginosa* is extracellular pathogen and thereby reacts with the immune system in a different way; moreover, it is not intestinal bacteria so that it might allow alternative vaccine administration ways. About the characters of T3SS of *P. aeruginosa* and the work already achieved in our laboratory, we will discuss in more details below (see in section C).

Also, *Yersinia* genus can also secret antigens through T3SS machinery. Even no study about the application of *Yersinia* T3SS in cancer vaccine design has been reported until now, the vaccine potential of recombinant *Yersinia enterocolitica* strain has been already investigated in different infectious models. Several studies have demonstrated the potential of the *Yersinia* outer protein E (YopE) to function as a carrier molecule for heterologous antigen delivery (403, 404). Fusion of these antigens to the first 138 amino acids of YopE can lead to the translocation of hybrid proteins into the cytosol of host cells and thereby elicit protective CD8+ T cell

responses (405-407). Furthermore, it was revealed recently that *Yersinia* T3SS is capable of delivering heterologous Ag with high molecular weight and complex structures, such as *E. histolytica* 170 kDa cysteine-rich proteins, to induce antigen-specific type 1 immune responses as well as promote the significant protection against invasive amoebiasis (408).

These current investigations highlight the potential use of the T3SS of bacteria for cancer vaccine development. Then, it should translate such proof of concept to effective therapeutics in humans.

B-IV Recent Strategies for Cancer Vaccine Improvement

One major challenge in the development of cancer vaccines is the immune-tolerance of tumor. In fact, many defined TAAs are self-proteins and therefore generally fail to prime efficient antitumor T cell responses. Thus, a bottleneck for developing successful cancer vaccines is to overcome potential mechanisms of immune suppression against antigenic but weakly immunogenic tumors (409). For this purpose, several strategies have been applied for improving the performances of cancer vaccines.

B-IV a Target of Co-inhibitory Pathway

A number of co-inhibitory molecules expressed on the surface of immune cells, cancer cells, and stromal cells play a role in decreasing immune responses to cancer. Thus, one strategy to enhance the outcome of an antitumor immune approach is to eliminate negative signals imparted to T-cells by co-inhibitory molecules, two of which, B7- H1, and CTLA- 4, have been widely investigated.

B7-H1 is the major ligand for programmed death 1 (PD-1) mediated immunosuppression. It is constitutively expressed on APCs and can be broadly induced on cells in both lymphoid tissues and non-lymphoid peripheral tissues following cellular activation (410). B7-H1 has been shown to promote evasion of

tumor immunity by promoting apoptosis of activated effector T-cells (411) and tumor resistance to T cell mediated lysis (412). Several studies in mouse models revealed that blockade of B7-H1 by mAb could enhance T cell responses and dramatically improves the efficacy of tumor vaccines (413, 414). Currently, anti-B7-H1 human mAbs are under clinical development and Phase I trial results showed that the blockade of B7-H1 by mAb was safe, well-tolerated, and induced a low rate of autoimmune-type side effects (415).

CTLA-4 is a co-inhibitory receptor expressed by activated T cells and Tregs. CTLA-4 is homolog of CD28 and can also bind to B7 on the surface of the APC with an even higher affinity than CD28. Binding of CTLA-4 to B7 initiates a negative signal cascade that leads to down-regulation of the T-cell response (416). Thereby, the blockade of CTLA-4 has been explored in cancer vaccine researches. Treatment with an anti-CTLA-4 mAb in murine models has been shown to improve tumor immunosurveillance and amplify the effects if cancer vaccines (416, 417). These preclinical results led to the ongoing clinical investigations of anti-CTLA-4 humanized mAbs, in which antiCTLA-4 has been used alone or in combination with other immune-mediated anticancer modalities, like cancer vaccines (418).

B-IV b Target of Regulatory T Cells

Treg cells are actively involved in an important mechanism of peripheral T-cell tolerance through their inhibition of self-reactive effector T-cells (419). As many define TAAs are antigens, Tregs mediated regulation is also a main reason for the decrease of antitumor immunity. In fact, the quantity of Treg cells has been seen increased in the tumors and peripheral blood of cancer patients (420, 421). Therefore, it is expected that depletion of these regulatory T cells would enhance antitumor immune responses.

Several studies in murine models showed that blockade or elimination of Treg cells by anti-CD25 mAbs strengthened antigen-specific T-cell responses, enhanced antitumor immunity and improve efficacy of antitumor vaccines, which leaded to the

tumor rejection (422, 423). However, a potential disadvantage of this approach is that effector T-cells also transiently up-regulate the expression of CD25 on their cell surface and therefore being at risk of depletion. One of the alternative choices for Treg cells deletion is denileukin diftitox (ONTAK(®), DAB(389) IL-2). Denileukin diftitox is a recombinant fusion protein of IL-2 and diphtheria toxin and capable of depleting cells that express high-affinity IL-2 receptor (424). In clinical trials, the application of denileukin diftitox has been shown to reduce Treg cells, enhance tumor-specific T-cell responses and improve the efficacy of vaccines in patients with different tumors (318, 425, 426).

B-IV c Diversified Prime-boost Vaccination Protocols

In recent years, it has been believed that the most effective immunization could be achieved through the association of different antigen delivery platforms.

Indeed, there exist a variety of vaccination vectors, but the action mode of each is not identical. Some pattern target cell mediated immune responses, while other modality aim at humoral immune responses. Some methodologies may be more effective in priming naive cells, while others may be more effective in enhancing memory cell function. But, even each antigen delivery pattern presents attractive properties, they also involve some limitations. For example, naked plasmid DNAs vaccines are safe and will not activate anti-vector immune response, however the DNA transfection efficacy via this approach is not high enough to prime efficient immune responses. Some of the most effective methods of immunization, like the use of some recombinant virus or bacteria, can be applied only a limited number of times because of host anti-vector responses, and also there are always the concerns of safety.

Thereby, combination of various antigen presentation systems would prime broad immune responses and achieve the best performance. The cooperation between different platforms, as part of prime-boost protocols, appears to be appropriate for therapeutic vaccinations against cancer. Numerous preclinical studies demonstrate the

advantages of diversified prime and boost protocols (427-430).

B-IV d Combination of Cancer Vaccines with Other Therapeutics

Antitumor effects induced by cancer vaccines may be difficult to obtain with large tumor masses for a variety of reasons, including (1) large tumor masses are difficult to penetrate by T cells; (2) the quantity of T cells generated by the host immune system would be far outnumbered by the cells in a large tumor mass; and (3) tumor cells produce immunoregulatory molecules that have the ability to suppress or inhibit T-cell function (431). Thereby, cancer vaccines might represent greatest performance in combination with other therapies, including radiotherapy, chemotherapy and cytokines.

Radiation is the standard of care for many types of cancer because of its direct cytotoxic effect on the tumor or its palliative effects on the patient. It has been recently reported that local irradiation of tumors with doses insufficient to induce tumor cell death could result in changes on the phenotype of the tumor cells that include the up-regulation of MHC, Fas, ICAM-1, and various tumor associated antigens (432-434). As a result of these changes, irradiated tumor cells are more susceptible to T cell mediated immune attack. In the clinical setting, the approach has been investigated in a phase II clinical trial in patients with localized prostate cancer, randomized to receive a PSA based poxviral vaccine plus radiotherapy versus radiotherapy alone (435).

Like radiotherapy, the use of various types of chemotherapy in combination with vaccines has resulted in enhanced antitumor immune responses. Although the mechanisms involved vary among the various types of cytotoxic drugs employed, in general, drugs can: (a) induce “immunogenic death” of tumor cells, leading to activation of DCs followed by antigen presentation to T cells (436, 437) or (b) modulate the phenotype of the tumor cells making them more susceptible to immune-mediated killing. For example, it has been shown that treatment of human colon carcinoma cell lines with 5-fluorouracil or cisplatin enhances their lytic

sensitivity to antigen-specific CD8+ cytotoxic T lymphocytes, by inducing expression of ICAM-1 and Fas (438).

Cytokines can promote the differentiation, activation, or recruitment of APC, therefore enhance antigen presentation and activation of antigen-specific T-cell responses, such as GMC-SF; or promote T-cell proliferation, activation, and effector function, like IL-2 (439), IL-7 (440), IL-12 (441) and IL-15 (442). These cytokines have been investigated in combination to cancer vaccine, significant enhanced antitumor immune responses have been observed.

C Type III Secretion System of Pseudomonas aeruginosa

C-I *P. aeruginosa* Bacteria

P. aeruginosa is a common environmental gram-negative bacillus. It acts as an opportunistic pathogen to humans under several particular circumstances, including: cystic fibrosis, immunosuppression (especially in iatrogenic cases), invasive mechanical ventilation, chronic bronchial diseases, nosocomial skin-piercing procedures (like surgery), and patients bearing various prostheses at mucosal interface (such as contact lenses or urinary catheters). Outside of these situations, due to the protection by physical barriers (mucous membranes, skin, mucociliary clearance, etc.), innate immunity (NKs, macrophages, etc.) and acquired immunity, *P. aeruginosa* is incapable of developing an infection.

In above mentioned situations, *P. aeruginosa* can be a pathogen. The implementation of its virulent factors are associated with direct cytotoxicity and local inflammatory reaction, which are often deleterious and can lead to poor functional outcome (for example, keratitis, destruction of lung parenchyma, etc.) or even vital prognosis (pneumonia, septic shock, etc). Many studies have suggested that the T3SS was probably the factor that contributed most to the virulence of this germ.

C-II T3SS

Gram-negative bacteria dominate six types of protein secretion systems: 1) type I secretion system, which transports proteins to the extracellular space through two bacterial membranes due to two pore-forming proteins that are linked between them by a periplasmic protein; 2) type II secretion system, which transports proteins from the periplasmic space to extracellular space via a protein embedded in the outer membrane; 3) T3SS, of which the secretion device traverses not only bacterial cell wall but also the membrane of a eukaryotic cell being in touch with the bacteria, and through which the toxins will be directly delivered into the cytoplasm of the eukaryotic cell; 4) and the system of type IV secretion, which delivers different types of substrates (DNA, proteins ...) in the extra-bacterial medium by a system similar to that used during the combination, and is not described in *P. aeruginosa*; (443) 5) type V secretion system (T5SS), which transports proteins from interior of a bacterial cell to its exterior by two steps: first, proteins can cross the inner membrane using the Sec system; then, beta-barrel domain of proteins will form a channel in the outer membrane, which allow the passenger protein to be secreted (444); 6) type VI secretion system (T6SS), which has been recently identified by the group of John Mekalanos in 2006 (445). The Type VI secretion system gene clusters contain from 15 to more than 20 genes. Hcp and VgrG have been shown to be nearly universally secreted substrates of the system. The secretion mechanism of this system has not yet been clearly identified.

The T3SS was originally described in *Yersinia* and *Salmonella.* Up now, T3SS has been found in the most Gram-negative bacilli and usually plays a dominant role for the virulence of these germs. The secretion apparatus of T3SS consists of about twenty remarkably preserved proteins, which, after assembling into a syringe-like pattern, can traverse the two bacterial membranes, insert into the membrane of a eukaryotic cell and allow the intra-cytoplasmic delivery of toxins. Generally, these transported toxins can modify the functions of target cell in the interest of bacteria, for example, the transformations of cytoskeleton and consequent modification of

phagocytosis and organelles transport; alterations of intracellular signaling for inducing apoptosis; decreasing of mediators production, induction of necrosis, etc.

As T3SS was introduced as an essential part to achieve this work, we describe later in more details the T3SS of *P. aeruginosa* and the progresses obtained in our laboratory in this field before this work.

C-II-a The Structure of T3SS

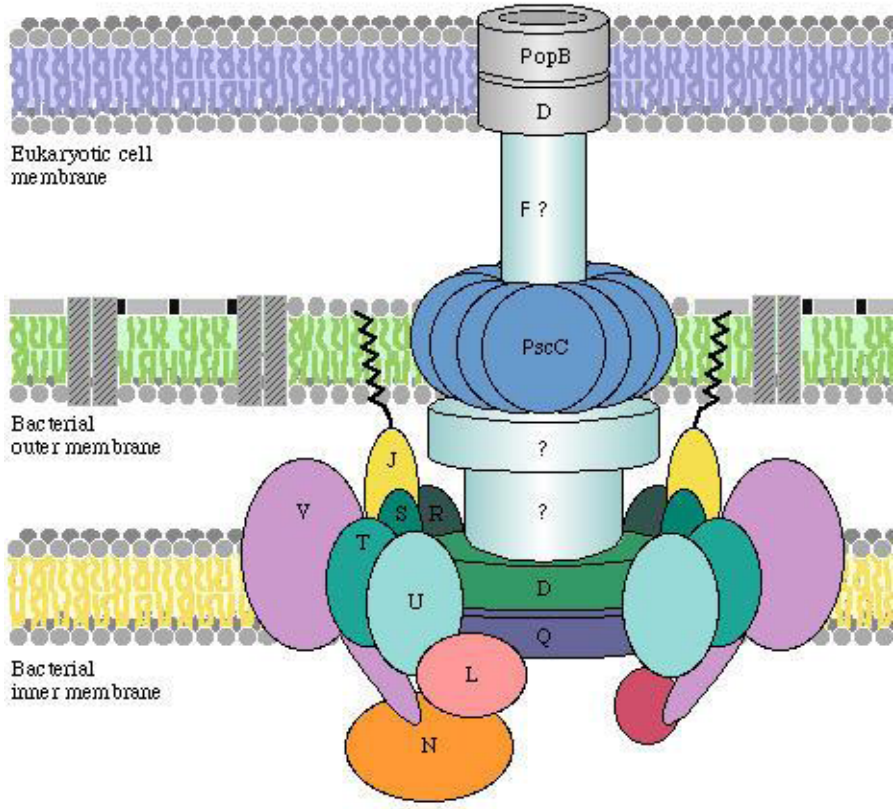

Figure 9: Type III Secretion System of *P. aeruginosa* (Adapted from Frank DW *et al.* (446)).

The secretion apparatus is embedded in bacterial membranes and continues with a needle-like structure that inserts terminally into the membrane of a eukaryotic cell, which allows the intracellular translocation of effectors.

The T3SS (Fig. 9) can be disassembled into two structures: 1) the secretion apparatus, which is a protein complex embedded in two bacterial membranes and the organization of which is very similar to that of the base of flagellate; 2) the

translocation apparatus, which is continuum to the secretion apparatus and can inserted into the membrane of a eukaryotic cell. The structural organization of this assembling complex as well as the involved proteins are quite retained from one bacterial genus to another so that the information collected in one genus (crystallography, protein-protein interaction, polymerization ...) could be transposed, at least in one part, to others. Thereby, the understandings of T3SS obtained from *S. typhimurium*, *Y. pseudotuberculosis*, *S. flexnerii* and *P. syringae* permit the deduction of T3SS structure in *P. aeruginosa*.

The part inserted into the inner membrane of the bacterial cell wall has a ring structure composed of proteins PscD, PscR, PscS, PscT, PscU and PscV. The part inserted into the outer membrane of the bacteria is a pore formed by the oligomerization of PscC. The two parts are connected by the protein PscJ, all of which form a pipe with the diameter of 50 Å (447).

Following the part crossing two bacterial membranes, there is a duct composed of a polymer of PscF protein and the end of this duct is the PcrV protein. Then, at the contact interface with a eukaryotic cell, two other proteins, PopB and PopD, will be transported by T3SS from the interior of bacteria to the cell membrane where they will insert and form a pore, after which T3SS is suitable for the intracellular translocation of effectors. During the translocation channel formation process, PcrV probably act as the chaperone which allows the oligomerization of PopB and PopD (446, 448). The role of PcrV has been proved by the application of anti-PcrV antibodies during the infections with *P. aeruginosa* (449).

C-II-b Effectors Delivered Through T3SS

Four toxins are secreted through the T3SS of *P. aeruginosa*: exotoxin (Exo) S, ExoT, ExoU and ExoY. Apart from these, PopB and PopD are also regarded as toxins, because their T3SS terminal pore forming activity was shown to be deleterious for the cells.

ExoS and ExoT

ExoS is a protein of 49 kDa and Exo T is a protein of 53 kDa. ExoS and ExoT share 76% amino acid identity, and are bifunctional effectors with N-terminal RhoGAP domains and C-terminal ADP-ribosyltransferase (ADPRT) domains. The GAP domains of either enzyme target small Rho-like GTPases, and can stimulate the reorganization of the actin cytoskeleton by inhibition of Rac and Cdc42 and stimulate actin stress fiber formation by inhibition of Rho (450-453), which probably allows bacteria to escape phagocytosis.

Activation of ADPRT activity needs a soluble eukaryotic protein, FAS (factor activating exoenzyme S) (454), which belongs to a highly conserved, widely distributed eukaryotic protein family, collectively designated as 14-3-3 proteins (455). Though both ExoS and ExoT have ADPRT activity, they ADP-ribosylate different substrates. ExoS has poly-substrate specificity and preferentially targets Ras superfamily proteins that are integral to cell signaling pathways (456), whereas ExoT has a more restricted host proteins and specially targets CrkI and CrkII, two host kinases that regulate focal adhesion and phagocytosis (457). ExoS ADPRT activity has effects on cell growth (DNA synthesis), morphology (cell rounding) and cellular adherence (458), whereas ExoS GAP activity plays an antiphagocytic role (459). The C-terminal part of Exo S possesses an ADP-ribosylation activity (ADPR) to the small G proteins of the Ras family. This ADPR activity inhibits transduction signal mediated by Ras, which could induce cell death.

Some studies have shown that proteins secreted through the T3SS of *Yersinia* express the secretion signal in N-terminal (460). In *P. aeruginosa*, the expression of a domain contained six amino acid in N-terminal in various toxins secreted by the T3SS (461) suggests that this is a secretion signal, but no work has so far confirmed this opinion. The group of D. Frank (462) deduced from their results that the secretion signal is contained in the first 99 amino acids of ExoS, however the proof was not so convincing. Our previous work has defined that the first 54 N-terminal amino acids of

ExoS form the ideal sequence allowing the secretion of the toxin via T3SS (3).

The *exoS* gene was found in 11-60% of the clinically obtained strains (especially in strains isolated from acute pneumonia, bacteremia and infection of burning). And, the *exoT* gene is expressed by nearly all strains.

ExoU

ExoU, parallely identified as PepA, is a protein of 72 kDa. ExoU is the most cytotoxic (461) and possesses lipase activity similar to patatin, human calcium independent phospholipase A2 (iPLA2) and cytosolic phospholipase A2 (cPLA2) (463).

ExoU is found in about one-third of clinical isolates, and these ExoU-expressing strains in 90% of cases are associated with severe disease (464). ExoU has been shown to cause fatality in a mouse model of lung infection (461), and expression of ExoU in *P. aeruginosa* strains lacking it increases virulence in a mouse model of acute pneumonia

Curiously, most strains of *P. aeruginosa* possess either the gene coding ExoS or the gene coding ExoU, but not both (465). The *P. aeruginosa* strain-CHA used as vector in our laboratory does not express ExoU.

ExoY

ExoY is an adenylate cyclase of 40 kDa and expressed by about 90 % *P. aeruginosa* strains. Active ExoY results in an elevation of intracellular cAMP and cell-morphology changes. And, ExoY only intoxicates host cells at sites of colonization due to the delivery mechanism of T3SS. Recently, studies showed that ExoY could inhibit *P. aeruginosa* invasion of epithelial cells coinciding with adenylate cyclase-mediated cytoskeleton disruption (466). However, ExoY does not seem to be important for infection (467).

PopB and PopD

The status of these two proteins is still ambiguous. Recent studies suggested that on one hand, they participate in the structure of the T3SS and are essential for the translocation of the toxins described above; on the other hand, the induction of T3SS by calcium depletion (see below) leads to their release into the environment as exotoxins likewise and endues them the role of pore-forming toxin (468).

Chaperones of T3SS Toxins

Chaperones are proteins that associate transiently with one or several substrates to prevent premature or incorrect intra-molecular or inter-molecular interactions. In T3SSs, before being secreted, some proteins are stored in the cytoplasm, which is not their final destination. These proteins need to be stabilized, separated from other interaction partners, and maintained in a secretion competent state with the help of chaperones (469). Thereby, T3SS chaperones have been suggested to i) act as anti-aggregation and stabilizing factors; ii) be secretion signal and set a secretion hierarchy; iii) function as anti-folding factors; iv) participate in the regulation of the T3SS (470).

In *P. aeruginosa*, several chaperones have been identified. SpcU has been defined as the specific *P. aeruginosa* chaperone for ExoU (471). In our previous work, ORF1 or SpsC has been suggested as the chaperone for ExoS (472).

C-II-c The Role of T3SS in *P. aeruginosa* Virulence

The T3SS appears to be predominant virulence factor during the acute phase of *P. aeruginosa* infection. It can induce the necrosis and apoptosis of various cell populations and lead to the impairment of phagocytes function. The damage caused by T3SS is more notable in lung infection.

T3SS can target a variety of cell populations, like dendritic cells, macrophages, neutrophils but also epithelial cells. The contact between these cells and the bacterium

can activate T3SS and subsequently either bring out cellular structure changes or induce apoptosis or necrosis of these cells. Different cellular damages induced by *P. aeruginosa* infection have been observed in several i*n vitro* experiments. For example, some ExoS+ strains as well as Exo U+ strains could develop cytotoxicity against macrophages and the cytotoxicity was T3SS-dependent (464); cytotoxicity against polymorphonuclear neutrophils, which can lead to rapid necrosis of the latter, is also T3SS-dependent dependent but ExoU-independent (473); moreover, the structure change in epithelial cells induced by the interaction with *P. aeruginosa* is still dependent on T3SS, and the presence of ExoU is crucial under this situation (474).

In animal models, the presence of T3SS is necessary for intra-pulmonary multiplication (474) and extra-pulmonary dissemination (467). Numbers of studies defined the roles of T3SS toxins. The experiments comparing a ExoU-ExoS+ strain and a ExoU-ExoS- strain demonstrated the decisive effection of ExoS relative to ExoT and ExoY; the comparison between a ExoU+ExoS-strain and a ExoU-ExoS+ strain revealed a decisive role of the two toxins in the intrapulmonary multiplication and a predominant role of ExoU in the dissemination extra-pulmonary (475). In some models, Exotic and ExoY haven’t play particular roles for *P. aeruginosa* virulence, and ExoT even presented a protective effect (474).

Clinical observations reported that the activation of T3SS during *P. aeruginosa* infection and the secretion of T3SS toxins were associated with poor clinical outcomes (476).

C-II-d Regulation of T3SS of *P. aeruginosa*

P. aeruginosa can be resistant to different environments that exert stresses to its metabolic resources or its physicochemical properties and expose potential dangerous elements to this germ. An important part of the genome of *P. aeruginosa* is dominated by regulatory genes, which could explain the powerful adaptability of *P. aeruginosa.* The T3SS plays an important role in the resistant ability of *P. aeruginosa* and it can be activated by at least three environmental signals (477, 478): i) *in vivo* contact with

eukaryotic host cells; ii) *in vitro* removal of calcium from medium; iii) the presence of serum (due to the existence of type III secretion factors, which were recently identified as albumin and casein (479).

T3SS of *P. aeruginosa* is tightly transcriptionally and post-transcriptionally regulated. All T3SS genes are coordinately regulated by ExsA, an AraC-like master transcriptional activator, which binds to a consensus sequence in the promoter region of these genes (480). Besides the major Exs-A dependent regulatory mechanism, several factors, including metabolic factors, quorum sensing (QS) systems, cyclic AMP with Vfr, PtrA, etc. (481) can also impact T3SS activation. However, their action approaches have not yet been perfectly understood.

ExsA-dependent Regulation

The regulator genes involved in this mechanism are *exsA, exsB, exsC, exsE* (assembled in *exsCEBA* operon) and *exsD* (locating in *exsD-pscBL* operon).

ExsA is a member of the AraC family of transcriptional regulators. ExsA functions as a central transcriptional regulator of T3SS because it is required for the expression of all T3SS genes, including itself, by binding to the consensus sequence TXAAAAXA, which is located approximately 51 or 52 bp upstream of the transcriptional start site of four operons (*pscN-U, popNpcr1234DR, pcrGVHpopBD, exsDpscB-L*) encoding the T3SS apparatus and translocation machinery, the operon *exsCEBA*, and the effector proteins (480).

exsB is not translated. Untranslated exsB region mediates either the stability or the translation of exsA (482).

ExsC, a type three secretion chaperone of ExsE, functions as an anti-anti-activator due to the fact that ExsC binds to and sequesters the anti-activator, ExsD, under T3SS-inducing conditions, thus freeing ExsA to activate the expression of T3SS genes (483).

ExsE is a secreted regulator of T3SS in *P. aeruginosa* (484). When the secretion channel is closed, ExsE is complexed with its chaperone (ExsC) in the cytoplasm, and transcription of the T3SS is repressed by sequestration of ExsA by ExsD. On the contrary, when the secretion channel is opened, ExsE is secreted, leaving ExsC free to interact with ExsD and releasing ExsA, thereby allowing liberated ExsA to activate transcription of the T3SS genes (see more details in later paragraph).

ExsD was identified as the first negative regulator of *P. aeruginosa* T3SS (485). Bacterial two-hybrid data indicated that ExsD interacts with the ExsA and functions as an antiactivator to regulate the expression of T3SS genes. ExsD is essential to prevent inappropriate expression of T3SS genes in conditions such as in the absence of the appropriate environmental cue for secretion or in mutants lacking a functional TTSA.

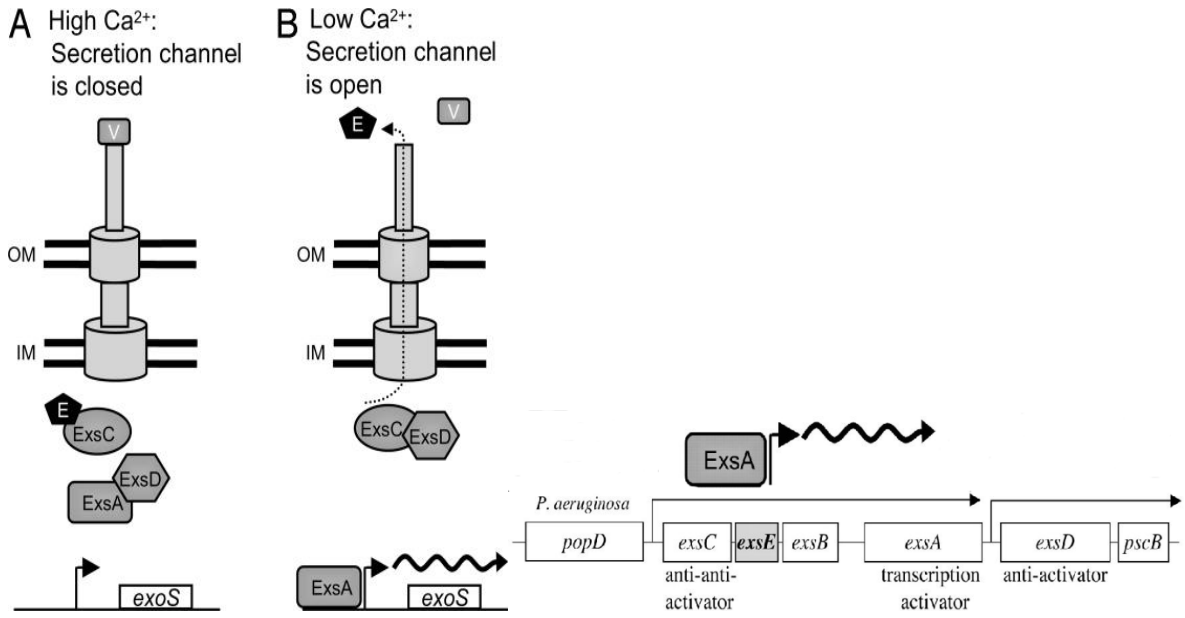

Figure 10: A Model for the Induction of the Type III Secretion Regulation.

In calcium depletion or cell contact situations, ExsE can be secreted trough T3SS, which can subsequently release ExsC and the inhibition of ExsA by ExsD. And finally, T3SS is activated. All proteins except ExsE (E) and PcrV (V) are indicated by name. Reprinted from Rietsch *et al.*, 2005 (484) and Urbanowski *et al.*, 2005 (486).

This complex regulatory network can be explained by figure 10 (484, 486). The first gene of the *exsD-pscBL* operon encodes ExsD which can inhibit ExsA activity by forming an inactive complex. ExsC is encoded by the first gene of the *exsCEBA* operon, Fixation of ExsD to ExsA can be prevented by the interaction between ExsC and ExsD. And, ExsE, encoded in the exsCEBA operon, can interact with ExsC and prevents it to bind with ExsD. Upon T3SS activation by calcium depletion or cell contact, ExsE is secreted through it, thus releasing ExsC and the inhibition of ExsA by ExsD.

ExsA-independent Regulation

The influences mediated by metabolic signals/stresses to T3SS can be demonstrated by several examples. Mutants lacking pyruvate dehydrogenase (*aceA* or *aceB*), or a glucose transport regulator (*gltR*) fail to induce the T3SS expression (487, 488). In the presence of histidine in the medium, excessive uptake and degradation of histidine due to the overexpression of histidine utilization genes, for instance *hutT*, abolishes the ability to induce exoS expression and rends the bacteria non-cytotoxic (489). A dose-dependent upregulation of T3SS gene expression is seen with increasing NaCl concentrations (478).

QS has been demonstrated to be crucial in the pathogenesis of *P. aeruginosa* infections and controls virulence factor gene expression (490, 491). Mutants lacking the *rhl* quorum sensing system show increased expression of T3SS genes and secretion of ExoS at an earlier stage during exponential growth (492, 493). Analysis of 5 *rhl* QS-deficient clinical isolates showed that, only one isolate produced normal T3SS effector proteins ExoS and ExoT, two isolates produced ExoT only, and two isolates produced no effector proteins (494).

Moreover, a membrane-associated adenylcyclase (CyaB), responsible for cAMP synthesis, and a cAMP-binding CRP homologue called Vfr are shown to be required for the expression of T3SS genes (488). Modulation of intracellular cAMP seems to

be an important mechanism controlling T3SS expression.

truA, gene encoding pseudouridinase enzyme, is required for the expression of type III secretory genes. Pseudouridination of tRNAs is proposed to be critical for the translation of T3SS genes or their regulators (495).

dsbA, gene encoding a periplasmic thiol/disulphide oxidoreductase affects expression of multiple virulence factors, including T3SS genes. This is probably a non-specific effect resulting from abnormal protein folding caused by the lack of disulphide bonds (496).

C-III Previously Achieved Work in Our Laboratory

We have been studying the T3SS of *P. aeruginosa* and trying to construct T3SS based antigen delivering bacterial strains for several years. The intracytoplasmic antigen delivery was carried out through the fusion of interest antigens with T3SS toxin ExoS. The development and application of bacterial vectors are still on going further. Previously achieved work focused on the determination of the necessary minimal sequence of ExoS for the secretion of an ExoS-antigen fusion protein, the development of different attenuated strains, the development of a plasmid construct in which ExsA was inserted in the dependence of an inducible promoter, and the application of attenuated bacterial vectors in anti-tumor vaccinations.

C-III-a Attenuation of Bacterial Strain

Firstly, two T3SS toxin genes (*exoS* and *exoT*) were deleted in CHA strain according to our published Cre-*lox* system (497) and this attenuated strain is called CHA-OST. *In vitro* experiments showed that the cytotoxicity of CHA-OST strain against bone-marrow-derived myeloid DCs from C57BL/6 mice the PLB985 cell line has been reduced to 50% of that of the parental strain (3). An international patent about this bacterium used as interest protein delivery vector has been applied in 2005 (WO 2005/049644).

Afterwards, other genes, *aroA* and two genes participating in the QS system (*lasI* and *rhlI*), have been targeted for further virulence attenuation. *aroA*-encoded 3-phosphoshikimate 1-carboxyvinyltransferase is a key enzyme in aromatic amino acid synthesis; the *aroA* deletion confers auxotrophy for aromatic amino acids and has been successfully used to elaborate attenuated *P. aeruginosa* strains for the purpose of anti-Pseudomonas vaccination. *lasI* and *rhlI* encode the two enzymes producing QS homoserine lactones 3-oxo-C12-HSL and C4-HSL, respectively; *P. aeruginosa* QS inactivation has been associated with virulence attenuation in various animal models for injury or illness, including those for pneumonia, burns, and pyelonephritis. Thereby, basing on Cre-*lox* system, CHA-OA (*ΔexoS ΔexoT ΔaroA*), CHA-OAL (*ΔexoS ΔexoT ΔaroA ΔlasI*), and CHA-ORL (*ΔexoS ΔexoT ΔrhlI ΔlasI*) have been constructed, of which the cytotoxicity has been significantly reduced (10).

C-III-b Construct of Inducible ExsA Containing Plasmid

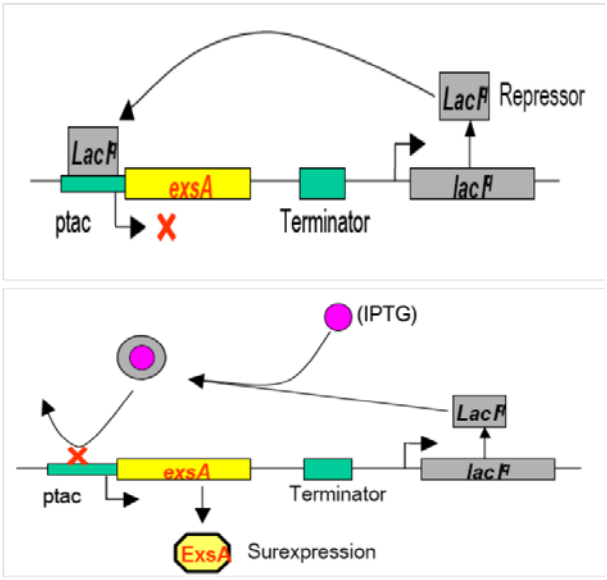

Figure 11: Inducible Protein Expression System.

The addition of IPTG can suppress the quiescent effect imparted by $LacI^{Q}$ to ptac, which can initiate exsA transcription and permit the expression of T3SS genes.

The sequence of the transcriptional activator-ExsA was cloned in a plasmid pEsxAind in dependence of the ptac promoter. This plasmid contains the sequence

encoding the repressor of ptac, $LacI^Q$. In non-induced situation, ptac is coupled with $LacI^Q$ and the transcription of ExsA is inhibited. With the presence of a ligand of $LacI^Q$ (lactose, isopropyl-β-D thiogalactopyranoside (IPTG)), the quiescent effect imparted by $LacI^Q$ to ptac can be eliminated, consequently *ExsA* gene will be transcribed and T3SS genes will be expressed (Fig. 11).

Studies realized in the laboratory shown that the addition of IPTG to the culture medium of one pExsAind transformed CHA strain effectively induced the transcription of the entire regulon in dependence of ExsA. And, the high-level protein secretion was only depended upon calcium depletion *in vitro* or contact with target cells *in vivo*.

C-III-c Determination of the Necessary Minimal Sequence for Protein Secretion (3)

In order to determine the region of the *P. aeruginosa* T3SS toxin ExoS required for the secretion and translocation of an ExoS-fused antigen, a series of fusion proteins containing different sequences of ExoS (the N-terminal 129, 96, 54, or 17aa of ExoS) and three reporter proteins that are not normally secreted by the T3SS (*P. aeruginosa* inhibitor of vertebrate lysozyme (IVY), *P. putida* catechol 2,3-dioxygenase (CDO), and green fluorescent protein (GFP)) were generated, respectively. The different fusion protein coding plasmids were transferred into *P. aeruginosa* strain CHA and the secretion of the fusion proteins was assessed after T3SS induction mediated by calcium depletion, a condition known to trigger T3SS.

It was observed that with the N terminal ExoS fragments of 129 or 96 aa, only IVY was efficiently secreted but not CDO or GFP. All reporter proteins fused with the N terminal 17 aa of ExoS were secreted only at low levels. However, high secretion levels when each of the reporter proteins was fused with the N terminal 54 aa of ExoS. Thereby, the first 54aa of Exo S is considered as the most optimal sequence for the secretion of fusion protein via T3SS.

C-III-d Application of Antigen Delivering Bacterial Vectors in Anti-tumor Vaccination

The antigen delivery efficiency and anti-tumor immunity activation ability of these attenuated T3SS based bacterial vectors have been evaluated not only *in vitro* but also in murine models and their performance has been confirmed;

Using OVA as model antigen, CHA-OST strain can provide 100% prophylactic protection against B16OVA tumor; CHA-ORL, CHA-OA, CHA-OAL can be applied *in vivo* at high dose but with the reduced anti-tumor efficiency (3, 10).

Moreover, our platform technology has been also demonstrated so efficient to break tolerance against self antigens. In murin glioblastoma model, CHA-OST-TRP2 strain can significant delay the tumor formation in prophylactic assay (6).

However, in different therapeutic experiments, the results were not so satisfied, maybe because of the non-optimized vaccination regime.

D The Objectives of This Work

Due to the endowed effective ability to deliver antigen to cytoplasm of APCs *in vivo*, T3SS based attenuated bacterial vectors, such as *Yersinia, Salmonella, Shigella*, attracted more and more attention for their potential interest in cancer vaccine development.

Basing on the studies of our laboratory during the last years, a great amount of characterizations of *P. aeruginosa* has been identified. In particular, T3SS antigen delivery induced immune response is MHC I restricted, CD8+ T cell mediated response. On the other hand, as the genome of *P. aeruginosa* has been identified and mapped, the attenuation of this germ through genetic manipulations has become realistic. Due to these progresses, we have developed live attenuated *P. aeruginosa* T3SS based vectors and applied them as antigen carriers for cancer vaccine design.

The ultimate objective of all cancer researches is that the results of these researches can be applied in human cancer treatment, which is also the important consideration for us. For this purpose, efficacy, safety (virulence attenuation), and the production process of bacterial vector complying with current good manufacturing practices grade are essential.

To respond to these requirements, in this study, we attempted to: 1) further enhance the performance of bacterial vectors; 2) validate bacterial vaccines efficacy in therapeutic vaccination mode; 3) generate safe, efficient vaccine candidate for clinical application on following good manufacturing practices; 4) evaluate bacterial vector in a humanized animal model; and 5) investigate a general issue for all bacterial/viral vaccine: pre-existing anti-vector immunity.

MATERIALS AND METHODS

A Bacterial Strains and Plasmids

Table 5: Bacterial Strains Used in This Study

Strains	Description	Source
E.coli		
DH5α	used for plasmid transformation	Invitrogen
S17-1	used for mating constructs into *P. aeruginosa*	Simon et al. 1983
P.aeruginosa		
CHA-OST	Mutant of CHA strain (a mucoid strain isolated from the lungs of a cystic fibrosis patient (498)) with the deletion of ExoS & ExoT encoding genes (CHA*ΔexoSΔexoT*)	(497)
CHA-OST S54-P-OVA	CHA-OST strain transformed with the plasmid pEAI S54-Padre-$OVA_{248-376}$	This study
CHA-OST S54-P-TRP2L	CHA-OST strain transformed with the plasmid pEAI S54-Padre-$TRP2_{125-376}$	This study
CHA-OST S54-TRP2L-P	CHA-OST strain transformed with the plasmid pEAI S54- $TRP2_{125-376}$-Padre	This study
CHA-OST S54-P-TRP2S	CHA-OST strain transformed with the plasmid pEAI S54-Padre-$TRP2_{291-376}$	This study
CHA-OST S54-TRP2S-P	CHA-OST strain transformed with the plasmid pEAI S54- $TRP2_{291-376}$-Padre	This study
CHA-OST S54-P-GP100	CHA-OST strain transformed with the plasmid pEAI S54-Padre-$GP100_{21-150}$	This study
CHA-OST- S54-P-HER2	CHA-OST strain transformed with the plasmid pEAI S54-Padre- $HER2_{340-507}$	This study
CHA-OST S54-P-OVA S54-P-TRP2L	CHA-OST strain transformed with the plasmid pEAI S54-Padre-$OVA_{248-376}$ S54-Padre-$TRP2_{125-376}$	This study
CHA-OST S54-P-TRP2L S54-P-OVA	CHA-OST strain transformed with the plasmid pEAI S54-Padre- $TRP2_{125-376}$ S54-Padre-$OVA_{248-376}$	This study
CHA-OST S54-P-TRP2L S54-P-GP100	CHA-OST strain transformed with the plasmid pEAI S54-Padre- $TRP2_{125-376}$ S54-Padre-$GP100_{21-150}$	This study
CHA-OST AroA LasI (CHA-OAL)	Mutant of CHA strain with the deletion of ExoS, ExoT, AroA & LasI encoding genes (CHA*ΔexoSΔexoTΔaroAΔlasI*)	(10)

CHA-OAL S54-P-OVA	CHA-OAL strain transformed with the plasmid pEAI S54-Padre-$OVA_{248-376}$	This study
CHA-CLIN1	CHA-OAL strain adapted in one chemically defined medium	This study
CHA-CLIN1 S54-P-OVA	CHA-CLIN1 strain transformed with the plasmid pEAI S54-Padre-$OVA_{248-376}$	This study
CHA-OST-*Δ*BD	Mutant of CHA strain with the deletion of ExoS, ExoT, PopB & PopD encoding genes (CHA*ΔexoSΔexoTΔpopBΔpopD*)	This study
CHA-OST-*Δ*BD S54-P-OVA	CHA-OST-*Δ*BD strain transformed with the plasmid pEAI S54-Padre-$OVA_{248-376}$	This study

Table 6: Plasmids Used in This Study

Plasmids	Description	Source
pExsAind	pUCP20 (Escherichia-Pseudomonas shuttle vector; $Ap^r(Cb^r)$ (499))-derived vector containing IPTG-inducible exsA; $Ap^r(Cb^r)$	(500)
pEAI-S54-$OVA_{248-376}$	Cloning of *orf1* sequence and *exoS54-ova*$_{248-376}$ fusion sequence in pExsAind plasmid	(3)
pEAI S54-Padre $OVA_{248-376}$	Derived from pEAI-S54-OVACter in which *exoS54-ova*$_{248-376}$ fusion sequence was replaced by *exoS54-padre-ova*$_{248-376}$ fusion sequence.	This study
pEAI S54-Padre-$TRP2_{125-376}$	Idem; *exoS54-padre-trp2*$_{125-376}$ fusion sequence.	This study
pEAI S54-$TRP2_{125-376}$-Padre	Idem; *exoS54-trp2*$_{125-376}$*-padre* fusion sequence	This study
pEAI S54-Padre-$TRP2_{291-376}$	Idem; *exoS54-padre-trp2*$_{291-376}$ fusion sequence	This study
pEAI S54 -$TRP2_{291-376}$-Padre	Idem; *exoS54-trp2*$_{291-376}$ *-padre* fusion sequence	This study
pEAI S54-Padre-$GP100_{21-150}$	Idem; *exoS54-padre-gp100*$_{21-150}$ fusion sequence	This study
pEAI-S54-PADRE-$HER2_{340-507}$	Idem; *exoS54-padre-her2*$_{340-507}$ fusion sequence	This study
pEAI S54-Padre-$OVA_{248-376}$ S54-Padre- $TRP2_{125-376}$	Idem ; whereas two independent fusion sequences, *exoS54-padre-ova*$_{248-376}$ and *exoS54-padre-trp2*$_{125-376}$, were sequentially cloned in pExsAind plasmid	This study
pEAI S54-Padre-$TRP2_{125-376}$	Idem; *exoS54-padre-trp2*$_{125-376}$ fusion	This study

S54-Padre-OVA$_{248-376}$	sequence and *exoS54-padre-ova$_{248-376}$* fusion sequence, sequentially.	
pEAI S54-Padre-TRP2$_{125-376}$ S54-Padre- GP100$_{21-150}$	Idem; *exoS54-padre-trp2$_{125-376}$* fusion sequence and *exoS54-padre-gp100$_{21-150}$* fusion sequence, sequentially.	This study
pEX100T mut *popBpopD*	pEX100Tlink-derived vector containing up- and downstream region of PopB-PopD and Gmlox; Ap^r, Gm^r	Madiha Derouazi, 2005

B Manipulations of Bacteria

B-I Manipulations of *Escherichia Coli*

B-I-a Bacterial Media

Luria Broth (LB) medium: Luria Broth Base (Miller) powder (Sigma): 25 g/L

H_2O QSP

Luria Broth (LB) plate: Luria Broth Base (Miller) powder: 25 g/L

Agar (Invitrogen): 10 g/L

H_2O QSP

Antibiotics (identical in liquid and solid media): Ampicillin (Sigma): 100 µg/mL

Gentamycin (Sigma): 10 µg/mL

B-I-b Transformation of *E.Coli* Strains with Plasmids

a) Thaw competent bacteria cells on ice;

b) Add 1-10 ng of plasmid DNA to 50 uL of competent cells and mix well. Incubate mixture on ice for 30 minutes;

c) Heat shock the cells for 30 seconds at 37C;

d) Place back on ice for 2 minutes;

e) Add 450 µL of pre-warmed SOC medium and shake at 250 rpm at 37°C for 1 hour to express the antibiotic resistance gene;

f) Spread 100 mL and 400 mL of transformed cells onto the pre-warmed LB plates containing appropriate antibiotic.

g) Incubate plates overnight at 37°C.

B-II Manipulations of *P. aeruginosa*

B-II-a Bacterial Media

Luria Broth (LB) medium: Luria Broth Base (Miller) powder: 25 g/L

H_2O QSP

Luria Broth (LB) plate: Luria Broth Base (Miller) powder: 25 g/L

Agar: 10 g/L

H_2O QSP

Pseudomonas Isolation Agar (PIA) plate: Difco Pseudomonas Isolation Agar: 45 g/L (Becton Dickinson)

Glycerol (VWR): 20 mL/L

H_2O QSP

Antibiotics: Carbecillin: 300 μg/mL in liquid medium, 600 μg/mL in solid medium

Gentamycin: 200 μg/mL in liquid medium, 400 μg/mL in solid medium

B-II-b Transformation of *P. aeruginosa* with Plasmids by Electroporation

a) Transfer 1 mL pre-cultured *P. aeruginosa* to a 1.5 mL micro-centrifuge tube and centrifuge at 13 000 rpm for 3 min.

b) Remove the supernatant, resuspend the cells in 500 μL cold sucrose (300 mM) and centrifuge at 13 000 rpm for 3 min.

c) Repeat the step 2 for 2 times.

d) Add 1-3 mL plasmid (10-30 ng) and mix well with the cell. Incubate on ice for 30 minutes.

e) Add the mixture in the cold electroporation cuvette (-20°C for 30 minutes) and pulse the cuvette at 1800 V, 5 ms (ElectroCell Manipulator ECM399 BTX system, Genetronic Inc, San Diego, CA).

f) Add immediately 450 mL of pre-warmed SOC medium and shake at 37°C for 1 hour.

g) Spread 100 mL and 400 mL of transformed cells onto the pre-warmed PIA plates containing appropriate antibiotic.

h) Incubate at 37°C overnight.

B-II-c *P. aeruginosa* T3SS Analysis

The T3SS of *P. aeruginosa* can be induced through the calcium depletion in the culture medium. We believe that this condition can artificially open the secretion channel of the T3SS, leading to 1) a transcriptional activation of all T3SS genes in dependence of ExsA; and 2) the secretion of toxins or toxin-protein fusion fragments. This calcium depletion can be achieved by incorporating a calcium chelator, EGTA (ethylene glycol bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid) of 5 mM, into culture medium. Moreover, we added also 20 mM $MgCl_2$ to the culture medium so that the bacteria have enough divalent cations. The detailed protocol is described as following.

After an overnight pre-culture in LB containing 300 µg/mL carbenicillin, the bacteria were resuspended at 0.2 OD600 in LB containing 300 µg/mL carbenicillin, 0.5 mM IPTG, 5 mM EGTA and 20 mM MgCl2 until the OD_{600} reaches a value between 1.5 and 2. Then, bacterial cultures were centrifuged at 13000 g for 10 min and the supernatant was recovered. The production of toxins or toxin-protein fusion fragments can be analyzed through an appropriated method (SDS-page, etc.).

B-III Adaptations of CHA-OAL Strain in One Chemically Defined Medium

At beginning, we compounded CHA-OAL strain adaptation medium basing M9 medium and Debell MR compounded medium (501). But the growth rate of bacteria was not high enough. After that, we further modified adaption medium by supplementing it with Mg^{2+} and Ca^{2+} and the composition of final CHA-OAL adaptation medium (M9-TheREx) is shown in table 7.

For CHA-OAL strain adaptation, the bacterial culture was begun in LB broth and the replacement of LB broth by modified M9 (M9-TheREx) broth for bacterial culture was progressive (100% LB —> 50% LB-M9-TheREx —> 20% LB-M9-TheREx —> 5% LB-M9-TheREx —> 2% LB-M9-TheREx —> 100% M9-TheREx). For each step, at least two days of adaptation was realized, finally OAL strain proliferate stably in 100% M9-TheREx medium. Except the culture medium, the manipulation protocols

for OAL strains are the same as that described in the section of Manipulations of *P. aeruginosa*.

Table 7: The Composition of Modified M9 (M9-TheREx) Medium

Extract of synthetic yeast…….	**4 g/L**
Tryptophan ……	**1 mmol/L (0,2 g/L)**
Glucose……	**14 mmol/L (2,5 g/L)**
Glycerol……...	**1%**
FeSO4……..	**0.4 g/L**
Citric acid……	**2 mmol/L (0,36g/L)**
M9 Salts Medium 5X (Sigma) approximate composition per liter	
Na2PO3 anhydre.....	**33.9 g**
KPO3.....	**15.0 g**
NaCl	**2.5 g**
NH4Cl.....	**5.0 g**
MgSO4…… ……	**1mmol/L (50mg/L)**
CaSo4…. ……..	**1mmol/L (50mg/L)**
H_2O……..	**qsp 1L**

B-IV Constitution of CHA-OST-ΔBD Mutant Strain

1. Preparation of *E.coli* S17-1 containing plasmid pKO-PopB & PopD: transform E.coli S17-1with pEX100T mut *popBpopD* (described before, B-I-b).
2. Mating between CHA-OST and E.coli S17-1- pKO-POP B & POP D

   a) Mix at 1:1 of 50 µL exponential phase ($OD_{600}\approx0.5$) CHA-OST and E.coli S17-1- pEX100T mut *popBpopD*. Deposit the mixture on the surface of LB agar without salt. Incubate the plate overnight in the incubator at 37° C.

   b) Transfer the bacteria into a micro-centrifuge tube containing 1 ml of LB.

   c) Spread 100 µL bacteria (OD600 is from 1 to 2) on the PIA (Gm_{400}) at 37°C, overnight.

   d) Incubate 50 colonies on the PIA containing 5% sucrose at 37°C, overnight. In theory, only mutants with double combination could survive in the presence of 5% sucrose, but in fact all mutants survive after overnight's incubation (with different size of the colonies).

e) Re-incubate these 50 colonies on the PIA (Gm_{400}) as well as PIA (Cb_{600}) at 37°C, overnight.

f) Select colonies growing on the PIA (Gm_{400}) but not on the PIA (Cb_{600}) for further verification by PCR

B-V Bacterial Vaccines Culture

Principally, *P. aeruginosa* vaccine strains were grown overnight at 37°C with shaking at 250 rpm in bacteria culture medium containing 300 µg/mL carbenicillin (Euromedex, France). Next morning, the bacteria were resuspended in fresh culture medium at OD_{600} of 0.2 in LB containing 300 µg/mL carbenicillin and 0.5 mM IPTG until the OD_{600} reaches value between 1.5 and 2. Finally, bacteria cells were washed and resuspended in PBS, pH 7.2 for mice injection.

The culture medium for CHA-OST and CHA-OALstrains was LB, and for adapted CHA-OAL (CHA-CLIN1) strains was M9-TheREx.

C Molecular Biology

C-I PCR

Polymerase chain reaction (PCR) is a common method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many billions of molecules. In this study, PCR has been used for either synthesizing DNA fragments for cloning or detecting the presence of one sequence. All the PCRs have been achieved as follows:

C-I-a Materials

a) Template DNA. The template can be a fragment of DNA, a preparation of genomic DNA or a plasmid. Up to 10 ng of DNA has been utilized per reaction,

b) Forward primer and reverse primer. Each primer should be 20-30 nucleotides in length, G+C content should be between 40 and 60%, with a balanced distribution of all four bases along the length of the primer and a low propensity to form stable secondary structures. Restriction sites can be added to the 5’ termini of

the primer and these primer-specific restriction sites are transferred to the termini of the target DNA during amplification. All primers used in this work were synthesized by Metabion, Germany and are shown in table 8.

Table 8: Primer Sequences Used in This Study

	Oligonucleotide Sequence
Primers used for cloning antigen in uni-antigen vector	
PADRE(Nter) forward	5'-gatcggccaagttcgtcgccgcctggaccctgaaggccgccgccggatccaaagcatg-3'
PADRE(Nter) reverse	5'-ctttggatccggcggcggccttcagggtccaggcggcgacgaacttggcc-3'
PADRE(Cter) forward	5'-gatccaaagcatgcgccaagttcgtcgccgcctggaccctgaaggccgccgccccatgwere-3'
PADRE(Cter) reverse	5'-gggcggcggccttcagggtccaggcggcgacgaacttggcgcatgctttg-3'
$TRP2_{125-376}$ forward (BamH I)	5'-aaggatccagacggaatatccattccctgact-3'
$TRP2_{125-376}$ reverse (Sph I)	5'-catgggcatgcttacttgcggaaggagtgagc-3'
$TRP2_{125-376}$ reverse (Bgl II)	5'-ggaagatcttacttgcggaaggagtgagccaag-3'
$TRP2_{291-376}$ forward (BamH I)	5'-aaggatccgactacaaccgccgggtcaca-3'
$TRP2_{291-376}$ reverse (Sph I)	5'-aagcatgccttgcggaaggagtgagccaagttatgaag-3'
$OVA_{248-376}$ forward (BamH I)	5'-gccggatccgatgaagtctcaggccttgag-3'
$OVA_{248-376}$ reverse (Sph I)	5'-catgggcatgcttaggcgttggttgcgatgt-3'
$OVA_{248-376}$ reverse (Bgl II)	5'-ggaagatcttaggcgttggttgcgatgt-3'
$GP100_{21-150}$ forward (BamH I)	5'-aaggatccctagaaggatccaggaatcaggac-3'
$GP100_{21-150}$ reverse (Sph I)	5'-aagcatgcttaagatgggcagggtccaccgt-3'
$HER2_{340-507}$ forward (BamH I)	5'-cgggatcccgagtgtgctatggtctgggc-3'
$HER2_{340-507}$ reverse (Sph I)	5'-acatgcatgcatgttactcgcccacacactcgtcct-3'
Primers used for cloning antigen in the second position in bi-antigen vector	
Universal forward primer (Bgl II)	5'-gaagatctcaggagaaggcaaccatcat-3'
S54-PADRE-$OVA_{248-376}$ reverse (Sph I)	5'-catgggcatgcttaggcgttggttgcgatgt-3'
S54-PADRE-$TRP2_{125-376}$ reverse (Sph I)	5'-catgggcatgcttacttgcggaaggagtgagc-3'
S54-PADRE-$GP100_{21-150}$ reverse (Sph I)	5'-catgggcatgctcttaagatgggcagggtccaccgt-3'

c) Thermostable DNA polymerase. The thermostable DNA polymerase used in this work was principally *PfuUltra*™ HF DNA polymerase, Stratagene, USA, which can synthesize PCR fragments with high fidelity due to its proofreading capacity. Using Pfu polymerase, the elongation rate is about 500 bp / min.

d) *PfuUltra*™ HF reaction buffer (Stratagene).

e) dNTP solution containing all four dNTPs (Invitrogen).

f) Thermal cycler (MJ MiniCyclerTM, watertown, USA).

g) In summary, standard PCR reaction compositions are as following:

Template DNA……1-10 ng
5 mM solution of four dNTPs……...2.5 µL
30 mM (pmol ml-1) forward primer…….0.5 µL
30 mM (pmol ml-1) reverse primer.....0.5 µL
PfuUltra™ HF DNA polymerase (2.5 U/µl)……0.5 µL
10X *PfuUltra*™ HF reaction buffer..... 5 mL
H_2O……...OSP 50 µL

C-I-b PCR Programming

PCR is an iterative process, consisting of three elements: denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence(s), and extension of the annealed primers by a thermostable polymerase.

a) The denaturation step at 95 °C (usually 30-60s) is required for obtaining single-stranded DNA molecules to which the primers will bind. In the first cycle of PCR, denaturation is sometimes carried out for 5 minutes to increase the probability that long molecules of template DNA are fully denatured.

b) The annealing step (usually 45 - 60 s) allows primers to hybridize with the matrix. Annealing is generally carried out 5°C lower than the melting temperature at which the oligonucleotide primers dissociate from their templates. The melting temperature (Tm) of oligonucleotide primers can be calculated using the following formula: Tm (°C) = 2(A+T) + 4(G+C), where (A+T) is the sum of the A and T residues in the oligonucleotide and (G+C) is the sum of G and C residues in the oligonucleotide.

c) The extension step permits DNA synthesis catalyzed by the thermostable polymerase. This step is carried out at 72°C in the case of *Pfu* DNA polymerase and the duration of this step is depended on the length of the amplifying sequence. For the last cycle of PCR, an extension time is generally longer than in the previous cycles, which is used to allow completion of all amplified products.

d) The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. In this study, PCR reactions generally

include more than thirty cycles which were carried out by incubating the tubes in Minicycler device (MJ Research, Watertown, MA).

e) One example of PCR amplification using *PfuUltra*™ HF DNA polymerase:

- 95°C 5 min
- 95°C 1 min
- 63°C 1 min
- 72°C 2 min (for 1 kb of length of the target DNA)
- Repeat steps 2-4 for another 34 cycles
- 72°C 10 min
- 4°C up to overnight

C-II Agarose Gel Electrophoresis

DNA is an anion. Due to this electric charge, DNA fragments can be migrated in an electric field. Electrophoresis through agarose gel is one simple, rapidly performed technique that has been used to identify, separate and purify DNA fragments in this study.

C-II-a Materials

- Agarose Ultrapure (Invitrogen)
- Electrophoresis buffer: 50 x TAE Solution, Euromedex (2 M Tris-Amino, 1 M Acetate, 50 mM EDTA, pH 8)
- Ethidium bromide: 10 mg/mL (20,000 X) (Sigma)
- Gel-loading buffer: Blue/Orange 6 X loading buffer (Promega)
- Molecular weight marker: 1 kb DNA ladder (100 ng/µL)(Promega)
- Gel-sealing tape
- Electrophoresis apparatus with chamber and comb (Embi Tec, San Diego, CA, USA)
- Electrophoresis power supply device (Apelex, Massy, France)
- UV device (Bioblock Scientific, Illkirch, France)
- Imaging device

C-II-b Methods

a) Prepare a solution of agarose in 1 X TAE buffer at an appropriate concentration (in general, 1% for the fragment of length more than 500 pb; 2% for the fragment of length less than 500 pb).

b) Heat the slurry in a microwave oven until the agarose dissolves. Add EB to the mixture to a final concentration of 0.5 μg/mL.

c) Shape gel in one mold containing an appropriate comb for forming the sample slots.

d) Mount the gel in the electrophoresis tank and add 1 X TAE buffer to cover the gel;

e) Mix the DNA samples with 6 ´ gel-loading buffer. Load the DNA marker and the sample mixture into the slots of the submerged gel.

f) Carry out the migration under 50-100 voltage.

g) Examine the gel by UV device or photograph the gel using the imaging device.

C-II-c Recovery of DNA from Agarose Gel

After the migration within agarose gel, the different DNA fragments can be recovered from gel by using the commercial gel extraction kit (QIAquick Gel Extraction kit (Qiagen, Courtaboeuf, France)). All procedures were performed according to the instructions of manufacturer.

C-III Cloning

In principle, closed circular plasmid DNA is cleaved with one or two restriction enzymes and ligated *in vitro* to foreign DNA bearing compatible termini. The products of the ligation reaction are then used to transform an appropriate strain of E. coli (DH 5α). The resulting transformed colonies are screened by PCR or by digestion with restriction enzymes to identify those that carry the desired DNA sequences. Finally, positive clones can be confirmed by commercial DNA sequencing.

C-III-a Plasmid Vectors Preparation

Purified plasmid DNA can be developed from bacteria, which generally involve three steps: growth of the bacterial culture, harvesting and lysis of the bacteria and purification of the plasmid DNA. In this work, generally 2-3 mL of bacteria were cultivated during 12-16 hours for DNA plasmid expression. Then, the extraction of plasmid was achieved by using commercial Kit (QIAprep Spin Miniprep kit (Qiagen, Courtaboeuf, France)). All procedures were performed according to the instructions of the manual of the kit.

C-III-b Restriction Enzyme Digestion

a) Materials:

- Restriction enzymes (Roche, Meylan, France or Invitrogen, Cergy pontoise, France)
- 10 X buffer for Restriction enzymes (Roche or Invitrogen)
- Plasmids
- Thermomixer (Eppendorf, Hamburg, Germany) or a water bath preset to 37°C

b) Standard recipe: Plasmid…….... 0.1-0.5 µg (1-5 µL)
Restriction enzymes (10 U ml-1)..... 0.5 mL
10 X buffer for restriction enzymes……1µL
H_2O.....QSP 10 µL

c) Method: Incubate the reaction mixture at 37°C for 1-3 hours. At the end, digestion can be checked by agarose gel electrophoresis (see C-II section).

C-III-c DNA Ligation

The ligation between digested plasmid vector and insert DNA has been achieved by using the commercial Kit (Rapid DNA ligation Kit, Roche, Meylan, France). All procedures were performed according to the instructions of the manual of the kit.

C-III-d Transformation

It was performed as described in Materials and Methods B-I-b and B-II-b.

C-III-e DNA Sequencing

It was performed at AGOWA, Germany by delivering 1 µg of plasmid (generally 10 µl of Miniprep) with appropriated sequencing primer.

D Protein Analysis

D-I Protein Precipitation

For precipitation of proteins, perchloric acid was added to supernatant at a final concentration of 15% and incubated at 4°C, overnight. The next day, precipitated proteins were centrifuged at 17000 g, 4°C for 30 min; proteins were washed two times with acetone (17000 g, 15 min), dried at room temperature.

D-II Protein Denaturation

Washed and dried proteins were resuspended in 60 µL denaturation buffer (0.5 M Tris-HCL, 0.6 M dithiothreitol, 10% SDS, 0.012% bromophenol blue, 30% glycerol). SDS is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. DTT is frequently used to reduce the disulfide bonds of proteins. This method allows the protein to preferentially migrate according to its mass but not its structure.

Dissolved in the denaturation buffer, protein can be analyzed immediately or conserved at -20°C. Before polyacrylamide gel migration, a further denaturation step was generally carried out by exposing the protein at 95°C for 5 min.

D-III SDS-PAGE (SDS-PolyAcrylamide Gel Electrophoresis)

In principle, the SDS-PAGE is realized in a vertically migrated gel composed of two parts. The upper part, also called stacking gel, is of thinner acrylamide (5%). The migration in this part allows all proteins to migrate at the same level after applying an electric field. The bottom part, also called resolving gel, is of concentrated acrylamide. The migration in this part permits the separation of the different proteins according to their molecular weight. The acrylamide concentration of the resolving gel can be

varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins. In this study, we usually used the gel of 12% or 15% acrylamide, we suggested that protein of molecular weight between 20 and 30 kDa are better visualized in a 15% acrylamide gel, and proteins of 30 to 40 kDa are better visualized in a 12% acrylamide gel.

D-III-a Materials

- Acrylamide (Purity 99.9%) / Bis-Acrylamide 40% Solution Sigma
- Stacking Gel Buffer, 0,5 M Tris-HCL, pH 6,8 Biorad
- Resolving Gel Buffer, 1,5 M Tris-HCL, pH 8,8 Biorad
- SDS Sigma
- Ammonium persulfate Sigma
- TEMED (N,N,N',N'-Tetramethylethylenediamine) Sigma
- Migaration buffer: (Tris-HCl 25 mM pH 8, glycine 200 mM, SDS 0,1%)

D-III-b SDS-Page Gel Compositions

	Stacking Gel	Resolving Gel
Acrylamide	5%	12% or 15%
SDS	1%	
Tris 0,5 M pH 6,8	25%	
Tris 1,5 M pH 8,8		25%
Ammonium persulfate 10% (w/v)	1%	
TEMED	1‰	
H_2O	QSP	

D-III-c Migration

Apply a voltage of 8 V/cm to the stacking gel and then increase the voltage to 15 V/cm to the resolving gel. Run it until the bromophenol blue reaches the bottom of the gel.

D-III-d Staining of SDS-Polyacrylamide Gels

In this study, we have stained SDS-polyacrylamides gels either through standard Coomassie Brilliant Blue staining methods or using a commercial rapid stainingsolution.

a) Standard Coomassie Brilliant Blue Staining: Coomassie Brilliant Blue binds nonspecifically to proteins but not to the gel, thereby allowing visualization of the proteins as discreet blue bands.

- Immerse the gel in the 0.25% Coomassie Brilliant Blue R 250 (Merck, Darmstadt, Germany) solution.
- Rotate slowly for a minimum of 1 hour at room temperature.
- Remove the staining solution destain the gel by soaking it in the methanol: glacial acetic acid: H2O (5:1:4, v/v/v) on a slowly rocking platform for 4-8 hours.
- Changing the destaining solution several times (Use methanol: glacial acetic acid: H2O 3:1:6, v/vv, for a more rapid destaining).
- Store indefinitely the fixed gel in H_2O containing 20 % glycerol before drying.

b) Rapid Staining Using One Commercial Staining Solution: PageBlue™ Protein Staining Solution, Euromedex. All procedures were performed according to the instructions of the manual of product.

E Immune response Monitoring

E-I CTLs Cytotoxicity Detection Assay

E-I-a *In Vitro* CTL Induction

a) Mice that have been vaccinated on day –14 and day –7 were sacrificed on day 0, and their spleens were removed and processed individually.

b) Prepare single cell suspension by grinding spleen and filtering the tissue through a cell strainer (70 µm; Becton Dickinson, Franklin Lakes, NJ).

c) Add splenocytes to ficoll solution (GE Healthcare/Amersham) and centrifuged at 800g for 20 minutes to obtain lymphocytes fraction.

d) Wash cells and resuspend lymphocytes in RPMI containing 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin, IL-2 30 U/mL.

e) Seat lymphocytes in a 6-well plate and incubate with mitomycin-C-treated tumor cells at the ration of 40:1 (L/T).

f) On day 3, add IL-2 to culture solution at 30 U/mL.

g) On day 6, non-adherent T lymphocytes were harvested for cytolytic assays.

E-I-b Cytotoxicity Assay

Inducted T lymphocytes were plated with target tumor cells (GL26) at different effector/target ratios (ranging from 10/1 to 60/1). The cytolytic activity of T cells was determined by measuring lactate dehydrogenase (LDH) release. The percentage of lysis was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Exp. Value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} * 100$$

- Positive control: target cells 100% lysed
- Negative control: only target cells

LDH release was measured according to the protocol of “Cytotoxicity Detection Kit (LDH)” (Roche, Indianapolis, In, USA). In general, tumor cells and CTL cells were co-incubated at 37°C, 5% CO2 in 200µl RPMI, 1% FBS during 4 hours, then 100 µL supernatant of each sample was transferred to an optically clear 96-well microplate and 100 µL detect reagent was added to each well. After that, the plate was incubated at room temperature for 30 min in the dark and the absorbance of samples was measured by an ELISA reader at 492 nm. For the 100% lysis control, tumor cells were treated with 100 µL Triton X-100 solution (2% in assay medium) during 4 hours.

E-II Treg Cells Detection through Flow Cytometry Analysis

a) Mice that have been vaccinated on day –14 and day –7 were sacrificed on day 0, and draining lymph nodes (DLN) in vaccination area were removed and

processed individually, and grinded to obtain the single cell suspension.

b) Incubate DLN lymphocytes with FITC-conjugated anti-CD4 Ab, PE-conjugated anti-CD25 Ab and APC conjugated anti-FoxP3 Ab on following the protocol of Miltenyi Biotec (Germany).

E-III IFN-γ Enzyme-Linked Immunosorbent Spot (ELISpot) Assay

a) Immunized mice were sacrificed and splenocytes were harvested as described before (E-I-a).

b) Splenocytes were washed and resuspended in RPMI containing 10% FBS at $8*10^6$ cells/mL.

c) Seat 50 μL/well of splenocytes to an IFN-γ capture ELISpot plate (MultiScreen HTS filter plates, Millipore, Danvers, MA).

d) Add 50 μL/well of 10 μg/mL OVA(SIINFEKL) peptide to plate and incubate at 37°C, 5%CO_2 for 18-20 hours. As positive control, splenocytes were incubated with 4 μg/mL Con A (Sigma); and as negative control, splenocytes were incubated with only medium (RPMI containing 10% FBS).

e) The next day, IFN-γ spots were developed according the manufacturer's protocol (Diaclone, France) and counted by an automated counter (Bioreader 4000 Pro, Biosys., Germany).

f) Results are reported as the number of spot-forming cells (SFC) per 4×10^6 splenocytes plus or minus the SD of triplicate animals.

E-IV Enzyme-Linked Immunosorbent Assay (ELISA)

The first day:

1. Coating: Dilute protein (OVA, Sigma) in 1X PBS buffer at concentration of 2.5 μg/ml. Distribute 100 μL/well of solution to 96-well polystyrene plate (BD, UK). Cover plate with plastic lid and incubate at 4°C overnight.

The second day:

1. Washing: Discard protein solution and wash plate three times with 0.05% tween - PBS.

2. Saturation: Distribute 300 µL of Tween 2% - BSA 2% - PAS buffer to each well and incubate 1 hour at R.T. with agitation.

3. Washing: same as previously.

4. Incubation of samples:

i) Preparation of samples: Dilute each sample at 1/1000 in Tween 0.05% - BSA 1% - PBS buffer.

ii) Distribute 100 µL of diluted pool samples in triple wells to plate and incubate plate at R.T. for 1h with agitation.

5. Washing: same as previously.

6. Incubation of secondary antibody: (Anti-Mouse IgG (whole molecule) − Biotin antibody produced in rabbit - IgG fraction of antiserum, buffered aqueous solution (Sigma B8520-1ML)).

Dilute secondary antibody at 1/1500 in Tween 0.05% - BSA 1% - SVF 1% - PBS buffer and distribute 100 µL to each well. Incubate the plate 1h at R.T. with agitation.

7. Washing: same as previously.

8. Incubation of avidin: (Avidin–Alkaline Phosphatase buffered aqueous solution (Sigma A7294-1ML))

Dilute avidin at 1/3000 in Tween 0.05% - BSA 1% - SVF 1% - PBS buffer and distribute 100 µL to each well. Incubate the plate 1 hour at R.T. with agitation.

9. Addition of substrate and dynamical detection: (4-Nitrophenyl phosphate disodium salt hexahydrate - tablet (Sigma N9389-50TAB))

i) Extemporaneous preparation: Dilute pNPP tablet in diethanolamine buffer (Diethanolamine reagent grade,≥ 98.0% (Sigma D8885-500G)) to concentration of 1mg/ml (for one plate: 2 tablets in 10 mL buffer)

ii) Distribute 100 µL to each well.

iii) Incubate plate in the darkness for 1h and read the OD at 405 nm with the reference at 620 nm.

F Manipulations of Eukaryote Cells

F-I *In Vitro* Antigen Presentation Assay

F-I-a Eukaryote Cell Lines

a) Murine myeloid dendritic cells (mDCs): mDCs used in this study were generated from the bone marrow of C57BL/6 mice (502) and were kindly provided by Dr Christian Villiers (INSERM-U823, France).

b) B3Z cells: The B3Z clone is a hybridoma between murine cell lines Z8 and B3. Z8 cell line expresses in its genome the β-galactosidase gene under the control of the IL2 promoter; B3 cell line is one CD8 + T lymphocyte clone that is specific for the ovalbumin peptide SIINFEKL (residues 257–264) in the context of H-2Kb. B3Z cells were cultivated in RPMI medium (Gibco. Invitrogen, France) supplemented with 10% FBS (Gibco. Invitrogen, France), 1% Penicillin-Streptomycin (GIBCO invitrogen, France), 800 µg/mL Geneticin (Invitrogen, Carlsbad, CA) and 50 µg/mL β-mercaptoethanol (Invitrogen, Carlsbad, CA).

F-I-b The Principle of Assay

As B3Z hybridoma cell line is specific for the ovalbumin peptide SIINFEKL, it could be activated when dendritic cells correctly received and processed the antigenic message (SIINFEKL epitope). Meanwhile, this activation is generally associated with the production of IL2. B3Z activation can be assessed by measuring the activity β-galactosidase.

F-I-c Methods

a) Wash and resuspend mDCs in RPMI medium supplemented with 2X glutamine (Gibco.Invitrogen), 20% FBS

b) Seated mDCs in flat-bottom 96-well plates at the concentration of $1*10^5$ cells/well.

c) Prepare bacterial cultures as previously described (see in B-V section).

d) Resuspend bacteria cells in RPMI and add to plate at multiplicity of infection (m.o.i) of 5.

e) Incubate bacteria cells with B3Z cell at 37°C, 5% CO_2 for 3 hours,

f) Add 10 µL of 250 µg/mL gentamicin (PAA Laboratories GmbH, Pasching, Austria) to each well and incubate plate at 37°C, 5%CO_2 for 30 min (step for killing bacteria).

g) Centrifuge the plate at 350 G for 10 min and discard the supernatant.

h) Add B3Z cells to plate at $1*10^6$ cells/well in RPMI medium supplemented with 10% FBS, 1% Penicillin-Streptomycin, 800 µg/mL Geneticin and 50 µg/mL β-mercaptoethanol for 16 hours.

i) Centrifuge the plate at 350 G for 5 min and discard the supernant.

j) Add 100 µL/well of lysis buffer (CPRG 0.15 mM; MgCl2 9 mM; Triton X-100 1%; β-mercaptoethanol 50 µM; PBS ;) and incubate the plate at 37°C for several hours.

k) Read the OD at 570 nm

F-II Tumor Cell Lines

Five tumor cell lines were used in this work: three of them, B16OVA, GL26 and GL261, were derived from C57BL6 mice; and two others, MC2 and MC2-HER2, were derived from human β2m–HLA-A2.1 (α1α2)–H-2Db (α3 transmembrane cytoplasmic) (HHD) transgenic mice.

B16OVA is derived from murine melanoma B16-F0 cell line. It was obtained by transfecting B16-F0 cell line with ovalbumin cDNA. B16-OVA cell line used in this work was kindly provided by Dr Georges Vassaux (Molecular Oncology Unit, Cancer Research UK Clinical Centre, Royaume-Uni). B16OVA cells were cultivated in DMEM medium (GIBCO, Invitrogen, France) containing 10% FBS, 1% Penicillin-Streptomycin and 800 µg/mL Geneticin G418.

GL26 and GL261 are two common murine glioma cell lines. These two cell lines were grown in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin.

MC-2 cell line relates to the original sarcomas that were induced by the

carcinogen methylcholanthrene (MC) in HHD mice; MC-2-HER-2 cell line was obtained by transfecting MC-2 cell line with full length HER-2 cDNA. Both cell lines were kindly provided by Dr Simona Vertuani (Immune and Gene Therapy Unit, Cancer Centrum Karolinska, Karolinska Hospital, Sweden). Cell lines were maintained in IMDM (GIBCO, Invitrogen, France) supplemented with 10% FBS, 1% Penicillin-Streptomycin. Geneticin G418 was added at 800 µg/mL to the medium for the MC-2/HER-2 cell line culture.

G Manipulations of Animals

G-I Mice

Female C57BL/6j mice were purchased from Janvier SA (Le Genest-Saint-Isle, France); homozygous HLA-A2.1 transgenic mice (human ß2m-HLA-A2.1 (α1α2)-H-2dB (α3 cytoplasmic domains of mouse)) were purchased from Charle River (France). All animals were experimented at 6-8 weeks of age. They were kept under pathogen-free conditions in the animal facility of the University Joseph Fourier (Grenoble, France). All animal experiments were approved by the Animal Experiment Committee of the Region and were performed in accordance with institutional and national guidelines.

G-II Animal Immunization

Mice were administered bacteria vaccines through subcutaneous (s.c.) injection. With CHA-OST-fusion protein strains, mice were injected in right flank at the dose of $5*10^6$ bacteria (100 µL)/time/mouse on following prophylactic (D-14/D-7) or various therapeutic vaccination schemas. With CHA-OAL-fusion protein strains, mice were injected either at the dose of $1*10^8$ bacteria (100 µL)/time/mouse in right frank or $5*10^7$ bacteria (50 µL)/position/time/mouse at 4 positions in right and left franks on following the different vaccination schema. All injections in mice were achieved with BD Micro-Fine™+ Insulin Syringes (BD, UK).

G-III Tumor Challenge

Before mice injection, all tumor cells were washed by PBS, pH 7.2 and then resuspended in PBS for injection. Immunized (prophylactic groups) or non-immunized (therapeutic groups) female C57BL/6j mice were injected s.c. in left flank on day 0 with B16OVA cells at the dose of $2*10^5$ cells (100 µL)/ mouse; with GL26 or GL261 cells at the dose of $1*10^5$ cells (100 µL)/ mouse. Immunized HHD mice were injected s.c. in left flank on day 0 with MC-2 or MC-2-HER-2 cells at the dose of $7*10^4$ cells (100 µL)/ mouse. All injections in mice were achieved with BD Micro-Fine™+ Insulin Syringes (BD, UK). The emergence and the dynamic growth of tumor mass were measured every 48 hours. When the diameter of tumor exceeded 10 mm, mice were sacrificed and this day corresponded to the period of survival. Analysis of data was realized with GraphPad Prism 5 software.

H Statistics

Bar graph analyses were evaluated by Student's t-test. Kaplan-Meyer survival curves of animals treated with different protocols were analyzed using the logrank test. A p-value below 0.05 between groups was considered to indicate a statistically significant difference.

RESULTS

Chapter 1
Optimization of Bacterial Vaccine Vectors

Résumé en Français :

Dans ce chapitre, nous avons tenté d'améliorer la performance de nos vecteurs bactériens en suivant différentes stratégies. Avant tout, un épitope (PADRE) spécifique des lymphocytes CD4+ Th a été ajouté aux antigènes. D'une part, cette addition est à l'origine d'une réponse immunitaire forte médiée par les CD8+ CTL. D'autre part, elle réduit la suppression médiée par les cellules Treg pour activer les cellules effectrices. Ainsi, une amélioration de la protection anti-tumorale *in vivo* a été obtenue. Par la suite, un modèle d'expression bi-antigénique a été appliqué aux vecteurs bactériens. Les vecteurs bi-antigéniques ont montré l'efficacité anti-tumorale supérieure que les vecteurs mono-antigéniques ; cependant autant que les vecteurs mono-antigéniques combinées. En outre, un vecteur potentiel activant l'immunité humorale a été construit par la délétion des deux protéines structurelles du SST3 : PopB et PopD. Des IgG spécifiques de l'antigène ont été détectés après des vaccinations avec un vecteur muté *ΔPopBΔPopD*.

In English:

A Improvement of Vaccine Efficiency by Adding CD4+ Th Helper Epitope (Pan-HLA-DR-binding Epitope (PADRE))

CD4+ T cells have been proved to play an important role during the priming and development of immune response. PADRE (AKFVAAWTLKAAA) is a widely-used MHC II-presented CD4+ T-helper epitope. It has been demonstrated to be a strong inducer of T helper cells in a variety of vaccine vector for providing help not only for the generation of CTL response, but also for the induction of a humoral immune response (503, 504). Particularly, PADRE is a promiscuous CD4+ Th determinant capable of binding with high affinity to most of DR alleles so that both human and murine MHC II molecules can bind this sequence. So, in this work, we attempted to improve the efficacy of our bacterial vaccines by adding PADRE as CD4+-T-cell

activator to the antigen delivery system.

A-I Construction of PADRE Containing Exogenous Antigen-expression Plasmids

The plasmid pExsAi-S54 (fig. 12-A) permits the cloning of target antigen in fusion with the first 54 amino acids of ExoS, which will allow the secretion of target antigen via T3SS of *P. aeruginosa*. pExsAI has been previously constructed by our laboratory. Basing on this vector, the PADRE coding sequence was inserted into the frame of fusion protein coding region. Since the location of insert might affect the transcription, translation, secretion through T3SS by bacteria, as well as the degradation of fusion protein by acid proteases in cytoplasm or by proteasome of APCs, PADRE coding sequence has been inserted either upstream or downstream of antigen cloning site.

The strategy for constructing PADRE Nter-containing exogenous antigen expression vector is presented in figure 12-B. Briefly, PADRE was integrated upstream of cloning site. A PADRE insert was synthesized to contain at 5' end a linker for the BamH I site of pEXSAi-S54, a new BamH I restriction site, and a linker for the SpH I site in pEXSAi-S54 at 3' end. Then this insert was ligated to BamH I/SpH I digested pEXSAi-S54 vector to form a new circular plasmid. As the pair of bases (marked in blue) was changed, the original BamH I restriction site was deleted. Therefore, there is only one BamH I/SpH I framing cloning site at 3' end of PADRE in the new plasmid. PADRE (Cter)-cloning vector where PADRE is integrated downstream of cloning site was also constructed by the same strategy (Fig. 12-C).

In order to evaluate T helper efficiency of PADRE in our bacterial vaccines, we chose TRP2, one well-known TAA expressed by both murine and human glioma, as target antigen. Two different constructions were made for TRP-2 ($TRP2L_{125-376}$, $TRP2S_{291-376}$). TRP2S included the strongest *in silico* predicted epitope ($TRP2_{363-372}$); TRP2L contained not only the predicted $TRP2_{363-372}$ but also one published $TRP2_{181-188}$ epitope (505). We cloned the two TRP2 sequences in our PADRE containing antigen expression vector (marked in blue in Fig 12-B&C).

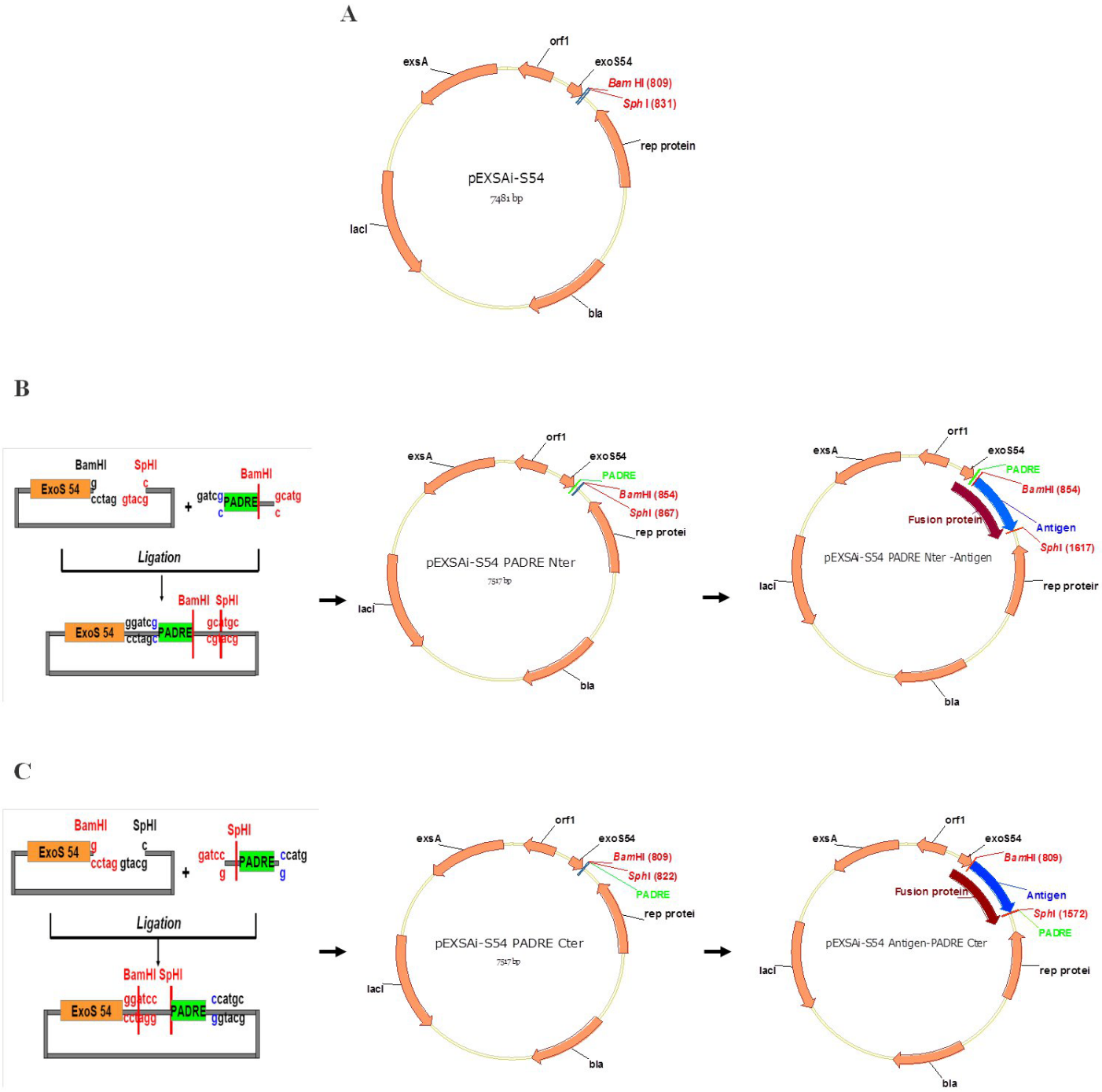

Figure 12: Construction of PADRE Containing Exogenous Antigen Expression Plasmids.

(A) PADRE containing plasmids were constructed from pEXSAi-S54 which comprises exoS54–necessary sequence for the secretion and translocation of toxins or toxin-protein fusion fragments; and multiple cloning sites (MCS) region containing BamHI/SpHI sites. (B) and (C) Strategy for constructing PADRE-containing exogenous antigen repression plasmids. We integrated PADRE either upstream or downstream of cloning site. Then, antigen comprising BamHI and SpHI sites could be integrated to PADRE-containing vector and form the fusion protein coding sequence with exoS54 and PADRE.

Both sequences were cloned at BamH I/Sph I restriction sites in protein coding region of PADRE-containing vector. The correct integrations of TRP2L and TRP2S were confirmed by commercial sequencing.

A-II *In Vitro* Evaluation of Protein Secretion Ability of PADRE Containing Vectors

P. aeruginosa can secret at least four effector proteins through its T3SS: ExoS, ExoT, ExoY and ExoU. The first 54 amino acids of ExoS is the necessary sequence for its secretion and translocation through T3SS. We transformed CHA-OST strain with the plasmid which can express the fusion protein beginning with the first 54 amino acids of ExoS (ExoS54), allowing the fusion protein to be also secreted and translocated by CHA-OST strain through T3SS. Besides, it is known that for the intracytoplasmic translocation through T3SS, two proteins play an important role: PopB and PopD which are also secreted upon T3SS induction and can be visualized by SDS-PAGE of cultured bacteria. When bacterial cells contact eukaryotic cells or under low calcium condition, T3SS is activated and PopB, PopD could insert into the membrane of eukaryotic cells and form the secretion and translocation channel.

Before vaccination, the secretion abilities of transformed strains – CHA-OST-PADRE-TRP2L and CHA-OST-PADRE-TRP2S have been verified in vitro through EGTA mediated calcium depletion in the culture medium, and the results are shown in figure 13. In this figure, the first lane represents CHA-OST-EI strain which was the control strain that contains non-antigen coding sequence. Thus, in the first lane we can see the two structural proteins – PopB and PopD (marked with arrows), but not target protein secretion. In the following lanes which represent CHA-OST-TRP2L, CHA-OST-PADRE(Nter)-TRP2L, CHA-OST-TRP2L-PADRE(Cter), CHA-OST-TRP2S, CHA-OST-PADRE(Nter)-TRP2S and CHA-OST-TRP2S-PADRE(Cter), besides PopB and PopD which indicate the correct activation of T3SS, we can also see the bands corresponding to the different target proteins (marked with asterisks). These results could not only confirm the T3SS secretion ability in vitro, but also predict the

in vivo protein delivery ability of our vaccine strains.

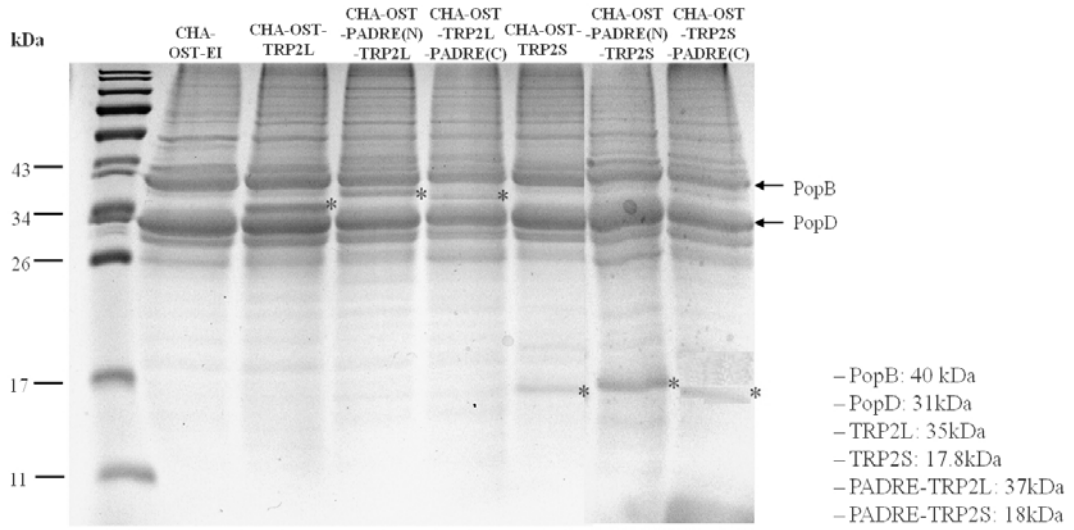

Figure 13: *In Vitro* Investigation of Fusion Proteins Secretion by PADRE Containing Exogenous Antigen Delivery Bacterial Vectors.

The secretion ability of PADRE containing exogenous antigen delivery strains has been verified before vaccination. For each strain, we can see two important structural proteins (marked with arrows)–PopB (top) and PopD (bottom) which form cytotoxins translocation channel in eukaryote cells membrane. CHA-OST-EI was the control strain which was transformed with pEXSAi-S54 without antigen, so in this strain, we can only observe PopB and PopD but not target protein. And in the other vaccine strains, we see fusion protein corresponding right molecular weight (marked with asterisks) as well as PopB and PopD. Thus, we can confirm the secretion ability of vaccination strains *in vitro* and also predict their *in vivo* antigen translocation abilities.

A-III Evaluation of Vaccination Efficiency *via* Immune Response Monitoring

A-III-a CTLs Cytotoxicity Detection Assay

CTL cytotoxicity detection assay measures *ex vivo* immune response activation. Here, two groups of vaccine strains were assessed. In the first group, CHA-OST-TRP2L, CHA-OST-PADRE(Nter)-TRP2L and CHA-OST-TRP2L-PADRE(Cter) strains have been investigated. In the second group, CHA-OST-TRP2S, CHA-OST-PADRE(Nter)-TRP2S and CHA-OST-TRP2S-PADRE(Cter) strains have been tested. In each group, mice injected with PBS were used as a negative control. We estimated the cytotoxicity of lymphocytes isolated from the mice by their ability to lyse GL26 cells after 6 days of *in vitro* induction.

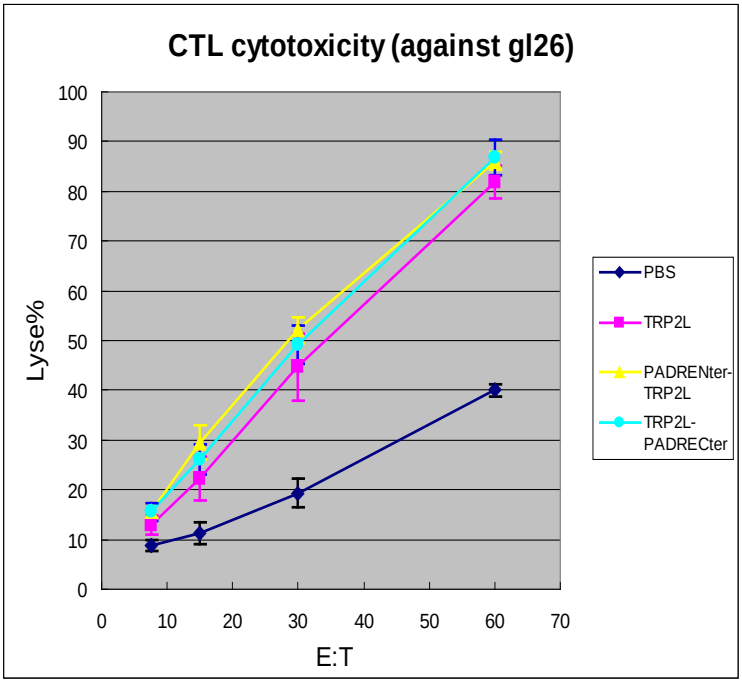

Figure 14: CTL Cytotoxicity Assessment for Investigating the Immune Response in Mice Immunized with TRP2L Delivering Strains.

Mice were injected with CHA-OST-TRP2L or CHA-OST-PADRE(Nter)-TRP2L or CHA-OST-TRP2L-PADRE(Cter) strains or PBS (negative control) two times on 14 days and 7 days before splenocytes sampling. Isolated lymphocytes were incubated with mitomycin-C-treated GL26 cells for 6 days for obtaining effector CTLs. Then, CTL assays were performed through measuring the LDH released by GL26 cells (Target, T) which were lysed by effector CTLs (Effector, E). The cytotoxicity is estimted by LDH release in % of that of total cell lysis. Data represented the mean +/- SD from 6 animals per group in triplicate (cumulative results of 2 independent experiments conducted using the same methodology).

Figure 14 presents the results of CTL assay for TRP2L group. We can see that with the increase of effector/target ratio, the lysis rate of GL26 cells increased in all samples, but the lines corresponding to immunized mice augment more obviously than that corresponding to control mice, which indicate the immune response activation in vaccinated mice. By comparing the different vaccine strains, we can find that CTLs cytotoxicity of mice injected with CHA-OST-PADRE(Nter)-TRP2L strain was stronger than that of mice injected with CHA-OST-TRP2L-PADRE(Cter) strain; and the later was still stronger than that of mice injected with CHA-OST-TRP2L, whereas the difference between strains is not evident.

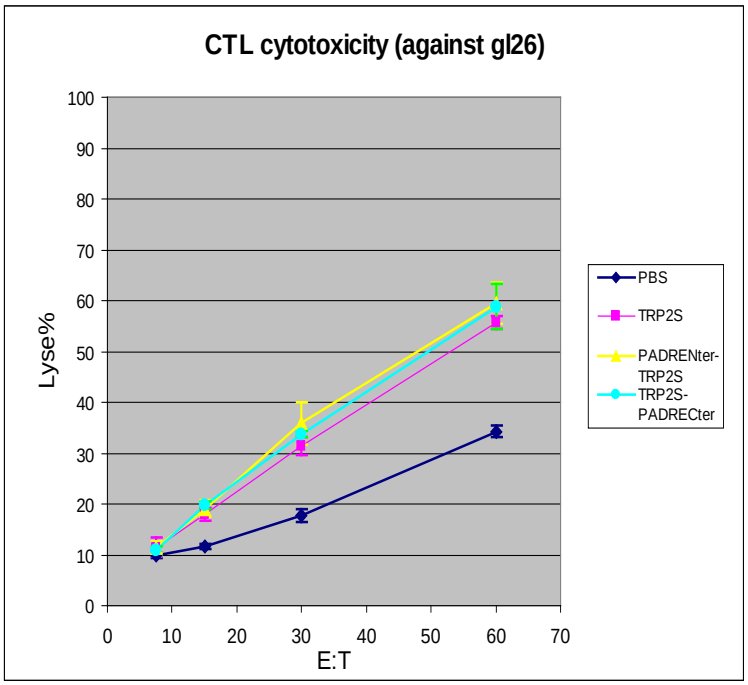

Figure 15: CTL Cytotoxicity Assessment for Investigating the Immune Response in Mice Immunized with TRP2S Delivering Strains.

Mice were injected with CHA-OST-TRP2S or CHA-OST-PADRE(Nter)-TRP2S or CHA-OST-TRP2L-PADRE(Cter) strains or PBS (negative control) two times on 14 days and 7 days before splenocytes sampling. The prinicle for CTL cytotoxicity assay is the same as that described in the legend of figure 14. Data represented the mean +/- SD from 6 animals per group in triplicate (cumulative results of 2 independent experiments conducted using the same methodology).

Figure 15 shows the results of cytotoxic CTL assay for TRP2S group. Similar to TRP2L group, the lysis rate also increased according to the E/T ratios, and we can also find the obvious difference between immunized mice and control mice, and the cytotoxicity of CTLs from mice injected with CHA-OST-PADRE(Nter)-TRP2S and CHA-OST-TRP2S-PADRE(Cter) are also a little stronger than that of mice injected with CHA-OST-TRP2S.

By comparing and combining the results of these two groups, we can confirm the anti-tumor immune response stimulated *in vivo* by bacteria strains, whereas the difference between different constructions was not so significant.

A-III-b Regulatory T (Treg) Cells Detection Assay

Treg cells, which are also called suppressor T cells, are a specialized subpopulation of T cells that act to suppress T cells responses and maintain the homeostasis of immune system. Recent researches proved that regulatory T cells are more defined by expression of the forkhead family transcription factor FoxP3. Expression of FoxP3 is required for regulatory T cell development and appears to control a genetic program specifying this cellular subset. Moreover, the large majority of FoxP3-expressing regulatory T cells are found within the MHC II restricted CD4-expressing (CD4+) helper T cell population and express high levels of the interleukin-2 receptor alpha chain (CD25). Therefore, those that express CD4+, CD25+ and Foxp3+ can be defined as regulatory T cells or "Tregs".

In order to investigate the immune modulating environment after bacterial vaccines injections, we also measured the quantity of regulatory T cells after vaccinations to estimate their impact to anti-tumor efficiency of our vaccines. In this trial, the investigated vaccine strains were also divided into two groups: the first group was composed of CHA-OST-TRP2L, CHA-OST-PADRE(Nter)-TRP2L and CHA-OST-TRP2L-PADRE(Cter) strains; and the second group contained CHA-OST-TRP2S, CHA-OST-PADRE(Nter)-TRP2S and CHA-OST-TRP2S-PADRE(Cter) strains. The situation of Treg in immunized mice was described by comparing with that in mice injected with PBS.

Figure 16 shows the results of Treg cells assessment in TRP2L group. We find that there was a general decrease of CD4+/CD25+/FoxP3+ regulatory T cells in DLN of immunized mice compared to that of negative control mice. The more obvious decrease of Treg cells in the lymph node was found in the mice immunized with CHA-OST-PADRE (Nter)-TRP2L strain, and the quantity of Treg cells in the lymph node of mice injected with CHA-OST-TRP2L strain decreased less.

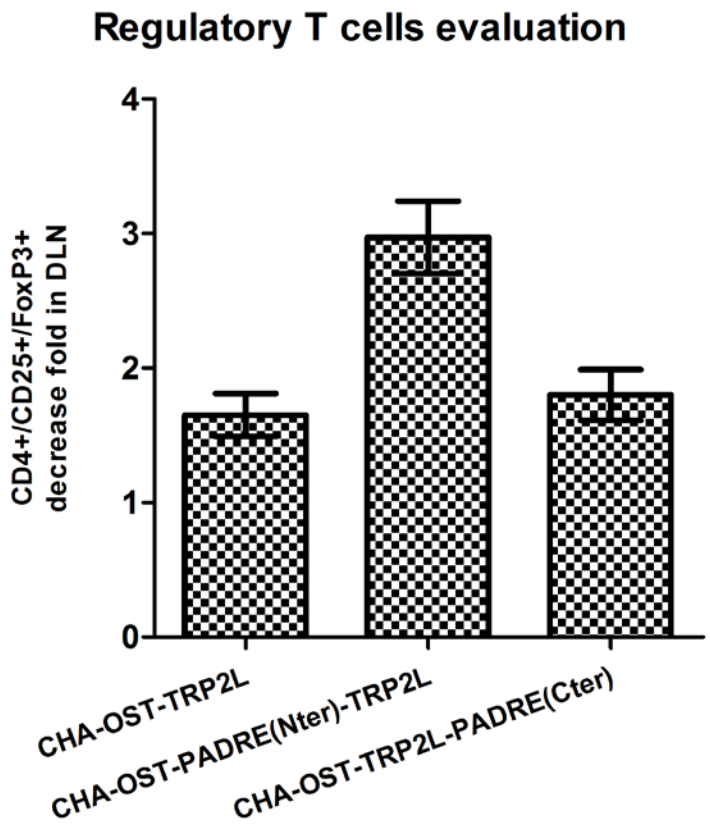

Figure 16: Evaluation of Regulatory T (Treg) Cells in Mice Immunized with TRP2L Delivering Strains.

Mice were injected with CHA-OST-TRP2L or CHA-OST-PADRE(Nter)-TRP2L or CHA-OST-TRP2L-PADRE(Cter) strains or PBS (negative control) two times on 14 days and 7 days before lymph nodes sampling. Lymphocytes isolated from draining lymph node (DLN) of mice were stained with fluoresein-conjugated antibodies anti-CD4+, anti CD25+ and anti FoxP3+. The CD4+/CD25+/FoxP3+ T cells were considered as regulatory T cells (Tregs) and detected by flow cytometry. The situation of Tregs cells in immunized mice were estimated by comparing with that in negative control mice. Data represented the mean +/- SD from 6 animals per group in triplicate (cumulative results of 2 independent experiments conducted using the same methodology).

Figure 17 shows the results of Treg cells assessment in TRP2S group. We can find the similar relation among the three vaccines as that in the TRP2L group: there were less Treg cells in lymph node of mice injected with CHA-OST-PADRE(Nter)-TRP2S strain and the Tregs in lymph node of mice injected with CHA-OST-TRP2S strain were more than that of other mice.

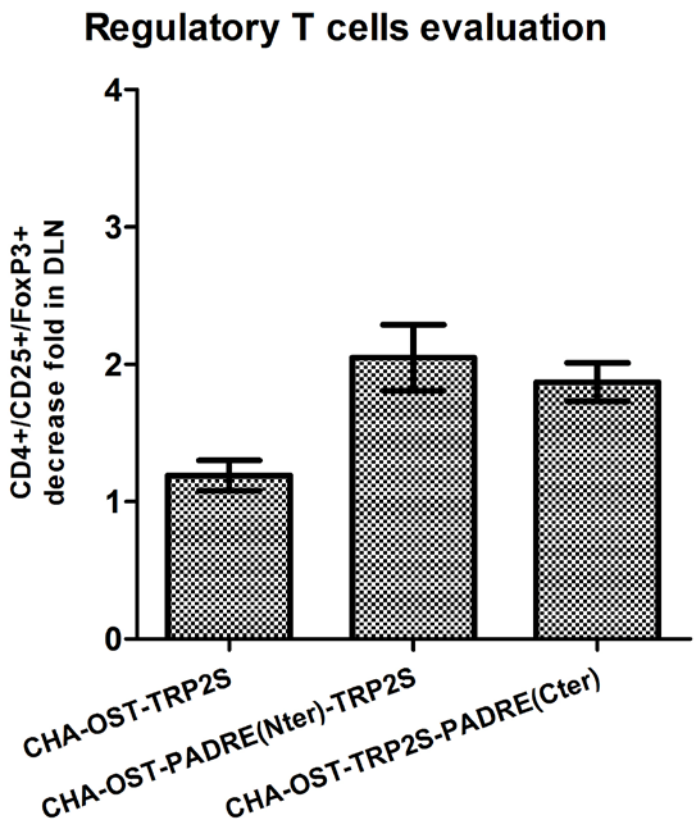

Figure 17: Evaluation of Regulatory T (Treg) Cells in Mice Immunized with TRP2S Delivering Strains.

Mice were injected with CHA-OST-TRP2S or CHA-OST-PADRE(Nter)-TRP2S or CHA-OST-TRP2S-PADRE(Cter) strains or PBS (negative control) two times on 14 days and 7 days before lymph nodes sampling. The prinicle Tregs assessment is the same as that described in the legend of figure 16. Data represented the mean +/- SD from 6 animals per group in triplicate (cumulative results of 2 independent experiments conducted using the same methodology).

A-IV Evaluation of Vaccination Efficiency *via* Tumor Challenge

Through this experiment, we estimated the true anti-tumor efficacy of PADRE containing exogenous antigen delivery vectors *via* their abilities to inhibit the formation and the development of tumor. For each vaccine, mice were vaccinated two times on 14 days and 7 days before the injection of tumor cells, and mice injected with PBS before tumor challenge served as negative control. $1*10^5$ GL26/ mouse were s.c. inoculated on day 0.

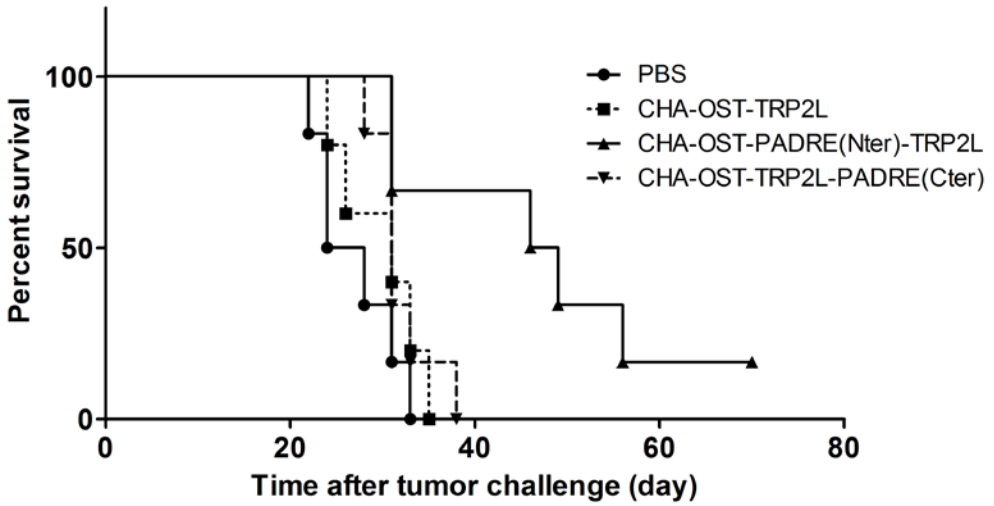

Figure 18: *In vivo* Evaluation of TRP2L Delivering Vectors.

Mice were s.c. immunized with different TRP2L delivering strains D-14/D-7 before s.c. GL26 tumor challenge. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p = 0.007 for CHA-OST-PADRE(Nter)-TRP2L *VS* PBS; p= 0.026 for CHA-OST-PADRE(Nter)-TRP2L *VS* CHA-OST-TRP2L; p= 0.022 for CHA-OST-PADRE(Nter)-TRP2L *VS* CHA-OST-TRP2L-PADRE(Cter); p = 0.259 for OST-TRP2L *VS* PBS; p = 0.106 for OST-TRP2L-PADRE(Cter) *VS* PBS; p = 0.548 for OST-TRP2L-PADRE(Cter) *VS* OST-TRP2L.

Figure 18 presents the results of survival period for the TRP2L group. We can see that, tumor emerged and progressed more rapidly in mice injected with PBS. The vaccines CHA-OST-TRP2L, CHA-OST-PADRE(Nter)-TRP2L and CHA-OST-TRP2L-PADRE(Cter) delayed tumor formation and tumor growth , but only CHA-OST-PADRE(Nter)-TRP2L vector statistically prevented the tumor formation. The statistic difference between CHA-OST-PADRE(Nter)-TRP2L strain and the other vaccines are significant (p= 0.007 for CHA-OST-PADRE(Nter)-TRP2L *VS* PBS; p= 0.026 for CHA-OST-PADRE(Nter)-TRP2L *VS* CHA-OST-TRP2L; p= 0.022 for CHA-OST-PADRE(Nter)-TRP2L *VS* CHA-OST-TRP2L-PADRE(Cter)). However the difference between CHA-OST-TRP2L, CHA-OST-TRP2L-PADRE(Cter) and PBS is not statistically significant.

Figure 19 shows the results for TRP2S group. We can also find the similar relation between vaccinated mice and control mice as that in the TRP2L group. In

general, tumor formed and developed more rapidly in control group (mice injected with PBS), all vaccine strains delayed more or less tumor formation and only CHA-OST-PADRE(Nter)-TRP2S statistically inhibited tumor emergence and development. The statistic difference between CHA-OST-PADRE(Nter)-TRP2S strain and CHA-OST-TRP2S strain is significant (p = 0.047), but not between CHA-OST-PADRE(Nter)-TRP2S and CHA-OST-TRP2L-PADRE(Cter) strains.

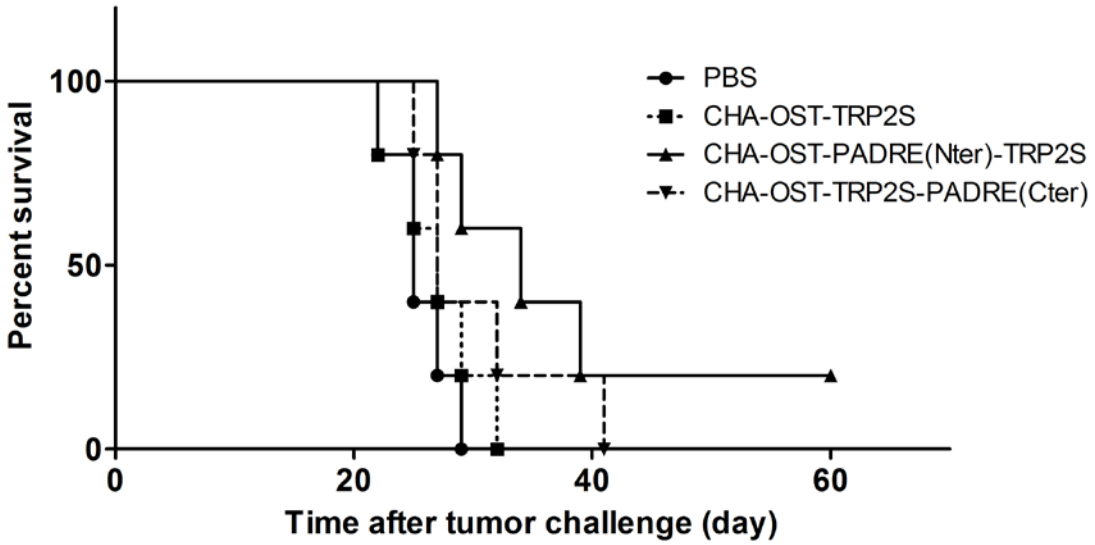

Figure 19: *In vivo* Evaluation of TRP2S Delivering Vectors.

Mice were s.c. immunized with different TRP2S delivering strains D-14/D-7 before s.c. GL26 tumor challenge. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p = 0.019 for CHA-OST-PADRE(Nter)-TRP2S *VS* PBS; p = 0.047 for CHA-OST-PADRE(Nter)-TRP2S *VS* CHA-OST-TRP2S; p= 0.945 for CHA-OST-PADRE(Nter)-TRP2S *VS* CHA-OST-TRP2S-PADRE(Cter); p = 0.683 for CHA-OST-TRP2S *VS* PBS; p = 0.125 for CHA-OST-TRP2S-PADRE(Cter) *VS* PBS; p = 0.366 for CHA-OST-TRP2S-PADRE(Cter) *VS* CHA-OST-TRP2S.

B Construction of Bi-antigen Delivery Vectors

In recent years, multi-epitope or multi-antigen expression pattern has been applied to enhance the potency of cancer vaccines. In order to strengthen our vaccine vectors, we also tried to apply multi-antigen expression pattern in our bacterial exogenous antigen delivery system.

B-I Construction of Bi-antigen Expression Plasmid

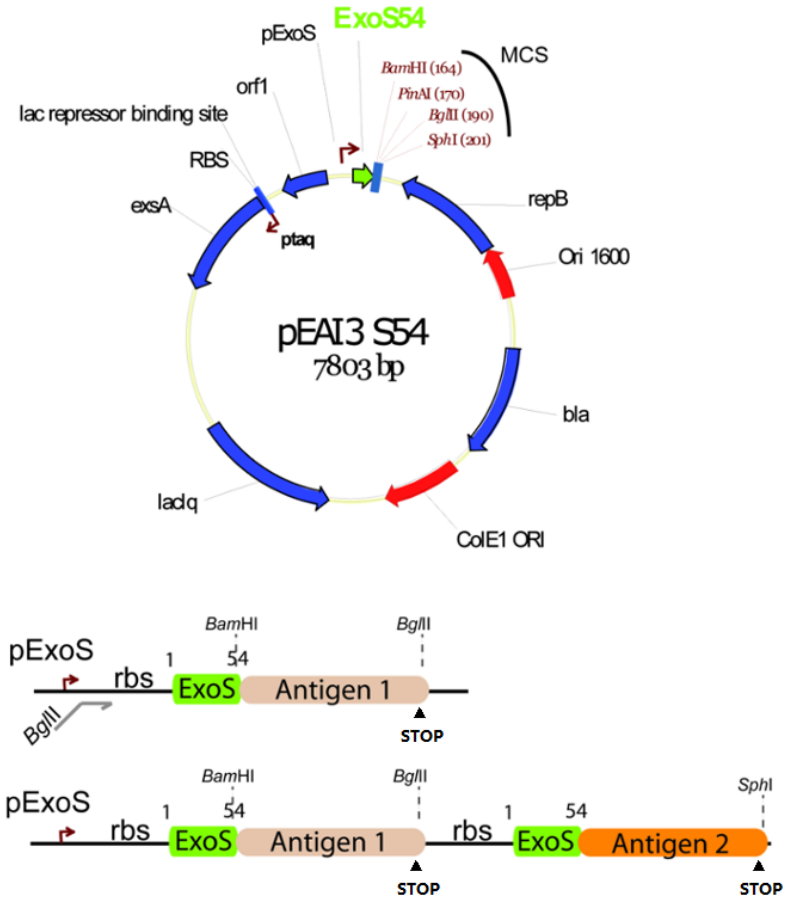

Figure 20: Construction of Bi-antigen Expressing Plasmid.

The bi-antigen co-expression vector was constructed basing single antigen expression vector (pEAI3 S54) in which the first antigen has been cloned at *BamH* I/*Bgl* II sites, the second S54-PADRE-antigen fusion protein expression cassette containing RBS site, fusion protein and STOP codon was added to single antigen expression plasmid at *Bgl* II/*Sph* I sites which located downstream to the first antigen.

Firstly, we constructed bi-antigen expression structure basing on PADRE containing single fusion protein expression plasmid. The principle is described in Fig 20.

Briefly, the first antigen was cloned at BamH I/Bgl II positions in multiple cloning sties (MCS) region in PADRE containing antigen expression plasmid (pEAI-S54-PADRE). The second S54-PADRE-antigen2 fusion protein expression

sequences containing ribosome binding site (RBS) + fusion protein2 + STOP codon were amplified by PCR using mono-antigen expression plasmids as templates. The PCR products were cloned as Bgl II/Sph I fragments into Bgl II/Sph I-digested pEAI-S54-PADRE-antigen1 at the downstream position of the first fusion protein expression cassette. Because each fusion protein expression cassette contains RBS and STOP-codon, two fusion proteins each containing Exo54 secretion TAG could be individually synthesized.

For constructing bi-antigen vectors, several antigens were chosen. Besides the model antigen ovalbumin, two melanoma associated antigens, TRP2 and GP100, were also targeted. Here, the TRP2 cloning sequence was $TRP2L_{125\text{-}376}$ that we have discussed in the previous chapter; and the GP100 cloning sequence was $GP100_{21\text{-}150}$ containing one nature murine $GP100_{25\text{-}33}$ epitope (506). Thereby, based on above principle, we constructed several bi-antigen expression plasmids: pEAI S54-Padre-$OVA_{248\text{-}376}$ S54-Padre-$TRP2L_{125\text{-}376}$, pEAI S54-Padre-$TRP2L_{125\text{-}376}$ S54-Padre-$OVA_{248\text{-}376}$ and pEAI S54-Padre- $TRP2L_{125\text{-}376}$ S54-Padre- $GP100_{21\text{-}150}$.

B-II *In vitro* Evaluation of Antigen Secretion Ability of Bi-antigen Vectors

For bi-antigen delivery application, we chose CHA-OST as the experimental strain and transformed it with above mentioned bi-antigen expression plasmids.

In order to evaluate bi-antigen vectors, we first assessed the antigen secretion ability of different strains *in vitro*. The results were shown in figure 21. Here, we used always CHA-OST-EI as the negative control and used relative single antigen vectors as the positive references. As we previously discussed, PopB and PopD are two important indicators of T3SS activation. In the figure 21, the two structure proteins (marked with arrows) can be observed in all lanes, which indicate the correct activation of T3SS. Besides, we can also see the bands corresponding to the different antigens (marked with asterisks) in all lanes except the first lane corresponding to CHA-OST-EI. So these results confirm the double antigen secretion ability of bi-antigen bacterial vectors. However, even both antigens could be secreted by

bi-antigen vectors, we found that when the antigen was cloned at the second position in bi-antigen vector, it was less secreted than when cloned alone in single-antigen vector.

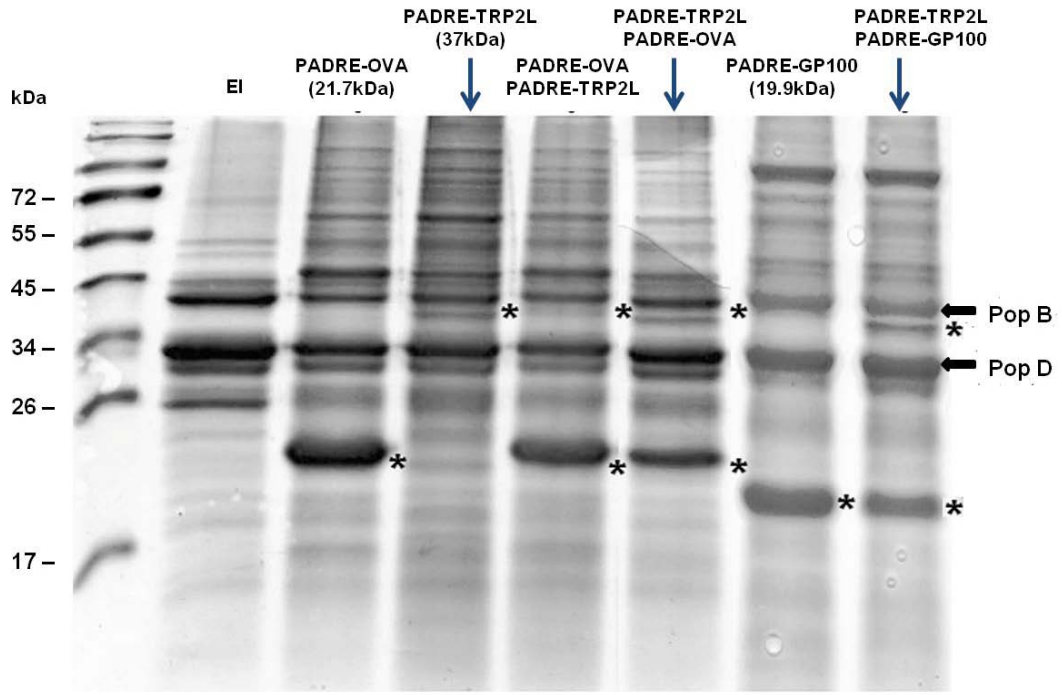

Figure 21: *In Vitro* Evaluation of Fusion Protein Secretion Ability of Bi-antigen Vectors.

T3SS-mediated fusion protein secretion by bi-antigen vectors was assessed by SDS-PAGE. The positions of PopB (top) and PopD (bottom) are marked with arrows; the positions of S54-PADRE-antigen fusion proteins are marked with asterisks.

B-III *In vitro* Evaluation of Antigen Translocation Ability of Bi-antigen Vectors

In previous work (3), it has been proved that the fusion protein delivery mediated by of *P. aeruginosa* could lead to DC maturation, activation and antigen presentation to specific CD8+ T lymphocytes. So, we evaluated in this work if bi-antigen vector has the same antigen translocation ability for a given antigen as mono-antigen vector. Furthermore, we wondered also if the antigen position could interfere with that process. To answer these questions, bi-antigen vector was evaluated through B3Z activation assay.

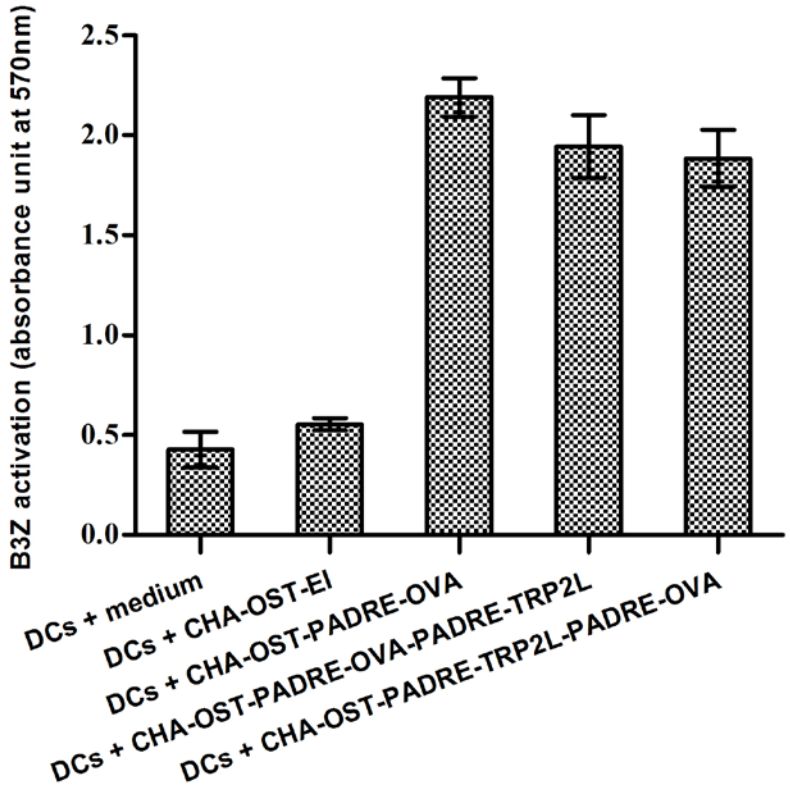

Figure 22: *In Vitro* Evaluation of T3SS-mediated Antigens Translocation by Bi-antigen Vectors through B3Z Activation Assay.

B3Z activation was assessed with a *β*-galactosidase enzymatic assay (absorbance at 570 nm). The bi-antigen vaccines vectors containing S54-PADRE-OVA fusion protein at the different position in bi-antigen expression plasmid (CHA-OST-PADRE-OVA-PADRE-TRP2L & CHA-OST-PADRE-TRP2L-PADRE-OVA) delivered the ovalbumin fragment through T3SS and was compared with three control vectors: CHA-OST-EI (negative control) and two single antigen vectors (CHA-OST-PADRE-OVA & CHA-OST-PADRE-TRP2L). Data represented the mean +/- SD from six animals per group in triplicate (cumulative results of two independent experiments conducted with the same methodology).

B3Z hybridoma cell line is specific for the ovalbumin peptide SIINFEKL (residues 257–264) in the context of H-2Kb and expresses the *β*-galactosidase gene under the control of the IL-2 promoter. B3Z activation indicates that dendritic cells have correctly received and processed the antigenic message. Therefore we chose $OVA_{248-376}$ as the model antigen and cloned it in either the first position or the second position in bi-antigen vector with $TRP2L_{125-376}$ as the second antigen. In figure 22, we can see that TCR-mediated B3Z activations were obtained after incubation with DCs exposed to CHA-OST-PADRE-OVA, CHA-OST-PADRE-OVA-PADRE-TRP2L and

CHA-OST-PADRE-TRP2L-PADRE-OVA strains, but no activation was found after incubation with DCs exposed to CHA-OST-EI control strain. These results indicated that as for antigen delivery and presentation, bi-antigen vectors appear to contribute slightly less to B3Z activation than mono-antigen vector although the difference was not statistically significant; nevertheless, no difference was found between CHA-OST-PADRE-OVA-PADRE-TRP2L and CHA-OST-PADRE-TRP2L-PADRE-OVA strains, which demonstrate that the antigen cloning position will not affect its delivery by bi-antigen vector.

B-IV Evaluation of Bi-antigen Vectors by e*x vivo* Immune Monitoring through IFN-γ ELISpot Assay

For evaluating the vaccination efficiency of bi-antigen bacterial vector, we investigated the antigen specific immune response after two times of injections of mice with either mono-antigen strain (CHA-OST-PADRE-TRP2L or CHA-OST-PADRE-GP100, $5*10^6$ bacteria / time) or bi-antigen strain (CHA-OST-PADRE-TRP2L-PADRE-GP100, $5*10^6$ bacteria / time). The T cell responses specific to the two TRP2 epitopes ($TRP2_{363-372}$ and $TRP2_{181-188}$) and one GP100 epitope ($GP100_{25-33}$) were monitored through IFN-γ ELISpot assay and this test has been repeated two times.

However, the results of this trial were not satisfying. In all immunized mice, no evident T cell responses against $TRP2_{363-372}$, $TRP2_{181-188}$ and $GP100_{25-33}$epitopes were detected (data not shown).

B-V *In vivo* Evaluation of Bi-antigen Vectors in Murine Tumor Models

B-V-a In Murine Glioma Model (GL261)

GL261 glioma cell line is one of the most frequently used murine brain tumor models and expresses both melanoma associated antigen TRP2 and GP100. To evaluate *in vivo* tumor protection efficiency induced by bi-antigen vector, we injected C57BL/6 mice with either mono-antigen strain (CHA-OAT-PADRE-TRP2L or CHA-OST-PADRE-GP100, $4*10^6$ bacteria / time), bi-antigen strain

(CHA-OST-PADRE-TRP2L-PADRE-GP100, $4*10^6$ bacteria/time) or combined mono-antigen strains (CHA-OST-PADRE-TRP2L+ CHA-OST-PADRE-GP100, $8*10^6$ bacteria in total/time). Here, we performed both prophylactic and therapeutic anti-GL261 assay. In both assays, the group injected with CHA-OST-EI strain was the negative control.

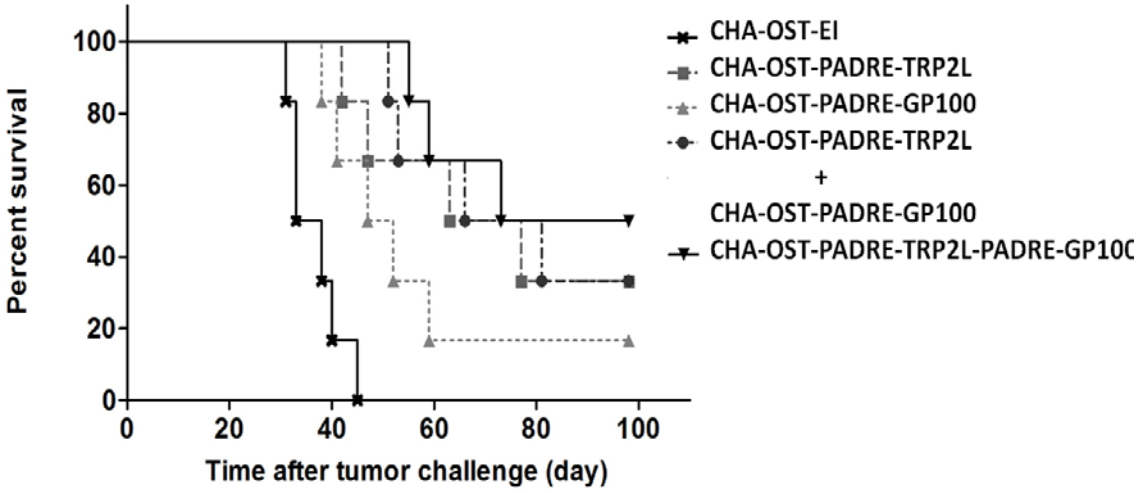

Figure 23: *In Vivo* Evaluation of Bi-antigen Vectors in Prophylactic Vaccination Model (in Murine GL261 Tumor Model).

Mice were s.c. injected with different CHA-OST-fusion protein strains on 14days and 7 days before s.c. GL261 tumor challenge. Kaplan-Meyer curves displayed survival data from groups of 12 mice (cumulative results of two independent experiments conducted with the same methodology). Statistical analysis: $p < 0.01$ for immunized groups *VS* CHA-OST-EI; $p = 0.044$ for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-PADRE-GP100; $p = 0.63$ for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-PADRE-TRP2L; and $p = 0.51$ for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* combined strains.

For the prophylactic assay, we injected C57BL/6 mice subcutaneously with the different strains at both 14 and 7 days before subcutaneous injection of $1*10^5$ GL261 cells, the results are shown in figure 23. In this assay, we can see that all mice injected with CHA-OST-EI developed rapidly a tumor and were sacrificed within 6 weeks after injection of GL261 cells; in all immunized groups, tumor formation has been significantly delayed (CHA-OST-PADRE-TRP2L *VS* CHA-OST-EI, $p = 0.0016$; CHA-OST-PADRE-GP100 *VS* CHA-OST-EI, $p = 0.0094$; combined strains *VS* CHA-OST-EI, $p = 0.0005$; CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS*

CHA-OST-EI, p = 0.0005). The bi-antigen strain injections and combined strains injections were more efficient to prevent tumor formation than mono-antigen strains. The difference between bi-antigen injected group and CHA-OST-PADRE-GP100 group was statistically significant ($p < 0.05$), whereas there was no significant difference among CHA-OST-PADRE-TRP2L injected group, bi-antigen strain injected group and combined strains injected group.

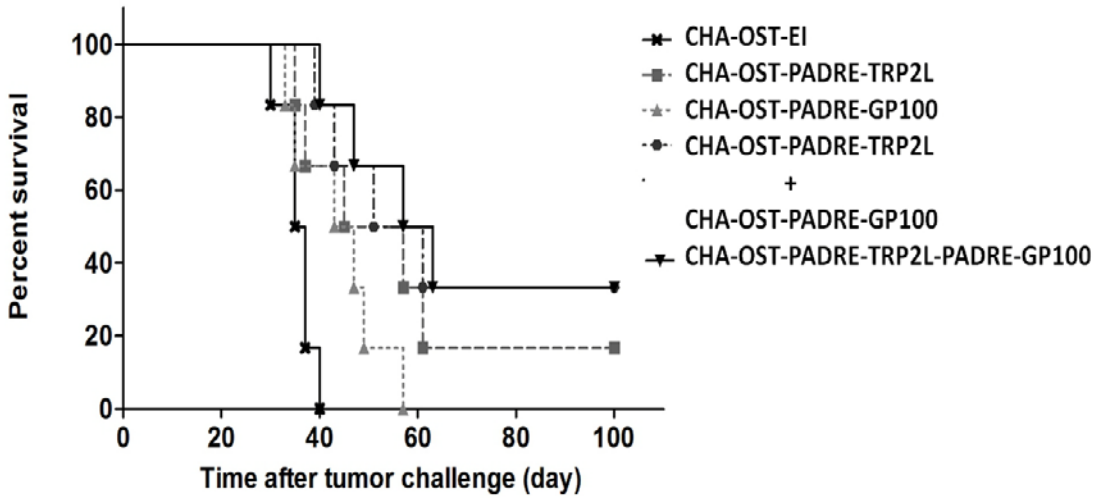

Figure 24: *In Vivo* Evaluation of Bi-antigen Vectors in Therapeutic Vaccination Model (in Murine GL261 Tumor Model).

Mice received a s.c. tumor challenge with GL261 tumor cells on day 0 and then were vaccinated with different CHA-OST-fusion protein strains on following D1/D5/D9/D13/D17/D21 therapeutic schema. Kaplan-Meyer curves displayed survival data from groups of 12 mice (cumulative results of two independent experiments conducted with the same methodology). Statistical analysis: p = 0.022 for CHA-OST-PADRE-TRP2L *VS* CHA-OST-EI; p = 0.054 for CHA-OST-PADRE-GP100 *VS* CHA-OST-EI; p = 0.0018 for combined strains *VS* CHA-OST-EI; p = 0.0014 for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-EI; p = 0.046 for CHA-OST-PADRE-TRP2L-PADRE-GP100 VS CHA-OST-PADRE-GP100; p = 0.50 for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-PADRE-TRP2L; and p=0.84 for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* combined strains.

For the therapeutic assay, we injected C57BL/6 mice subcutaneously with $1*10^5$ GL261 cells on day 0 and then with the different strains on following the therapeutic schema containing six injections with 4 days of interval (described in the next chapter). The results are shown in figure 24. We can observe similar results that those obtained in prophylactic assay. CHA-OST-PADRE-TRP2L strain injections,

combined strains injections and bi-antigen strain injections provided the significant tumor protection (CHA-OST-PADRE-TRP2L *VS* CHA-OST-EI, p = 0.032; combined strains *VS* CHA-OST-EI, p = 0.0018; CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-EI, p = 0.0014). The CHA-OST-PADRE-GP100 strain injections slightly delayed the tumor formation whereas the difference was not significant (CHA-OST-PADRE-GP100 *VS* CHA-OST-EI, p = 0.055). Combined strains and bi-antigen strain were more efficient than mono-antigen strain. Moreover, the differences between CHA-OST-PADRE-TRP2L injected group, bi-antigen strain injected group and combined strains injected group were not remarkable, while the difference between bi-antigen injected group and CHA-OST-PADRE-GP100 group was statistically significant ($p < 0.05$).

B-V-b In Murine Melanoma Model (B16)

Transplantable murine melanomas are well-established models for the study of experimental cancer therapies. Melanomas express different tumor-associated Ags, such as TRP2, GP100, MAGE, etc., which are potential targets for novel designs of therapeutic cancer vaccines. Many immunotherapeutic protocols have been tested using the murine B16 melanoma cell line that originated in the C57BL/6 (H-2b) mouse strain. So, in this study, we have also investigated our bi-antigen vectors in murine B16 model. The investigated vaccine strains were same as that assessed in GL261 model.

In the prophylactic assay, C57BL/6 mice were subcutaneously injected with the different strains at both 14 and 7 days before subcutaneous injection of $2*10^5$ B16 cells, the results are shown in figure 25. We can find that immunization with single antigen strains slightly delayed the tumor formation, but the difference was not significant; moreover, the combined injection mode or administration with bi-antigen vector can provide a slight optimization, but the difference between single antigen vectors, combined strains and bi-antigen strain was neither statistically significant. However, just the slight optimization provided by combined strains or bi-antigen

strains leads to the significant differences between immunized groups and non-immunized control group. (CHA-OST-PADRE-TRP2L *VS* CHA-OST-EI, p = 0.32; CHA-OST-PADRE6GP100 *VS* CHA-OST-EI, P = 0.17; combined strains *VS* CHA-OST-EI, p = 0.046; CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-EI, p = 0.037)

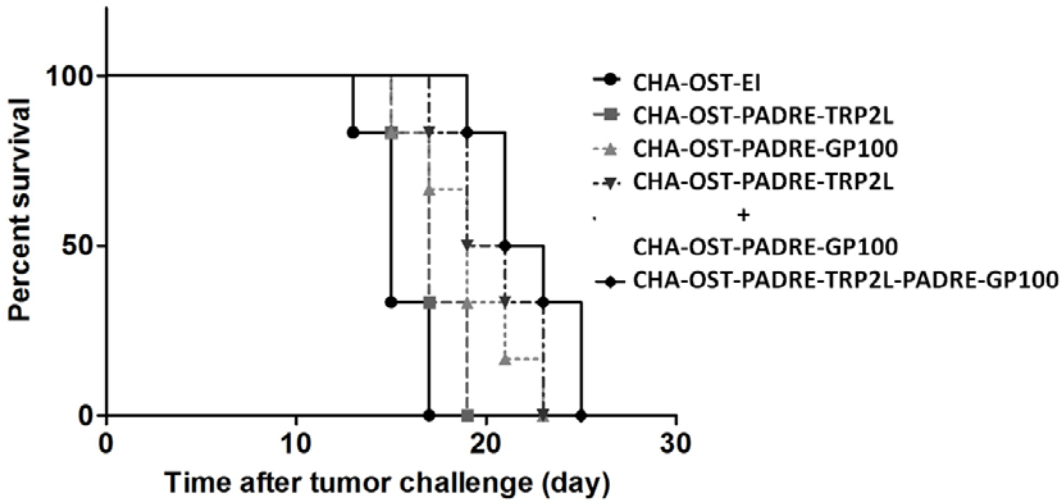

Figure 25: *In Vivo* Evaluation of Bi-antigen Vectors in Prophylactic Vaccination Model (in Murine B16 Tumor Model).

Mice were s.c. injected with different CHA-OST-fusion protein strains on 14 days and 7 days before s.c. B16 tumor challenge. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p =0.32 for CHA-OST-PADRE-TRP2L *VS* CHA-OST-EI; P =0.17 for CHA-OST-PADRE6GP100 *VS* CHA-OST-EI; p =0.046 for combined strains *VS* CHA-OST-EI; p =0.037 for CHA-OST-PADRE-TRP2L-PADRE-GP100 *VS* CHA-OST-EI.

In B16 melanoma model, as the tumor protection provided by bacterial vaccines was not so significant in prophylactic assay, we didn't go on with the therapeutic assay.

In vivo tumor challenge results obtained in both tumor models clearly indicated that vaccinations with two antigens are more efficient than vaccinations with one antigen and that this bivalence could be obtained with only one strain expressing both antigens delivered through T3SS. Moreover, the bi-antigenic strain contributed to the injection of fewer amounts of bacteria, 4.10^6 *VS* 8.10^6 for the combination of the both single antigen expression strain.

C Construction of Humoral Response Activating Vector

We have previously demonstrated that antigen delivery through the T3SS of *P. aeruginosa* would target at MHC-I-restricted antigen presentation pathway and be capable of trigger CD8 + CTL responses (3, 6). Thereby, in order to trigger a broader anti-tumor immune response and enhance the vaccination efficacy, we looked for elaborating the vectors which target humoral immune response arm of antitumour immunity.

C-I Strategy

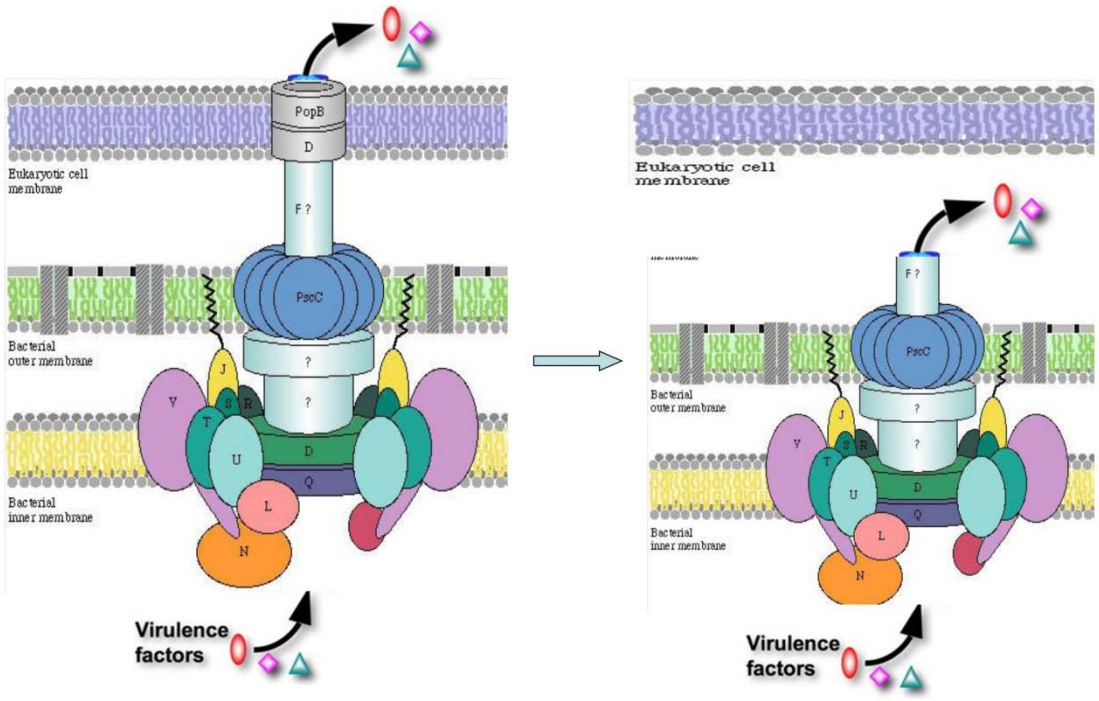

Figure 26: Strategy for Humoral Response Activating Bacterial Vector Construction (Adapted from Frank DW *et al.* (446)).

For the toxin translocation through *P. aeruginosa* T3SS, tow structure proteins- PopB and PopD are crucial. When bacterial cells contact eukaryotic cells or present in low calcium condition, T3SS is activated and PopB and PopD could insert into the membrane of eukaryotic cells and form the secretion and translocation channel. We supposed that the intra-cytoplasmic translocation of toxins should be blocked and the toxins would be secreted to extra-cellar space, which might induce an antigen specific humoral immune response.

As we have previously discussed, the T3SS was the principal factor that contributed most to the virulence of *P. aeruginosa*. Particularly, for the

intra-cytoplasmic delivery of toxins, two structure proteins – PopB and PopD are needed. Thereby, we supposed that even T3SS is activated, if PopB and PopD are not present, the intra-cytoplasmic translocation of proteins should be blocked. In this condition, *P. aeruginosa* acts as a general exogenous pathogen. The pathogen-associated proteins might be captured within bacteria by APCs through phagocytosis/pinocytosis or be secreted into extra-cellular space. Thereby, antigen specific humoral response might be activated. Basing on this hypothesis, we suggested that *P. aeruginosa* ⊿PopB⊿PopD mutant can be used as antigen delivery vectors for humoral immune response activation. This strategy is briefly described by figure 26.

C-II Construction of CHA-OST-⊿PopB⊿PopD (⊿BD) Mutant

The mutagenesis was performed by the method developed by L. Quénée (497). The plasmids used for allelic exchange were developed by M. Derouazi in 2005. Firstly, we realized the *popBpopD* gene deletion in the mutant CHA-OST in order to obtain the CHA-OST-⊿BD strain.

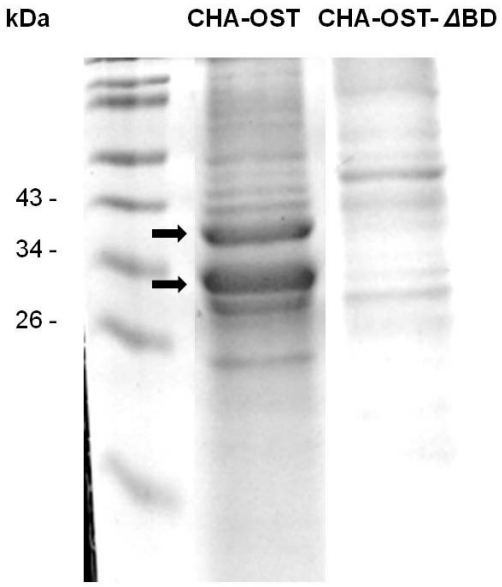

Figure 27: Verification of CHA-OST-⊿BD Strain by SDS-PAGE.

PopB (top) and PopD (bottom) were detected in the bacterial culture of CHA-OST strain (marked with arrows) but not in that of CHA-OST-⊿BD strain.

Then, we verified the CHA-OST-*Δ*BD strain by PCR technique. However, the results of several assays were incredible. In repeated verifications, there were always the bands of non-specific amplification. As PopB and PopD can be visualized by SDS-PAGE of cultured bacteria, we chose the alternative method to investigate the mutant strain.

In figure 27, we can see in the lane which represents the culture of CHA-OST strain, two bands corresponding to PopB and PopD proteins exist, but they are not present in the lane which represents the culture of CHA-OST-*Δ*BD strain.

C-III T3SS Investigation of CHA-OST-*Δ*BD Strain

In order to investigate the T3SS of CHA-OST-*Δ*BD strain, we transformed it with pEAI S54-Padre-$OVA_{248-376}$. As the T3SS evaluation studies described before, we assessed T3SS activation of CHA-OST-*Δ*BD strain in calcium deleting medium and verified the antigen expressing and secretion ability of this strain. Here, CHA-OST-EI strain served as the negative control and CHA-OST-OVA strain was used as positive reference. We tested the three strains in two conditions, either vaccinations condition in which we added only IPTG to induce antigen expression but no calcium deletion was realized; or *in vitro* T3SS activating condition in which calcium was depleted by EGTA and magnesium was added to maintain divalent cations.

In figure 28, we can see that PopB and PopD proteins can be observed in the lanes corresponding to CHA-OST-EI and CHA-OST-PADRE-OVA strains in both conditions, the bands are more evident in T3SS activating condition than in vaccination condition. However, in both conditions, in the lanes corresponding to CHA-OST-*Δ*BD-PADRE-OVA strain, PopB and PopD are not present; meanwhile, OVA protein was secreted when T3SS has been activated. These results demonstrate: 1) the successful deletion of Pop B and Pop D proteins in CHA-OST-*Δ*BD mutant; 2) the fusion protein expression and secretion ability of this strain; and 3) the good

carrying of fusion protein by CHA-OST-*Δ*BD before T3SS activation.

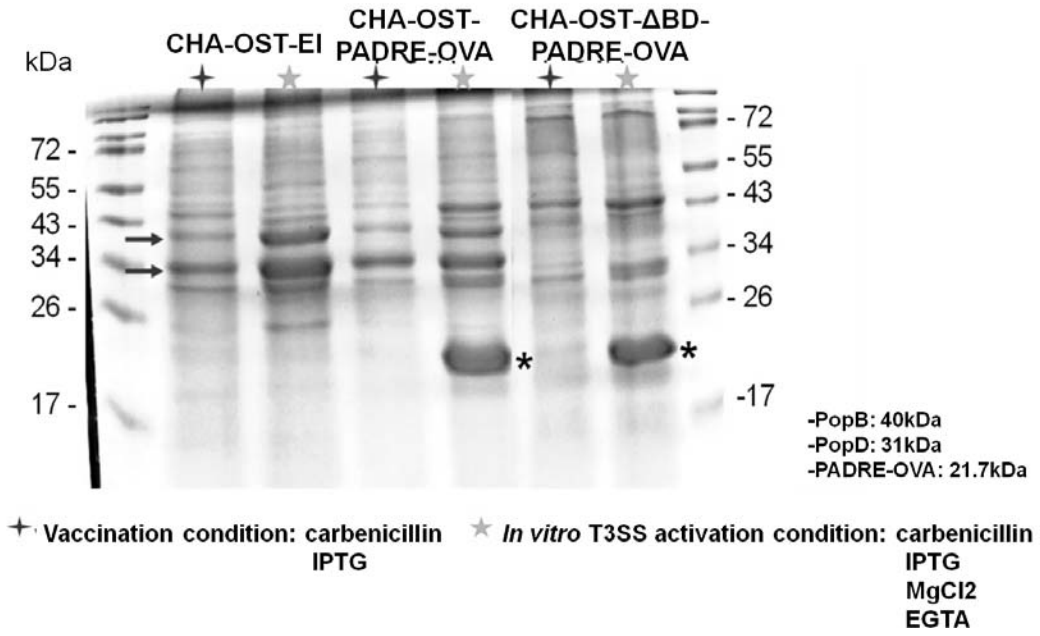

Figure 28: *In Vitro* Evaluation of Fusion Protein Expression and Secretion Ability of CHA-OST-*Δ*BD Vector.

T3SS-mediated fusion protein expression and secretion by CHA-OST-*Δ*BD vector was investigated in two conditions: 1) vaccinations condition, in which only IPTG was added to induce antigen expression but no calcium depletion was realized; 2) *in vitro* T3SS activating condition, in which calcium was depleted by EGTA and magnesium was added to maintain divalent cations. The positions of PopB (top) and PopD (bottom) are marked with arrows; the positions of S54-PADRE-antigen fusion proteins are marked with asterisks.

C-IV Investigation of Humoral Response Induced by CHA-OST-*Δ*BD Vector

To investigate humoral response activating ability of CHA-OST-*Δ*BD vector, we used OVA as model antigen. Here, CHA-OST-EI strain served as negative control, CHA-OST-PADRE-OVA was used as the reference, and commercially ordered OVA protein supplemented with complete/non-complete adjuvant was used as the positive reference. In CHA-OST-EI and CHA-OST-PADRE-OVA groups, mice were injected with $5*10^6$ bacteria /time; in OVA group, mice were injected with pure protein at 5 μg/time. For CHA-OST-*Δ*BD-PADRE-OVA strain, two quantities were assessed: $5*10^6$ bacteria /time (L) or $1*10^7$ bacteria /time (H). Moreover, one more group in

which mice were injected with combined strains ($5*10^6$ CHA-OST-PADRE-OVA + $5*10^6$ CHA-OST-⊿BD-PADRE-OVA / time) was also added in this assay. In this trial, mice were administered 3 injections with 14 days of interval and the antibody detection was realized 14 days after the last injection. For each group, six mice were experimented.

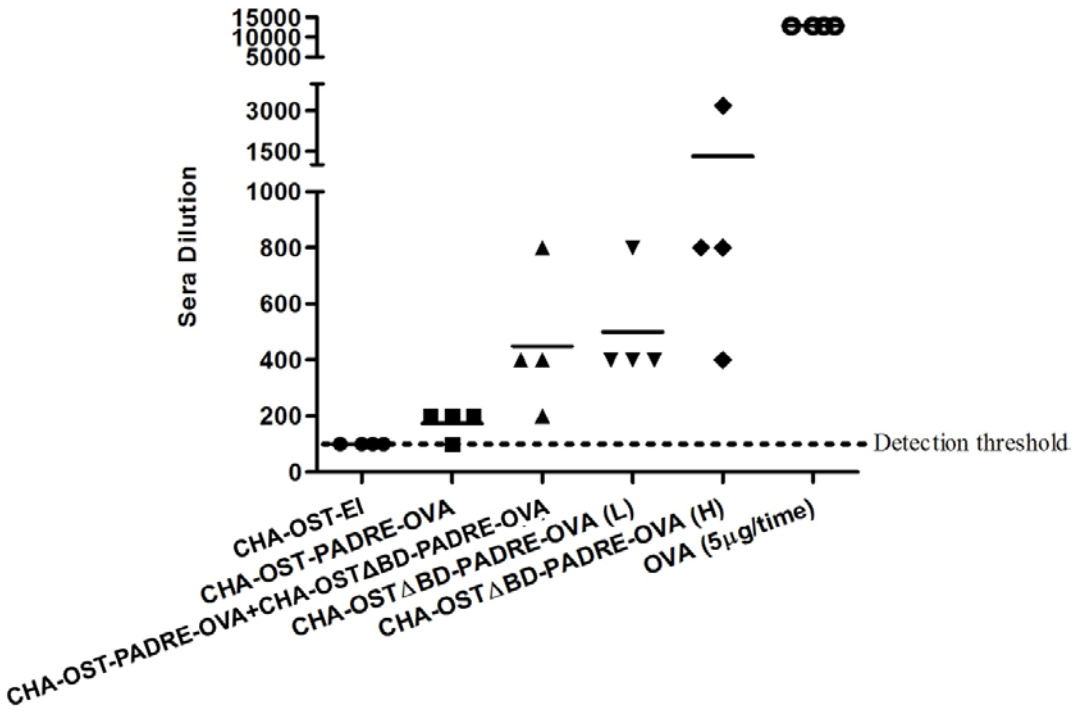

Figure 29: Evaluation of the Humoral Response Activating Ability of CHA-OST-⊿BD Vector.

Mice were subcutaniously injected three times at a 14 days of interval with OST-PADRE-OVA ($5*10^6$/time) or CHA-OST-⊿BD-PADRE-OVA at low ($5*10^6$/time) or high ($1*10^7$/time) dose or combined strains (CHA-OST-PADRE-OVA ($5*10^6$/time) + CHA-OST-⊿BD-PADRE-OVA ($5*10^6$/time)). Antibody detection was realized 14 days after the last injection. Mice injected with CHA-OST-EI ($5*10^6$/time) were used as negative control and mice injected with pure OVA protein (5µg/time) supplemented with complete/non-complete adjuvant were used as positive control. Data represented the mean with individual values from six animals per group in duplicate.

In figure 29, we can see that: 1) no evident antigen specific humoral response was activated by CHA-OST-PADRE-OVA injections, which confirm that T3SS mediated antigen delivery is MHC I pathway restricted; 2) some response were observed in CHA-OST-⊿BD-PADRE-OVA strain injected groups and the response was bacteria dose dependent, which reveals that the humoral response activating

potential of this mutant vector; whereas the response was not statistically significant (P= 0.08, one way ANOVA); 3) moreover, by comparing CHA-OST-*Δ*BD-PADRE-OVA low dose injected group and combined strains injected group, no obvious interference between different strains was observed, which means the there was no interference and competition between the different strains.

D Discussion

D-I Addition of CD4+ Th Epitope – PADRE

Several MHC class II restricted peptides have been identified to be potentially helpful for the development of vaccines. PADRE was firstly developed by Alexander in 1994 (507). We chose PADRE as CD4+-T-cell activator to enhance the vaccine performance because this peptide has been demonstrated as a strong inducer of T helper cells in a variety of vaccine vectors for not only the generation of CTL response, but also the induction of a humoral immune response (251, 504, 508, 509). Furthermore, different to other MHC II restricted peptides, PADRE is a promiscuous CD4+ Th determinant capable of binding with high affinity to most of DR alleles so that both human and murine MHC II molecules can bind this sequence. Thereby, it would be not only an ideal CD4+ T cell activator for cancer immunotherapy within murine model but also a promising candidate for human anti-tumor vaccines development.

Since the location of insert encoding the target antigen might affect the transcription, translation, secretion through T3SS by bacteria, as well as the degradation of fusion protein by acid proteases in cytoplasm or by proteasome of APCs, PADRE coding sequence has been inserted either upstream or downstream of antigen cloning site. We first investigated *in vitro* fusion proteins expression and secretion ability of PADRE containing vector. We found that the insertion of PADRE epitope didn’t interfere the fusion protein expression and secretion ability of bacterial vectors, neither the position of PADRE did (Fig. 13).

Then, we have proved the optimization effect of PADRE in our vaccines, which is more significant when PADRE is synthesized upstream of antigen, i.e. in PADRE (Nter) – cloning vector. CTL experiments (Fig. 14 and Fig. 15) demonstrated the obvious immune responses stimulated by our bacterial vectors. Moreover, through tumor challenge experiments, we found that PADRE(Nter)-cloning vector can significantly improve the anti-tumoral efficacy of vaccines; the emergence of tumor was delayed and the progression of tumor was decreased. All observations are consistent in the groups of different constructions of TRP2 antigen (TRP2L, Fig. 18 and TRP2S, Fig. 19). However, in CTL experiments, although the cytotoxicity curve corresponding CHA-OST-PADRE(Nter)-Antigen was slightly higher than that representing the other vaccines, the difference was not significant. We think that this could be due to the timing of detection. As we know, cell-mediated response to acute infection can generally be divided into four phases: 1) CTLs activation/expansion phase: this phase lasts generally 7-8 days after the infection, during which naïve CTL precursors are primed and exponentially proliferate; 2) CTLs contraction phase: this phase lasts about 2-3 weeks after activation/expansion phase, during which 90%-95% effector T cells undergo apoptosis ; 3) memory T cell maintenance phase: during this phase, memory T cells could be maintained at stable levels throughout the life of the mouse and for many years in human; 4) the rapid CTLs-recalling response following re-exposure to the pathogen (90, 510). In this study, we detected CTL cytotoxicity two weeks after vaccination, which corresponded to the beginning of the contraction phase; meanwhile the programming and maintenance of CD8+ cells by CD4+ T cells will be more obvious during the maintenance/memory phase. Thus, it is possible that the difference between vaccines shown by CTL experiments was not evident at this time point. However, through tumor challenge experiments, we can see the obvious difference between different vaccine constructions 35-45 days after vaccination, which supports our hypothesis to the results of CTL experiments. In order to verify PADRE efficiency by CTLs assay, later experiments should be performed at prolonged detection time or ELISpot assays should be used.

On the other hand, regulatory T cells have been proved to be the suppressor of effector T cells. They can not only induce immune tolerance to self-antigen, but also suppress the immune response to non-self antigens, such as microbial antigens, tumor antigens, etc. (511). Thus, T regulatory cells are considered as a restriction factor for anti-tumor immunotherapy and inactivation of T regulatory cells has been suggested as an approach to improve immunogenicity of anti-tumor vaccines (512). Therefore, in this study, in order to better evaluate the immune response stimulated by bacterial vectors and further estimate the impact of Tregs to our vaccines, we have also measured the percentage of regulatory T cells in draining lymph node after vaccination. Through Tregs detection assay (Fig. 16 and Fig. 17), we can find in both groups (TRP2L and TRP2S) that there were a lower level of Tregs in mice injected with CHA-OST-PADRE(Nter)-Antigen. So we can think that the suppression effect of immune system to activated effector cells was the weakest in mice injected with CHA-OST-PADRE(NTer)-Antigen. Thereby, except for the co-stimulation effect of CD4+ cells, this result can further confirm and better explain why the immune response stimulated by vaccine CHA-OST-PADRE(Nter)-Antigen is the strongest and why the anti-tumor action of CHA-OST-PADRE(Nter)-Antigen is the most efficient. Moreover, as deletion of T regulatory cells has been suggested as an approach to improve immunogenicity of anti-tumor vaccines. It is possible that we construct anti-Tregs vaccine and combine it with PADRE-antigen vaccines in later experiment. With less impact of Tregs cells, the anti-tumor efficiency of PADRE-Antigen vaccines might be more improved.

Moreover, in this study, we have found that between the two tested TRP2 constructions, TRP2L always functioned better than TRP2S. Vaccines containing TRP2L fragment can stimulate stronger immune response than TRP2S vaccines, which was demonstrated by both CTL assay and tumor challenge experiment. Comparing antigen sequences, we think the better efficiency of TRP2L might be due to the fact that TRP2L contains longer antigen sequence than TRP2S, in which it might exist more potential, non-identified CD8+ and CD4+ epitopes. So, in further

experimentation, we need to construct the antigen sequences of different length between TRP2L and TRP2S and verify their immunogenicity by vaccination, so as to identify the potential epitopes and better explain the difference between TRP2L and TRP2S.

D-II Construction of Bi-antigen Delivery Vectors

In recent years, multi-epitope or multi-antigen expression pattern has been applied to enhance the potency of cancer vaccines. Because vaccinations through this approach could induce a broader cellular immune response while making cancer cells more visible for T cells, it is likely to be more efficacious than immunization with a vaccine for a single antigen (513). Numerous studies demonstrated the efficient tumor prevention mediated by different types of vaccine vectors based on this pattern, such as multi-epitope DNA vaccine (514), dendritic cell (DC)-based vaccine (515), multi-peptide vaccine (516), multi-antigen containing virus vector (517). Also, the efficiency of this pattern has been applied not only in animal experiments but also in human trials (515, 518, 519). But so far, little correspondent results have been gained in bacterial vaccines domain. Thereby, we would like to strengthen the immune response activating ability of our bacterial vectors by applying the multiple-antigen expression pattern.

In this study, we developed bi-antigen delivering bacterial vector using one *P. aeruginosa* mutant strain - CHA-OST. Bi-antigen cloning vector was constructed from the single antigen expression plasmid, each fusion protein expression cassette contains RBS and STOP-codon and thus two fusion proteins could be co-transcribed and individually translated (Fig. 20). However, we wounded if the position of antigen encoding genes on plasmid with interfere with the relative secretion of each protein. So, we first investigated *in vitro* fusion proteins expression and secretion ability of bi-antigen vector. The results shown by several bi-antigen strains were identical: both fusion proteins could be well expressed and secreted by bi-antigen vectors; however, the protein secretion mediated by bi-antigen vectors is position dependent, fusion

proteins were slightly less secreted when they were cloned at the second position probably due to a lower level of translation (Fig. 21). We think that there might also be a competition between these two proteins as for the secretion through T3SS. It is possible that the protein encoded by the first fusion protein expression cassette in bi-antigen vectors holds the dominant position during T3SS secretion process.

As we were not sure if the position – dependent protein secretion will further affect the antigen delivery processed by bi-antigen vectors and antigen specific immune response activation, we used OVA as the model antigen and cloned it at both positions in bi-antigen vector to investigate the influence of antigen cloning position to antigen delivery and presentation. Trough B3Z activation assay (Fig. 22), we didn't find evident difference evoked by OVA cloning position, which indicated that the DCs activation and antigen presentation were not directly antigen position dependent. However, bi-antigen vectors provided slightly lower B3Z activation than single antigen vector. We hypothesize that because the assay was realized *in vitro*, the quantity of DCs was limited and saturation of MHC I presentation must have occurred, which resulted in the competition between the two antigens as for binding to MHC I molecules and limited the presentation of individual antigen. But this would not be a problem for *in vivo* assay. For this reason, it needed to further evaluate bi-antigen vector through *in vivo* tumor challenge.

In the *in vivo* experiments, we tested bi-antigen vector expressing two self antigens TRP2 and GP100 in two natural tumor model (Glioblastoma (GL261) and melanoma B16) and the results were encouraging. In GL261 model, both in prophylactic and in therapeutic assay (Fig. 23 and Fig. 24), bi-antigen strains provided a more efficient protection. Tumor onset was more effectively delayed and tumor growth progression was more evidently reduced in bi-antigen strain vaccinated group than in single-antigen strain vaccinated groups. And, bi-antigen strain demonstrated a similar performance as combined single-antigen strains. In B16 model (Fig. 25), similar results were obtained; bi-antigen strains provided a more efficient tumor

protection than each signal antigen delivery strain and demonstrated the similar performance to combined single-antigen strains. More interesting, both single antigen delivery vector haven't provide significant tumor protection, but bi-antigen delivery vector did.

Here, we also found that the anti-tumor protection contributed by our bacterial vectors is weaker in B16 model than in GL216. We hypothesized that this result might be related to the intrinsic characters of B16 tumor. It is well known that B16 is a poorly immunogenic cell line and express very low class I levels and hence low levels of antigens, which is an important mechanism for B16 to escape from T-cell killing and also might be one potential explanation for the weaker B16 protection obtained with our bacterial vaccines. Even then, strengthened antitumor effect provided by bi-antigen expression pattern has been demonstrated even in this poorly immunogenic tumor model.

All above results reveal the advantages of bi-antigen strains. The first advantage is a better efficiency: vaccinations with bi-antigen vector containing GP100 and TRP2L provided a better tumor protection than both single-antigen immunizations. The second advantage is simplicity: in contrast to the individual preparation for two vaccines, bi-antigen vector is processed in one time. Furthermore, through this study, the feasibility and the efficacy of multiple antigen delivery via our bacterial vectors have been confirmed. Here, we have only investigated bi-antigen delivery pattern, it is possible that, in future studies, more than two antigens could be combined in our bacterial vaccines to induce a broader immune response; and more significant tumor protection might be obtained.

However, in *ex vivo* immune monitoring essay, no evident responses to two TRP2 epitopes and one GP100 epitope have been detected. Our hypothesis for this phenomenon is that the peptide specific immune response activation is dependent on protein processing machinery of APCs as well as tumor cells. Indeed, different studies reported that several CTL raised against high-affinity binding peptides did not

recognize tumor cells expressing the targeted antigen, mainly because their protein processing machinery did not display the peptides (520, 521). Thereby, in our experiment, both the processing of translocated antigen by APCs and the processing of TAAs by tumor cells might affect the immune response to certain peptides. Whatever, the performance of our bacterial vectors has been proved by *in vivo* tumor challenge, which reveals the distinct advantage of our immunogenic platform: our bacterial vector can deliver whole protein and its application as cancer vaccine would not be restricted by CD8+ T peptides identification and hence to MHC I.

D-III Construction of Humoral Response Activating Vectors

To date, most of anti-tumor vaccination strategies have aimed to trigger CD8+ CTLs responses, as CTLs recognize tumor cells presenting peptides from TAAs through MHC-I molecules and the immune response stimulated by this way is more specific and more efficient. In fact, both in animal and clinical studies, the strageties that focused on the use of MHC class I-restricted tumor Ags and CD8+-directed immunization could truly activate antigen specific CTL responses, but the overall cell mediated responses were weak and not so efficient to eliminate cancer cells and control tumor growth. The limited outcome is mainly due to the immune-escape mechanisms established by advanced tumors that inhibit effector T cells or prevent full T cell activation (22, 522). In recent studies, interest has been directed to target at multiple arms of immunity to generate a strong, broad and long-lasting antitumor immunity; incorporation of humoral and cellular immunity may represent further improvement of current T cell-based cancer immunotherapy.

As it has been proved that the immune response activated by *P. aeruginosa* T3SS based vectors is the MHC-I-restricted CD8+ T cells mediated response, we would like to elaborate bacterial vectors that could target at anti-tumor humoral immunity. This idea has been realized through the structural and functional knowledge of *P. aeruginosa* T3SS. As PopB and PopD are two indispensible T3SS structure proteins that insert to the mammalian cells membrane and form the channel allowing toxin

translocation, we suggested that the intra-cytoplasmic translocation of protein should be terminated when these two proteins were deleted. Bacteria can be captured by APCs through phagocytosis or pinocytosis and the pathogen-associated proteins would be targeted to MHC II pathway or secreted in extracellular environment; and antigen specific humoral response might be activated through T_h2 pathway (Fig. 26).

However, we were not sure if the structural change of T3SS will affect the antigen expression and carrying ability of *P. aeruginosa* ⊿PopB⊿PopD mutant, thereby we first evaluated this mutant strain *in vitro* in two conditions: vaccine preparation condition and T3SS activation condition (Fig. 28). The protein of interest has only been detected in T3SS activation condition, which reveals the good antigen carrying ability of *P. aeruginosa* ⊿PopB⊿PopD mutant.

Then, the humoral response activating ability of ⊿PopB⊿PopD mutant was assessed by antigen specific IgG titration (Fig. 29). We found that OVA specific IgG can be detected in CHA-OST-⊿BD-PADRE-OVA strain injected groups but not in CHA-OST-PADRE-OVA strain injected group and the intensity of this response was bacteria dose dependent. These results on one hand demonstrated that the response activated by *P. aeruginosa* T3SS delivery mechanism is cellular component mediated response; on the other hand affirmed our hypothesis that *P. aeruginosa* ⊿PopB⊿PopD mutant is powerful to activate the antigen specific humoral response. However, the humoral response detected in this experiment is moderate. For the weak humoral response, our hypothesis is that the quantity of antigen delivered by CHA-OST-⊿BD-PADRE-OVA strain is not sufficient to activate significant humoral response. It has been previously detected in our laboratory that $5*10^8$ of *P. aeruginosa* vaccine vector can express and secret about 1 µg interest protein, in this trial, we injected $5*10^6$ CHA-OST-⊿BD-PADRE-OVA per mice each time, which means mice were immunized with OVA at the level of about 0.01 µg/time. This immunization quantity is much weak than that administered in positive control group (5µg/mouse/time). As the humoral response was bacteria dose dependent, the

significant humoral response might be occurred when the quantity of delivered protein is augmented. This can be achieved by using one avirulent strain which can be administered at high dose.

Furthermore, in order to validate the efficacy of this mutant, some supplementary experiments need to be realized. For example, as we discussed above, the humoral response activated by *P. aeruginosa* ⊿PopB⊿PopD mutant is possibly T_h2 pathway mediated, the activation of antigen specific CD4+ T_h cells should be evaluated. Moreover, the potential improvement of CD8+ T-cell mediated responses should be investigated. Finally, the anti-tumor efficiency of this mutant should be validated by tumor challenge in animal model.

Chapter 2
Definition of the Therapeutic Vaccination Schema

Résumé en Français:

Dans nos travaux précédents, l'efficacité anti-tumorale prophylactique des vecteurs de *P. aeruginosa* a été prouvée. Dans l'objectif d'aller vers des applications chez l'homme, leurs performances anti-tumorales doivent être évaluées en mode thérapeutique. Plusieurs schémas thérapeutiques contenant deux injections ont été testés en modèle B16OVA murin. Bien que la protection existe, ces protocoles de vaccination doivent être optimisés. Ainsi, dans ce chapitre, afin d'optimiser l'efficacité thérapeutique de nos vaccins et de déterminer un schéma plus idéal pour la vaccination thérapeutique, nous avons essayé de moduler la fréquence et l'intervalle d'injection. Les résultats *in vivo* et *in vitro* ont montré qu'une fréquence et un intervalle appropriés d'immunisation sont importants pour programmer la réponse immune primaire contre l'antigène.

In English:

In our previous work, the prophylactic anti-tumor efficiency of *P. aeruginosa* vectors has been proved (3, 6). For clinical application purpose, their anti-tumor protection performance should be confirmed in therapeutic vaccination mode.

A Optimization of Therapeutic Vaccination Scheme

A-I Previously Tested Therapeutic Vaccination Schema

Previously, several two-injection therapeutic schedules in murine B16OVA model have been tested (10). Although protection occurred, these vaccination protocols should be optimized. Among these therapeutic vaccination schemas, D+0/D+7 schema demonstrated the better anti-tumor protection. So at the beginning of this work, we assessed one more time this scheme through the tumor challenge in B16OVA tumor model. Here, CHA-OST-PADRE-OVA strain ($5*10^6$/time) was used as vaccine vector.

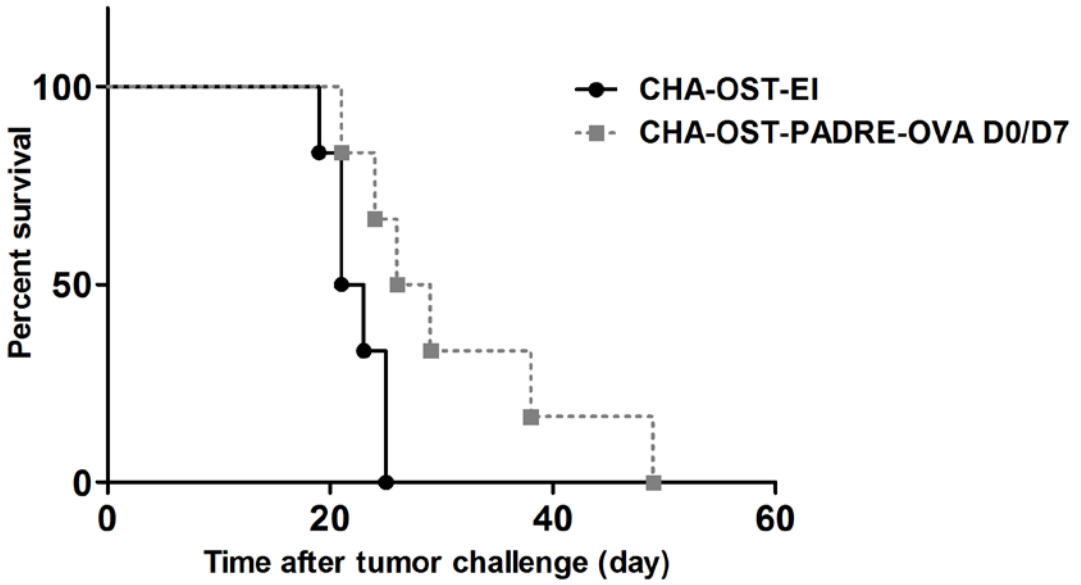

Figure 30: Evaluation of the Therapeutic Anti-tumor Efficiency of *P. aeruginosa* Vector on Following D+0/D+7 Schema.

Mice were inoculated with B16OVA cells on day 0 and subcutaneously injected with CHA-OST-PADRE-OVA strain ($5*10^6$/time) on day 0 and day 7. CHA-OST-EI was used as negative control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p=0.03 for CHA-OST-PADRE-OVA (D+0/D+7) *VS* CHA-OST-EI.

In accordance with previous experiments, tumor protection was provided by two times of therapeutic vaccinations this time (p=0.03) (Fig. 30). These survival data were saved as the reference for further optimization.

A-II Optimization Strategy

Some recent anti-tumor researches based on different vaccine vectors demonstrated encouraging therapeutic results using OVA as model antigen and their vaccination schedules contained more vaccination times or shorten vaccination interval (523-526). Thus, in order to optimize the therapeutic potency of our vaccines and determine an ideal therapeutic vaccination schema, we tried to increase vaccination times or reduce vaccination interval. In this trial, CHA-OST-PADRE-OVA strain was used as vaccination strain.

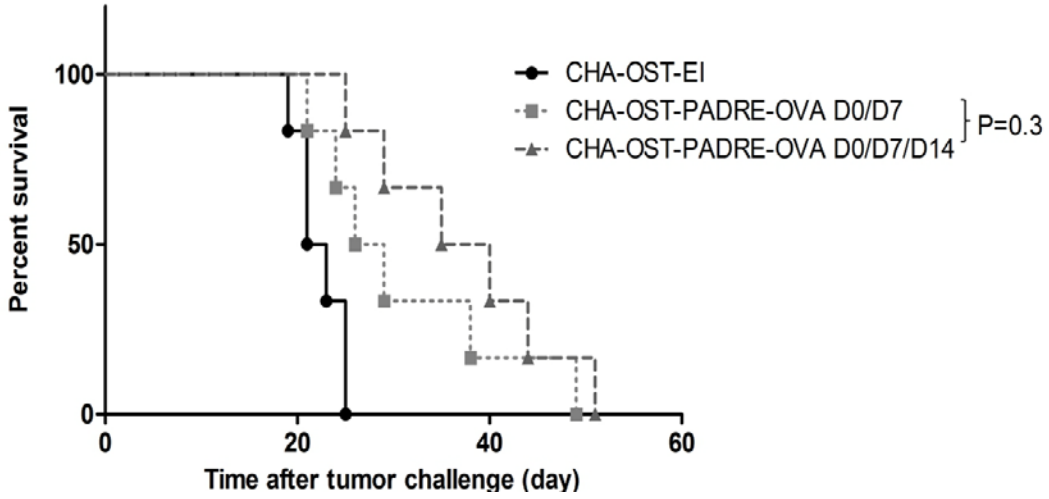

Figure 31: Optimization of the Therapeutic Anti-tumor Efficiency of *P. aeruginosa* vector by Increasing Vaccination Frequency.

Mice were inoculated with B16OVA cells on day 0 and subcutaneously injected with CHA-OST-PADRE-OVA strain ($5*10^6$/time) on day+0, day+7 and day+14. CHA-OST-EI was used as negative control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p=0.03 for schema D+0/D+7/D+14 *VS* schema D+0/D+7.

Firstly, we increased the vaccination frequency without changing vaccination interval. Mice were first challenged with B16OVA tumor cells on day 0 and then injected three times with CHA-OST-PADRE-OVA strain on day 0, +7 and +14. The results were shown in figure 31. Here, we can see that increasing the number of vaccination can slightly delay the tumor formation, but the difference was not significant (p=0.3)

Then, we tried to reduce the vaccination interval but not changing the vaccination numbers. That means mice were first challenged with B16OVA tumor cells on day 0 and then injected three times with CHA-OST-PADRE-OVA strain on day 1 and +5. Tumor protection provided by this schema was shown in figure 32. Similar to preceding experiment, reducing the interval of vaccination can slightly delay the tumor formation, but no significant difference was not observed (p=0.59)

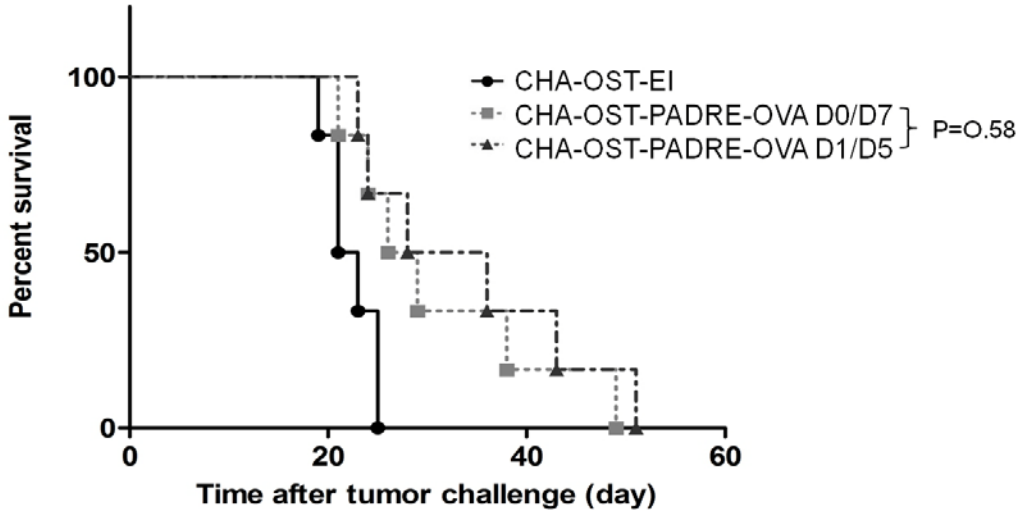

Figure 32: Optimization of the Therapeutic Anti-tumor Efficiency of *P. aeruginosa* Vector by Reducing Vaccination Interval.

Mice were inoculated with B16OVA cells on day 0 and subcutaneously injected with CHA-OST-PADRE-OVA strain ($5*10^6$/time) on day+1 and day+5. CHA-OST-EI was used as negative control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p=0.59 for schema D+1/D+5 *VS* schema D+0/D+7.

Thereby, for obtaining a significant optimization, we attempted to change the vaccination schedule in both parameters – vaccination number and vaccination interval. In this condition, mice were always first challenged with B16OVA tumor cells on day 0 and then injected with CHA-OST-PADRE-OVA strain on day +1/+5/+9/+13 or +1/+5/+9/+13/+17/+21. This time, encouraging results were obtained. In figure 33, we can see that by augmenting vaccination number and reducing vaccination interval at the same time, tumor protection was improved and the optimization was vaccination related; the statistically significant change was observed in the mice injected six times with 4 days interval. Here, it has to be noticed that we vaccinated the mice 6 times because before the 7^{th} vaccination, there were already some mice were sacrificed with the tumor exceeded 10mm of diameter. Moreover, thinking about the toxicity of CHA-OST strain and possible immunity impairment caused by the bacteria administration, we didn't try much shorter interval.

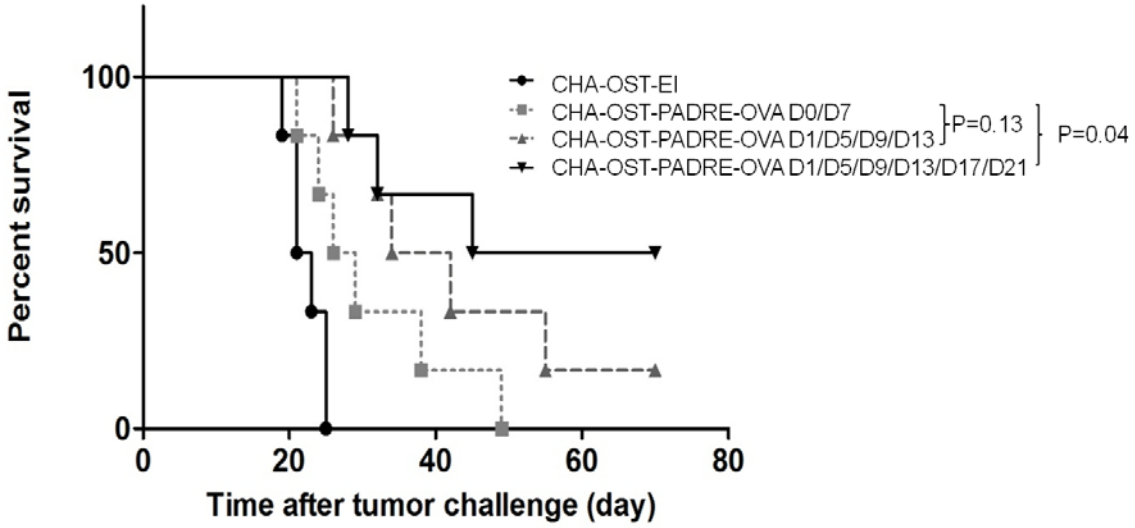

Figure 33: Optimization of the Therapeutic Anti-tumor Efficiency of *P. aeruginosa* Vector by Increasing Vaccination Frequency and Reducing Vaccination Interval.

Mice were inoculated with B16OVA cells on day 0 and subcutaneously injected with CHA-OST-PADRE-OVA strain ($5*10^6$/time) on following D+1/D+5/D+9/D+13 or D+1/D+5/D+9/D+13/D+17/D+21 schema. CHA-OST-EI was used as negative control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p=0.13 for schema D+1/D+5/D+9/D+13 *VS* schema D+0/D+7; p=0.04 for schema D+1/D+5/D+9/D+13/D+17/D+21 *VS* schema D+0/D+7.

B Confirmation of the Optimization Strategy through Immune Response Monitoring

As we published before (3, 6), the immune response activated by T3SS delivery is CD8+ cytotoxic T lymphocytes (CTLs) mediated immune response. Therefore, in order to confirm the *in vivo* findings and our optimization strategy, we have also assessed the dynamic evolution of acute CD8+ T cell response activated by different vaccination protocols through ELISpot assay.

In this trial, we used always CHA-OST-PADRE-OVA as vaccination strain. In each group, there were 3 mice and the experiment was repeated twice. Each time, we quantified antigen specific CD8+ T cells at three time points. The first measurement was realized just before the injections. The second time point was 4 days after the last

injection of each group, a lag corresponding to the peak values of activated CD8+ T cell responses. On day25, when we measured the peak value of T cell response in D1/D5/D9/D13/D17/D21 group, we would also like to know remaining T cell response in D1/D5 group. As this day corresponded to a lag of 20 days after the last injection in D1/D5 group, we chose 20 days after the last injection as the third measurement time point for all groups. By combining results measured at different time points, we can evaluate the dynamic change of acute T cell response for each group. In figure 34, we can see the typical two phases of T cell response in all groups: an expansion phase followed by a contraction phase. Moreover, through increasing injection frequency and reducing injection interval, the immune response expansion phase was prolonged, the peak value of T cell response was increased and the T cell response contraction phase was delayed. These results confirmed that more frequent and continuous injection could reinforce adapted T cell response.

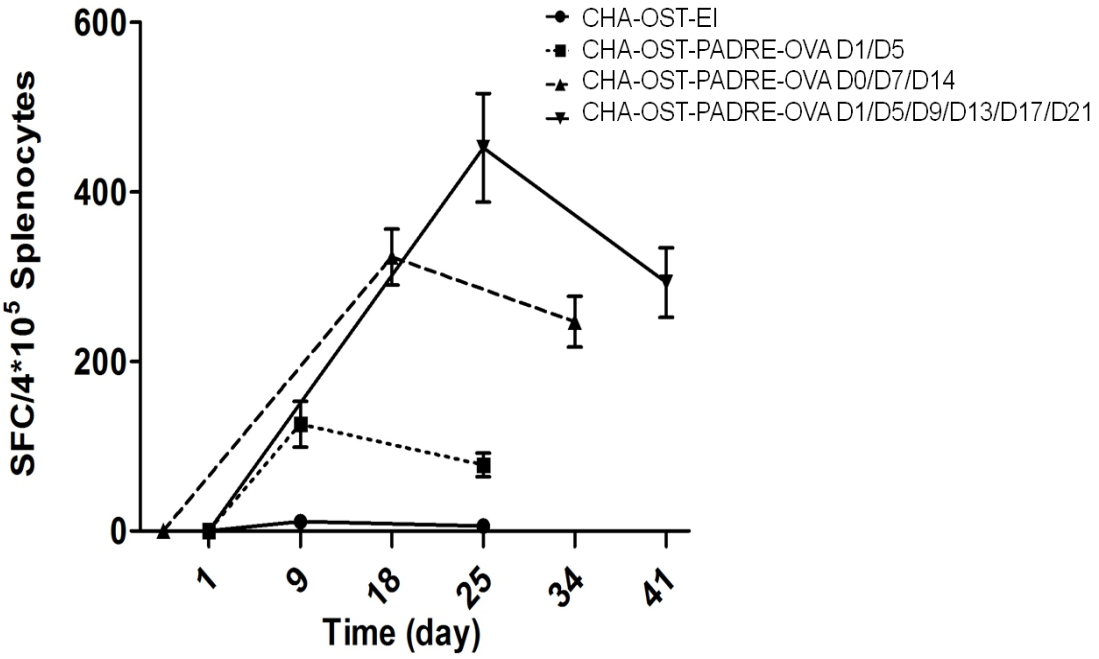

Figure 34: Dynamic Change of CD8+ T Cells Mediated Responses Activated by Different Immunization Protocols.

The CD8+ immune response was assessed through ELISpot at three time points: 1) before the first injection; 2) 4 days after the last injection; 3) 20 days after the last injection. CHA-OST-EI was used as negative control. Data represented the mean +/- SD from six animals per group (cumulative results of two independent experiments conducted with the same methodology).

C Further Evaluation of Therapeutic Vaccination Schemas through Function Evaluation of Memory CTLs

The ultimate purpose of cancer vaccine administration is to generate long lasting immune memory to prevent tumor recurrence. To prevent tumor cells from becoming established tumor, it is critical that the vaccine-induced tumor-specific memory T cells can immediately sense tumor cells emergence and be rapidly reactivated and expanded to kill the malignant cells in initial stage. Thereby, in order to further evaluate the efficiency of our bacterial vector, we have also investigated memory T cell mediated response after immunizations and its potential to prevent tumor recurrence.

First, we examined whether the memory T cells can sense the inoculation of B16OVA tumor cells and be reactivated. Three months after the last vaccination, we inoculated mice with B16OVA tumor cells and quantified recalled CTLs' response. Generally, 1 month after pathogen infection, pathogen related CD8+ T cell response should enter memory T cells dominating phase and little effector CTLs could be detected (89). Thereby, we believe that the CTLs quantified here could demonstrate the reactivation and expansion ability of memory T cells. In figure 35, we demonstrated by ELISpot analysis that 3 months after the last vaccination, only few CTLs were remaining in all groups and there was no evident CTLs amount difference among the different groups; but 7 days after the inoculation of B16OVA cells which express the specific antigen, there were significant CTLs increasing in all immunized groups but not in negative control group and the most significant change was found in D1/D5/D9/D13/D17/D21group.

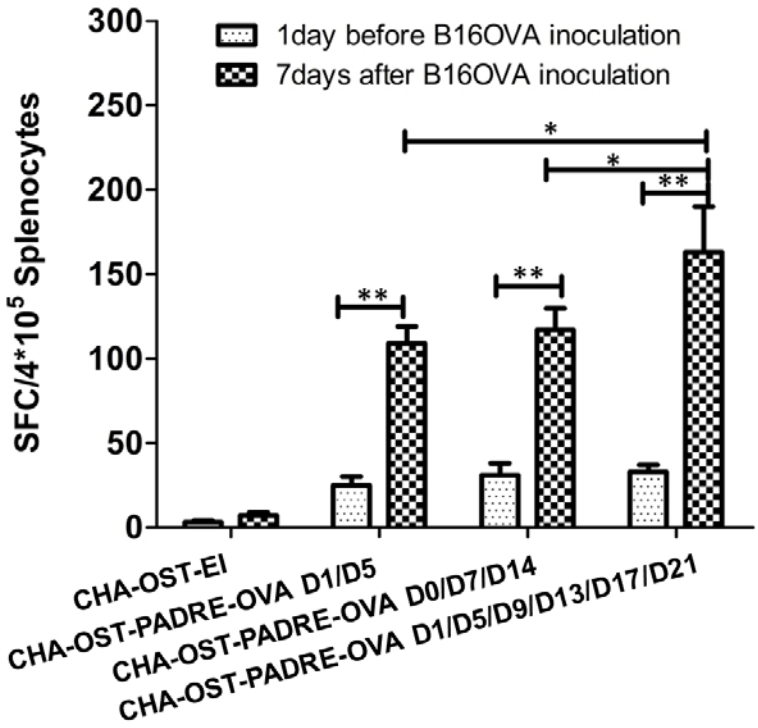

Figure 35: Function Evaluation of Memory CTLs Induced by Different Immunization Protocols through Effector CTLs Recalling Assessment Realized by ELISpot.

Mice (n=3 per group) were vaccinated with the different immunization protocols. B16OVA tumor cells were s.c. injected 3 months after the last injection. The CTLs recalling response was assessed by ELISpot at two time points: 1) one day before B16OVA cells inoculation; 2) 7 days after B16OVA cells inoculation. CHA-OST-EI was used as negative control. Data represented the mean+/- SD. * $p<0.05$; ** $p<0.01$.

To confirm these findings, we also verified long term protection against tumor after bacterial vector immunization in animal model. The results in figure 36 showed that by comparing to negative control group, there is a significant long term tumor rejection in all groups (schema D1/D5 *VS* CHA-OST-EI, p=0.0029; schema D0/D7/D14 *VS* CHA-OST-EI, p=0.0014; schema D1/D5/D9/D13/D17/D21 *VS* CHA-OST-EI, p=0.0006). The tumor rejection was more significant in the group which has been vaccinated 6 times with 4 days of interval (schema D1/D5/D9/D13/D17/D21 *VS* schema D1/D5, p=0.004; schema D1/D5/D9/D13/D17/D21 *VS* schema D0/D7/D14, p=0.1917). These results suggest that bacterial vector induced long lasting T cells able to sense the B16OVA tumor cell challenge and immediately expand to a significantly higher level and execute a strong

effector function. We hypothesize that this phenomenon corresponds to a good induction of memory T cells by our vaccination; the best vaccination scheme for that purpose is D1/D5/D9/D13/D17/D21 vaccination schedule.

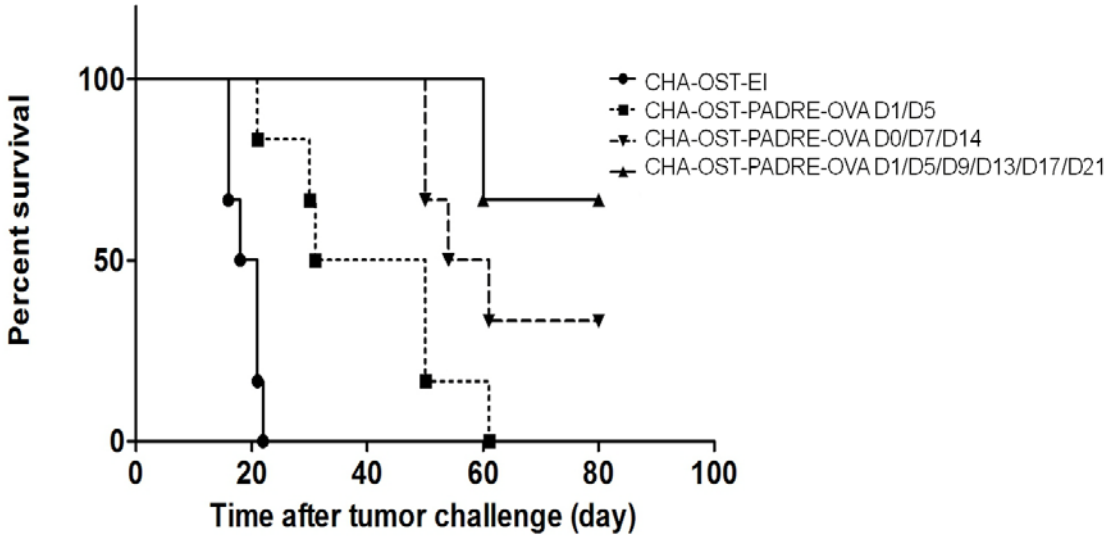

Figure 36: Function Evaluation of Memory CTLs Induced by different immunization Protocols through Tumor Challenge.

Kaplan-Meyer curves displayed survival data of mice (n=6) vaccinated with various immunization protocols and challenged with B16 OVA tumor cells three months after the last vaccination. CHA-OST-EI was used as negative control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: $p<0.01$ for immunized groups VS CHA-OST-EI; p=0,004 for schema D1/D5/D9/D13/D17/D21 *VS* schema D1/D5; p=0.1917 for schema D1/D5/D9/D13/D17/D21 *VS* schema D0/D7/D14.

The data presented in this chapter lead to the conclusion that our bacterial vector could provide efficiently therapeutic anti-tumor protection and that the D1/D5/D9/D13/D17/D21 schedule is more efficient in both short term and long term response than the other schema.

D Discussion

In our previous work, the potential use of *P. aeruginosa* attenuated strains as carriers for antitumor vaccination purposes has been only reported in prophylactic experiments (3, 6, 10). As the ultimate objective of our bacterial vaccines development is clinical application, their tumor protection efficiency should be

evaluated and confirmed in therapeutic mode.

We have tested the therapeutic vaccination schema with two injections at 7-days of interval, although tumor protection occurred, the results were not satisfying. So, in order to verify the therapeutic efficiency of our vaccines, we need to establish one efficient vaccination strategy at first.

It has been well accepted that the CD8+ T-cell response is a dynamic process and acute CTLs response contains expansion and contract phase (90). Recently, one progressive-differentiation model has been proposed for the sustained expansion during the primary response to antigen. According to the progressive-differentiation model , accumulated stimulation of CD8+ T cells by antigen may induce successive rounds of division/differentiation (527). As we have discussed in previous paragraphs, that the immune response activated by *P. aeruginosa* T3SS based vectors is the MHC-I-restricted CD8+ T cells mediated response, we hypothesized that the change in vaccination frequency or injection interval could modify CD8+ immune response. The results of *in vivo* tumor challenge assays obtained with OVA antigen demonstrated that altering only the vaccination frequency or the injection interval couldn’t carry expected results; significant improvement of therapeutic vaccination efficiency has been realized by changing both parameters (Fig. 31-33). In fact, continuous and more frequent immunizations could more effectively prime the immune response (Fig. 34). With the increase of vaccination frequency and the reduction of vaccination interval, the expansion phase has been obviously prolonged, the peak value of specific anti OVA CD8+ T cells amount was enhanced and CD8+ T response persisted for longer time. Both *in vitro* and *in vivo* results demonstrated that appropriate immunization frequency and interval are both important for programming primary immune response to antigen.

Furthermore, in the fight of the immune system against cancer, a long lasting and effective CD8+ T cell memory is also crucial, because this T cell population can sense the emergence of tumor cells and control tumor recurrence. Therefore, in this work we

also estimated the long term efficacy of our bacterial vectors by inoculation of B16OVA tumor cells three months after bacterial vectors immunization. The results obtained from both *ex-vivo* immune monitoring (Fig. 35) and *in vivo* tumor challenge (Fig. 36) demonstrated that the T cell response induced by our bacterial vector was able to sense the residual tumor cells and effectively inhibiting the tumor recurrence.

Our bacterial vectors demonstrate the therapeutic efficiency not only in artificial B16OVA model, but also in natural tumor model (GL261 and B16) of which the results are presented and discussed in result-chapter 1.

Based on these preliminary results, we can conclude that through modulating the frequency and the interval of injections, the anti-tumor therapeutic efficiency of our *P. aeruginosa* vaccines could be successfully optimized. As the bacterial vector used in this experiment was CHA-OST which is not totally avirulent, the toxicity of this bacteria strain and the possible immunity impairment caused by the bacteria administration were indeed the considerations for us. Therefore, we haven't tried the vaccination schedule with the interval less than 4 days. In future experiments using other more attenuated strains, as well as different antigens, the therapeutic vaccination strategies containing different immunization frequencies and variable intervals should be individually evaluated. It is possible that vaccination efficiency could be more enhanced.

Chapter 3
Improvements for Good Manufacturing Practices (G.M.P.) Compliance

Résumé en Français:

L'objectif ultime de toutes les recherches du cancer est que les résultats de ces recherches puissent être appliqués dans le traitement du cancer humain. De façon similaire, le développement de nos vaccins anti-tumoraux basés sur des bactéries vivantes est également orienté vers les applications cliniques. Dans ce chapitre, avant tout, un candidat (CHA-CLIN1) qui pourrait être appliqué en clinique a été généré par l'adaptation dans un milieu chimiquement défini d'un mutant (CHA-OAL) de P. aeruginosa totalement avirulent. Ensuite, la très faible infectiosité de cette nouvelle souche a été surmontée par l'injection des souris à de multiples emplacements. Les résultats in vivo et in vitro ont montré qu'une injection à de multiples emplacements est plus performante qu'une injection dans un emplacement unique. L'immunisation dans des emplacements multiples avec le vecteur CHA-CLIN1 pourrait donner des efficacités prophylactique et thérapeutique proches de celles du vecteur CHA-OST.

In English:

The ultimate objective of all cancer researches is that the results of these researches can be applied in human cancer treatment. In a similar way, the development of our bacteria based cancer vaccines should also be oriented towards clinical applications. For this purpose, one ideal bacterial vaccine should represent the best compromise between virulence attenuation and efficiency. Moreover, for good manufacturing practices reason, bacterial vectors should be produced in chemically define medium with constant growth performance to ensure the quality of the product. That is the reason why we initiated the work presented in this chapter.

A Adaptation of CHA-OAL Mutant in One Chemically Defined Medium

A-I Definition of Bacteria Culture Medium

M9 medium (Miller, *et al.* 1972) is one of the standard minimal medium for the cultivation of bacteria. DeBell RM *et al* compounded one chemically defined medium

which has been proved to be ideal for Exotoxin A production by *P. aeruginosa* (501). So, the initial culture medium for CHA-OAL strain adaptation was prepared based on these media. However, the growth rate of bacteria was not high enough (Fig. 37).

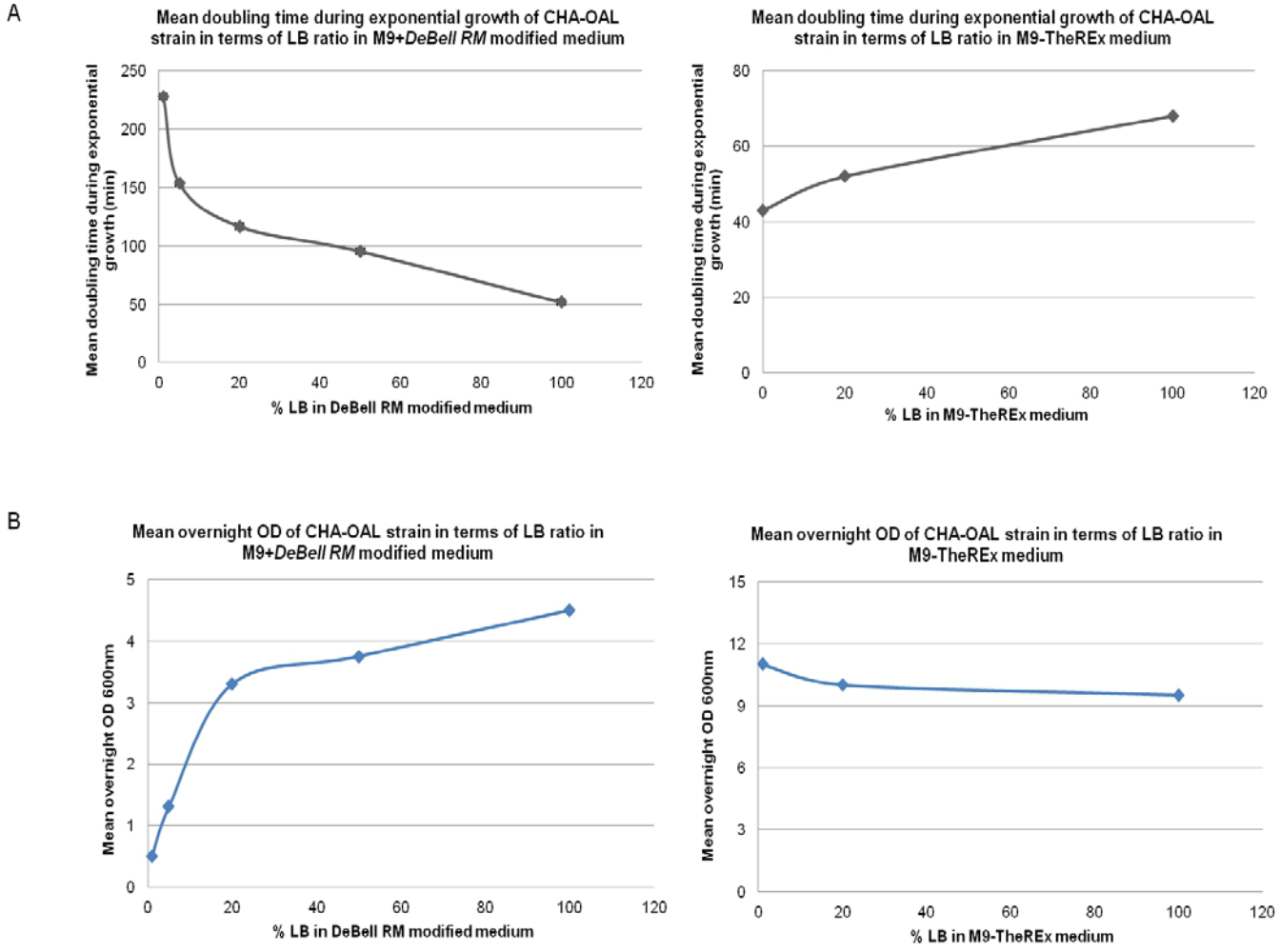

Figure 37: Adaptation of CHA-OAL Strain in One Chemically Defined Medium.

The initial culture medium for CHA-OAL adaptation was prepared basing on M9 medium and DeBell RM prepared chemically defined medium in which Mg2+ and Ca2+ were of low level. The growth rate of bacteria was not high enough. CHA-OAL strain was later adapted in the medium supplemented with 1 mmol/L Mg^{2+} and Ca^{2+} and growth rate of bacteria was optimized. After adaptation by progressive medium replacement, the growth of CHA-OAL strain in M9-TheREx medium was constant. (A) Adaptation of CHA-OAL strain in Mg^{2+} and Ca^{2+} poor (left) and abundant (right) medium was described by mean doubling time during exponential growth (min) in terms of LB percentage in culture medium; (B) Adaptation of CHA-OAL strain in Mg^{2+} and Ca^{2+} poor (left) and abundant (right) medium was described by mean overnight OD (600 nm) in terms of LB percentage in culture medium.

Some studies revealed that elevated levels of Mg2+ and Ca2+ could help *P. aeruginosa* growth and proteins production (528, 529). Hence, we further modified the adaptation medium by supplementing it with Mg^{2+} and Ca^{2+} and the composition of the modified M9 medium (M9-TheREx) is shown in table 7. In figure 37, we can see that, when CHA-OAL strain was being adapted in the medium with low level of Mg^{2+} and Ca^{2+}, with the decreasing of LB proportion in culture medium, the amplification speed of bacteria also decreased: overnight OD value was reduced and doubling time during exponential growth was prolonged. However, when CHA-OAL strain was being adapted in M9-TheREx medium, both parameters were optimized. After adaptation by progressive medium replacement, the growth of CHA-OAL strain in M9-TheREx medium was constant and even faster than in LB broth (mean doubling time during exponential growth: 43 min in M9-TheREx and 68 min in LB). In order to distinguish with original CHA-OAL strain, we renamed the adapted strain as CHA-CLIN1.

A-II Investigation of Micro-biological Characters of CHA-CLIN1 Strain as Antigen Delivery Vector

A-II-a Toxicity Detection

Table 9: *In Vivo* Toxicity Test of CHA-OST and CHA-CLIN1 Mutants

Mutant	Dose	Mortality
CHA-OST (in LB)	10^5 10^6 10^7	0/6 0/6 4/6
CHA-OAL (in LB)	10^7 10^8 10^9	0/6 0/6 0/6
CHA-CLIN1 (in M9-TheREx)	10^7 10^8 10^9	0/6 0/6 0/6

In order to verify if the adaption and the new growth condition have modified the

virulence of CHA-CLIN1 strain, we assessed the *in vivo* toxicity of strains either cultivated in LB or M9-TheREx medium by observing mortality after one s.c. injection of 10^7, 10^8, or 10^9 bacteria to 6-week-old female C57BL/6 mice. The toxicity of the new CHA-CLIN1 strain was also compared with the toxicity of CHA-OST strain (table 9). It can be observed that the toxicity of CHA-CLIN1strain in M9-TheREx is very low compared to the toxicity of CHA-OST strain and is at the same level of the previously published CHA-OAL strain.

A-II-b T3SS Analysis in CHA-CLIN1 Strain

After that, we also investigated if T3SS characters of CHA-CLIN1 strain were modified by both adaptation and growth in M9-TheREx medium.

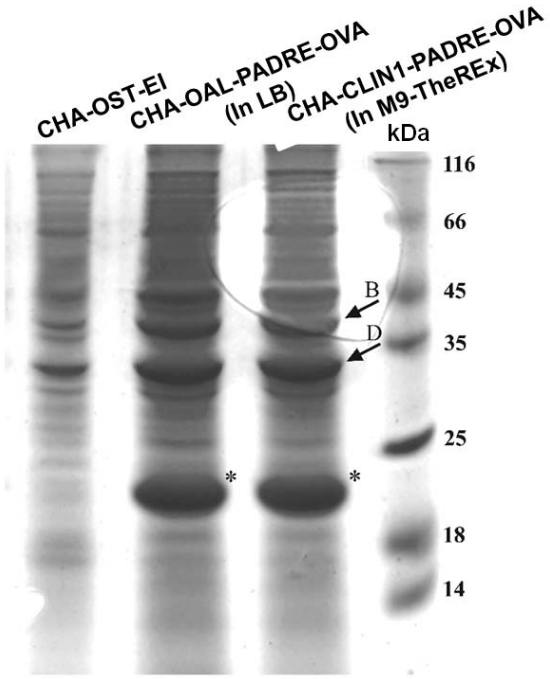

Figure 38: T3SS Evaluation of CHA-CLIN1 Strain.

M9-TheREx medium cultivated CHA-CLIN1 strain was transformed with pEAI-S54-Padre-$OVA_{248-376}$ and its antigen expression and secretion ability was assessed by SDS-PAGRE. The positions of PopB (top arrow) and PopD (bottom arrow) are marked with arrows; the positions of S54-PADRE-antigen fusion proteins are marked with asterisks.

In this assay, we used OVA as model antigen. Thus, we transformed CHA-CLIN1 strain with pEAI-S54-Padre-$OVA_{248-376}$ and verified the fusion protein

secretion in both LB and M9-TheREx medium. The results are presented in figure 38. Compared to CHA-OAL strain, CHA-CLIN1 strain grown in M9-TheREx conserved well its antigen expression and secretion ability. Moreover, the presence of PopB and PopD which indicates the activation of T3SS was correct.

B Optimization of the Vaccination Efficiency of CHA-CLIN1 Strain

In our previous work, we used CHA-OAL strain as vaccination vector *in vivo* for tumor protection in the B16OVA melanoma murine model (10), but the results indicated that CHA-OAL strain with two injections was less efficient than CHA-OST strain. So, we tried to optimize the efficiency of CHA-CLIN1 strain in this work.

B-I Definition of an Ideal Vaccination Dose

To define an ideal vaccination dose, we immunized mice with different doses of CHA-CLIN1-PADRE-OVA bacteria (10^7, 10^8, or 10^9 bacteria/time) on day -14 and day -7. And, CD8+ T cells activation was assessed through ELISpot assay on day 0. In this assay, immunization with the CHA-OST-PADRE-OVA strain ($5*10^6$ bacteria /time on D-14 and D-7) served as positive reference.

In figure 39, we can see that 10^8 CHA-CLIN1-PADRE-OVA/time primed the strongest CD8+ T response among the three doses, but the vaccination efficiency of CHA-CLIN1 strain was always significantly weaker than that of CHA-OST.

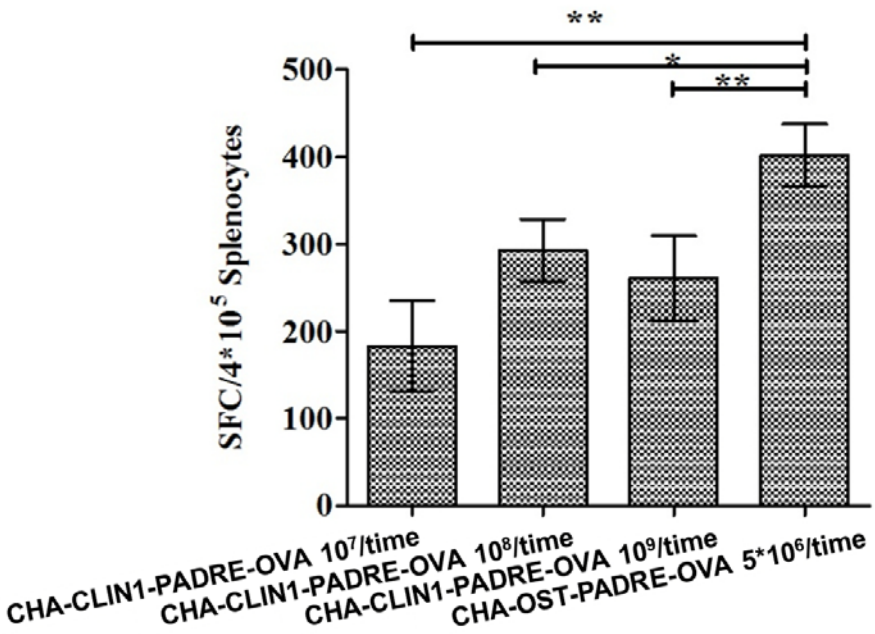

Figure 39: Definition of the Most Efficient Dose of CHA-CLIN1 Strain through ELISpot Assay.

C57BL/6 mice were injected in right flank with CHA-CLIN1-PADRE-OVA strain at the dose of 10^7 or 10^8 or 10^9 cells/time or with CHA-OST-PADRE-OVA strain at the dose of $5*10^6$ cells/time D-14/D-7 days before ELISpot assay. Data represented the mean +/- SD from three animals per group, *p<0.05, **p<0.01.

B-II Definition of an Efficient Vaccination Mode

In recent studies, mutations in the aroA gene have been applied to pathogens, such as *Salmonella typhi* (530), *Aeromonas hydrophila* (531) and *P. aeruginosa* (532), for the live, attenuated vaccine strains production. Because *aroA* gene encodes an enzyme essential for the synthesis of aromatic amino acids which are generally not available in host tissues, the deletion of *aroA* gene both restrains the bacterial growth and inhibits the pathogenic dissemination in mammalians, which could also limit the immunogenicity and efficiency of vaccine (533).

So, we tried to optimize CHA-CLIN1 strain efficiency through two approaches: 1) vaccination of mice at multiple loci (four s.c. injections in right and left flanks, $5*10^7$ bacteria/position/time, on D-14/D-7) and 2) vaccination with more injections and reduced time between two injections ($1*10^8$ bacteria/time, on

D-16/D-12/D-8/D-4). For the two vaccination protocols, the amount of injected bacteria was identical. The effects of the different optimization strategies were firstly estimated by ELISpot mediated CD8+ T cell response quantification on day 0 and the results were shown in figure 40.

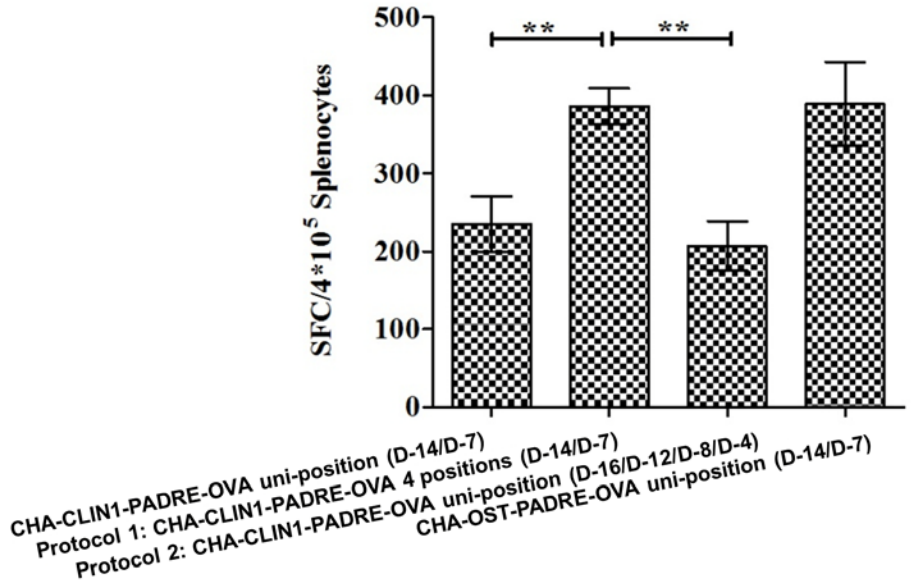

Figure 40: Strategy for Optimizing the Vaccination Efficiency of CHA-CLIN1 Strain.

Protocol 1: C57BL/6 mice were injected twice at four positions in right and left flanks with CHA-CLIN1-PADRE-OVA strain of $5*10^7$ cells/position/time 14 days and 7 days before ELISpot assay; Protocol 2: C57BL/6 mice were injected four times with four days of interval at single position in right flank with CHA-CLIN1-PADRE-OVA strain at the dose of 10^8cells/time before ELISpot assay. The immune responses primed following the different protocols were compared with that activated by standard CHA-OST-PADRE-OVA injections. Data represented the mean+/- SD from three animals per group **$p<0.01$.

We observed that there were no remarkable optimization through increasing injection frequency and reducing injection interval. However the multi-position injection with CHA-CLIN1-PADRE-OVA strain significantly optimized the vaccination efficiency and primed a nearly identical CD8+ response as CHA-OST-PADRE-OVA strain.

B-III *In vivo* Evaluation of the Vaccination Efficiency of CHA-CLIN1 Strain

In order to confirm the optimization strategy, we further assessed *in vivo* B16OVA tumor protection mediated by CHA-CLIN1-PADRE-OVA vaccinations at either uni-position or multi-positions. Here, both prophylactic and therapeutic anti-B16OVA assay were performed. CHA-OST-EI strain was used as negative control and CHA-OST-PADRE-OVA strain was used as positive reference.

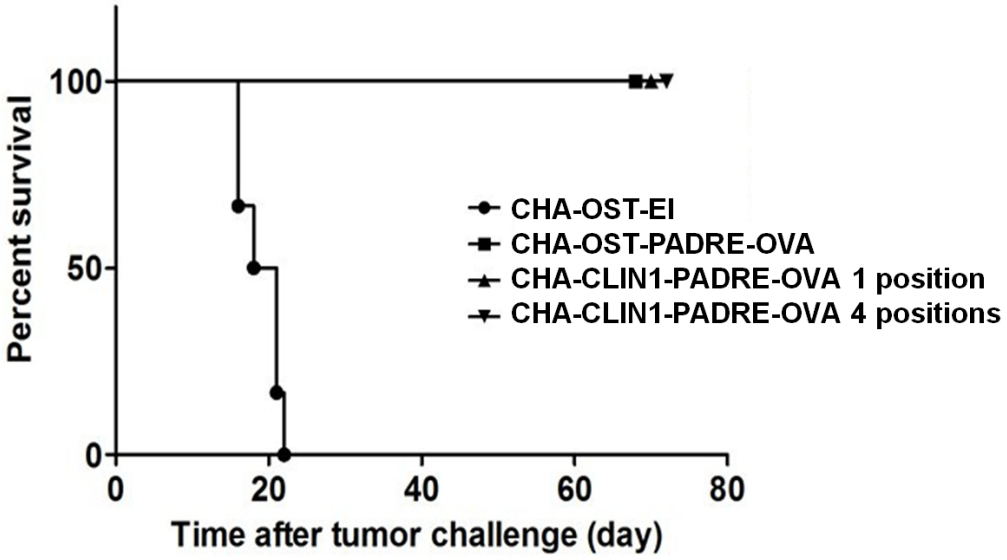

Figure 41: *In Vivo* Evaluation of the Optimized Vaccination Protocol for CHA-CLIN1 Strain in Prophylactic Vaccination Mode.

C57BL/6 mice were injected twice at uni-position with CHA-CLIN1-PADRE-OVA strain at the dose of $2*10^8$ cells/time or at four positions in right and left flanks at the dose of $5*10^7$ cells /position /time on 14 days and 7 days before B16OVA tumor challenge. CHA-OST-EI is the negative control. CHA-OST-PADRE-OVA is the positive control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: $p<0.01$ for immunized groups *VS* EI, no statistically significant difference between immunized groups.

Figure 41 presents the results of prophylactic assay. In contrast to CHA-OST-EI strain injected group, all immunized groups demonstrated efficient anti-tumor protections, no mouse developed tumor in all groups. CHA-CLIN1-PADRE-OVA vaccinations based on different protocols demonstrated the same efficacy as CHA-OST-PADRE-OVA strain.

Figure 42 shows the results of therapeutic assay. All immunized groups presented tumor rejections with slight differences between the three immunized group,

CHA-CLIN1-PADRE-OVA vaccinations at four positions were slightly more efficient than CHA-OST-PADRE-OVA vaccinations which were still better than CHA-CLIN1-PADRE-OVA vaccinations at one position, whereas the differences between the three groups there were not statistically significant

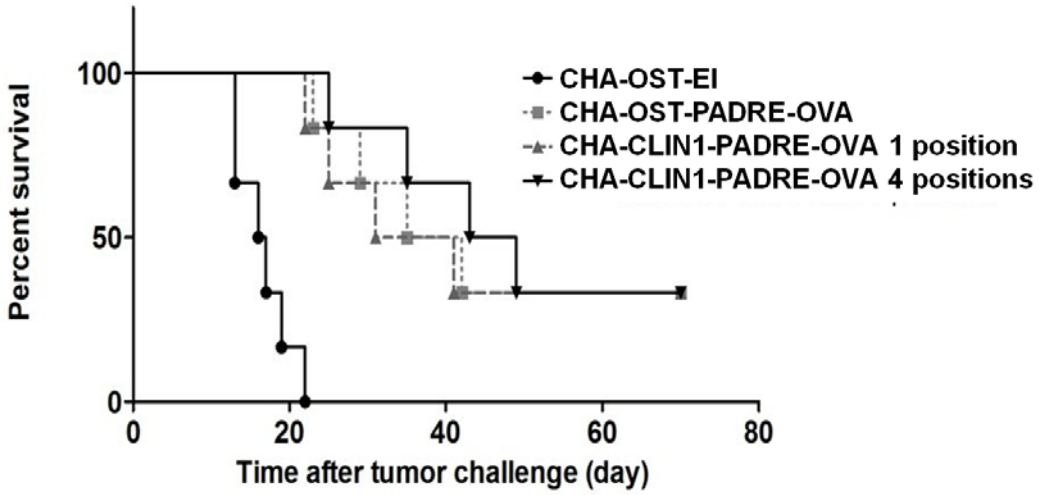

Figure 42: *In Vivo* Evaluation of the Optimized Vaccination Protocol for CHA-CLIN1 Strain in Therapeutic Vaccination Mode.

C57BL/6 mice received a s.c. tumor challenge with GL261 tumor cells on day 0 and then were vaccinated with at uni-position with CHA-CLIN1-PADRE-OVA strain at the dose of $2*10^8$ cells/time or at four positions in right and left flanks with CHA-CLIN1-PADRE-OVA strain at the dose of $5*10^7$ cells/position/time on following D1/D5/D9/D13/D17/D21 therapeutic schema. CHA-OST-EI is the negative control. CHA-OST-PADRE-OVA is the positive control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: $p<0.01$ for immunized groups *VS* EI; no statistically significant difference between immunized groups.

C Discussion

In clinical trials, for bacteria based vaccines, safety (virulence attenuation), efficacy, and the production process of bacterial vector complying with current good manufacturing practices grade are indispensible. In our previous work (10), CHA-OAL strain, one *P. aeruginosa*-attenuated mutant, was constructed and characterized with greatly reduced toxicity. Hence, it could be an ideal candidate for clinical trials. However, all the encouraging results of the CHA-OAL strain were

obtained in a complex not chemically defined LB broth. Moreover, the CHA-OAL strain demonstrated uncontrollable growth defects in this medium (data not shown). Moreover, its efficiency at high doses has not yet been assessed. All these preliminary results emphasize the need to: i) develop a new chemically defined medium; ii) adapt the CHA-OAL strain to that medium; and iii) define an appropriate vaccination mode to validate its vaccination efficiency.

C-I Adaptation of CHA-OAL Strain in One Chemically Defined Medium

M9 is one of the standard minimal medium for bacterial culture. In recent fundamental studies, M9 medium has been applied in different bacterial species culture, such as *Escherichia coli B*, *Salmonella Typhimurium*, and *Pseudomonas putida* (534-536). On the other hand, DeBell RM *et al.* compounded one chemically defined medium in which L-amino acids, basal and trace salts, glucose and glycerol are important compositions; and this chemically defined medium has been proved to be ideal for exotoxin A production by *P. aeruginosa* (501). Thus, we attempted to adapt CHA-OAL vector basing on these media. However, the preliminary results were not satisfactory; the growth rates of bacterial cells were low and the overall bacteria proliferation was poor (Fig. 37).

Several studies have shown that elevated levels of Mg^{2+} and Ca^{2+} may promote *P. aeruginosa* growth (528, 529). However, in the initial adaptation medium, Mg^{2+} and Ca^{2+} were not abundant. Thereby, we added 1mM $MgSO_4$ and 1mM $CaSO_4$ and also chemically defined synthetic yeast extracts to M9 medium, yielding modified M9 (M9-TheREx) media (Table 7). After adaptation by progressive medium replacement, the growth of CHA-OAL strain in M9-TheREx medium was greatly increased and even better than in LB broth (Fig. 37). These results revealed the influrence of divalent cations to *P. aeruginosa* proliferation.

On the other hand, *in vitro* T3SS evaluation demonstrated that the adaptation and culture in M9-TheREx medium did not affect 2 important phenotypes of bacteria:

toxicity (Table 9) and protein expression and secretion capacities (Fig. 38).

As the adaptation process was long and led to faster growth of the bacteria, we decided to rename this strain—CHA-CLIN1.

C-II Optimization of the Vaccination Efficiency of CHA-CLIN1 Strain

Recently, auxotrophic strains (notably auxotrophic for aromatic amino acids) have been generated to obtain safer attenuated vaccines in immunotherapy studies. Because the *aroA* gene encodes an enzyme essential for the synthesis of aromatic amino acids that are generally not available in host tissues, the deletion of *aroA* could limit the pathogenic growth and dissemination in mammalians (533). Indeed, we previously verified the dissemination of the CHA-OAL strain after an SC injection, no bacteria were found in the organs such as blood, liver, spleen, and kidney (10). We suggest that this absence of dissemination could restrain the immunogenicity and efficiency of vaccine.

Thereby, in this experiment, we used different vaccination modes to improve the efficiency of the CHA-CLIN1 strain: vaccination at shorten interval in order to modulate immune response; or vaccination at multiple loci in order to mimic bacteria dissemination. The results of immune monitoring assay (Fig. 40) demonstrated that a simple increase in vaccination frequency did not significantly change the experimental results, whereas the vaccinations at multiple loci could artificially mimic the dissemination of bacteria and led to a reinforced immune response. And this finding is in accordance with that of *in vivo* tumor challenge: multiple loci injections demonstrated a better performance than uni-position injections and executed nearly the same prophylactic and therapeutic efficiencies as the CHA-OST strain (Fig. 41 and 42). Here, the difference was not statistically significant, for this point, our hypothesis is that OVA is one of the most powerful rejection antigens; the immune response is so strong to cover up the difference provoked by the different protocols. Therefore, we should continue to investigate our optimization strategy using self-antigen.

Chapter 4
Application of Bacterial Vectors in Humanized Murine Model

Résumé en Français:

Dans ce chapitre, dans le but d'évaluer le potentiel de notre vecteur bactérien dans l'immunothérapie humaine, nous avons utilisé le vecteur CHA-OST pour délivrer un antigène basé sur les séquences humaines d'HER-2 dans un premier modèle de souris humanisées (human ß2m-HLA-A2.1 (α1α2)-H-2dB (α3 transmembranaire cytoplasmique), HHD). La protection anti-tumorale a été obtenue après une immunisation par une double injection de la souche CHA-OST-PADRE-HER-2.

In English:

In order to estimate the potential of our bacterial vector for human immunotherapy, we would like to apply the vector to deliver antigen based on human sequences in one first level humanized mice model.

The human β2m–HLA-A2.1 (α1α2)–H-2Db (α3 transmembrane cytoplasmic) (HHD) transgenic mice are H-2Db-/- β2m-/- double knockout and could express only HLA class I molecules to force the mouse CD8T cell repertoire to make use of the transgenic HLA class I molecules both at the thymic and peripheral levels. Phenotypic and functional analyses of their peripheral CD8+ T cell repertoire indicate that HHD monochains support thymic positive selection of CD8+ CTL and activate virus-specific HLA-A2.1–restricted CTL in the periphery (537). So, we try to estimate the anti-tumor response activating efficiency of our bacterial vector in this animal model.

In this trial, we choose human HER-2 (ErbB-2) as the model antigen. HER-2 is a 185-kDa glycoprotein member of the epidermal growth factor receptor family of tyrosine kinases which is over-expressed in 25–40% of all breast cancers and in a variety of other tumors such as ovarian, gastric and colorectal carcinomas.

A Construction of HER-2 Expression Vector

The sequence encoding the positions 340-507 of human HER-2 protein was cloned at BamH I/Sph I sites in our PADRE containing single antigen expression plasmid. The sequence that we chose contains two published HLA-A2 restricted HER-2 epitopes: HER-$2_{369-377}$ and HER-$2_{435-443}$ (538, 539). The correct cloning was confirmed by commercial sequencing.

In this assay, we used CHA-OST strain as the vaccination vector and transformed it with pEAI-S54-Padre-HER-$2_{340-507}$ plasmid. As usual, before the animal experimentations, we first verified *in vitro* the protein expression and secretion state of HER-2 containing vector. The results were as we expected and were shown in figure 43. T3SS was well activated and HER-2 fusion protein was well expressed and secreted.

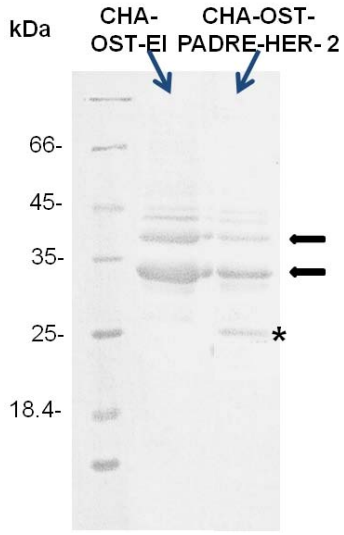

Figure 43: *In Vitro* Evaluation of Fusion Protein Secretion Ability of CHA-OST-PADRE-HER2 Strain.

The fusion protein expression and secretion ability of CHA-OST-PADRE-HER-2 strain was assessed by SDS-PAGE. The positions of PopB (top) and PopD (bottom) are marked with arrows; the position of S54-PADRE-HER2 fusion protein is marked with asterisk.

B Ex Vivo *Monitoring the Immune Response Activated by HER-2 Expressing Vector*

For evaluating the immunization efficiency of our bacterial vector in humanized murine model, we investigated the antigen specific immune response after two times of CHA-OST-PADRE-HER-2 strain injections. The T cell responses specific to the two published HER-2 epitopes (HER-$2_{369-377}$ and HER-$2_{435-443}$) were monitored through IFN-γ ELISpot assay and this test has been repeated two times.

However, the results of this trial were not satisfying, no evident T cell responses against HER-$2_{369-377}$ and HER-$2_{435-443}$ epitopes were detected (fig. 44).

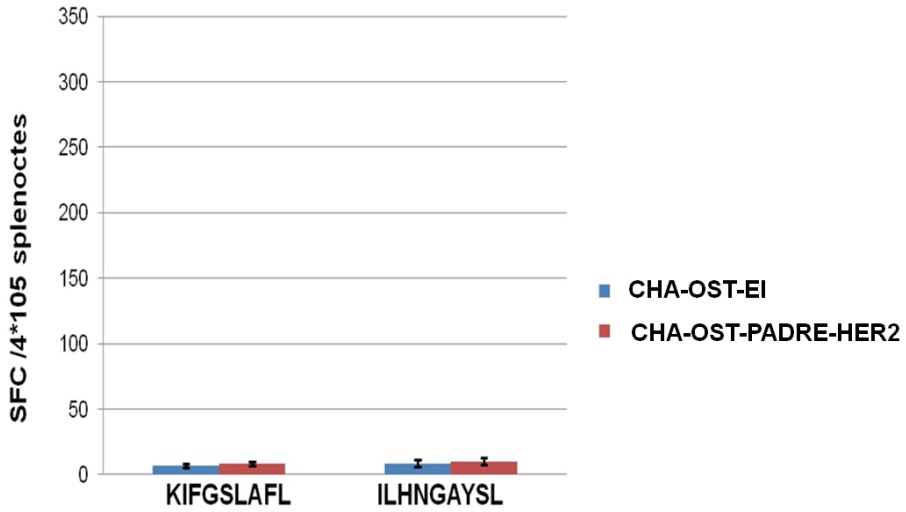

Figure 44: Evaluation of the Vaccination Efficiency of Bacterial Vector in Humanized Murine Model through *Ex Vivo* Immune Monitoring Assessed by IFN-γ ELISpot Assay.

HHD mice were injected with CHA-OST-PADRE-HER-2 strain at 5*106 bacteria/ time on day-14 and day-7. The T cell responses specific to the two HER-2 epitopes (HER-$2_{369-377}$ and HER-$2_{435-443}$) were assessed on day 0 through IFN-γ ELISpot assay. CHA-OST-EI was used as negative control. Data represented the mean +/- SD from six animals per group (cumulative results of two independent experiments conducted with the same methodology).

C In Vivo *Evaluation of HER-2 Expressing Vector in HHD Transgenic*

Mice

CHA-OST-PADRE-HER-2 strain was further evaluated in humanized murine model through tumor challenge experiment. HHD mice were s.c. injected with CHA-OST-PADRE-HER-2 strain two times on day -14 and day -7, then s.c. inoculated with $7*10^4$ MC-2-HER-2 cells on day 0. Here, mice administered with CHA-OST-EI strain were used as negative control. For each group, 6 mice were experimented.

In figure 45, we can see that in contrast to *ex vivo* immune response monitoring test, the results of this trial were positive. Immunizations with CHA-OST-PADRE-HER-2 strain significantly delayed the tumor formation (p=0.05 for CHA-OST-PADRE-HER-2 versus CHA-OST-EI), which demonstrate that our bacterial vector are also efficacious in this humanized murine model.

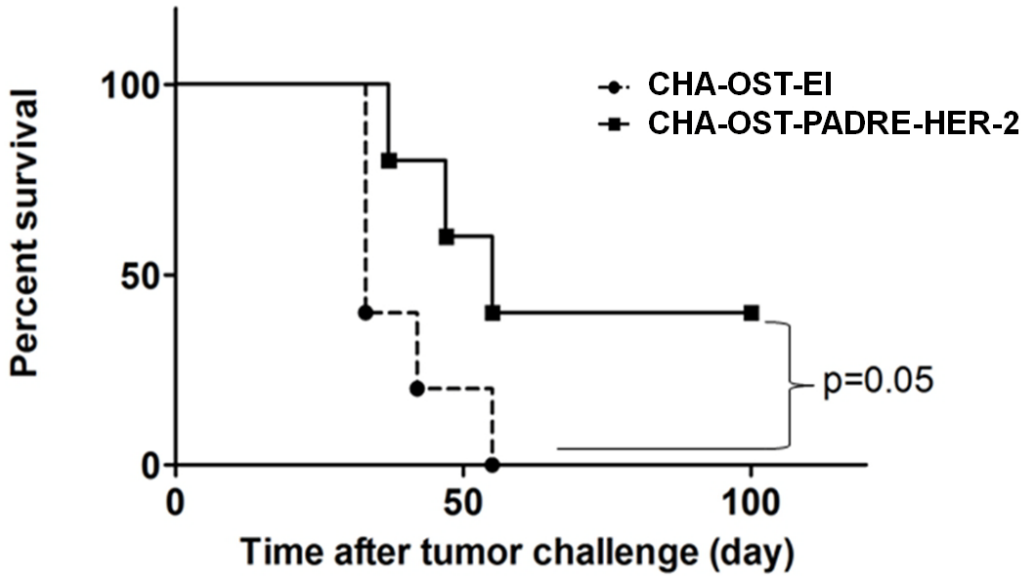

Figure 45: *In Vivo* Evaluation of the Vaccination Efficiency of Bacterial Vector in HHD Murine Model.

HHD mice were s.c. injected with CHA-OST-PADRE-HER-2 on D-14/D-7 before s.c. MC2-HER2 tumor challenge. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: p=0.05 for CHA-OST-PADRE-HER-2 *VS* CHA-OST-EI.

D Discussion

The experiments realized in HHD mice permitted the investigation of our bacterial vector in one first level humanized animal model.

The anti-tumor performance of our bacterial vaccine vector was revealed by *in vivo* tumor challenge. The tumor formation was significantly delayed in immunized HHD mice (Fig. 45).

However, in immune monitoring assay, no evident immune response has been detected to two published HER-2 epitopes (Fig. 44). There are two possible explanations for these results. First, as we have previously discussed in bi-antigen vector chapter, the peptide specific immune response activation depends on protein processing machinery of APCs as well as tumor cells. Both the processing of translocated antigen by APCs and the processing of TAAs by tumor cells might affect the immune response to certain peptides. Thereby, the good epitopes might be not the predicted ones. Second, some studies reported that HER-2 can induce multiple defects in antigen presentation, such as inefficient antigen processing, down regulation of MHC class I expression, so that HER-2 expressing carcinomas are poorly recognized by CTLs and the protection in MC2-HER-2 model is independent of cellular component but humoral component depended (540-542). In this case, the anti-tumor effect observed in above tumor challenge assay is possible provide by humoral immunity. There might be HER-2 Ags released from lysed bacteria to the extracellular space, pinocytosed by APCs and presented through MHC II pathway.

Thereby, in order to find out the true mechanism, more experiments need to be realized: 1) to investigate the humoral response activation after immunization; 2) to apply humoral immunity activating vector (CHA-OST-*Δ*BD) in this model to verify if the protection in MC2-HER-2 model is indeed humoral component dependent; 3) to combine CHA-OST and CHA-OST-*Δ*BD vectors and verify if strengthened efficiency will occur.

Chapter 5 The Influence of Pre-existing Anti-vector Immunity to Vaccination Efficacy

Résumé en Français:

Une immunité anti-vecteur préexistante représente un obstacle pour la plus part des vaccins basés sur les bactéries ou les virus. Dans ce chapitre, nous avons étudié ce problème potentiel pour notre vecteur bactérien. L'essai du challenge tumoral a montré qu'une administration répétée du vecteur bactérien ne fait pas diminuer l'efficacité de la vaccination.

In English:

Even attenuated bacteria has been widely proposed as a vaccine carrier, one of the major issue for the use of bacterial vaccine vector is that pre-existing anti-bacteria immunity might affect its ability of eliciting effective immune responses against heterologous passenger antigens through different manners, like faster elimination of the vaccine carrier, induction of regulatory T cells, etc.

Thereby, in the experiment presented in this chapter, we have addressed this consideration with repeated immunization utilizing CHA-OST strain that should not specifically stimulate peptide-specific CTL responses. And then, we investigated the influence of pre-existing anti-vector immunity to vaccine efficacy through tumor challenge assessment.

A Investigation of the Influence of Pre-existing Anti-vector Immunity to Vaccination Efficacy

The strategy of investigation is described in figure 46. Briefly, the experimentation timing was as following: 1) anti-vector immunity priming: 3 months before the tumor challenge, mice were administered with 2 injections with the interval of 7 days; 2) anti-vector immunity boosting time point: 2 months before the tumor challenge, mice were administered 4 injections with the interval of 4 days; 3) anti-tumor prophylactic vaccination: mice were administered with two injections on

14 days and 7 days before the tumor challenge; 4) Tumor challenge: mice were s.c. inoculated with $2*10^5$ B16OVA cells/mice.

Four groups were involved in this trial: 1) Group 1: mice injected with 100µl PBS/time on following the experimental schedule described above. 2) Group 2: mice injected with $5*10^6$ CHA-OST/time on following the experimental schedule described above. 3) Group 3: mice injected with $5*10^6$ CHA-OST/time for anti-vector immunity priming and boosting; and immunized with $5*10^6$ CHA-OST-PADRE-OVA/time for the induction of antitumor immunity. 4) Group 4: mice injected with $5*10^6$ CHA-OST-PADRE-OVA/time on day -14 and day -7 before tumor challenge, which served as the positive control. Here, the group 1 and group 2 on one hand acted as the negative control for immunized groups; on the other hand served to verify if the repeated injections of *P. aeruginosa* strains would interfere the dynamic formation of B16OVA tumor. In each group, 6 mice were experimented.

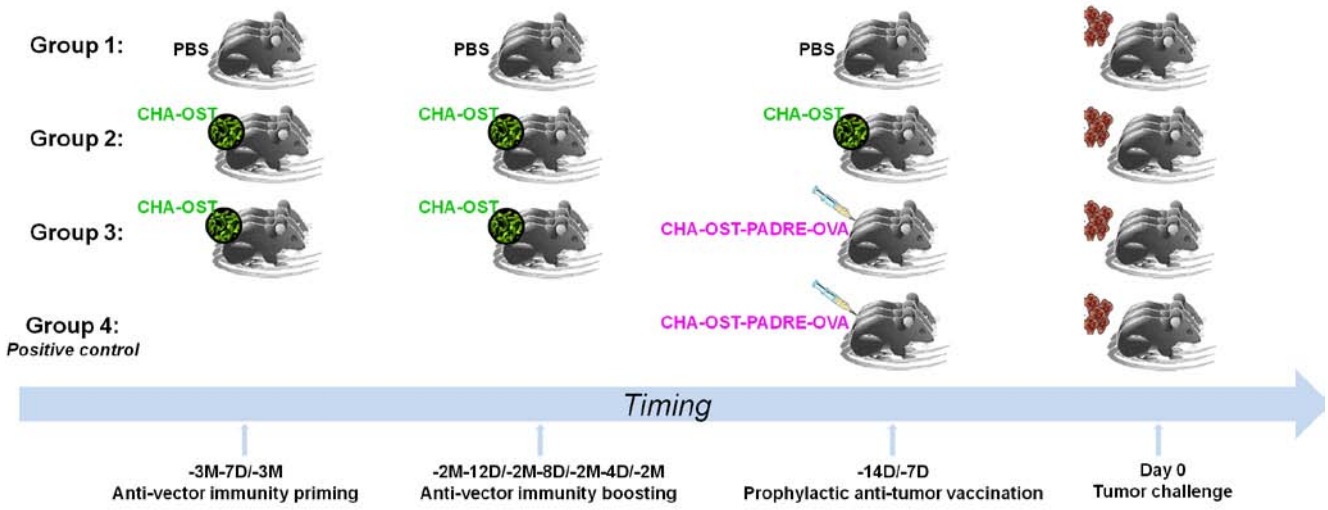

Figure 46: Strategy for Investigating the Influence of Pre-existing Anti-vector Immunity to Bacteria Vaccine Efficiency.

The figure 47 presents the results of tumor challenge. We can see that, there is no difference between the group 3 in which mice have been repeatedly administered with CHA-OST-PADRE-OVA strain before tumor challenge and the positive control group

(group 4), in both groups tumor has been completely rejected and all mice were always survival, which indicates that pre-existing anti- *P. aeruginosa* immunity has not affect its ability of eliciting effective immune responses against heterologous passenger antigens. On the other hand, there was also no difference between PBS injected group and CHA-OST injected group, which means that the repeated injections of *P. aeruginosa* strain will not interfere the dynamic formation of tumor. Thereby, these results support the use of *P. aeruginosa* as a candidate vaccine vector.

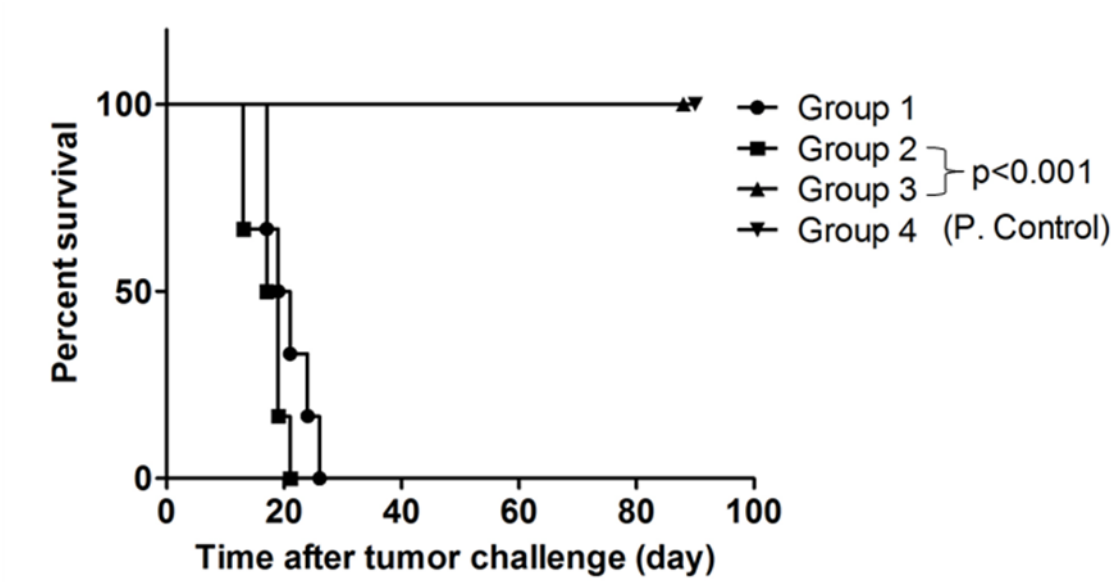

Figure 47: *In Vivo* Investigation of the Influence of Pre-existing Anti-vector Immunity to the Anti-tumor Efficiency of Bacterial Vector.

This experiment was designed for investigating the influence of pre-existing anti-vector immunity to vaccination efficiency. Four groups of mice were involved and the experimentation timing is described in figure 46. Mice in Group 3 were repeatedly injected with $5*10^6$ CHA-OST/time for induction of anti-vector immunity and with $5*10^6$ CHA-OST-PADRE-OVA/time for prophylactic vaccination before tumor challenge. Mice only injected with 100µL PBS/time (Group 1) or $5*10^6$ CHA-OST/time (Group 2) before tumor challenge were used as negative control. And, mice administered with $5*10^6$ CHA-OST-PADRE-OVA / time only on 14 days and 7 days before the tumor challenge were used as positive control. Kaplan-Meyer curves displayed survival data from groups of 6 mice. Statistical analysis: $p<0.0001$ for groups 3&4 *VS* groups 1&2; no difference was found between group 3 and group 4.

B Discussion

The pre-existing anti-vector immunity is one major obstacle for the most bacteria or virus based vaccines, because it might accelerate the elimination of the vaccine carriers and limit the activation of effective immune responses against heterologous passenger antigens.

Directly through tumor challenge assay (Fig. 47), no evident impairment of vaccination efficiency has been detected after repeated bacterial vector administration. For this finding, there are several possible explanations: 1) *P. aeruginosa* is one acute infectious pathogen, the immune response to pathogen related Ags could be activated in short term and before the bacteria are eliminated, thereby the influence of pre-existing anti- *P. aeruginosa* immunity is not so evident; 2) the strain used in this experiment is one attenuated mutant strain, the immune defense to this vector may not be so strong; 3) the antigen used in this experiment is OVA which is a one of the most powerful rejection antigens and the immune response to OVA is strong so that the immune impairment to the bacterial vector has been covered up; 4) the vaccination efficiency has been verified in prophylactic mode in which the protection is generally more evident than in therapeutic mode, particularly using OVA.

Thereby, in order to confirm the influence of pre-existing anti-*P. aeruginosa* immunity to vaccination efficacy, more experiments need to be realized: 1) to quantify pre-existing anti-vector humoral and cellular immune response; 2) to verify the vaccination efficiency using self antigen; 3) to verify the vaccination efficiency through immune monitoring assay; 4) to verify the vaccination efficiency in therapeutic vaccination mode.

CONCLUSION AND PERSPECTIVES

En Français:

En raison de l'efficacité pour délivrer des antigènes directement dans le cytoplasme des CPAs *in vivo*, les vecteurs bactériens atténués tels que *Yersinia*, *Salmonella*, *Shigella*, et qui sont basés sur les propriétés du système de sécrétion de type 3 (SSTT) ont attiré de plus en plus d'attention grâce à leur potentiel dans le développement des vaccins contre le cancer. *P. aeruginosa* utilise cet appareil pour délivrer ses effecteurs toxiques au cours d'une infection. Auparavant, plusieurs souches atténuées de *P. aeruginosa* ont été obtenues dans notre laboratoire et leur utilisation potentielle comme vecteur de vaccination anti-tumorale a été démontrée dans des modèles murins (3, 10, 11).

L'objectif du travail de cette thèse est de renforcer le vecteur vaccinal basé sur le SST3 de *P. aeruginosa* et d'essayer de développer des vecteurs bactériens vaccinaux pour des applications cliniques. Pour se faire, nous avons effectué plusieurs séries d'expériences afin : d'améliorer l'efficacité de vaccination du vecteur bactérien, de proposer un processus de préparation du vecteur conformément aux bonnes pratiques de fabrication actuelles et d'évaluer le potentiel de ce vecteur pour l'immunothérapie humaine dans un modèle animal humanisé.

Dans un premier temps, la performance de notre vecteur bactérien a été améliorée en utilisant différents modèles de tumeurs murines et en suivant différentes stratégies. Avant tout, un épitope (PADRE) spécifique des lymphocytes CD4+ Th a été ajouté aux vecteurs. D'une part, cette addition est à l'origine d'une réponse immunitaire forte médiée par les CD8+ CTL. D'autre part, elle réduit la suppression médiée par les cellules Treg pour activer les cellules effectrices. Ainsi, une amélioration de la protection anti-tumorale *in vivo* a été obtenue. Par la suite, un modèle d'expression bi-antigénique a été appliqué au vecteur bactérien. La délivrance du vecteur bi-antigénique a révélée plusieurs avantages par rapport a un vecteur d'un antigène singulier. Parmi ces avantages l'efficacité et la simplicité. Le vecteur bi-antigénique peut atteindre une efficacité de vaccination équivalente à celle obtenue pour les deux

vecteurs combinés de chaque antigène, mais administré en une seule dose qui est sûre. En outre, un vecteur potentiel activant l'immunité humorale a été construit par la délétion des deux protéines structurelles du SSTT: PopB et PopD. Des IgG spécifiques de l'antigène ont été détectés après des vaccinations avec un vecteur muté *ΔPopBΔPopD*. Cette réponse humorale est dépendante de la dose des bactéries.

Dans un deuxième temps, la performance thérapeutique du vecteur bactérien a été optimisée par la modulation de la fréquence des injections et l'intervalle qui les sépare. Les résultats *in vivo* et *in vitro* ont montré qu'une fréquence et un intervalle appropriés d'immunisation sont importants pour programmer la réponse immune primaire contre l'antigène. Grâce à cela, la performance thérapeutique de notre vecteur bactérien a été confirmée dans des modèles naturels de tumeurs. En outre, l'efficacité à long terme de notre vecteur bactérien a été évaluée pour la première fois. Nos résultats démontrent que la réponse médiée par les cellules T mémoires et induite par le vecteur bactérien est capable de détecter les cellules tumorales résiduelles et inhibe efficacement la récurrence de la tumeur. La modulation de la fréquence et l'intervalle d'injection pourrait conduire à une efficacité variable à long terme.

Dans un troisième temps, un candidat qui pourrait être appliqué en clinique a été généré par l'adaptation dans un milieu chimiquement défini d'un mutant (CHA-OAL) de *P. aeruginosa* totalement avirulent. Nous avons constaté que les cations divalents (Mg^{2+} et Ca^{2+}) joue un rôle crucial dans la prolifération de *P. aeruginosa*. Comme la délétion du gène *aroA* dans la souche CHA-CLIN1 pourrait limiter sa dissémination et ainsi limiter l'immunogénicité et l'efficacité de ce vecteur, nous avons essayé d'injecter des souris avec cette souche à de multiples emplacements pour reproduire artificiellement la dissémination des bactéries. Grâce à cela, la très faible infectieusité de cette nouvelle souche a été surmontée. Les résultats *in vivo* et *in vitro* ont montré qu'une injection à de multiples emplacements est plus performante qu'une injection dans un emplacement unique. Une immunisation dans des emplacements multiples avec le vecteur CHA-CLIN1 pourrait donner des efficacités prophylactique et thérapeutique proches par rapport au vecteur CHA-OST.

Dans un quatrième temps, et dans le but d'évaluer le potentiel de notre vecteur bactérien dans l'immunothérapie humaine, nous avons utilisé le vecteur CHA-OST pour délivrer un antigène basé sur les séquences humaines d'HER2 dans un premier modèle de souris humanisées (HHD). Les résultats du challenge tumoral sont encourageants. Ils ont permis de mettre en évidence la possibilité d'utiliser nos vecteurs bactériens afin d'évaluer, d'optimiser et de définir des séquences d'antigènes humains pour des essais cliniques ultérieurs.

Enfin, une immunité anti-vecteur préexistante représente un obstacle pour la plus part des vaccins basés sur les bactéries ou les virus. Nous avons étudié ce problème potentiel dans ce travail et des résultats intéressants ont été obtenus. Les essaies du challenge tumoral ont montré qu'une administration répétée du vecteur bactérien ne fait pas diminuer l'efficacité de la vaccination.

Par ailleurs, il est sûr que certaines de ces expériences doivent être refaites et certains de ces résultats ont besoin d'être reproduits.

En outre, certaines de ces stratégies devraient être optimisées comme la construction d'un vecteur contenant plus d'antigènes pour induire une réponse immunitaire plus forte et plus large et assure un rejet plus efficace de la tumeur. La performance d'activation de l'immunité humorale devrait également être améliorée en utilisant une autre souche atténuée pouvant délivrer une grande quantité d'antigènes.

Pour simuler une réponse immunitaire plus semblable à celle de l'homme, nous pouvons vérifier ces concepts dans certains modèles avancés d'animaux humanisés, tel que la ligné murine NSG (ou NOD scid gamma) (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ*) qui est parmi les les souches les plus immunodéprimés des souris du laboratoire consanguinées décrite à ce jour. Les souris NSG sont dépourvus des cellules T matures, des cellules B et des cellules NK. Elles sont également déficientes dans de multiples voies de signalisation de cytokines et ont de nombreux défauts dans l'immunité innée (542). Ces immunodéficiences des souris NSG permettent la greffe d'un large éventail de cellules humaines primaires afin de reconstituer un répertoire

immunitaire humain. Dans ce modèle animal, nous pourrons obtenir des preuves plus convaincantes pour des futures applications cliniques.

De plus, les stratégies d'administration des antigènes doivent être combinées. Le concept actuel de l'immunothérapie contre le cancer consiste à ce que les vaccins efficaces contre le cancer devraient être capables de cibler : i) les cellules T CD4+ et CD8+ pour générer une immunité anti-tumorale forte et de longue durée, ii) des moyens multiples de l'immunité qui pourrait être important pour lutter contre le fait de contourner la réponse immunitaire à différents niveaux par la tumeur. À cet égard, nous devrions étudier les stratégies de vaccination hétérologues et combinées.

In English:

Due to the endowed effective ability to deliver antigen to cytoplasm of APCs *in vivo*, T3SS based attenuated bacterial vectors, such as *Yersinia, Salmonella, Shigella*, attracted more and more attention for their potential interest in cancer vaccine development. *P. aeruginosa* exploits also this endowed apparatus to deliver its effector toxins during infection of mammalian. Previously, several attenuated *P. aeruginosa* strains have been established in our laboratory and their potential as the carriers for anti-tumor vaccination purpose has been reported in murine models (3, 10, 11).

The objective of the work presented in this manuscript is to strengthen *P. aeruginosa* T3SS based vaccine vectors and direct the development of bacterial vaccine vectors toward clinical applications. For this purpose, we carried out several series of experiments to improve the vaccination efficiency of bacterial vectors; to realize the production process of bacterial vectors complying with current good manufacturing practices grade; and to evaluate the potential of bacterial vectors for human immunotherapy in one humanized animal model.

First, the performance of our bacterial vector has been improved in different murine cancer models by several strategies. First of all, one CD4+ Th epitope - PADRE has been added to bacterial vectors. The addition of PADRE has been observed to on one hand induce stronger CD8+ CTL mediated immune responses and on the other hand attenuate Treg cells mediated suppression to activated effector cells. Thereby, improved *in vivo* anti-tumor protections have been obtained. Second, bi-antigen expression pattern has been applied to bacterial vectors. Bi-antigen delivery vectors demonstrate several advantages than single-antigen delivery vectors, including: efficiency and simplicity. Meanwhile, bi-antigen delivery vectors can achieve the equal vaccination efficiency as combined single-antigen delivery vectors, but be administered at the safer dose. Moreover, one potential humoral immunity activating vector has been constructed through the deletion of two T3SS structural

proteins - PopB and PopD. Antigen specific IgG has been detected after the immunizations with one *⊿*PopB*⊿*PopD mutant vector and this humoral response was bacteria dose dependent.

Second, the therapeutic performance of bacterial vector has been optimized by modulating injection frequency and interval. Both *in vitro* and *in vivo* results demonstrated that appropriate immunization frequency and interval are equally important for programming primary immune response to antigen. Through this way, the therapeutic performance of our bacterial vector has been confirmed in natural tumor models. Besides, in this work, the long term efficacy of our bacterial vector has been evaluated for the first time. Our results demonstrate that the memory T cell response induced by bacterial vector was capable of sensing the residual tumor cells and effectively inhibiting the tumor recurrence. The modulation of injection frequency and interval might lead to variable long term efficiency.

Third, one clinically applicable candidate has been generated by adapting one totally avirulent *P. aeruginosa* mutant (CHA-OAL) in a chemically defined medium. Through this experiment, we found that divalent cations (Mg^{2+} and Ca^{2+}) make a crucial role for *P. aeruginosa* proliferation. Furthermore, as the deletion of *aroA* gene in CHA-CLIN1 strain could limit its dissemination and thereby restrain the immunogenicity and efficiency of this vector, we tried to inject mice with this strain at multiple loci to artificially mimic the dissemination of bacteria. Through this way, the poor infectivity of this new strain has been overcome. Both *in vitro* and *in vivo* results demonstrated that multiple loci injections demonstrated a better performance than uni-position injections; and multiple loci immunizations with CHA-CLIN1 vector executed nearly the same prophylactic and therapeutic efficiencies as CHA-OST vector.

Fourth, in order to estimate the potential of our bacterial vector for human immunotherapy, we use the CHA-OST vector to deliver antigen based on human HER-2 sequences in one first level humanized murine model (HHD mice). These results from this experimental model show the possibility to apply our bacterial

vectors for further investigation, optimization and definition of human antigen sequences in relative clinical trials.

Finally, as the pre-existing anti-vector immunity is one major obstacle for the most bacteria or virus based vaccines, we have also investigated the potential issue in this work. Attractive results have been obtained. Through tumor challenge assay, no evident impairment of vaccination efficiency has been detected after repeated bacterial vector administration.

Next, certainly, some of these experiments need to be repeated and some of these results require to be reproduced.

Moreover, some of these strategies should be more optimized, such as the construction of a delivery vector containing more antigens to induce a strong and broad immune response and provide more efficient tumor rejection. The humoral immunity activation performance should also be enhanced by using one more attenuated strain which can deliver a large quantity of antigens.

And then, for simulating the immune response more similar to humans', we can verify these concepts in some advanced humanized animal models, for example, NSG(or NOD scid gamma) (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ*) murine line which is among the most immunodeficient strains of inbred laboratory mice described to date. NSG mice lack mature T cells, B cells, and natural killer (NK) cells. They are also deficient in multiple cytokine signaling pathways and have many defects in innate immunity (543). The compound immunodeficiency in NSG mice permit the engraftment of a wide range of primary human cells and thereby the reconstitution of a human immune repertoire. In this animal model, we can obtain more convincing proofs for future clinical applications.

Furthermore, antigen delivery strategies should be combined. The current concept of cancer immunotherapy is that effective cancer vaccines should be capable of targeting both CD4+ and CD8+ T cells to generate a strong and long-lasting antitumor immunity and targeting multiple arms of immunity may be important to counteract

tumor immune-escape at different levels. In this respect, we should investigate combined heterologous vaccination strategies.

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Annex

Publications:

Derouazi M, **Wang Y,** Marlu R, Epaulard O, Mayol JF, Pasqual N, Le Gouellec A, Polack B, Toussaint B. « Optimal epitope composition after antigen screening using a live bacterial delivery vector: application to TRP-2 ». *Bioeng Bugs* 2010 Jan-Feb; 1(1):51-60.

Abstract:

Immunotherapeutic approaches, based on the generation of tumor-specific cytotoxic T-lymphocytes (CTL), are currently emerging as promising strategies of anti-tumor therapy. The potential use of attenuated bacteria as engineered vectors for vaccine development offers several advantages, including the stimulation of innate immunity. We developed an attenuated live bacterial vector using the type III secretion system (TTSS) of Pseudomonas aeruginosa to deliver in vivo tumor antigens. Using an inducible and rapid expression plasmid, vaccination with several antigens of different length and epitope composition, including TRp-2, gp100 and MUC18, was evaluated against glioma tumor cells. We observed similar CTL immunity and T-cell receptor (TCR) repertoire diversity with the vaccines, TRP2(125-243), TRP2L(125-376) and TRP2S(291-376). However, only immunization with TRP2L(125-376) induced significant anti-tumor immunity. Taken together, our data indicate the importance of the epitopes composition and/or peptide length of these peptides for inducing cytotoxic T-lymphocyte (CTL) mediated immunity. Characteristics that consistently improved anti-tumor immunity include: long peptides with immunodominant and cryptic CD8(+) epitopes, and strong CD4(+) Th epitopes. Our bacterial vector is versatile, easy-to-use and quick to produce. This vector is suitable for rapid screening and evaluation of antigens of varying length and epitope composition.

Yan Wang, Audrey Le Goue¨llec, Hichem Chaker, Hoda Asrih, Benoit Polack, and Bertrand Toussaint. « Optimization of Antitumor Immunotherapy Mediated by Type III Secretion System-based Live Attenuated Bacterial Vectors ». Accepted by *Journal of Immunotherapy* on Dec 19, 2011.

Abstract:

Recently, due to their effective ability to deliver antigen to antigen-presenting cells in vivo, type III secretion system-based attenuated bacterial vectors have increasingly attracted attention for their potential interest in cancer vaccine development. We have previously developed live attenuated *Pseudomonas aeruginosa* type III secretion system-based vectors to deliver in vivo tumor antigens. In this work, we improved the

performance of these bacterial vectors through several approaches in different murine cancer models involving non–self-antigens or self-antigens. First, by modulating injection frequency and interval, bacterial vaccination activated immune response could be enhanced and the in vivo therapeutic efficacy of bacterial vaccines could be improved. The optimized vaccination scheme induced long-lasting CD8+ T cells' response. Second, a dual antigen delivery pattern was successfully applied in our bacterial vectors. Compared with a single antigen delivery vector, biantigen delivery vectors demonstrated several advantages including better tumor rejection efficiency, simplicity of use, and safety. Third, 1 more attenuated mutant-CHA-OAL strain that is totally avirulent in mice was further adapted to grow in a chemically defined medium to comply with current good manufacturing processes. The poor infectivity of this new strain could be overcome by vaccinations at multiple loci, yielding an efficiently improved vaccination performance. Taken together, our results highlight the potential of our live attenuated *P. aeruginosa* vectors for applications in relevant clinical trials.

Conference:

Y. Wang, M. Derouazi, R. Marlu, O. Epaulard, Nicolas Pasqual, B. Polack and B. Toussaint. « Optimal epitope composition after antigen screening using a live bacterial delivery vector- Application to TRP2 » (poster). The 2nd European Congress of Immunology, Berlin, Germany, 2009-09.

Résumé en Français

En raison de l'efficacité pour délivrer des antigènes directement dans le cytoplasme des CAPs *in vivo*, les vecteurs bactériens atténués et basés sur les propriétés du système de sécrétion de type 3 (SST3) attirent de plus en plus l'attention grâce à leur potentiel dans le développement des vaccins contre le cancer. *Pseudomonas aeruginosa* est un pathogène opportuniste responsable d'infections graves chez les personnes immunodéprimées, les grands brûlés et les patients atteints de la mucoviscidose. Cette pathogénicité repose sur de nombreux facteurs de virulence dont le SST3. Dans nos travaux précédents, le potentiel de souches atténuées de *P. aeruginosa* dans le domaine de la vaccination anti-tumorale a été démontré. Dans ce travail, nous avons optimisé des vecteurs vaccinaux basés sur le SST3 de *P. aeruginosa* pour des applications cliniques.

Dans un premier temps, la performance de ces vecteurs bactériens a été améliorée en utilisant différents modèles de tumeurs murines. Ceci par : 1) l'ajout d'un épitope spécifique des lymphocytes CD4+ Th aux vecteurs; 2) l'application d'un modèle d'expression bi-antigénique aux vecteurs; 3) la construction de vecteurs induisant une réponse humorale. Dans un deuxième temps, la performance thérapeutique du vecteur bactérien a été optimisée par la modulation de la fréquence des injections et l'intervalle qui les sépare. Cette performance a été confirmée dans des modèles différents de tumeurs murines. Dans un troisième temps, un candidat qui pourrait être appliqué en clinique a été généré par l'adaptation d'un mutant (CHA-OAL) de *P. aeruginosa* totalement avirulent dans un milieu chimiquement défini. La très faible infectiosité de cette souche a été surmontée par en vaccinant à des emplacements multiples. Par la suite, le potentiel du vecteur bactérien dans l'immunothérapie humaine a été également évalué- dans un premiers temps-dans un modèle de souris humanisées (HHD). Enfin, nous avons observé qu'une immunité anti-vecteur pré-existante n'a pas d'effet sur l'efficacité de la vaccination par le vecteur bactérien.

L'ensemble de nos résultats a mis en évidence le potentiel de nos vecteurs vivants et atténués de *P. aeruginosa* pour des applications dans des essais cliniques pertinents.

Mots clés : L'immunothérapie du cancer, Vaccin bactérien vivant atténué, Système de Sécrétion de Type III, *Pseudomonas aeruginosa*.