UNIVERSITÉ PARIS DESCARTES
FACULTÉ DE MÉDECINE

CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE

UNIVERSITE PARIS DESCARTES

THESE

Pour obtenir le grade de

DOCTEUR EN SCIENCES

de la Vie et de la Santé

Ecole Doctorale: Gc2ID

Discipline: Immunologie

Présentée et soutenue publiquement par:

PHAM VAN Linh

le 30 juin 2010

Titre:

Modulation de la réponse immunitaire par des agonistes de la voie de signalisation TLR/IL-1R dans le modèle d'asthme

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Liste des abréviations

ADN	Acide désoxyribonucléique
AP-1	Activating protein-1
ARN	Acide ribonucléique
ARNdb	ARN double brin
ARNm	ARN messager
ARNsb	ARN simple brin
BCG	Bacille de Calmette et Guérin
CARD	Caspase recruitment domain
CD	Cluster de différenciation
CMH	Complexe majeur d'histocompatibilité
CPA	Cellule présentatrice de l'antigène
DC	Cellule dendritique
DT1	Diabète de Type 1
FoxP3	Forkhead box P3
GM-CSF	Granulocyte Macrophage -Colony Stimulating Factor
ICAM	Molécule d'adhésion intercellulaire
ICOS	Inducible costimulator
IFN	Interféron
IgE	Immunoglobuline E
IKK	IκB Kinase
IL-	Interleukine-
IPS-1	Interferon-beta promoter stimulator 1
IRAK	IL-1 R associated kinase
IRF	interferon regulatory factor
ITAM	immunoreceptor-based tyronise activation motif
JNK	Janus kinase
LBA	Lavage bronchoalvéolaire
LPS	Lipopolysaccharide
LRR	Répétition riche en leucine
LTB4	Leucotriène B4
LTC4	Leucotriène C4
MBP	Major basic protein

Mda-5	Melanoma differentiation-associated gene 5
mDC	DC myéloïde
MyD88	Myeloid differenciation factor 88
NF-κB	Facteur nucléaire κB
NK	Natural killer
NKT	Lymphocyte T NK
NLR	NOD-like receptor
ODN	Oligodeoxynucléotide
OVA	Ovalbumine
PAMP	Motif moléculaire spécifique des pathogènes
PBMC	Cellules mononucléaires du sang périphérique
pDC	DC plasmacytoïde
PGD2	Prostaglandin D2
PGN	Peptidoglycane
Poly(A:U)	Acide polyadenylique acid-polyuridylique
Poly(I:C)	Acide polyinosinique-polycitydilique
PRR	Pathogen recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-like receptor
RSV	Virus respiratoire syncytial
SNP	Polymorphisme d'un nucléotide simple
SOCS	Suppressor of cytokine signaling
STAT	Signal Transducers and Activator of Transcription
TCR	récepteur des cellules T
TGF-β	transforming growth factor-β
Th	T helper
TIR	Toll/IL-1R
TLR	Toll-like receptor /récepteur Toll-like
TNF-α	Tumor necrosis factor-α
TRAF 6	Tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
Treg	lymphocyte T régulateur $CD4^{+}$ $CD25^{+}$
TRIF	TIR domain-containing adapter inducing IFN-β

INTRODUCTION

I. MALADIE ASTHMATIQUE

Considéré actuellement comme une maladie inflammatoire bronchique chronique, l'asthme représente un problème majeur de santé publique par sa fréquence élevée dans la plupart des pays du monde et par ses coûts socio-économiques. Par ses caractères complexes, variables et évolutifs, la définition de la maladie se base sur des caractéristiques physiopathologiques et cliniques. Bien que de nombreuses connaissances et progrès concernant la maladie viennent ces dernières décennies des études cliniques, pharmaceutiques et de la recherche expérimentale, plusieurs aspects de la physiopathologie de cette maladie ne sont pas encore connus précisément et plusieurs questions restent à élucider. Les traitements actuellement disponibles sont efficaces et permettent de contrôler les symptômes dans un nombre très significatif de cas. Pourtant, des progrès sont encore nécessaires dans ce domaine, car le contrôle de l'asthme est, en réalité, encore loin d'être optimal. Ces progrès attendus viendront certainement par l'éducation thérapeutique des patients, par la formation médicale continue et l'implication des pouvoirs publics, mais surtout des nouvelles connaissances dans la compréhension de la maladie, du développement et de la mise au point de nouveaux outils thérapeutiques.

A. DEFINITION ET GENERALITES

1. Définition de l'asthme

L'asthme est une maladie bronchique dont la définition reste encore symptomatique et descriptive. Les changements dans la définition de cette maladie au cours des dernières décennies sont le résultat de l'évolution des connaissances, notamment physiopathologiques. L'aspect prédominant observé dans l'histoire de la maladie est constitué par les épisodes de dyspnée, accompagnés souvent de toux, survenant en particulier pendant la nuit. La respiration sifflante (wheezing) les ronchi, les sibilants perçus à l'auscultation pulmonaire sont les signes

cliniques typiques. L'aspect pathologique dominant de la maladie est l'inflammation des voies respiratoires associée de façon variable au changement de structure de ces dernières, responsable essentiellement des conséquences fonctionnelles observées dans l'asthme *(Fig.1).* Ainsi, l'asthme est défini par un groupe d'experts internationaux comme *"un désordre inflammatoire chronique des voies respiratoires dans lequel sont impliqués plusieurs types cellulaires et leurs médiateurs. L'inflammation chronique est associée à une hyperréactivité des voies respiratoires qui entraine des épisodes récurrents de respiration sifflante, de dyspnée, de sensation d'oppression thoracique, et/ou de toux particulièrement pendant la nuit ou au petit matin. Ces épisodes sont souvent associés à une obstruction extensive de degré variable, souvent réversible spontanément ou sous l'effet d'un traitement".* (WHO/NHLBI workshop report, 2009)

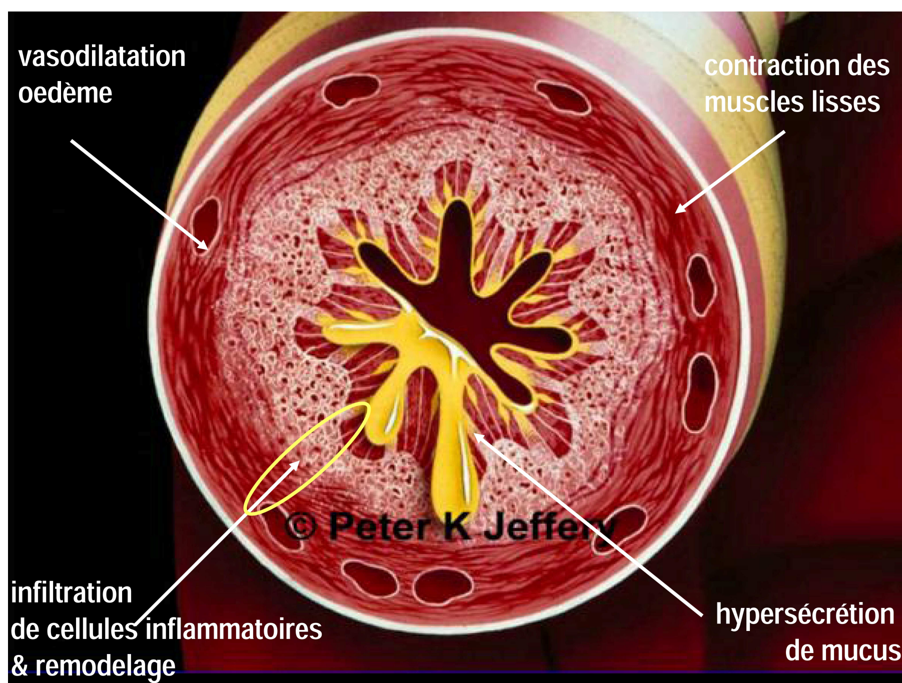

***Figure** 1*. Coupe schématique d'une bronche d'un patient asthmatique

D'un point de vue pédagogique, cette définition pourrait être présentée dans un autre ordre commençant par la clinique (épisodes récidivants de dyspnée, d'oppression thoracique paroxystique et sifflante, à tendance nocturne, réversible spontanément ou sous l'effet d'un traitement), la physiologie

respiratoire (obstruction bronchique variable et réversible, hyperréactivité bronchique caractéristique) pour envisager un diagnostic précis, puis continuant par la physiopathologie (inflammation bronchique polymorphe) pour que le traitement soit adapté au mieux. Par ailleurs, la définition présentée ci-dessus n'est pas totalement satisfaisante car elle ne prend pas en compte les facteurs étiologiques qui déclenchent ou précipitent les crises d'asthme, en particulier l'allergie. D'un point de vue épidémiologique, cette définition s'avère aussi problématique, ce qui pourrait, en cas d'application, entrainer un recensement d'autres affections présentant un chevauchement clinique et fonctionnel avec l'asthme, en particulier les bronchopneumopathies chroniques obstructives (BPCO). En l'absence de marqueur biologique sensible et spécifique de type pathognomonique, il est difficile de proposer une définition consensuelle (Bourdin et al, 2006).

2. Données épidémiologiques

L'asthme constitue dans le monde entier l'une des pathologies chroniques les plus fréquentes. Il représente une cause de morbidité importante dans les pays développés en raison de sa prévalence élevée qui a tendance à augmenter depuis ces dernières décennies, et une préoccupation majeure dans les pays en voie de développement pour des raisons économiques et humanitaires. La fréquence de l'asthme varie considérablement selon les pays et il est difficile de déterminer avec précision le nombre d'asthmatiques dans le monde entier. Le GINA (*Global Initiative for Asthma)* et l'OMS estiment que l'asthme atteint environ 300 millions de personnes dans le monde, que sa fréquence augmente régulièrement depuis 40 ans et que sa mortalité reste inquiétante, avec plus de 250000 morts par an dans le monde (Bourdin et al, 2006; Pearce et al., 2007). Avec 15 millions d'années de vie compromises annuellement par la maladie, selon l'estimation de l'OMS, la charge de la maladie représente à l'échelle mondiale 1% de la charge totale induite par toutes les maladies (WHO/NHLBI workshop report, 2009).

Le rapport garçon/fille varie de 1,5 à 3,3 avant la puberté, il devient légèrement inférieur à 1 à l'âge adulte. Ainsi, pendant et après la puberté, il y a plus de femmes asthmatiques que d'hommes (Fagan et al., 2001). Trois quarts des sujets ont eu leur première crise avant l'âge de 20 ans. Un second pic d'apparition de la maladie se situe aux environs de la cinquantaine: ce sont les asthmes dits tardifs, en général (mais pas toujours) nonallergiques. Trente à 40% des asthmes de l'enfant deviennent asymptomatiques au passage à l'âge adulte. Les facteurs prédisant la persistance de l'asthme sont la sévérité, le début précoce et le caractère allergique de l'asthme d'une part, le début précoce de la puberté et le surpoids d'autre part (Guerra et al., 2004).

A titre d'exemple, la prévalence de l'asthme chez les enfants des États-Unis est passée de 3,6% en 1980 à 5,8% en 2003 *(Fig.2)*. L'asthme est la troisième cause d'hospitalisation chez les personnes de moins de 18 ans aux États-Unis, dépassé seulement par la pneumonie et les accidentés (Eder et al., 2006). En 1964, 19% des enfants australiens ont, d'après leurs parents, manifesté des symptômes asthmatiques ou une respiration sifflante à un moment quelconque au cours de leurs 7 premières années de vie alors qu'ils étaient 46% en 1990 (Peat et al., 1992; Robertson et al., 1991).

La difficulté des études épidémiologiques de l'asthme est liée aux méthodes utilisées pour en poser le diagnostic. Les études effectuées simplement au moyen de questionnaires rapportent des fréquences souvent plus élevées que celles incluant des mesures des débits ou du test d'hyperréactivité bronchique. Parmi les nombreuses études épidémiologiques consacrées à l'asthme, peu ont été fondées sur une méthodologie rigoureuse, standardisée, permettant des comparaisons dans les différents pays du monde. Chez l'enfant, l'étude ISAAC *(International Study of Asthma and Allergies in Childhood)* avec ses trois phases, représente l'étude coopérative multicentrique qui répond le mieux à ces critères et qui est la seule étudiant l'épidémiologie de l'asthme et de l'allergie pédiatrique à l'échelle mondiale et avec mobilisation et participation record (314 centres dans 106 countries, avec près de deux millions d'enfants). La phase I de

l'étude ISAAC a été conçue pour permettre des comparaisons de la prévalence des symptômes de l'asthme dans des pays de différentes régions du monde. Au cours de la phase III, la première phase d'enquête a été répétée après un intervalle de 5-10 ans chez les enfants âgés de 6-7 ans en vue d'évaluer la tendance au fil du temps. Ainsi, la prévalence des symptômes d'asthme actuel, rapportée pour les enfants de 13-14 ans de l'étude ISAAC, varie de 1 à 36% pour l'ensemble des pays étudiés et de 10 à 18% en France (Bourdin et al, 2006; Pearce et al., 2007).

À l'échelle mondiale, la moyenne de cette prévalence a changé légèrement de 13,2% à 13,7% dans le groupe 13-14 ans (augmentation moyenne de 0,06% par an) et de 11,1% à 11,6% dans le groupe de 6-7 ans (augmentation moyenne de 0,13% par an). En Europe occidentale, la prévalence est restée stable chez les enfants âgés de 13-14 ans, mais a augmenté de 0,20% par an chez les enfants âgés de 6-7 ans.

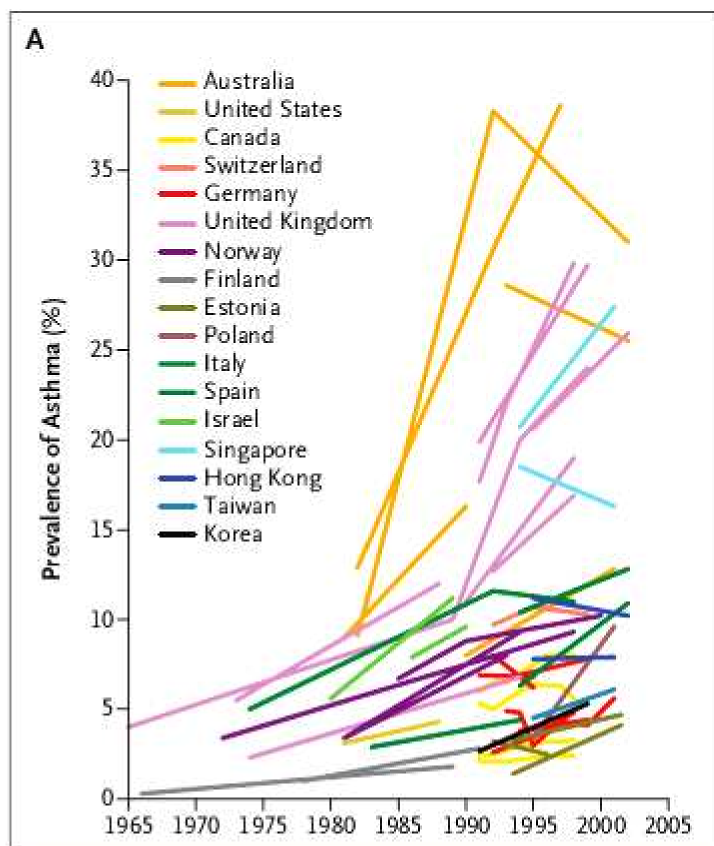

***Figure** 2.* Prévalence de l'asthme dans le monde

D'après: Waltraud Eder *et al. N Engl J Med 2006*

Pour les autres régions du monde, chez les enfants âgés de 13-14 ans et de 6-7 ans, les changements respectifs aux deux groupes d'âges sont: en Océanie (-0,39% et -0,21%), en Amérique latine (+0,32% et 0,07%); En Europe du nord et Europe de l'Est (+0,26% et 0,05%), en Afrique (+0,16% et 0,10%), en Amérique du Nord (+0,12% et 0,32%), en Méditerranée orientale (-0,10% et 0,79%), en Asie-Pacifique (+0,07% et -0,06%) et dans le Sous-continent indien (+0,02% et 0,06). Ces résultats indiquent que les différences dans la prévalence des symptômes d'asthme ont réduit, en particulier dans le groupe de 13-14 ans, l'augmentation de la fréquence dans les régions où celle-ci était faible.

Au Vietnam, une étude portant sur les enfants de deux écoles à Hanoï utilisant le questionnaire de l'étude ISSAC a rapporté les pourcentages d'enfants ayant la respiration sifflante dans le passé, au cours des derniers 12 mois, ayant un asthme considérés ou diagnostiqués de 24.9%, 14.9%, 12.1% et 13.9% respectivement (Nga et al., 2003). Une autre étude dans le cadre de l'étude ISSAC effectuée à Ho Chi Minh ville, grande agglomération urbaine du pays, a montré un pourcentage d'enfants de 12 à 13 ans ayant les symptôme d'asthme très élevé (29,1%), beaucoup plus que celui rapporté précédemment (10%), faisant de cette ville l'endroit où la fréquence de l'asthme est la plus élevée de l'Asie.

Les augmentations de la prévalence des symptômes d'asthme en Afrique, en Amérique latine et dans certaines parties de l'Asie indiquent que la différence dans la prédominance de l'asthme à l'échelle mondiale est réduite et la charge mondiale de l'asthme continue à augmenter (Pearce et al., 2007). Aussi dans le cadre de l'étude ISAAC, les analyses économiques et écologiques ont rapporté récemment une association inversée significative, déjà bien remarquée précédemment, entre le niveau de vie et la prévalence de l'asthme dans laquelle les symptômes de l'asthme ont tendance à être plus répandus dans les pays et les communautés à revenu élevé dans les deux groupes d'âge étudiés (Lai et al., 2009). La tendance est inversée pour la fréquence des symptômes d'asthme sévère parmi ceux qui présentent des symptômes d'asthme actuel.

Dans l'étude longitudinale European Community Respiratory Health Survey (ECRHS, 1996) réalisée chez des adultes de 20-44 ans, on observe, comme dans l'étude ISAAC, des pays qui possèdent une prévalence très élevée, comme l'Angleterre (7,5 à 8,4%), la Nouvelle-Zélande (9 à 11,3%), l'Australie (11,9%), et des pays dont la prévalence est très basse, comme l'Islande (3,4%) et la Grèce (2,9%) (ECRHS, 1996). En France, pour cette tranche d'âges, les fréquences varient de 2,7 % à Grenoble, à 3,5 % à Montpellier et 4,0 % à Paris et les fréquences de ceux qui manifestent des symptômes d'asthme dans leur vie varient de 7,4% à Grenoble, 9,2 % à Montpellier et 9,3 % à Paris, soit plus du double des fréquences des symptômes d'asthme actuel (Neukirch et al., 1995).

Un gradient Est-Ouest (avec des prévalences plus basses à l'Est) et un gradient Nord-Sud (avec des prévalences plus basses au Sud) sont retrouvés dans les deux études, ISSAC et ECRHS (Bourdin et al, 2006). Plusieurs autres études réalisées dans le monde montrent depuis les quatre dernières décennies que la fréquence de l'asthme s'accroit d'environ 6-10% par an chez l'enfant (Bourdin et al, 2006; Evans, III et al., 1987; Latvala et al., 2005). Un plateau semble avoir été atteint dans certains de ces pays (Anderson, 2005; Toelle et al., 2004). Le pourcentage accru des enfants diagnostiqués ou ayant des symptômes d'asthme reflète une prise de conscience de cette maladie et/ou des changements de pratiques de diagnostic, l'augmentation de l'offre, mais aussi, probablement, une réelle aggravation de la morbidité de la maladie.

La mortalité de l'asthme se produit la plupart du temps durant les années d'adolescence. La surmortalité mondiale de l'asthme de la fin des années 1970 a mis en question le rôle de β2-agonistes. En 1989 une étude cas-témoins décrivait une association entre fénotérol et décès par asthme en Nouvelle-Zélande de 1981 à 1989, association rapidement étendue à l'ensemble des β2-agonistes. Cependant, une méta-analyse des études cas-témoins concluait que seuls les β2-agonistes utilisés en nébulisation constituaient un facteur de risque de surmortalité, la mortalité augmentant radicalement lorsque plus de 20 mg par mois d'équivalents albutamol

étaient utilisés. Or la prescription des nébulisations est réservée aux cas d'asthme les plus sévères. Ce ne sont donc pas les β2-agonistes, mais bien la sévérité de l'asthme pendant une exacerbation aiguë qui constitue le principal facteur de risque de mortalité (Mullen et al., 1993). Dans une enquête australienne de mortalité liée à l'asthme chez les enfants, les attaques d'asthme mortelles étaient les plus fréquentes (53%) dans la catégorie d'âge de 12 à 15 ans, souvent par manque de connaissance des patients sur la maladie ou le retard dans l'identification de la sévérité, de la prise en charge des attaques d'asthme (Martin et al., 1995).

3. Charges socio-économiques de la maladie

L'asthme est l'une des maladies les plus coûteuses dans les pays développés. Le coût pour la société peut se diviser en coûts directs, indirects et coûts intangibles, associés aux aspects psychosociaux de l'asthme. Les coûts directs comprennent les hospitalisations, les traitements en salle d'urgence, les consultations médicales, les frais de laboratoires, les médicaments et les autres thérapeutiques, représentent 35-60 % des dépenses. Les coûts indirects résultent des pertes financières non médicales dues à la maladie: absentéisme scolaire et professionnel, perte de productivité au travail, invalidité et décès.

La qualité de vie des enfants asthmatiques symptomatiques est altérée, qu'il s'agisse de l'activité scolaire, de la pratique du sport et des loisirs, des relations familiales. La mesure la plus précise de l'impact social de l'asthme chez l'enfant est vraisemblablement l'absentéisme scolaire dont l'asthme est l'une des principales causes, entraînant jusqu'à 25 % de l'absentéisme scolaire dans certains pays. Les enfants asthmatiques ont aussi des difficultés à s'adapter au milieu scolaire, à suivre l'enseignement et à communiquer. Chez l'adulte, le retentissement de l'asthme sur la qualité de vie et les performances socioprofessionnelles n'est pas moins important. Les jours d'absence au travail par semestre à cause de l'affection, du degré du handicap, sont directement proportionnels à la sévérité de l'asthme.

4. Facteurs inpliqués dans le développement et l’expression de la maladie

a) Facteurs de l’hôte

La développement de l'asthme est le résultat de la conjonction des facteurs génétiques de l’hôte (possiblement déterminants) et des facteurs de l’environnement (favorisants). Le composant héréditaire dans l’asthme n'est pas simple. Les données actuelles montrent que plusieurs gènes sont impliqués dans la pathogénèse de l'asthme et qui pourraient être différents dans différents groupes ethniques. La diversité et le poids respectif de ces différents facteurs expliquent les multiples phénotypes. Il est fort probable que c'est l'association de plusieurs gènes à effets modérés qui importe et que de multiples associations existent, expliquant également l'hétérogénéité clinique de l'asthme. Les études des familles et les analyses des associations cas-témoin ont identifié un nombre de régions chromosomiques qui sont associées à la susceptibilité de l’asthme. Outre les gènes qui prédisposent à la susceptibilité de la maladie, il y a également les gènes qui sont associés à la réponse à différent traitements comme les glucocorticostéroides, les β2-agonistes, les antagonistes des leukotriènes…

Études familiales

Les études d'agrégation familiale pour l'asthme et les maladies qui lui sont associées (atopie, hyperréactivité bronchique, rhinite allergique) démontrent qu'il existe une agrégation familiale de ces phénotypes. 1177 enfants ont été suivis pendant 20 ans ; 11,5 % des enfants sans parent asthmatique développent un asthme contre 1/3 si l'un des parents est asthmatique (risque relatif de l'ordre de 3) et 1/2 si les deux parents le sont (risque relatif de l'ordre de 4) (Burrows et al., 1995).

Études de criblage du génome et des gènes candidats

Les études familiales de génétique moléculaire sont les seules capables d'approcher physiquement les gènes de susceptibilité de l'asthme et d'en analyser les modifications par rapport aux sujets non malades. Des liens ont été retrouvés dans les familles d'asthmatiques

et/ou d'atopiques sur un grand nombre de chromosomes *(tableau 1)* (CSGA, 1997; Daniels et al., 1996; Dizier et al., 2000; Marsh et al., 1994; Wjst et al., 1999).

Tableau 1: Principaux gènes liés à l'asthme et aux allergies respiratoires

Régions chromosomiques	**Phénotypes associés**
1p32-34	Asthme, IgE
2pter	Asthme, IgE
2q14	Asthme
2q33	Asthme
3q21	Asthme
4q35	HRB
5p15	Asthme
5q31-33	Asthme, IgE totales
6p21-23	Asthme, IgE totales et spécifiques, éosinophilie
7p11	Asthme
7p15.2	IgEtotales, éosinophilie, HRB, asthme
7q11-22	Asthme
8p21-23	Asthme
9q31	Asthme, IgE spécifiques
11p15	Asthme
11q13	Asthme, IgEtotales, atopie, TC
12q14	Asthme, IgE totales
13q14	Atopie, asthme, IgE totales
14q11.1	IgEspécifiques, TC
14q11-13	Asthme
16p12	IgE totales et spécifiques
16q22-24	IgE totales, HRB, asthme
17p11.1-11.2	Asthme
17q12-21	Asthme, TC
19q13	Asthme
20p13	Asthme
21q21	Asthme

HRB:hyperréactivité bronchique
TC: tests cutanés positifs aux principaux pneumallergènes.

Études des polymorphismes génétiques

De nombreux gènes sont polymorphes, c'est-à-dire présentent une modification minime d'une (ou plus rarement plusieurs) paires de bases par rapport à la séquence normale. Ces variants expliqueraient la majorité des maladies génétiques dites complexes telles que l'asthme, l' atopie, ou encore le diabète et l'hypertension artérielle, ainsi que la plupart des réponses aux thérapeutiques.

Dans l'asthme, la plupart des gènes connus pour modifier la susceptibilité de la maladie ont été identifiés grâce à des études qui ont cherché à analyser les variants comme les polymorphismes d'un simple nucléotide (SNP) qui influencent l'inflammation allergique de l'asthme ou qui sont liés à des phénotypes de celui-ci. Les premiers variants décrits ont concerné le gène codant pour le récepteur FceRI-β: une substitution de l'isoleucine en position 181 en leucine (variant I181L) et une substitution de l'acide glutamique en position 237 en glycine (variant E237G). La présence du variant E237G expose à un risque accru d'atopie, de rhinite, d'hyperréactivité bronchique, d'asthme (Shirakawa et al., 1994). Le polymorphisme TLR2/-16934T protège les enfants de fermiers de la survenue d'asthme, de sensibilisation aux pneumallergènes et de rhinite allergique (Eder et al., 2004b). Le variant IL4C-589T (responsable d'une expression accrue d'IL4) est associé à un fort taux d'IgE, un asthme allergique plus fréquent, une fonction respiratoire légèrement altérée (Rosenwasser et al., 1995) et répondant peu aux glucocorticoïdes. Certains asthmatiques ne répondent pas favorablement à l'action des β2-mimétiques du fait de la présence de mutations dans le gène du récepteur β2-adrénergique (Molimard and Marthan, 2000) et répondent mieux aux glucocorticoïdes s'ils possèdent un variant du gène du récepteur CRHR1 (Tantisira et al., 2004c). De nombreux autres polymorphismes ont été associés à l'asthme ou aux phénotypes associés ou à la réponse aux traitements (Vercelli, 2008). Schématiquement, les polymorphismes des gènes impliqués dans l'asthme pourraient être classés en fonction des étapes où ils interviennent, ou des incidences qu'ils génèrent. Le polymorphisme des gènes de l'immunité innée sera traité dans le chapitre suivant.

- *Ceux qui interviennent dans la différentiation et la fonction effectrice des cellules T_h2*

  Ce deuxième groupe inclut des gènes qui régulent directement la synthèse de l'IgE et d'autres mécanismes effecteurs et d'amplification dépendant des cytokines de type Th2. La polarisation Th2 à partir des cellules $CD4^+$ naïves nécessite l'induction de GATA-binding protein 3 (GATA3) par des signaux émis par les

interactions IL-4-IL-4R et sous l'influence de STAT6. L'activité de GATA3, switch principal de la polarisation Th2, est inhibée par le facteur de transcription T-bet, switch principal de la différenciation Th1, par interaction entre GATA3 et son ADN cible (Hwang et al., 2005). Ainsi, des variants de GATA3 (Pykalainen et al., 2005), TBX21 (qui code T-bet) (Tantisira et al., 2004b), IL4 (Basehore et al., 2004; Kabesch et al., 2003b), IL4RA (Loza and Chang, 2007), STAT6 (Schedel et al., 2004) et IL12B (codant l'IL-12p40) (Morahan et al., 2002), ont été récemment décrits comme étant associés à l'asthme et à l' allergie, ainsi qu'à la réponse à la corticothérapie.

L'IL-13 est la principale cytokine de l'inflammation allergique dans l'asthme. En effet, l'IL-13 est suffisante pour expliquer tous les signes majeurs de l'asthme dans les modèles d'asthme expérimental (hyperréactivité bronchique, infiltration de cellules inflammatoires, hypersécrétion de mucus et fibrose) (Grunig et al., 1998; Wills-Karp et al., 1998). De plus, l'IL-13 avec l'IL-4, sont les cytokines activement impliquées dans la synthèse d'IgE. L'IL-13 et ses récepteurs sont fortement exprimés dans les voies respiratoires des patients atteints d'asthme (Lordan et al., 2002). Ainsi, le gène codant l'IL13 est aussi l'un des gènes candidats les plus étudiés dans l'asthme et l'allergie. L'IL13 Arg130Gln est un SNP qui induit le remplacement d'une Arginine en position 130 par une Glutamine et l'expression d'un variant d'IL-13 dont l'activité biologique est augmentée (Vladich et al., 2005). Ce polymorphisme est associé également à une augmentation des IgE totales sériques, de l'asthme (Heinzmann et al., 2000), et de l'atopie (He et al., 2003). Un SNP du promoteur du gène codant l'IL-13, IL13-1112CT, conduit à une expression accrue de l'IL-13 dans les cellules Th2 (Hummelshoj et al., 2003; van der Pouw Kraan TC et al., 1999). Par ailleurs, il existe une liaison génétique entre ces deux polymorphismes et ceux qui portent les deux expriment une concentration élevée d'un variant d'IL-13 hyperactif.

La liaison croisée des récepteurs de haute affinité FceRI est le principal stimulus pour la sécrétion de cytokines Th2 par les mastocytes et les basophiles. Les SNP de FcεRIβ, qui codent pour la chaîne β de FceRI, sont trouvés parmi les polymorphismes les plus incriminés (Shirakawa et al., 1994) influençant l'expression des récepteurs (Donnadieu et al., 2003) et la libération de manière IgE-dépendante des médiateurs inflammatoires par les mastocytes. La phase effectrice de l'inflammation allergique repose également sur l'interaction entre l'IL-5 dérivée des cellules Th2 et son récepteur (IL-5RA) sur les progéniteurs éosinophiles. L'IL-5 entraîne le développement des éosinophiles, qui peuvent être ensuite recrutés dans les voies respiratoires et devenir à leur tour une source importante de cytokines Th2. Des SNP de l'IL5 et IL5RA (Kabesch et al., 2007; Namkung et al., 2007) sont trouvés associés modérément à des phénotypes particuliers de la maladie.

- *Les cellules épithéliales:* L'expression de SPINK5, qui joue un rôle dans la protection contre les protéases libérées par les mastocytes ou les allergènes, est limitée à l'épithélium. Des mutations dans le gène de SPINK5 peuvent conduire à un syndrome de Netherton, trouble autosomique récessif rare qui se caractérise par le caractère défectueux de la fonction épithéliale. Un SNP (SPINK5 Glu420Lys, rs2303067) a été signalé comme étant fortement associé à l'asthme (Chavanas et al., 2000; Walley et al., 2001). Filaggrin et la S100 protéine sont impliqués dans le maintien de l'intégrité épithéliale dans les voies respiratoires. L'association d'un polymorphisme du gène profilaggrine sur le segment chromosomique 1q13 et l'asthme a été décrite (Ying et al., 2006b).

- *La fonction pulmonaire:* Parmi les gènes candidats, il y a des gènes dont les polymorphismes ont été décrits comme étant associés à la fonction pulmonaire, au

remodelage des voies aériennes et à la gravité de la maladie. Ce groupe comprend les gènes ADRB2 (Drysdale et al., 2000), TNF (Moffatt and Cookson, 1997), TGFB1 (Silverman et al., 2004), LTC4S (Sayers et al., 2003), TBXA2R (Shin et al., 2003), ALOX5 (Drazen et al., 1999), NOS1 (Gao et al., 2000) qui codent respectivement pour le récepteur β2 adrénergique, le TNFα, le TGFβ1, les enzymes pour la synthèse de LTC4, le récepteur de thromboxane A2, 5-lipooxygénase et l'enzyme pour la synthèse de NO. Certains de ces gènes sont les cibles pour les études d'identification des gènes qui conditionnent les réponses au traitement (Vercelli, 2008).

b) Facteurs environnementaux

Bien que les allergènes intérieurs et extérieurs soient bien connus pour provoquer des exacerbations de l'asthme, leur rôle spécifique dans le développement de cette maladie n'est pas encore entièrement résolu. Les études de cohortes à la naissance ont montré que la sensibilisation aux allergènes d'acariens, de squames de chat, de chien (Sporik et al., 1990; Wahn et al., 1997) et d'Aspergillus (Hogaboam et al., 2005) sont des facteurs de risque indépendants de symptomes asthmatiques chez les enfants jusqu'à 3 ans. Toutefois, la relation entre l'exposition aux allergènes et la sensibilisation chez les enfants n'est pas simple. Il dépend des allergènes, de la dose, de la durée d'exposition, de l'âge des enfants, et probablement aussi de la génétique. Pour certains allergènes, tels que ceux issus des acariens dans la poussière de maison et des cafards, la prévalence de la sensibilisation semble être en corrélation directe avec l' exposition (Huss et al., 2001; Wahn et al., 1997). Bien que certaines données suggèrent que l'exposition aux allergènes d'acariens de la poussière de maison puisse avoir un lien de causalité dans le développement de l'asthme (Sears et al., 2003), d'autres études ont remis en question cette interprétation (Sporik et al., 1990; Charpin et al., 1991). Pour les cas concernant des allergènes d'animaux domestiques, certaines études épidémiologiques ont constaté que l'exposition précoce à ces animaux peut protéger un enfant contre une sensibilisation allergique ou le développement de

l'asthme (Gern et al., 2004; Ownby et al., 2002; Platts-Mills et al., 2001), mais d'autres suggèrent que l'exposition peut augmenter le risque de sensibilisation allergique (Almqvist et al., 2003; Celedon et al., 2002; Melen et al., 2001; Ownby et al., 2002) La prévalence de l'asthme , réduite chez les enfants élevés en milieu rural, pourrait être liée aux contacts différentiels avec des endotoxines dans ces environnements (Braun-Fahrlander, 2003).

Le cas du rôle des agents infectieux dans l'asthme sera traité dans le chapitre suivant.

Plusieurs substances ont été impliquées dans les différentes formes d'asthme professionnel (Braun-Fahrlander, 2003; Malo et al., 2004). Ces substances comprennent de très petites molécules telles que les isocyanates, irritants qui peuvent causer une hyperréactivité bronchique, les agents chimiques, ou des produits biologiques végétaux et animaux qui stimulent la production d'IgE. L'asthme professionnel apparaît essentiellement chez l'adulte, et est estimé à l'origine d'environ 1 cas d'asthme sur 10 chez ces sujets (Nicholson et al., 2005). L'atopie et le tabagisme peuvent augmenter le risque d' asthme professionnel.

L'exposition à la fumée du tabac est associée au déclin accéléré de la fonction pulmonaire chez les personnes souffrant d'asthme, à l'exacerbation de l'asthme et peut rendre les patients moins sensibles au traitement par les glucocorticoïdes (Chalmers et al., 2002; Chaudhuri et al., 2003; Lazarus et al., 2007). L'exposition prénatale ou post natale à la fumée du tabac est associée à des effets nocifs mesurables dont un plus grand risque potentiel à développer des symptômes de type asthmatique dans les premières années de vie de l'enfant.

Le rôle de la pollution asmosphérique dans le déclenchement des symptômes d'asthme reste controversé (WHO/NHLBI workshop report, 2009; American Thoracic Society., 2000). Les enfants élevés dans un environnement pollué ont une fonction pulmonaire diminuée (Gauderman et al., 2004), mais la relation avec le développement de l'asthme n'est pas apparente.

Le rôle de l'alimentation, en particulier, en relation avec le développement de l'asthme, a fait l'objet de nombreuses études. En général, les don nées révèlent que les nouveaux-nés nourris au lait de vache ou avec des protéines végétales ont une incidence plus élevée de respiration sifflante dans leur petite enfance par rapport à ceux nourris au lait maternel (WHO/NHLBI workshop report, 2009).

B. REACTION INFLAMMATOIRE ASTHMATIQUE

1. Introduction des allergènes et phase d'induction

Les cellules dendritiques (DC) conventionelles expriment de nombreux récepteurs de reconnaissance des pathogènes (PRR) dont les récepteurs Toll-like (TLR), domaine d'oligomérisation de fixation des nucléotides et des récepteurs lectine de type C (Sandor and Buc, 2005a; Sandor and Buc, 2005b; Sandor and Buc, 2005c; Suarez et al., 2008). Comme la plupart des allergènes inhalés sont contaminés par des lipopolysaccharides (LPS) et peptidoglycanes, ils peuvent exercer des effets adjuvants en activant les DC. Étant donné que de nombreux allergènes, notamment Derp2, sont membres de la famille des protéines de liaison des lipides MD2-like et que plus de 50% des allergènes majeurs sont des protéines liant des lipides, cette similarité pourrait aussi expliquer l'immunogénicité de ces allergènes. En l'absence de contamination des ligands de TLR, certains allergènes peuvent activer les DC en déclenchant des récepteurs activés par des protéase (PAR). Ils sont présents sur les cellules épithéliales, fibroblastes, cellules musculaires lisses, cellules endothéliales, et sur un certain nombre de cellules inflammatoires. En dehors des effet sur l'état d'activation des DC, les PAR sont également impliqués dans la prolifération des cellules mésenchymateuses participant ainsi ainsi au remodelage des voies respiratoires (Berger et al., 2001).

La stimulation des TLR et des PAR conduit aux événements qui aboutissent à la production de chimiokines qui attirent les éosinophiles, les neutrophiles, les monocytes et les DC

dans les voies respiratoires et à la production de cytokines qui peuvent induire la maturation des DC et la polarisation Th2 (Kiss et al., 2007). Dans cette phase initiale, les cellules résidantes des voies respiratoires et aussi les cellules recrutées secrètent de nombreux médiateurs chimioactifs *(Fig.3)*. La lymphopoiétine stromale thymique (TSLP), facteur de croissance granulomonocytaire (GM-CSF), l'interleukine (IL)-25 et IL-33 représentent les plus importants *(Fig.4)*.

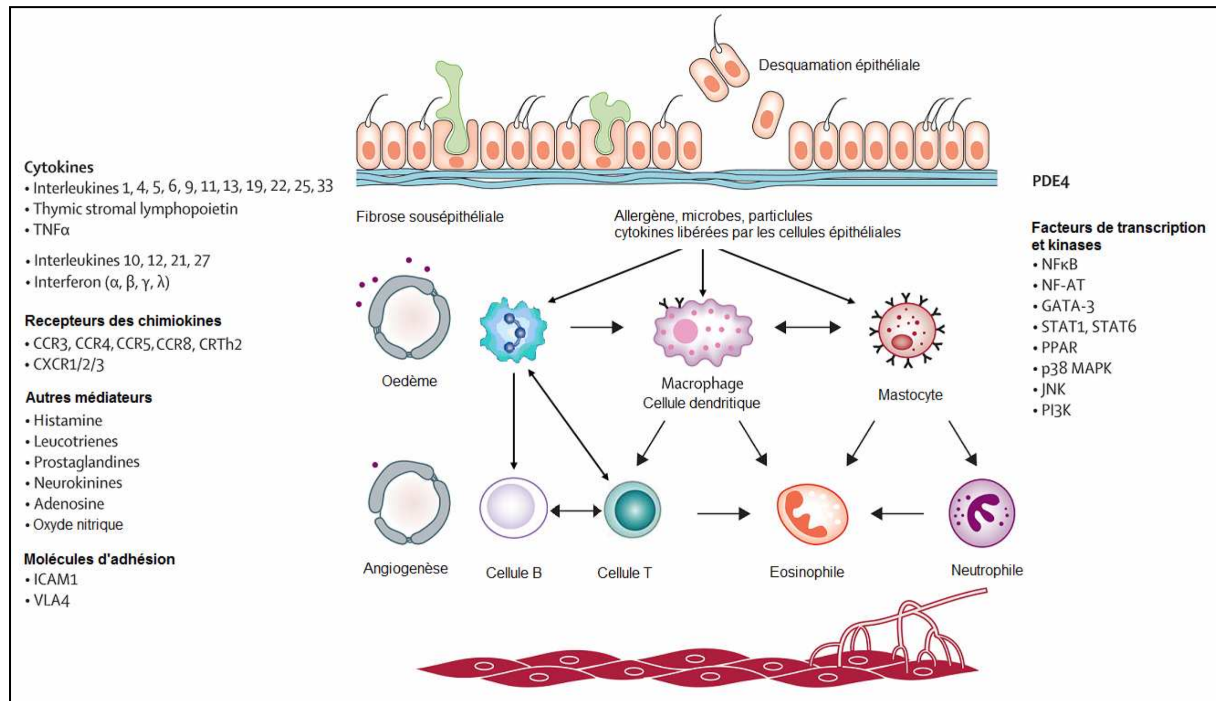

Figure 3. Schéma récapitulatif des acteurs cellulaires et moléculaires impliqués dans l'asthme

TSLP est une cytokine caratérisée récemment avec une forte capacité de modulation des réponses immunitaires. La cytokine est fortement exprimée dans les cellules épithéliales des voies aériennes des patients asthmatiques (Hammad and Lambrecht, 2008; Liu et al., 2007). Les DC stimulées par TSLP favorisent la différenciation des cellules T pour produire des cytokines de type Th2 et produisent des chimiokines CCL17 (TARC) et CCL22 (MDC) pour recruter ces cellules (Ito et al., 2002; Wang et al., 2006; Barnes, 2008a; Holgate and Polosa, 2008). Il a été démontré que TSLP pouvait induire la différenciation Th2 à partir des T CD4+ naïfs directement, en l'absence des DC conduisant à la production d'IL-4 (Omori and Ziegler, 2007). En dehors de

ses effets sur les DC et sur les cellules TCD4+ naïves, TSLP pourrait également activer les mastocytes pour produire des cytokines effectrices des réponses Th2 (Holgate and Polosa, 2008). Les souris déficientes pour le gène de TSLP ne parviennent pas à développer une réponse Th2 (Adcock et al., 2008).

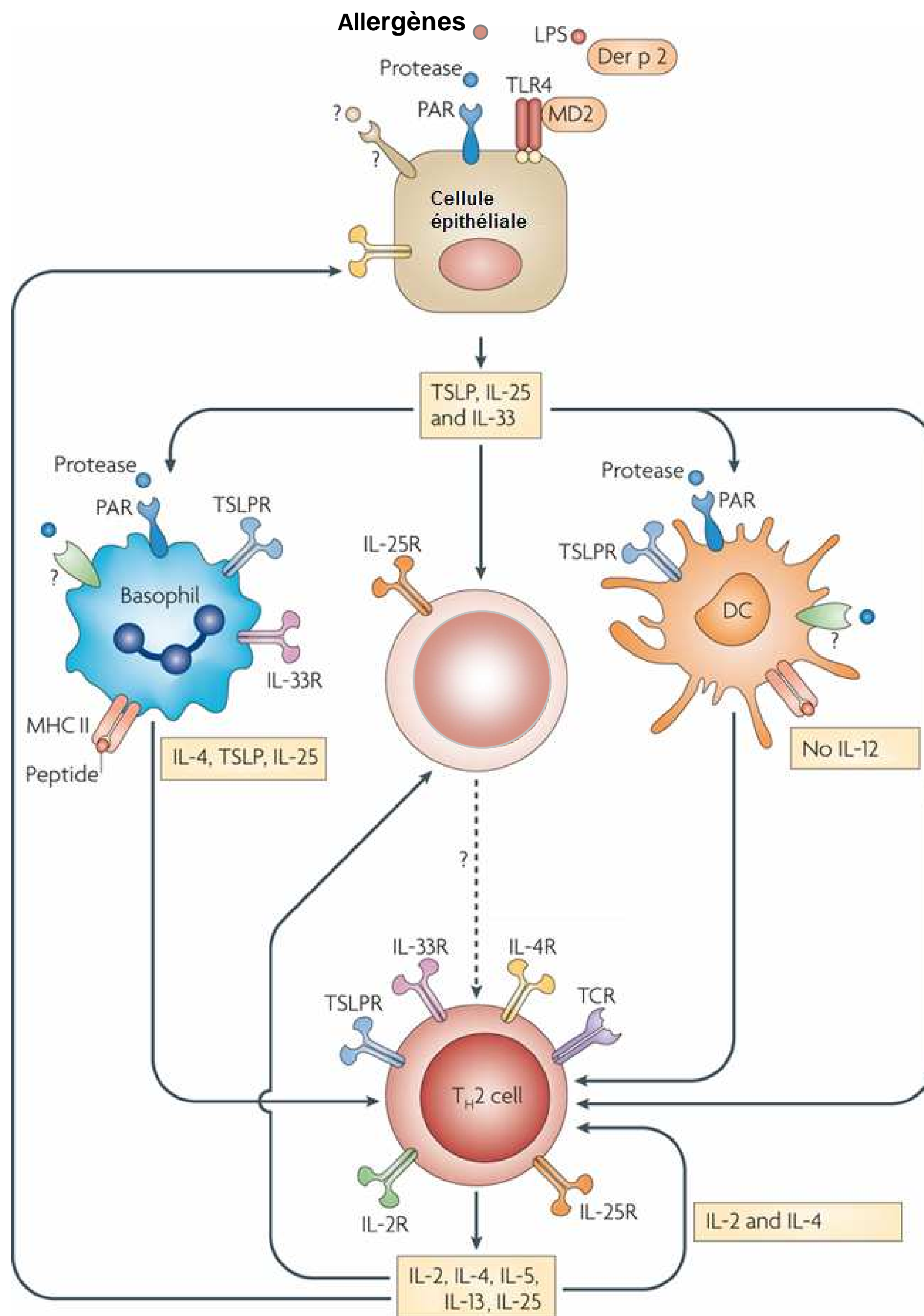

***Figure** 4.* Cytokines impliquées dans l'initialtion de la réponse immunitaire dans l'asthme. Adapté d'après Zhu *et al. Nature Reviews Immunology 2010*

GM-CSF est une autre cytokine importante qui est produite par les cellules épithéliales des voies respiratoires à l'exposition aux allergènes protéolytiques. D'autres sensibilisants connus pour leur capacité d'induire des réponses Th2, telles que les particules de diésel de gaz d'échappement, les particules de l'air ambiant et la fumée de cigarette peuvent également induire la libération de GM-CSF par les cellules épithéliales (Bleck et al., 2006). GM-CSF stimule la prolifération et la différenciation des précurseurs des neutrophiles, des éosinophiles et des monocytes. Il active également fonctionnellement des formes matures correspondantes et peut induire la libération de métabolites de l'acide arachidonique et la production accrue de produits intermédiaires réactifs de l'oxygène (ROI) (Holgate and Polosa, 2008).

IL-25 (IL-17E) est produite par des cellules épithéliales ainsi que d'autres cellules innées, comme les éosinophiles, les basophiles et les lymphocytes Th2. Dans un modèle d'asthme expérimental, IL-25 ARNm est exprimé dans les poumons et le blocage de l'IL-25 par le récepteur soluble réduit le recrutement des éosinophiles et des cellules Th2 dans les voies respiratoires. En revanche, la surexpression de l'IL-25 dans le poumon ou l'administration systémique de l'IL-25 aux souris, induit une hyperéosinophilie sanguine et une augmentation des taux sériques d'IgE et augmente la production de cytokines Th2 et le recrutement des éosinophiles dans les voies aériennes (Oppmann et al., 2000). Le récepteur de l'IL-25, IL-17RB, est fortement exprimé sur les lymphocytes iNKT CD4 (+) naïfs et activés. Des cellules iNKT IL-17RB (+) produisent de grandes quantités de cytokines Th2, substantiellement augmentées par la stimulation IL-25 (Stock et al., 2009).

Des travaux expérimentaux ont montré que dans cette phase d'induction, les mastocytes résidant dans les voies respiratoires sont activés sous l'effet de TLSP libéré essentiellement par les cellules épithéliales. Pourtant, sous cet effet, les mastocytes produisent et libèrent des cytokines (IL-5, IL-6, IL-13) mais pas de médiateurs préformés contenus dans leurs granules (Allakhverdi et al., 2007a). Aussi dans cette phase initiale, plusieurs

chimiokines comme IL-8, TARC, MDC, éotaxine, GM-CSF et aussi IL-5, IL-3 sont libérées, et par conséquent, plusieurs types cellulaires (cellules T, éosinophiles, basophiles essentiellement) seront produits et recrutés dans la muqueuse bronchique. Le rôle dans le développement de l'asthme de l'IL-33, autre cytokine produite aussi par les cellules épithéliales sera présenté en détail dans la partie suivante.

2. Phase de réaction asthmatique

a. Acteurs cellulaires

Cellules épithéliales des voies respiratoires

Si les cellules épithéliales des voies aériennes sont connues pour jouer un rôle important dans l'immunité innée comme barrière physique, elles sont maintenant reconnues comme des cellules fondamentales dans le développement des réponses allergiques. Par leur aptitude à produire des cytokines (IL-25, IL-33, TSLP surtout), elles sont activement impliquées dans le développement des manifestations d'asthme d'une manière directe, indépendamment des cellules Th2 (Barrett and Austen, 2009). Les preuves s'accumulent suggérant que l'épithélium des voies respiratoires des sujets asthmatiques présente des anomalies phénotypiques et fonctionnelles comparé à celui des sujets non asthmatiques.

Conceptuellement, l'épithélium bronchique est dans une position unique pour traduire les interactions gènes-environnement. Dans des circonstances normales, l'épithélium forme une barrière très stricte presque imperméable et présente une grande capacité de réparation en cas de blessure. Il est maintenant clair que dans l'asthme, l'épithélium bronchique est plus fragile, avec une perte facile de cellules cylindriques en raison des perturbations à la fois des jonctions serrées et des desmosomes (Barbato et al., 2006). La perméabilité de l'épithélium asthmatique est augmentée, conduisant à un meilleur accès des allergènes inhalés, des polluants, et d'autres irritants pour les

cellules basales et les tissus des voies respiratoires sous-jacentes. Cette perte de fonction de barrière peut refléter une anomalie génétique. L'association du polymorphisme du gène profilaggrine sur le segment chromosomique 1q13 et l'asthme a été décrite (Ying et al., 2006a). Filaggrin et le S100 protéine sont impliquées dans le maintien de l'intégrité épithéliale des voies respiratoires.

L'atteinte de l'épithélium bronchique est caractéristique des patients asthmatiques. Dans l'asthme, non seulement les épithéliales sont plus sensibles aux dégâts, mais les processus de réparation sont également compromis (Knight and Holgate, 2003; Holgate, 2008). L'épithélium des voies respiratoires dans les biopsies bronchiques chez des patients souffrant d'asthme exprime moins le marqueur de prolifération PCNA (proliferating cell nuclear antigen), ce qui traduit une expression accrue de p21waf, un inhibiteur de kinase qui régule négativement la croissance cellulaire (Puddicombe et al., 2003). Pourtant, une autre étude a rapporté une potentialité proliférative des cellules épithéliales plus importante, associée à forte expression de l'ARNm des PCNA (Kicic et al., 2006).

Une fois les cellules épithéliales endommagées, le processus de réparation se déclenche. Toutefois, la réparation entraîne la production d'une variété de cytokines et de facteurs de croissance dont les effets cumulatifs constituent les changements structuraux caractéristiques de l'asthme, surtout dans les formes plus chroniques et sévères.

La prodution de cytokines et chimiokines par les cellules épithéliales représente également un des domaines de recherches actuellement très actifs pour répondre non seulement à la question concernant la pathogénèse de l'asthme, mais aussi à celle du développement des réponses immunitaires proallergiques de type Th2 (Barrett and Austen, 2009). Les cellules épithéliales des asthmatiques présentent un niveau anormal d'expression spontanée de certaines cytokines et chimiokines comme GM-CSF, IL-8, IL-6, prostaglandin 2, ainsi qu'après stimulation par des

cytokines Th2 ou des facteurs environnementaux (Davies et al., 1995; Nakamura et al., 1996; Chung, 2006). Les anomalies des cellules épithéliales pourraient être intrinsèques chez les sujets prédisposés à l'atopie parce que certaines de ces anomalies sont conservées après plusieurs passages successifs en culture.

Sous la stimulation des allergènes et/ou des microbes (virus, bacteries, champignons), des particules stimulantes, les cellules épithéliales libèrent les cytokines comme TLSP, IL-25, GM-CSF qui déclenchent et amplifient les réponses immunitaires caractéristiques (Hammad and Lambrecht, 2008; Barrett and Austen, 2009).

Mastocytes

Les mastocytes ont un rôle clé dans l'asthme. Ils sont concentrés dans les tissus de la muqueuse et sont recrutés à la surface des voies aériennes par le facteur de cellules souches (SCF, ligand de c-kit) libéré par les cellules épithéliales. CXCL8 et CXCL10, produits par les cellules musculaires lisses des voies aériennes, sont importants dans le recrutement des mastocytes en agissant avec leurs récepteurs, et CXCR2/CXCR3, respectivement. Inversement, les mastocytes sécrètent CCL19 qui, par son récepteur CCR7, stimule la migration des cellules lisses bronchiques et contribue à hyperplasie des muscles lisses (Kaur et al., 2006).

L'activation des mastocytes, en particulier par leur récepteur de haute affinité des IgE (FcεRI), conduit à la dégranulation et la libération d'histamine, tryptase et d'autres protéases, l'héparine et certaines cytokines (TNF-α, l'IL-4 et l'IL-5), et induit également la synthèse de médiateurs eicosanoïdes (LTC4, LTD4, LTE4, PG2 et TXA2), la transcription de cytokines et de chimiokines qui seront sécrétées durant une période maximale de 72 heures après l'induction de cette réponse (Okayama et al., 2003). Ces médiateurs sont de puissants agents bronchoconstricteurs et augmentent la perméabilité microvasculaire. Le blocage des IgE à l'aide de l'anticorps monoclonal *Omalizumab* conduit à l'atténuation marquée de la

bronchoconstriction induite par les allergènes (Ong et al., 2005). PGD2 et LTD4 interagissent avec les récepteurs de surface cellulaire sur les éosinophiles, les macrophages, les basophiles, les mastocytes et les cellules Th2. Ces médiateurs sont libérés également par divers déclencheurs environnementaux.

Plusieurs études ont décrit l'implication des mastocytes durant la pathogenèse de l'asthme (Taube et al., 2004), même si d'autres études ont montré que les caractéristiques de l'asthme peuvent se développer en l'absence de mastocytes ou d'IgE (Kraneveld et al., 2005). Dans un modèle murin d'asthme chronique, il a été montré que l'exposition à de multiples antigènes entraînait une activation des mastocytes à la fois via une voie dépendante de FcRγ (via les IgE et/ou les IgG1) et indépendante de FcRγ. Cette activation des mastocytes contribuerait à la majoration d'une hyperréactivité bronchique et à une inflammation chronique, y compris à l'infiltration des éosinophiles et des lymphocytes et une hyperplasie des cellules gobelet (Yu et al., 2006). Les mastocytes sont également une source importante de métalloprotéinases de la matrice 3 et 9 (MMP-3, MMP-9) qui, par leur interaction avec les protéines de la matrice et les protéoglycanes, ont également été incriminées dans le remodelage de la paroi des voies respiratoires (Wenzel et al., 2003). D'autres études ont proposé que le développement de la phase tardive de l'asthme puisse être dépendante du TNF-α produit par les mastocytes (Kim et al., 2007).

Basophiles

Les basophiles sont des granulocytes qui proviennent des progéniteurs CD34+. Ils complètent leur maturation dans la moelle osseuse puis en sortent sous leur forme mature. Si les mastocytes résident dans les tissus, les basophiles y sont très peu présents. Les basophiles ont une demi-vie courte de quelques jours environ. Bien que les basophiles aient été souvent décrits comme des cellules inflammatoires circulantes qui apparaissaient en réponse à une

stimulation par les IgE, il a été montré que ces cellules peuvent aussi s'accumuler durant d'autres types de réponses immunitaires. Compte tenu du faible pourcentage de basophiles circulant dans le sang périphérique, il est possible que le rôle de ces cellules soit sous-estimé dans les conditions allergiques. Les basophiles ont été détectés dans des biopsies bronchiques de patients asthmatiques (Nouri-Aria et al., 2001; Kepley et al., 2001). Il est admis que les médiateurs libérés par ces cellules peuvent avoir des conséquences importantes voire dramatiques pour les patients puisque une libération accrue d'histamine pourrait induire un choc anaphylactique (Falcone et al., 2006). Il semble que les basophiles soient les principales cellules qui produisent l'IL-4 et IL-13 après stimulation allergénique et favorisent ainsi le développement de la réponse Th2 *(Fig.5)*. En outre, il a été démontré que, par liaison de leurs récepteurs FcεRI avec les IgE, les basophiles sécrètent de l'IL-25 qui a des effets sur les cellules Th2 mémoires (Wang et al., 2006). Des basophiles activés produisent également le TSLP (Min and Paul, 2008). Les basophiles ont une expression élevée des récepteurs de l'IL-33 T1/ST2 et produisent de l'IL-4, de l'IL-6, de l'IL-13 et de l'histamine en réponse à cette cytokine (Schneider et al., 2009a; Kondo et al., 2008a). Les basophiles peuvent également amplifier les réponses Th2 en produisant de l'histamine qui inhibe la réponse Th1 (Schneider et al., 2004). L'histamine favorise le développement des cellules Th2 (Sirois et al., 2000) et la production d'IL-5, IL-13 et IL-10 par les cellules Th2 (Osna et al., 2001). *In vitro*, les basophiles sont capables d'induire la commutation de l'isotype à IgE sur des cellules B.

Un anticorps spécifique des basophiles, Ba103, a récemment été généré. Une seule injection de cet anticorps est suffisante pour dépléter les basophiles en périphérie pendant 10 jours mais n'a aucun effet sur les mastocytes (Sokol et al., 2008). La déplétion des basophiles peut réduire considérablement la réponse de type Th2 antiparasitaire.

Très récemment, des études distinctes ont démontré que les basophiles expriment les molécules du complexe majeur d'histocompatibilité de type II et peuvent, en l'absence de DC, présenter les antigènes aux cellules T CD4 naïves *in vitro* et *in vivo,* confirmant le rôle majeur des basophiles dans les réponses immunitaires de type Th2 et leur conférant une place majeure en tant que cellules présentatrices de l'antigène (Wynn, 2009).

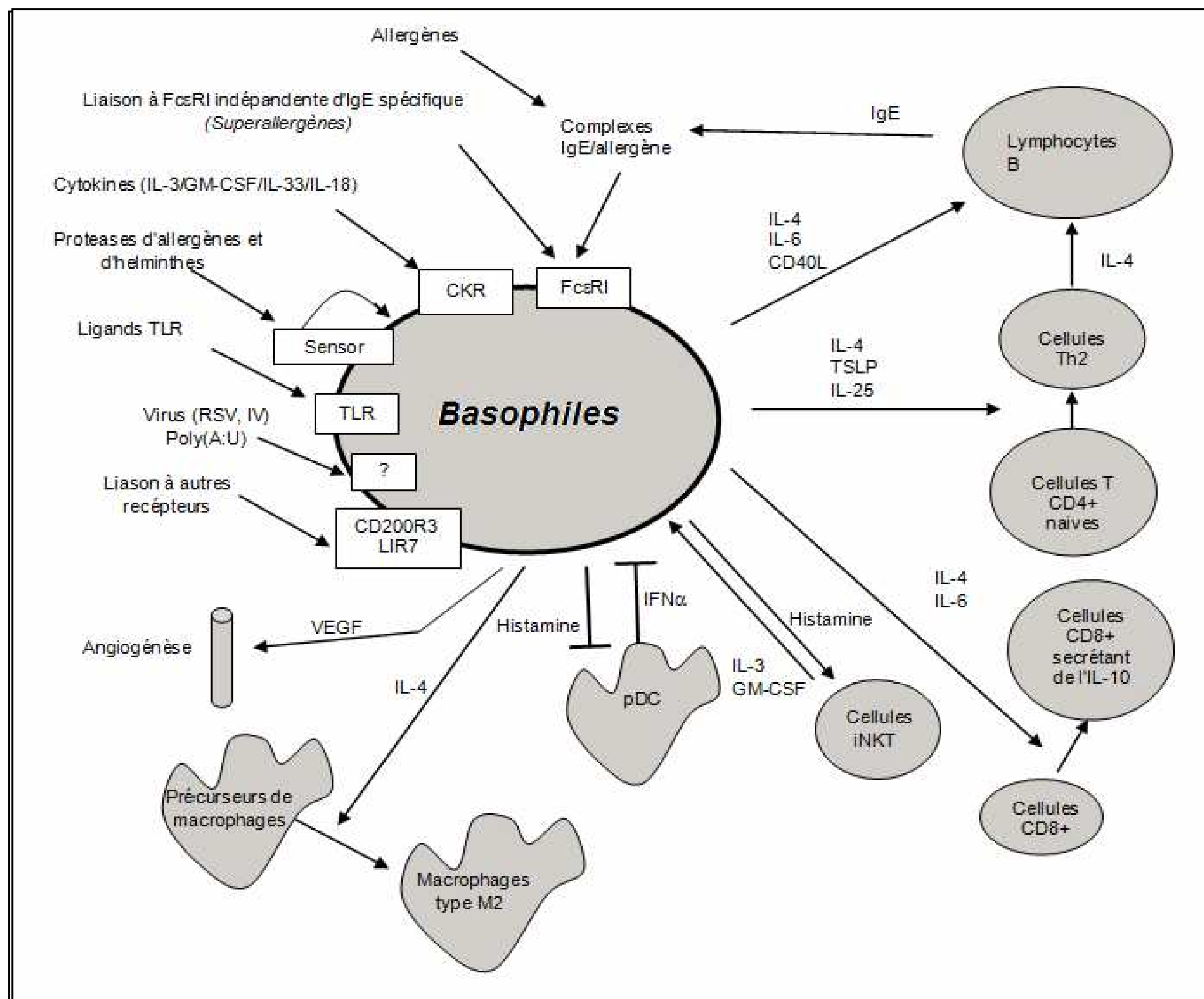

***Figure** 5.* Schéma récapitulatif des activités potentielles des basophiles. Adapté d'après Dy et al. *In press*

<u>*Eosinophiles*</u>

L'inflammation qui se produit dans l'asthme est souvent décrite comme éosinophilique. Les éosinophiles sont présents non seulement dans la paroi des voies respiratoires, mais aussi dans l'asthme mal contrôlé dans les expectorations et le liquide de lavage bronchoalvéolaire

(Holgate and Polosa, 2008). Ces cellules sont recrutées à partir de la moelle osseuse en tant que précurseurs sous les effets de facteurs incluant PGD2, leucotriènes (LT), cytokines et chimiokines (IL-3, IL-5, GM-CSF, éotaxine 1, 2 et 3, RANTES) (Holgate and Polosa, 2008; Sehmi et al., 2003). Plusieurs cytokines régulent l'éosinophilie. Dans une première étape, la stimulation de la production et la mobilisation des éosinophiles de la moelle osseuse est principalement contrôlée par l'IL-5, des chimiokines telles que RANTES/CCL5 et l'éotaxine, tandis que l'IL-4 et l'IL-13 interviendraient pour favoriser la transmigration des éosinophiles à partir du réseau vasculaire vers les compartiments tissulaires en augmentant l'expression des molécules d'adhésion de l'endothélium (Woltmann et al., 2000). Le leucotriène B4, les leucotriènes cystéinyl (LTC4, LTD4 et LTE4) et la prostaglandine (PG) D2 interviennent également dans l'accumulation et la migration des éosinophiles (Fujishima et al., 2005). Une fois présents dans les tissus, les éosinophiles contribuent à la manifestation des symptômes par le relargage de leurs granules contenant différents médiateurs proinflammatoires comme MBP *(major basic protein)*, ECP *(eosinophil cationic protein)*, EDN *(eosinophil-derived neurotoxin)* et EP *(eosinophil peroxydase)*. Ils ont également la capacité de générer des médiateurs eicosanoïdes tels que la prostacycline (PGI2) et les leucotriènes. La MBP provoque une dégranulation des mastocytes et la libération d'histamine et de leucotriènes qui, à leur tour, vont causer une bronchoconstriction (Rothenberg, 1998). Elle induit aussi un dysfonctionnement des fonctions vagales au niveau des récepteurs muscariniques (Piliponsky et al., 2001). À l'activation, les éosinophiles libèrent des superoxydes qui potentiellement induisent des lésions tissulaires et également des cytokines et des chimiokines (Maddox and Schwartz, 2002). Il a été récemment démontré que la EDN des éosinophiles modulait les DC pour amplifier la réponse Th2 en stimulant TLR2 (Yang et al., 2008). Par la capacité à générer des TGF-β et induire la prolifération des fibroblastes, la synthèse du collagène, et la

maturation des myofibroblastes, ils contribuent également au remodelage tissulaire bronchique (Williams and Jose, 2000).

Bien que le rôle effecteur des éosinophiles ait été montré dans différents modèles expérimentaux d'asthme, il existe des arguments suggérant un rôle protecteur de ces cellules dans la défense de l'hôte tel qu'il a été montré durant une infection par le RSV (Rosenberg et al., 2007).

Neutrophiles, monocytes et macrophages

Certains asthmatiques ont des neutrophiles dans leurs expectorations. En général, l'asthme associé aux neutrophiles tend à être plus grave, probablement à cause de la destruction tissulaire accrue et du remodelage des voies aériennes (Holgate and Polosa, 2006).

Le nombre des monocytes et des macrophages dans les poumons des patients atteints d'asthme est augmenté. Ils sont dérivés de monocytes circulants qui migrent vers les poumons en réponse à l'action chimiotactique des chimiokines tels que CCL2 (MCP1), agissant sur le CCR2, le CXCL1 (GROα), et sur le CXCR2 (Sandor and Buc, 2005c). Bien que ces cellules constituent une source de leucotriènes, de ROI, et d'une variété d'enzymes lysosomales, leur rôle précis dans des lésions tissulaires et dans la pathogenèse de l'asthme reste à élucider. Dans l'asthme réfractaire à la corticothérapie, les monocytes et les macrophages sont considérés comme ayant un rôle important (Loke et al., 2006).

Lymphocytes T

Les cellules T activées jouent un rôle important dans l'asthme. Pendant une longue période, la réponse des lymphocytes T dans l'asthme a été interprétée dans le cadre du fameux paradigme Th1/Th2 dans lequel le développement des cellules Th2 est considéré comme capital et essentiel dans le développement de l'asthme allergique. La participation des cellules T dans la pathogenèse de l'asthme allergique a été élargie pour inclure la contribution des cellules T régulatrices (Treg), des lymphocytes NKT et de la sous population T nouvellement décrite, Th17.

- *Lymphocytes Th2*

Les cellules Th2 jouent un rôle central dans l'inflammation allergique. Une fois coordonnées et stimulées, elles augmentent l'expression d'un cluster de gènes sur le chromosome 5q31-33 comprenant les gènes codant l'IL-3, IL-4, IL-5, IL-9, IL-13 et GM-CSF. Ces cytokines sont impliquées dans la commutation de classe d'immunoglobulines des cellules B vers la synthèse d'IgE (IL-4 et IL-13), le recrutement des mastocytes (IL-4, IL-9 et IL-13), et la stimulation, maturation des éosinophiles (IL-3, IL-5 et GM-CSF) et des basophiles (IL-3), cellules effectrices essentielles de la réponse allergique (Barnes, 2008a). Les cellules Th2 différenciées sont recrutées dans le site inflammatoire par la production des chimiokines CCL1, CCL2, CCL11 et CCL17 (TARC) (Teran, 2000).

Le facteur de transcription GATA3 est crucial pour la différenciation des cellules Th2, des cellules T naïves et la sécrétion de cytokines de type Th2 *(Fig.6)*. L'expression de GATA3 est contrôlée par STAT6 *(signal transducer and activator of transcription 6)*, qui est induit par la liaison de l'IL-4 à son récepteur (Barnes, 2008b).

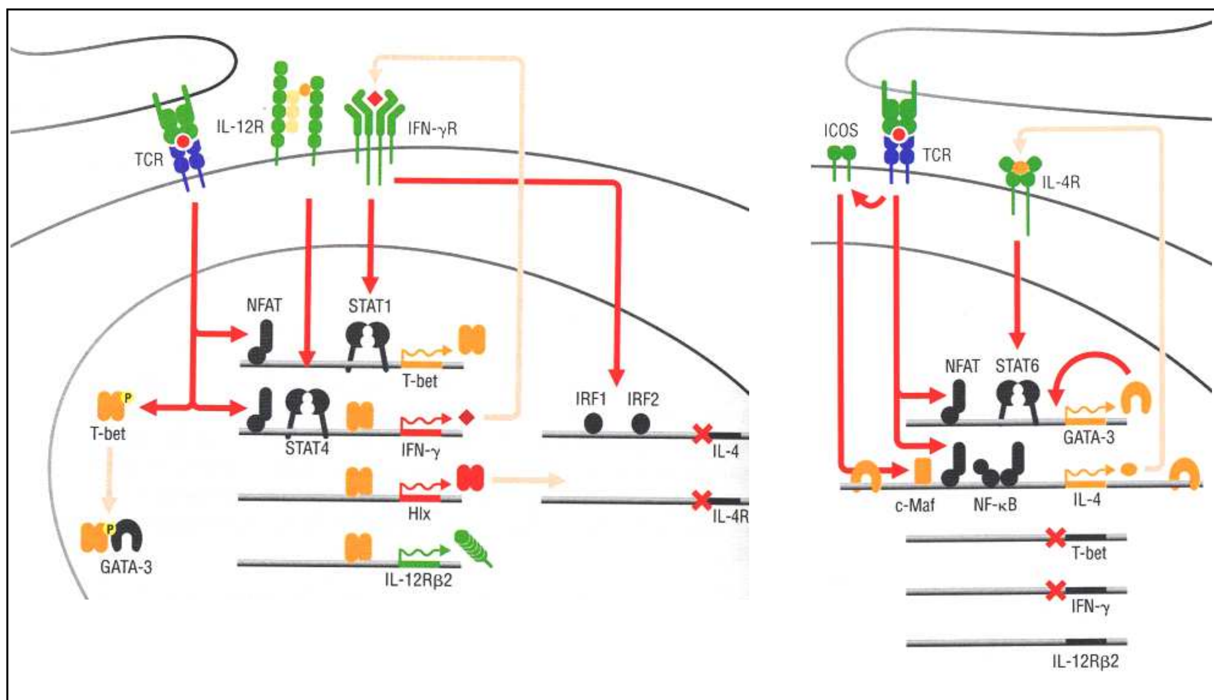

Figure 6. Balance Th1-Th2. *Immunité Ed deboeck 2009*

Le facteur de transcription essentiel pour la différenciation des cellules Th1 est T-bet *(Fig.6)*. L'expression de T-bet est contrôlée par STAT1, induit par la liaison de l'IFN-γ à son récepteur. Lorsqu'il est phosphorylé, T-bet peut s'associer à GATA-3 et inhiber sa fonction. L'IL-27 augmente également l'expression de T-bet et par conséquent diminue la fonction de GATA-3 (Barnes, 2008a).

- *Lymphocytes T régulateurs*

Il existe des arguments attestant l’existence d’une population de cellules T régulatrices (Treg) qui se sont développées durant la maturation des cellules T dans le thymus (appelées cellules T régulatrices naturelles) ou en réponse à une stimulation particulière en périphérie (cellules T régulatrices induites) (Bluestone and Abbas, 2003). Les Treg dites naturelles constituent 5 à 10% des lymphocytes CD4+ de la périphérie. Ces cellules expriment constitutivement et fortement la molécule CD25 (cellules T $CD25^{high}$), la molécule CTLA-4 (cytotoxic T-lymphocyte antigen-4), le GITR (glucocorticoid-induced tumor necrosis factor receptor) et le facteur de transcription Foxp3 (forkhead box protein 3) (Nomura and Sakaguchi, 2007). Contrairement aux autres cellules T CD4+, ces cellules ne prolifèrent pas et ne produisent pas de cytokines lorsqu’elles sont stimulées avec un antigène *in vitro*, mais suppriment la prolifération et la production de cytokines par d'autres cellules T appelées effectrices. Le marqueur CD25 n’est pas spécifique de ces cellules car il est aussi exprimé par les CD4+ activées, tout comme GITR et CTLA-4. Un bon marqueur phénotypique est le facteur de transcription FoxP3, puisque la suppression ou la réduction de FoxP3 abroge leur capacité suppressive et inversement, l’expression ectopique de la molécule FoxP3 aux effecteurs $CD25^{-}$ confère des propriétés suppressives à ces cellules (Fontenot et al., 2003). Des travaux récents ont montré que l’expression de FoxP3 inhibe le développement des cellules effectrices produisant de l’IL-17 (Th17) en empêchant l'expression du facteur de transcription ROR-γt (Zhou et al., 2008). L’expression du facteur FoxP3 est lui-même inhibé

par l'expression du facteur de transcription des Th1 T-bet ou du facteur de transcription des Th2 GATA3 (Wei et al., 2007; Mantel et al., 2007). Les cellules FoxP3+ exercent des fonctions suppressives via la production de cytokines telles que l'IL-10 et le TGF-β (Nomura and Sakaguchi, 2007; Sakaguchi and Powrie, 2007). Il a été montré que ces cellules exercent également des fonctions suppressives via la production d'IL-35 (Collison et al., 2007a). Ces cellules peuvent exercer leur fonctions régulatrices sur différents types cellulaires, directement ou via des effets sur les DC. Les cellules T $CD25^{high}$ peuvent aussi supprimer directement les fonctions effectrices des mastocytes (Gri et al., 2008).

A la différence des effets des lymphocytes T régulateurs naturels non spécifiques de l'allergène, les lymphocytes T régulateurs dits "induits" limiteraient les réponses allergiques par des actions spécifiques de l'allergène. Chez la souris, les cellules T déjà converties à la différenciation d'une lignée effectrice (Th2, Th17) ou au phénotype mémoire, préservent encore leur plasticité et, dans certaines conditions, pourraient se redifférencier pour exprimer FoxP3 et adopter un phénotype régulateur (Kim et al., 2010; Oboki et al., 2008). Une étude récente a proposé une classification du pool des cellules T régulatrices humaines en trois sous populations, $CD45RA^{+}FOXP3^{lo}$ (Treg quiescent), $CD45RA^{-}FOXP3^{hi}$ (Treg activé) et $CD45RA^{-}FOXP3^{lo}$ (Treg induit). Cette dernière population pourrait se redifférencier des cellules T mémoires en périphérie (Miyara et al., 2009). Il a été suggéré que le déclenchement de l'asthme chez l'homme pourrait être lié à un développement insuffisant des lymphocytes T régulateurs ou/et une inhibition de leur fonction (Provoost et al., 2009; Mamessier et al., 2005; Robinson, 2009). Inversement, des travaux récents montrent que l'immunothérapie spécifique utilisant l'allergène impliqué dans l'asthme pourrait induire des cellules T régulatrices produisant de l'IL-10 (Tr1), ce qui conduirait à une atténuation de l'inflammation allergique (Francis et al., 2008; Radulovic et al., 2008; Akdis and Akdis, 2009; Mobs et al., 2010; Larche, 2007; Bohle et al., 2007). Toutefois, d'autres études récentes sur des protocoles

utilisant des adjuvants comme les motifs CpG, ont montré des avantages cliniques indépendants de la production d'IL-10, indiquant que d'autres mécanismes sont mis en jeu dans cette situation (Nagata and Nakagome, 2010). Des recherches supplémentaires portant sur la différenciation dynamique *in vivo* des Treg dans l'interaction avec d'autres populations cellulaires ainsi que sur le rôle de différentes sous population de Tregs en immunothérapie dans le contexte de la maladie asthmatique sont encore nécessaires.

- *Lymphocytes NKT*

Les lymphocytes NKT (Natural Killer T) constituent une sous-population de lymphocytes T qui exprime à la fois des marqueurs de T et de NK. Ces cellules sont soit CD4+, soit CD4-CD8- et une petite population de NKT humains est CD8+. Ils expriment un TCRαβ avec une chaîne α invariante. Chez la souris, la chaine invariante du TCR de ces cellules est de type Vα14Jα18 alors que chez l'homme, ils expriment la chaîne Vα24Jα18 (Park et al., 2000). On suppose que le ligand endogène qui assure la sélection est l'isoglobotrihexosylcéramide (iGb3), un produit de dégradation de lipides de la membrane *(Fig.7)*. Les NKT répondent à des antigènes glycolipidiques présentés par la molécule CMH de classe I non polymorphique CD1d, en produisant rapidement de grandes quantités de cytokines telles l'IL-4, l'IL-13, l'IL-10 et l'IFN-γ (Park et al., 2000).

Récemment, l'équipe de Trottein a démontré que l'activation des DC par les agonistes des TLR7/8 et TLR9 induisait la production d'IFN de type I et de glycosphingolipides, deux phénomènes nécessaires à l'activation des lymphocytes iNKT (Vanhoutte et al., 2008). Première indication que les agonistes des TLR peuvent induire l'expression de(s) ligand(s) endogène(s) des lymphocytes NKT, ce travail permet de mieux appréhender le paradoxe entre le répertoire TCR restreint de ces cellules et leur implication dans des situations infectieuses très variées.

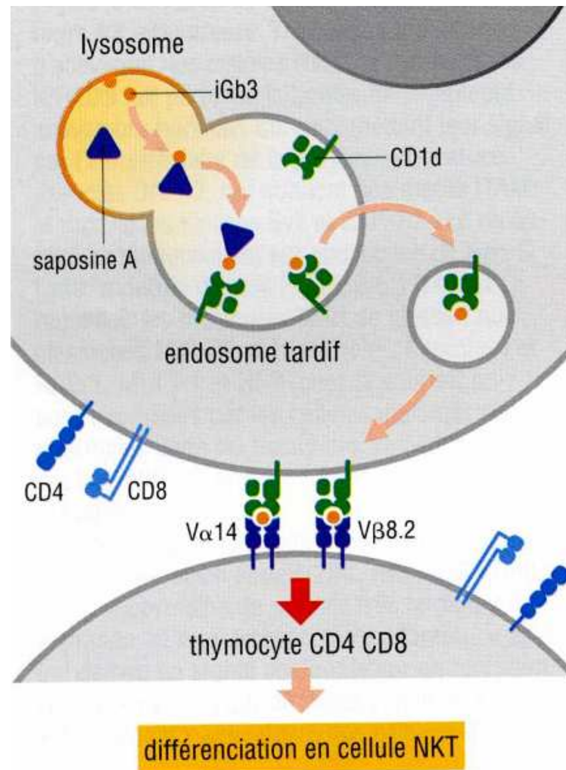

Figure 7. Ligand endogène supposé impliqué dans la sélection thymique des cellules NKT.
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Par la production rapide de cytokines, ces cellules peuvent inhiber ou amplifier les réponses immunes. Des observations expérimentales ont suggéré un rôle délétère des cellules NKT dans le développement de l'asthme. Il a été rapporté que les souris déficientes pour les cellules NKT développent un asthme allergique d'une ampleur très réduite (Akbari et al., 2003; Lisbonne et al., 2003; Bilenki et al., 2004). Ces données ont été confortées par des observations cliniques (Akbari et al., 2006; Hamzaoui et al., 2006; Pham-Thi et al., 2006) ce qui a même conduit certaines équipes à proposer que les cellules NKT pourraient être l'acteur essentiel responsable du développement de l'asthme (Umetsu and DeKruyff, 2010). Pourtant ces données et arguments ne paraissent pas encore suffisants pour établir une conclusion. D'autres études expérimentales ne confirment pas l'observation concernant le rôle indispensable des NKT dans le développement des réponses d'asthme (Apostolou et al., 1999; Brown et al., 1996; Korsgren

et al., 1999). L'étude utilisant les souris déficientes pour la β2-microglobuline, qui n'expriment ni les molécules du CMH I, y compris le CD1d, ni les cellules CD8 et NKT, a montré que l'hyperréactivité bronchique n'est pas affectée chez ces souris (Zhang et al., 1996). En revanche, une fois activées par l'α-GalCer, ces cellules inhibent le déclenchement de l'asthme (Morishima et al., 2005; Matsuda et al., 2005; Hachem et al., 2005) ce qui suggère leur rôle potentiellement régulateur.

Ainsi, le rôle spécifique des lymphocytes NKT dans le développement de l'asthme est complexe. En effet, leur action dans le déclenchement de la réponse asthmatique dépendra de leur statut d'activation mais aussi des collaborations avec d'autres populations T conventionnelles.

- *Lymphocytes Th17*

En 2000, Infante-Duarte et coll. ont montré que les cellules T productrices d'IL-17 constituaient une population distincte des cellules Th1 et des cellules Th2 chez les souris et les humains (Infante-Duarte et al., 2000). Ivanov et al. ont identifié ROR-γt comme un facteur de transcription essentiel pour la différenciation des cellules Th17 (Ivanov et al., 2006). Chez la souris, l'IL-23 est nécessaire à la différenciation induite par l'IL-6 et le TGF-β des cellules Th17 (McGeachy and Cua, 2007) *(Fig.8)*. Chez l'homme, le rôle du TGF-β est remplacé par l'IL-1β. Il a été récemment décrit que l'IL-35, produite par les lymphocytes T régulateurs FoxP3+, supprimait la réponse des cellules Th17, (Niedbala et al., 2007; Collison et al., 2007b). Très rapidement après leur description, il a été démontré par l'étude des souris déficientes pour le gène codant l'IL-17R que les cellules Th17 étaient indispensables à la phase d'induction (sensibilisation) du développement des réponses asthmatiques expérimentales (Schnyder-Candrian et al., 2006).

Figure 8. Les cytokines impliquées dans le développement des cellules Th17.
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L'augmentation des ARNm de l'IL-17 et la sécrétion de cette protéine dans les poumons, le lavage broncho-alvéolaire (LBA) et le sérum des patients asthmatiques ont été montrées (Wong et al., 2001; Molet et al., 2001). De plus, le niveau d'IL-17 est étroitement corrélé au degré de sévérité de l'hypersensibilité bronchique chez les patients asthmatiques (Barczyk et al., 2003). L'IL-17 potentialise l'activation des fibroblastes, des cellules épithéliales et des cellules musculaires lisses. Ainsi, l'IL-17 favorise la production d'IL-6, d'IL-8, d'IL-11 et de CXCL1/Groα par les fibroblastes bronchiques humains (Molet et al., 2001). La combinaison d'IL-17F et d'IL-23 peut stimuler la production des cytokines inflammatoires telles que l'IL-1β et le TNF-α par les éosinophiles (Cheung et al., 2008).

- *Lymphocytes B*

La production des anticorps IgE et le déclenchement des réponses allergiques

Les cellules B jouent un rôle important dans les maladies allergiques, y compris l'asthme allergique, pour la synthèse des IgE spécifiques aux allergènes sous l'influence des réponses dérégulées à cellules Th2. Chez la souris, les cytokines de type Th2, l'IL-4 ou l'IL-13, stimulent la commutation isotypique vers l'IgG1 et vers l'IgE. Chez l'homme, l'IL-4, induit la commutation vers l'IgE et vers un isotype d'IgG (IgG4) qui interagit d'une manière peu

efficace avec les récepteurs Fcγ et active faiblement le complément *(table 12)*. Le contact avec l'allergène dans la phase de sensibilisation induit la synthèse par les cellules B des anticorps IgE spécifiques de l'allergène. Cette phase met de deux à trois semaines pour se développer pleinement Ces anticorps se fixent rapidement à leurs récepteurs de haute affinité (FcεRI) présents essentiellement sur les mastocytes et les basophiles (DeFranco, 2009).

Table 12: Sélection des isotypes d'anticorps chez la souris

Cytokine	Isotype sélectionnés
IL-4	IgG1, IgE
IFN-γ	Ig2a
TGF-β	IgA, IgG2b

Lors d'un contact ultérieur avec l'allergène inhalé, la réponse est déclenchée par le pontage des molécules d'IgE liées aux FcεRI, ce qui entraine la dégranulation immédiate des mastocytes et des basophiles avec libération des médiateurs dont l'histamine et de protéases suivie par la synthèse et la libération des médiateurs lipidiques et des cytokines inflammatoires peu de temps après *(Fig9)*. La réponse inflammatoire précoce provoque le recrutement de nombreuses populations cellulaires, y compris les basophiles, les éosinophiles et les cellules Th2 aux sites inflammatoires.

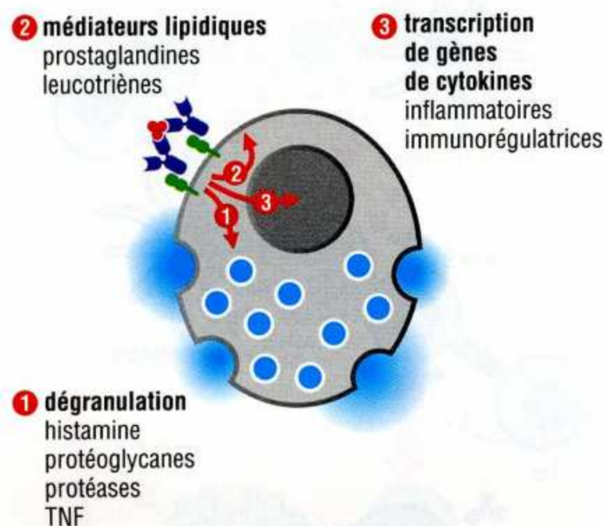

Figure 9: L'activation des mastocytes déclenche une réaction allergique
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Régulation de la synthèse d'IgE

Le FcεRII (CD23) est une protéine membranaire de type II appartenant à la famille des lectines de molécules d'adhérence. FcεRII existe sous deux formes, CD23a et CD23b. Les deux isoformes de CD23 se trouvent sur les cellules B. Le CD23a est exprimé de façon constitutive, alors que CD23b est induit par des facteurs tels que l'IL-4 et la combinaison de CD40L avec l'IL-4. Structurellement, CD23 présente un seul domaine transmembranaire suivi d'un domaine extracellulaire. La liaison de CD23 à l'IgE fournit un signal régulateur négatif à la production d'IgE qui en fait arrêter la synthèse (Lamers and Yu, 1995). Le domaine extracellulaire de CD23 est sensible à la protéolyse, conduisant à la libération de fragments solubles. ADAM10 est la principale protéase endogène qui libère le CD23 soluble (Weskamp et al., 2006). CD23 se lie aux IgE par l'intermédiaire d'un deuxième ligand, CD21 (Aubry et al. 1994) et CD23 soluble est considéré comme un facteur de régulation positive de la synthèse des IgE par la co-liaison aux IgE membranaires et à CD21. Il a été montré que Der p 1, l'antigène majeur des acariens clive de manière sélective CD23 et favorise ainsi la synthèse d'IgE (Schulz et al., 1998). CD23 soluble se lie également à CD11b/CD18 (Mac-1) et à CD11c/CD18 (CR4), ce qui favorise la libération de médiateurs pro-inflammatoires comme l'IL-1β, IL-6 et TNF-α. Les complexes IgE-allergènes liés à CD23 exprimé par les cellules B activées facilitent la présentation des antigènes aux cellules T (Gould and Sutton, 2008). L'interaction entre CD23 et HLA-DR dans la membrane cellulaire B est impliquée dans le transport des complexes IgE-allergène-CD23 aux endosomes, où les peptides dérivés d'allergènes seront chargés dans les rainures des molécules CMH (Karagiannis et al., 2001). Par ce procédé les cellules B exprimant CD23 sont alors en mesure de présenter une variété de peptides dont les structures sont parfois lointaines de celles correspondant aux molécules des cellules B.

Comme la synthèse des IgE est dépendante des cytokines libérées par les cellules Th2, elle est régulée par les facteurs qui influencent le développement et les activités de ces dernières. C'est le cas par exemple des cellules Th1, Th17, des lymphocytes NKT, et des T régulateurs. Les cellules épithéliales, les cellules dendritiques, les basophiles participent également à la régulation de la production des IgE par les cellules B.

b. Médiateurs de l'inflammation asthmatique

De nombreux médiateurs sont impliqués dans la physiopathologie de l'asthme. Ils sont sécrétés généralement par différentes populations cellulaires, et inversement chaque population cellulaire sécrète plusieurs médiateurs. Ces molécules ont des effets multiples et souvent redondants sur les voies aériennes et sur les cellules présentes localement pour induire des modifications pathologiques caractéristiques de la maladie: oedème, hypersécrétion de mucus, recrutement des cellules immunitaires, lésions tissulaires, hyperréactivité bronchique. La multiplicité des acteurs rend très difficile l'établissement du rôle de chacun de ces médiateurs dans la physiopathologie de l'asthme.

Histamine

L'histamine est sécrétée essentiellement par les basophiles et les mastocytes. Elle a une propriété bronchoconstrictrice puissante par son action directe sur les récepteurs H1 exprimés sur les cellules musculaires lisses. Elle entraîne aussi une extravasation vasculaire également dépendante des récepteurs H1, et par conséquent, un oedème muqueux qui participe à l'obstruction bronchique. Des recherches récentes ont identifié un rôle déterminant du récepteur H4 exprimé par les cellules immunitaires et du transporteur Oct3 dans la régulation de l'histamine (Schneider et al., 2005). L'importance de ces deux dernières molécules dans l'asthme n'est pas encore clairement établie.

Chimiokines

Le recrutement de cellules inflammatoires dans les voies respiratoires par des chimiokines est un processus essentiel au développement de l'asthme. Les chimiokines agissent par l'intermédiaire de leurs récepteurs spécifiques et sont fréquemment ciblées en recherche thérapeutique *(Fig10).*

L'expression de CCR3 par les mastocytes, les basophiles les éosinophiles et les cellules Th2 et la production de ses ligands, CCL11 (éotaxine), CCL24 (éotaxine-2), et CCL26 (éotaxine-3), sont augmentées dans l'asthme et sont impliquées dans le recrutement des éosinophiles (Medina-Tato et al., 2006).

CCR4 est exprimé essentiellement sur des cellules Th2, mais aussi sur une sous population des cellules Th17, des cellules NKT, des basophiles *(Fig.9).* Le nombre de cellules Th2 exprimant CCR4 ainsi que l'expression de ses deux principaux ligands CCL17 (TARC) et CCL22 (MDC) dans les voies aériennes asthmatiques sont augmentés après stimulation allergénique (Kallinich et al., 2005; Panina-Bordignon et al., 2001; Thomas et al., 2003). Cependant, les souris déficientes en CCR4 ne présentent pas de changement dans le recrutement des cellules dans les poumons ou dans l'induction de l'hyperréactivité bronchique (Conroy et al., 2003). Pourtant, des anticorps dirigés contre CCL17 et CCL22 ont été efficaces pour diminuer l'inflammation dans des modèles animaux d'asthme (Medina-Tato et al., 2006).

CCR5 fonctionne comme un récepteur de chimiokine exprimé principalement sur les lymphocytes Th1, les macrophages, les cellules dendritiques mais aussi les cellules NKT. Les chimiokines qui sont des ligands naturels de ce récepteur sont MIP-1α, MIP-1β et RANTES (également connu sous le nom CCL3, CCL4, CCL5 respectivement). Dans un modèle animal d'asthme, RANTES/CCL5 diminue l'inflammation des voies respiratoires induite par les allergènes (Thomas et al., 2003; Chvatchko et al., 2003).

CCR8 est exprimé sur les cellules Th2. Les souris déficientes en CCR8 ont une forte réduction de l'infiltration des éosinophiles et une diminution de l'hyperréactivité bronchique induite par les allergènes. Des antagonistes de CCR8 et de son ligand CCL1 ont été récemment développés (Ghosh et al., 2006).

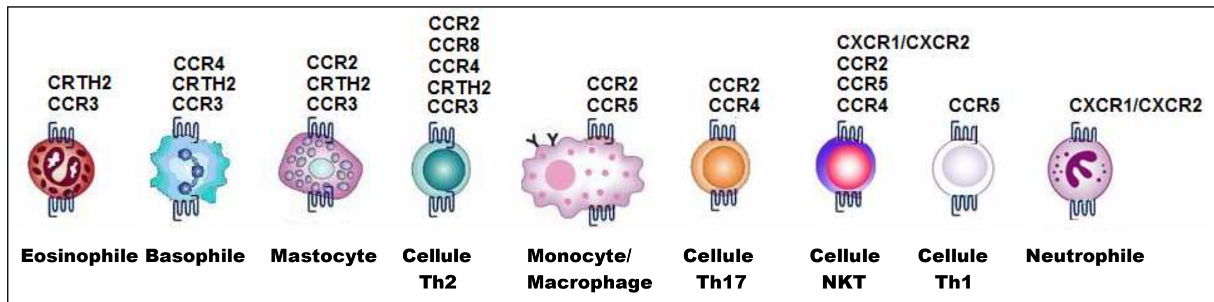

Figure 10. L'expression de CCR3, CRTH2, CCR4, CCR5, CCR8, CCR2 et CXCR1/CXCR2 sur les principaux acteurs cellulaires impliqués dans l'inflammation asthmatique

La molécule chémoattractante CRTH2 (chemoattractant receptor-homologous molecule expressed on TH2) est exprimée sur les cellules Th2, mais aussi sur les mastocytes, les basophiles, les éosinophiles, représente un récepteur de haute affinité pour la PGD2, et sert ainsi de médiateur pour la migration des cellules Th2, des mastocytes, des basophiles, des éosinophiles vers les sites inflammatoires. De nombreux antagonistes sélectifs CRTH2 ont été développés et les résultats des essais cliniques sont attendus avec intérêt (Ly and Bacon, 2005; Pettipher et al., 2007).

CXCL8 (IL-8) est une chimiokine importante pour la chimiotaxie et l'activation des neutrophiles, et des macrophages. Cette chimiokine agit sur ses deux principaux récepteurs (CXCR1 et CXCR2) dont elle induit fortement l'expression, essentiellement sur les neutrophiles mais aussi, à un niveau plus faible sur les cellules NKT (Thomas et al., 2003). L'expression de CXCR1/CXCR2 et de CXCL8 est augmentée dans les biopsies prélevées chez des patients souffrant d’asthme sévère. Des antagonistes de CXCR1/CXCR2 ont été rapportés efficaces dans le traitement des exacerbations graves ou chez les patients atteints d'asthme sévère (Qiu et al., 2007).

CCR2, qui est exprimé sur les monocytes et les lymphocytes Th2, les mastocytes, une sous-population de lymphocytes Th17, et à un faible niveau sur les cellules NKT, est aussi une cible pour la thérapeutique dans l'asthme (Barnes, 2004).

Leucotriènes

Ces médiateurs sont impliqués dans la maladie asthmatique. Ils sont synthétisés par de nombreux types cellulaires. L'enzyme nécessaire à leur synthèse est la 5-lipooxygénase dont l'activation induit la synthèse de LTB4, puissant agent chimioattractant pour les neutrophiles. Les LTC4, LTD4 et LTE4 qui ont tous un effet bronchoconstricteur puissant et induisent une hyperréactivité bronchique, augmentent la perméabilité vasculaire et stimulent la sécrétion de mucus. L'efficacité d'antagonistes spécifiques du LTD4 ou d'inhibiteurs de synthèse des leucotriènes a permis de démontrer que les LT participent à la bronchoconstriction induite par un effort ou l'exposition à un allergène chez les asthmatiques. Ils sont également efficaces dans les tests de provocation à l'aspirine, ce qui indique que le LTD4 est un agent bronchoconstricteur important au cours de l'asthme à l'aspirine. Actuellement, les leukotriènes constituent le seul médiateur dont l'inhibition ait un effet thérapeutique (Adcock et al., 2008; Leff, 2001; Dahlen, 2006).

Monoxyde d'azote

Le monoxyde d'azote (NO) est produit de façon constitutive dans les voies aériennes. Le NO endogène est le médiateur qui agit comme un frein sur la bronchoconstriction dépendant du système cholinergique (Ward et al., 1993). Le NO régule également les mouvements ciliaires et la perméabilité vasculaire (Barnes and Kharitonov, 1996). L'augmentation de la production de NO chez les asthmatiques a été décrite comme étant associée à une hyperexpression de la NO synthase inductible par les cellules épithéliales des voies aériennes (Kharitonov et al., 1994).

Cependant, l'inhalation brève de NO n'a qu'un effet bronchodilatateur très modéré chez l'asthmatique et l'inhalation d'inhibiteurs de la NO synthase n'a pas d'effet sur la bronchocontriction chez les patients asthmatiques (Yates et al., 1995). Le NO exhalé pourrait être considéré comme un index de l'inflammation des voies aériennes (Singh and Evans, 1997).

Dans la pathogénèse de l'asthme, le NO est associé à l'éosinophilie, et à l'inflammation des voies aériennes (Bochner and Busse, 2005a; Benbernou et al., 1997a). Le NO favorise la différenciation des cellules Th2 et augmente la production d'IL4 tout en inhibant la prolifération et la production d'IL2 et d'IFN-γ par les lymphocytes Th1 (Taylor-Robinson et al., 1994; Chang et al., 1997). Le NO pourrait participer à la réponse inflammatoire en se combinant à des anions superoxydes pour aboutir à la formation d'ions peroxynitrites qui peuvent avoir un effet toxique direct sur les voies aériennes (Barnes and Kharitonov, 1996).

Cytokines

Les cytokines impliquées dans les réponses inflammatoires asthmatiques ont été décrites avec les cellules qui les produisent. Une attention particulière a été donnée dans nos travaux au rôle de l'IL-33 que nous traiterons ici plus en détail.

L'IL-33 est un membre de la famille de l'IL-1 récemment identifié comme le ligand naturel de ST2, son récepteur (IL-1RL1, T1, fit-1, IL-33Rα) *(Fig.11)*. L'IL-33 est synthétisée par les cellules épithéliales, par les cellules endothéliales, les cellules musculaires lisses et les adipocytes sous forme d'un précurseur de 30 kDa (pro-IL-33). (Moussion et al., 2008; Paul and Zhu, 2010). Il a été récemment rapporté que la cytokine est clivée *in vivo* par la calpaïne pour libérer sa forme active de 18 kDa (Hayakawa et al., 2009).

Son récepteur, qui existe sous forme transmembranaire (ST2) et sous forme soluble (leurre), est exprimé principalement sur les mastocytes matures, sur les basophiles, les cellules T $CD4^+$ Th2, les cellules NK et NKT chez la souris et chez l'homme (Smithgall et al., 2008; Kondo et al., 2008b; Lambrecht et al., 2000). L'administration d'IL-33 aux souris induit la production d'IgE, d'IL-5 et d'IL-13 et s'accompagne de changements histopathologiques dans les poumons et dans le tractus gastro-intestinal. In vitro, l'IL-33 polarise les lymphocytes T $CD4^+$ en une population de cellules T qui produisent de l'IL-5 et de l'IL-13 en présence de l'antigène et d'APC. Cette polarisation nécessite la molécule ST2 et MyD88, et est indépendante de l'IL-4 et de STAT6. Cette différenciation est également dépendante de la phosphorylation de la protéine kinase activée par les mitogènes (MAPK) et NF-κB, mais pas de l'induction de GATA3 ou de T-bet (Schmitz et al., 2005; Kurowska-Stolarska et al., 2008).

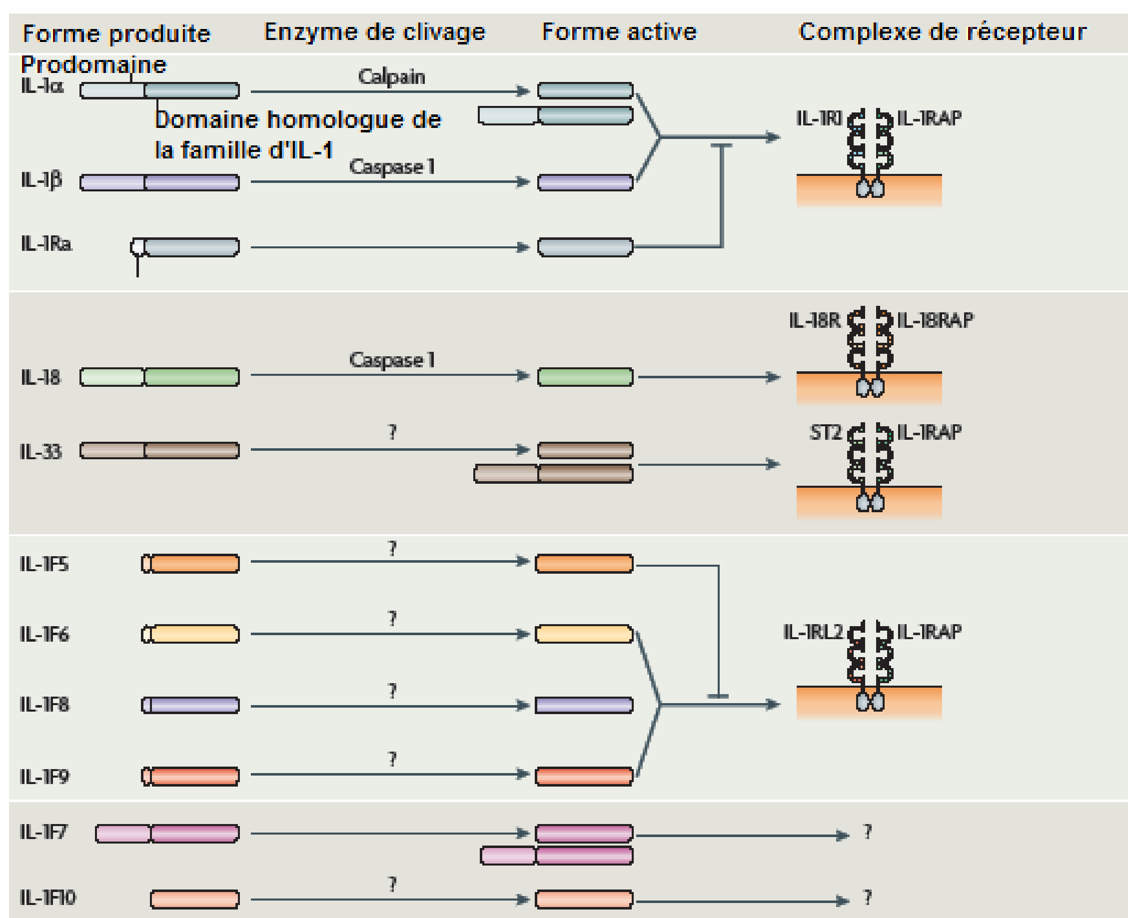

Figure 11. Représentation schématique des membres de la famille de IL-1 et leurs récepteurs
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Bien que l'expression de ST2 soit fortement augmentée sur les cellules Th2, l'absence d'IL-33 n'affecte pas la différenciation des cellules Th2 ni leurs fonctions effectrices (Hoshino et al., 1999a). Ces arguments suggèrent que l'IL-33 pourrait induire une population de cellules T distincte des cellules Th2, et favorisant la production d'IL-5 et d'IL-13 indépendamment de l'IL-4. Par ailleurs, un traitement par l'IL-33 des souris RAG-$2^{-/-}$, déficientes en cellules T et B, induit encore une hyperréactivité bronchique et une inflammation des poumons identiques à celles des réponses asthmatiques développées sur des souris contrôles de type sauvage. Cette évidence suggère d'une part, que la principale cible de l'IL-33 est différente du lymphocyte Th2 et d'autre part, que l'IL-33 favorise la réponse de cellules innées résidant dans les voies respiratoires durant les réponses inflammatoires asthmatiques.

Les cellules qui sont ciblées par l'IL-33 et participent aux réponses de type Th2 ont été identifiées comme les mastocytes, les basophiles, les cellules NK et NKT et les cellules dendritiques (Smithgall et al., 2008; Kondo et al., 2008b; Lambrecht et al., 2000; Rank et al., 2009; Schneider et al., 2009b). L'étude des basophiles fraichement isolés de la moelle osseuse qui expriment ST2 constitutivement a montré que ces cellules répondent à la stimulation par l'IL-33 en produisant une quantité substantielle d'histamine, d'IL-6 et d'IL-4 dans des proportions qui diffèrent du profil des cytokines produites en réponse à l'IL-3 (Kondo et al., 2008b; Schneider et al., 2009b).

II. INFECTION ET ASTHME

A. DETECTION DES INFECTIONS PAR LES RECEPTEURS DE L'IMMUNITE INNEE

1. Récepteurs de reconnaissance des pathogènes

a. Famille TLR

Les récepteurs Toll-like (TLR), de par leur capacité à détecter des motifs moléculaires conservés spécifiques des pathogènes (PAMP), jouent un rôle essentiel dans la mise en place de la réponse immunitaire chez les vertébrés. Conservés tout au long de l'évolution, les TLR sont exprimés par un grand nombre de types cellulaires, conférant ainsi aux cellules, y compris non immunitaires, la capacité à distinguer le soi du non-soi. Ils sont donc les premiers détecteurs des infections tant bactériennes que virales, fongiques ou parasitaires et peuvent orienter la réponse immune vers un phénotype tant T helper (Th)1 que Th2, Th17 ou T régulateur (Treg). Leur aptitude à activer les cellules présentatrices de l'antigène (CPA), notamment les cellules dendritiques (DC), place ces récepteurs à l'interface entre l'immunité innée et l'immunité adaptative. Décrits initialement pour leur capacité à induire une réponse inflammatoire anti-infectieuse, la voie de signalisation TLR/IL-1R est aujourd'hui connue pour intervenir dans de nombreuses maladies y compris dans l'asthme ouvrant de nouvelles perspectives dans le champ thérapeutique. Notre étude porte essentiellement sur la modulation de l'asthme par la stimulation de la voie de signalisation TLR/IL-1R et notamment sur la place des cellules NKT, des cellules T régulatrices, des cellules Th17 et des basophiles dans cette pathologie.

Découverte et répartition des TLR

Les TLR sont des récepteurs transmembranaires de type I dont la spécificité est déterminée génétiquement. Initialement décrits chez la drosophile, des analogues du récepteur Toll sont aujourd'hui connus chez les plantes et les invertébrés. Hautement conservés chez les mammifères, les TLR ont été décrits chez l'homme et chez la souris, exprimés par un grand

nombre de types cellulaires. Jusqu'à présent, 10 et 12 TLR fonctionnels ont été identifiés chez l'homme et chez la souris, respectivement, avec les TLR1 à TLR9 conservés chez les deux espèces. Le TLR10 de la souris n'est pas fonctionnel à cause d'une insertion des rétrovirus et les TLR11 à TLR13 ont été perdus dans le génome humain. Les études de souris déficientes en TLR ont démontré que chaque TLR a une fonction distincte en termes de motifs moléculaires (PAMPs) qu'ils reconnaissent et de réponses immunitaires induites (Kawai and Akira, 2010).

Malgré quelques différences inter espèces, les cellules humaines et murines présentent des profils d'expression de ces récepteurs semblables. Parmi les TLR, les TLR3, TLR7, TLR8 et le TLR9 représentent les TLR exprimés sur les membranes endosomiques et spécialisés dans la détection des acides nucléiques microbiens tandis que les TLR1, TLR2, TLR4, TLR6 sont présents sur la membrane cytoplasmique et détectent des motifs composants de paroi bactérienne et des proteines de l'enveloppe virale (Tregoning and Schwarze, 2010; DeFranco, 2009).

Les cellules phagocytaires et CPA professionnelles, monocytes/macrophages et DC, expriment la quasi-totalité des TLR quoique sur différentes sous populations. Ainsi, chez la souris, les TLR1 à 6 sont présents esentiellement sur les DC myéloïdes (mDC), alors que l'expression des TLR7 et TLR9 au niveau détectable est restreinte aux DC plasmacytoïdes (pDC). Les monocytes/macrophages, quant à eux, expriment l'ensemble des TLR, à l'exception du TLR3 présent préférentiellement sur un sous type mDC ($CD8_{\alpha}^{+}$) (Muzio et al., 2000). Les cellules B expriment également de nombreux TLR, de même que les cellules T, les cellules natural killer (NK), les lymphocytes T-NK invariants (iNKT), les basophiles, les éosinophiles et les mastocytes (Varadaradjalou et al., 2003).

Outre les cellules immunitaires, les TLR sont présents sur de nombreux autres types cellulaires qui contribuent aux réponses inflammatoires, notamment les cellules épithéliales des surfaces des muqueuses intestinales et respiratoires qui sont à l'interface avec le milieu extérieur (Wang et al., 2002).

Reconnaissance des motifs moléculaires conservés des pathogènes par TLR

Lors des infections bactériennes, le TLR4 sera préférentiellement activé par les bactéries à Gram négatif via le LPS *(Fig12)* alors que le TLR2 associé au TLR1 ou TLR6 répondra aux lipopeptides et à l'acide lipotéichoïque des bactéries à Gram positif (Hoshino et al., 1999b; Ozinsky et al., 2000). Ces deux récepteurs coopèrent également entre eux et avec d'autres TLR pour détecter des pathogènes. Les TLR5 et TLR11 reconnaissent respectivement la flagelline, composant principal des flagelles (Hayashi et al., 2001), et les bactéries uropathogènes (UPEC) (Zhang et al., 2004), alors que TLR9 est stimulé par des motifs CpG hypométhylés de l'ADN bactérien (Hemmi et al., 2000). L'étude de la réponse immune induite par la présence de Mycobacterium tuberculosis a mis en évidence la participation de TLR2, TLR4 et TLR9 (Abel et al., 2002; Quesniaux et al., 2004).

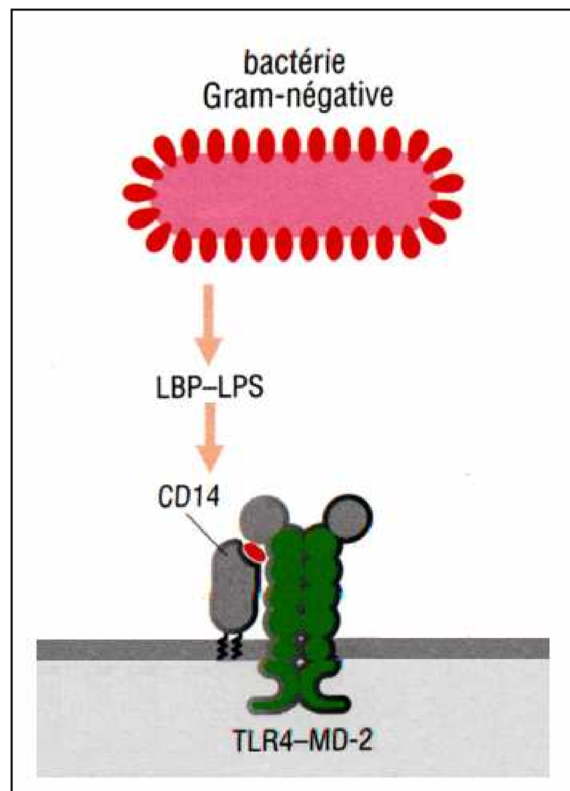

Figure 12. Le TLR4 reconnaît les bactéries à Gram négatif via le LPS

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Les virus stimulent également le système immunitaire via différents TLR: les TLR2, TLR4, TLR6 sont impliqués dans l'interaction des cellules hôtes des protéines de l'enveloppe virale, en particulier pour le cas du virus respiratoire syncytial (Kurt-Jones et al., 2000; Murawski et al., 2009). L'acide ribonucléique double brin (dsRNA) de certains virus ou la forme intermédiaire générée lors des phases de réplication virale active le TLR3 (Wang et al., 2004) alors que les TLR7 et TLR8 reconnaissent l'ARN viral simple brin (ssRNA) (Heil et al., 2004) et le TLR9 reconnaissent l'acide désoxyribonucléique génomique viral (Lund et al., 2003). La question de la discrimination entre acides nucléiques endogènes et exogènes reste donc ouverte mais il est possible d'imaginer que la distinction ne soit pas opérée au niveau des TLR mais en amont, par d'autres molécules qui seraient responsables de l'internalisation et de la dégradation de ces acides nucléiques, les rendant alors accessibles aux TLR qui à leur tour, induiraient une réponse immunitaire, indépendamment de leur nature.

Comme nous nous sommes intéressés au rôle de la stimulation de TLR7 dans l'asthme, nous présenterons ici quelques données de la littérature sur ce récepteur. TLR7 appartient à un groupe des TLR spécialisés dans la reconnaissance des acides nucléiques avec TLR3, 8 et 9. TLR7 n'était précédemment connu que pour la reconnaissance de composés synthétiques. Néanmoins il est désormais associé à la détection des ARNsb viraux et impliqué dans certaines infections virales.

La famille des imidazoquinolines, agonistes synthétiques de TLR7, a démontré leur capacité à induire des cytokines inflammatoires, notamment l'IFN-α. Les composés de cette famille possèdent une structure similaire aux acides nucléiques. Outre ses activités antivirales par l'induction des IFN, ce composé a été testé pour l'activité anticancéreuse étant donné sa

capacité à induire l'apoptose sur plusieurs types de cellules. Par ailleurs, des composés synthétiques non viraux mais analogues de ces ARNsb, comme le Poly(U) peuvent aussi activer les cellules immunitaires via TLR7 (Diebold et al., 2004).

Le resiquimod (4-amino-2-ethoxymethyl-α,α-dimethyl-1H-imidazo[4,5- c]quinolin-1-ethanol, R848,) est un analogue de l'imiquimod. Il induit 50 à 100 fois plus de cytokines essentiellement Th1 que son analogue (Megyeri et al., 1995). Le principal type cellulaire activé par le resiquimod est la cellule dendritique mais il est également capable d'activer d'autres types de cellules telles que les monocytes, les cellules NK (Hart et al., 2005). Il est utilisé comme adjuvant de vaccination pour son effet stimulateur sur la réponse immunitaire (Vasilakos et al., 2000).

Bien que TLR7 et TLR8 soient fortement homologues, il existe une spécificité dans la reconnaissance des ARNsb. Des études chez des souris déficientes pour TLR7 ont montré son rôle dans l'induction d'IFN de type I en réponse au virus A de la grippe et à celui de la stomatite vésiculaire (VSV) (Lund et al., 2003; Diebold et al., 2004). TLR8 reconnaît préférentiellement les ARNsb riches en guanine et uridine dérivés du VIH (Heil et al., 2004).

L'activation de TLR7 recrute la protéine MyD88 qui forme un complexe avec IRAK1, IRAK4 et TRAF6. Cette dernière protéine va conduire à l'activation de NF-κB et d'IRF5, IRF7. La stimulation de TLR7 induit différentes voies de signalisation qui aboutissent à la production de deux grands groupes de cytokines: cytokines inflammatoires et IFN de type I. La reconnaissance des agonistes se fait dans l'endosome, mais la signalisation est cytosolique comme pour tous les autres TLR.

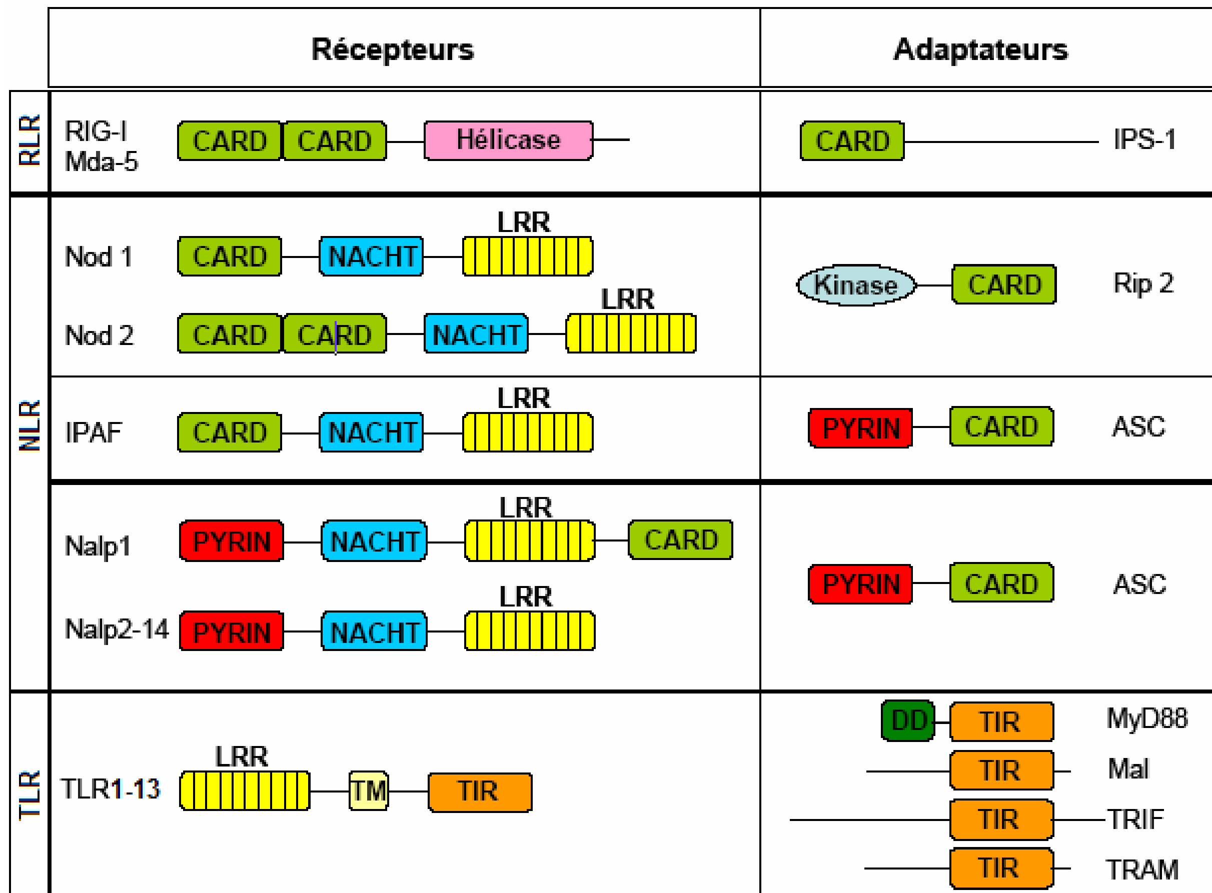

Figure 13. Représentation schématique des récepteurs de l'immunité innée et des protéines adaptatrices comportant des domaines CARD, PYRIN et TIR. Abbréviations : TLR, Toll-like receptor; RLR, RIG-like receptor; NLR, Nod-like receptor; CARD, Caspase recruitment domain ; LRR, Leucine rich repeat; TM, transmembrance domain; TIR, Toll-IL-1 related domain; DD, Death domain; RIG-I, retinoic acid-inducible gene I; Mda-5, melanoma differentiation-associated gene 5; Nod, Nucleotide-binding oligomerization domain-containing protein; Nalp, NACHT, LRR and PYD domain-containing protein; IPS-1, IFN-promotor stimulator-1; Rip-2, receptor interacting protein-2; ASC, apoptosis-associated speck-like; MyD88, myeloid differentiation primary-response gene 88; Mal, MyD88-adaptor-like protein; TRIF, TIR-domain-containing adaptor protein inducing IFN-β; TRAM, TRIF-related adaptor molecule. Adapté d'après Werts *et al. Cell death and differentiation* 2006.

Outre les TLR qui détectent efficacement les composants microbiens à l'extérieur des cellules et à l'intérieur des endosomes, deux autres familles de récepteurs de l'immunité innée ont été identifiés, les NLR (NOD-like receptors) et les RLR (RIG-like receptors) *(Fig.13)*.

b. Famille NLR

Les membres de cette famille de molécules cytosoliques ont été regroupés pour leur structure similaire qui comporte une région NACHT permettant la liaison de nucléotides et l'oligomérisation et un domaine LRR en C-terminal reconnaissant les ligands (Kufer et al., 2005; Ting et al., 2008; Chae et al., 2003). Les ligands de ces protéines intracytosoliques restent largement inconnus. Certaines ont cependant été impliquées dans les réponses inflammatoires, notamment NOD1 et NOD2 reconnaissant des composants du peptidoglycane, l'acide diaminopimélique et muramyldipeptide; Ipaf, la flagelline; NALP3, l'acide muramyldipeptide, l'ARNdb et les ADN viraux et bactériens *(Fig.14)*. NOD1 a un spectre d'expression étendu y compris sur les cellules épithéliales, NOD2 est exprimée notamment par les macrophages et cellules dendritiques mais aussi par les cellules NK et les cellules épithéliales de certains tissus (Athie-Morales et al., 2008; Shigeoka et al., 2010; DeFranco, 2009).

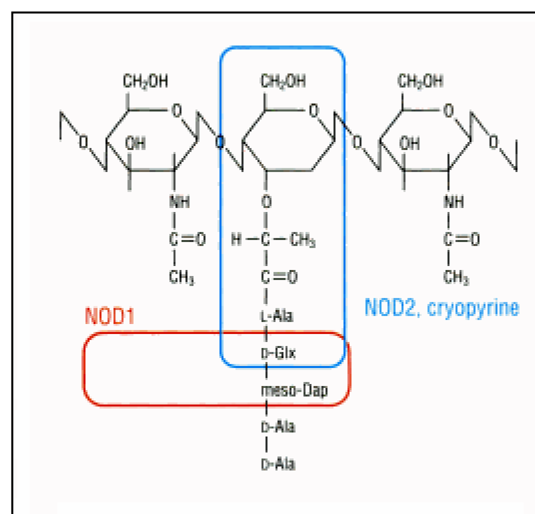

Figure 14. Ligands bactériens des molécules NOD1, NOD2 et NALP3. *Immunité Ed deboeck 2009*

c. Famille RLR

Parallèlement à la découverte de la famille NLR, de nombreux travaux ont mis en évidence l'existence d'une réponse anti-virale indépendante des voies TLR suggèrant l'existence de senseurs cytoplasmiques détectant les virus en cours de réplication, et identifiés

ultérieurement comme appartenant à la famille des RLR, dont font partie les protéines RIG-I et Mda5 (Kang et al., 2002; Diebold et al., 2003; Yoneyama and Fujita, 2004; Yoneyama et al., 2004). Dans les infections respiratoires, RIG-I est activée par les paramyxovirus, les virus de la grippe, le virus respiratoire syncytial, et le métapneumovirus humain (hMPV), tandis que Mda5 est activé par les picornavirus comme les rhinovirus *(Fig.15)*. Les paramyxovirus, les virus de la grippe sont également détectés par la molécule NALP3, de la famille NLR (Tregoning and Schwarze, 2010; DeFranco, 2009).

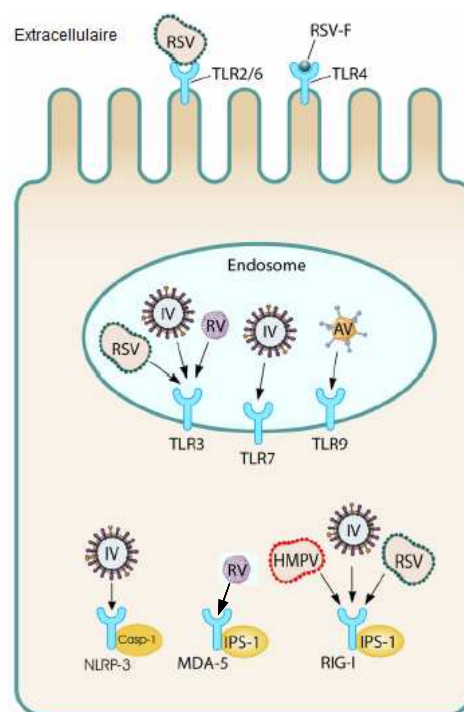

Figure 15. Détecteurs de l'infection virale respiratoire. Adapté d'après Tregoning *et al, 2010.* RSV: virus respiratoire syncytial; IV: virus influenza; RV: rhinovirus; AV: adenovirus; HMPV: métapneumovirus humain

2. Signalisation des TLR

Les voies de signalisation décrites ci-dessous ont été particulièrement bien documentées mais restent assez générales. En effet, elles ne rendent pas compte de la grande diversité des réponses observées *in vitro* et *in vivo* après stimulation des TLR. De nombreuses études se sont attachées à déterminer les spécificités des voies en aval des différents TLR. L'objet de notre étude n'étant pas leur description détaillée, nous ne décrirons pas cette incroyable complexité d'interactions protéiques.

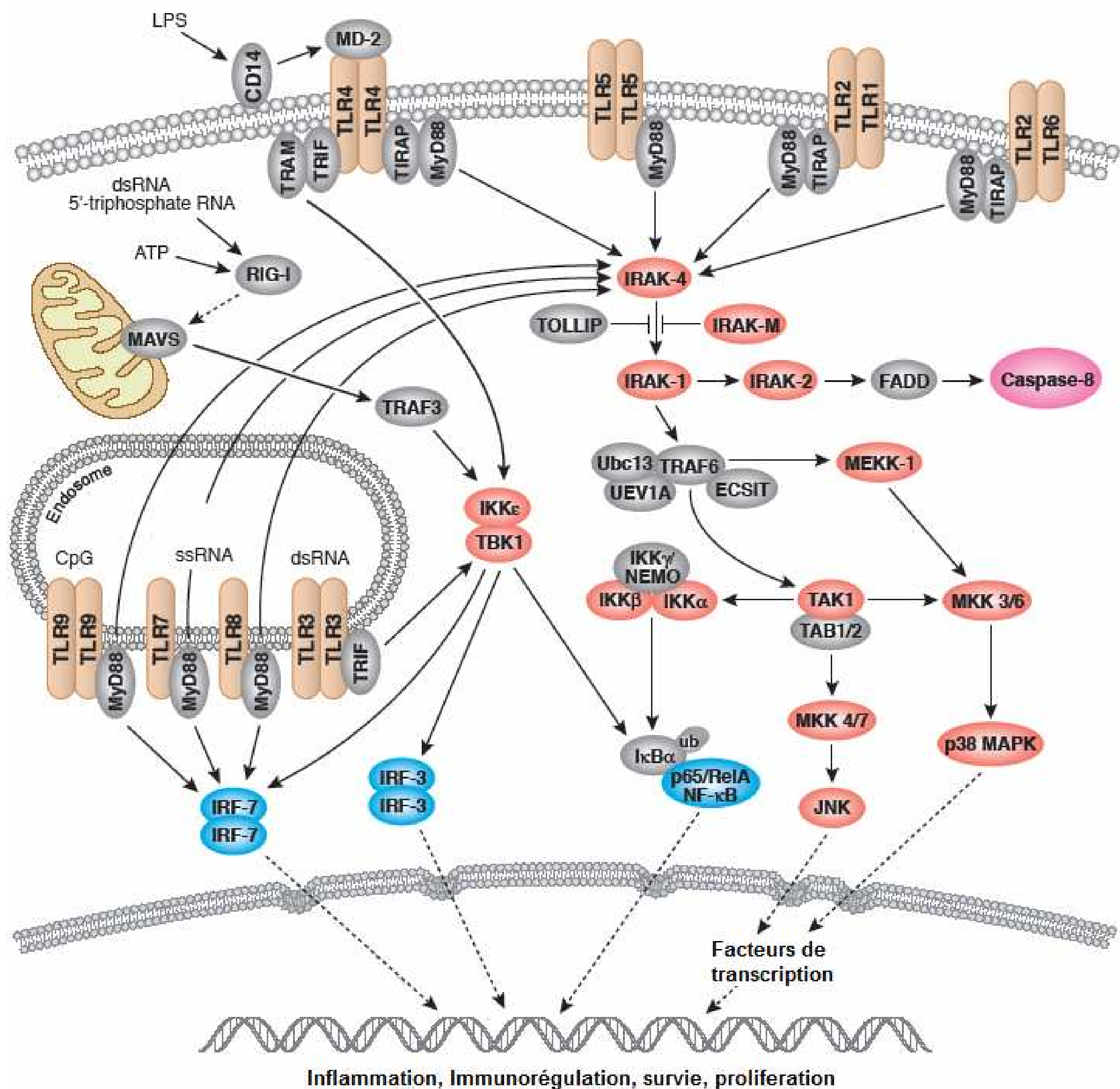

Figure 16. Les deux grandes voies de signalisation décrites sur les CPA **:** La voie dépendante de MyD88 utilisée par tous les TLR à l'exception de TLR3 et la voie dépendante de TRIF utilisée par TLR3 et TLR4. *Cell signaling technology 2010*

a. La voie en aval de MyD88

Cette voie d'activation conduit à la libération de NF-κB qui migre dans le noyau et initie la transcription des gènes codant pour les cytokines proinflammatoires et les IFN de type I (Wang et al., 2001). MyD88 participe également à la liberation du facteur de transcription AP-1 (Wang et al., 2001), à l'activation de différents membres de la famille des IRF, IRF5, IRF7 (Kawai et al., 2004; Kawai and Akira, 2005). En aval de TLR7, 8 et 9,

MyD88 est nécessaire à l'activation d'IRF7. Ces trois récepteurs sont très présents sur les cellules dendritiques plasmacytoïdes. Sur cette population cellulaire, l'activation d'IRF7 induit la forte production d'IFNα (Kawai et al., 2004). Il a été montré également que IRF1 était activable par MyD88 , ce qui permet sa translocation nucléaire et l'induction des gènes de l'IFN-β, de l'IL-12p35 et de la NO synthase inductible (Negishi et al., 2006). L'activation de la voie dépendante de MyD88 induit la transcription de nombreux gènes, et certains d'entre eux ont un rôle crucial dans la modulation de transcription NF-kB-dépendante *(Fig.16)*. Les protéines IκB IκBζ et C/EBPδ agissent en collaboration avec NF-kB pour favoriser la production de l'IL-6 alors que IκB-NS supprime l'induction de l'IL-6, et du TNFα en modulant la liaison de NF-kB p65 à son ADN cible (Kawai and Akira, 2010).

b. La voie en aval de TRIF

Cet adaptateur a été décrit pour l'instant dans les voies de signalisation en aval de TLR3 et de TLR4 et répresente le seul recruté par TLR3. Il est capable d'activer IRF3 via TBK1 et NF-κB et de conduire à la production d'IFN-β et de cytokines proinflammatoires (Yamamoto et al., 2003b; Yamamoto et al., 2003a). Les molécules RIP1 et TRAM sont essentielles pour l'activation de cette voie (Meylan et al., 2004; Fitzgerald et al., 2003). TRAF3 et NAP-1 ont été impliqués dans la régulation de l'activation de TBK1 par TRIF (Hacker et al., 2006; Oganesyan et al., 2006). A la suite de la reconnaissance de l'agoniste, la signalisation est induite, conduisant à l'activation de facteurs de transcription, responsables de l'expression de nombreux gènes *(Fig.16)*.

Un point commun entre tous les TLR est l'induction de cytokines proinflammatoires et de médiateurs immunologiques dépendant notamment de protéines NF-κB, AP1 et des IRF. Ce type de réponse a pour l'objectif d'éliminer des pathogènes.

3. Rôle des récepteurs innés dans l'induction des réponse immunitaires

Ainsi, des récepteurs membranaires (TLR) et cytosoliques (NLR, RLR) coopèrent pour mettre en place une protection anti-infectieuse. La défense antivirale repose essentiellement sur les TLR3, TLR7, TLR8 et TLR9 exprimés majoritairement par les pDC mais aussi les TLR2, TLR4, TLR6 exprimés préférentiellement par les mDC et induisant la production massive des IFN de type I. Les RLR sont exprimés par de nombreux autres types cellulaires et activent, via NF-kB et IRF3, la production d'IFN de type I dans les cellules infectées. Les récepteurs Ipaf, NALP1 et NALP3, regroupés au sein de l'inflammasome, induisent la caspase-1, protéine clé dans l'activation des cytokines proinflammatoires de la famille de l' IL-1, alors que NOD1, NOD2 et les TLR peuvent induire les précurseurs de ces cytokines par la voie NF-κB (Martinon and Tschopp, 2005; O'Neill et al., 2003). De plus, l'activation des TLR, par des mécanismes encore mal définis, peut potentialiser la formation et l'activation des inflammasomes (Kahlenberg et al., 2005).

Les signaux de détection déclenchent rapidement des réponses immunitaires en produisant des interférons et des cytokines inflammatoires comme l'IL-1, l'IL-6, l' IL-18 et éventuellement l'IL-33. Les cytokines inflammatoires ainsi que des lésions tissulaires causées par les microbes vont induire la production d'autres cytokines, essentiellement par les cellules résidantes, en particulier les cellules épithéliales.

Les TLR interviennent à tous les stades d'une réponse immunitaire, du plus précoce (détection des microorganismes invasifs et initiation de la réponse innée) au plus tardif (atténuation de la réponse et retour à l'état physiologique de veille). Cette capacité exceptionnelle des TLR à diriger la réponse immune est due, entre autres, à leur expression ubiquitaire sur des cellules de l'organisme, dont les cellules non immunitaires.

La capacité des TLR à activer les différentes cellules de l'immunité innée est largement documentée et cette seule caractéristique suffirait à conférer à cette classe de récepteurs une place importante dans le domaine de l'immunologie. Les DC une fois activées migrent vers les ganglions lymphatiques où elles activent les cellules T en présentant les antigènes et en fournissant différents signaux de co-stimulation qui vont orienter la polarisation des cellules T vers un phénotype effecteur (Th1, Th2 ou Th17) ou régulateur (Treg, NKT) (Kawai and Akira, 2010; de Jong et al., 2002). Cela leur confère la capacité unique d'induire les réponses immunes adaptatives ou la propriété régulatrice. Le point de départ de ces phénomènes de maturation débutant très souvent par la stimulation des TLR par leurs agonistes respectifs, ces récepteurs occupent une place prépondérante à l'interface entre les réponses innées et adaptatives.

Il est généralement admis que les différents effecteurs T permettent de lutter contre l'ensemble des pathogènes invasifs, les cellules Th1 étant plus particulièrement recrutées dans le cadre de microorganismes intracellulaires, les cellules Th2 permettant de contrôler essentiellement les infections fongiques et parasitaires. Plus récemment sont apparues les cellules Th17 qui seraient impliquées dans la défense contre les pathogènes extracellulaires. Ces trois sous populations de cellules effectrices se différencient à partir de cellules T naïves (Th0) activées par les DC, sous l'influence de différents profils cytokiniques induits en réponse à l'activation des TLR dans l'interaction entre l'aggresseur et les cellules résidantes qui servent de barrière et les DC. L'IFNγ et l'IL-12 jouent un rôle essentiel dans la différenciation Th1, l'IL-4 favorise le développement de la lignée Th2, alors que l'IL-6 et de le TGF-β sont impliqués dans la différentiation de la lignée Th17 (Bettelli et al., 2007).

Les agonistes des TLR ont prouvé leur efficacité dans l'induction des réponses CTL et sont largement utilisés en tant qu'adjuvants dans les stratégies de vaccination, entre autres pour lever la tolérance en présence de cellules T régulatrices CD4+CD25+ *in vitro* et *in vivo*

(Yang et al., 2004). Les lymphocytes B expriment également différents TLR, et ce de façon constitutive pour les cellules murines naïves. A l'instar de leur double rôle dans l'activation des lymphocytes T, les TLR contrôlent et modulent les réponses B, indirectement via les DC et macrophages producteurs de cytokines et autres facteurs d'activation des cellules B, et directement par le double engagement du récepteur des cellules B (BCR) et des TLR présents sur les lymphocytes. Les TLR et la protéine MyD88 sont indispensables à l'établissement de l'immunité humorale (Pasare and Medzhitov, 2005a; Pasare and Medzhitov, 2005b) et la stimulation des TLR lymphocytaires contrôle la prolifération, la commutation de classe isotypique et la différenciation en plasmocyte (Ruprecht and Lanzavecchia, 2006; Genestier et al., 2007).

Si les Treg jouent un rôle crucial dans le maintien de la tolérance périphérique et contrôlent les réponses immunitaires afin de prévenir tout dommage tissulaire, des études récentes ont révélé qu'ils peuvent également freiner l'induction de ces mêmes réponses. Il apparaît donc évident que l'activité de ces cellules doit être finement contrôlée pour permettre la mise en place d'une protection efficace contre les infections. Cette régulation implique certes des signaux endogènes humoraux et cellulaires (cytokines et molécules de costimulation sur les CPA), mais récemment de nombreuses équipes ont montré que les pathogènes eux-mêmes intervenaient dans ce phénomène via les TLR exprimés par les lymphocytes Treg (Caramalho et al., 2003; Kubo et al., 2004). A l'heure actuelle, il est établi que TLR2 joue un rôle prépondérant dans la modulation de ces cellules, et ce de façon directe. Non seulement les souris déficientes en TLR2 présentent un défaut numérique en lymphocytes Treg, mais encore la stimulation par ses agonistes induit la prolifération et augmente les capacités suppressives de ces cellules (Zanin-Zhorov et al., 2006; Sutmuller et al., 2006). Liu et collaborateurs ont proposé un élégant modèle d'activation séquentielle des lymphocytes T conventionnels et des Treg par la stimulation de TLR2. Durant les phases précoces de l'infection, la stimulation du récepteur par les PAMP induirait la prolifération des

lymphocytes Treg et T conventionnels, mais seuls ces derniers seraient activés dans un premier temps. Au niveau des cellules Treg, l'activation du TLR2 induit une perte transitoire de l'induction de Foxp3, molécule clé de leur activité. A l'inverse, la reconnaissance des PAMP par le TLR2 des lymphocytes T conventionnels stimule la production de grandes quantités d'IL-2, phénomène qui contribuerait à inhiber transitoirement l'activité suppressive des lymphocytes Treg. Ce n'est que dans un second temps que les cellules régulatrices regagneraient leur potentiel suppresseur permettant de limiter la réponse immune (Liu et al., 2006). Cet effet dans le contrôle des lymphocytes Treg ne semble pas être généralisé pour d'autres TLR. Si la stimulation du TLR5 des cellules Treg humaines augmente leurs capacités suppressives (Crellin et al., 2005), celle des TLR8 et TLR9 ont des effets opposés (Peng et al., 2005). De plus, le rôle du TLR4 sur l'activité des lymphocytes Treg reste controversé (Caramalho et al., 2003; Kubo et al., 2004; den Haan et al., 2007; Pasare and Medzhitov, 2003; Fehervari and Sakaguchi, 2004; Lewkowicz et al., 2006). Les mécanismes de cette particularité du TLR2 n'ont pas encore été élucidés mais sa place centrale dans le contrôle des cellules régulatrices pourrait être reliée à la reconnaissance de ligands endogènes et/ou exogènes qui participeraient à l'induction d'un état de tolérance et à la maintenance des cellules Treg.

Les lymphocytes iNKT contribuent aux réponses cellulaires et humorales de par leur auto-réactivité et leur capacité à produire rapidement et massivement des cytokines tant Th1 que Th2. Exprimant un répertoire TCR restreint Vα14-Jα18, ces cellules sont sélectionnées par la molécule CD1d reconnaissant des ligands glycolipidiques dont certains d'origine bactérienne (Fischer et al., 2004; Kinjo et al., 2005; Kinjo et al., 2006; Sriram et al., 2005; Mattner et al., 2005) et interviennent dans de nombreuses situations infectieuses tant d'origine virale que bactérienne, parasitaire ou fongique, souvent de façon bénéfique, mais parfois aussi préjudiciable pour l'hôte. Les lymphocytes iNKT peuvent être activés par l'IL-12 et l'IL-18

produites par des DC activées par du LPS, même en l'absence de stimulation par des antigènes présentés par le CD1d (Nagarajan and Kronenberg, 2007). Les voies d'activation des lymphocytes iNKT dépendantes du TCR sont également sujettes à l'influence des stimulations TLR. D'une part la présence de pathogènes ou PAMP augmente l'expression de CD1d sur les CPA (Berntman et al., 2005; Skold et al., 2005; Raghuraman et al., 2006), d'autre part la présence de cytokines seules n'est pas toujours suffisante pour activer les cellules iNKT. Etant donné le profil d'expression relativement restreint des TLR pour chaque type cellulaire, la coopération entre différentes sous-populations de DC peut être requise pour une activation optimale des lymphocytes iNKT (Montoya et al., 2006). Cette observation soulève la question de la nature du ligand endogène des cellules iNKT qui serait exprimé par les DC activées.

Pressenti pendant quelque temps comme candidat, l'isoglobotrihexosylcéramide (iGb3) est aujourd'hui sujet à controverse quant à son importance réelle dans la génération et l'activité des lymphocytes iNKT (Zhou et al., 2004; Porubsky et al., 2007). Il reste toutefois envisageable que ce ligand endogène soit exprimé par les CPA après stimulation des voies TLR, hypothèse qui permettrait d'expliquer le paradoxe entre un répertoire TCR restreint et un rôle dans des situations infectieuses très variées des cellules iNKT.

Les TLR interviennent également à la phase ultime de la réponse immune, l''induction de l'apoptose des cellules cibles, et constituent des facteurs clés de la défense anti-infectieuse ou antinéoplasique (Maratheftis et al., 2007; Lehner et al., 2007; Salaun et al., 2006). De nombreux microorganismes ont mis en place des stratégies de survie impliquant le détournement ou l'inhibition des voies TLR pour circonvenir le système immunitaire. Ces phénomènes d'échappement, qui se traduisent généralement par un environnement immunomodulateur caractérisé par une importante production d'IL-10, ont été particulièrement étudiés dans le cadre des infections parasitaires et fongiques que nous n'aborderons pas ici.

B. Relation entre infections et asthme

Bien qu'il existe une prédisposition hériditaire à l'asthme, l'augmentation de l'incidence de l'asthme dans les pays développés au cours de dernières décennies reflète l'influence importante de facteurs de l'environnement. Ce phénomène a conduit à la formulation de l'hypothèse de l'hygiène qui énonce que l'amélioration des conditions d'hygiène est associée à la fréquence croissante des troubles immunologiques de nature allergique ou autoimmune. A l'inverse, l'observation clinique mais aussi des données épidémiologiques ont rapporté le cas de déclenchement et/ou d'exacerbation de l'asthme au cours des épisodes d'infection respiratoire en particulier pour les infections virales ou d'autres types d'infections (DeFranco, 2009; WHO/NHLBI workshop report, 2009). Conceptuellement, une infection virale peut être facilitée et exacerbée chez les personnes présentant une prédisposition asthmatique en raison d'un biais de leur fonction immunitaire vers des réponses pro-Th2, conduisant à une production réduite d'IL-12 et d'IFN-γ, et par conséquent, une plus faible immunité antivirale. Inversement, l'infection virale active les cellules épithéliales et d'autres cellules résidantes de la muqueuse bronchique, qui peuvent à leur tour favoriser ou exacerber l'asthme. Ces différents aspects seront développés ci-dessous.

1. Hypothèse de l'hygiène

Ce concept du rôle protecteur des infections n'est pas nouveau. Dans les années 60, l'étude portant sur le risque de sclérose en plaque a suggéré que ce risque augmente chez les personnes ayant grandi dans des demeures dont les standards sanitaires étaient particulièrement élevés (Leibowitz et al., 1966). Deux décennies plus tard, l'observation que le risque de rhinite allergique était inversement corrélé à l'ordre de naissance au sein de la fratrie et à la taille de la famille, il a été proposé que l'exposition aux infections au cours de la petite enfance pouvait jouer un rôle dans la prévention de cette maladie allergique (Strachan, 1989). Ainsi naquit l'hypothèse

dite “de l’hygiène” postulant une corrélation inverse entre la fréquence des maladies infectieuses et le risque de développement des pathologies allergiques ou autoimmunes. Bien que certaines études n'aient pas confirmé un effet bénéfique du contact avec des agents infectieux (Krause et al., 2003; Ota et al., 2003), de nombreuses observations épidémiologiques parmi lesquelles celles des études utilisant le questionnaire ISAAC et portant sur les enfants "à la ferme", ont apporté des arguments en faveur de cette hypothèse (Ma et al., 2009; Pelosi et al., 2005; Von Ehrenstein et al., 2000; Shirakawa et al., 1997; Herz et al., 2000; Aaby et al., 2000; Hopfenspirger and Agrawal, 2002). Ainsi, au cours des trente dernières années, l’incidence de l’asthme, de la rhinite, de la dermatite atopique, du DT1 et de la maladie de Crohn n’a cessé d’augmenter dans les pays industrialisés alors que la fréquence des maladies infectieuses a continuellement diminué, suite à l’utilisation généralisée de l’antibiothérapie et de la vaccination, mais également de l’amélioration des conditions socio-économiques et d’hygiène (Bach, 2002). Il existe certes une prédisposition génétique aux désordres immunitaires. Cette observation semble refléter une forte influence des facteurs environnementaux.

Les études sur l’asthme dans des modèles animaux sont assez nombreuses, ce qui a permis de comprendre les modulations que les agents infectieux pourraient engendrer sur la réponse immune de type asthmatique développée expérimentalement. Le modèle le plus fréquemment utilisé est l’allergie à l’ovalbumine, dans lequel on observe un recrutement important d’éosinophiles dans les poumons, une production des IgE spécifiques de l'allergène et une hyperréactivité bronchique, symptômes très proches de ceux de l’asthme chez l'homme.

En accord avec le rapport de l'effet protecteur de la vaccination par le BCG sur le développement de l'atopie chez les enfants (Shirakawa et al., 1997; Aaby et al., 2000), dans un modèle d'asthme, la préimmunisation avec Mycobacterium bovis juste après leur naissance a fait diminuer les IgE produites après immunisation avec l’ovalbumine, effet accompagné d'une atténuation des changements histopathologiques (Hopfenspirger and Agrawal, 2002; Ozdemir et

al., 2003; Tukenmez et al., 1999). Administrées chez des souris déjà immunisées, les mycobactéries n'influencent pas la sensibilisation à l'antigène mais exercent des effets bénéfiques même si les souris ont déjà développé une inflammation allergique. En effet, l'administration intranasale de BCG après une première stimulation diminue l'hyperréactivité bronchique, le recrutement des éosinophiles et le niveau d'IL-5 dans les lavages bronchopulmonaires (Hopfenspirger and Agrawal, 2002). La protection vis-à vis de l'asthme expérimental a été rapportée également quand le Mycobacterium bovis avait été traité par la chaleur ou par un processus de lyophilisation (Wang and Rook, 1998; Major et al., 2002; Lagranderie et al., 2008). Le mécanisme responsable de la protection a été également exploré (Lagranderie et al., 2010). D'autres espèces bactériennes comme Listeria monocytogenes, Propionibacterium acnes, Bordetella pertussis, Chlamydia sont également protectrices dans différents modèles d'asthme (Liu et al., 2003; Braga et al., 2003). Le rôle bénéfique de la microflore a également été rapporté dans d'autres modèles. L'administration d'antibiotiques qui modifie la composition des germes commensaux induit une augmentation des IgE et des réponses Th2 (Bashir et al., 2004).

2. Les infections dans le déclenchement/exacerbation de l'asthme: rôle clé de l'immunité innée

Les données épidémiologiques ont montré que les agents infectieux associés aux réponses asthmatiques sont souvent de nature virale (Busse, 1993), et rarement bactérienne (Kraft, 2000). Quelques virus tels que le virus respiratoire syncytial (VRS), le rhinovirus (RV), le virus de la grippe A et le virus parainfluenza sont souvent associés à l'asthme (Newcomb and Peebles, Jr., 2009).

L'association de l'infection par le VRS et l'asthme chez l'enfant a été décrite depuis de nombreuses années. Des études chez l'homme et chez l'animal ont démontré l'impact de ce virus sur l'exacerbation de l'asthme (Gershwin, 2006). Plus récemment, les infections à rhinovirus (RV) ont été rapportées fortement associées au développement d'une respiration

sifflante persistante (Kusel et al., 2007). Les études portant sur des patients souffrant d'asthme atopique infectés avec RV ont montré une association entre ces deux entités (Newcomb and Peebles, Jr., 2009). Bien que le VRS et RV représentent les virus les mieux caractérisés dans les épisodes d'exacerbation chez les enfants, le RV est l'agent viral le plus souvent associé aux crises graves d'asthme chez les patients adultes (Lemanske, Jr. et al., 2005).

Table 2. Pathogènes les plus fréquemment identifiés dans les épisodes d'exacerbation de l'asthme

Pathogènes	**Acide nucléique**	**(%) des épisodes d'exacerbation**
Adenovirus	ARNdb nu	<1
Coronovirus	ARNsb capsulé, (+)	26-40
Métapneumovirus humain	ARNsb capsulé, (-)	7
Influenza	ARNsb capsulé, (-)	2-9
Virus respiratoire syncytial	ARNsb capsulé, (-)	1-12
Rhinovirus	ARNsb noncapsulé, (+)	34-75
Mycoplasma pneumoniae	AND/ARN	18-46
Chlamydia pneumoniae	AND/ARN	2-12

De multiples études ont montré que près de 80% des exacerbations de l'asthme chez les enfants et aussi chez les adultes ont été précédées par une infection virale des voies respiratoires, dont la majorité est associée avec le rhinovirus (Kling et al., 2005; Mallia and Johnston, 2006). Récemment, le métapneumovirus humain (nouveau variant du virus parainfluenza) a été associé à une exacerbation grave de l'asthme chez l'enfant (Schildgen et al., 2004).

Les infections des voies respiratoires inférieures par le VRS sont associées à une augmentation du risque de respiration sifflante à l'âge de 6 ans. Le risque diminue avec l'âge des enfants (jusqu'à l'âge de 11 ans) et il n'y a pas d'association avec l'atopie. (Stein et al., 1999). Il est établi que l'infection à VRS provoque le développement et souvent la persistance d'une réponse Th2. Il a été démontré que les IgE spécifiques à VRS étaient associées à une

hyperréactivité des voies aériennes à la méthacholine, une induction des cytokines et chimiokines inflammatoires comme TSLP, IL-4, IL-5, IL-6, IL-13, TNF-α ainsi que CCL5, MIP-1α et MCP-1, et une synthèse accrue de prostaglandines et de leucotriènes. L'activation des mastocytes, des éosinophiles, des cellules dendritiques et des lymphocytes T a été bien documentée après une infection virale associée à des épisodes d'exacerbation de l'asthme (Gershwin, 2006; Ogra, 2004; Esnault et al., 2008).

L'infection à rhinovirus augmente aussi l'expression de multiples chimiokines, y compris RANTES (CCL5) (Schroth et al., 1999), qui favorise l'infiltration des éosinophiles (Kameyoshi et al., 1992). Dans cette infection, l'expression d'iNOS et de NO est également augmentée (Sanders et al., 2004). Dans la pathogénèse de l'asthme, le NO est associé à l'éosinophilie, à l'inflammation des voies aériennes et à la différenciation des cellules Th2 (Bochner and Busse, 2005b; Benbernou et al., 1997b).

Les cellules épithéliales des bronchioles stimulées par des cytokines proinflammatoires (IL-1β, TNF-α), par le peptidoglycane bactérien (PGN), par l'acide lipotéchoïque (LTA) des bactéries Gram-positifs ou encore par des ARN double brin, produisent du TSLP (Allakhverdi et al., 2007b). La production de TSLP est également observée après une infection par RSV (Wang et al., 2009) ou RV (Kato et al., 2007). In vitro, le RV16 induit fortement la production de TSLP par les cellules épithéliales humaines et cette production est dépendante de NF-kappaB et d'IRF-3 (Lee and Ziegler, 2007). Avec son large spectre d'activité sur un éventail d'acteurs cellulaires (cf. partie décrivant de TLSP dans la réaction inflammatoire asthmatique), le TSLP est proposé comme un médiateur important dans le déclenchement et/ou l'exacerbation de l'asthme (Esnault et al., 2008).

Ainsi, les infections virales activent les cellules épithéliales qui, dans un environnement inflammatoire de type Th2, produisent des quantités importantes de TSLP et cette cytokine

pro-allergique amplifie la réponse Th2 préexistante. Cette cytokine pourrait jouer un rôle à la fois dans le déclenchement et dans l'exacerbation de l'asthme.

Parmi les autres mécanismes qui ont été proposés pour participer à l'exacerbation de l'asthme par les virus respiratoires, on peut citer les augmentations de l'expression de neuropeptides survenant après infection par VRS (Gershwin, 2006; Newcomb and Peebles, Jr., 2009; Tregoning and Schwarze, 2010).

Mycoplasma pneumoniae et Chlamydia pneumoniae, ont été décrites comme des bactéries atypiques et ont souvent été associées à une exacerbation de l'asthme chez les enfants et les jeunes adultes, même si leur implication dans l'exacerbation de cette pathologie reste à confirmer (Hansbro et al., 2004; Newcomb and Peebles, Jr., 2009). Des études récentes ont également décrit M. pneumoniae ainsi que C. pneumoniae dans la pathogenèse de l'asthme chronique (Gershwin, 2006).

Les données épidémiologiques montrent que l'exposition aux allergènes fongiques est associée à l'exacerbation de l'asthme. C'est le cas de nombreux champignons aéroportés comme les espèces d'Alternaria, Aspergillus, Penicillium et Cladosporium dont l'exposition peut survenir à l'intérieur, ou à l'extérieur des habitats (Denning et al., 2006). Outre leur allergénicité, la plupart de ces allergènes fongiques possèdent une activité protéase qui leur permet d'activer les DC, en stimulant des récepteurs de protéases (PAR), et différentes cellules comme les cellules épithéliales, les fibroblastes, les cellules musculaires lisses, pour induire la production de TSLP qui, à son tour, amplifie les réactions allergiques (Kouzaki et al., 2009).

Parmi les infections parasitaires, les helminthes déclenchent de fortes réponses immunitaires pro-Th2 qui se caractérisent par l'augmentation du nombre d'éosinophiles, l'activation des mastocytes muqueux et un taux élevé d'IgE. D'un côté, l'état atopique semble conférer une résistance aux infections parasitaires. Des niveaux élevés d'IgE et une éosinophilie importante réduisent le taux de fécondité des vers et la fréquence de réinfection

après un traitement (Quinnell et al., 2004). D'un autre côté, la réponse immunitaire développée par les mécanismes de défense antiparasitaire pourrait exacerber l'asthme. Cependant, ces observations ne peuvent pas être facilement généralisées puisque la défense antiinflammatoire développée vis-à-vis de certains parasites arrive parfois à moduler voire diminuer l'asthme (Leonardi-Bee et al., 2006; Yazdanbakhsh and Matricardi, 2004).

3. Polymorphisme des détecteurs innés des pathogènes et asthme

L'immunité innée est maintenant considérée comme jouant un rôle important dans le déclenchement de la réponse immunitaire et dans l'immuno-régulation. La synthèse des IgE et l'inflammation allergique sont influencées par des polymorphismes dans les gènes qui codent pour les récepteurs de reconnaissance des motifs ou les molécules accessoires présentes à la surface des cellules telles que la molécule CD14, les récepteurs Toll-like 2 (TLR)2 , TLR4, TLR6, TLR10, dans le cytosol comme les molécules NOD1 (CARD4) et NOD2 (CARD15), ou sur la membrane endosomique (TLR7, TLR8, TLR9). Les polymorphismes des gènes codant pour ces récepteurs sont associés à des maladies autres que l'asthme et les allergies ce qui explique le rôle fondamental de l'immunité innée (Vercelli, 2008). Il est à noter que selon les critères environnementaux utilisés pour définir les cohortes de sujets, des résultats contraditoires ont été publiés quand au polymorphisme des gènes de l'immunité innée.

Le LPS est la fraction bioactive. Cette structure est commune à toutes les bactéries gram-négatives et a été utilisée comme substitut de la charge microbienne de l'environnement. La molécule CD14 se trouve à la surface des monocytes et des macrophages mais aussi sous forme soluble (sCD14) dans le sérum et les sécrétions. Cette molécule interagit avec le LPS délivré par LBP (LPS-binding protein) pour former un complexe impliqué dans l'initiation de la réponse immunitaire innée à une infection bactérienne, via l'activation du complexe TLR4-MD-2 *(Fig12)*. Un polymorphisme fréquent dans la région promotrice du CD14, caractérisé par un remplacement de base C (allèle C) par T (allèle T) en position 159 en amont du début de la transcription du gène codant pour CD14 ou 260 de l'emplacement de la traduction (CD14/-159 ou CD14/-260), a été

associé à une élévation de sCD14 et un risque d'atopie diminué (Baldini et al., 1999). Depuis cette première description, plus de 200 études évaluant l'association de ce polymorphisme aux phénotypes de l'asthme, mais aussi à d'autres maladies, ont été publiées et l'effet initialement décrit n'était pas uniformément reproduit. Il a été démontré au moins dans cinq études indépendantes que l'exposition à un niveau élevé aux endotoxines dans l'environnement domestique avait un rôle protecteur contre le développement de l'atopie parmi les porteurs de l'allèle C (Zambelli-Weiner et al., 2005; Eder et al., 2005; Simpson et al., 2006; Williams et al., 2006; Williams et al., 2008; Simpson and Martinez, 2010). Cela signifie que l'influence de l'environnement sur une population devrait être considérée en se référant à l'étude du génotype de celle-ci. Cette complexité est biologiquement plausible. Notre système immunitaire inné reconnaît le LPS par le complexe trimoléculaire CD14/TLR4/MD2. Ainsi, le facteur de l'environnement ou le génotype n'est pas suffisant à lui seul pour causer des maladies complexes comme l'asthme et l'atopie et celles-ci sont plutôt le résultat de l'interaction de l'un avec l'autre. Ces résultats concernant le polymorphisme de CD14 et de TLR4 constituent un exemple typique non seulement de la relation entre les facteurs génétiques et environnementaux, mais aussi du contexte de détermination multigénique d'une maladie ou d'un phénotype particulier.

Il a été proposé que les produits microbiens présents dans le milieu agricole pourraient interagir avec TLR2 et cette observation est soutenue par l'observation d'une augmentation de l'expression de ce récepteur à la surface des CPA du sang périphérique chez les enfants d'agriculteurs par rapport aux enfants qui n'ont pas été élevés dans une exploitation agricole. Cette interaction pourrait avoir un effet protecteur vis-à-vis de l'asthme et l'allergie. Le polymorphisme TLR2/-16934 du gène codant pour TLR2 est mentionné comme un déterminant majeur de prédisposition à l'asthme et à l'atopie chez les enfants d'agriculteurs. De plus, il a été constaté qu'une variation génétique dans les TLR4/+4434 est inversement associée au niveau d'IgE spécifiques chez les enfants fortement exposés aux endotoxines

(Eder et al., 2004a; Lauener et al., 2002). Une autre étude a montré, dans une population adulte en Allemagne, que seuls les sujets avec l'allèle mineur G de TLR4/+4434 ont une association inverse avec l'exposition aux endotoxines et à la poussière de maison mesurée par la réponse bronchique à la métacholine (Werner et al., 2003). Le polymorphisme Asp299Gly substitué dans TLR4, a été associé à une diminution du risque de la réactivité bronchique (Fageras et al., 2004). Dans une étude effectuée dans la population afro-américaine, il a été décrit un polymorphisme de TLR6 dans lequel la Ser249 est remplacée par la Pro, associé à une diminution du risque d’asthme (Tantisira et al., 2004a). Le polymorphisme de TLR10, avec la 1031G remplacée par la A et la 2322A par la G, a été montré associé à la pathologie. Ce résultat a été reproduit dans une autre étude (Lazarus et al., 2004). En outre, le polymorphisme dans l’IRAK4, qui est important dans les voies de signalisation IL-1R/TLR/MyD88, montre une forte association entre les IgE et l'asthme (Tewfik et al., 2009).

L’étude du polymorphisme du gène TLR3 est important puisqu’il est impliqué dans la réponse à certains virus qui déclenchent la bronchiolite respiratoire chez l’enfant, et qui pourraient augmenter le risque d'asthme. Plusieurs polymorphismes, exoniques et introniques ont été décrits; cependant, ceux-ci n'ont pas été associés à l'asthme ou à la sécrétion d'IgE dans l'étude des familles asthmatiques au Japon (Noguchi et al., 2004). Les associations des polymorphismes pour TLR7 (rs179008) et TLR8 (rs2407992) à l’asthme et d’autres maladies atopiques ont été identifiés (Moller-Larsen et al., 2008). Un polymorphisme pour TLR9 avec la A-1237T remplacée par la C, a été associé à un risque accru d'asthme dans une population Euro-américaine (Lazarus et al., 2003).

Le polymorphisme du gène de la molécule NOD1 a été montré avoir une forte association avec la présence de l'asthme avec des niveaux élevés d'IgE (Hysi et al., 2005). Le polymorphisme de NOD2 est également associé à la gravité de certaines maladies atopiques et à la prévalence de l'asthme et ce avec une élévation des taux sériques d'IgE (Kabesch et al., 2003a).

OBJECTIFS DU TRAVAIL

L'asthme est déclenché par des allergènes de l'environnement, des irritants ou par des infections chez les sujets génétiquement prédisposés. Il se caractérise par une inflammation des voies respiratoires qui est fortement associée à une hyperréactivité bronchique et aux manifestations cliniques. L'inflammation asthmatique est caractérisée, pour toutes les formes de la maladie (d'origine allergique ou non allergique), par une infiltration des cellules innées et des lymphocytes T et par la libération de cytokines et chimiokines. Ces médiateurs sont, en grande partie, responsables du développent, du maintien, de l'exacerbation et ensuite du retour à l'homéostasie. La relation entre les agents infectieux et l'asthme est complexe sachant que les agents infectieux peuvent déclencher, exacerber ou protéger cette pathologie et celle-ci implique initialement des réponses immunitaires innées. L'étude de la modulation de la réponse immunitaire responsable de l'inflammation bronchique asthmatique, peuvent à terme permettre de développer de nouvelles stratégies thérapeutiques.

Bien que les réponses immunitaires de type Th2 représentent un aspect caractéristique de l'asthme allergique, les connaissances récemment acquises sur l’asthme attribuent aux cellules résidantes des voies respiratoires et d’une manière générale, aux cellules immunitaires innées recrutées et à leurs composants spécifiques, un rôle de plus en plus important à tel point qu'ils peuvent assurer par elles seules, en absence totale des réponses immunes adaptatives, les réactions pathologiques pathognomoniques de l'asthme.

L’IL-33 (IL-1F11) est la cytokine de la famille de l'IL-1R la plus récemment décrite. Cette cytokine favorise la polarisation des cellules T naives vers les cellules Th2 et cette fonction est associée avec la production des cytokines comme l'IL-4, l'IL-5 et l'IL-13. L’IL-33 favorise la réponse des mastocytes et des basophiles de façon dépendante et indépendante de la stimulation du récepteur de haute affinité pour l'IgE et induit la production d'IL-4, d'IL-6 et d'IL-13. Bien que cette cytokine puisse induire la production de cytokines Th2, son rôle dans le développement des cellules Th2 a été montré dispensable. En revanche, en absence totale

des cellules responsables des réponses immunitaires adaptatives, l'IL-33 est suffisante pour générer tous les aspects des réponses asthmatiques chez la souris. Dans le contexte de l'immunorégulation, nous avons examiné le role de l'IL-33 sur la population de lymphocytes NKT qui se situe à l'interface entre l'immunité innée et immunité adaptative. Les résultats obtenus sont décrits dans l'article n°1.

Les Th17, la population T effectrice qui a un rôle physiologique dans la défense immunitaire contre les bactéries extracellulaires et les infections fongiques ainsi qu'une implication dans l'autoimmunité a été identifiée récemment. La caractérisation du développement, la fonction et une description très rapide leur implication dans le développement de l'asthme, a posé la question sur sa place dans le contexte global du développement des réponses inflammatoires asthmatiques, leur activité en phase effectrice, la relation de cette population avec d'autres populations T. Les cellules NKT sont considérées comme une population soit effectrice soit régulatrice selon leur implication dans la modulation de pathologies autoimmunes ou allergiques. Ces cellules sont également impliquées dans la défense anti-infectieuse vis-à-vis de certains pathogènes. Dans l'asthme, ces cellules ont été décrites, chez l'homme et dans les modèles murins, comme des cellules effectrices bien que certaines évidences et arguments suggère un role différent. En effet, la production rapide et modulable d'une quantité importante des cytokines telle que l'IFN-γ est en accord avec une fonction régulatrice de cette population dans l'asthme. Compte tenu que de nombreux facteurs peuvant influencer le développement des cellules Th17, nous avons testé si les cellules NKT pourraient influencer non seulement la balance Th1/Th2 mais éventuellement le développement et/ou les activités des cellules Th17. La partie des résultats concernant cet aspect est présentée dans l'article n°2.

Les polynucléaires basophiles sont identifiés depuis très longtemps et sont souvent décrits en parallèle avec les mastocytes, dans la phase précoce des réactions allergiques. Bien

que leur rôle dans l'asthme puisse être considéré comme redondant avec celui des mastocytes, il n'a jamais été validé ou invalidé et par conséquent, cette population cellulaire n'était pas officiellement reconnue dans cette fonction. Pour cette raison, nous avons testé les réponses fonctionnelles des basophiles en réponse à différents agonistes TLR et testé leur implication après stimulation dans le modèle d'asthme expérimental. Les résultats obtenus sont présentés dans l'article n°3 et nous discuterons de l'implication des basophiles dans l'asthme et notamment dans les situations où les réponses asthmatiques sont exacerbées.

Les TLR représentent une des principales classes de récepteurs de reconnaissance des pathogènes du système immunitaire inné et jouent un rôle initiateur des réponses immunitaires de l'organisme. La relation entre les agents infectieux et l'asthme est confinée essentiellement sur les interactions entre les agonistes TLR et les acteurs cellulaires impliqués dans l'asthme. Il est communément admis que la stimulation d'un TLR est associée avec la production des IFN et des cytokines proinflammatoires qui favorisent la différentiation des cellules Th1. C'est dans la pathologie allergique que l'hypothèse de l'hygiène a été initialement formulée pour expliquer les effets protecteurs des infections ou des agents infectieux vis-à-vis des manifestations allergiques. Pourtant, des études épidémiologiques ainsi que des données expérimentales montrent que la relation entre les infections et les allergies en général, l'asthme en particulier ne peux pas être expliquée en se basant seulement sur l'équilibre des populations effectrices Th1/Th2. Aussi, nous avons développé des études en postulant que la stimulation des TLR pourrait non seulement induire des réponses immunitaires innées mais aussi favoriser le développement de cellules immnorégulatrices ce qui pourrait expliquer l'effet bénéfique dans les désordres immunopathologiques et dans l'effet protecteur observé dans le modèle d'asthme. La partie des résultats obtenus dans cette approche est présentée dans l'article n°4.

Le résiquimod (R848) est une molécule de la famille des imidazoquinolines, un analogue de l'imiquimod et se comporte comme un agoniste TLR7. Il possède la capactité d'induire des cytokines essentiellement Th1 chez l'homme ainsi que chez la souris. Par sa capacité à stimuler le système immunitaire de l'hôte, cet agoniste TLR est décrit comme un adjuvant dans la vaccination. Nous avons ici étudié l'effet immunomodulateur de R848 dans le modèle d'asthme. Les résultats obtenus sont décrits dans l'article n°4, 5, 6.

Dans nos études expérimentales, le potentiel rôle régulateur de la stimulation des récepteurs de la famille TLR/IL-1 a été exploré dans le modèle d'asthme allergique. Nous avions postulé que la stimulation des réponses "innées" dans certaines conditions influence non seulement les cellules effectrices mais aussi les cellules régulatrices ce qui aboutit soit à la protection voire à la suppression des réponses pathologiques, ou *inversement*, à l'exacerbation de ces dernières. La stimulation de la voie de signalisation TLR/IL-1R par des agonistes naturels, microbiens ou synthétiques module les fonctions de certaines cellules effectrices et/ou régulatrices impliquées dans cette pathologie telles que les basophiles, les lymphocytes NKT, les cellules de type Th2, les cellules Th17 et les lymphocytes T régulateurs. Pour réaliser ces expériences, nous avons adapté le protocole du modèle d'asthme expérimental aux questions posées. Les conséquences de ces réponses immunitaires seront discutées dans le contexte de l'asthme.

L'identification des mécanismes cellulaires et moléculaires pouvant aboutir à la protection ou la suppression ou inversement l'exacerbation de l'asthme sont fondamentaux pour la connaissance de cette pathologie.

RESULTATS

Article n°1

Interaction directe entre l'IL-33, cytokine proTH2 et les lymphocytes NKT invariants (iNKT) et NK dans la production d'IFNγ

Elvire Bourgeois, Linh Pham Van, Michel Samson, Séverine Diem, Anne Barra, Stéphane Roga, Jean-Marc Gombert, Elke Schneider, Michel Dy, Pierre Gourdy, Jean-Philippe Girard, André Herbelin.

L'IL33 a été identifiée récemment comme une cytokine exerçant des fonctions pro-Th2, ce qui soulève la question de son effet sur les lymphocytes NKT invariants (iNKT), qui sont des producteurs importants d'IL4. Dans ce travail, nous rapportons le fait que l'IL-33, administrée pendant 7 jours chez la souris, entraîne un doublement du nombre de cellules iNKT dans la rate et le foie. Cette augmentation résulte d'un effet direct, puisque les cellules iNKT purifiées expriment de façon constitutive le récepteur T1/ST2 et répondent à l'IL-33 par une expansion *in vitro* et une activation fonctionnelle. Contrairement à l'effet Th2 attendu, l'IL-33 induit une augmentation préférentielle d'IFN-γ plutôt que la production d'IL-4 après engagement du TCR et ce dépendant de l'IL-12 endogène. En association avec l'IL-12, l'IL-33 induit également l'augmentation de la production d'IFN-γ par les cellules NK. Considérés dans leur ensemble, ces résultats suggèrent que l'IL-33 peut être considérée comme un facteur de co-stimulation des réponses cellulaires de l'immunité innée et cette production de cytokines par les cellules NK et NKT pourrait représenter un des mécanismes de régulation immunitaire.

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The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-γ production

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IL-33 has recently been identified as a cytokine endowed with pro-Th2 functions, raising the question of its effect on invariant natural killer T cell (iNKT), which are potent IL-4 producers. Here, we report a two-fold increase of iNKT-cell counts in spleen and liver after a 7-day treatment of mice with IL-33, which results from a direct effect, given that purified iNKT cells express the T1/ST2 receptor constitutively and respond to IL-33 by *in vitro* expansion and functional activation. Conversely to the expected pro-Th2 effect, IL-33 induced a preferential increase in IFN-γ rather than IL-4 production upon TCR engagement that depended on endogenous IL-12. Moreover, in combination with the pro-inflammatory cytokine IL-12, IL-33 enhanced IFN-γ production by both iNKT and NK cells. Taken together these data support the conclusion that IL-33 can contribute as a co-stimulatory factor to innate cellular immune responses.

Key words: Cytokines · Inflammation · Natural killer cells · Natural killer T cells · Th1/Th2 cells

Introduction

IL-33 (or IL-1F11) has recently been identified as a ligand of the orphan T1/ST2 receptor, a member of the IL-1 receptor (IL-1R) family [1] that was initially described as a nuclear factor, nuclear factor from high endothelial venules, abundantly expressed by endothelial cells in lymphoid tissues [2, 3].

IL-33 induces its biological effects through a heterodimeric complex comprising the T1/ST2 receptor [1] and the IL-1R accessory protein (IL-1RAcP), another member of IL-1R family [4, 5]. T1/ST2 engagement triggers a signalling pathway that requires MyD88 and NF-κB [1, 4, 6]. It has long been known that T1/ST2 is expressed primarily in mast and Th2 cells and is associated with important Th2 effector functions [7–9]. Accordingly, IL-33 has been found to promote Th2 cytokine production by mast cells and polarized T cells *in vitro,* and to induce pulmonary and mucosal Th2 inflammation when administered *in vivo* [1].

iNKT cells constitute a distinctive subpopulation of mature αβ-T cells bearing an invariant TCR α-chain together with NK-cell receptors [10, 11]. They recognize glycosphingolipid Ags presented by CD1d, a non-classical class I-like Ag-presenting molecule, and respond rapidly to TCR stimulation with α-galactosylceramide (α-GC) by generating a number of cytokines,

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particularly IFN-γ and IL-4 [10, 11]. In most disease models in which iNKT cells have been implicated their beneficial or detrimental effects have been ascribed to either Th1 or Th2 cytokines [10, 11]. It has also been established that the balance between these two profiles depends essentially on the microenvironment, which favours IL-4 or IFN-γ production [12–17].

Given its previously established pro-Th2 functions, IL-33 seemed a plausible candidate for the regulation of iNKT-cell activities, prompting us to investigate whether it could directly interact with this regulatory cell subset to drive IL-4 production. Starting from the observation that the incidence of iNKT cells was increased in spleen and liver of mice injected with IL-33, we examined how this treatment affected their functional status in terms of activation, as well as IFN-γ and IL-4 production. We found that IL-33 is a potent co-stimulator of iNKT cells that induces, in combination with IL-12, a preferential increase of IFN-γ production *in vivo*.

Results

IL-33 targets and activates iNKT cells to increase their numbers both *in vivo* and *in vitro*

Since repeated injections of IL-33 promote striking pathological modifications associated with Th2 differentiation [1], we addressed the question whether iNKT cells, which are a potent source of IL-4, were affected by this treatment. To this end, after seven daily i.p. injections with IL-33, spleen and liver iNKT cells were counted as α-GC-loaded CD1d TT^+ cells expressing an intermediate level of TCR-β. Mice treated with IL-33, developed splenomegaly, as previously described [1] as well as hepatomegaly (lymphocyte counts: IL-33: $1.92 \pm 0.15 \times 10^5$ *versus* $0.99 \pm 0.14 \times 10^5$ after vehicle, $p<0.005$). IL-33 promoted a nearly two-fold increase in iNKT-cell counts in both spleen and liver (Fig. 1A, middle panels), concomitant with cellular activation, attested by up-regulation of the early activation marker CD69 (Fig. 1A, right panels). In the same experimental set-up, IL-33 did not modify mainstream T-cell (defined as α-GC-loaded CD1d TT^- TCR-β^+ cells) counts, even though it led to a modest increase in the expression of CD69. The incidence of iNKT cells in spleen and liver was not altered by the treatment with IL-33 (Fig. 1A, left panels; Fig. 1B), probably because of the concomitant increase of B cells (data not shown), and the significant decrease in the percentage of mainstream spleen T cells (Fig. 1A, left panels).

We next performed *in vitro* experiments to assess whether IL-33 contributed directly to iNKT-cell expansion. To address this question, we evaluated its effect on IL-7-induced thymocyte proliferation, an experimental procedure leading to the expansion of both iNKT and mainstream mature T cells ([18]; Fig. 2, panel A *versus* panel H). Even though IL-33 did not support the survival of mature thymocytes on its own (data not shown), it clearly increased the number of iNKT cells after a 5-day exposure to IL-7 (IL-7+IL-33: $2.82 \pm 0.34 \times 10^6$ *versus* IL-7: $1.07 \pm 0.14 \times 10^6$, $p<0.05$). Furthermore, iNKT-cell frequency was significantly higher in cultures supplemented with IL-33 (Fig. 2B), whereas that of mainstream T cells remained unchanged (Fig. 2C), indicating a preferential effect of IL-33 on the iNKT-cell fraction. Once again, as *in vivo*, the surface expression of the early activation marker CD69 was up-regulated preferentially on iNKT cells (Fig. 2D) compared with mainstream T cells (Fig. 2E). Lastly, in support of a direct interaction, iNKT cells sorted from thymocyte cultures with IL-7, expressed the T1/ST2 receptor (Fig. 2F) and proliferated in response to IL-33 (Fig. 2G). Note that freshly isolated thymic iNKT cells (Fig. 2H) expressed the T1/ST2 receptor constitutively

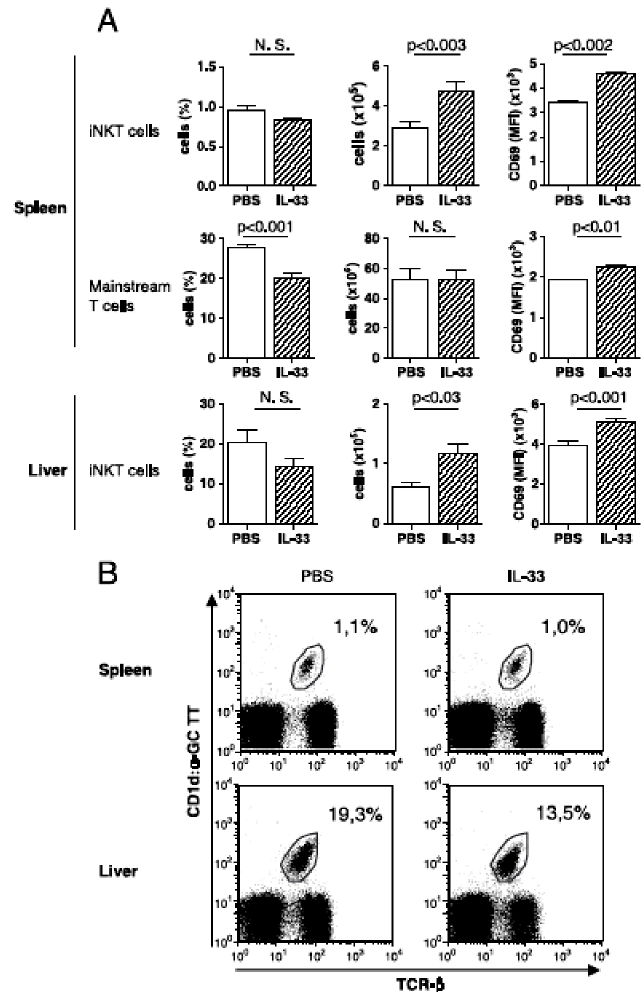

Figure 1. IL-33 targets and activates iNKT cells to increase their number *in vivo*. iNKT and mainstream T cells were defined as α-GC-loaded CD1d TT^+ TCR-β^+ cells and α-GC-loaded CD1d TT^- TCR-β^+ cells, respectively. Wild-type C57Bl/6 mice received PBS (white columns) or IL-33 (hatched columns) daily for 7 days. (A) iNKT and mainstream T-cell percentages (left panels), iNKT and mainstream T-cell counts (middle panels), and MFI of the early activation marker CD69 (PE labelled) on iNKT and mainstream T cells (right panels) in spleen and liver MNC. Data are mean±SEM from 5 to 6 mice per group; NS, not statistically significant. Statistical significance was established by Student's unpaired t-test. (B) A representative flow cytometry plot defining the two T-cell populations in spleen and liver MNC assessed in (A). The values indicate the percentages of iNKT cells corresponding to the indicated (outlined) population.

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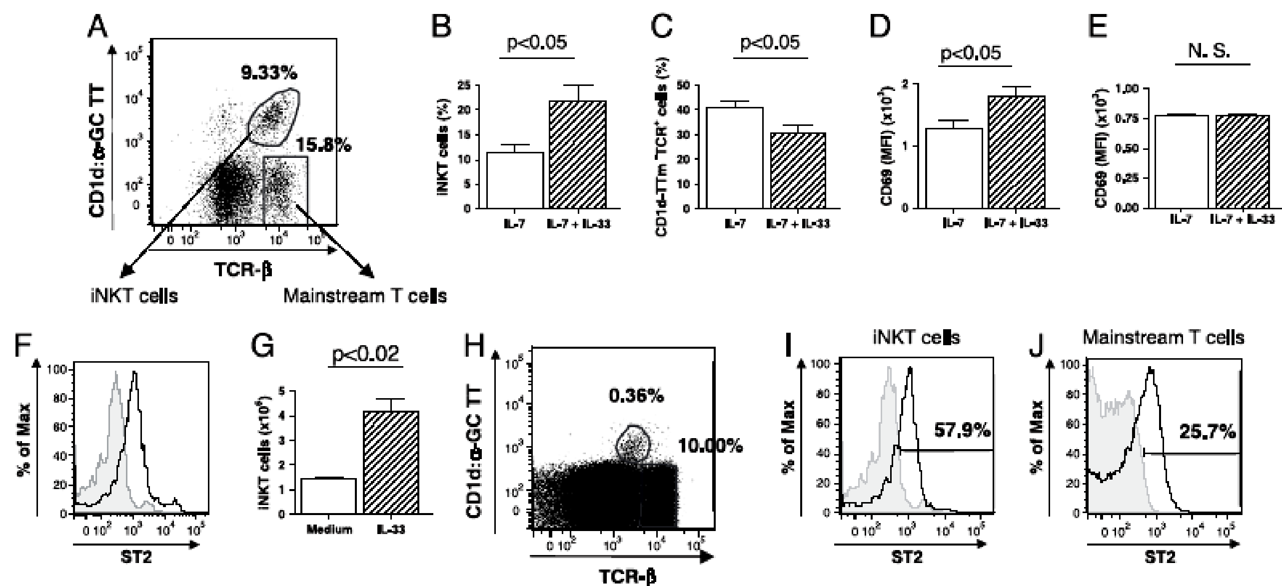

Figure 2. IL-33 contributes directly to iNKT cell activation and expansion. (A) Flow cytometry plot showing the gating and percentages of iNKT (α-GC-loaded CD1d TT$^+$ TCR-β$^+$) and mainstream T (α-GC-loaded CD1d TT$^-$ TCR-β$^+$) cells isolated from wild-type C57Bl/6 mice after a 5-day culture of thymocytes with IL-7. Percentages of (B) iNKT and (C) mainstream T cells, as well as, (D and E) their respective surface expression levels of CD69 (PE labelled) after a 5-day culture of thymocytes, isolated from wild-type C57Bl/6 mice, with IL-7 or IL-7 plus IL-33 (D, iNKT cells; E, mainstream T cells). (F) Expression of the T1/ST2 receptor on iNKT cells (bold line) relative to isotype control (thin line) after a 5-day culture of thymocytes with IL-7; one representative experiment out of three. (G) iNKT cells were sorted from thymocyte cultures supplemented with IL-7 and then incubated (1×10^6 cells) for further 48 h with or without IL-33. (B–E, G) Data represent the means ± SEM of three to four separate experiments, each carried out with four to six pooled thymi from wild-type C57Bl/6 mice. (H) Flow cytometry plot showing the gating and percentages of iNKT and mainstream T cells *ex vivo* from C57Bl/6 mice. Expression of the T1/ST2 receptor (bold lines) relative to isotype controls (thin lines) on (I) iNKT and (J) mainstream T cells. Values in histograms are the percentages of cells expressing either the T1/ST2 receptor. (H–J) One representative experiment out of three is shown. Statistical significance was established by Wilcoxon or Student's t-test, as appropriate.

(Fig. 2I) and the percentage of T1/ST2$^+$ cells in this population was higher than in mainstream T cells (Fig. 2J). *IL-33R* gene expression was further confirmed by real-time RT-PCR since both *ST2* and *IL-1RAcP* transcripts were consistently detected in purified thymic iNKT cells using 23.3 and 26.9 threshold cycles, respectively. A similar result was obtained with spleen and liver iNKT cells that did likewise express the T1/ST2 receptor constitutively, as assessed by flow cytometry (Fig. 3A) and real-time RT-PCR (data not shown).

IL-33 directly enhances IL-4 and IFN-γ production by iNKT cells upon TCR engagement

One of the original features of iNKT cells consists in their capacity to release large amounts of both Th1 and Th2 cytokines, mainly IFN-γ and IL-4, following TCR engagement. We examined the effect of IL-33 on this particular Th1/Th2 cytokine profile, by measuring IFN-γ and IL-4 production by total spleen cells stimulated with the specific iNKT-cell ligand α-GC. We observed a more than two-fold augmentation of IL-4 levels in these conditions (Fig. 3B), in agreement with the previously reported pro-Th2 effect of IL-33 on other cell populations, such as mast cells [5, 6, 19, 20] and Th2 lymphocytes [1, 4]. Surprisingly, IL-33 induced much more IFN-γ than IL-4 since it induced on average a 30-fold increase in response to α-GC, which is 15 times more than the effect on IL-4. The enhancement was strictly iNKT-cell-dependent since IFN-γ was detected at very low levels and IL-4 was virtually absent in the supernatants of cells recovered from the iNKT-cell-deficient mice (Fig. 3B). The up-regulation of IFN-γ and IL-4 production by IL-33 alone or in combination with α-GC was also abrogated in the absence of the MyD88 adaptor molecule (Fig. 3B), a member of the IL-33 signalling receptor complex [1, 4, 6]. The preferential effect of IL-33 on IFN-γ was maintained when splenocytes from BALB/c rather than C57Bl/6 mice were used (Fig. 3C), showing that the pro-Th1 effect of IL-33 persists in strains biased towards Th2-mediated immunity.

We further investigated the effect of IL-33 on cytokine production by iNKT cells *in vivo*. As shown in Fig. 4, both IFN-γ and IL-4 were rapidly and markedly increased in the serum of mice having received IL-33 together with α-GC, as compared with α-GC-injected controls. To prove that iNKT cells were responsible for this cytokine production, we analysed IFN-γ expression in single cells using intracellular staining. As shown in Fig. 4B, IL-33 augmented not only the percentage of IFN-γ$^+$ cells among the gated iNKT subset of the spleen, but also upregulated their CD69 surface expression (Fig. 4C).

We confirmed that IL-33 targeted iNKT cells specifically by sorting them electronically from both spleen and liver, using the α-GC-loaded CD1d TT and the receptor CD5 for positive selection (Fig. 5A). DC loaded with α-GC (DC α-GC) promoted a substantial IL-4 production by sorted α-GC-loaded CD1d TT$^+$

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$CD5^+$ spleen cells that was about two-fold higher in the presence of IL-33. Once again, IFN-γ rather than IL-4 production was enhanced, as attested by its ten-fold increase relative to control cultures set up with DC α-GC alone.

As shown in Fig. 5B and C, a significant but less marked increase in cytokine production in response to IL-33 was obtained when freshly isolated α-GC-loaded CD1d TT^+ $CD5^+$ cells from spleen and liver were stimulated with coated anti-CD3 mAb instead of α-GC-loaded DCs, suggesting a possible contribution of DC. IL-33 enhanced the effect of anti-CD3 mAb at both suboptimal (1–2 μg/mL) and optimal (5–10 μg/mL) concentrations (data not shown) and promoted preferential IFN-γ production, which increased by a factor 10 instead of 2 for IL-4. This ratio remained the same at different doses of anti-CD3 mAb (data not shown). Lastly, IL-33 amplified the proliferation of sorted liver or spleen iNKT cells in response to TCR engagement (Fig. 5, right panels) in accordance with a direct interaction.

Endogenous IL-12 contributes to IL-33-dependent enhancement of IFN-γ production by iNKT cells

It has been reported that α-GC-loaded DC produce IL-12 upon interaction with iNKT cells, which in turn enhances their IFN-γ production [12]. To examine whether this mechanism was involved in our experimental protocol, we performed DC/iNKT co-culture experiments in the presence of neutralizing anti-IL-12 mAbs. As shown in Fig. 6A, the blockade of endogenous IL-12 largely reduced IFN-γ levels generated during incubation of iNKT cells stimulated with α-GC-loaded DC and IL-33, whereas IL-4 production remained unchanged. As a definite proof for the implication of IL-12, we repeated the experiments in C57Bl/6 mice in which the IL-12 p40 chain had been deleted (Fig. 6B). Once again, the loss of IL-12 reduced IFN-γ but not IL-4 production upon costimulation with α-GC and IL-33.

IL-33 enhances IL-12-induced IFN-γ production by iNKT and NK cells

iNKT cells participate not only in acquired but also in innate immune responses, since, like classical NK cells, they can be fully activated and produce large amounts of IFN-γ without TCR engagement, after exposure to the pro-inflammatory cytokine IL-12 [12, 13, 21]. When assessed in this experimental set-up, IL-33 markedly enhanced the synthesis of IFN-γ by sorted α-GC-loaded CD1d TT^+ $CD5^+$ cells from spleen and liver, stimulated with IL-12 (Fig. 7A). However, it is important to note that the sorting of iNKT cells using α-GC-loaded CD1d TTs implies a TCR engagement, which may have conferred responsiveness to IL-33, as previously reported [22]. To avoid this stimulation, we purified $NK1.1^+CD5^+$ iNKT cells and found that unlike their α-GC-loaded CD1d TT^+ counterpart (see Fig. 5B and C), they produced neither IFN-γ nor IL-4 spontaneously or in response to IL-33 alone but conserved their reactivity to IL-12 and its enhancement by IL-33 (Fig. 7B). These data are in accordance with a possible involvement in early immune responses occurring independently from TCR engagement. Consistent with this view, we found that other typical IL-12 responder cells, namely the NK- ($NK1.1^+CD3^-$) cell population, expressed the T1/ST2 receptor constitutively, in both spleen and liver (Fig. 8A). Furthermore, its functionality was assessed by the remarkable 250-fold increase of IFN-γ production that occurred when IL-33 was added to sorted $NK1.1^+CD5$ cells from spleen and liver stimulated with IL-12 (Fig. 8B).

Discussion

Our study provides the first evidence that IL-33 directly targets immunoregulatory iNKT cells to increase their state of activation and their incidence in spleen and liver after a 7-day *in vivo* treatment. This effect resulted from a direct interaction, as assessed by *in vitro* experiments with purified iNKT cells which express the T1/ST2 receptor constitutively and are expanded and functional in response to IL-33.

Based on the current view that IL-33 exerts its biological activity by promoting a Th2 immune response [1], we expected that it would also drive a preferential IL-4 production by iNKT cells. However, it turned out that exposure to IL-33 privileged the production of IFN-γ production in response to TCR engagement, revealing a marked pro-Th1 effect that was enhanced on average 30-fold, that is 10–15 times the increase of IL-4, as well as that of other pro-Th2 cytokines, namely IL-5 and IL-13 (data not shown). Finally, the capacity to promote a pro-Th1 cytokine profile constitutes a general feature of IL-33 applying likewise to C57Bl/6 and BALB/c mice, which are biased towards a pro-Th1 and a pro-Th2 response, respectively.

It is not yet clear whether this activity is restricted to iNKT cells or applies to conventional T-cell populations. Our preliminary data indicate that IL-33 can also amplify IFN-γ production by conventional $CD4^+$ memory T cells, sorted as $CD44^+$ $CD62L^-CD4^+$ α-GC-loaded CD1d $tetramer^-$ (TT) cells, in response to primary TCR stimulation (data not shown). The discrepancy between these findings and those reported by Schmitz *et al.* [1] might be explained by the use of polarized Th1 and Th2 cells rather than freshly isolated, naturally activated $CD4^+$ T cells, as in our experimental setup.

The preferential IFN-γ production induced by IL-33 in iNKT cells indicates that the immunomodulatory functions of this newly discovered cytokine might actually be more complex than initially assumed. Depending on the local cytokine environment it might indeed behave like an enhancer of Th1-mediated inflammation, as is the case in our study where endogenous Il-12 mediates the enhancement of IFN-γ production by iNKT cells in response to IL-33 both *in vitro* and *in vivo*. Further investigations are now needed to determine to what extend the contrasting activities of iNKT cells regarding Th1 [21] *versus* Th2 [23] adaptive immune responses are regulated by endogenous IL-33 *in vivo*. Our results

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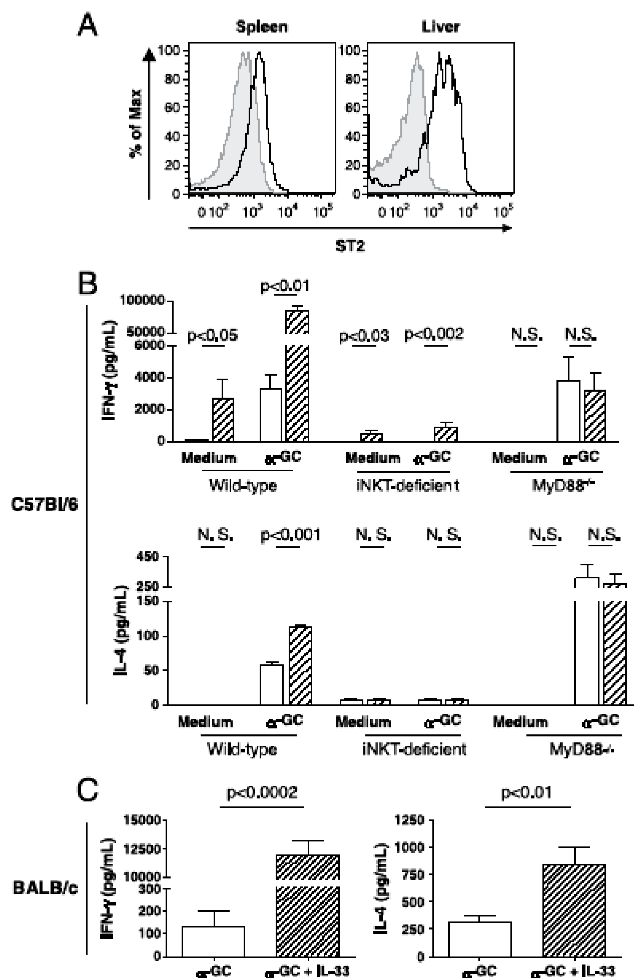

Figure 3. IL-33 enhances both IL-4 and IFN-γ production by splenocytes in response to the specific iNKT cell ligand α-GC: dependence on MyD88. (A) *Ex vivo* expression of the T1/ST2 receptor by spleen and liver iNKT cells (bold line) relative to their isotype controls (thin line) from wild-type C57Bl/6 mice. One representative experiment out of three is shown. Total splenocytes from (B) wild-type, iNKT-deficient (Jα18$^{-/-}$) or MyD88$^{-/-}$ C57Bl/6 mice or (C) wild-type BALB/c mice were cultured for 48 h with or without α-GC, and in the presence (hatched columns) or absence of (white columns) of IL-33. IFN-γ and IL-4 concentrations in the supernatants were quantified by ELISA. Data represent means±SEM of three separate experiments, each carried out with three pooled organs for each group of mice. NS: not significant statistical significance was established by Wilcoxon or Student's t-test, as appropriate.

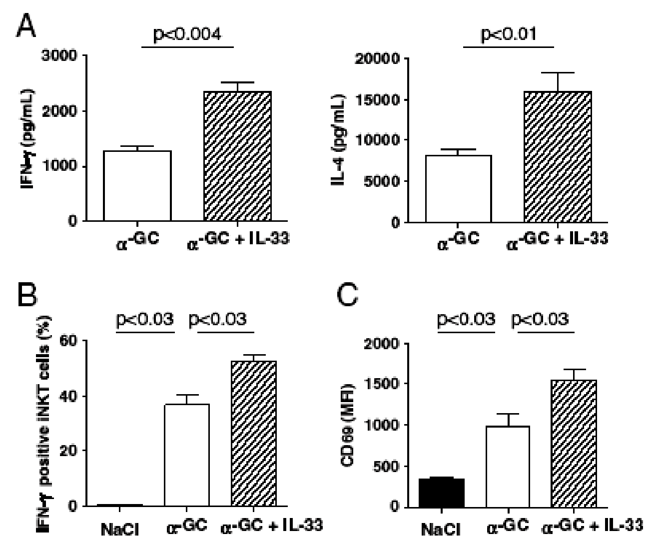

Figure 4. IL-33 amplifies both IFN-γ and IL-4 production by iNKT cells following α-GC *in vivo* challenge. Wild-type C57Bl/6 mice received a single i.p. injection of NaCl (black columns) or 2 μg α-GC in the absence (white columns) or presence of 4 μg of IL-33 (hatched columns) and were sacrificed 2 h later. (A) Serum IFN-γ and IL-4 concentrations were quantified by ELISA. (B, C) iNKT cells isolated from mononuclear liver cells were analysed for (B) intra-cytoplasmic IFN-γ (PE labelled) or (C) CD69 (FITC labelled) surface membrane expression. Data are means±SEM from 3 to 6 mice per group. Statistical significance was established by Student's unpaired t-test.

also support the notion that IL-33 may contribute not only to acquired but also to innate immune responses by targeting iNKT cells. Indeed, we demonstrate that IFN-γ production can be induced without TCR cross-linking, provided that the proinflammatory cytokine IL-12 is present during stimulation. Rapid regulation of ST2 expression during human NK-cell activation by IL-12 is one mechanism that has been proposed to explain the ability of IL-33 and IL-12 to synergistically induce IFN-γ [24]. However, we detected no up-regulation of ST2 surface expression on mouse iNKT cells in response to IL-12 both *in vitro* and *in vivo* (data not shown), suggesting that other mechanisms must be involved.

The capacity of IL-12-induced IFN-γ production by iNKT cells exposed to IL-33 is of potential interest in a patho-physiological setting, considering the well-established regulatory functions of this cell population in anti-infectious and anti-tumor immune responses [10, 11, 13, 25]. The potential involvement of the IL-33/ST2 pathway in innate cellular immune responses is also supported by our observation that IL-33 targets not only iNKT but also NK cells, both *in vitro* and *in vivo*, and by previous reports on the release of pro-inflammatory mediators by mast cells in response to IL-33 [5, 20, 26]. It is not yet clear how IL-33 increases cytokine production by iNKT or NK cells. According to our *in vitro* data, the interaction with its receptor alone is not sufficient to induce IFN-γ secretion, which failed to occur in the absence of TCR or IL-12 signalling in cultures set up with naive iNKT (CD5^{+} NK1.1^{+}) (Fig. 7B) or NK cells (Fig. 8). The same conclusion applies to IL-4 production (data not shown), suggesting that IL-33 acts as a cofactor rather than an inducer *per se*. Yet, somewhat paradoxically, IL-33 administration alone activated both iNKT (Fig. 1) and NK cells (data not shown) *in vivo*. It is conceivable that this occurs indirectly through endogenous factors generated by other target cells of IL-33. This hypothesis is consistent with the fact that IFN-γ could only be detected in response to IL-33 alone in cultures set up with whole spleen cells (Fig. 3B and C) but not with sorted iNKT cells. It remains to be determined whether endogenous IL-33 provides a general means of amplifying inherent iNKT-cell activities, by enhancing both IL-4 and IFN-γ secretion, as

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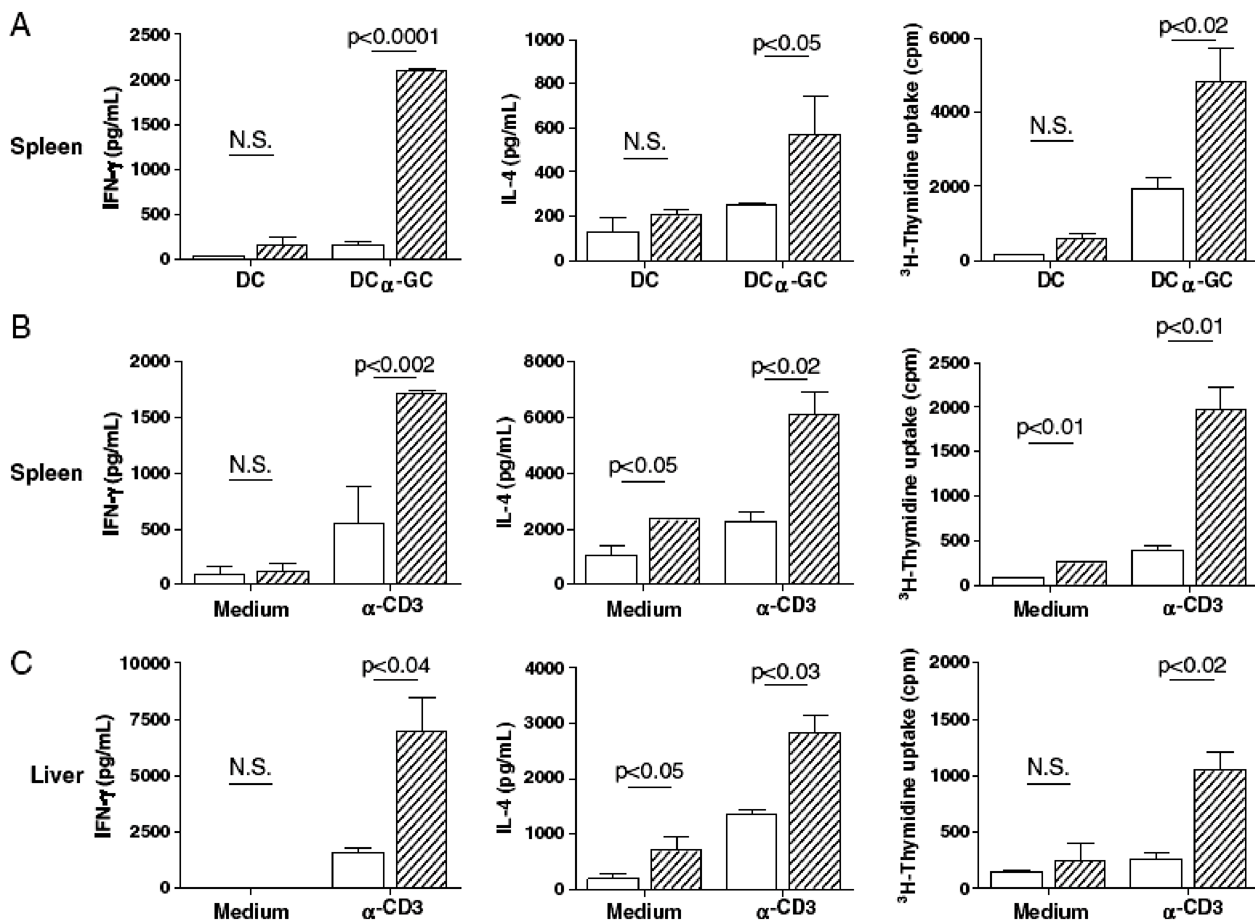

Figure 5. IL-33 directly induces a preferential increase in IFN-γ rather than IL-4 production by iNKT cells upon TCR engagement. Freshly sorted iNKT (α-GC-loaded CD1d TT$^+$ CD5$^+$) cells from (A, B) spleen and (C) liver from wild-type C57Bl/6 mice were cultured for 48 h with (A) α-GC-loaded (DC α-GC) or unloaded DC (DC) or (B, C) on plates coated with or without anti-CD3 mAb, and in the presence (hatched columns) or absence (white columns) of IL-33. IFN-γ (left panels) and IL-4 (middle panels) concentrations in the supernatants were quantified by ELISA. Proliferation rate (right panels) was determined by the incorporation of ^{3}H-thymidine. Data represent means±SEM of three separate experiments, each carried out with 8–10 pooled organs for each group of mice. NS: not significant. Statistical significance was established by Wilcoxon or Student's t-test, as appropriate.

previously proposed for IL-12 [16, 17]. This hypothesis is consistent with the constitutive surface expression of T1/ST2 receptors by iNKT cells.

As to the molecular events leading to the pro-Th1 effect, our data support the conclusion that IL-33 amplifies the signalling pathway initiated by TCR cross-linking or IL-12 since it has no effect on its own. It remains to be elucidated whether this occurs through the recruitment of MyD88 adaptor protein, which we found to be required for the activity of IL-33.

Another important issue concerns the relevance of our results in humans. According to preliminary experiments, IL-33 amplifies the expansion of iNKT cells in PBMC in response to the α-GC ligand and enhances the production of IFN-γ by IL-12-activated NK cells (J. M. G. and A. B.; unpublished data). These data, along with those recently reported by Smithgall *et al.* [24], support a similar mechanism of action in mice and humans.

In conclusion, we demonstrate a new pro-Th1 activity of IL-33, which can act as a co-stimulatory factor in innate cellular immune responses. Our findings challenge the prevailing opinion that IL-33 is strictly a pro-Th2 cytokine and provide further evidence for the importance of the microenvironment and the cellular context for determining both Th1 and Th2-oriented immune responses.

Materials and methods

Reagents

Murine rIL-12, rIL-33 and anti-IL-12 mAb (clone C17.8) were purchased from R&D Systems (Abingdon, UK). α-GC (KRN 7000) was provided by the Pharmaceutical Research Laboratory of Kirin Brewery (Gunma, Japan). Fluorochrome-conjugated anti-βTCR (clone H57-597), anti-CD3 (clone 500-A2), anti-CD4 (clone RM4-5), anti-CD5 (clone 55-7.3), anti-CD69 (clone H1-2F3), anti-CD44 (IM7), anti-IFN-γ (clone XMG1.2) and

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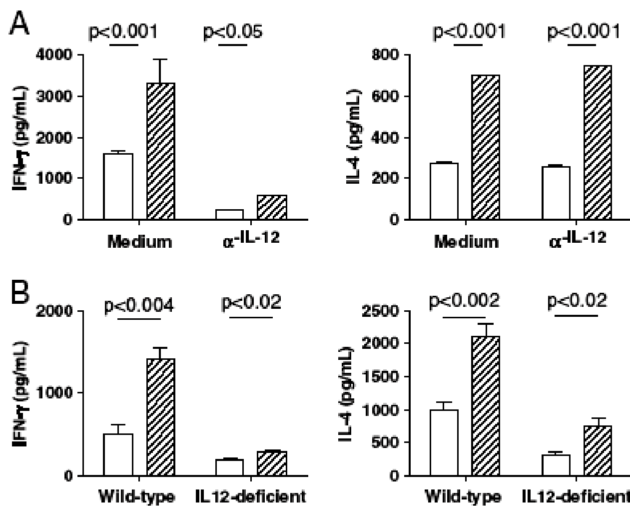

Figure 6. IL-12 contributes to the enhancement of IFN-γ production but not that of IL-4 in response to the co-treatment of α-GC and IL-33. (A) Freshly sorted iNKT (α-GC-loaded CD1d TT^{+}CD5^{+}) cells from the spleens of wild-type C57Bl/6 mice were cultured for 48h with α-GC-loaded (DC α-GC), in the presence (hatched columns) or absence (white columns) of IL-33 with or without neutralizing anti-IL-12 mAb. IFN-γ (left panels) and IL-4 (right panels) concentrations in the supernatants were quantified by ELISA. Statistical significance was established by Wilcoxon or Student's t-test, as appropriate. (B) Wild-type and mutant C57Bl/6 IL-12 p40$^{-/-}$ mice received a single i.p. injection of NaCl or IL-33 (data not shown) or 2 μg α-GC in combination with 4 μg of IL-33 (hatched columns) or alone (white columns), and were sacrificed 2h later. IFN-γ (left panels) and IL-4 (right panels) concentrations in sera were quantified by ELISA. The concentrations of IL-4 and IFN-γ were less than 20pg/mL in mice injected with NaCl or IL-33 alone. Statistical significance was established by Student's unpaired t-test.

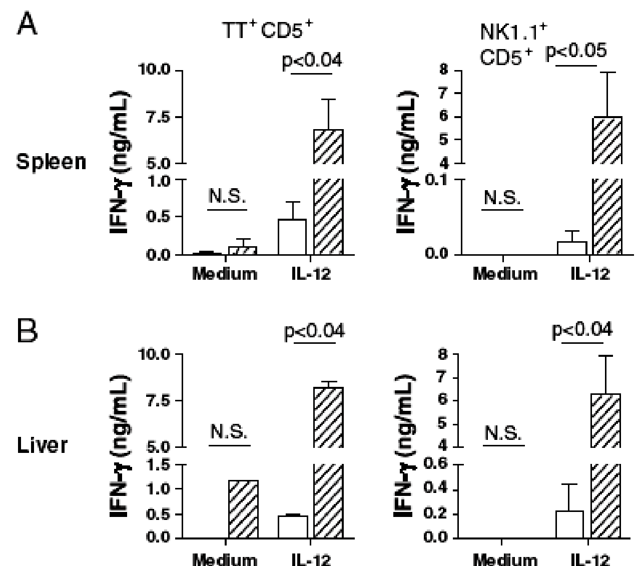

Figure 7. IL-33 enhances IL-12-induced IFN-γ production by iNKT cells without TCR engagement. Freshly sorted splenic (upper panels) and liver (bottom panels) (A) α-GC-loaded CD1d TT^{+}CD5^{+} or (B) NK1.1^{+}CD5^{+} cells from wild-type C57Bl/6 mice were cultured for 48h with or without IL-12, in the presence (hatched columns) or absence (white columns) of IL-33. IFN-γ in supernatants was quantified by ELISA. Data represent the means±SEM of three separate experiments, each carried out with 8–10 pooled organs. NS: not significant. Statistical significance was established by Wilcoxon or Student's t-test, as appropriate.

anti-NK1.1 (clone PK136), and corresponding isotype controls were from BD Pharmingen (San Diego, CA). The FITC-conjugated anti-T1/ST2 and its FITC-conjugated isotype control Ab were obtained from MD Bioscience and BD Pharmingen, respectively. APC-conjugated α-GC-loaded CD1d TT was obtained through the NIH tetramer facility.

Production of human rIL-33

The human cDNA encoding IL-33 aa 112–270 [3] was subcloned into expression vector pET-15b (Novagen), and human rIL-33 was produced in *E. coli* BL21pLysS (Novagen) and purified on Ni-NTA agarose (Qiagen), according to the manufacturer's instructions.

Mice and *in vivo* treatments

Seven- to eight-wk-old mice were used in this study. Wild-type, mutant Jα$^{-/-}$18, MyD88$^{-/-}$ female mice on a C57Bl/6 genetic background and wild-type female mice on a BALB/c genetic background were bred and maintained in our animal facility under specific pathogen-free conditions. Mutant C57Bl/6 IL-12 p40$^{-/-}$ mice and their wild-type male controls from the Jackson Laboratory were provided by Jean-François Arnal (Institut National de la Santé et de la Recherche Médicale, U589, Toulouse, France). The *in vivo* efficiency of IL-33 was controlled by measuring the increase of the hematopoietic cell counts in the spleen of treated mice. Based on preliminary experiments using a seven-day treatment with various doses of IL-33 (1, 2, 4 and 8 μg daily) followed by sacrifice 12 h after the last injection, we currently injected a dose of 4 μg per day and per mouse, which was sufficient to induce reproducibly a maximal splenomegaly, as previously described by Schmitz *et al.* [1]. In another series of experiments, mice received a single i.p. injection of 2 μg α-GC in combination or not with 4 μg of IL-33, and were sacrificed 2 h later. Animal experiments were performed according to the French institutional committee.

Cell preparation

All cell preparations were carried out in complete RPMI ((RPMI 1640; Invitrogen Life Technology, Grand Island, NY) supplemented with 10% FCS, antibiotics and 2-ME). After perfusion with PBS, livers were homogenized through a 100-μm cell strainer and washed. Parenchymal cells were removed by centrifugation at 50g for 5 min. The suspension was centrifuged over a 35% Percoll (Amersham Biosciences Europe, Orsay, France) gradient and mononuclear cells (MNC) were isolated by harvesting the

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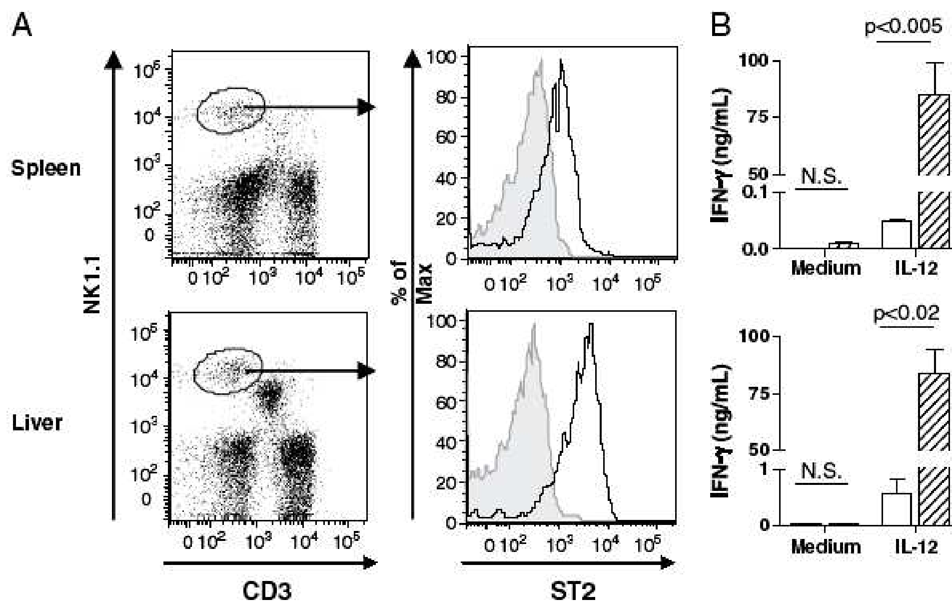

Figure 8. IL-33 enhances IL-12-induced IFN-γ production by NK cells. (A) Flow cytometry plot showing gating for splenic and liver NK ($CD5^-NK1.1^+$) cells isolated from wild-type C57Bl/6 mice and ex *vivo* expression of the T1/ST2 receptor (bold line) by these NK cells relative to the isotype control (thin line). One representative experiment out of three is shown. (B) Freshly sorted splenic (top panel) and liver (bottom panel) NK ($CD5^-NK1.1^+$) cells from wild-type C57Bl/6 mice were cultured for 48 h with or without IL-12 in the presence (hatched columns) or absence (white columns) of IL-33. IFN-γ concentrations in the supernatants were quantified by ELISA. Data represent the means±SEM of three separate experiments, each carried out with 8–10 pooled organs. Statistical significance was established by Wilcoxon or Student's t-test, as appropriate. NS: not significant.

interphase. Spleen cells and thymocytes recovered after homogenization and lysis of RBC in ammonium chloride buffer were suspended in complete RPMI 1640.

Staining for flow cytometry, FACS sorting and cell purification

Cells were re-suspended in staining buffer (PBS with 10% FCS) and anti-CD16/32 (BD Pharmingen) to block non-specific binding. Membrane labelling, as well as intracellular cytokine staining, was performed as described before [16]. At least 1500 events gated among the population of interest were analysed on a FACS Canto cytometer using BD FACSDiva software (BD Bioscience). The proportion of α-GC-unloaded CD1d TT^+ cells among gated β-TCR^+ T cells was always below 0.05, 0.1, and 0.5% in thymus, spleen and liver, respectively. iNKT cells were sorted from thymus, liver or spleen as α-GC-loaded CD1d TT^+ $CD5^+$ cells, while NKT and NK subsets were distinguished and sorted from the liver or spleen as $CD5^+NK1.1^+$ and $CD5^-NK1.1^+$ cells. In some experiments, thymic iNKT cells were sorted after a 5-day expansion of whole thymocytes with IL-7 (see section *Cell culture and cytokine assays*). Prior to sorting, freshly isolated thymocytes or splenocytes were enriched for iNKT and/or NK cells by magnetic depletion of CD8, CD11b, CD62L and CD19 cells (Invitrogen Life Technology), according to the manufacturer's instructions. Sorted cells were routinely >97% pure.

Cell culture and cytokine assays

Activation and expansion of thymic iNKT cells were assessed by flow cytometry after a 5-day culture of whole thymocytes (20×10^6/well) with or without IL-33 (10 ng/mL) in the presence of IL-7 (40 ng/mL), as previously described [18]. Bone marrow-derived DC were prepared as reported [19]. On day 6, DC were loaded or not with α-GC (100 ng/mL) for 18 h. A total of 2.5×10^4 sorted iNKT, NK and NKT cells were cultured for 48 h in 200 μL complete RPMI with or without coated anti-CD3 mAb (10 μg/mL, BD Pharmingen), α-GC-loaded or unloaded DC (5.0×10^3), in the presence or absence of IL-33 (10 ng/mL) or IL-12 (20 ng/mL) or both, and with or without anti-IL-12 mAb (5 μg/mL) in round-bottomed 96-well plates at 37°C and 5% CO_2. In another set of experiments, total splenocytes (0.5×10^5 cells/well) were incubated for 48 h with or without α-GC (100 ng/mL) in the presence or absence of IL-33 (10 ng/mL). IL-4 and IFN-γ in supernatants were quantified using standard sandwich ELISA, as previously described [18]. Cells were pulsed [^{3}H]thymidine, then harvested and thymidine uptake was assessed as described [14].

RNA isolation and real-time RT-PCR

Total RNA was extracted from thymic iNKT and T lymphocytes or from liver, spleen and thymic iNKT cells using the SV Total RNA isolation Kit® (Promega, Charbonnieres-lesbains, France) and then subjected to a reverse transcription reaction using

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high-capacity cDNA archive kit® (Applied Biosystem, Foster City, CA). A total of 30 ng total complementary DNA was used as a template for amplification with primers specific for T1/ST2 (for.: 5′TACCAGGGTGGAGCCTACT3′, rev.: 5′GCCCAACCTTCTACCTC-CTC3′); IL-1RacP (for.: 5′TGAGCTTTTTCATCCCCTTG3′, rev.: 5′ATAGATCTGGGGTGGCAATG3′) and 18S (for.: 5′CGCCGCTA GAGGTGAAATTC3′, rev.: 5′TTGGCAAATGCTTTCGCTC3′) used at a 250 nM final concentration. The real-time quantitative PCR were performed as previously described [16].

Statistical analysis

Data are expressed as means $\pm$ SEM. Mean differences between experimental groups were evaluated by Student's unpaired *t*-test. Paired statistical analysis of the *in vitro* effect of IL-33 was performed according to Wilcoxon or Student's *t*-test, as appropriate.The *p*-values under 0.05 were considered statistically significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G. *et al.*, IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005. 23: 479–490.

2 Baekkevold, E. S., Roussigne, M., Yamanaka, T., Johansen, F. E., Jahnsen, F. L., Amalric, F., Brandtzaeg, P. *et al.*, Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am. J. Pathol.* 2003. 163: 69–79.

3 Carriere, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., Bouche, G. and Girard, J. P. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor *in vivo*. *Proc Natl Acad Sci USA*. 2007. 104: 282–287.

4 Chackerian, A. A., Oldham, E. R., Murphy, E. E., Schmitz, J., Pflanz, S. and Kastelein, R. A., IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J.Immunol.* 2007. 179: 2551–2555.

5 Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U., IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl. Acad. Sci. USA* 2007. 104: 18660–18665.

6 Ho, L. H., Ohno, T., Oboki, K., Kajiwara, N., Suto, H., Iikura, M., Okayama, Y. *et al.*, IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *J. Leukoc. Biol.* 2007. 82: 1481–1490.

7 Coyle, A. J., Lloyd, C., Tian, J., Nguyen, T., Erikkson, C., Wang, L., Ottoson, P. *et al.*, Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med.* 1999. 190: 895–902.

8 Lohning, M., Stroehmann, A., Coyle, A. J., Grogan, J. L., Lin, S., Gutierrez-Ramos, J. C., Levison, D., *et al.*, T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl. Acad. Sci. USA* 1998. 95: 6930–6935.

9 Xu, D., Chan, W. L., Leung, B. P., Huang, F., Wheeler, R., Piedrafita, D., Robinson, J. H. and Liew, F.Y., Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 1998. 187: 787–794.

10 Kronenberg, M. and Gapin, L., The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2002. 2: 557–568.

11 Bendelac, A., Savage, P. B. and Teyton, L., The biology of NKT cells. *Annu. Rev. Immunol.* 2007. 25: 297–336.

12 Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S., Ohta, A., Ohmi, Y., Sato, M. *et al.*, The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 1999. 189: 1121–1128.

13 Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y. *et al.*, Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 1997. 278: 1623–1626.

14 Leite-De-Moraes, M. C., Hameg, A., Pacilio, M., Koezuka, Y., Taniguchi, M., Van, K. L., Schneider, E. *et al.*, IL-18 enhances IL-4 production by ligand-activated NKT lymphocytes: a pro-Th2 effect of IL-18 exerted through NKT cells. *J. Immunol.* 2001. 166: 945–951.

15 Hameg, A., Gouarin, C., Gombert, J. M., Hong, S., Van, K. L., Bach, J. F. and Herbelin, A., IL-7 up-regulates IL-4 production by splenic NK1.1+ and NK1.1- MHC class I-like/CD1-dependent CD4+T cells. *J. Immunol.* 1999. 162: 7067–7074.

16 Zhu, R., Diem, S., Araujo, L. M., Aumeunier, A., Denizeau, J., Philadelphe, E., Damotte, D. *et al.*, The Pro-Th1 cytokine IL-12 enhances IL-4 production by invariant NKT cells: relevance for T cell-mediated hepatitis. *J. Immunol.* 2007. 178: 5435–5442.

17 Brigl, M., Bry, L., Kent, S. C., Gumperz, J. E. and Brenner, M. B., Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 2003. 4: 1230–1237.

18 Herbelin, A., Gombert, J. M., Lepault, F., Bach, J. F. and Chatenoud, L., Mature mainstream TCR alpha beta+CD4+thymocytes expressing L-selectin mediate "active tolerance" in the nonobese diabetic mouse. *J. Immunol.* 1998. 161: 2620–2628.

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19 Allakhverdi, Z., Smith, D. E., Comeau, M. R. and Delespesse, G., Cutting edge: the ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J. Immunol.* 2007. 179: 2051–2054.

20 Iikura, M., Suto, H., Kajiwara, N., Oboki, K., Ohno, T., Okayama, Y., Saito, H. *et al.*, IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest.* 2007. 87: 971–978.

21 Cui, J., Watanabe, N., Kawano, T., Yamashita, M., Kamata, T., Shimizu, C., Kimura C. *et al.*, Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Valpha14 natural killer T cells. *J. Exp. Med.* 1999. 190: 783–792.

22 Nagarajan, N. A. and Kronenberg, M., Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J. Immunol.* 2007. 178: 2706–2713.

23 Burdin, N., Brossay, L. and Kronenberg, M., Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 1999. 29: 2014–2025.

24 Smithgall, M. D., Comeau, M. R., Yoon, B. P., Kaufman, D., Armitage, R. and Smith, D.E., IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 2008. 8: 1019–1030.

25 Takeda, K., Hayakawa, Y., Atsuta, M., Hong, S., Van, K. L., Kobayashi, K., Ito, M. *et al.*, Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12. *Int. Immunol.* 2000. 12: 909–914.

26 Moulin, D., Donze, O., Talabot-Ayer, D., Mezin, F., Palmer, G. and Gabay, C., Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine* 2007. 40: 216–225.

Abbreviations: **α-GC:** α-galactosylceramide · **IL-1RAcP:** IL-1R accessory protein · **iNKT cell:** invariant natural killer T cell · **MNC:** mononuclear cells · **TT:** tetramer

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Article n°2

Les lymphocytes NKT invariants (iNKT) inhibent le développement de la lignée TH17

Lennart T. Mars, Luiza Araujo, Philippe Kerschen, Séverine Diem, Elvire Bourgeois, Linh Pham Van, Nadège Carrié, Michel Dy, Roland S. Liblau, André Herbelin

Les cellules T se différencient en sous-populations effectrices distinctes en réponse à un pathogène. Les lymphocytes NKT invariants (iNKT), restreints par CD1d, qui se trouvent à l'interface entre l'immunité innée et l'immunité adaptative, orientent ce processus : ils peuvent soit amplifier, soit bloquer les réponses Th1 et Th2. La nouvelle population de cellules T CD4(+) auxiliaire Th17 identifiée récemment est impliquée dans les défenses contre certains microbes, dans l'autoimmunité et dans le pathogénèse de l'asthme. Pour analyser l'influence des cellules iNKT sur la différenciation des cellules T naïves, nous avons utilisé un modèle de transfert adoptif de cellules TCD4(+) spécifiques d'antigène. L'activation des cellules T CD4(+)CD25(-)CD62L(+) transférées à des souris receveuses, permet normalement l'expansion et la différenciation Th17. Cette étude a établi que l'activation *in vivo* des cellules iNKT empêchait le développement des cellules T naïves vers la lignée Th17. Cette réponse Th17 revenait à la normale lors du transfert adoptif de cellules iNKT dans des souris Jα18(-/-), confirmant le fait que les cellules iNKT contrôlent le compartiment Th17. Ces résultats concordent avec l'observation d'une augmentation des réponses Th17 chez des souris dépourvues de cellules iNKT. Les expériences de neutralisation cytokinique *in vivo* ont révélé le rôle de l'IL-4, de l'IL-10 et de l'IFN-γ dans cette régulation. Les résultats suggèrent que les cellules iNKT jouent un rôle important dans le contrôle de la lignée Th17 et constituent une barrière naturelle régulant les réponses Th17.

Invariant NKT cells inhibit development of the Th_{17} lineage

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T cells differentiate into functionally distinct effector subsets in response to pathogen encounter. Cells of the innate immune system direct this process; CD1d-restricted invariant natural killer T (iNKT) cells, for example, can either promote or inhibit Th_1 and Th_2 responses. Recently, a new subset of $CD4^+$ T helper cells, called Th_{17}, was identified that is implicated in mucosal immunity and autoimmune disorders. To investigate the influence of iNKT cells on the differentiation of naïve T cells we used an adoptive transfer model of traceable antigen-specific $CD4^+$ T cells. Transferred naïve $CD25^-CD62L^+$ $CD4^+$ T cells were primed by antigen immunization of the recipient mice, permitting their expansion and Th_{17} differentiation. This study establishes that in vivo activation of iNKT cells during T-cell priming impedes the commitment of naïve T cells to the Th_{17} lineage. In vivo cytokine neutralization experiments revealed a role for IL-4, IL-10, and IFN-γ in the iNKT-cell-mediated regulation of T-cell lineage development. Moreover, by comparing IL-17 production by antigen-experienced T cells from unmanipulated wild-type mice and iNKT-cell-deficient mice, we demonstrate an enhanced Th_{17} response in mice lacking iNKT cells. This invigorated Th_{17} response reverts to physiological levels when iNKT cells are introduced into $J\alpha18^{-/-}$ mice by adoptive transfer, indicating that iNKT cells control the Th_{17} compartment at steady state. We conclude that iNKT cells play an important role in limiting development of the Th_{17} lineage and suggest that iNKT cells provide a natural barrier against Th_{17} responses.

autoimmune encephalomyelitis | immune regulation | iNKT cells | multiple sclerosis

To respond to diverse microbial infections, T cells differentiate into functionally distinct subsets that secrete unique combinations of cytokines (1). Recently, a novel subset of T helper (Th) cells was identified—called Th_{17} cells—that produce IL-17A, IL-17F, and IL-22 (2). Th_{17} cells protect the host against extracellular pathogens encountered at mucosal surfaces, but they also play a detrimental role in experimental models of multiple sclerosis, as well as in human inflammatory bowel disease and psoriasis (2). In mice, cytokines TGF-β and IL-6 initiate the differentiation of T cells into the Th_{17} lineage by inducing expression of a transcription factor called retinoic acid receptor-related orphan nuclear receptor (ROR)-γt and of the IL-23 receptor (3–6). IL-21 and IL-23 further support the differentiation of Th_{17} cells, permitting IL-22 expression (6–10). In addition to providing autocrine support to the cells that produce them, some lineage-specific cytokines can impede the development of other Th lineages. For example, IL-21 secretion by Th_{17} cells inhibits Th_1 cytokines (11), whereas the reciprocal regulation of Th_1 and Th_2 responses is mediated by IL-4 and IFN-γ (12), both of which antagonize Th_{17} differentiation (13, 14). Upon pathogen encounter, innate immune cells critically influence Th differentiation. For example, triggering of Toll-like receptors (TLRs) on dendritic cells (DCs) drives IL-12 production, which favors Th_1 differentiation. Similarly, upon engagement of their receptors dectin-1 or nucleotide oligomerization domain 2 (Nod2), DCs produce IL-6 and IL-23, which orientate T cells to the Th_{17} lineage (15–17). Other cells of the innate immune system that influence Th differentiation include invariant natural killer T cells (iNKT), a CD1d-restricted T-cell population that expresses an invariant T-cell receptor α-chain by using a $V\alpha14$-$J\alpha18$ rearrangement in mice and a $V\alpha24$-$J\alpha18$ rearrangement in humans. Like conventional T cells, iNKT cells comprise distinct functional subsets. Notably, $NK1.1^{pos}$ iNKT cells produce IL-4 and IFN-γ, whereas $NK1.1^{neg}$ iNKT cells produce IL-17 and IL-21 (18–21). The iNKT cell response is elicited by the presentation of pathogen-derived or endogenous glycolipid antigens by CD1d-expressing antigen-presenting cells (APCs) (22). iNKT cells can support and sustain Th_1 responses by activating NK cells, which secrete IFN-γ, thus facilitating DC maturation and IL-12 production (22). Under different inflammatory conditions, iNKT cell activation favors Th_2 differentiation by producing IL-4, thereby conferring clinical benefit in animal models of organ-specific autoimmune diseases (23–27).

The ability of iNKT cells to regulate the differentiation of conventional Th_1 and Th_2 cells raises the question of whether they might also influence the Th_{17} lineage. On one hand, the $NK1.1^{neg}$ iNKT cell subset that produces IL-17 and IL-21 might support and sustain Th_{17} responses (18–21); on the other hand, the clinical benefit provided by the activation or enrichment of iNKT cells in Th_{17}-driven models of organ-specific autoimmune diseases suggests that iNKT cells might control pathogenic Th_{17} responses (23–26, 28, 29). This study investigated the influence of iNKT cells on the Th_{17} response. We found that activation of iNKT cells in vivo impedes commitment of naïve $CD4^+$ T cells to the Th_{17} lineage. Moreover, when comparing the Th_{17} compartment between wild-type and iNKT-cell-deficient mice, we found that antigen (Ag)-experienced $CD4^+$ T cells produce more IL-17 when they are isolated from iNKT-cell-deficient mice ($J\alpha18^{-/-}$). This invigorated Th_{17} response in the $J\alpha18^{-/-}$ mice can be corrected by the adoptive transfer of iNKT cells. Consequently, our data reveal that iNKT cells control the Th_{17} lineage in healthy, unmanipulated mice and illustrate the importance of iNKT cells both in regulating Th_{17} lineage development and in the persistence of the Th_{17} population.

Results and Discussion

iNKT Cells Regulate IL-17 Production by $CD4^+$ T Cells. To evaluate the influence of iNKT cells on Th_{17} cells, we first compared the capacity of $CD4^+$ T cells from wild-type and iNKT-cell-deficient

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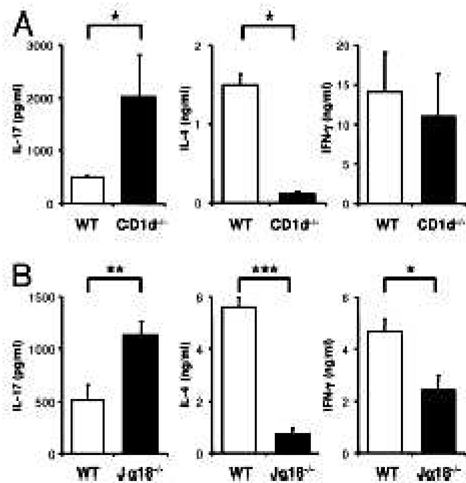

Fig. 1. iNKT cells negatively regulate IL-17 production by $CD4^+$ T cells. Purified $CD4^+$ T cells were stimulated for 72 h with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. (*A*) Release of IL-17 (*Left*), IL-4 (*Middle*), and IFN-γ (*Right*) by $CD4^+$ T cells from (*A*) wild-type (WT) or $CD1d^{-/-}$ C57BL/6 mice or (*B*) wild-type (WT) or $J\alpha18^{-/-}$ C57BL/6 mice was assayed by ELISA. Each data point represents the mean ± SEM of 2 (*A*) or 3 (*B*) experiments.

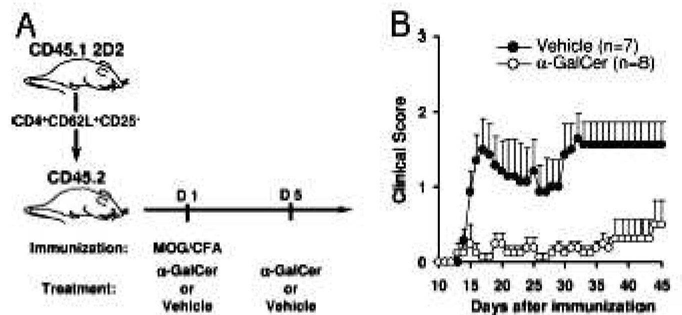

Fig. 2. In vivo treatment with α-GalCer prevents EAE. (*A*) The 2D2 transfer model uses traceable MOG-specific naïve $CD4^+$ T cells purified from CD45.1 congenic 2D2 mice. Twenty-four hours after transfer of 10^5 $CD62L^+CD25^-$ $CD4^+$ T cells, the recipient CD45.2 C57BL/6 mice were immunized with MOG_{35-55} in the presence either of α-GalCer or the PBS/DMSO solvent (vehicle). (*B*) The average clinical score from α-GalCer-treated mice (open circles, $n = 8$) and vehicle-treated controls (filled circles, $n = 7$) from 2 independent experiments is presented.

($CD1d^{-/-}$, $J\alpha18^{-/-}$) animals to produce IL-17. Splenic $CD4^+$ T cells purified from unmanipulated C57BL/6 mice were stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 mAbs in the presence of IL-23. Under such APC-free conditions, IL-23 promoted the expansion of preexisting IL-17-producing cells, but did not permit de novo Th_{17} differentiation of naïve $CD4^+$ T cells (4, 6, 30). $CD4^+$ T cells from the iNKT-cell-deficient $CD1d^{-/-}$ mice produced up to 3-fold more IL-17 than $CD4^+$ T cells from wild-type mice (Fig. 1*A*), suggesting that a CD1d-dependent T-cell population controls the Th_{17} lineage. By contrast, the production of IL-4 was significantly reduced in $CD4^+$ T-cell cultures from $CD1d^{-/-}$ mice, indicative of the absence of iNKT cells, which produce large amounts of IL-4 in response to TCR stimulation (31). To unequivocally assess the implication of CD1d-restricted iNKT cells we determined the cytokine production of stimulated $CD4^+$ T cells from $J\alpha18^{-/-}$ mice that selectively lack iNKT cells. Similar to the phenotype observed with $CD1d^{-/-}$ mice, $CD4^+$ T cells from $J\alpha18^{-/-}$ mice produced more IL-17 as compared with $CD4^+$ T cells from wild-type mice (Fig. 1*B*), confirming that iNKT cells limit IL-17 production in vitro. Stimulated $CD4^+$ T cells from $J\alpha18^{-/-}$ mice also produced less IL-4 than their wild-type counterparts (Fig. 1*B*) compatible with the absence of iNKT cells. A less pronounced reduction in IFN-γ secretion was equally observed. To assess if the impact of iNKT cells on the Th_{17} lineage extends to other mouse strains we compared the cytokine profile of stimulated $CD4^+$ T cells from wild-type and $J\alpha18^{-/-}$ NOD mice, and observed a comparable inhibition of IL-17 secretion in the presence of iNKT cells (NOD: 864 ± 110 pg/ml vs. $J\alpha18^{-/-}$: 2707 ± 379 pg/ml; $P = 0.03$). Interestingly, the $CD4^+$ T-cell cultures from NOD mice produced more IL-17 than C57BL/6-derived cultures, potentially reflecting the autoimmune process developing in NOD mice.

Activating iNKT Cells in Vivo Inhibits Autoantigen-Specific Th_1 and Th_{17} Responses. To investigate the influence of iNKT cells on the Th_{17} lineage in vivo, we used the mouse model of experimental autoimmune encephalomyelitis (EAE), in which immunization with a peptide fragment of myelin oligodendrocyte glycoprotein (MOG) induces myelin-specific Th_1 and Th_{17} cells that synergistically contribute to the demyelinating lesions in the CNS (32, 33). In this model, activation of iNKT cells ameliorates the disease by reducing the intensity of the pathogenic T-cell response (24–26). To study the differentiation of autoreactive T cells at the single-cell level, we introduced a traceable population of naïve MOG_{35-55}-specific $CD4^+$ T cells into the mice before inducing EAE (Fig. 2*A*). These $CD62L^+CD25^-$ autoreactive $CD4^+$ T cells were purified from CD45.1 congenic, 2D2 transgenic mice expressing an $I\text{-}A^b$-restricted TCR (Vα3.2-Vβ11) specific for MOG_{35-55} on $CD4^+$ T cells. These transferred MOG-specific T cells underwent an expansion of up to 3,700-fold, 9 days after immunization with MOG_{35-55} (Fig. S1). Analysis of the intracellular cytokine profile of the congenic ($CD45.1^+$) MOG-specific T cells by FACS enabled us to quantify IFN-γ-producing Th_1 and IL-17-producing Th_{17} cells (Fig. S1*A*). Both the Th_1 and Th_{17} responses peaked 9 days after immunization in the spleen (Fig. S1*B*) and draining lymph nodes (LNs; data not shown), but the number of Th_{17} cells was significantly inferior to that of Th_1 cells. These mice developed moderate clinical signs of EAE, on average, 17 days after immunization (Fig. 2).

To validate the regulatory capacity of iNKT cells in this model, we treated immunized mice with 2 injections of α-GalCer (Fig. 2*A*), an exogenous glycolipid that closely resembles the microbial glycosphingolipids that are agonists of iNKT cells (34). These injections significantly reduced the severity of EAE when compared with the vehicle-injected control mice (Fig. 2*B*). In vitro recall responses to MOG_{35-55} revealed that α-GalCer injections inhibited the antigen-specific secretion of IL-17 (Fig. 3*A*), IFN-γ, and TNF-α (data not shown) by splenocytes. This was not due to loss of MOG-specific $CD45.1^+$ $CD4^+$ T cells, which were only slightly reduced after α-GalCer treatment (Fig. 3*B*). Instead, activation of iNKT cells with α-GalCer markedly reduced both the frequency (Fig. 3 *C* and *D*) and the absolute numbers (vehicle: $17 \pm 6 \times 10^3$ vs. α-GalCer: $2 \pm 1 \times 10^3$; $P = 0.001$) of Th_{17} cells as assessed by analysis of intracellular cytokines in the transferred $CD45.1^+$ $TCRV\alpha3.2^+$ $CD4^+$ T cells. A similar inhibition of Th_{17} differentiation was observed amongst endogenous $CD45.1^-$ $CD4^+$ T cells (data not shown). Interestingly, although the activation of iNKT cells also reduced the frequency (Fig. 3*E*) and slightly reduced the absolute numbers (vehicle: $34 \pm 12 \times 10^3$ vs. α-GalCer: $8 \pm 2 \times 10^3$; $P = 0.1$) of Th_1 cells, its impact on the Th_1 response appeared more modest than on the Th_{17} response. This inhibition of the MOG-specific Th_{17} and Th_1 responses is attributable to iNKT cells because α-GalCer treatment of $J\alpha18^{-/-}$ mice had no significant influence on the expansion and differentiation of autoreactive T cells (Fig. 3 *B*, *D*, and *E*). Similar results were obtained with cells from the draining LNs, where α-GalCer

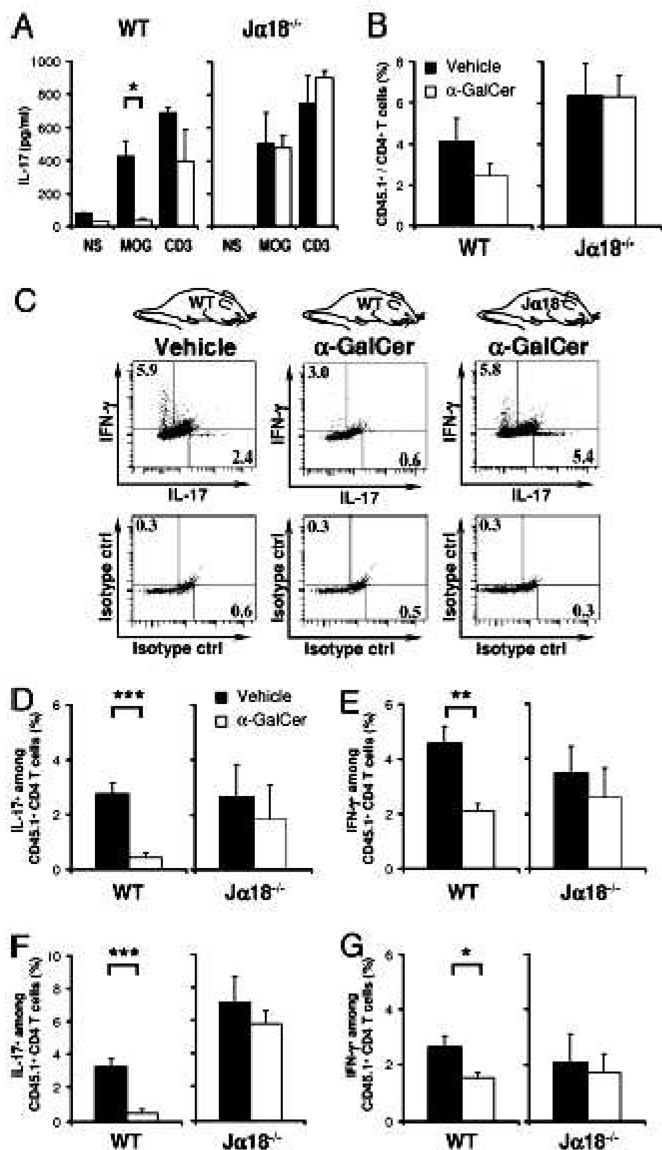

Fig. 3. Activation of iNKT cells by α-GalCer blocks the commitment of naïve MOG-specific T cells to the Th_1 and Th_{17} lineages. By using the 2D2 transfer model described in Fig. 2, we assessed MOG-specific T-cell differentiation in immunized C57BL/6 (WT) and Jα18$^{-/-}$ recipient mice treated with α-GalCer or vehicle. (*A*) Nine days after immunization, we assessed the in vitro recall response by splenocytes from α-GalCer-treated recipients (white bars; WT, *n* = 4; Jα18$^{-/-}$, *n* = 4) or vehicle-treated recipients (black bars; WT, *n* = 4; Jα18$^{-/-}$, *n* = 4). IL-17 release was measured from unstimulated (NS), MOG_{35-55}-stimulated (MOG; 100 μg/ml), and anti-CD3 mAb-stimulated (CD3) cultures after 72 h. (*B*) The number of CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells was established in mice treated with α-GalCer (white bars; WT, *n* = 11; Jα18$^{-/-}$, *n* = 4) or vehicle (black bars; WT, *n* = 11; Jα18$^{-/-}$, *n* = 4). α-GalCer slightly reduced the proportion of 2D2 CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells in the spleens of WT but not Jα18$^{-/-}$ mice. (*C–G*) The MOG-specific Th_{17} and Th_1 differentiation was analyzed by establishing the intracellular IL-17 and IFN-γ production of CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells from WT and Jα18$^{-/-}$ recipient mice treated with α-GalCer or vehicle. Th_{17} differentiation is indicated by the frequency of IL-17-producing 2D2 T cells in the spleen (*C* and *D*) and draining LNs (*F*). Th_1 differentiation is indicated by the frequency of IFN-γ-producing 2D2 T cells in the spleen (*C* and *E*) and draining LNs (*G*). Intracellular cytokine analyses were also performed after antigen-specific restimulation with MOG_{35-55} and revealed a similar inhibition of Th_1 and Th_{17} responses by α-GalCer treatment, although the number of cytokine-positive cells was generally lower. Cytokine secretion in (*A*) presents 1 representative experiment of 3 performed. Intracellular cytokine analyses in (*B–G*) present 3 independent experiments for the WT mice and 1 experiment for Jα18$^{-/-}$ mice.

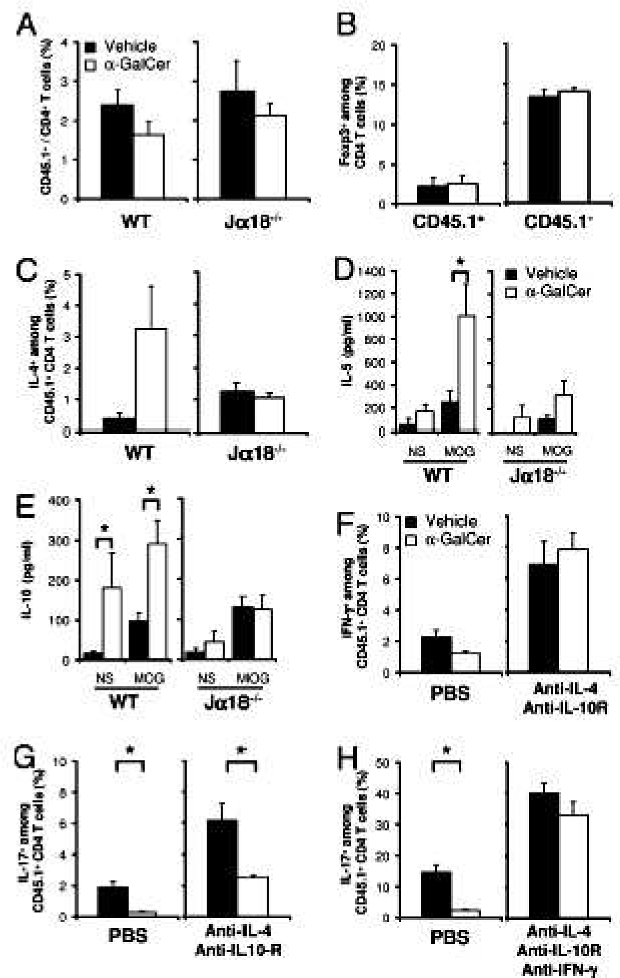

Fig. 4. Activation of iNKT cells by α-GalCer regulates the Th_1 and Th_{17} responses by immune deviation and IL-10 production. By using the 2D2 transfer model described in Fig. 2, we assessed MOG-specific T-cell differentiation in the draining LNs of immunized C57BL/6 (WT) and Jα18$^{-/-}$ mice. Nine days after immunization, the intracellular IFN-γ, IL-17, IL-4, and IL-10 expression by CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells was assessed in α-GalCer-treated mice (white bars; WT, *n* = 11; Jα18$^{-/-}$, *n* = 4) and vehicle-treated mice (black bars; WT, *n* = 11; Jα18$^{-/-}$, *n* = 4). (*A*) The effect of α-GalCer on the proportion of 2D2 CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells in the draining LNs of recipient mice. (*B*) The frequency of Foxp3-positive cells among 2D2 CD45.1$^+$TCRVα3.2$^+$ and endogenous CD45.1$^-$ CD4$^+$ T cells. (*C*) The proportion of IL-4-positive cells among 2D2 CD45.1$^+$TCRVα3.2$^+$ CD4$^+$ T cells increased upon α-GalCer treatment of WT, but not Jα18$^{-/-}$ mice, indicating an enhanced Th_2 differentiation in the presence of iNKT cells but not in their absence. (*D* and *E*) The release of IL-5 (D) and IL-10 (*E*) during the in vitro recall response was assessed in α-GalCer-treated mice (white bars; WT, *n* = 4; Jα18$^{-/-}$, *n* = 4) or vehicle-treated mice (black bars; WT, *n* = 4; KO, *n* = 4). Cytokine release in unstimulated (NS) and MOG_{35-55}-stimulated (100 μg/ml) cultures was measured after 72 h by Luminex immunoassay. (*F–H*) The role of IL-4, IL-10, and IFN-γ in regulating T-cell differentiation was evaluated in vivo by injecting neutralizing mAbs. Th_1 (*F*) and Th_{17} (G-H) commitment was assessed in mice treated with vehicle or α-GalCer that had received mAbs neutralizing IL-4 and IL-10R (*F* and *G*), or IL-4, IL-10R, and IFN-γ (*H*). The single-cell analysis presents 3 separate experiments for the WT mice (*A* and *C*) and a single experiment for the Jα18$^{-/-}$ mice (*A* and *C*). For the cytokine secretion (*D* and *E*), 1 representative experiment of 2 is shown. The cytokine neutralization data (*F–H*) are from 1 experiment involving 4 individual mice per group for each neutralization protocol.

treatment influenced expansion only modestly (Fig. 4*A*), but blunted IFN-γ and IL-17 production by MOG-specific CD4$^+$ T cells in an iNKT-cell-dependent manner (Fig. 3 *F* and *G* and Fig. S2). Taken together, our data show that activation of iNKT cells

in vivo inhibits differentiation of naïve autoreactive CD4+ T cells into pathogenic Th_1 and Th_{17} effector cells, and that the inhibitory effect is most pronounced on the Th_{17} lineage.

iNKT Cell-Induced IL-4 and IL-10 Inhibit the Th_1 Response, and IL-4, IL-10, and IFN-γ Are Required to Control the Th_{17} Response. The therapeutic efficacy of α-GalCer in EAE depends on the production of anti-inflammatory cytokines IL-4 and IL-10 (24, 25). To assess whether α-GalCer promotes the commitment of CD4+ T cells to the IL-4-producing Th_2 or IL-10-producing Tr_1 lineages, we analyzed the intracellular cytokine-profile of the MOG-specific T-cell response. In addition, given the reciprocal relationship between Th_{17} and inducible regulatory T cells (3, 4, 35), we enumerated the frequency of Foxp3+ CD4+ T cells. No variation in the proportion of Foxp3+ CD4+ T cells was observed among congenic 2D2 T cells and in the endogenous CD4+ T-cell pool (Fig. 4*B*). Evidence of immune deviation was obtained, as the proportion of IL-4-producing congenic 2D2 CD4+ T cells (and endogenous Th_2 cells; data not shown) augmented significantly in α-GalCer-treated wild-type mice when compared with the vehicle-treated mice (Fig. 4*C* and Fig. S2), and their restimulation in vitro is associated with elevated levels of the Th_2-associated cytokine IL-5 (Fig. 4*D*). By contrast, α-GalCer treatment of $J\alpha18^{-/-}$ mice had no effect on the frequency of Th_2 cells and their associated cytokines (Fig. 4 *C* and *D*), indicating that the Th_2 bias seen in wild-type mice depended on iNKT cells. Interestingly, an enhanced production of IL-10 was observed in cultures from α-GalCer-treated mice (Fig. 4*E*) irrespective of MOG_{35-55} restimulation. In addition, no detectable increase in the frequency of IL-10-producing MOG-specific 2D2 T cells (Fig. S2) was observed, suggesting that iNKT cells can promote IL-10 production by non-T cells.

To assess whether IL-4 and IL-10 contributed to the inhibition of Th_1 and Th_{17} differentiation by the in vivo α-GalCer treatment, we neutralized the effects of both IL-4 and IL-10 with mAbs injected i.p. Under these cytokine-blocking conditions, the proportion of Th_1 2D2 cells tripled and the regulatory effect of iNKT cell activation by α-GalCer on Th_1 cells was fully blocked (Fig. 4*F* and Fig. S3*A*). Concomitantly, the proportion of Th_{17} cells increased. Nevertheless, α-GalCer treatment still significantly limited the Th_{17} response (Fig. 4*G* and Fig. S3*A*). Given the capacity of iNKT cells to produce copious amounts of IFN-γ (22), the increase in IFN-γ-producing Th_1 cells after IL-4 and IL-10R neutralization, and the capacity of IFN-γ to antagonize Th_{17} differentiation (13, 14), we asked whether IFN-γ could be an additional factor involved in the regulation of Th_{17} commitment by iNKT cells. Neutralizing IFN-γ in vivo doubled the proportion of Th_{17} 2D2 CD4+ T cells in both PBS and α-GalCer-treated mice (Fig. S3*B*). Nevertheless, α-GalCer remained efficient in reducing the frequency of Th_{17} cells, indicating that IFN-γ is necessary but not sufficient for the iNKT-cell-mediated regulation of the Th_{17} response (Fig. S3*A*). Importantly, neutralizing IL-4, IL-10, and IFN-γ simultaneously tripled the proportion of Th_{17} cells and fully abrogated the capacity of α-GalCer to inhibit Th_{17} commitment (Fig. 4*H* and Fig. S3*A*). Blocking IL-4 + IL-10R and/or IFN-γ also prevented the EAE-protective effect of α-GalCer, rendering these mice susceptible to severe disease (Table S1). We conclude that iNKT-cell-induced IL-4 and IL-10 production mediates the inhibition of the Th_1 response, whereas the regulation of Th_{17} commitment by iNKT cells requires IL-4, IL-10, and IFN-γ.

iNKT Cells Regulate the Th_{17} Compartment at Steady State. Having established that iNKT cells control Th_{17} differentiation, we aimed to assess whether this cellular mechanism is of importance in controlling the Th_{17} compartment under steady-state conditions in vivo. To this end, we compared IL-17 production by Ag-experienced CD4+ T cells from wild-type and $J\alpha18^{-/-}$ mice in vitro. Splenic CD62L− CD4+ T cells were purified by FACS sorting. In addition, using the CD1d-αGalCer tetramer we eliminated iNKT cells to exclude their potential in vitro effects. Polyclonal activation in vitro revealed a 2-fold enhancement of IL-17 production when the Ag-experienced T cells were isolated from $J\alpha18^{-/-}$ mice when compared with those isolated from wild-type mice (Fig. 5*A*). No influence on the IFN-γ response was observed (cells from wild-type mice produced 3,150 pg/ml IFN-γ, those from $J\alpha18^{-/-}$ mice produced 3,155 pg/ml IFN-γ). IL-17 production by Ag-experienced CD4+ T cells was not affected by neutralization of IL-4 and IFN-γ in vitro (Fig. 5*B*), consistent with the reported resistance of committed Th_{17} cells to IL-4- and IFN-γ-mediated regulation (13, 14). These findings suggest that iNKT cells control the Th_{17} compartment in vivo at steady state as they limit the IL-17-producing capacity of Ag-experienced CD4+ T cells.

To investigate this novel function of iNKT cells in vivo, we reconstituted iNKT-deficient nonirradiated $J\alpha18^{-/-}$ mice with iNKT cells by adoptive transfer of 10^6 CD1d:α-GalCer tetramer-positive TCR-αβ thymocytes from wild-type mice. We assessed the impact of this iNKT cell reconstitution on the Th_{17} population after 21 days, a period that is probably insufficient for the Th17 compartment to be replenished by de novo activated naïve T cells at steady state. Thus, the data likely reflect the impact of the introduced iNKT cells on preexisting Ag-experienced cells. CD1d:α-GalCer tetramer staining revealed efficient grafting of wild-type iNKT cells in $J\alpha18^{-/-}$ mice as indicated by the 4% frequency of CD1d:α-GalCer tetramer-positive cells among liver mononuclear cells (Fig. 5*C*). Strikingly, this grafting of wild-type iNKT cells fully reversed the enhanced IL-17 production by CD4+ T cells from $J\alpha18^{-/-}$ mice (Fig. 5*D*), demonstrating that iNKT cells control the Th_{17} compartment at steady state. This effect was specific for the Th_{17} lineage because we observed no alteration in IFN-γ production by the Th_1 compartment (Fig. 5*E*). No reciprocal induction of Foxp3+ regulatory T cells was observed after iNKT-cell reconstitution (data not shown). To address whether the transferred iNKT cells impacted the frequency of Th_{17} cells in vivo we enumerated the proportion of IL-17-producing CD4+ T cells using intracellular cytokine staining. The reconstitution of iNKT cells reduced the frequency of Th_{17} cells almost to the level observed in unmanipulated WT mice (Fig. 5*F*). These splenic CD4+ T-cell cultures from reconstituted mice express on average 0.05% of iNKT cells (Fig. 5*C*), making an in vitro effect by iNKT cells unlikely. As such, our findings reveal a previously undescribed function for iNKT cells in regulating the size of the Th_{17} pool in vivo at steady state.

Conclusions. Th_{17} cells contribute to the host defense against extracellular pathogens, but excessive Th_{17} responses or those targeting self-antigens can provoke severe tissue injury (2, 36). Mechanisms controlling the Th_{17} subset might therefore be important to preserve the integrity of tissues. Our study reveals the importance of iNKT cells in regulating Th_{17} lineage commitment and persistence. We show that activation of iNKT cells with α-GalCer during priming of the CD4+ T-cell response prevents the differentiation of naïve CD4+ T cells toward the Th_{17} lineage without impairing T-cell expansion. The mechanism used by iNKT cells to abrogate Th_{17} commitment by naïve CD4+ T cells requires IL-4, IL-10, and IFN-γ. No reciprocal influence on Foxp3+ T cells was observed. After α-GalCer treatment IL-4 expression is likely initiated by iNKT cells and sustained by autoreactive Th_2 cells that regulated the Th_{17} response. α-GalCer activation of iNKT cells and the ensuing NK response are expected sources of IFN-γ, with a moderate impact of IFN-γ by the conventional Th_1 response due to its efficient inhibition by α-GalCer. Our data, therefore, suggest an important role for innate IFN-γ in regulating Th_{17} development. These mechanisms were effective in limiting CNS tissue damage in the paralytic mouse model of EAE, in which autoimmune Th_1 and

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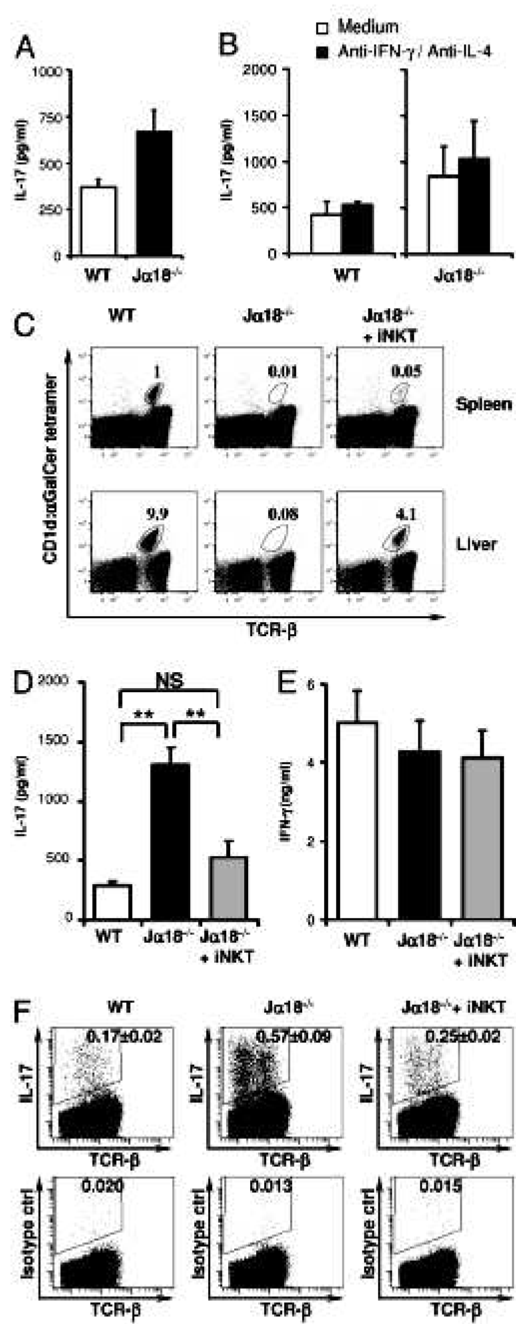

Fig. 5. iNKT cells inhibit IL-17 production by CD4⁺ T cells at steady state. (*A*) CD62L⁻ α-GalCer-CD1d tetramer^{neg} CD4⁺ T cells from wild-type (WT) or Jα18$^{-/-}$ C57BL/6 mice were sorted by FACS and stimulated with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. IL-17 levels were measured after 72 h. (*B*) As in (*A*) but in the presence or absence of neutralizing mAbs against IL-4 and IFN-γ. These mAbs failed to significantly enhance IL-17 production by Ag-experienced CD4⁺ T cells. Data are from 3 (*A*) or 2 (*B*) individual experiments using 2–4 pooled spleens per group. (*C*) Jα18$^{-/-}$ recipient mice received thymic iNKT cells (Jα18$^{-/-}$ + iNKT), and 3 weeks later the proportion of the CD1d:α-GalCer tetramer⁺ TCRβ⁺ (iNKT) cells (gated on mononuclear cells) in the spleen (*Upper*) and liver (*Lower*) of recipient mice was compared with that of the control wild-type (WT) and non-reconstituted Jα18$^{-/-}$ mice. A representative FACS plot is shown for each group. (*D–G*) Jα18$^{-/-}$ recipient mice received thymic iNKT cells as in (*C*). Three weeks later, MACS-purified splenic CD4⁺ T cells from the recipient and control mice were cultured with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. IL-17 (*D*) and IFN-γ (*E*) secretion were measured 72 h later in cultures derived from recipient (Jα18$^{-/-}$ + iNKT, $n = 6$) and control (WT, $n = 6$; Jα18$^{-/-}$, $n = 6$) mice. The frequency of Th_{17} cells (*F*) was assessed under identical conditions in cultures derived from iNKT-reconstituted (Jα18$^{-/-}$ + iNKT, $n = 2$) and control (WT, $n = 4$; Jα18$^{-/-}$, $n = 6$) mice. Data represent the mean ± SEM of n individual mice per group.

Th_{17} responses contribute to demyelinating lesions in the CNS (32, 33). This observation might also be of relevance for microbial infections where iNKT cells are activated by either pathogen-derived or endogenous glycolipid antigens presented in the hydrophobic groove of CD1d (37–39). It is conceivable that the activation of iNKT cells during infections might similarly influence T-cell differentiation and prevent the induction of an excessive Th_{17} response, thereby limiting secondary tissue damage.

Importantly, we show that iNKT cells also impact on the Th_{17} pool in vivo in unmanipulated mice. We reveal that iNKT cells restrain IL-17 production by CD4⁺ T cells in both C57BL/6 and autoimmune-prone NOD mice. Strikingly, the enhanced IL-17 production observed in iNKT-cell-deficient mice was fully reversed by the transfer of thymic iNKT cells. This was associated with the capacity of the transferred iNKT cells to reduce the frequency of Th_{17} cells in vivo, restoring a Th_{17} frequency similar to that observed in WT mice. Because no effect on IFN-γ production by antigen-experienced Th_1 cells was observed, it is assumed that iNKT cells specifically control the persistence of Ag-experienced Th_{17} cells. Under steady-state conditions, iNKT cells are activated and functionally engage dendritic cells and B cells (40, 41), which is thought to be of importance to prepare the host for future pathogen encounter. Our findings suggest that, in addition, iNKT cells impact on Ag-experienced CD4⁺ T cells at steady state, implying a role for iNKT cells in shaping the T-cell response before pathogen exposure. Given the role of the Th_{17} cell subset in inflammatory tissue damage, its confinement by iNKT cells might provide a barrier that limits predisposition to inflammatory diseases and supports iNKT cells as a therapeutic target in Th_{17} mediated diseases (42).

Materials and Methods

Mice, EAE Induction, and in Vivo Treatments. C57BL/6 mice (Charles River Laboratories), CD1d$^{-/-}$ mice (43), Jα18$^{-/-}$ mice (44), 2D2 MOG-TCR mice (45), and NOD mice were housed under specific pathogen-free conditions. All experimental protocols were approved by the local ethics committee and are in compliance with European Union guidelines. EAE was induced by s.c. immunization with 50 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; Mimotopes) emulsified in CFA (Difco) and 2 i.v. injections of pertussis toxin (day 0, 200 ng; day 2, 400 ng; List Biological Laboratories). Disease severity was monitored daily (28). Where indicated, mice were treated with two 4-μg doses of α-GalCer (Kirin Brewery) on day 0 (emulsified in the MOG_{35-55} CFA mixture) and day 4 (i.p.) or with anti-IL-4 (11B11), anti-IL10R (1B1), or anti-IFN-γ (XMG1.2) mAbs injected i.p. at a dose of 0.5 mg/mouse on days −3, −2, 0, 2, 5, and 8 for 2D2 differentiation, or days −2, −1, 0, 3, (0.5 mg/mouse) and 5, 9, 13, 17, and 21 (0.15 mg/mouse) for EAE.

T-Cell Purification and Adoptive Transfer. Splenic CD4⁺ T cells were isolated (>94% pure) by MACS positive selection (Miltenyi Biotec). Subsequently, conventional Ag-experienced CD4⁺ (CD1d:α-GalCer tetramer^{neg} CD62L⁻ CD4⁺) T cells were isolated by FACS (>97% pure). MOG-specific naïve CD62L⁺ CD25⁻ CD4⁺ cells were purified from CD45.1 congenic 2D2 mice by MACS negative selection (Miltenyi Biotec) by using anti-CD8 (YTS169), anti-B220 (RA3–6B2), anti-Mac1 (CI:A3–1), and anti-CD25 (7D4) mAbs followed by positive selection with anti-CD62L-coated beads, providing >80% CD4⁺ T cells, of which 95% expressed the transgenic 2D2 TCR. For reconstitution studies, freshly isolated thymocytes were enriched for iNKT cells by CD8 depletion (Invitrogen Life Technology); 3%−5% of recovered thymocytes were CD1d:α-GalCer tetramer⁺ TCRβ⁺ iNKT cells. The equivalent of 1.0×10^6 iNKT cells was adoptively transferred i.v. into Jα18$^{-/-}$ mice.

Intracellular and Intranuclear Cytokine Staining and Flow Cytometry. Single-cell suspensions from LNs (0.5×10^6/well) or spleen (3×10^6/well) were stimulated for 4 h with PMA (0.5 μg/ml), ionomycin (1 μg/ml; Sigma), and GolgiPlug™ (BD Biosciences). After FcγR blockade (mAb 2.4G2), cells were stained with fluorescently labeled mAbs: anti-TCR Vα3.2 (RR3–16), anti-CD4 (RM4–5), anti-CD45.1 (A20), and anti-cytokine mAbs against IL-10 (JES5–16E3), IFN-γ (XMG1.2), IL-17 (TC11–18H10), IL-4 (BVD4–1D11), or IgG1 isotype control (A110–1) using a Cytofix/Cytoperm™ Plus Kit (BD Biosciences) or an intranuclear Foxp3 detection kit (eBiosciences). Labeled cells were analyzed with a

LSRII flow cytometer (Becton Dickinson) using the BD FACS-Diva software. Cell sorting was performed using a FACSAria Flow Cytometer (Becton Dickinson).

In Vitro Cytokine Production. 2.0×10^5 $CD4^+$ T cells or 0.5×10^5 Ag-experienced $CD4^+$ T cells were cultured with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (10 μg/ml) mAbs (BD Biosciences) and IL-23 (10 ng/ml; R&D Systems). When indicated, anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) mAbs (BD Biosciences) were added. For in vitro recall responses, single-cell suspensions of LN cells (0.5×10^6/well) or splenocytes (1×10^6/well) were cultured with the indicated stimuli. Supernatants were analyzed for IFN-γ, IL-17A, or IL-4 by Duoset ELISA (R&D Systems) or tested for 10 different cytokines (IFN-γ, TNF-α, CXCL10, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and IL-17A) by using LINCOplex Multiplex immunoassays and Luminex instrumentation (Millipore).

Statistical Analyses. Data are presented as means of *n* individual mice per group ± SEM, unless otherwise stated. The Mann-Whitney *U* test was used, except for cytokine production, which was analyzed using the unpaired Student *t* test. Kinetics of EAE development was analyzed using the log rank test; EAE incidence and mortality using a χ^2 test; and severity using the Mann-Whitney *U* test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

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1. Mosmann TR (1988) Directional release of lymphokines from T cells. *Immunol Today* 9(10):306–307.
2. Ouyang W, Kolls JK, Zheng Y (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28(4):454–467.
3. Bettelli E, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441(7090):235–238.
4. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24(2):179–189.
5. Ivanov II, et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126(6):1121–1133.
6. Zhou L, et al. (2007) IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8(9):967–974.
7. Nurieva R, et al. (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448(7152):480–483.
8. Korn T, et al. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448(7152):484–487.
9. Liang SC, et al. (2006) Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203(10):2271–2279.
10. Zheng Y, et al. (2007) Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445(7128):648–651.
11. Mehta DS, Wurster AL, Grusby MJ (2004) Biology of IL-21 and the IL-21 receptor. *Immunol Rev* 202:84–95.
12. Murphy KM, Reiner SL (2002) The lineage decisions of helper T cells. *Nat Rev Immunol* 2(12):933–944.
13. Park H, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6(11):1133–1141.
14. Harrington LE, et al. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6(11):1123–1132.
15. van Beelen AJ, et al. (2007) Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 27(4):660–669.
16. Schnare M, et al. (2001) Toll-like receptors control activation of adaptive immune responses. *Nat Immunol* 2(10):947–950.
17. LeibundGut-Landmann S, et al. (2007) Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 8(6):630–638.
18. Michel ML, et al. (2007) Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* 204(5):995–1001.
19. Harada M, et al. (2006) IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. *J Exp Med* 203(13):2929–2937.
20. Coquet JM, et al. (2008) Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci USA* 105(32):11287–11292.
21. Rachitskaya AV, et al. (2008) Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J Immunol* 180(8):5167–5171.
22. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. *Annu Rev Immunol* 25:297–336.
23. Miyamoto K, Miyake S, Yamamura T (2001) A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413(6855):531–534.
24. Singh AK, et al. (2001) Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194(12):1801–1811.
25. Jahng AW, et al. (2001) Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194(12):1789–1799.
26. Furlan R, et al. (2003) Activation of invariant NKT cells by alphaGalCer administration protects mice from MOG35–55-induced EAE: Critical roles for administration route and IFN-gamma. *Eur J Immunol* 33(7):1830–1838.
27. Yoshimoto T, Bendelac A, Watson C, Hu-Li J, Paul WE (1995) Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science* 270(5243):1845–1847.
28. Mars LT, et al. (2002) Cutting edge: V alpha 14-J alpha 281 NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. *J Immunol* 168(12):6007–6011.
29. Grajewski RS, et al. (2008) Activation of invariant NKT cells ameliorates experimental ocular autoimmunity by a mechanism involving innate IFN-gamma production and dampening of the adaptive Th1 and Th17 responses. *J Immunol* 181(7):4791–4797.
30. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 278(3):1910–1914.
31. Yoshimoto T, Paul WE (1994) CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179(4):1285–1295.
32. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM (2008) IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med* 205(7):1535–1541.
33. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM (2008) Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14(3):337–342.
34. Kawano T, et al. (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278(5343):1626–1629.
35. Korn T, et al. (2008) IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci USA* 105(47):18460–18465.
36. Burchill MA, et al. (2003) Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with Borrelia burgdorferi. *Infect Immun* 71(6):3437–3442.
37. Mattner J, et al. (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434(7032):525–529.
38. Kinjo Y, et al. (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434(7032):520–525.
39. Kinjo Y, et al. (2006) Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* 7(9):978–986.
40. Galli G, et al. (2003) CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* 197(8):1051–1057.
41. Vincent MS, et al. (2002) CD1-dependent dendritic cell instruction. *Nat Immunol* 3(12):1163–1168.
42. Mars LT, et al. (2004) Therapeutic manipulation of iNKT cells in autoimmunity: Modes of action and potential risks. *Trends Immunol* 25(9):471–476.
43. Mendiratta SK, et al. (1997) CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6(4):469–477.
44. Cui J, et al. (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278(5343):1623–1626.
45. Bettelli E, et al. (2003) Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197(9):1073–1081.

Article n°3

L'ARN double-brin poly(A:U) stimule les basophiles et exacerbe l'asthme allergique

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Nous avons exploré la réponse des basophiles murins aux agonistes TLR. Cette population cellulaire a été générée *in vitro* à partir de cellules de moelle osseuse. Parmi les agonistes testés (poly(I:C), poly(A:U), R848, CpG-ODN), seul l'ARN double-brin poly(A:U) a stimulé efficacement les basophiles et induit une production importante d'histamine, d'IL-4 et d'IL-6. L'administration intrapéritonéale de poly(A:U) *in vivo* induit une mobilisation des basophiles puisqu'il a augmenté leur fréquence dans le sang et le foie. Nous avons testé la capacité du poly(A:U) à stimuler l'asthme allergique dans un modèle induit par l'OVA et les résultats ont montré que cet ARN active les basophiles et amplifie la réponse d'asthme allergique. Nous avons observé que cet agoniste avait maintenu la survie des progéniteurs des basophiles.. Par l'étude de la signalisation impliquée dans cette réponse, nous avons montré que cette voie était indépendante des voies de signalisation TLR (MyD88 et TRIF) ou RLR (IPS-1) dans les basophiles. Etant donné que le poly(A:U) est considéré comme un mimique viral, nous proposons que ces résultats puissent refléter les effets de certains virus pulmonaires qui sont bien connus pour exacerber l'asthme allergique.

Activation of basophils by the double-stranded RNA poly(A:U) exacerbates allergic inflammation

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Abbreviations used in this paper: ***BALF***: Bronchoalveolar Lavage Fluid; ***BM***: bone marrow, ***dsRNA***: double stranded polyribonucleic acid; ***poly(A:U):*** polyadenylic:polyuridylic acid; ***TLR***: Toll-like receptor

ABSTRACT

We explored the response of murine basophils generated in vitro from bone marrow cells. to TLR agonists. Among the molecules tested [poly(I:C), poly(A:U), R848, CpG-ODN], only the double-stranded RNA poly(A:U) effectively stimulated basophils and induced significant production of histamine, IL-4 and IL-6. Intraperitoneal administration of poly(A:U) in vivo induces mobilization of basophils since increased their frequency in blood and liver. We tested the ability of poly(A:U) to stimulate allergic asthma in a model induced by OVA and the results showed that this RNA activates basophils and amplifies asthmatic responses. We found that this agonist had maintained the survival of progenitor basophils. By studying the signaling involved in this response, we showed that this effect was independent of TLR (MyD88 and TRIF) or RLR (IPS-1) signaling pathways in basophils. Given that poly (A:U) is considered a viral mimic, we propose that these results may reflect the effects of certain viruses which are lung known to exacerbate allergic asthma.

INTRODUCTION

Basophils are the least abundant granulocytes, representing less than 1% of peripheral blood leukocytes. For this reason, together with their poor phenotypic and morphological characterization and lack of animal models, their specific physiological role is not fully understood. Even though mast cells and basophils are both considered primary effector cells of bronchial asthma, there is compelling immunological, biochemical and pharmacological evidence that they exert distinct functions at the onset and development of the asthma phenotype [1]. Basophils are the major early producers of interleukin (IL)-4 and IL-13, T helper (T_H)2-type cytokines crucial for initiating and maintaining allergic responses. Moreover, in agreement with their putative effector functions, basophils are clearly associated with fatal asthma [2] and detected in nasal lavage fluid after allergen challenge of patients with allergic rhinitis [3]. This notion is further supported by the correlation between gain of function mutations of the IL-4R and disease exacerbation [4, 5].

Murine basophils are most frequent in the bone marrow where they originate and most likely complete their maturation, conversely to mast cells, which differentiate in the periphery [6]. They constitute an excellent source of IL-4, IL-6 and histamine, in response to growth factors like IL-3 and GM-CSF or calcium ionophore and IgE[7,8,9], in accordance with the prevailing notion that they are critical for the development of T_H2 immune responses, either directly through their T_H2 cytokine profile or indirectly through the release of histamine, which inhibits T_H1 responses[9]. Recent studies have shown that basophil activation is not only promoted by antigen-specific IgE crosslinking, but can be caused in non-sensitized individuals by parasitic antigens, lectins and viral superantigens, either by direct crosslinking of the FcεR or binding to non-specific IgE antibodies [10]. Furthermore, novel IgE- and FcεR-independent routes of stimulation have been reported, consistent with the assumed regulatory functions of basophils in innate immunity [11]. In further support of this notion, several recent

studies provide evidence they can also act as antigen-presenting cells [12-14] , in particular in the case of an allergen-induced T_H2 response.

It is not clear so far whether pathogens contribute directly to activation and recruitment of basophils *in vivo* by triggering pathogen-associated molecular pattern recognition pathways. Toll-like receptor 2 (TLR2) and TLR4 expression has been reported for human basophils, which responded selectively to the TLR2 ligand peptidoglycan by producing IL-4 and IL-13 but not histamine[15,16], while they failed to do so upon exposure to the TLR4 agonist LPS. TLR2, TLR4 and TLR6 transcripts have been detected in murine basophils and T_H2 cytokine expression in response to peptidoglycan and LPS[17] has been reported. However, the activation of basophils by TLRs *in vivo* and its functional relevance remains to be investigated and their direct implication in allergic asthma has not been demonstrated as yet.

Upper respiratory tract viruses, especially rhinovirus and respiratory syncytial virus, are the most common and important cause of asthma exacerbation. They generate replication-mediated double-stranded (ds)RNA in infected cells which serves as a ligand for pattern-recognition receptors (PRR). We postulated that this type of interaction might lead to increased T_H2 cytokine production by basophils accounting for their adverse effect in allergic asthma. To verify this hypothesis, we tested the effect of dsRNA and a number of TLR ligands on typical basophil activities, using purified populations derived from bone marrow cultures. We found that double stranded polyadenylic:polyuridylic acid [poly(A:U)] activated basophils both *in vitro* and *in vivo* to produce histamine and T_H2 cytokines, such as IL-4, IL-6 and IL-13 in a MyD88- TRIF- and IPS-1-independent fashion. Furthermore, we demonstrate the direct implication of basophils in allergic inflammation exacerbation, by means of either adoptive transfer or depletion with a specific antibody, and assess the physiological relevance of poly(A:U) in this model by showing the aggravation of disease hallmarks when basophils were exposed to the dsRNA before transfer.

MATERIALS AND METHODS

Mice, cytokines and reagents. This study was performed with male and female 7-9-wk-old mice. C57BL/6J mice were purchased from CERJ (Les Genest St. Isle, France). MyD88$^{-/-}$, TRIF$^{-/-}$ and IPS-1$^{-/-}$ mutants, kindly provided by Dr. S. Akira, were back-crossed for 12 generations into a C57BL/6J. Jα18$^{-/-}$ mice (C57BL/6J background) were originally provided by Dr. T. Taniguchi and bred in our animal facility under specific pathogen-free conditions, as were NOD mice. FcεRI$\alpha^{-/-}$ mutants were a gift from Dr. S. Mecheri (Pasteur Institute, Paris). TRIF$^{-/-}$, MD-2$^{-/-}$, TLR4$^{-/-}$, TLR7$^{-/-}$ and TLR9$^{-/-}$ mice were provided by the CDTA (Orléans, France). These mutant strains were all backcrossed on a C57BL/6J background.

Cytokines, antibodies, TLR agonists and other reagents. Murine recombinant IL-3 was purchased from R&D Systems (Lille, France). The following appropriately labeled antibodies were used: anti-mouse CD117 (2B8), CD49b (Hmα2), FcεRIα (MAR-1), CD69 (H1.2F3) and isotype controls (BD Pharmingen, Le-Pont-de-Claix, France). The poly(A:U) used was purchased from Sigma-Aldrich (St.-Quentin Fallavier, France), Midland (Midland, TX) or prepared in the laboratory using polyA (Fluka/ Sigma-Aldrich, Lyon, France) and polyU (MP Biomedical, Illkirch, France) as described before [24]. The TLR3 and TLR7 agonists, namely poly(I:C) and R848, were from InVivoGen (Toulouse, France). The TLR4 agonist lipopoysaccharide (LPS) was purchased from Alexis Biochemicals (Paris, France). RNase A was purchased from Sigma-Aldrich.

Preparation of RNA and RT-PCR analysis. RNAs from splenocytes or sorted basophils were extracted with TRIzol reagent (Invitrogen, Cergy-Pontoise, France). Ten μg of transfer RNA were added prior to RNA extraction and reverse transcription was performed with total RNA. TLR1, 2, 3, 4, 5, 6, 7, 8, 9 and 11 expression was determined by PCR analysis carried

out on a MWG AG Biotech Primus 96 apparatus using sense and antisense primers (Sigma Genosys, Table 1) under the following cycling conditions: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Double-amplification of 35 cycles each was performed.

	Sense primers	**Antisense primers**
TLR1	5'-TCTCTGAAGGCTTTGTCGATACA	5'-GACAGAGCCTGTAAGCATATTCG
TLR2	5'-CATTGGGTGGAGAACCTCATGGTCCAG	5'-CTAGGACTTTATTGCAGTTCTCAGATT
TLR3	5'-CGGATTCTTGGTTTCAAGGAAATAGAC	5'-TAATGTGCTGAATTCCGAGATCCAAG
TLR4	5'-CAAGAACATAGATCTGAGCTTCAACCC	5'-CTGTCCAATAGGGAAGCTTTCTAGAG
TLR5	5'-GCTATGCTTTGAAGAAAGAGACTTCAT	5'-TAGGAAATGGTTGCTATGGTTCGCAA
TLR6	5'-AACAGGATACGGAGCCTTGA	5'-CCAGGAAAGTCAGCTTCGTC
TLR7	5'-TTCCGATACGATGAATATGCACG	5'-TGAGTTTGTCCAGAAGCCGTAAT
TLR8	5'-GGCACAACTCCCTTGTGATT	5'-CATTTGGGTGCTGTTGTTTG
TLR9	5'-CCGCAAGACTCTATTTGTGCTGG	5'-TGTCCCTAGTCAGGGCTGTACTCAG
TLR11	5'-TTGATGTATTCGTGTCCCACTGC	5'-CCACTCTTTCTCTCCTCTTCCTCG

Sorting of BM-derived basophils and flow cytometry. Basophil-enriched populations were generated from total BM cells cultured for 7-9 days with IL-3, as described before (). This population contained on average 25-40% basophils defined as c-kit$^-$FcεRIα$^+$CD49b$^+$ cells. To avoid activation by FcεR staining, they were electronically sorted as c-kit$^-$CD49b$^+$ cells using a FACSAria cell sorter (BD). Purity was >98% upon reanalysis of aliquots stained with anti-FcεRIα mAb. After FcγR blockade by CD16/32, basophils were stained in different organs with CD49b-APC, c-kit-FITC and FcεRIα-PE mAbs and analyzed in a FACSCanto™ flow cytometer using FACSDiva software (BD). Basophils were also sorted after 6 days of culture and incubated at a concentration of 10^6 per ml in the presence of 10 ng/ml IL-3 or 100 μg/ml of poly(A:U) in 24-well plates. Viable cells were counted daily using trypan blue exclusion.

Cell cultures. For cytokine and histamine assays, cells from bone marrow, liver, lung and sorted basophils were recovered as previously described[37]. The cells were suspended in MEM

medium (Invitrogen) supplemented with 10% horse serum (Invitrogen) instead of FCS, which contains histaminase[38]. They were adjusted to a final concentration of 10^7 and 2.5 x 10^6 liver and bone marrow cells per ml, respectively, and exposed for 24 h to different stimuli, as stated in Results, relative to medium controls. In some cases poly(A:U) was pretreated with RNase A (100 µg/ml) for 5 h at 37°C and 10^{-6} M. Supernatants were collected and stored at -20°C for cytokine and histamine assays.

Cytokine and histamine assays. IL-4, IL-5, IL-6, IL-13 eotaxin, and TARC were quantified in culture supernatants, BALF and liver using ELISA kits from R&D according to the manufacturer's instructions. Histamine concentrations in cell supernatants were currently determined by an automated continuous flow fluorometric technique (lower limit of sensitivity: 50 pg/ml), as previously described[39].

In vivo treatment. Female C57BL/6J or NOD mice received daily i.p. injections of 100 µg of poly(A:U) or NaCl for 5 days. 24 h after the last injection, they were anesthetized with ketamine (35 mg/kg; Sigma-Aldrich) before collecting blood from the retro-orbital sinus. After sacrifice, basophils were identified among mononuclear cells from different organs upon staining with CD49b-APC and FcεRIα-PE mAbs.

Experimental allergic asthma. Male NOD mice were sensitized by *i.p.* injections of 100 µg OVA on day 0, 2, and 4. Thereafter, from D10 to D15, they received a daily treatment with aerosolized OVA at a concentration of 20 mg/ml or NaCl for 20 min using an ultrasonic nebulizer (Ultra-Neb99, DeVilbiss). 1 h later they were given 50 µg poly(A:U) or NaCl *i.n.*

Adoptive transfer or depletion of basophils. Jα$18^{-/-}$ mice immunized according to the above protocol received 10^6 bone marrow-derived sorted basophils after a 2 h exposure to poly(A:U) or culture medium alone by *i.v* injection 1 h before the first OVA aerosol challenge (6 mice per group). For basophil depletion, immunized male NOD mice were injected *i.p* with 5 µg of

Ba103 antibody per mouse twice a day from D7 to D9. Phenotypic and functional analysis of basophils was performed after sacrifice on D16.

Statistics. All data are expressed as means ± SD or SEM. Statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$) was established by Student's t test, using GraphPad Prism5 software.

Repeat administration of poly(A:U) increases peripheral basophil recruitment.

Basophils are present at very low levels in mice outside the bone marrow. We examined whether poly(A:U) could promote their peripheral recruitment following 5 daily *i.p* injections at a dose of 100 µg per mouse. On day 6, we analyzed $CD49b^{+}Fc\varepsilon RI\alpha^{+}$ cells in different organs and found increased frequencies among mononuclear cells recovered from both liver and blood (Fig. 1*A*). The effect was particularly striking in the liver where the percentage of basophils rose from 0.1 to 1%, resulting in increased responsiveness to IL-3 in terms of IL-4 and IL-6 production (Fig. 1*B*).

Poly(A:U) aggravates allergic asthma through activation and recruitment of basophils.

Basophils are believed to carry out important effector functions during allergic inflammation, but have not been detected so far in bronchoalveolar fluid (BALF) in murine experimental allergic asthma. We used NOD mice, which develop a more pronounced allergic response than the C57BL/6 strain [18], to assess whether the incidence and activity of basophils increased during disease progression. Knowing that viral infections can exacerbate the pathology in asthmatic patients[19, 20], we postulated that as a dsRNA recognized by pathogen, poly(A:U) might exert a similar effect through its ability to activate and recruit basophils to the periphery. To mimic viral respiratory tract infections, poly(A:U) was given *i.n.* to immunized mice 1 h after the OVA challenge from day 10 to 15. In these conditions, we detected a significant increase in the incidence of pulmonary basophils that has not been

reported in this model before (Fig. 2*A*). It occurred already in mice challenged with OVA alone and increased substantially after administration of poly(A:U), concurrently with a rise of the hyperreactivity (AHR), an increase of infiltrating cells such as eosinophils in BALF and an enhanced production of the pro-T_H2 cytokines IL-4, IL-5, IL-6 and IL-13, along with the chemokine eotaxin (Fig. 2*B, C* and *D*).

To establish that basophils increased disease symptoms directly, we carried out adoptive transfers of purified cells into Jα18-deficient mice, which contain less medullary basophils than their wild-type controls (0.28 ± 0.01% versus 0.6 ± 0.04%, respectively). Basophils were purified from enriched populations generated during 7-9 days of culture in the presence of IL-3, followed by electronic sorting of $CD49b^+c\text{-}kit^-$ cells, which yielded a >95% pure population upon reanalysis (Fig. 3*A*). Sorted cells were treated for 2 h with or without 100 μg/ml of poly(A:U) prior to retro-orbital injection, 1 h before the first aerosol challenge with OVA of mice previously immunized on day 0, 2 and 4 with 100 μg of OVA. The treatment with 20 mg OVA in aerosol was continued from day 10 to day 15, followed by sacrifice on day 16. As shown in Fig. 3*B*, these mice, which are known for mounting a poor T_H2 response, develop modest asthma symptoms in terms of eosinophil infiltration and secretion of eotaxin and TARC, which become more severe in the group of mice having received unstimulated basophils and are further aggravated after exposure to poly (A:U) prior to injection.

Basophil depletion attenuates allergic asthma syndromes.

We chose the inverse strategy, namely basophil depletion, to validate the transfer data. To this end, we injected the monoclonal antibody Ba103[21] from day 7 to 9, twice a day. The treatment was very effective since it decreased OVA-induced basophil recruitment to the liver from 1.9% to 0.1% (Fig. 4*A*). Basophil depletion resulted in a less marked asthma response evidenced by reduced eosinophil recruitment to BALF and lower pulmonary cytokine and

chemokine production (Fig. 4*B*). Poly(A:U)-induced total cell and eosinophil infiltration was abolished after depletion of basophils, proving their critical role in cellular recruitment (Fig. 4*C*). Taken together, these data support the conclusion that a synthetic analogue of double stranded viral antigen can target basophils directly to promote their typical pro-T_H2 activities and increase their recruitment *in vivo*, thereby exacerbating experimental allergic asthma.

Poly(A:U) increases the life span of basophils.

We addressed the question whether poly(A:U) improved basophil survival. In keeping with this assumption, the loss of $CD49b^+Fc\varepsilon RI\alpha^+$ cells during a 48-h culture of bone marrow cells, relative to those plated initially, was less pronounced in the presence of poly(A:U) than in controls set up in culture medium alone (Fig. 5*A*). Accordingly, bone marrow cells that had been exposed to poly(A:U) produced more IL-6 and histamine in an RNA-specific manner than their unstimulated counterpart (Fig. 5*B*), while IL-4 production was not detected in these conditions. It is also noteworthy that gated poly (A:U)-induced basophils contained a higher proportion of cells expressing CD69, an early activation marker (Fig. 5*C*). The capacity of poly(A:U) to protect bone marrow basophils against cell death was confirmed with purified cells derived from culture (BMDB), which until day 4 survived nearly as well in the presence of poly(A:U) as of IL-3 (Fig. 5*D*), whereas the dsRNA did not support basophil differentiation from BM cells.

Poly(A:U) targets basophils to promote cytokine and histamine production.

Since the dsRNA poly(A:U) has been described as a putative TLR3/TLR7 agonist, we examined the expression of TLR mRNAs by RT-PCR analysis using basophils purified from enriched populations generated during 7-9 days of culture in the presence of IL-3, followed by electronic sorting of $CD49b^+c\text{-}kit^-$ cells. We observed that these cells expressed transcripts for all TLRs tested (Fig. 6*A*). However, their responses in terms of IL-4 and IL-13 production was much more limited since they responded only to poly(A:U) but not to the TLR3 agonist

poly(I:C), and agonists of 7 and 9, namely R848 or single- stranded poly(U) and ODN-CpG, respectively, were likewise ineffective (Fig. 6*B*). Poly(A:U)-induced cytokine production was dose-dependent, increasing from 10-100 μg/ml (Fig. 6*C*) and failed to occur after treatment with RNase A, as illustrated by IL-4 production in Fig. 6*D*, which proves its RNA specificity. RNase A had no effect on its own and did not modify IL-3-induced histamine and cytokine production (data not shown).

Poly(A:U) activates basophils through a FcεRI-independent route and a MyD88-, TRIF- and IPS-1-independent signaling pathway.

Basophil activation by poly(A:U) did not rely on IgE, as it was maintained in FcεRIα-deficient mice (Fig. 7*A*). It was also not mediated through endogenous IL-3 or GM-CSF since basophils recovered from mice lacking both the common and the IL-3-specific β chain required for IL-3, IL-5 and GM-CSF signal transduction, remained responsive (data not shown). As expected, poly(A:U)-induced IL-4 production by basophil-enriched BM cells was not mediated through TLR4 or 9 since it was not impaired in the corresponding KO mice (data not shown). More surprisingly, poly(A:U) remained active on purified basophils recovered from mice in which the gene encoding MyD88 or TRIF had been disrupted (Fig. 7*B* and *C*). Since these adaptor molecules are requisite for TLR-induced signaling, the implication of the putative dsRNA receptors TLR3 and/or TLR7 was ruled out. This was also true for basophils purified from IPS-1-deficient mice that generated IL-4 in response to poly(A:U) (Fig. 7*D*). These data were analyzed using different preparations of poly(A:U) either available commercially or prepared from a mixture of poly(A) and poly(U). Only a MyD88-dependency was observed using a preparation of LPS-contaminated poly(A:U) that was no more detectable when a LPS-free preparation of poly(A:U) was used (data not shown). It is to

note that, in vitro, LPS is a poor agonist of basophil activation since very high concentrations of pure LPS (1 μg/ml) are required to induce low amounts of IL-4 (data not shown).

DISCUSSION

Our study provides the first evidence that the double-stranded RNA poly(A:U) targets basophils to promote their activation in terms of histamine and TH2 cytokine production and increase their survival. Using a murine model of allergic asthma, we established likewise that this effect is relevant in vivo since it enhances the peripheral recruitment and functions of basophils, which in turn contribute directly to the severity of the disease by enhancing asthma syndromes like eosinophil infiltration and TH2-type cytokine production.

The sensors through which basophils recognize pathogens during innate immune responses have not been thoroughly investigated so far. As reported by others [17, 22], purified cells of this lineage express a wide range of TLRs on transcriptional levels, but apart from poly(A:U), a putative TLR3/TLR7 agonist, none of their selective ligands induced a consistent biological activity. Poly(A:U) promoted concomitant histamine and cytokine production, similarly to IL-3, the most efficient inducer of basophil activities. However, a contribution of endogenous growth factor could be ruled out since the activity persisted in mice deficient for both β_c and β_{IL-3} chains, which fail to respond to IL-3, IL-5 and GM-CSF. The effect of poly(A:U) was also independent from FcεR expression or classical TLR transduction cascades involving MyD88 or TRIF. Since the IPS-1 (CARDIF) pathway has been previously implicated in dsRNA recognition and has been recently described in response to poly(A:T) stimulation, we tested whether this pathway could be induced by poly(A:U). However, results obtained in vitro established the IPS-1-independency. Although we have tested the potential signaling pathway induced by RNA, we could not identified the pathway through which viral infections might contribute to basophil activation. In order to eliminate

the potential doubt concerning a potential LPS contamination in poly(A:U), we have observed that a high concentration of pure LPS induced only low amounts of cytokine production and cannot induce the results observed with poly(A:U). Furthermore, we still observed basophil activation using two other preparations of LPS-free poly(A:U) and this activity was lost following RNase A treatment. However, we cannot exclude that bacterial endotoxins may exacerbate cytokine production of basophils induced by dsRNA.

In vivo treatment with poly(A:U) enhanced the incidence of basophils in the blood and in the liver where they conserved their functional characteristics, i.e. their capacity to produce histamine and cytokines in response to IL-3. Furthermore, we provide the first demonstration for increased infiltration of basophils in the lung of mice suffering from allergic asthma and prove by adoptive transfer that they mediate OVA-induced eosinophil irecruitment and cytokine/chemokine production. These activities were further upregulated upon exposure to poly(A:U) before transfer, suggesting that they were patho-physiologically relevant. Conversely, *in vivo* basophil depletion by Ba103 antibody alleviated disease syndromes, thus validating the transfer data. In this context, it is noteworthy that the exacerbated asthma syndromes developed by some mouse strains, such as NOD vs. C57BL/6 mice[18] are associated with increased basophil activity in terms of IL-4 and histamine production, while the opposite is true in mice displaying a low T_H2 response (Jα18 KO mice vs. WT mice). This observation makes sense if one takes the regulatory functions of basophils during allergic airway inflammation into account.

The exacerbation of asthma symptoms, such as increased eosinophil infiltration in BALF in response to poly(A:U), was associated with increased production of several cytokines known for their pro-inflammatory functions, such as IL-13, a central mediator of allergic asthma [33] and IL-6[34]. The role of IL-4 in asthma exacerbation is particularly well

established, owing to its capacity to induce the IgE switch, upregulate the expression of vascular cell adhesion molecule-1 (VCAM-1), enhance eosinophil transmigration across the endothelium, and increase mucus secretion and differentiation of T_H2 lymphocytes[35]. Indeed, increased susceptibility to allergic asthma has been linked to polymorphisms in the IL-4 gene promoter and IL-4R signaling, a finding that has already given rise to clinical trials evaluating the effect of naturally occurring antagonists [36]. In conclusion, we postulate that the asthma exacerbation associated with viral infections might be mediated at least in part by basophils through their capacity to skew the antiviral response toward a T_H2 cytokine profile. Our finding provides new insights into the complex mechanisms that regulate the severity of allergic diseases and their paradoxical aggravation by viral infection.

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REFERENCES

1. Casolaro,V., Spadaro,G., & Marone,G. Human basophil releasability. VI. Changes in basophil releasability in patients with allergic rhinitis or bronchial asthma. Am. Rev. Respir. Dis. 142, 1108-1111 (1990).

2. Koshino,T. et al. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. Clin. Exp. Allergy 23, 919-925 (1993).

3. Iliopoulos,O. et al. Histamine-containing cells obtained from the nose hours after antigen challenge have functional and phenotypic characteristics of basophils. J. Immunol. 148, 2223-2228 (1992).

4. Noben-Trauth,N. et al. An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. Proc. Natl. Acad. Sci. U. S. A 94, 10838-10843 (1997).

5. Kaplan,M.H., Schindler,U., Smiley,S.T., & Grusby,M.J. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity. 4, 313-319 (1996).

6. Arinobu,Y., Iwasaki,H., & Akashi,K. Origin of basophils and mast cells. Allergol. Int. 58, 21-28 (2009).

7. Schneider,E., Rolli-Derkinderen,M., Arock,M., & Dy,M. Trends in histamine research: new functions during immune responses and hematopoiesis. Trends Immunol. 23, 255-263 (2002).

8. Schneider,E. et al. Murine hematopoietic progenitor cells produce IL-6 in response to IgE. Exp. Hematol. 23, 353-361 (1995).

9. Schneider,E. et al. IL-3-induced coexpression of histidine decarboxylase, IL-4 and IL-6 mRNA by murine basophil precursors. Exp. Hematol. 27, 1010-1018 (1999).

10. Falcone,F.H., Zillikens,D., & Gibbs,B.F. The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. Exp. Dermatol. 15, 855-864 (2006).

11. Oh,K., Shen,T., Le,G.G., & Min,B. Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils. Blood 109, 2921-2927 (2007).

12. Sokol,C.L. et al. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. Nat. Immunol. 10, 713-720 (2009).

13. Perrigoue,J.G. et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. Nat. Immunol. 10, 697-705 (2009).

14. Yoshimoto,T. et al. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. Nat. Immunol. 10, 706-712 (2009).

15. Schramm,G. et al. Cutting edge: IPSE/alpha-1, a glycoprotein from Schistosoma mansoni eggs, induces IgE-dependent, antigen-independent IL-4 production by murine basophils in vivo. J. Immunol. 178, 6023-6027 (2007).

16. Bieneman,A.P., Chichester,K.L., Chen,Y.H., & Schroeder,J.T. Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion. J. Allergy Clin. Immunol. 115, 295-301 (2005).

17. Yoshimoto,T. & Nakanishi,K. Roles of IL-18 in basophils and mast cells. Allergol. Int. 55, 105-113 (2006).

18. Araujo,L.M. et al. Exacerbated Th2-mediated airway inflammation and hyperresponsiveness in autoimmune diabetes-prone NOD mice: a critical role for CD1d-dependent NKT cells. Eur. J. Immunol. 34, 327-335 (2004).

19. Jackson,D.J. et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am. J. Respir. Crit Care Med. 178, 667-672 (2008).

20. Stensballe,L.G. et al. The causal direction in the association between respiratory syncytial virus hospitalization and asthma. J. Allergy Clin. Immunol. 123, 131-137 (2009).

21. Obata,K. et al. Basophils are essential initiators of a novel type of chronic allergic inflammation. Blood 110, 913-920 (2007).

22. Komiya,A. et al. Expression and function of toll-like receptors in human basophils. Int. Arch. Allergy Immunol. 140 Suppl 1, 23-27 (2006).

23. Kang,D.C. et al. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc. Natl. Acad. Sci. U. S. A 99, 637-642 (2002).

24. Sugiyama,T. et al. Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. Int. Immunol. 20, 1-9 (2008).

25. Hornung,V. et al. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314, 994-997 (2006).

26. Siren,J. et al. Retinoic acid inducible gene-I and mda-5 are involved in influenza A virus-induced expression of antiviral cytokines. Microbes. Infect. 8, 2013-2020 (2006).

27. Paz,S. et al. Induction of IRF-3 and IRF-7 phosphorylation following activation of the RIG-I pathway. Cell Mol. Biol. (Noisy. -le-grand) 52, 17-28 (2006).

28. Barral,P.M. et al. Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: Key regulators of innate immunity. Pharmacol. Ther.(2009).

29. Sarkar,D., Desalle,R., & Fisher,P.B. Evolution of MDA-5/RIG-I-dependent innate immunity: independent evolution by domain grafting. Proc. Natl. Acad. Sci. U. S. A 105, 17040-17045 (2008).

30. Kato,H. et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. 205, 1601-1610 (2008).

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31. Ablasser,A. et al. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat. Immunol.(2009).

32. Su,Z.Z., Sarkar,D., Emdad,L., Barral,P.M., & Fisher,P.B. Central role of interferon regulatory factor-1 (IRF-1) in controlling retinoic acid inducible gene-I (RIG-I) expression. J. Cell Physiol 213, 502-510 (2007).

33. Wills-Karp,M. et al. Interleukin-13: central mediator of allergic asthma. Science 282, 2258-2261 (1998).

34. Yokoyama,A. et al. Circulating interleukin-6 levels in patients with bronchial asthma. Am. J. Respir. Crit Care Med. 151, 1354-1358 (1995).

35. Ying,S. et al. Associations between IL-13 and IL-4 (mRNA and protein), vascular cell adhesion molecule-1 expression, and the infiltration of eosinophils, macrophages, and T cells in allergen-induced late-phase cutaneous reactions in atopic subjects. J. Immunol. 158, 5050-5057 (1997).

36. Borish,L.C. et al. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. J. Allergy Clin. Immunol. 107, 963-970 (2001).

37. Zhu,R. et al. The Pro-Th1 cytokine IL-12 enhances IL-4 production by invariant NKT cells: relevance for T cell-mediated hepatitis. J. Immunol. 178, 5435-5442 (2007).

38. Dy,M., Lebel,B., & Schneider,E. Presence of histaminase in fetal calf sera. J. Immunol. Methods 48, 261-265 (1982).

39. Schneider,E. et al. Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. J. Exp. Med. 202, 387-393 (2005).

LEGENDS TO FIGURES

FIGURE 1. Poly(A:U) targets basophils in vivo. *A*, Poly(A:U)-induced basophil recruitment to the periphery. NOD mice received daily *i.p.* injections of 100 µg of poly(A:U) or NaCl for 5 consecutive days. 24 h later, basophils were analyzed in blood and liver. The dot plots represent a typical result obtained with 1 mouse out of 5. Data are means ± SEM. *B,* Functional activation of hepatic basophils in response to poly(A:U). Mononuclear liver cells (10^7/ml) from NOD mice having received poly(A:U) or saline as stated above were incubated with IL-3 for 24 h before measuring IL-4 and IL-6 production. Significant changes between PBS and Poly(A:U)-treated mice (n=5 mice per group); ** $p \leq 0.01$; *$p \leq 0.05$.

FIGURE 2. Increased basophil levels coincide with asthma exacerbation. *A,* Increased basophil recruitment to the lung of immunized mice having received poly(A:U). NOD mice were immunized according to the protocol described in Materials and Methods. One group was challenged with NaCl followed by *i.n.* NaCl inoculation, a second group was challenged with OVA followed by *i.n.* administration of either NaCl or 50 µg of poly(A:U) 1h after OVA aerosol challenge from D10-D14. Basophil recruitment was analyzed in the lungs. *B,* Mice treated with poly(A:U) showed an increased AHR as compared to control mice. AHR to Mch was measured 24hrs after the last challenge. *C,* Mice treated with poly(A:U) showed an increased eosinophilia and IL-4 and eotaxin production as compared to control mice. These experiments were performed twice using 4 to 6 mice per group. One representative experiment is shown.

FIGURE 3. Adoptive transfer of basophils exacerbates allergic inflammation. *A,* Preparation of purified bone marrow-derived basophils (BMDB). $CD49b^+c\text{-}kit^-$ cells were electronically sorted from basophil-enriched populations generated during 7-9 days of culture in the presence of IL-3. *B,* After immunization, $J\alpha18^{-/-}$ mice were divided into 3 groups, one which received no further treatment and served as control and the other two injected either with 10^6

sorted basophils that had been pretreated or not with poly(A:U). Total cells and eosinophils in BALF, eotaxin and TARC levels were quantified in lung. n= 6. ** $p \leq 0.01$; * $p \leq 0.05$.

FIGURE 4. Basophil depletion alleviates allergic inflammation. *A,* Reduced eosinophil counts in BALF after basophil depletion. Mice received two daily injections of 5 μg Ba103 for three days before OVA aerosol challenge. Basophil depletion was verified in the liver by flow cytometry and eosinophils were counted in BALF. *B,* Decreased T_H2 cytokine levels in BALF of basophil-depleted immunized and OVA-challenged mice. Cytokines were measured in BALF 24 h after the last challenge. *C,* Abrogation of OVA- or OVA+poly(A:U)-induced increase of eosinophil infiltration after basophil depletion. These experiments were performed twice using 4 to 6 mice per group. One representative experiment is shown. **$p \leq 0.01$; *$p \leq 0.05$.

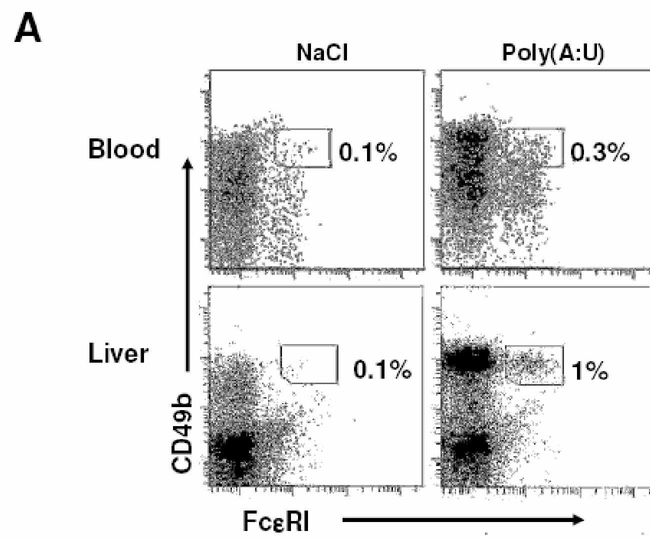
FIGURE 5. Poly(A:U) promotes basophil activation and increased survival *in vitro*. *A,* Increased basophil counts in the presence of poly(A:U). Bone marrow cells from C57BL/6J mice were incubated 48 h at a concentration of 2.5 x 10^6 cells/ml in MEM + 10% horse serum with or without 100 μg/ml of poly(A:U) pretreated or not with RNase A. The incidence of basophils identified as $CD49^+Fc\varepsilon RI\alpha^+$ cells was determined before and after incubation by double staining. *B*, Increased histamine and IL-6 levels in BM cell supernatants after a 48-h exposure to poly(A:U), n=5. *C,* Poly(A:U)-induced upregulation of the early activation marker CD69. CD69 expression was analyzed among gated basophils after a 48-h incubation, n= 4. *D,* Increased basophil survival in response to poly(A:U). 10^6 sorted basophils per ml were cultured with 10 ng/ml of IL-3 or 100 μg/ml of poly(A:U) in RPMI with 10% FCS. Cellular survival was measured daily by counting viable cells by trypan blue exclusion. Data are means ± SD. One representative experiment out of 2 is shown. ***$p \leq 0.001$; **$p \leq 0.01$; * $p \leq 0.05$.

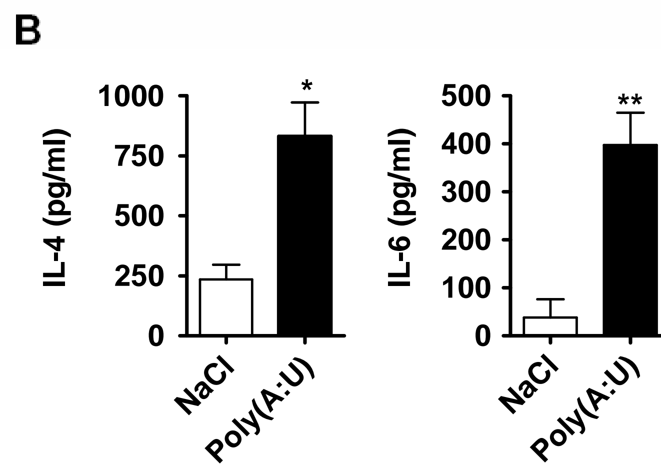
FIGURE 6. Basophils are activated by the dsRNA poly(A:U). *A,* TLR mRNA expression**.** Sorted c-kit^-CD49b^+ basophils were analyzed by RT-PCR for transcripts encoding TLR1 to 11 using the sense and antisense primers listed in Table I of Materials and Methods. Total

splenocytes and H_2O were used as positive and negative controls. *B,* Cytokine production by BMDB stimulated with poly(A:U) or TLR agonists. 10^6 sorted basophils/ml were incubated with or without TLR agonists at the two doses indicated. IL-4 and IL-13 were measured in supernatants collected 24 h after stimulation. Each histogram represents one of at least four independent experiments. *C,* Poly(A:U)-induced histamine and IL-4 production. BMDB were incubated with or without poly(A:U) (100 µg/ml) or IL-3 (10 ng/ml) for 24 h and histamine was measured in supernatants by fluorometric detection. Data are means ± SEM. Each histogram represents one of at least four independent experiments. IL-4 production was measured after 24 h in supernatants, as compared with controls set up in culture medium or stimulated with IL-3. Data are means ± SEM; Each histogram represents one of at least five independent experiments. One representative experiment out of two is shown. ** $p \leq 0.01$; * $p \leq 0.05$.

FIGURE 7. FcεR-, MyD88-, TRIF- and IPS-1-independent activation of basophils by poly(A:U). BMDB prepared from WT or deficient mice were incubated for 24 h with or without 100 µg of poly(A:U) or 10 ng/ml of IL-3. Each histogram represents one of at least three independent experiments.

Figure 1





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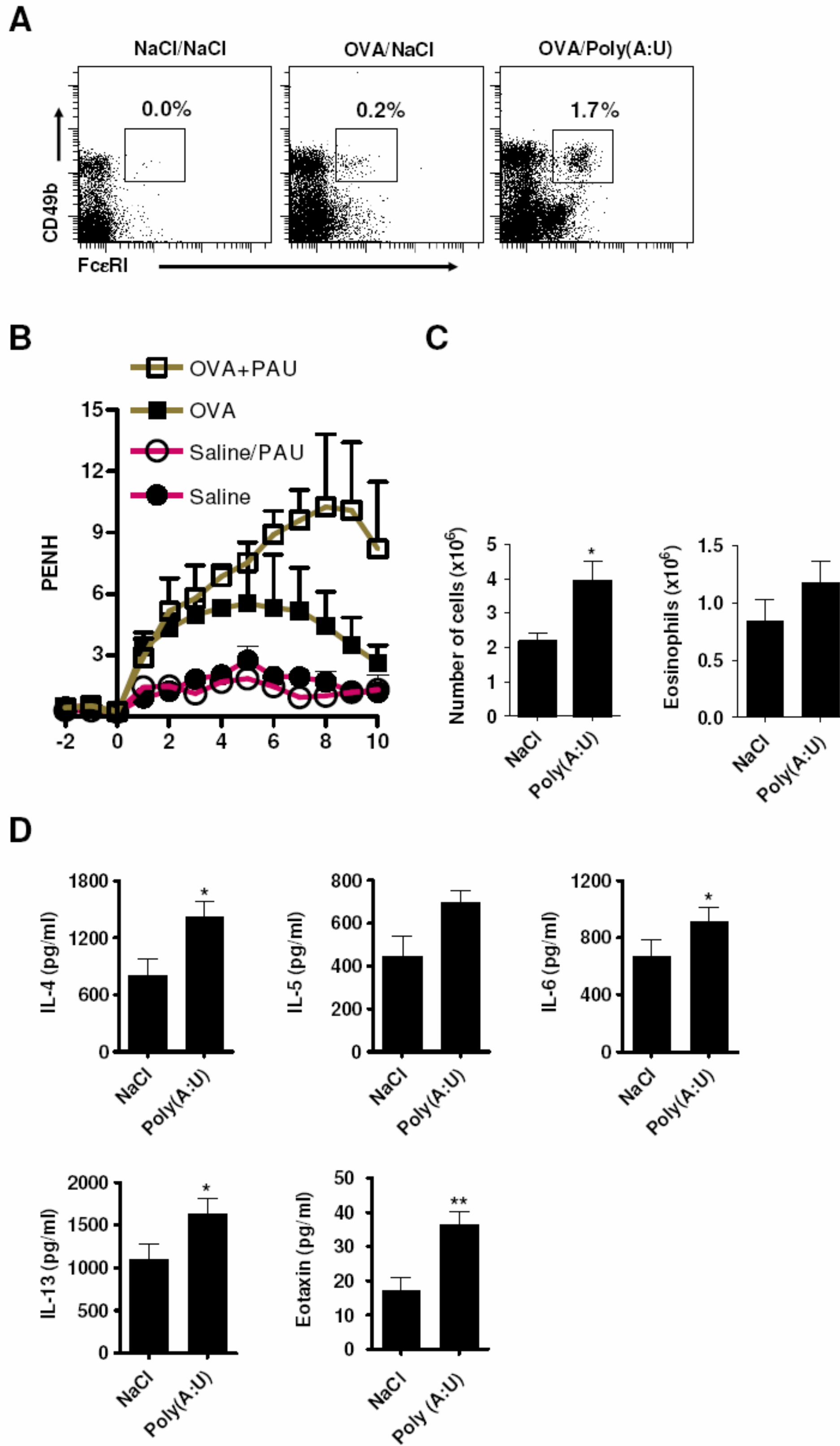

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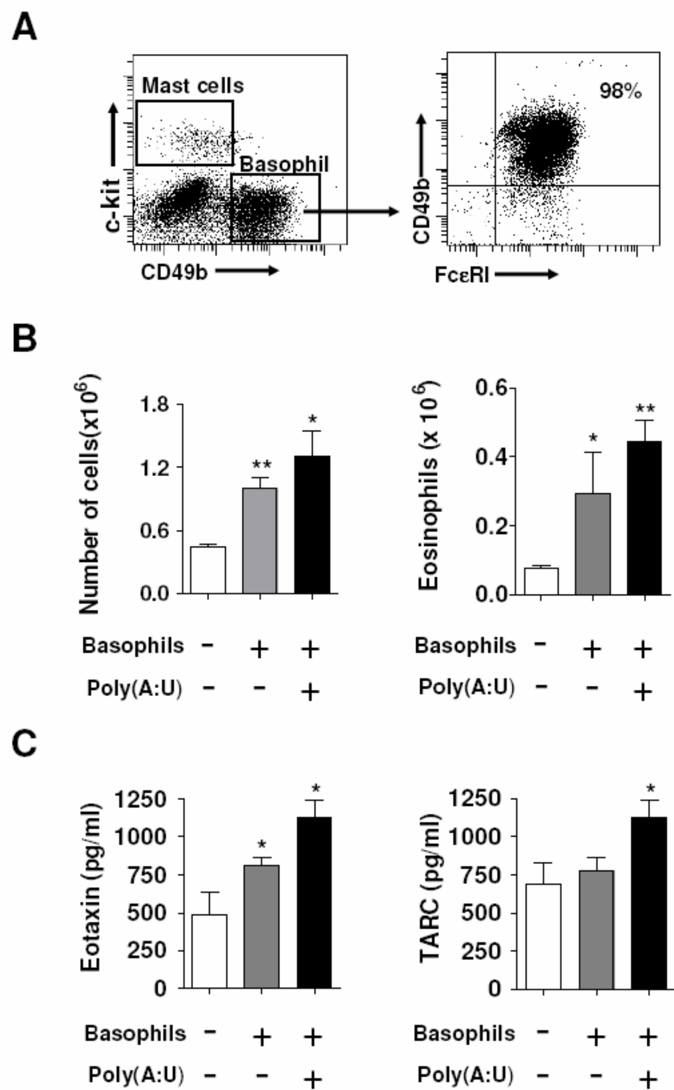

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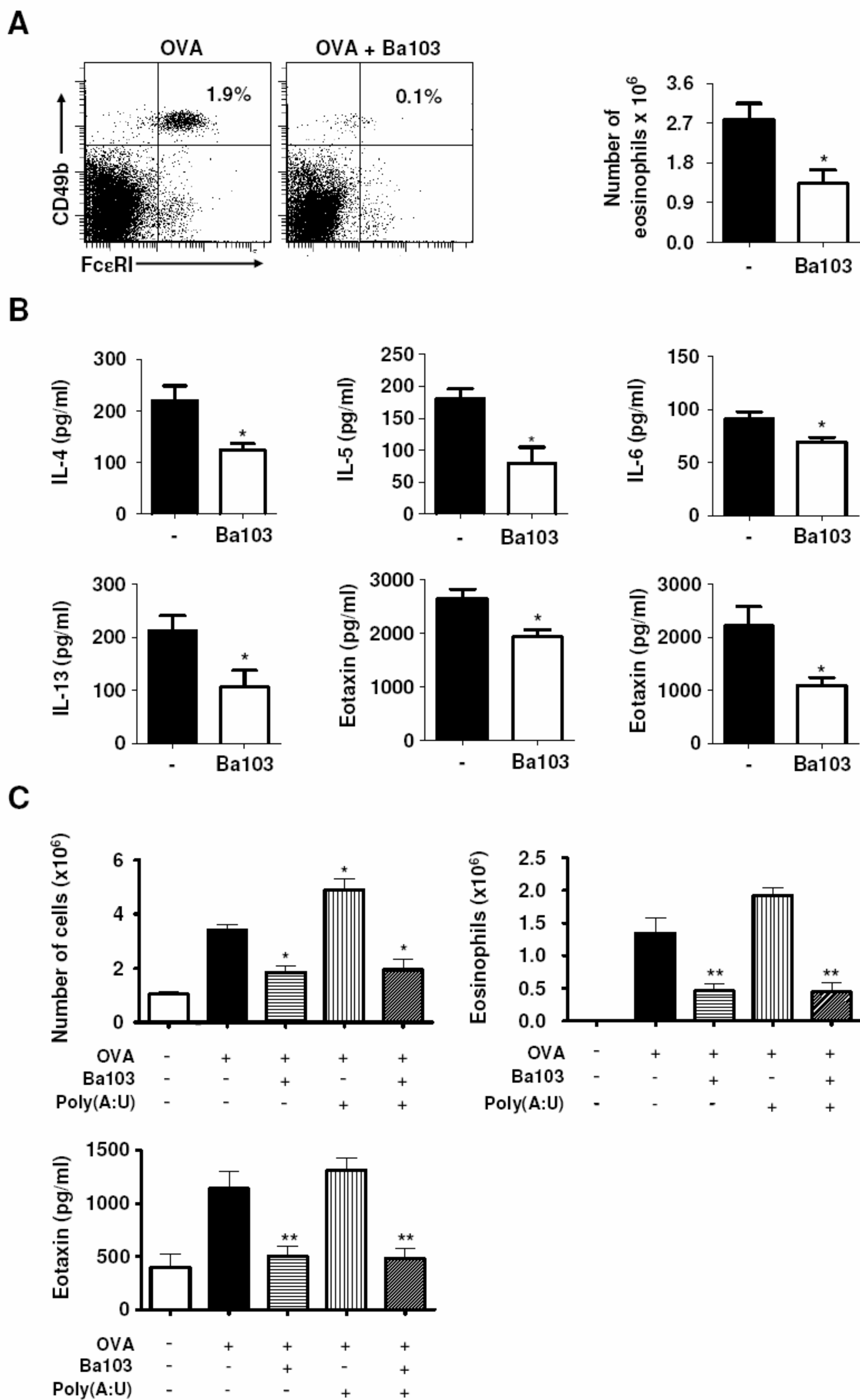


Figure 5

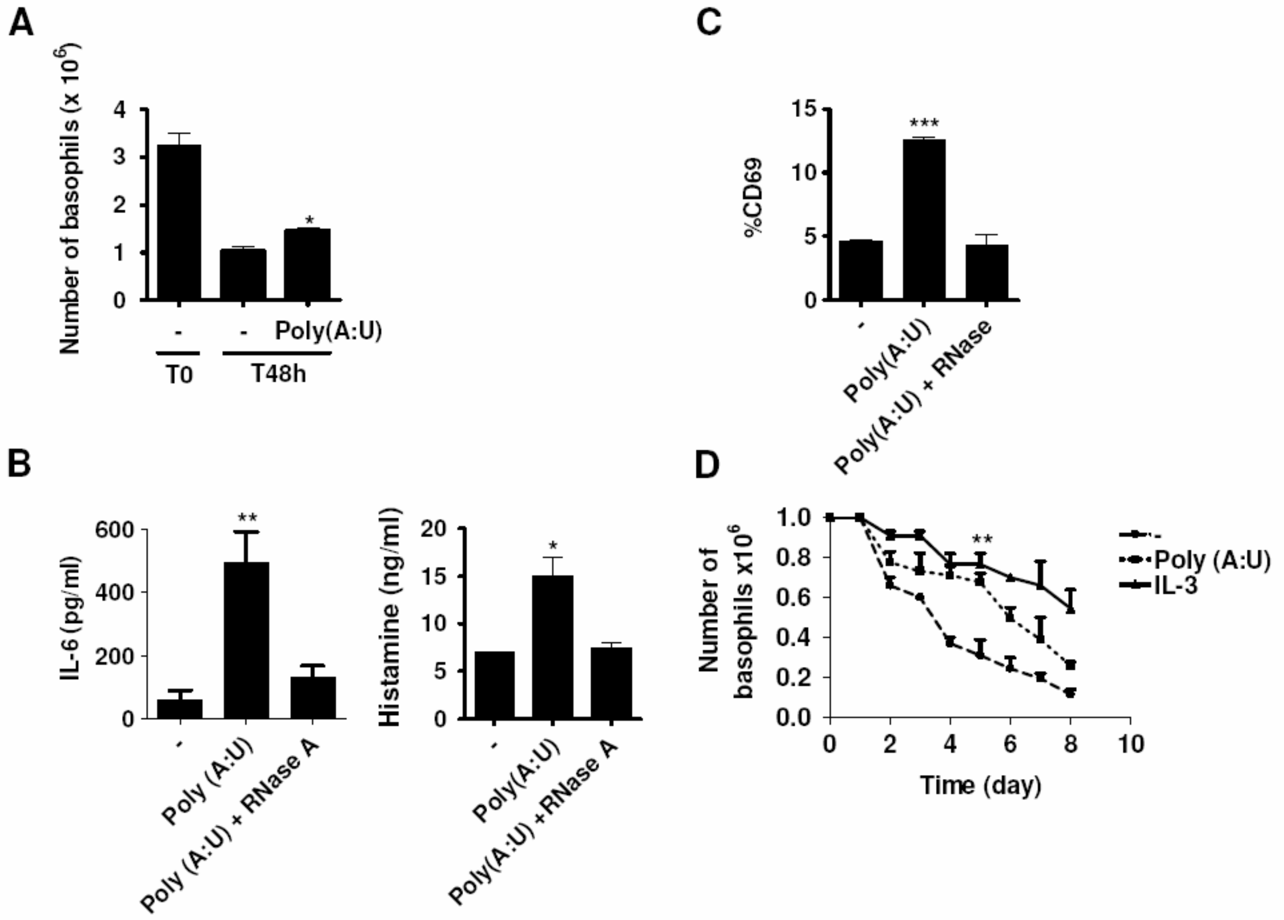
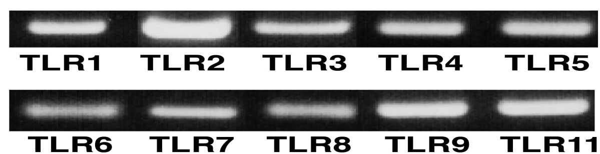


Figure 6

A



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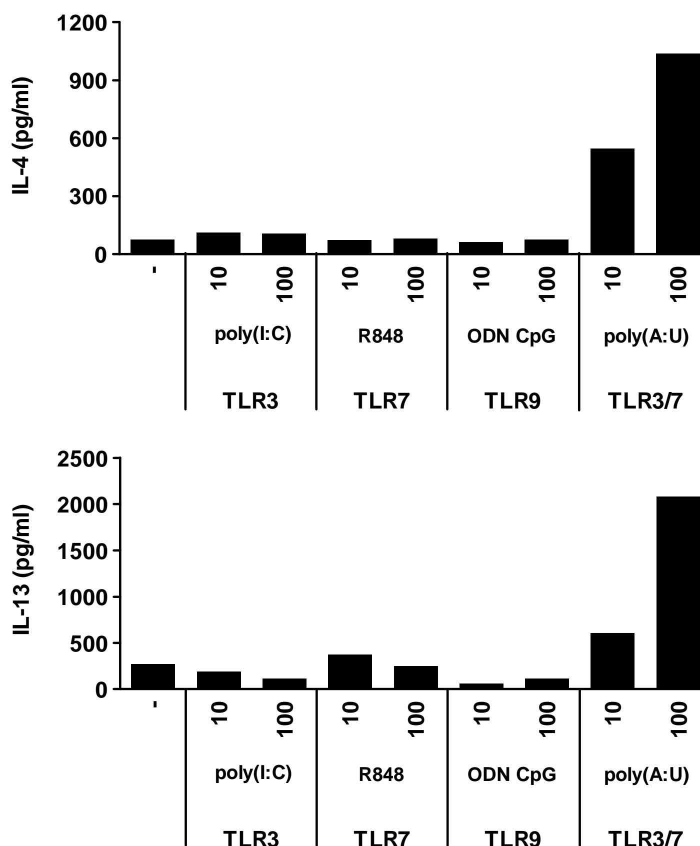

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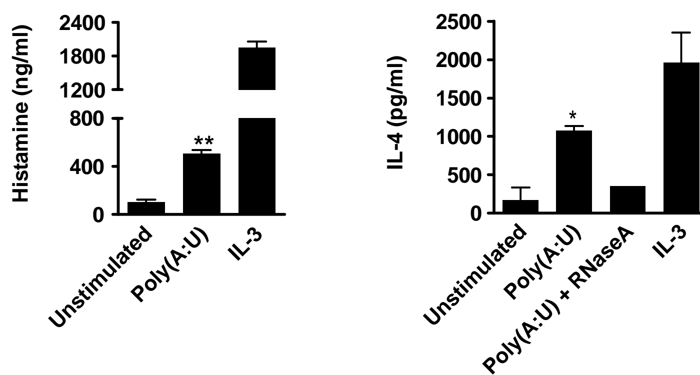
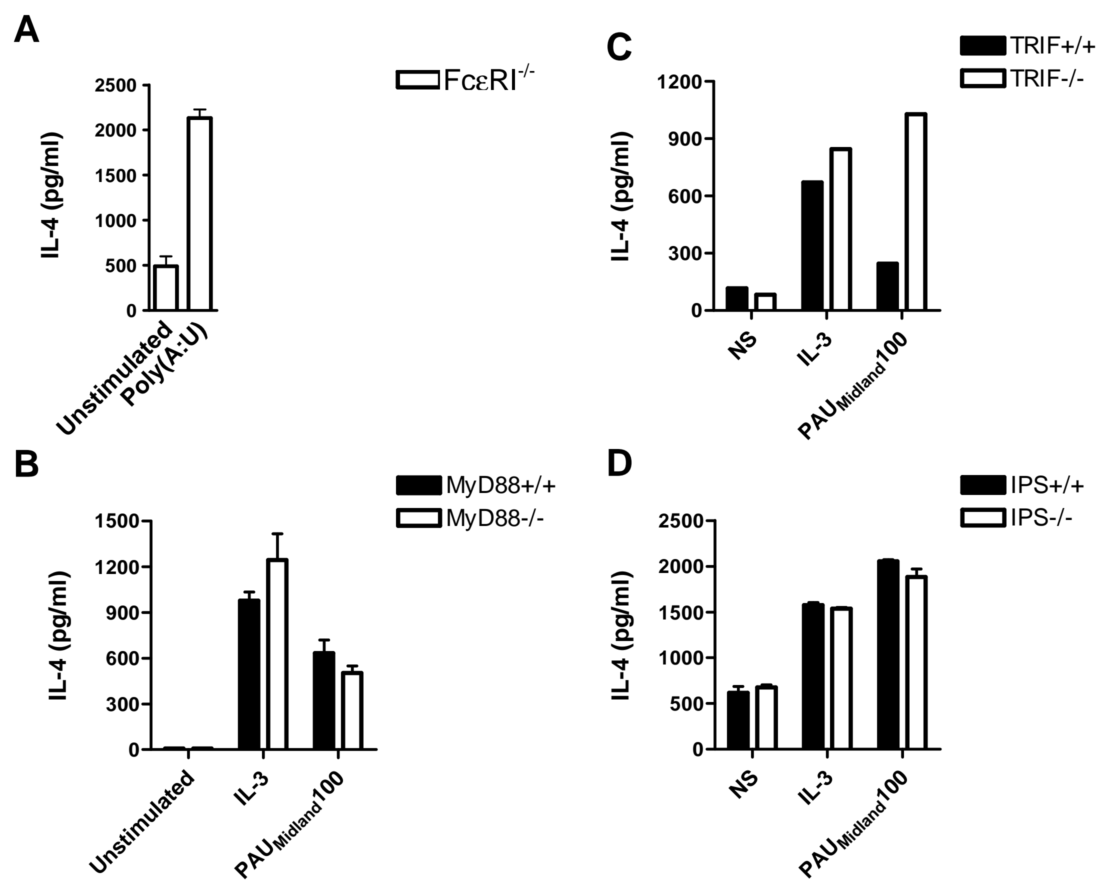


Figure 7



Article n•4

La stimulation TLR induit la suppression de l'asthme allergique expérimental et du diabète chez la souris NOD

Aude Aumeunier, Françoise Grela*, Abdulraouf Ramadan*, Linh Van Pham, Emilie Bardel, Alejandro Gomez Alcala, Pascale Jeannin, Shizuo Akira, Jean-François Bach, Nathalie Thieblemont*

(*) Ces auteurs ont contribué également à ce travail

Nous avons postulé que la stimulation TLR pouvait cibler les cellules T régulatrices dont le rôle a largement été décrit dans la prévention des maladies allergiques et autoimmunes. Notre étude a démontré que le traitement par des structures purifiées de pathogènes, agonistes des TLR, suffit à protéger des souris du développement de l'asthme. Parallèlement, le traitement des souris NOD avec la P40, le Poly(I:C), le LPS et le R848 a conféré une protection contre le développement du diabète de type 1, ou une ralentissement du développement de celui-ci. Ce travail a également mis en évidence le fait qu'un traitement par certains agonistes TLR induisait le recrutement des cellules CD4+CD25+Foxp3+ dans le sang et que certains agonistes TLR étaient capables d'induire la production d'IL-10 et de TGF-β dans le sérum. Nous avons ainsi montré que la stimulation TLR est essentielle dans l'induction de cellules et de cytokines immunomodulatrices et pourrait être impliquée dans la protection des maladies allergiques et autoimmunes par les infections.

SYSTEMIC TOLL LIKE RECEPTOR STIMULATION SUPPRESSES EXPERIMENTAL ALLERGIC ASTHMA AND AUTOIMMUNE DIABETES IN NOD MICE

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Running Title: *TLR and the hygiene hypothesis*

Keywords

Toll-like receptor; MyD88; autoimmune diabetes; allergic asthma, cytokines, regulatory T cells, NK T cells probiotics, hygiene hypothesis

Competing interests

The authors have declared no competing interests exist.

ABSTRACT

Background: Infections are often associated with exacerbation of allergic diseases or their complications. Paradoxically, epidemiological and experimental data have shown that infectious microorganisms can also prevent this pathology. We have tested whether Toll-like receptor (TLR) stimulation can recapitulate the protective effect of infectious agents on allergy and autoimmunity.

Methods and Findings: Here, we performed a systematic study of these disease-modifying effects of a set of natural or synthetic TLR agonists using two experimental models, OVA-induced asthma and spontaneous autoimmune diabetes. In the same models, we also investigated the effect of probiotics. Additionally, we examined the effect of the genetic invalidation of MyD88 on the development of spontaneous diabetes and of allergic asthma. We demonstrate that multiple TLR agonists prevent allergy and autoimmunity, namely ovalbumin-induced allergic asthma and spontaneous autoimmune diabetes in non-obese diabetic mice when administered parenterally. Probiotics which stimulate TLRs, also protect from these two diseases. The physiological relevance of these results is suggested by the major acceleration of ovalbumin-induced asthma in MyD88 invalidated mice. Our results strongly indicate that these TLR-mediated effects involve immunoregulatory cytokines such as IL-10 and TGF-β and different subsets of regulatory T cells, notably $CD4^+FoxP3^+$ T cells for TLR4 agonists and NK T cells for TLR3 agonists.

Conclusions: These observations provide a plausible explanation for the hygiene hypothesis according to which the decline of infections in western countries contribute to the increased prevalence of allergy and autoimmunity. They also open new therapeutic perspectives for the prevention of these pathologies.

INTRODUCTION

There is compelling evidence to indicate a central role of Toll-like receptors (TLRs) in the stimulation of innate and adaptive immunity when applied at the site of the immune response [1–3]. More limited but convincing observations suggest a possible role of TLRs in the triggering of allergic and autoimmune diseases [4–9]: such induction could not be obtained in several experimental models after genetic invalidation of certain TLRs or one of their adaptor molecules, namely MyD88 [6,7,10,11].

On the other hand, more unexpected observations have indicated that systemic TLR stimulation can prevent the onset of allergic and autoimmune diseases when it is implemented early enough in the natural history of the disease. Thus, administration of the TLR4 agonist LPS and of the TLR9 agonist CpG has been shown to prevent spontaneous diabetes onset in the non obese diabetic (NOD) mouse [12,13]. Similarly, administration of various TLR agonists may prevent onset of ovalbumin (OVA)-induced allergic asthma [14–20], even if in this model, the same agonists may exceptionally show the opposite effect depending on experimental conditions[21–24]. TLR-mediated prevention of allergic and autoimmune diseases could represent one of the major mechanisms underlying the hygiene hypothesis according to which the major increase of these diseases observed in western countries over the last three decades is secondary to the decline of infections [25–27]. Originally elaborated in the context of an increased susceptibility to allergy we proposed that the hypothesis could also applied to autoimmune diseases [28].

One may postulate that the TLR ligands that are present in numerous pathogens have a general non specific inhibitory effect on allergic and autoimmune responses. This would

explain that a decrease of infections is associated with a reduction of the inhibitory effects thus leading to an increase in allergic and autoimmune diseases. Validating the hygiene hypothesis in the clinical setting is a complex issue. A major problem is that some particular infections may trigger/ exacerbate either allergic or autoimmune diseases and that the nature of the infections contributing to protection is still ill-defined. This is why so far the best direct evidence in support of the hygiene hypothesis has been collected from experimental animal models such as the NOD mouse in which a variety of pathogens (living pathogens or bacterial extracts) totally prevent autoimmune diabetes onset [29–32] (reviewed in [28]). The study of bacterial extracts, which are easier to use and analyze as compared to living pathogens, is complicated by the multiplicity of their components.

Here, we have tested whether TLR stimulation can recapitulate the protective effect of infectious agents on allergy and autoimmunity using a single mouse strain namely the NOD mouse to avoid biases due to differences in genetic background. To that aim we first performed a systematic screening of a set of natural or synthetic TLR agonists in two experimental models, OVA-induced asthma and autoimmune diabetes. Secondly, in the same models we investigated the effect of probiotics. We also examined the effect of the genetic invalidation of MyD88 on the development of spontaneous diabetes, as previously performed by the group of A. Chervonsky [33], and extended the analysis to the allergic asthma model.

Collectively, results presented in this study demonstrate that systemic TLR stimulation by both probiotics and TLR agonists is efficacious in preventing from both allergy and autoimmunity.

MATERALS AND METHODS

Mice

Conventional NOD mice (K^d, $I\text{-}A^{g7}$, and D^b) were bred in our animal facility at the Hôpital Necker as well as $CD28^{-/-}$ NOD mice (a kind gift from J.A. Bluestone, UCSF, San Francisco, CA), $CD1d^{-/-}$ (a kind gift from M. Kronenberg, La Jolla institute, San Diego, CA) and $IL\text{-}4^{-/-}$ (a kind gift from D. Mathis and C. Benoist, Josslin Center, Boston, MA). We backcrossed C57BL/6 $MyD88^{-/-}$ mice into the NOD genetic background (N13). Seven-week-old female C57Bl/6 mice were purchased from Janvier.

Ethics Statement

All experiments have been conducted in accordance with European Union Council Directives (86/609/EEC) and with institutional guidelines (INSERM: Institut National de la Santé et de la Recherche Médicale). The animal facility has an agreement delivered by the *Prefecture de Police* of Paris, France.

TLR agonists and probiotics

The P40 protein of *Klebsiella pneumoniae* was purified as previously described [34]. We obtained purified LPS from *S. minnesota* and R848 from Alexis Biochemicals (Paris, France). Poly(I:C) was purchased from Invivogen (Toulouse, France) and Sigma-Aldrich (St Louis, MO) as well as lipid A from *S. Minnesota*. The probiotic preparation VSL#3 containing *bifidobacterium, lactobacillium* and *streptococcus* was purchased from Sigma-tau (Ivry-sur-Seine, France).

The ovalbulmin-induced airway inflammation model and treatments

On day 0 mice were sensitized with 100 µg of chicken egg OVA (Sigma-Aldrich) in 1.6mg aluminium hydroxide *i.p.* in a volume of 200µl. Then they were challenged with 50 mg/ml OVA upon aerosol exposure on three consecutive days (days 7–9) to induce allergic

airway inflammation. Controls received a NaCl solution. The TLR agonists P40, Poly(I:C), LPS or R848 were injected *i.p.* 24 hrs and 1 hr before the first challenge. We assessed lung function by measuring AHR 24 hrs after the last challenge by delivering an aerosol of Mch (Sigma-Aldrich) for 1min at 150mM to mice placed in a plethysmographic chamber (EMKA technologies, Paris, France). The index of airflow obstruction was expressed as enhanced pause (Penh). After AHR assessment, mice were killed for further analysis. For probiotic treatment, VSL#3 preparation (5. 10^9 bacteria/mouse in 100 µl PBS) was administered by gavage 5 days a week during 6 weeks before the OVA immunization. In these experiments control mice were treated with PBS.

An infraoptimal protocol was also set for $MyD88^{+/+}$ and $MyD88^{-/-}$ NOD mice. Here mice were immunized with only 50µg of OVA in 1.6mg of aluminium hydroxide and challenged by aerosol exposure with a single reduced dose of OVA (20mg/ml) on day 7. Samples were collected 48hrs after the challenge for further analyses. In some experiments a monoclonal antibody specific for the IL-10 receptor, that neutralizes IL-10 activity (1B1 2.1C4 clone) was administered *i.p.* 48 hrs and 1 hr before the first challenge.

For adoptive transfer experiments, 5.10^6 $CD4^+$ cells were purified from total spleen cells following magnetic bead sorting (Myltenyi Biotec, Paris, France) recovered from either probiotic-treated or untreated control NOD mice. The cells were injected *i.v.* to syngeneic recipients that had already been immunized with OVA and which received the $CD4^+$ cell infusion 1 hr before the first challenge.

Bronchoalveolar lavage

Mice were anesthetized with urethane (Sigma-Aldrich) administered *i.p.* The lungs were cannulated through the trachea to perform the bronchoalveolar lavage. Cellular fractions

recovered were processed for differential staining by cytospin centrigugation. The BALF as well as lung homogenates were analyzed for cytokine and chemokine content by ELISA (R&D Systems, Lille France) according to manufacturers' specifications.

Monitoring for autoimmune diabetes and treatment of NOD mice

We treated NOD mice with TLR agonists or PBS and monitored weekly for clinical signs of diabetes using Gluko-Test reagent sticks, to detecting glucose in urine samples (Boehringer Mannheim, Meylan, France). When needed, glycemia was also measured in a drop of blood collected from the tail vein and using a Reflolux S glucometer (Boehringer Mannheim). NOD mice were also treated with probiotics (VSL#3; 5.10^9 bacteria/mouse in 100□l PBS) delivered orally by gavage 5 times a week starting at 4 weeks of age. In these experiments control mice received PBS.

Histological analysis

Pancreas were collected when needed, fixed in 4% formaldehyde and paraffin-embedded. Serial 2-µm sections were stained with hematoxylin and eosin to scored for islet mononuclear cell infiltration.

***In vitro* cultures**

For cytokine production $2x10^5$ splenocytes from MyD88$^{+/+}$ and MyD88$^{-/-}$ C57Bl/6 mice were cultured in complete medium: RPMI supplemented with antibiotics and 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France) in the absence or presence of TLR agonists at varying doses. After 48 (for IL-10) to 72 (for TGF-β) hrs of culture at 37°C, supernatants were harvested. For TGF-β, responses to TLR agonists were compared to control cultures performed in serum-free medium. When needed peritoneal macrophages were collected 48 hrs following *i.p.* injection with 2 ml of thioglycollate broth (BioMérieux, Craponne, France). Macrophages were

then cultured in presence of probiotics for 24 hrs at 37°C. All supernatants were harvested and stored at -20°C until cytokines were dosed by ELISA (R&D systems, Lille, France).

Circulating cytokine analysis

NOD mice were injected with TLRs agonists or fed with probiotics for two weeks (see details in the results section). Twenty four hrs after the end of treatment, serum samples were collected and levels of circulating TGF-β and IL-10 were measured by ELISA (R&D systems).

Flow cytometry analysis

Spleen cells were recovered following treatment with TLR agonists or probiotics (see details in the results section). Cell suspensions were stained with antibodies to CD25 (labelled to phycoerythrin (PE)) and CD4 (labelled to phycoerythrin fluorescein isothiocyanate (FITC)) (BD Biosciences, Pont de Claix, France). Then cells were fixed and labelled with the Foxp3 kit (BD Biosciences) according to manufacturer's instructions. Samples were collected on a FACSCantoII cytometer (BD Biosciences). Data were gated on mononuclear cells with forward- and side-scatter properties using the FACS Diva Software.

Statistical analysis

Diabetes incidence was plotted using the Kaplan-Meier method, i.e., nonparametric cumulative survival plot. Statistical comparison between curves was performed using the logrank (Mantel-Cox) test that provided the corresponding χ^2 values. When needed, statistical comparison of mean values was performed using Student's t test. In the allergic model, the difference between groups was calculated with the Mann-Whitney U test for unpaired data (GraphPad Prism Software, La Jolla, CA). Differences were considered significant when $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

RESULTS

Effect of TLR agonists on allergic asthma and autoimmune diabetes in NOD mice

We tested the effect of agonists of TLR2 (P40 protein of *Klebsiella pneumoniae* and the Pam_3Cys lipopeptide), TLR3 (double-stranded RNAs polyinosinic:polycytidylic acid, Poly(I:C)), TLR4 (lipopolysaccharide (LPS) and lipid A) and TLR7 (resiquimod (R848)) on experimental allergic asthma and autoimmune diabetes in the NOD mouse. In the asthma model, NOD mice were immunized intraperitoneally on day 0 with OVA in presence of alum, challenged one week later with 3 consecutive OVA aerosol administrations and analyzed 24 hrs after the last challenge. Following this protocol, mice presented allergic inflammation and airway hyper-responsiveness (AHR) to methacholine (Mch). Allergic inflammation resulted in an increase of cell recruitment including eosinophils in the bronchoalveolar lavage fluid (BALF), and of cytokine and chemokine production, Interleukin (IL-)4 and eotaxin respectively, in the lung. The TLR2, TLR3, TLR4 and TLR7 agonists were administered 24 hrs and 1hr before the first challenge. In treated mice a significant decrease in AHR as well as in eosinophil counts in BALF and levels of IL-4 and eotaxin in the lung were observed (Figure 1). Levels of IL-5, IL-13, IL-17 and TARC in the lungs were also decreased (data not shown).

In the spontaneous autoimmune diabetes model, TLR agonists were injected once a week intraperitoneally (*i.p.*) starting at four weeks of age for 20 consecutive weeks. In these conditions, most of the TLR agonists tested were protective (Figure 2A). It is interesting that, for a given TLR, some agonists were not protective although their efficiency at stimulating the TLR pathway *in vitro* has been well demonstrated. Thus, lipid A, which is the lipid portion of LPS, was not protective whereas LPS was (Figure 2A). Most agonists required a long treatment (20 consecutive weeks) for inducing protection, with the exception of P40 which was also protective after a shorter 10-week treatment (Figure 2B). TLR agonists were protective when treatment was started early in disease development, between 4 and 10 weeks

of age. A delayed treatment did no longer protect from diabetes (Figure 2C); disease aggravation was never observed.

In protected animals the histological analysis of pancreata showed a reduction in destructive islet infiltration (i.e. invasive insulitis) (Figure 2D). It appeared that depending on the agonist the effect observed was either a global prevention of the infiltration (in the case of P40) or a control of insulitis progression (in the case of LPS, R848 and Poly(I:C)) with high proportion of infiltrated islet showing a benign form of non invasive peripheral insulitis (Figure 2D).

Contrasted effects of MyD88 invalidation on allergic asthma and autoimmune diabetes

In order to study if exogenous or endogenous pathogens (intestinal commensal bacteria) modulate OVA-induced allergic asthma or diabetes, we studied the effect of MyD88 deficiency on these diseases. At variance with results described above which showed a striking parallelism in the pharmacological effects of the TLR agonists in both conditions, impact of MyD88 deficiency on asthma and diabetes was strikingly different. Thus, MyD88$^{-/-}$ mice immunized with OVA died (with asphyxia) within few hours following the first or the second aerosol challenge with OVA as compared to wild-type MyD88$^{+/+}$ mice which regularly survived the three challenges (Figure 3A). Given these results MyD88$^{-/-}$ mice were immunized and challenged with lower doses of OVA (50 µg as compared to 100 µg for immunization and 20 mg/ml as compared to 50 mg/ml for challenge) to allow a significant mouse survival. Results shown on Figure 3B indicate that following this infraoptimal OVA stimulation BALF from MyD88$^{-/-}$ mice contained significantly increased numbers of total leucocytes and eosinophils as compared to controls.

Contrasting with the exacerbated airway allergic response the incidence of autoimmune diabetes was drastically reduced in MyD88$^{-/-}$ mice (Figure 3C). None of these

mice developed diabetes by 30 weeks of age; a nearly complete prevention of insulitis was also observed (Figure 3D).

Effect of TLR agonists on immune regulatory pathways

Using both *in vitro* and *in vivo* models we investigated the implication of immune regulatory cytokines and of subsets of regulatory lymphocytes in the protective effect of TLR2, TLR3, TLR4 and TLR7 agonists.

In vitro, the 4 agonists induced the production of either TGF-β (for P40, Poly(I:C) and LPS) or IL-10 (all 4 agonists) by spleen cells (Figure 4A). *In vivo*, serum concentrations of TGF-β and IL-10 were measured 24 hrs after the agonists' injection. As shown in Figure 4B, an increased level of IL-10 and TGF-β was observed after treatment with TLR2, 3 or 7 agonists. We also observed that the administration of a single dose of LPS or R848 increased the number of regulatory $CD4^{+}CD25^{+}Foxp3^{+}$ T cells in the spleen 24hrs after injection whereas Poly(I:C) did not (Figure 4C and data not shown).

To directly evaluate *in vivo* the role of $CD4^{+}CD25^{+}Foxp3^{+}$ and NK T cells in the protection induced by TLR3 and TLR4 agonists we took advantage of $CD28^{-/-}$ and $CD1d^{-/-}$ NOD mice that are deficient in these two subsets respectively. Results shown in Figure 4D demonstrate that the protective effect of LPS was not observed in $CD28^{-/-}$ NOD mice but was still present in $CD1d^{-/-}$ NOD mice. Conversely, the protective effect of Poly(I:C) was observed in $CD28^{-/-}$ NOD mice but not in $CD1d^{-/-}$ NOD mice. Interestingly, the TLR3 agonist-mediated protection that was dependent on the presence of NK T cells was abrogated in $IL\text{-}4^{-/-}$ NOD mice which was not the case for the protective effect of LPS, whose protective effect did not depend on NK T cells (Figure 4D).

Effect of probiotic bacteria on allergic asthma and autoimmune diabetes

We studied the capacity of probiotic bacteria to protect from both experimental allergic asthma and autoimmune diabetes.

In the allergic asthma model we administered VSL#3, a commercial combination of probiotic bacteria (*bifidobacterium, lactobacillium* and *streptococcus*), orally for 6 weeks before the first i.p. OVA immunization. In the spontaneous diabetes model, treatment was given once a week for 20 consecutive weeks starting at 4 weeks of age. Results demonstrated significant protection in the two models (Figure 5A,B). The protective effect was dose-dependent (data not shown).

In vitro, the same probiotic preparation induced TNF-α and IL-10 production by macrophages, an effect that was MyD88-dependent as shown by the absence of production of both cytokines by cells obtained from MyD88$^{-/-}$ mice (Figure 5C).

The TLR dependency of the probiotic bacteria effect was further confirmed *in vivo* in the allergic asthma model since MyD88$^{-/-}$ mice were insensitive to treatment, using the low OVA dose described above, compatible with mouse survival (Figure 5D). In MyD88$^{-/-}$ mice results showed no effect of probiotic bacteria treatment on neither eosinophil recruitment nor IL-4 production (Figure 5D).

Interestingly, in the sera of mice protected following probiotic bacteria administration increased levels of TGF-β were detected (Figure6A). We also detected in the spleen of protected mice an increased frequency of CD4^{+}CD25^{+}FoxP3^{+} T cells (Figure 6B).

In keeping with the *in vitro* data described above the protective effect of probiotic bacteria was IL-10-dependent. In fact, administration of an anti-IL-10 receptor monoclonal antibody before the first aerosol OVA challenge completely abolished the therapeutic effect (Figure 6C).

Finally, the probiotic protective effect was transferable by T cells. Adoptive transfer of $CD4^+$ splenocytes from probiotic-treated mice into OVA-challenged wild-type NOD mice significantly inhibited AHR (Figure 6D).

DISCUSSION

Here we show that a wide spectrum of agonists for various TLRs administered systematically inhibits both allergic and autoimmune responses extending in a comprehensive fashion previous isolated reports. These results are at variance with observations made after using TLR agonists at the site of antigen administration in normal and pathological conditions as discussed in the introduction.

The study of $MyD88^{-/-}$ mice provided different results in asthma and diabetes, which initially surprised us in view of the similarity of the effects of all TLR agonists in the two animal models. The acceleration of OVA-induced asthma in $MyD88^{-/-}$ mice is in agreement with the pharmacological effects of TLR agonists in this model, corroborating their postulated mode of action and supporting the notion that environmental infectious agents contribute to the modulation of allergic reactions through TLR stimulation. These data are in keeping with the previous report showing that $MyD88^{-/-}$ mice present a Th2 skewed balance with increased IgE levels [35]. It was more difficult to explain the complete prevention of diabetes observed in $MyD88^{-/-}$ NOD mice reported by Chervonsky and observed in our study [33]. The first interpretation is that MyD88 is mandatory for diabetes development. Another possibility is that MyD88 deficiency favors the development of infections that inhibit diabetes onset according to the hygiene hypothesis. The latter interpretation is supported by Chervonsky's data showing that specific pathogen-free (SPF) $MyD88^{-/-}$ mice are protected from diabetes (as in our own study) but germ-free $MyD88^{-/-}$ mice show normal diabetes incidence [33]. It may be interesting to mention here that invalidation of the CD28 gene also shows contrasting effects on diabetes and asthma with acceleration of diabetes and reduction of

asthma [36,37]. Finally, it appears that our results showing diabetes prevention with TLR agonists represent the only evidence for TLR involvement in the control of the diabetogenic response in NOD mice.

Results obtained in our studies in the diabetes model point to several non mutually exclusive mechanisms underlying asthma and diabetes prevention by TLR agonists. These include: 1) the production of immune regulatory cytokines that was observed both *in vitro* and *in vivo*, 2) the involvement of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells indicated by the loss of protection observed in $CD28^{-/-}$ NOD mice that are devoid of $CD4^{+}CD25^{+}$T cells [38], and 3) the involvement of NKT cells demonstrated by the absence of diabetes protection in $CD1d^{-/-}$ NOD mice that are deprived of NKT cells [39]. Among the TLR agonists protective for diabetes, it is interesting to highlight that the regulatory mechanisms involved differed. Thus, TLR3 stimulation by Poly(I:C) required the presence of NKT cells but not that of $CD4^{+}CD25^{+}FoxP3^{+}$ T cells. Conversely, TLR4 stimulation by LPS required the presence of $CD4^{+}CD25^{+}FoxP3^{+}$ T cells but not that of NKT cells. It is also striking that diabetes protection by TLR3 stimulation required the presence of IL-4 which was not the case for TLR4-induced protection. It is to note that the role of $CD4^{+}CD25^{+}$T cells could not be tested in the allergic asthma model since $CD28^{-/-}$ mice do not develop OVA-induced asthma [37].

Finally, the role of cytokines is difficult to associate with a given TLR since TLR-induced cytokine production, which was consistently observed, varied with each agonist: one should note however that TLR2, TLR3 and TLR7 agonists preferentially stimulated IL-10 and TGF-β production which was not the case for LPS.

Our results using probiotic were performed to approach a more physiological situation of a TLR-mediated inhibition of allergic asthma as assessed by the absence of protection in $MyD88^{-/-}$ NOD mice. This model allowed us to perform complementary mechanistic studies

that showed tranfer of protection by $CD4^+$ T cells, stimulation of IL-10 production and loss of the protective effect following administration of a neutralizing anti-IL-10 receptor antibody.

The association of a defined mechanism with a specific TLR is further complicated by the fact that for a given TLR distinct agonists do not show the same pharmacological profile: for instance in the case of TLR4, LPS is protective whereas lipid A is not. The whole of these data pave the way for a new pharmacology of TLR stimulation in allergy and autoimmunity with contrasting effects depending not only on the nature of the TLR receptor but also on that of the specific ligand.

If it were confirmed that TLR stimulation modulates the function of regulatory $CD4^+CD25^+FoxP3^+$ T cells or NKT cells, it would be of central importance to determine whether this effect is direct or indirect (e.g. through dendritic cells [40,41]). Studies from different laboratories indicate the presence of TLRs on various subsets of regulatory T cells with however some contradictory data [42–44]. One should also note that mechanisms other that those discussed above could operate [45,46], notably IL-10 production by B cells [47–49].

The relevance of these results for the evolving epidemiology of asthma and autoimmune type 1 diabetes is intriguing for the search of new preventive treatments of these diseases. Results presented in this manuscript indicate that TLR ligands present in infectious agents could contribute to the protection afforded by these agents against these diseases according to the hygiene hypothesis. Compelling evidence suggests indeed that the reduction of infections is an important factor explaining the increased incidence on allergic and autoimmune diseases[28]. The role of TLRs in infection-mediated protection of immune diseases does not exclude other mechanisms notably lymphocyte competition for homeostatic factors which develop between the immune responses mounted by infectious agents and the allergic and autoimmune responses raised by strong antigens[50]. At the therapeutic level, our

results suggest the possibility of using TLR ligands and probiotics in the prevention of allergic and autoimmune diseases inasmuch safety is fully documented. Preliminary data requiring further confirmation have been reported for probiotics in atopic dermatitis [51–54]. One would certainly prefer, in the future, to use well-defined chemical synthetic TLR agonists as those used in our work. Probiotics present the interest of direct accessibility and low toxicity but suffer from poor standardization. TLR agonists are probably more potent but are confronted with the risk of stimulating undesirable immune responses which would necessitate safety studies.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AA, FG, AR, LPV, EB and NT. Performed the experiments: AA, FG, AR, LPV, EB and AGA. Analyzed the experiments: AA, FG, AR, LPV, EB, AGA and NT. Contributed reagents/materials/analysis tools: P.J. and S.A. Wrote the paper: AA, FG, JFB and NT.

REFERENCES

1. Blander JM, Medzhitov R (2006) On regulation of phagosome maturation and antigen presentation. Nat Immunol 7: 1029-1035.

2. Kumar H, Kawai T, Akira S (2009) Pathogen recognition in the innate immune response. Biochem J 420: 1-16.

3. Beutler B (2009) Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev 227: 248-263.

4. Anders HJ, Vielhauer V, Eis V, Linde Y, Kretzler M, Perez de LG, Strutz F, Bauer S, Rutz M, Wagner H, Grone HJ, Schlondorff D (2004) Activation of toll-like receptor-9 induces progression of renal disease in MRL-Fas(lpr) mice. FASEB J 18: 534-536.

5. Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, Odermatt B, Conrad C, Ittner LM, Bauer S, Luther SA, Uematsu S, Akira S, Hengartner H, Zinkernagel RM (2005) Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 11: 138-145.

6. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 416: 603-607.

7. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, Christensen SR, Shlomchik MJ, Viglianti GA, Rifkin IR, Marshak-Rothstein A (2005) RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. J Exp Med 202: 1171-1177.

8. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN (2009) House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med 15: 410-416.

9. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, Karp CL (2009) Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature 457: 585-588.

10. Marty RR, Dirnhofer S, Mauermann N, Schweikert S, Akira S, Hunziker L, Penninger JM, Eriksson U (2006) MyD88 signaling controls autoimmune myocarditis induction. Circulation 113: 258-265.

11. Su SB, Silver PB, Grajewski RS, Agarwal RK, Tang J, Chan CC, Caspi RR (2005) Essential role of the MyD88 pathway, but nonessential roles of TLRs 2, 4, and 9, in the adjuvant effect promoting Th1-mediated autoimmunity. J Immunol 175: 6303-6310.

12. Serreze DV, Hamaguchi K, Leiter EH (1989) Immunostimulation circumvents diabetes in NOD/Lt mice. J Autoimmun 2: 759-776.

13. Quintana FJ, Rotem A, Carmi P, Cohen IR (2000) Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. J Immunol 165: 6148-6155.

14. Sel S, Wegmann M, Sel S, Bauer S, Garn H, Alber G, Renz H (2007) Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. J Immunol 178: 7805-7813.

15. Akdis CA, Kussebi F, Pulendran B, Akdis M, Lauener RP, Schmidt-Weber CB, Klunker S, Isitmangil G, Hansjee N, Wynn TA, Dillon S, Erb P, Baschang G, Blaser K, Alkan SS (2003) Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. Eur J Immunol 33: 2717-2726.

16. Camateros P, Tamaoka M, Hassan M, Marino R, Moisan J, Marion D, Guiot MC, Martin JG, Radzioch D (2007) Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. Am J Respir Crit Care Med 175: 1241-1249.

17. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC, Liew FY (2005) TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. J Immunol 174: 7558-7563.

18. Moisan J, Camateros P, Thuraisingam T, Marion D, Koohsari H, Martin P, Boghdady ML, Ding A, Gaestel M, Guiot MC, Martin JG, Radzioch D (2006) TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. Am J Physiol Lung Cell Mol Physiol 290: L987-L995.

19. Tulic MK, Wale JL, Holt PG, Sly PD (2000) Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 22: 604-612.

20. Hollingsworth JW, Whitehead GS, Lin KL, Nakano H, Gunn MD, Schwartz DA, Cook DN (2006) TLR4 signaling attenuates ongoing allergic inflammation. J Immunol 176: 5856-5862.

21. Jeon SG, Oh SY, Park HK, Kim YS, Shim EJ, Lee HS, Oh MH, Bang B, Chun EY, Kim SH, Gho YS, Zhu Z, Kim YY, Kim YK (2007) TH2 and TH1 lung inflammation induced by airway allergen sensitization with low and high doses of double-stranded RNA. J Allergy Clin Immunol 120: 803-812.

22. Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, Raz E (2004) Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol 172: 2739-2743.

23. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 196: 1645-1651.

24. Murakami D, Yamada H, Yajima T, Masuda A, Komune S, Yoshikai Y (2007) Lipopolysaccharide inhalation exacerbates allergic airway inflammation by activating mast cells and promoting Th2 responses. Clin Exp Allergy 37: 339-347.

25. Shah U, Walker WA (2002) Pathophysiology of intestinal food allergy. Adv Pediatr 49: 299-316.

26. Strachan DP (1989) Hay fever, hygiene, and household size. BMJ 299: 1259-1260.

27. Conrad ML, Ferstl R, Teich R, Brand S, Blumer N, Yildirim AO, Patrascan CC, Hanuszkiewicz A, Akira S, Wagner H, Holst O, von ME, Pfefferle PI, Kirschning CJ, Garn H, Renz H (2009) Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78. J Exp Med 206: 2869-2877.

28. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 347: 911-920.

29. Sadelain MW, Qin HY, Lauzon J, Singh B (1990) Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. Diabetes 39: 583-589.

30. Zaccone P, Raine T, Sidobre S, Kronenberg M, Mastroeni P, Cooke A (2004) Salmonella typhimurium infection halts development of type 1 diabetes in NOD mice. Eur J Immunol 34: 3246-3256.

31. Alyanakian MA, Grela F, Aumeunier A, Chiavaroli C, Gouarin C, Bardel E, Normier G, Chatenoud L, Thieblemont N, Bach JF (2006) Transforming growth factor-beta and natural killer T-cells are involved in the protective effect of a bacterial extract on type 1 diabetes. Diabetes 55: 179-185.

32. Takei I, Asaba Y, Kasatani T, Maruyama T, Watanabe K, Yanagawa T, Saruta T, Ishii T (1992) Suppression of development of diabetes in NOD mice by lactate dehydrogenase virus infection. J Autoimmun 5: 665-673.

33. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 455: 1109-1113.

34. Jeannin P, Bottazzi B, Sironi M, Doni A, Rusnati M, Presta M, Maina V, Magistrelli G, Haeuw JF, Hoeffel G, Thieblemont N, Corvaia N, Garlanda C, Delneste Y, Mantovani A (2005) Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. Immunity 22: 551-560.

35. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R (2001) Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2: 947-950.

36. Lenschow DJ, Herold KC, Rhee L, Patel B, Koons A, Qin HY, Fuchs E, Singh B, Thompson CB, Bluestone JA (1996) CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. Immunity 5: 285-293.

37. Kimzey SL, Liu P, Green JM (2004) Requirement for CD28 in the effector phase of allergic airway inflammation. J Immunol 173: 632-640.

38. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12: 431-440.

39. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van KL (1997) CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity 6: 469-477.

40. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, Capron M, Ryffel B, Faveeuw C, Leite de MM, Platt F, Trottein F (2007) Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. Immunity 27: 597-609.

41. Nyirenda MH, O'Brien K, Sanvito L, Constantinescu CS, Gran B (2009) Modulation of regulatory T cells in health and disease: role of toll-like receptors. Inflamm Allergy Drug Targets 8: 124-129.

42. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J (2003) Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med 197: 403-411.

43. Pasare C, Medzhitov R (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299: 1033-1036.

44. Liu H, Komai-Koma M, Xu D, Liew FY (2006) Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proc Natl Acad Sci U S A 103: 7048-7053.

45. Zuany-Amorim C, Sawicka E, Manlius C, Le MA, Brunet LR, Kemeny DM, Bowen G, Rook G, Walker C (2002) Suppression of airway eosinophilia by killed

Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 8: 625-629.

46. Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT (2004) Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. Nat Immunol 5: 1149-1156.

47. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF (2008) A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 28: 639-650.

48. Bouaziz JD, Yanaba K, Tedder TF (2008) Regulatory B cells as inhibitors of immune responses and inflammation. Immunol Rev 224: 201-214.

49. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM (2002) B cells regulate autoimmunity by provision of IL-10. Nat Immunol 3: 944-950.

50. Bourgeois C, Stockinger B (2006) CD25+CD4+ regulatory T cells and memory T cells prevent lymphopenia-induced proliferation of naive T cells in transient states of lymphopenia. J Immunol 177: 4558-4566.

51. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 357: 1076-1079.

52. Kalliomaki M, Salminen S, Poussa T, Arvilommi H, Isolauri E (2003) Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet 361: 1869-1871.

53. Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, Paerregaard A (2003) Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol 111: 389-395.

54. Ruemmele FM, Bier D, Marteau P, Rechkemmer G, Bourdet-Sicard R, Walker WA, Goulet O (2009) Clinical evidence for immunomodulatory effects of probiotic bacteria. J Pediatr Gastroenterol Nutr 48: 126-141.

FIGURE LEGENDS

Figure 1. Stimulation of TLR pathways prevents allergic inflammation and airway hyper-responsiveness.

NOD mice were treated as described in the Methods section. Briefly, mice immunized with OVA on day 0 were challenged with OVA or NaCl (controls) on days 7, 8 and 9. Each TLR agonist or phosphate-buffered saline (PBS) was administered 24 hrs and 1 hr before the first challenge. OVA-challenged mice were treated with PBS or (**A**) P40 (200µg/challenge/mouse), a TLR2 agonist, (**B**) Poly(I:C) (100µg/challenge/mouse), a TLR3 agonist, **(C)** LPS (100µg/challenge/mouse), a TLR4 agonist and **(D)** R848 (100µg/challenge/mouse), a TLR7 agonist. AHR to Mch was measured 24hrs after the last challenge and total cell as well as eosinophils in BALF and cytokine and chemokine concentrations in lungs. Mice treated with TLR agonists showed a decreased AHR, eosinophilia and IL-4 and eotaxin production as compared to control mice (* $p < 0.05$; ** $p < 0.01$). These experiments were performed twice using 4 to 6 mice per group. One representative experiment is shown.

Figure 2. Stimulation of TLR pathways prevents spontaneous autoimmune diabetes in NOD mice.

A. Female NOD mice were injected *i.p.* with 200µg P40, 100µg Poly(I:C), 5µg LPS from *S. minnesota*, 5µg Lipid A, 10µg R848 or PBS once a week, starting at 3-4 weeks of age and for 20 consecutive weeks. Mice were monitored weekly for the advent of glycosuria (diabetes). Significant prevention from diabetes was observed with most TLR agonists tested (* $p < 0.05$; ** $p < 0.01$) but not with Lipid A. Each panel represents one of independent experiments using 8 mice per group. **B.** A shorter treatment with the TLR2 agonist P40 administered between 4 and 14 weeks also induced significant protection from diabetes (** $p < 0.01$). **C.** Treatment with Poly(I:C) was highly protective if started up to 10 weeks of age but not

later (at 16 weeks of age). **D.** Histological examination of hematoxylin and eosin stained pancreas sections recovered from the various experimental groups was performed (n=8 per group). Islet infiltration (insulitis) was scored by deducing the proportion of non-infiltrated islets (healthy islets) and of islets showing a non destructive peripheral insulitis (peri-insulitis) or an invasive/ destructive insulitis (destructive insulitis). The relative degree of islet inflammation in mice treated with P40, Poly(I:C), LPS or R848 is shown in a cumulative histogram as compared to PBS-treated controls.

Figure 3. MyD88$^{-/-}$ NOD mice are sensitive to airway allergic inflammation but resistant to the development of autoimmune diabetes.

As compared to MyD88$^{+/+}$ NOD mice, MyD88$^{-/-}$ NOD mice were hypersensitive to experimental allergic asthma. **A.** The left panel shows the data observed in MyD88$^{+/+}$ (n=25) and MyD88$^{-/-}$ (n=35) mice immunized with our conventional protocol, namely immunization with 100 µg of OVA on day 0 and challenge with 50mg/ml of OVA or NaCl on days 7, 8 and 9. Results are expressed in percentage of survival. The right panel shows the data observed when using an infraoptimal protocol according to which MyD88$^{+/+}$ (n=10) and MyD88$^{-/-}$ (n=15) mice were immunized with 50µg of OVA on day 0 and challenged only once with 20mg/ml of OVA or NaCl on day 7. Results are also expressed in percentage of survival. **B.** Results show that, using the infraoptimal immunization and challenge protocol, the eosinophil recruitment in BALF was more important in MyD88$^{-/-}$ as compared to MyD88$^{+/+}$ mice (* $p < 0.05$). **C.** Monitoring for the cumulative incidence of spontaneous diabetes showed that MyD88$^{-/-}$ NOD mice (n=25) were fully protected from disease (littermate female MyD88$^{+/+}$ NOD mice (n=60) showed a normal disease incidence reaching 80% by 30 weeks of age) (** $p < 0.01$). **D.** Histological examination of hematoxylin and eosin stained pancreas sections recovered from 20-week-old MyD88$^{+/+}$ and MyD88$^{-/-}$ NOD mice (n=8 per group) showed that a great majority of islets in MyD88$^{-/-}$ NOD mice were insulitis free.

Figure 4. Stimulation of the TLR/MyD88 pathway modulates immune regulatory cytokines and lymphocyte subsets.

A. *In vitro* stimulation of C57BL/6 mouse spleen cells with varying doses of different TLR agonists (P40, 1 or 20μg/ml; Poly(I:C), 1 or 10μg/ml; LPS, 0.1 or 1μg/ml; R848 0.1 or 1μg/ml) induced the production of cytokines such as IL-10 (at 48 hrs) and TGF-β (at 72 hrs). Study of splenocytes from MyD88$^{+/+}$ or MyD88$^{-/-}$ C57BL/6 mice confirmed that the effect is dependent on the MyD88 pathway. Results are expressed as mean cytokine level ± SD. Results are representative of three independent experiments. **B.** Circulating levels of IL-10 and TGF-β were detected following *in vivo* administration of TLR agonists. NOD mice were injected *i.p* 20μg of P40, 100μg of Poly(I:C) or 10μg of R848 (n=8 per group). Control mice were injected with saline (PBS). Sera were collected 24 hours after the injection and cytokine levels were measured by ELISA (*p < 0.05; ** p < 0.01; *** p < 0.005). **C.** Representative flow cytometry plots representing proportions of CD25^{+}FoxP3^{+} T cells (examined on gated CD4^{+} cells) in the spleen of mice 24hrs after injection of 5μg LPS or 10μg R848 (n=5 per group). **D.** The same conventional treatment protocol (described in Figure 2) with the TLR4 agonist LPS (5 μg/week/mouse) and the TLR3 agonist Poly(I:C), that protected wild type NOD mice from diabetes, was applied to female NOD mice invalidated for CD28 (CD28$^{-/-}$), CD1d (CD1d$^{-/-}$) and IL-4 (IL-4$^{-/-}$). Results obtained showed that the Poly(I:C)-induced protective effect was maintained in CD28$^{-/-}$ NOD mice (*p < 0.05) but not in CD1d$^{-/-}$ and IL-4$^{-/-}$ NOD mice. As a mirror-like image the LPS-induced protective effect was maintained in CD1d$^{-/-}$ and IL-4$^{-/-}$ NOD mice (* p < 0.05) but not in CD28$^{-/-}$ NOD mice. One representative experiment out of two is shown.

Figure 5. Probiotic administration prevents from both allergic asthma and autoimmune diabetes: a TLR/MyD88 pathway-dependent effect.

A. NOD mice received 5 days a week for 6 weeks a preparation of probiotics (VSL#3, 5.10^9 bacteria/mouse) and underwent the conventional OVA immunization/challenge protocol previously described. AHR as well as eosinophil counts in BALF and IL-4 levels in lung homogenates were measured. Results showed that probiotic treatment significantly prevented from experimental allergic asthma (** $p < 0.01$; *** $p < 0.005$). **B.** The same probiotic preparation (VSL#3; 5.10^9 bacteria/mouse) was administered orally by gavage to female NOD mice three times a week starting at 4 weeks of age (n=8 per group). Results obtained demonstrated a very significant disease protection (*** $p < 0.005$). **C.** *In vitro* incubation for 24 hrs of peritoneal macrophages from NOD mice with increasing concentrations of the VSL#3 probiotic preparation induced a dose-dependent production of TNF-α and IL-10. The MyD88 dependency of the effect was demonstrated by the lack of effect when macrophages from MyD88$^{-/-}$ mice were analyzed. **D.** The probiotic-induced protection from allergic airway inflammation was MyD88-dependent as illustrated by the comparative results obtained in MyD88$^{+/+}$ mice. The effect was illustrated here by the data on eosinophil counts in BALF showing that MyD88$^{-/-}$ mice (immunized and challenged according to the infraoptimal protocol, see figure 3) were completely refractory to the probiotic-treatment effect as compared to MyD88$^{+/+}$ mice (** $p < 0.01$). Results are representative of one experiment out of two.

Figure 6. Probiotic treatment and immune regulation

A. The VSL#3 probiotic preparation was administered orally to female NOD mice 5 days a week for 2 weeks (n=5 per group). Twenty four hrs after the last administration sera were collected and circulating TGF-β was measured: increased levels were found in mice treated with the active compound as compared to controls (* $p < 0.05$). **B.** Female NOD mice were treated with the VSL#3 probiotic preparation according to the same protocol described in A

(n=5 per group). Twenty four hrs after the end of treatment, spleens were recovered and $CD25^+FoxP3^+$ T cells (examined on gated $CD4^+$ cells) were analyzed in spleen. Representative flow cytometry plots are shown. **C.** NOD mice received 5 days a week for 6 weeks the VSL#3 probiotic preparation and then underwent the conventional OVA immunization/challenge protocol previously described. Results obtained showed that the probiotic-protective effect was completely reversed (both in terms of reduction of AHR (** $p < 0.01$) and of eosinophil recruitment in BALF (* $p < 0.05$, ** $p < 0.01$)) following IL-10 neutralization upon administration of an anti-IL-10 receptor monoclonal antibody prior to the first challenge. One representative experiment out of two is shown. **D.** Experimental allergic asthma was induced according to the conventional OVA immunization/ challenge protocol already described in normal NOD recipient mice transferred with $CD4^+$ cells purified from the spleen of probiotic- or control-treated syngeneic mice. Purified $CD4^+$ cells were transferred 24 hrs before the first challenge. Results obtained showed that both AHR and eosinophil recruitment in BALF were significantly decreased (** $p < 0.01$ for both parameters) in recipients of $CD4^+$ cells recovered from probiotic-treated donors. One representative experiment out of two is shown.

Figure 1

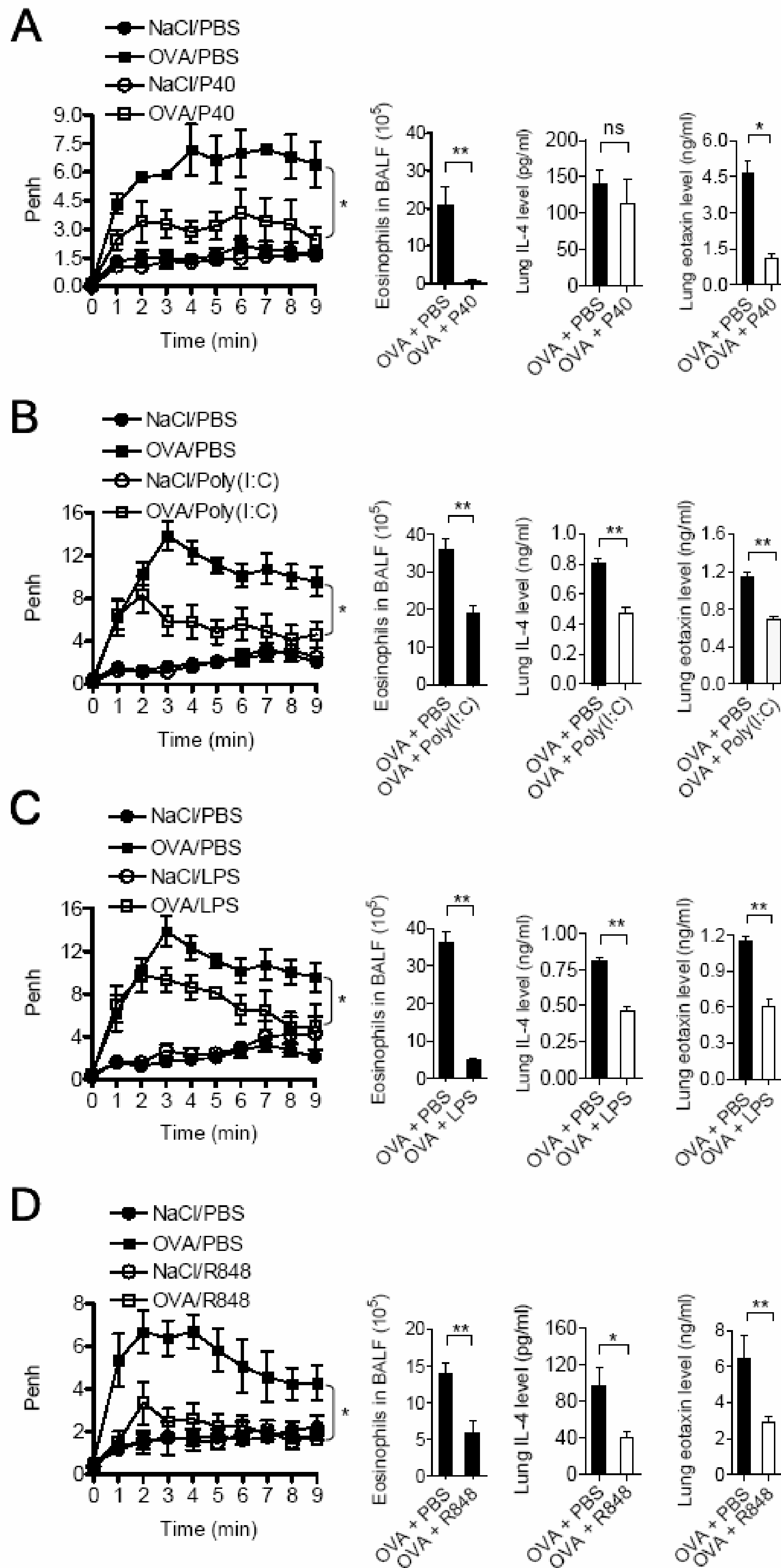


Figure 2

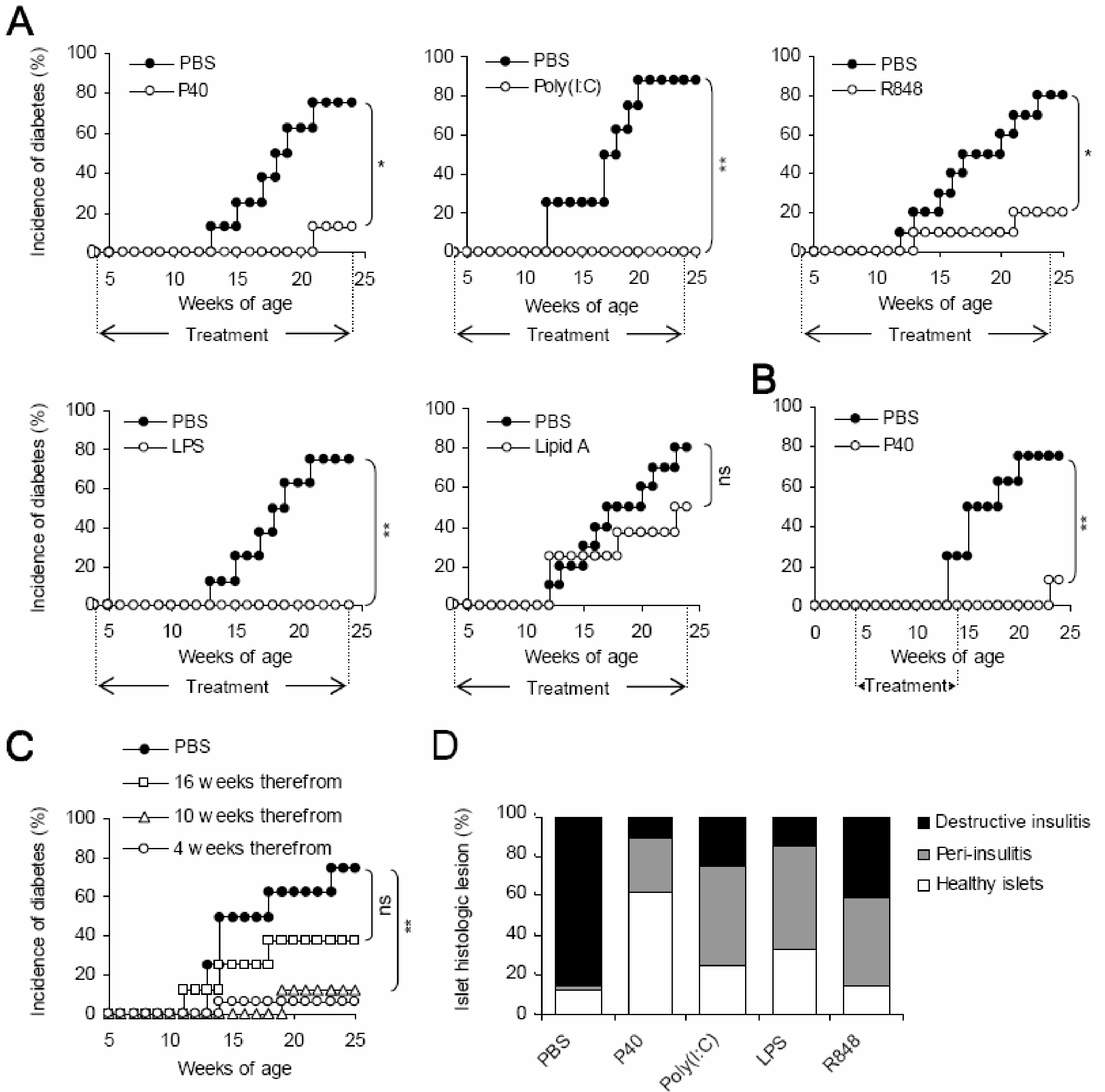
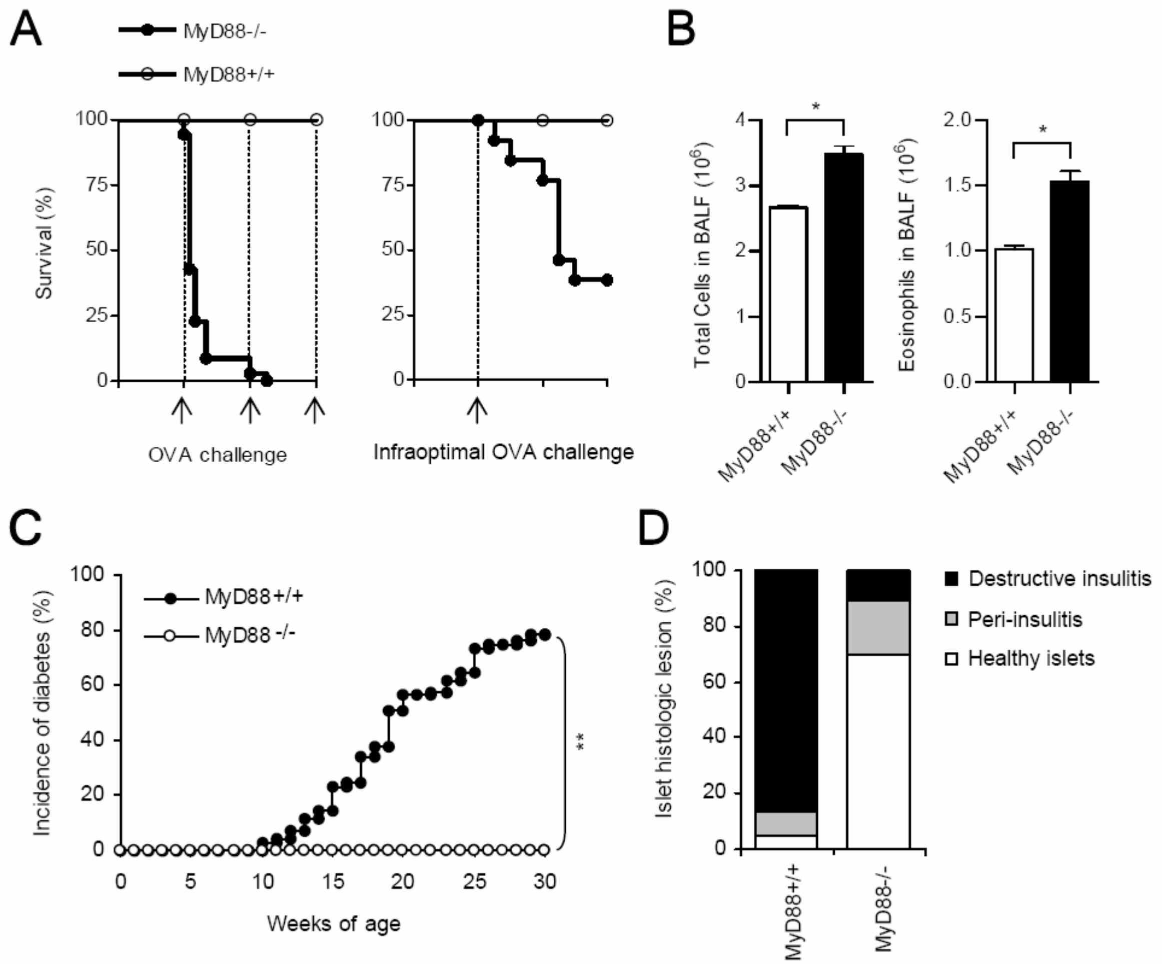


Figure 3



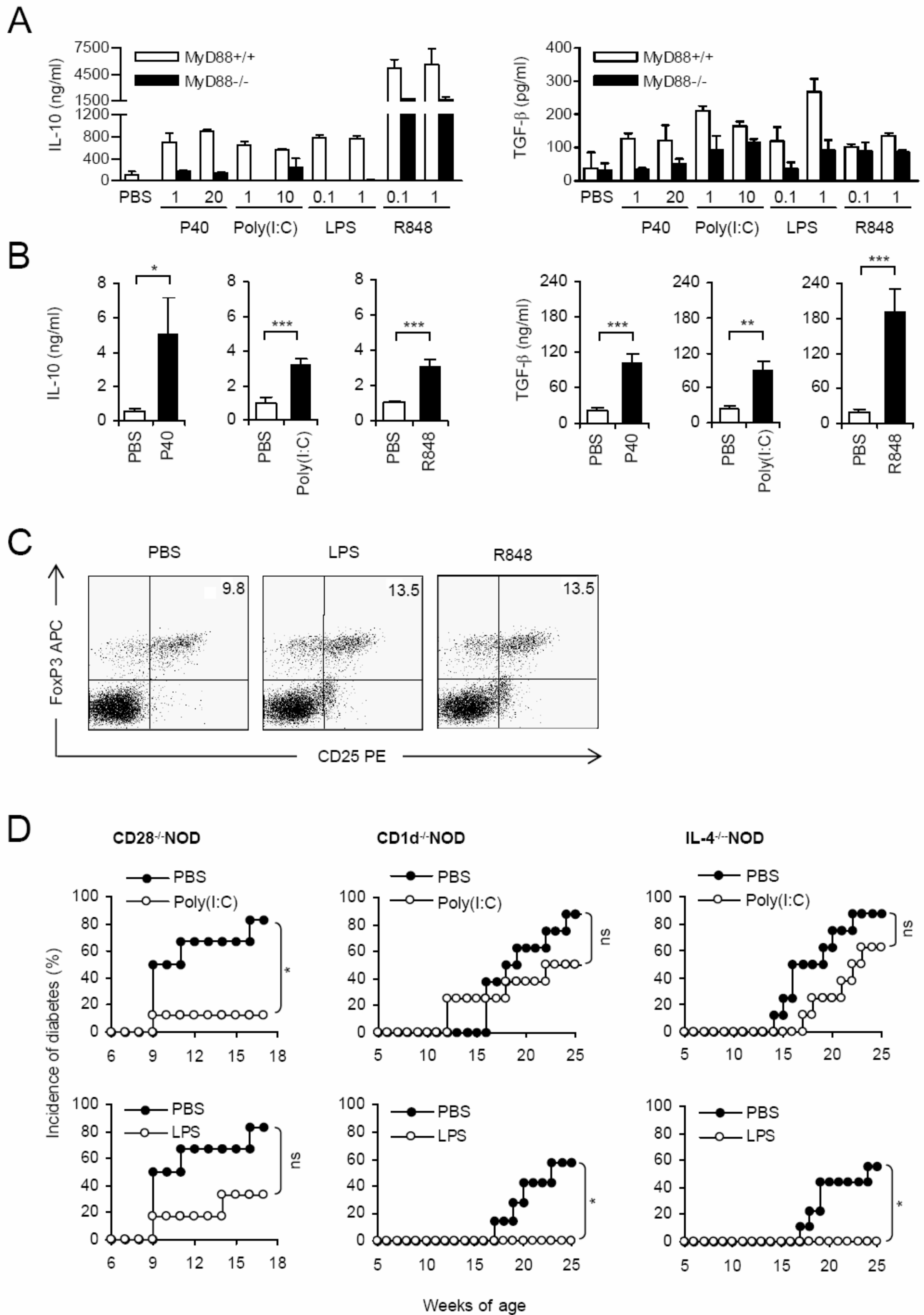


Figure 4
A
IL-10 (ng/ml)
MyD88+/+
MyD88-/-
TGF-β (pg/ml)
PBS
P40
Poly(I:C)
LPS
R848
B
IL-10 (ng/ml)
TGF-β (ng/ml)
C
PBS
LPS
R848
9.8
13.5
13.5
FoxP3 APC
CD25 PE
D
CD28-/-NOD
CD1d-/-NOD
IL-4-/-NOD
Incidence of diabetes (%)
Weeks of age
ns

Figure 5

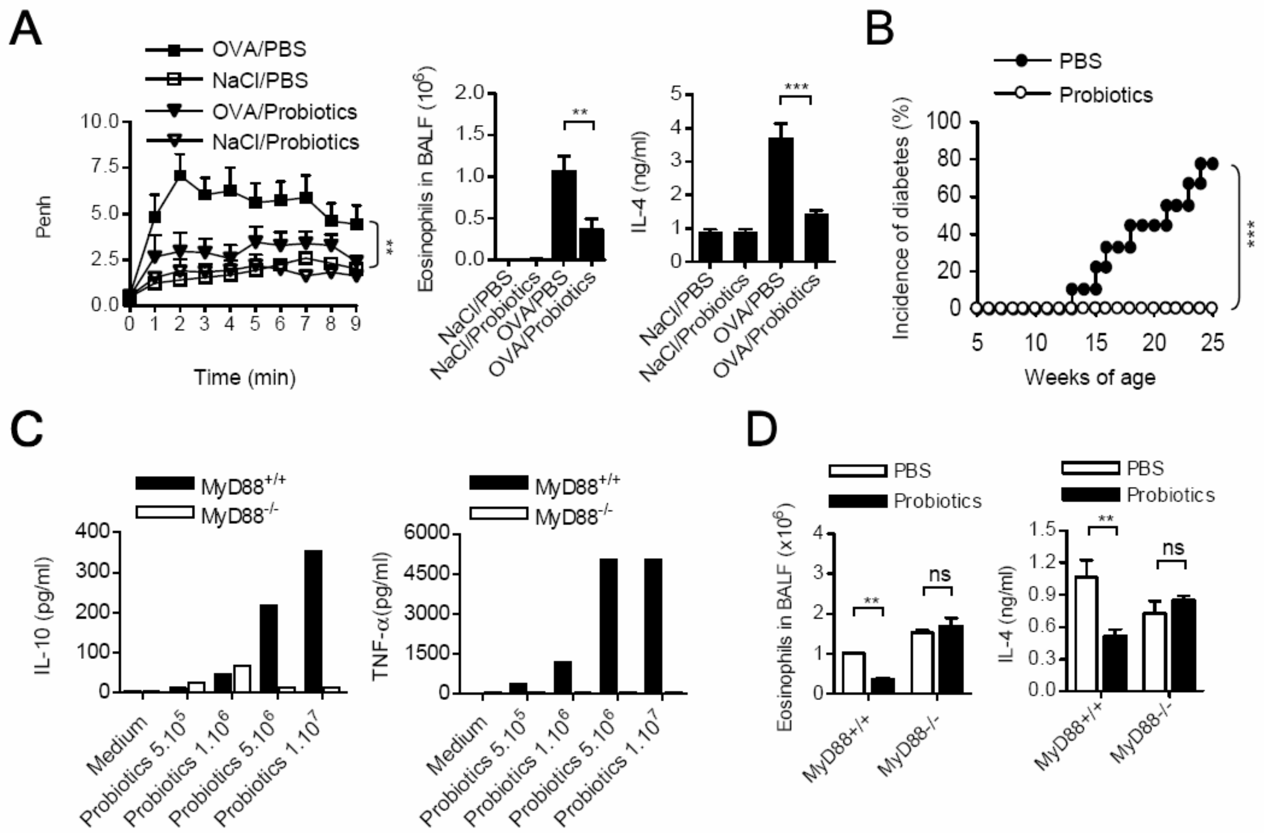
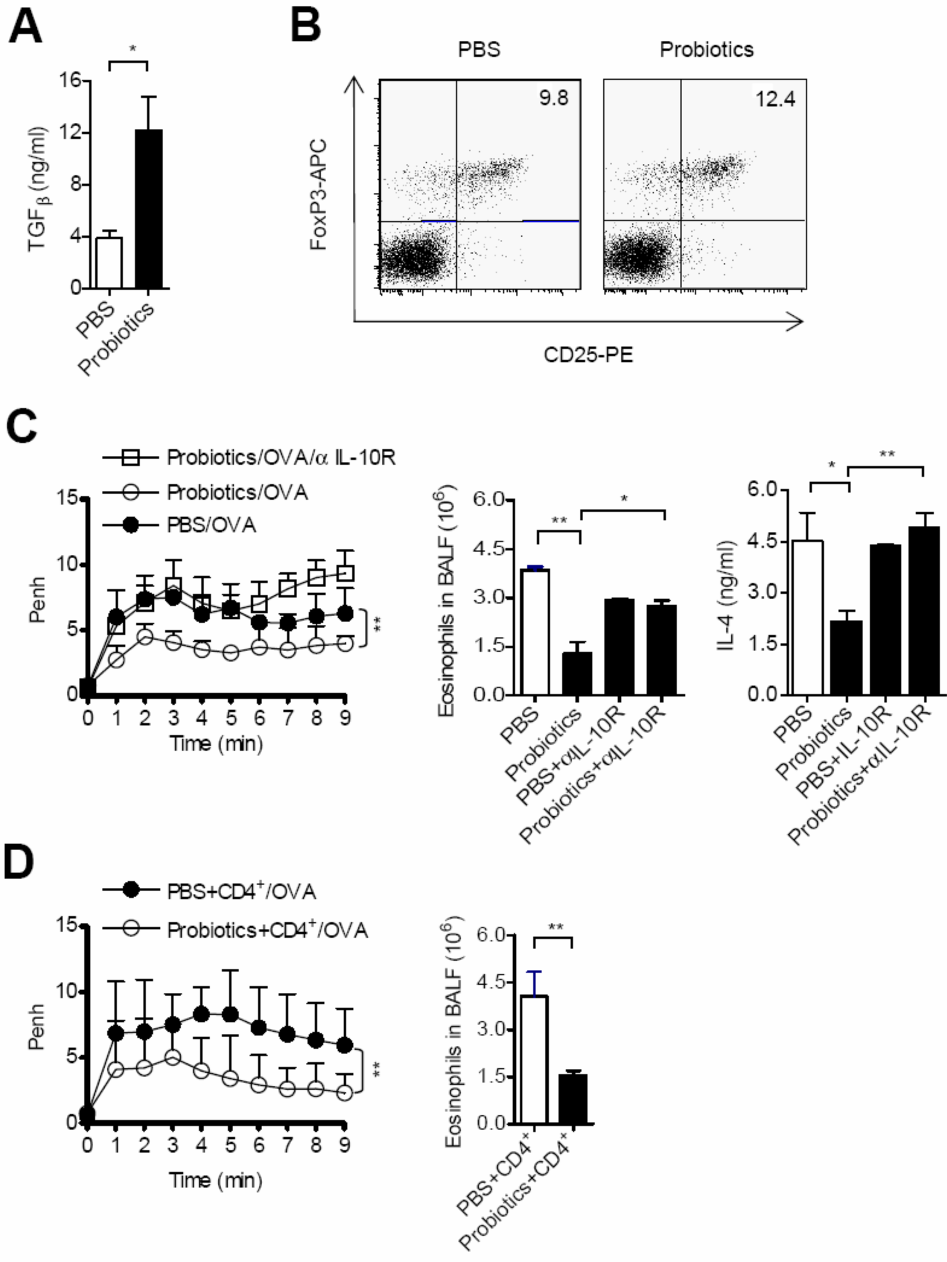


Figure 6



Article n°5

L'agoniste TLR7 (R848) atténue l'inflammation allergique en mettant en jeu les lymphocytes NKT invariants (iNKT) producteurs d'IFN-γ

Françoise Grela, Aude Aumeunier, Emilie Bardel, Linh Pham Van, Jeroen Vanoirbeek, Michel Dy, André Herbelin, Nathalie Thieblemont

Il a été bien démontré que la stimulation de TLR7 non seulement déclenche des réponses antivirales, mais également atténue l'asthme expérimental. Connaissant l'implication des lymphocytes T Natural Killer invariants (iNKT) dans ces deux situations, nous avons postulé que ces cellules pouvaient contribuer à l'effet anti-inflammatoire des ligands TLR7. Nous montrons, dans ce travail, que les symptômes d'asthme allergique sont effectivement atténués après transfert adoptif de splénocytes de souris activés par R848, agoniste du TLR7, à condition que ces cellules proviennent de souris sauvages et non de souris $J\alpha 18^{-/-}$ déficientes en iNKT. Cela prouve l'implication spécifique de cette population cellulaire régulatrice. De plus, nous avons montré que l'IFNγ jouait un rôle crucial dans cet effet protecteur puisque cet effet est perdu lorsque les cellules proviennent d'animaux déficients en IFNγ. En faveur d'une implication directe de l'activation des iNKT par la voie TLR, nous avons mis en évidence une augmentation rapide des niveaux sériques d'IFNγ associée à une expression accrue de CD69 à la surface des cellules iNKT. Enfin, nous d émontrons que les cellules iNKT expriment TLR7 et répondent in vitro au R848 par la production importante d'IFNγ en présence d'IL12. Ces résultats confirment que les cellules iNKT productrices d'IFNγ contribuent à l'amélioration de l'asthme allergique induite par le traitement par les ligands TLR7.

The Toll-Like Receptor 7 agonist R848 alleviates allergic inflammation by targeting invariant Natural Killer T cells to produce IFN-γ

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Running title: TLR7 agonist activates iNKT cells

Keywords: Toll-like receptor 7; iNKT cells; IFN-γ; allergic inflammation

ABSTRACT

It has been documented that Toll-like receptor 7 (TLR7) stimulation triggers not only antiviral responses but also alleviates experimental asthma. Considering the implication of invariant Natural Killer T (iNKT) cells in both situations, we postulated that they might contribute to the anti-inflammatory effect of TLR7 ligands. We show here that allergic inflammation is effectively attenuated upon adoptive transfer of spleen cells activated by the TLR7 agonist R848, provided that they are obtained from wild type and not from iNKT cell-deficient $J\alpha18^{-/-}$ mice, which proves the specific involvement of iNKT cells. Furthermore, we provide evidence that IFN-γ is critical for the protective effect, which is lost when transferred iNKT cells are sorted from IFN-γ-deficient mice. In support of a direct activation of iNKT cells through TLR7 signaling *in vivo*, we observed a prompt increase of serum IFN-γ levels, associated with upregulation of CD69 expression on iNKT cells. Moreover, we demonstrate that iNKT cells effectively express TLR7 and respond to R848 *in vitro* by producing high levels of IFN-γ in the presence of IL-12, consistent with the conclusion that their contribution to the alleviation of allergic inflammation upon treatment with TLR7 ligands is mediated through IFN-γ.

INTRODUCTION

Asthma is one of the most common chronic diseases in children in developed countries and has become a major public health issue. The hygiene hypothesis formulated by Strachan in 1989 claims that the decrease of infections during early childhood is responsible for the increased prevalence of allergic diseases (1). However, even though some epidemiological data support this assumption it has not been definitively proven so far. Substantial positive evidence has been provided by the observation that infections and non-pathogenic agents such as lactobacilli reduced the frequency of atopy (2,3). Furthermore, in experimental animal models it has been demonstrated that deliberate infections can prevent the onset of allergic diseases. Initially, it was argued that in developed countries the lack of microbial burden in early childhood, which normally favors a strong Th1-biased immunity, redirects the immune response toward a Th2 phenotype and therefore predisposes the host to allergic responses. However, more recent studies revealed that the mechanisms are actually far more complex, highlighting the role of specialized regulatory T cell subsets in the control of the Th1/Th2 balance.

Toll-like receptors (TLR) recognize pathogen-derived molecular patterns (PAMPs) of infectious agents, which trigger particular signaling pathways that influence the orientation of the immune response. Among these, TLR7 acts as a sensor of simple-stranded RNAs and imidazoquinolin family compounds, such as R848, also called resiquimod (4,5). This compound has already been shown to protect mice from asthma, by decreasing its typical symptoms, namely airway hyperresponsiveness (AHR), eosinophil recruitment to the lung and Th2 responses. It has also been reported for its ability to abrogate airway remodeling in a chronic asthma model (6). The efficacy of R848 in preventing the development of allergic inflammation in rodent models has been well established and some cellular and molecular mechanisms have been described. For example, it has been reported that the TLR7 agonist

inhibits the production of OVA-specific IgE and IgG1 antibodies and abolishes all hallmarks of experimental asthma, including AHR and allergic airway inflammation. The protective effect persisted, at least partially, in IL-12p35$^{-/-}$ mice, disappearing only when IL-10 was likewise deficient (7). A recent study suggested that IFN-γ-producing CD8^{+} T cells may contribute to the anti-allergic effect of R848, even though it was not abolished in IFN-γ-deficient mice (8). Camateros *et al.* reported that the expression profile of genes involved in natural killer (NK) cell-mediated cytotoxicity and NK cell recruitment to the lung was altered following R848 treatment, but could not establish a causal relationship between NK cell recruitment/activation and treatment efficiency (9).

Because invariant Natural Killer T (iNKT) cells, which express both NK markers and an invariant Vα14-Jα TCR chain, play a key role in the development of asthma, we hypothesized that they might take part in R848-induced asthma protection (10,11). iNKT cells exert deleterious or beneficial effects, depending on the stage of the immune response they are activated in. For example, they can aggravate allergic asthma by enhancing AHR and eosinophilia during the effector phase, but confer protection at disease onset, once they have been activated by their specific ligand α-galactosylceramide (α-GalCer) (12-14).

In our present study, we addressed the potential role of iNKT cells in the alleviation of allergic inflammation by TLR7 stimulation. We provide the first evidence that these regulatory cells are activated *in vivo* by R848, as evidenced by upregulation of their CD69 surface expression and prompt IFN-γ production. Using adoptive transfer experiments, we demonstrate that iNKT cells are responsible for asthma protection by donor cells from wild type mice having received a treatment with R848, as their counterpart from iNKT cell-deficient Jα18$^{-/-}$ mice had no such effect.

MATERIALS AND METHODS

Animals

6-8-week-old specific pathogen-free, C57BL/6J mice were purchased from Janvier (Les Genest St. Isle, France). $TLR7^{-/-}$ and $J\alpha18^{-/-}$ (kindly provided by Dr. Shizuo Akira and by Dr. T. Taniguchi, respectively) and $IFN\text{-}\gamma^{-/-}$ (Jackson Laboratories, Bar Harbor, ME) were bred in our animal facilities under specific pathogen-free conditions. Animal experiments were performed according to the recommendations of the French Institutional Committee.

Reagents

R848 was purchased from Alexis (Paris, France). Phorbol Myristate Acetate (PMA) and ionomycin (Iono) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Murine recombinant IL-12 was from R&D Systems (Lille, France). Fluorochrome-conjugated anti-CD5 (clone 55-7.3), anti-NK1.1 (clone PK136), anti-TCRβ (clone H57-597) and anti-CD69 (clone H1.2F3) were from BD Pharmingen (Le Pont de Claix, France). Allophycocyanin-conjugated CD1d/α-GalCer loaded tetramers were prepared in our laboratory from the murine CD1d/β_2-microglobulin expression vector (kindly provided by Sidobre and Kronenberg, CA) loaded with α-GalCer or provided by the NIH tetramer core Facility.

Protocol of immunization and challenge

Mice were immunized by i.p. injection of 100 µg of ovalbumin (OVA) (Sigma-Aldrich) adsorbed onto 1.6 mg alum adjuvant (Merck, Fontenay-sous-Bois, France) in 0.4 ml saline solution. Seven days later, mice were challenged on 3 consecutive days (D7, D8, D9) with aerosolized OVA (5% saline solution) or saline alone for 20 min.

Sorting of NKT cells

Splenocytes from WT or IFN-$\gamma^{-/-}$ mice were depleted of $CD8^+$, $CD62L^+$, $Mac1^+$ and $CD19^+$ cells after labeling with the corresponding mAbs (clone 53.67, Mel14, M1/70, 1D3, respectively) using anti-rat Ig-coated magnetic beads (Invitrogen Dynal®, Cergy-Pontoise, France) and negatively enriched cells were stained with anti-NK1.1-PE and anti-CD5-APC antibodies (BD Pharmingen) before performing cell sorting. Alternatively, cells were stained with fluorescently labeled CD1d/α-GalCer-tetramer and αβ-TCR antibodies. $CD5^+NK1.1^+$ or CD1d/α-GalCer-$tetramer^+TCR^{int}$ cells were sorted using a FACSVantage (BD).

Adoptive transfer

5.10^6 splenocytes or 5.10^4 sorted $CD5^+$ $NK1.1^+$ NKT cells from WT, $J\alpha 18^{-/-}$ or IFN-$\gamma^{-/-}$ C57BL/6 mice having received either PBS or 150 µg R848 were injected *i.v.* into C57BL/6 mice immunized with OVA, 1 hour before the first challenge on D7.

Measurement of AHR

AHR was assessed 24 h after the last challenge by delivering an aerosol of methacholine (Mch) (Sigma-Aldrich) for 1 min at 150 mM to unrestrained, conscious mice placed in a plethysmographic chamber (EMKA Technologies, Paris, France). The index of airflow obstruction was expressed as enhanced pause (Penh), calculated as: Penh = Te [(expiratory time)-Tr (relaxation time)]/Tr x [Pef (peak expiratory flow)/Pif (peak inspiratory flow)]. The values of Penh were expressed per minute and are the average of three determinations recorded every 20s. For reasons of simplification, the area under the curve (A.U.C.) was calculated for 7 min after Mch inhalation. The resulting A.U.C. values are indicated in graphs and represent the quantitative expression of AHR.

Bronchoalveolar lavage fluid (BALF) and lung homogenates

Immediately after evaluating AHR, mice were anesthetized by an *i.p.* injection of urethane (2 g/kg body mass, Sigma Aldrich). A tracheal canula was inserted and bronchoalveolar lavage was performed by instillation of sterile PBS. Lungs were then perfused with a saline solution through the pulmonary artery and then homogenized in sterile PBS. Differential cell counts were determined in BALF after May-Grünwald/Giemsa (Merck) staining of cells on cytospin slides and IL-4 and IL-5 levels were measured in lung homogenates by ELISA (R&D Systems).

Ex vivo iNKT cell analysis

WT and Jα18$^{-/-}$ mice were injected *i.p.* with 10 µg of R848 followed by collection of sera and spleens 2 h later. Splenocytes were stained and analyzed by flow cytometry. IFN-γ and IL-4 concentrations in sera were quantified by ELISA (R&D systems) and expressed as pg/ml based on a regression curve established for each assay with recombinant murine cytokines.

In vitro iNKT cell stimulation assays

2.10^4 purified cells (purity > 99%) were cultured in 200 µl RPMI medium supplemented with 10% inactivated FCS, 1% Penicillin-Streptomycin and 5.10^{-5} M 2-ME. Cells were then stimulated with rIL-12 and R848, PMA+Iono or medium alone. IFN-γ levels in supernatants were measured by ELISA (R&D Systems) after 48h of stimulation.

RT-PCR analysis

RNA from splenocytes or sorted CD5$^+$ NK1.1$^+$ NKT cells was extracted with TRIzol reagent (Invitrogen). Ten µg of transfer RNA was added prior to RNA extraction and reverse transcription was performed on total RNA. TLR7 expression was determined by PCR analysis performed on a MWG AG Biotech Primus 96 apparatus using the sense 5'-TTCCGATACGATGAATATGCACG-3’ and antisense 5'-TGAGTTTGTCCAGAAGCCGTAAT-3’ primers (Sigma Genosys) under the following cycling conditions: denaturation at 94°C for

1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Double-amplification of 35 cycles each was performed.

Flow cytometry

After Fcγ-R blockade with 2.4G2, splenocytes were labeled using anti-CD5, anti-NK1.1 antibodies CD1d/α-GalCer tetramer-APC, anti-TCRβ-FITC and anti-CD69-PE at appropriate dilutions. For intracellular TLR7 staining they were fixed and permeabilized using a Cytofix/Cytoperm kit, according to the manufacturer's instructions (BD Biosciences), followed by incubation with rabbit polyclonal anti-TLR7 antibody (Alexis Biochemicals) and goat PE-labeled anti-rabbit IgG (BD). Normal rabbit polyclonal IgG was used as negative control. Cells were acquired on a FACSCantoII flow cytometer (BD Biosciences) and analyzed in the lymphocytes gate defined by forward- and side-scatter properties, using FACSDiva Software.

Statistical analysis

Results are presented as mean values ± SEM and statistical significance was established by Mann-Whitney's test. P values < 0.05 were regarded as significant (*, $P < 0.05$; **, $P < 0.01$). All statistical analyses were performed with Graph Pad Prism 5.0 (GraphPad software).

RESULTS

R848-induced protection against allergic inflammation can be transferred to immunized recipient mice.

We set out to confirm the protective effect of R848 against the onset of ovalbumin (OVA)-induced allergic inflammation in our experimental protocol. As expected, OVA-immunized C57BL/6 mice treated with R848 1 hour prior to OVA challenge versus saline controls, developed less AHR, recruited fewer infiltrating cells to the lung, especially eosinophils, and produced less Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13, as well as chemokines like eotaxin-1 (Fig. 1*A* and data not shown).

We then devised an adoptive transfer protocol to evaluate the potential role of iNKT cells in the protective effect. To this end, we transferred splenocytes from donors having previously received a treatment with R848 (OVA-WT R848) relative to saline controls (OVA-WT) before the first OVA challenge of the recipients. We observed a significant decrease in AHR, total cell and eosinophil recruitment, as compared to mice injected with splenocytes from PBS-treated mice (Fig. 1*B*), which validated the experimental approach and proved that R848-targeted immune cells actively mediated the reduction of allergic inflammation in recipient mice. Nevertheless, no modulation in the Th2 cytokines was observed (data not shown).

IFN-γ-producing iNKT cells account for R848-induced protection in allergic inflammation.

To assess the role of iNKT cells in the protection conferred by spleen cells from R848-treated mice, we compared donor cells from wild type and iNKT cell-deficient $J\alpha18^{-/-}$ C57BL/6 mice for their anti-allergic action. We found that AHR as well as total cell and eosinophil infiltration in BAL were no longer attenuated when splenocytes from R848-treated $J\alpha18^{-/-}$ mice were transferred (Fig. 2, *A* and *B*).

Since it has been reported that protection in allergic inflammation models mediated by iNKT cells is IFN-γ-dependent (12-14), we examined whether this cytokine was also involved in our model. Hence, we transferred splenocytes from IFN-γ-deficient R848-treated mice and found that, like their iNKT-cell-deficient counterpart, they failed to reduce AHR and eosinophilia in BAL (Fig. 2, *C* and *D*), supporting the conclusion that alleviation of allergic inflammation induced by TLR7 activation depended both on iNKT cells and IFN-γ production.

To verify whether iNKT cells can provide active protection on their own, we injected purified cells after electronic sorting from R848-treated WT donors (OVA-NKT R848). These cells suppressed allergic inflammation as efficiently as total splenocytes, while iNKT cells from untreated donors had no such effect in OVA-challenged mice (OVA-NKT; Fig. 3). The key role of IFN-γ in this process was established by transferring iNKT cells from R848-treated IFN-γ-deficient mice (Fig. 3). The lack of effect proved that the treatment with R848 alleviated allergic syndromes through iNKT cell-derived IFN-γ.

TLR7 stimulation activates iNKT cells to produce IFN-γ in vivo.

We investigated the ability of the TLR7 agonist R848 to activate iNKT cells *in vivo* by measuring the production of IFN-γ and the expression of the activation marker CD69 on the cell surface. A strong increase of IFN-γ levels was detected in sera of R848-treated mice only 2 hours after *i.p.* injection, as compared to PBS-treated controls (Fig. 4*A*). This response was iNKT cell-dependent since $J\alpha18^{-/-}$ mice failed to respond to the TLR7 agonist. The proportion of iNKT cells, identified as CD1d/α-GalCer-tetramer^{+}αβ-TCR^{+} splenocytes, was not modified by the treatment that induced a strong upregulation of the mean fluorescence intensity (MFI) of the early activation marker CD69 relative to control mice (Fig. 4*B*).

R848 targets directly iNKT cells to promote IFN-γ production.

We next addressed the question whether R848 targeted iNKT cells directly to induce IFN-γ production *in vitro*. Such a mechanism was plausible, as TLR7 was expressed on both transcriptional and protein levels in sorted cells, using RT-PCR analysis and intracellular staining (Fig. 5, *A* and *B*).

Nonetheless, R848 alone did not induce IFN-γ production by purified $CD5^{+}NK1.1^{+}$ iNKT cells. Given that TLR7 stimulation promotes a strong inflammatory response, we postulated that inflammatory signals might be required for priming, enabling iNKT cells to produce IFN-γ in response to a stimulation, as reported before (15-18). This turned out to be the case since addition of the inflammatory cytokine IL-12 during exposure to R848 did effectively result in IFN-γ production by iNKT cells (Fig. 5*C*), as compared with PMA + Iono as a positive control. Sorted CD1d/α-GalCer-$tetramer^{+}$αβ-TCR^{+} cells responded similarly, with higher background values (Fig. 5*D*). As shown in Fig. 5*E*, R848-induced IFN-γ production was inhibited in iNKT cells sorted from $TLR7^{-/-}$ mice, which proved the specific involvement of this signaling pathway. Taken together, our results demonstrate that, in an appropriate inflammatory environment, primed iNKT cells respond to R848 stimulation by producing IFN-γ

DISCUSSION

In the present study we provide evidence for an IFN-γ-dependent contribution of iNKT cells to the alleviation of experimental allergic inflammation induced by *in vivo* treatment with the TLR7 agonist R848. This particular T cell population has been previously reported for reducing disease syndromes, such as AHR and eosinophilia, by inhibiting effector cells after activation by a single injection of its cognate ligand α-GalCer (12-14). The IFN-γ-dependent alleviation of asthma symptoms by R848 given shortly before OVA challenge is reminiscent of this effect.

Herein we demonstrate for the first time that iNKT cells are activated by TLR7 stimulation, thus providing protection against allergic inflammation. This mechanism of action is consistent with previous studies documenting the activation of multiple genes expressed by NK and iNKT cells upon TLR7 activation (9). It is also in agreement with the protective role ascribed to IFN-γ in R848-induced asthma protection in a rodent model (8). Obviously, these data do not exclude the participation of other regulatory cell populations in the final outcome of the treatment with R848. For instance, TLR7 stimulation activates both dendritic and NK cells and induces Th1 responses that generate high amounts of IFN-γ, IL-10 and IL-12 to counteract the allergic Th2 cytokine profile. In favor of this hypothesis, it has been shown that the TLR3 and TLR7 agonists poly(I:C) and R848 protect mice from allergic asthma, airway inflammation, goblet cell hyperplasia and AHR in an IL-12-dependent manner, while both IL-12 and IL-10 were required for the reduction of eosinophil and lymphocyte counts in BALs (7). Other investigators have implicated IFN-γ in the anti-allergic effect of R848 following the conversion of human allergen-specific $CD4^+$ Th2 lymphocytes into IFN-γ producing cells. This effect was associated with increased production of IL-12, IFN-α, IL-18, TNF-α, IL-10 and IL-15 by $CD14^+$ cells, as well as expansion of IFN-γ-

producing $CD3^-CD16^+$ NK cells (19). Note that additional mechanisms of action might come into play, involving notably plasmacytoid dendritic cells and/or suppressive regulatory T cells (20-22).

This is the evidence that iNKT cells can become functional in response to the TLR7 agonist R848 in an inflammatory environment, without TCR engagement. However, in physiological conditions, DCs are most likely co-actors in iNKT cell activation by providing large amounts of pro-inflammatory molecules. Several reports have described a DC-dependent activation of iNKT cells by microbial products, targeting TLR4, 7 and 9 (17,18,23). Indeed, CpG ODNs activate iNKT cells only when plasmacytoid and myeloid DCs are present to provide TLR9-induced type I IFNs and the CD1d required for TCR engagement, respectively. On the other hand, IFN-γ production by iNKT cells exposed to LPS depends on IL-12 and IL-18, generated by DCs (17,23). It has also been shown that iNKT failed to respond to the TLR4 agonist in the absence of DCs.

R848 drives iNKT cell activation through the TLR7/MyD88 pathway, in accordance with a role during early innate immune responses to infection. Stimulation of TLR7 may enable iNKT cells to control viral replication, as already reported following exposure to viruses, like HIV-1, hepatitis C virus (HCV), hepatitis B virus (HBV) or herpes simplex virus type 1 (24-27). This novel mechanism of activation may explain the response to MCMV infection by iNKT cells reported by Wesley *et al.* (28) who suggest that they sense exogenous stimuli through a mechanism that does not require CD1d.

Taken together, our findings uncover a new mechanism of iNKT cell activation through which infections can modulate allergic responses, contributing to a better understanding of the complex interactions mediated through this regulatory cell population.

ACKNOWLEDGMENTS

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REFERENCES

1. Strachan, D. P. 1989. Hay fever, hygiene, and household size. BMJ 299:1259-1260.

2. Kalliomaki, M., S. Salminen, H. Arvilommi, P. Kero, P. Koskinen, and E. Isolauri. 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 357:1076-1079.

3. Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. Science 275:77-79.

4. Jurk, M., F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford, and S. Bauer. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat.Immunol. 3:499.

5. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526-1529.

6. Camateros, P., M. Tamaoka, M. Hassan, R. Marino, J. Moisan, D. Marion, M. C. Guiot, J. G. Martin, and D. Radzioch. 2007. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. Am.J.Respir.Crit Care Med. 175:1241-1249.

7. Sel, S., M. Wegmann, S. Sel, S. Bauer, H. Garn, G. Alber, and H. Renz. 2007. Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. J.Immunol. 178:7805-7813.

8. Xirakia, C., O. Koltsida, A. Stavropoulos, A. Thanassopoulou, V. Aidinis, P. Sideras, and E. Andreakos. 2010. TLR7-triggered Immune Response in the Lung Mediates Acute and Long-lasting Suppression of Experimental Asthma. Am.J.Respir.Crit Care Med.

9. Camateros, P., C. Kanagaratham, J. Henri, R. Sladek, T. J. Hudson, and D. Radzioch. 2009. Modulation of the allergic asthma transcriptome following resiquimod treatment. Physiol Genomics 38:303-318.

10. Lisbonne, M., S. Diem, K. A. de Castro, J. Lefort, L. M. Araujo, P. Hachem, J. M. Fourneau, S. Sidobre, M. Kronenberg, M. Taniguchi, P. Van Endert, M. Dy, P. Askenase, M. Russo, B. B. Vargaftig, A. Herbelin, and M. C. Leite-de-Moraes. 2003. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. J.Immunol. 171:1637-1641.

11. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. Dekruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat.Med. 9:582-588.

12. Matsuda, H., T. Suda, J. Sato, T. Nagata, Y. Koide, K. Chida, and H. Nakamura. 2005. alpha-Galactosylceramide, a ligand of natural killer T cells, inhibits allergic airway inflammation. Am.J.Respir.Cell Mol.Biol. 33:22-31.

13. Hachem, P., M. Lisbonne, M. L. Michel, S. Diem, S. Roongapinun, J. Lefort, G. Marchal, A. Herbelin, P. W. Askenase, M. Dy, and M. C. Leite-de-Moraes. 2005. Alpha-galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-gamma. Eur.J.Immunol. 35:2793-2802.

14. Morishima, Y., Y. Ishii, T. Kimura, A. Shibuya, K. Shibuya, A. E. Hegab, T. Iizuka, T. Kiwamoto, Y. Matsuno, T. Sakamoto, A. Nomura, M. Taniguchi, and K. Sekizawa. 2005. Suppression of eosinophilic airway inflammation by treatment with alpha-galactosylceramide. Eur.J.Immunol. 35:2803-2814.

15. Leite-de-Moraes, M. C., G. Moreau, A. Arnould, F. Machavoine, C. Garcia, M. Papiernik, and M. Dy. 1998. IL-4-producing NK T cells are biased towards IFN-gamma production by IL-12. Influence of the microenvironment on the functional capacities of NK T cells. Eur.J.Immunol. 28:1507-1515.

16. Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat.Immunol. 4:1230-1237.

17. Nagarajan, N. A. and M. Kronenberg. 2007. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J.Immunol. 178:2706-2713.

18. Tyznik, A. J., E. Tupin, N. A. Nagarajan, M. J. Her, C. A. Benedict, and M. Kronenberg. 2008. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. J.Immunol. 181:4452-4456.

19. Brugnolo, F., S. Sampognaro, F. Liotta, L. Cosmi, F. Annunziato, C. Manuelli, P. Campi, E. Maggi, S. Romagnani, and P. Parronchi. 2003. The novel synthetic immune response modifier R-848 (Resiquimod) shifts human allergen-specific CD4+ TH2 lymphocytes into IFN-gamma-producing cells. J.Allergy Clin.Immunol. 111:380-388.

20. Zuany-Amorim, C., E. Sawicka, C. Manlius, A. Le Moine, L. R. Brunet, D. M. Kemeny, G. Bowen, G. Rook, and C. Walker. 2002. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat.Med. 8:625-629.

21. Stock, P., T. Kallinich, O. Akbari, D. Quarcoo, K. Gerhold, U. Wahn, D. T. Umetsu, and E. Hamelmann. 2004. CD8(+) T cells regulate immune responses in a murine model of allergen-induced sensitization and airway inflammation. Eur.J.Immunol. 34:1817-1827.

22. Lagranderie, M., M. Abolhassani, J. A. Vanoirbeek, C. Lima, A. M. Balazuc, B. B. Vargaftig, and G. Marchal. 2010. Mycobacterium bovis bacillus Calmette-Guerin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. J.Immunol. 184:1062-1070.

23. Paget, C., T. Mallevaey, A. O. Speak, D. Torres, J. Fontaine, K. C. Sheehan, M. Capron, B. Ryffel, C. Faveeuw, d. M. Leite, F. Platt, and F. Trottein. 2007. Activation

of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. Immunity. 27:597-609.

24. van Dommelen, S. L. and M. A. Degli-Esposti. 2004. NKT cells and viral immunity. Immunol.Cell Biol. 82:332-341.

25. Kakimi, K., L. G. Guidotti, Y. Koezuka, and F. V. Chisari. 2000. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J.Exp.Med. 192:921-930.

26. Grubor-Bauk, B., A. Simmons, G. Mayrhofer, and P. G. Speck. 2003. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. J.Immunol. 170:1430-1434.

27. Ashkar, A. A. and K. L. Rosenthal. 2003. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. J.Virol. 77:10168-10171.

28. Wesley, J. D., M. S. Tessmer, D. Chaukos, and L. Brossay. 2008. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. PLoS.Pathog. 4:e1000106.

FIGURE 1. Protection against allergic inflammation can be transferred by splenocytes from mice having received R848. **A**, Mice immunized with OVA were treated on 3 consecutive days with R848 and challenged with OVA. AHR to metacholine was measured 24 h after the last challenge with OVA or NaCl. OVA-challenged mice were treated (OVA-R848; n=5) or not (OVA; n=5) with R848. Total cell and eosinophil recruitment was measured in BALF. **B**, WT OVA-immunized recipient mice were transferred with splenocytes from PBS (OVA-WT; n=5) or R848-treated WT donor mice (OVA-WT R848). AHR, total cell and eosinophil recruitment were evaluated one day after the last challenge. Each panel represents one of at least three independent experiments. *, $P < 0.05$ and **, $P < 0.01$.

FIGURE 2. iNKT cells and IFN-γ are implicated in R848-induced protection in allergic inflammation. *A,* Mice were transferred with PBS- or R848-treated WT splenocytes (OVA-WT or OVA-WT R848, respectively; n=5 per group) or R848-stimulated $J\alpha18^{-/-}$ splenocytes (OVA-$J\alpha18^{-/-}$ R848; n=5). AHR was measured 24 hours after the last challenge. B, Total cell and eosinophil recruitment in BAL was no longer modulated in the OVA- $J\alpha18^{-/-}$ R848 group. *C*, AHR was measured in mice injected with WT or IFN-$\gamma^{-/-}$ splenocytes treated or not with R848: OVA-WT, OVA-WT R848 and OVA-IFN-$\gamma^{-/-}$ R848 (n=5 per group). *D,* R848-stimulated IFN-$\gamma^{-/-}$ splenocytes failed to decrease total cell and eosinophil numbers in BAL. Th2-type cytokine modulation was observed in neither experiment. Each panel represents one of at least three independent experiments. *, $P < 0.05$ and **, $P < 0.01$.

FIGURE 3. iNKT cells producing IFN-γ account for R848-induced protection in experimental allergic inflammation. Sorted $CD5^+$ $NK1.1^+$ iNKT cells from WT or IFN-$\gamma^{-/-}$ R848-treated mice (OVA-NKT R848 and OVA-NKT IFN-$\gamma^{-/-}$ R848 respectively; n=5 per group) or WT PBS-treated mice (OVA-NKT; n=5) were transferred to OVA-immunized WT

recipients. R848-activated NKT cells reduced AHR (*A*), total cell and eosinophil recruitment (*B)*, conversely to their IFN-γ-deficient counterpart. Each panel represents one of at least two independent experiments. **, $P < 0.01$.

FIGURE 4. R848 induces iNKT cell activation and rapid IFN-γ production. *A*, WT and $J\alpha18^{-/-}$ mice were injected *i.p.* with 10 μg of R848 or PBS. Sera were collected 2 h after injection when IFN-γ levels were measured by ELISA. *B*, Splenocytes from R848- or PBS-injected mice were stained with fluorescently labeled CD1d/α-GalCer-tetramer and anti-αβ-TCR and anti-CD69 antibodies. Cell surface expression of CD69 marker was increased on iNKT cells from R848-treated mice (shaded histogram) compared to PBS-injected mice (blank histogram). The CD69 MFI was significantly increased in the R848-treated group (n=4). Each panel represents one of at least two independent experiments.

FIGURE 5. iNKT cells respond to R848 by producing IFN-γ in a TLR7-dependent manner *in vitro*. *A*, mRNA expression of TLR7 by sorted $CD5^{+}NK1.1^{+}$ iNKT cells was evaluated by RT-PCR. Total splenocytes and H_2O were used as positive and negative control, respectively. *B*, Intracellular TLR7 expression was evaluated in iNKT cells by flow cytometry using rabbit polyclonal antibody against TLR7 and goat PE-labeled anti-rabbit IgG (shaded histogram). Normal rabbit polyclonal IgG (white histogram) was used as negative control. *C*, Sorted $CD5^{+}$ $NK1.1^{+}$ iNKT cells were stimulated with a TLR7 agonist: 1 μg/ml R848 or 10^{-8} M PMA + 10^{-6} M ionomycin and cultured in the absence or presence of 10 ng/ml of IL-12. IFN-γ production was evaluated in supernatants by ELISA 48h after stimulation. *D*, Sorted $tetramer^{+}$ iNKT cells were incubated in the presence of IL-12 and R848 and biological activity was quantified as described in panel C. *E*, Sorted iNKT cells from $TLR7^{+/+}$ or $TLR7^{-/-}$ mice were incubated in the presence of IL-12 and R848 and biological activity was quantified as described in panel C. Each panel represents one of at least two independent experiments.

Figure 1

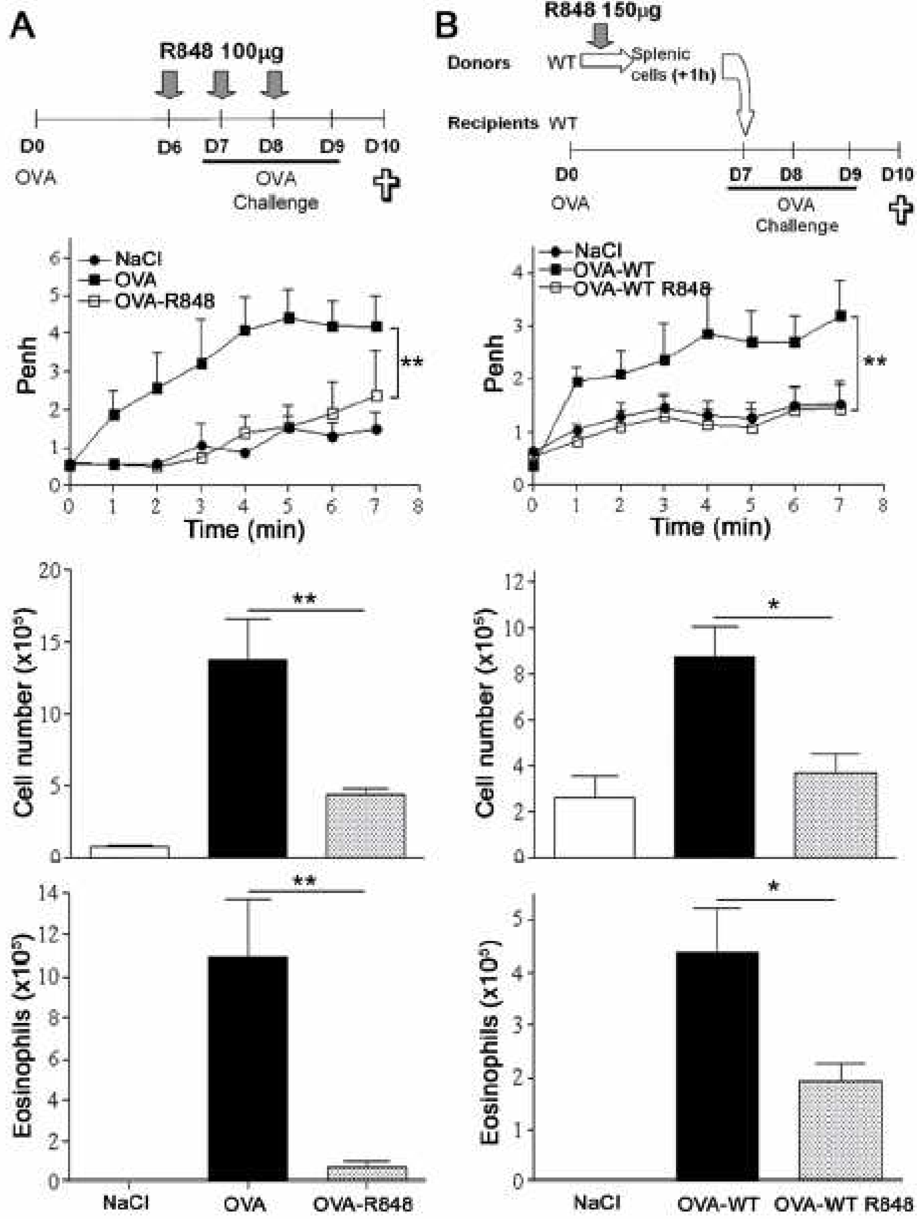
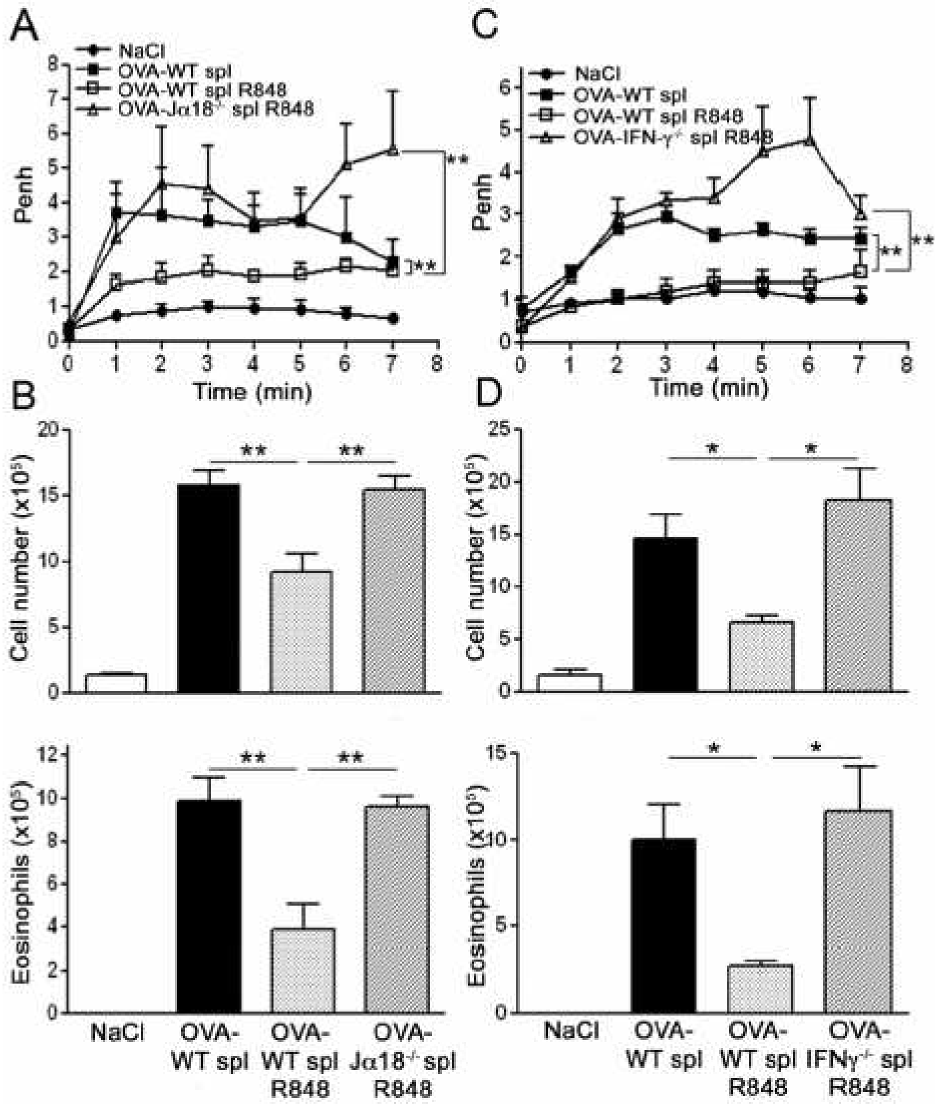


Figure2



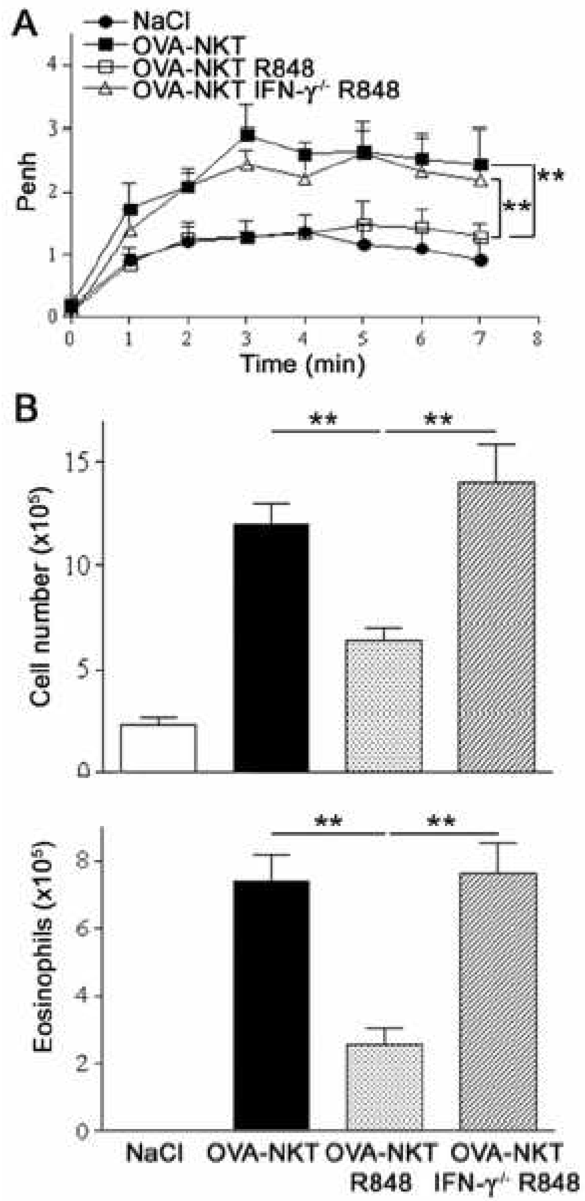
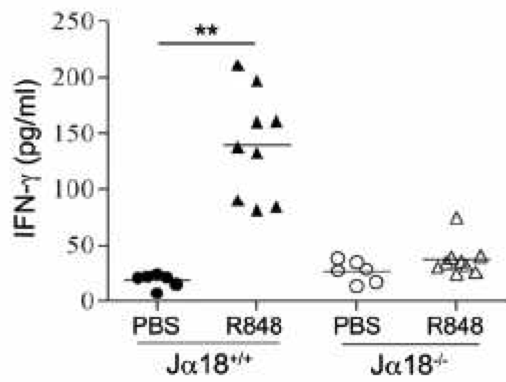


Figure3
A
NaCl
OVA-NKT
OVA-NKT R848
OVA-NKT IFN-γ-/- R848
Penh
Time (min)
**
**
B
Cell number (x10^5)
**
**
Eosinophils (x10^5)
**
**
NaCl
OVA-NKT
OVA-NKT R848
OVA-NKT IFN-γ-/- R848

Figure 4



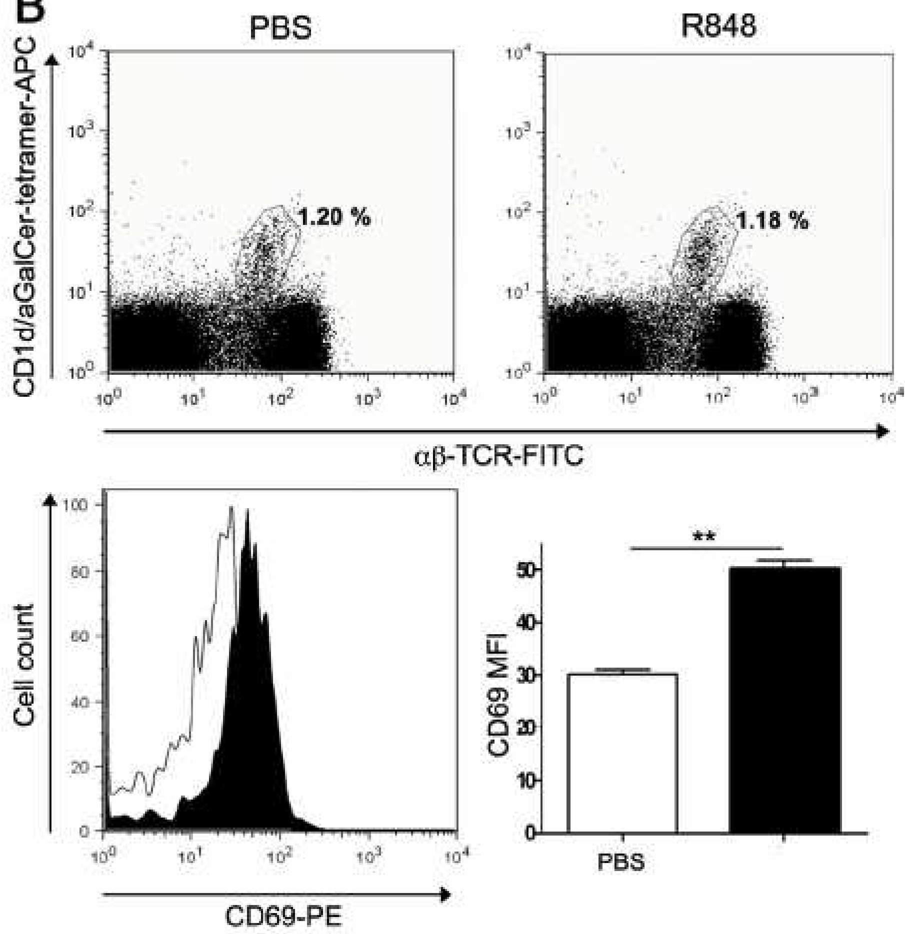
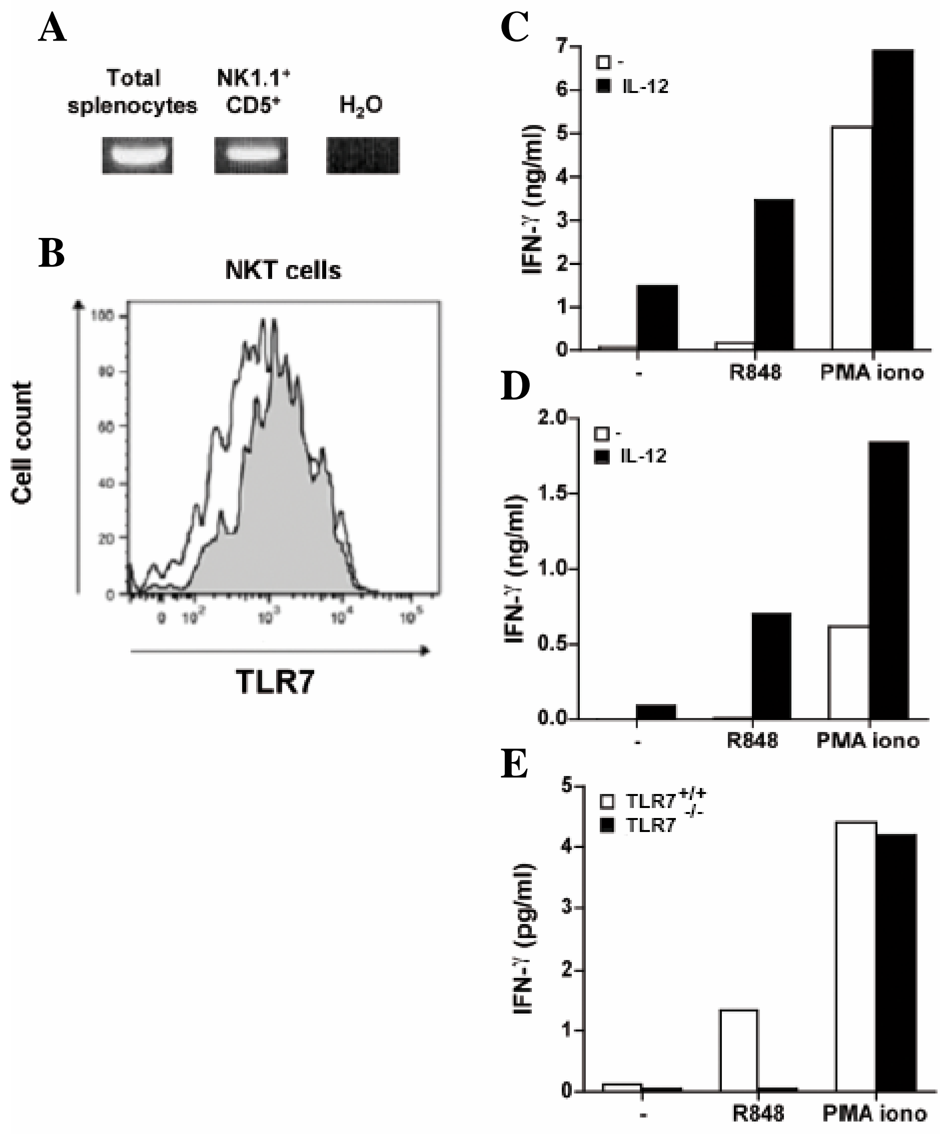


Figure 5



Suppression des réponses d'asthme par la stimulation de TLR-7

Linh Pham Van, Emilie Bardel, Jeroen Vanoirbeek, Michel Dy, Nathalie Thieblemont.

Il est maintenant largement démontré que l'exposition à l'environnement et les infections influencent l'évolution de l'asthme qui est étroitement contrôlé par les cellules effectrices et régulatrices ainsi que par les cytokines telles que l'IL-10 et le TGF-β. Dans ce travail, nous avons cherché à déterminer si la stimulation de TLR7, qui a un rôle protecteur dans de nombreux protocoles expérimentaux, pouvait aussi avoir un effet suppresseur au cours de l'asthme déjà établi. Nous avons montré que le traitement par R848, agoniste TLR7, atténuait les symptômes d'asthme allergique par un mécanisme impliquant les cellules T régulatrices. En effet, cette suppression est abolie après déplétion des cellules CD25+, qui se développent dans les poumons des souris traitées. L'effet suppresseur, dépend partiellement du TGF-β. Nos résultats ouvrent de nouvelles perspectives thérapeutiques ciblant les cellules T régulatrices dans la suppression de l'asthme allergique.

Suppression of established asthma responses via TLR7 stimulation

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Running Title: TLR7 stimulation suppresses allergic asthma

Keywords: Toll-like receptor; allergic asthma, regulatory T cells; TGF-β

Abbreviations: AHR, Airway Hyperreactivity; BALF, bronchoalveolar lavage fluid; iNKT cells, invariant Natural Killer cells; NOD: non-obese diabetic; OVA, ovalbumin; phosphate-buffered saline; Penh, Enhanced Pause; R848: resiquimod; TLR: Toll-like receptor;

ABSTRACT

Background: It is widely acknowledged that environmental exposure to allergens and infectious agents influence the evolution of asthma syndromes, which are tightly controlled by effector and regulatory cells as well as cytokines like IL-10 and/or TGF-β. In this context, the recognition of pathogen-associated motives through TLR activation pathways plays a critical role with important consequences on disease progression and outcome.

Objectives: We addressed the question whether stimulation of TLR7 that has been shown to be protective in several experimental protocols, can also suppress asthma syndromes once the disease is established.

Methods: We used an OVA-induced experimental model of murine allergic asthma, in which R848 was injected at the end of a series of challenges with aerosolized OVA. Airway hyperreactivity, recruitment of infiltrating cells and cytokine production were measured in the lung of mice having received R848 or not. The suppressive role of regulatory T cells (Tregs) was assessed by cell depletion, while the effect of endogenous TGF-β and IL-10 was evaluated after neutralization with antibodies.

Results: We found that treatment with the TLR7 agonist R848 attenuated allergic symptoms through a mechanism that required Tregs, as assessed by the expansion of this population in the lung of mice having received R848 and the loss of suppression after its *in vivo* depletion. The suppression of asthma symptoms was largely dependent on TGF-β with a minor contribution of IL-10.

Conclusion: Treatment with the TLR7 agonist R848 suppresses established allergic asthma through a TGF-β-dependent mechanism opening new therapeutic opportunities for pharmacological targeting of Tregs.

INTRODUCTION

Epidemiological studies have established that in recent decades the prevalence of allergic asthma has significantly increased in developed countries. Emerging evidence attests that early life events, including exposure to allergens and infections, are critical in programming effective regulatory pathways to maintain pulmonary homeostasis. Toll-like receptor (TLR) signaling contributes prominently to $CD4^+$ T cell activation by connecting innate and acquired immunity. Mouse models of allergic asthma have long been used to dissect the immunological mechanisms leading to asthma, and despite the heterogeneity of experimental protocols in terms of strain differences, type and dose of antigen, time and route of administration, most studies have provided evidence for a protective effects of natural or synthetic TLR2, TLR4, TLR9 ligands in allergen-induced lung inflammation [(1-4)]. Similarly, the synthetic ligand of TLR7, resiquimod (or R848), has been reported for preventing typical respiratory syndromes (airway hyperresponsiveness) and allergic inflammation (recruitment of eosinophils to the lung, production of IgE antibodies and Th2-driven cytokine production) [(5-7)] in treated mice. In the rat, R848 has been shown to inhibit the inflammatory reaction and to abrogate airway remodeling [(8)]. These protective effects were observed after administration of R848 during the sensitization phase, while in mice having already mounted a primary allergic response treatment with R848 resulted in a marked reduction of secondary reactions following repeat allergen aerosol challenges mediated through IL-12 and IL-10 [(7;8)]. Although IFN-γ-producing NK cells have been held responsible for R848-induced asthma protection, it is clear by now that they cannot solely account for the protection that is abolished neither by their depletion nor in IFN-γ-deficient mice [(9;10)].

Major cellular components driving asthmatic reactions include eosinophils and $CD4^+$ Th2 cells generating a number of soluble mediators[(11)]. Different regulatory T cells contribute to lung homeostasis, particularly $CD4^+CD25^+$ regulatory T cells [(12)]. Naturally occurring cells of this lineage develop in the thymus, while their induced counterpart originates from conventional T cells in peripheral lymphoid tissues. Several subsets of regulatory T cells with distinct suppressive functions have been described [(13-15)]. Knowing that $Foxp3^+$ Tregs increase

following microbial stimulation and inhibit allergen-induced lung pathologies in a manner that is not antigen-specific, it can be assumed that they take part in the process that leads to reduced asthma and allergy development upon infection. Regulatory T cells may exert their suppressive functions via cell-to-cell contact as well as cytokines [(16)]. Among these, IL-10 and TGFβ, which are both produced by Tregs are well acknowledged for their role in allergic inflammation. Indeed, IL-10 is an essential suppressive factor during allergic responses in the lung[(17)], while TGF-β ensures pulmonary homeostasis by inhibiting both Th2 and Th1 responses and has been implicated in the control of allergic airway inflammation [(18;19)]. The regulatory potential of other cell populations, such as the $CD8^+$ T cells, IL-17-producing cells or Natural Killer T cells (NKT) cells [(16)] has also been documented.

In the present study, we investigated the effect of the TLR7 agonist R848 in a murine experimental model of established allergic asthma. We provide evidence that this compound targets pulmonary Tregs enabling them to exert a protective effect mediated through TGF-β production.

MATERIAL AND METHODS

Animals. C57BL/6J mice were purchased from CERJ (Les Genest St. Isle, France). Jα18$^{-/-}$ mice on a C57BL/6 genetic background (kindly provided by Dr. T. Taniguchi, Japan) and non-obese diabetic (NOD) (K^{d}, I-A^{g7}, D^{b}) mice were bred in our animal facility under specific pathogen-free conditions. All experiments have been conducted in accordance with European Union Council Directives (86/609/EEC) and with institutional guidelines (INSERM: Institut National de la Santé et de la Recherche Médicale). The animal facility is accredited by an agreement delivered by the *Prefecture de Police* of Paris, France.

Reagents. R848 was commercially obtained from Alexis Biochemicals (Paris, France). Rat monoclonal antibody specific to the mouse IL-10 receptor (1B1 2.1C4 clone) was gently provided by A. O'Garra (DNAx, Palo Alto, CA) and monoclonal antibody to human TGF-β that cross-reacts and neutralizes murine TGF-β (2G7 clone) by C.J.M. Melief (Leiden University Medical Center, Leiden, the Netherlands). These mAbs have been reported before as blocking either IL-10R or TGF-β in different protocols [(20-22)]. Depletion of CD25 cells were performed using the purified anti-CD25 mAb PC61 (rat IgG1). A rat monoclonal anti-IgG antibody was used as negative control.

Induction of experimental asthma and *in vivo* treatment with R848. Eight to nine week-old male C57BL/6 or NOD mice were immunized by an *i.p.* injection of 100 µg of chicken egg OVA (Sigma-Aldrich, Saint Quentin Fallavier, France) adsorbed onto 1.6 mg aluminium hydroxide (Merck, Fontenay-sous-Bois, France) in a volume of 0.4 ml. Seven days later, mice were challenged on 6 consecutive days (D7, D8, D9, D10, D11, D12) with aerosolized OVA diluted in 0,9% saline solution (5% w/v). R848 was injected *i.p.* one hour before the challenge on D11 and 12 at the dose of 100 µg/injection. Twenty-four hours after the last challenge, AHR was measured, mice were sacrificed and samples were collected for further analysis.

Depletion of Tregs *in vivo*. Intraperitoneal injection of 200 µg PC61 resulted in more than 90% depletion of $CD4^+CD25^+$ T cells in spleen and lung within 48 hours. Mice were treated *i.p.* with 200 µg PC61 at days 9 and 11 (2 hours after OVA challenges). Since in preliminary experiments we could not establish a significant difference between mice having received 200 µg rat IgG1 isotype control or PBS, we limited the control groups to mice injected with PBS.

Measurement of airway hyperreactivity. AHR was evaluated 24 h after the last challenge by both whole body plethysmography (EMKA Technologies, Paris, France) and an invasive forced oscillation technique measuring airway resistance and compliance in anaesthetized and ventilated mice with a FlexiVent system (FlexiVent; SCIREQ, Montreal, Québec, Canada). Plethysmography measurement was performed with unrestrained, conscious mice receiving an aerosol of metacholine (Sigma-Aldrich) for 1 minute at 150 mM (C57BL/6 mice) or 100 mM (NOD mice). The index of airflow obstruction was expressed as enhanced pause (Penh), calculated as: Penh = Pause x [Pef (peak expiratory flow)/Pif (peak inspiratory flow)], Pause = Te [(expiratory time)-Tr (relaxation time)]/Tr. The values of Penh were expressed per minute and are the average of three determinations recorded every 20s. For simplification, the area under the curve (A.U.C.) was calculated for 10 minutes. The resulting AUC. values represent the quantitative expression of AHR are indicated in graphs. The data obtained by this technique were confirmed by measuring airway resistance and compliance to methacholine with a FlexiVent system 24 h after the last challenge.

Collection and analysis of bronchoalveolar lavage fluid (BALF). Immediately after AHR measurement, mice were anesthetized by *i.p.* injection of urethane (2 g/kg body mass, Sigma Aldrich). Airways were washed with sterile PBS. Total and differential cell counts were carried out in BALFs after May-Grünwald/Giemsa (Merck) staining of cells on cytospin slides.

Isolation of lung mononuclear cells (MNC). To obtain intraparenchymal lung MNC, the lung was minced, and incubated in RPMI 1640 supplemented with 5% FCS, 100 U penicillin/streptomycin, 10 mM HEPES, 50 µM 2-ME, 20 mM L-glutamine containing 20 U/ml collagenase and 1 g/ml DNase type I. After incubation for 60 min at 37°C, any remaining intact tissue was eliminated by passing through a 75 µm filter and cells were collected by centrifugation. The cell pellet was suspended in 5 ml of 40% Percoll and layered onto 5 ml of 70% Percoll, centrifuged (2,400 rpm, 4°C, 30 min) and then collected.

Cytokine and chemokine analysis

Samples were collected and cytokines (IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN-γ) and chemokines (eotaxin, tarc) were assayed using Duoset ELISA kits (R&D systems).

Cytometry analysis

Cells were stained with PE-conjugated anti-CD25 and FITC-conjugated anti-CD4 (BD Biosciences, Pont de Claix, France) before fixation and intracellular labelling using a Foxp3 staining kit (BD Biosciences) according to manufacturer's instructions. Cells were acquired on a FACSCantoII cytometer (BD Biosciences), gated on mononuclear cells according to forward- and side-scatter properties and analyzed using FACS Diva and Flowjo software.

Statistical analysis. Results are presented as mean values ± SEM and statistical significance was established by Mann-Whitney's test when appropriate (GraphPad Prism Software, La Jolla, CA). Analysis of variance test was used to compare differences between groups for data of AHR followed by Bonferrroni correction within each set of comparison. Differences were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

RESULTS

R848 treatment suppresses ongoing established allergic inflammation.

We examined the effect of the TLR7 agonist R848 on established allergic symptoms, by injecting the compound at the end of a series of antigen challenges. In these experimental conditions we found that hyperresponsiveness was drastically reduced whether it was evaluated by plethysmography or lung resistance and compliance measurements when R848 was injected during the last aerosol administration on D11 and D12 (Fig 1, *A*). Total cell and eosinophil recruitment into BALF was likewise decreased in mice having received R848, as were the concentrations of Th2-type cytokines and chemokines, such as IL-4, IL-5, IL-13, eotaxin, determined in lung homogenates (Fig 1, *B, C*). IL-10 and IL-17 dropped likewise to control levels likewise decreased, whereas IL-12, and IFN-γ were also observed (Fig 1, *C*).

Regulatory T cell counts increase in the lung of R848-treated mice and mediate the suppression of asthma symptoms.

Tregs being the most likely mediators of R848-induced asthma suppression, we evaluated whether their incidence was modified in the lung of R848-treated mice relative to saline controls. We observed a significant increase in the percentage of both $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells in mice having received the TLR7 agonist (Fig. 2), indicating that this population was recruited upon activation. To verify its contribution to the anti-allergic effect of R848, we depleted $CD25^+$ T cells from mice having developed allergic asthma before injecting R848. Preliminary experiments showed that the antibody PC61 was relatively inefficient, even at a high dose (1 mg), in depleting $CD4^+CD25^{high}$ Tregs from C57BL/6 mice (data not shown), while in the NOD strain, an almost complete depletion was achieved at a relatively small dose within two days post injection, providing optimal conditions. Since NOD mice develop OVA-

induced asthma symptoms more easily than C57BL/6 mice, we assessed the suppressive effect of R848 in this strain used for further studies with the same protocol.

OVA-sensitized and -challenged NOD mice were depleted for $CD25^{+}$ cells by *i.p.* injection of PC61 (200 μg) at day 9 before treatment with R848 or saline. As shown in Fig. 3, more than 90 % of pulmonary $CD4^{+}CD25^{high}$ T cells were depleted in these conditions. R848-induced suppression of asthmatic symptons after OVA challenge was fully reversed in the absence of Tregs that had no aggravating effect on their own, whether in terms of AHR (Fig 4, *A*), total cell recruitment into BALF, eosinophilia (Fig 4, *B*), or increased levels of Th2-type cytokine and chemokine production in the lung (Fig 4, *C*). It can therefor be concluded that Tregs are critical for the suppression of allergic asthma induced by R848.

The suppression of asthma symptoms in mice treated with R848 is TGF-β-dependent.

Both IL-10 and TGF-β are recognized as important mediators of suppressive functions of Tregs, prompting us to examine their role in our model. To this purpose, mice were injected *i.p.* with neutralizing mAbs directed against TGF-β (2.5 mg per mouse) or IL-10 receptor (500 μg) at day 11 and 12, one hour after saline or OVA challenge. It turned out that the suppressive effect of R848 treatment assessed by decreased AHR (Fig 6, *A*), total cell recruitment in BALF, eosinophilia (Fig 5, *B*) and Th2-type pulmonary cytokine and chemokine production (Fig 5, *C*) was lost in mice treated with neutralizing anti-TGF-β mAbs. By contrast, a significant suppression persisted in mice having received the blocking anti-IL-10R mAbs, in terms of decreased hyperreactivity and eosinophilia, as well as IL-4 and IL-13 release in the lung. However, the production of some cytokines and chemokines such as eotaxin, IL-5, IL-10 and IFN-γ differed significantly from controls, suggesting that IL-10 may be at least, partially involved. Nonetheless, the major contribution to the suppressive effect of R848 is undoubtedly provided by TGF-β.

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DISCUSSION

In the present study we demonstrate that treatment with the TLR7 agonist R848 suppresses the symptoms of established asthma through a mechanism involving TGF-β. R848 has previously been reported for its capacity to prevent disease onset when given before allergen challenge [7;10]. However, knowing that the original encounter with the sensitizing allergen remains undefined and consecutive challenge is usually unpredictable, the prevention of the primitive event that leads to sensitization and/or suppression of established asthma would be more valuable from the therapeutic point of view. We found that administration of R848 before immunization did not protect against disease onset (personal unpublished data). Hence, we set up an acute asthma model with well-established allergic hallmarks to assess whether TLR7 stimulation could suppress as well as prevent the syndromes. Our protocol differed from those currently used for asthma prevention, insofar as R848 was given at the end rather than at the beginning of a series of OVA challenges when disease symptoms were manifest [6-8] Suppression of established asthma upon administration of the TLR7 agonist has been claimed by other investigators [7]. Yet, in this study mice received R848 between a first and a second set of allergen challenges at an interval of 3 to 4 weeks. It is therefore possible that the effect might actually have been preventive rather than suppressive since at the time of the treatment with the TLR7 agonist the asthma symptoms induced by the first series of challenges had probably faded. In a recent study, the protective treatment was given 1 day before a set of three challenges [10]. In this setting, the authors claimed to suppress asthma when mice were additionally stimulated with the antigen one week before the second set of challenges. It is thus difficult to discriminate whether the treatment is protective or really suppressive.

Tregs being the most obvious candidates for asthma suppression, we found that their depletion did effectively restore the allergic symptoms alleviated by the injection of R848. The

removal of this cell population did not significantly aggravate the disease in the asthma-prone NOD mice *per se*, indicating that it is not inherently suppressive in this type of disorder. In agreement with this result, similar studies performed with other murine strains susceptible to asthma, such as A/J, BALB/c also showed that Treg depletion resulted only in a modest increase of airway eosinophilia without enhancement of AHR or Th2-type cytokine production [(23;24)].

Thus, treatment with R848 rendered Tregs functionally competent to counteract disease symptoms. This observation is in accordance with the concept that regulatory responses can be induced by TLR stimulation, which seems to be a natural strategy to prevent an excessive immune response. Since plasmacytoid dendritic cells (pDCs) are also activated through TLR7 stimulation, it can be assumed that they are also involved in R848-induced asthma suppression, as recently demonstrated for the suppressive effects induced by *Mycobacterium bovis* Bacillus Calmette-Guérin [(14)], which depend on both pDCs and Tregs.

Tregs can exert their suppressive action through cell-to-cell contact or immunosuppressive cytokines like IL-10 or TGF-β. Most studies have assigned a central role to IL-10 alone or in combination with TGF-β in the control of allergic airway inflammation. In our model the suppressive effect was predominantly mediated by TGF-β since its neutralization by specific antibodies abolished R848-induced asthma symptoms completely, while inhibiting the effect of IL-10 by receptor blockade did not restore the airway hyperresponsiveness. In humans, IL-10 is produced mainly by subsets of natural and induced Tregs that express low levels of CD25 (IL-10-producing $CD4^+Foxp3^+CD25^{low}ICOS^+$ nTreg and induced Tr1 cells). These cells appear to inhibit only T cell proliferation by blocking the function of APCs and are more prone to apoptosis in an inflammatory environment or in culture compared to other TGF-β-producing subsets. In our experimental conditions, pulmonary IL-10 levels were consistently lower in mice treated with R848 than in untreated asthmatic controls. In addition to its ability to inhibit effector T cell proliferation and functions, to induce the release of mediators by APCs and

apoptosis in numerous cell types, TGFβ influences the lineage specificity of effector T cell subsets depending on the concurrent presence of maturation and polarization factors. It might therefore be hypothesized that the alleviation of asthma symptoms by the TLR7 agonist might result from its capacity to redirect allergen-specific T cell responses. It is noteworthy that treatment with R848 normalized the IL-17 levels that were substantially increased in the lung of asthmatic mice relative to saline controls. TGF-β is known to be crucially involved at the interface between Treg and Th17 cell differentiation pathways through its ability to induce their respective signature transcription factor FoxP3 and ROR-γ-t. The other pro-inflammatory cytokines generated in our experimental setting modulate preferentially the Th17 differentiation pathway.

Exposure to pathogens as well as TLR agonists modulates the development of regulatory pathways. Immune reprogramming remains an exciting prospect. Our study has revealed an immune regulatory mechanism in lung homeostasis induced by TLR7 stimulation that can be exploited for improving therapies for asthma.

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REFERENCES

(1) Akdis CA, Kussebi F, Pulendran B, Akdis M, Lauener RP, Schmidt-Weber CB et al. Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. Eur J Immunol 2003; 33(10):2717-26.

(2) Hessel EM, Chu M, Lizcano JO, Chang B, Herman N, Kell SA et al. Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. J Exp Med 2005; 202(11):1563-73.

(3) Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC et al. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. J Immunol 2005; 174(12):7558-63.

(4) Revets H, Pynaert G, Grooten J, De BP. Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses. J Immunol 2005; 174(2):1097-103.

(5) Moisan J, Camateros P, Thuraisingam T, Marion D, Koohsari H, Martin P et al. TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. Am J Physiol Lung Cell Mol Physiol 2006; 290(5): L987-L995.

(6) Quarcoo D, Weixler S, Joachim RA, Stock P, Kallinich T, Ahrens B et al. Resiquimod, a new immune response modifier from the family of imidazoquinolinamines, inhibits allergen-induced Th2 responses, airway inflammation and airway hyper-reactivity in mice. Clin Exp Allergy 2004; 34(8): 1314-20.

(7) Sel S, Wegmann M, Sel S, Bauer S, Garn H, Alber G et al. Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. J Immunol 2007; 178(12):7805-13.

(8) Camateros P, Tamaoka M, Hassan M, Marino R, Moisan J, Marion D et al. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. Am J Respir Crit Care Med 2007; 175(12):1241-9.

(9) Camateros P, Kanagaratham C, Henri J, Sladek R, Hudson TJ, Radzioch D. Modulation of the allergic asthma transcriptome following resiquimod treatment. Physiol Genomics 2009; 38(3):303-18.

(10) Xirakia C, Koltsida O, Stavropoulos A, Thanassopoulou A, Aidinis V, Sideras P et al. TLR7-triggered Immune Response in the Lung Mediates Acute and Long-lasting Suppression of Experimental Asthma. Am J Respir Crit Care Med 2010.

(11) Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation.Immunity 2009; 31(3):425-37.

(12) Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. J Exp Med 2005; 202(11):1539-47.

(13) Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. Curr Opin Immunol 2003; 15(6):627-33.

(14) Lagranderie M, Abolhassani M, Vanoirbeek JA, Lima C, Balazuc AM, Vargaftig BB et al. Mycobacterium bovis bacillus Calmette-Guerin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. J Immunol 2010; 184(2):1062-70.

(15) Zuany-Amorim C, Sawicka E, Manlius C, Le MA, Brunet LR, Kemeny DM et al. Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells. Nat Med 2002; 8(6):625-9.

(16) Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. Immunity 2009; 31(3):438-49.

(17) Leech MD, Benson RA, De VA, Fitch PM, Howie SE. Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. J Immunol 2007; 179(10):7050-8.

(18) Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T et al. Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. J Immunol 2007; 178(3):1433-42.

(19) Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes BT et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. J Clin Invest 2004; 114(1):28-38.

(20) Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S et al. CD4+. J Immunol 2003; 170(5):2516-22.

(21) Taher YA, van Esch BC, Hofman GA, Henricks PA, van Oosterhout AJ. 1alpha,25-dihydroxyvitamin D3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-beta. J Immunol 2008; 180(8):5211-21.

(22) Vissers JL, van Esch BC, Hofman GA, Kapsenberg ML, Weller FR, van Oosterhout AJ. Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. J Allergy Clin Immunol 2004; 113(6):1204-10.

(23) Hadeiba H, Locksley RM. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. J Immunol 2003; 170(11):5502-10.

(24) Jaffar Z, Sivakuru T, Roberts K. CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. J Immunol 2004; 172(6):3842-9.

FIGURE LEGENDS

FIG 1. R848 treatment suppresses allergic responses in mice. **(A)** C57BL/6 mice were sensitized by *i.p.* injection of OVA at day 0 and challenged at days 7/8/9/10/11/12 by aerosols with OVA or NaCl. At days 11 and 12, mice were treated *i.p.* with R848 (100 μg) or PBS one hr before the OVA aerosol exposures. Airway responsiveness to metacholine were assessed 24 hrs after the last challenge with OVA or NaCl by measurement of lung resistance (R), compliance (C) and, on alternative experiment, the whole body plethysmography. **(B)** Mice were killed at day 13, BALF and lung were collected. Total cells, eosinophils, macrophages and lymphocytes were counted in BALF. **(C)**. IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IFN-γ, eotaxin, tarc were measured by ELISA in lung homogenates. Results are expressed as mean of 4-5 mice/group. Similar results were observed in 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG 2. Suppression of asthma by R848 is associated with an expansion of Treg in the lungs. PBS- and R848-treated mice were analyzed by flow cytometry for the expression of CD4, CD25 and Foxp3. R848 injection increases the percentage of Treg cells in lung reflected by the CD25 expression profile and the proportion of CD4+CD25high T cells (upper panel) as well as modulation of Foxp3 expression (lower panel) gated on CD4+ T cells infiltrated in lung. Representative plots and histograms from each group were indexed. The representative histograms for CD25 (upper) and FoxP3 (lower) expression from PBS-injected (dotted line) R848-treated (solid line) groups were overlaid. Significant differences were obtained for both CD25 ($p < 0.01$) and FoxP3 expression ($p < 0.05$).

FIG 3. Depletion of $CD25^{+}FoxP3^{+}$ T cells in lung using anti-CD25 antibody. Treg cell depletion was performed by an *i.p.* injection of PC61 (200 μg) at day 9 on one of two groups of OVA-sensitized and -challenged mice (**A**) as well as on one from two groups of R848-treated OVA-sensitized and -challenged mice (**B**). The sensitization and challenges of OVA and the R848 treatment were performed as previously described. In both groups treated with anti-CD25 mAbs, analyses by flow

cytometry revealed a depletion of more than 90% of $CD25^{+}FoxP3^{+}$ T cells (lower dot plots) compared to those of their corresponding control group (upper dot plots).

FIG 4. R848 mediated suppression of allergic responses depends on the presence of Treg cells. NOD mice were sensitized and challenged by OVA as previously described. Treg cell depletion was performed by an *i.p.* injection of PC61 (200 µg) at day 9 on OVA-sensitized and -challenged mice that would be treated or not with R848. The treatment by R848 was performed at days 11, 12 with dose, route, and timing identically to previous description on OVA-sensitized and -challenged mice that had been injected PC61 or not. The suppression effects of R848 treatment on airway hyperresponsiveness to methacholine **(A)**, increased total number of cells in BALF and eosinophil recrutement to the lung **(B)**, and increase levels of Th2-driven cytokines and chemokines induced in lung **(C)** was observed on in NOD mice treated by R848 but this effect was lost in those received anti-CD25 depleting antibody injection. Mice were pooled in the NaCl-challenged control group since treatment of R848 or PC61 induced no significant modifications. Results are expressed as mean of 4-5 mice/group. Similar results were obtained in 2 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG 5. Suppression of OVA-induced allergic responses is dependent of TGF-β. C57BL/6 mice were sensitized and challenged by OVA as previously described. The treatment by R848 or PBS was performed with dose, route, and timing identically to previous description on sensitized and challenged mice. Blocking antibody anti-IL10R (500 µg) and neutralizing antibody anti-TGF-β (2.5 mg) were injected i.p. at day 11 and 12 one hr after saline or OVA-challenge. The suppression effects of R848 treatment on airway hyperresponsiveness to methacholine (A), increased total number of cells in BALF and eosinophil recrutement to the lung (B), and increased

levels of Th2-driven cytokines and chemokines induced in lung (C) was assessed at day 13. The mice in the NaCl-challenged control group were pooled since treatment of R848 or antibodies induced no significant modifications. Results are expressed as mean of 4-5 mice/group. Similar results were obtained in 2 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 1

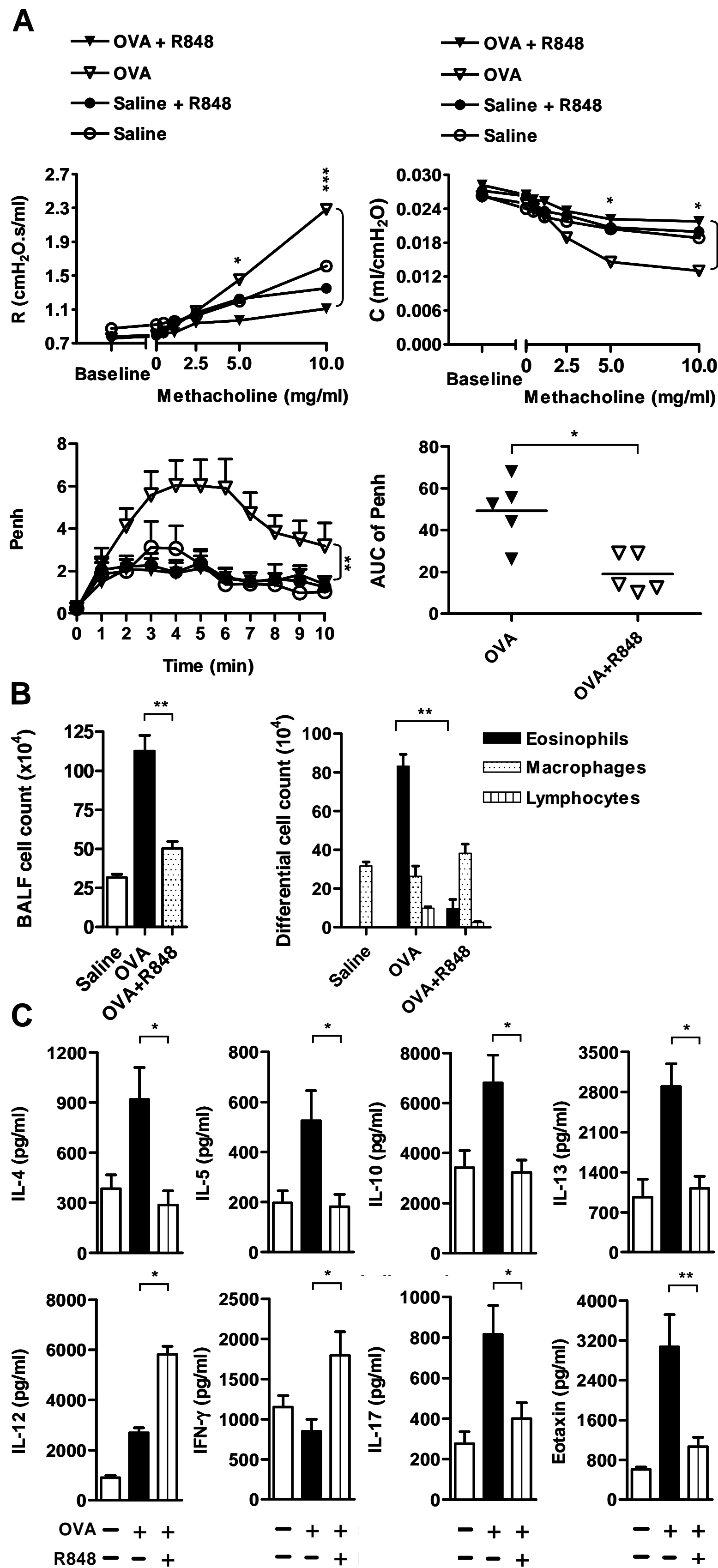


Figure 2

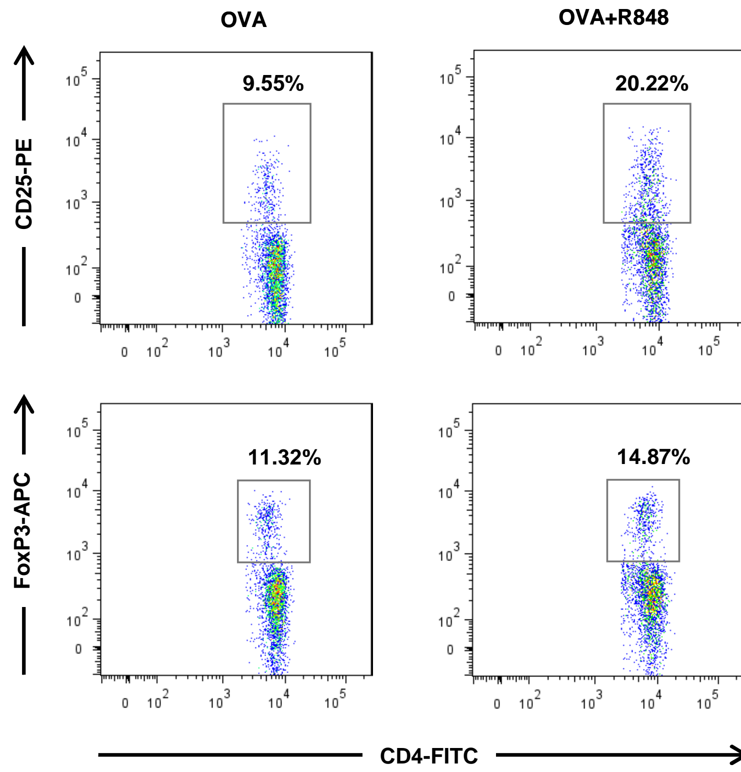


Figure 3

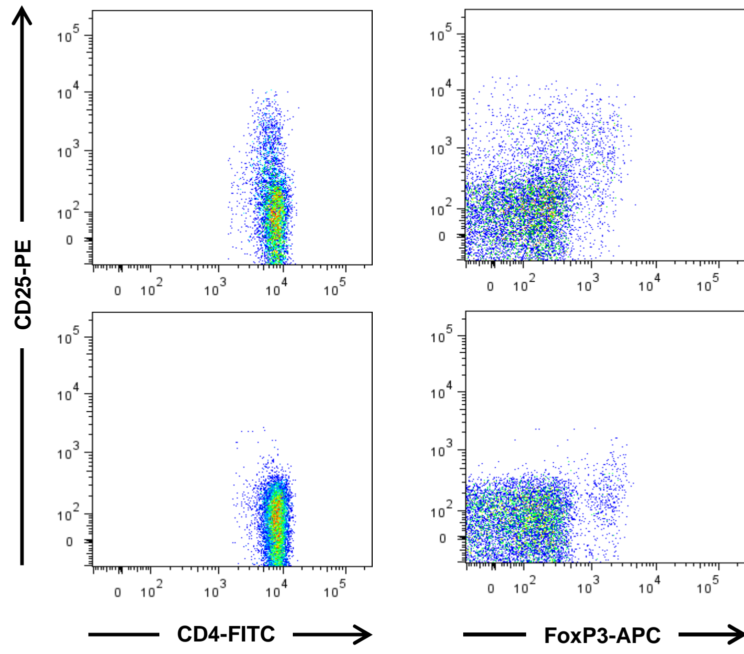


Figure 4

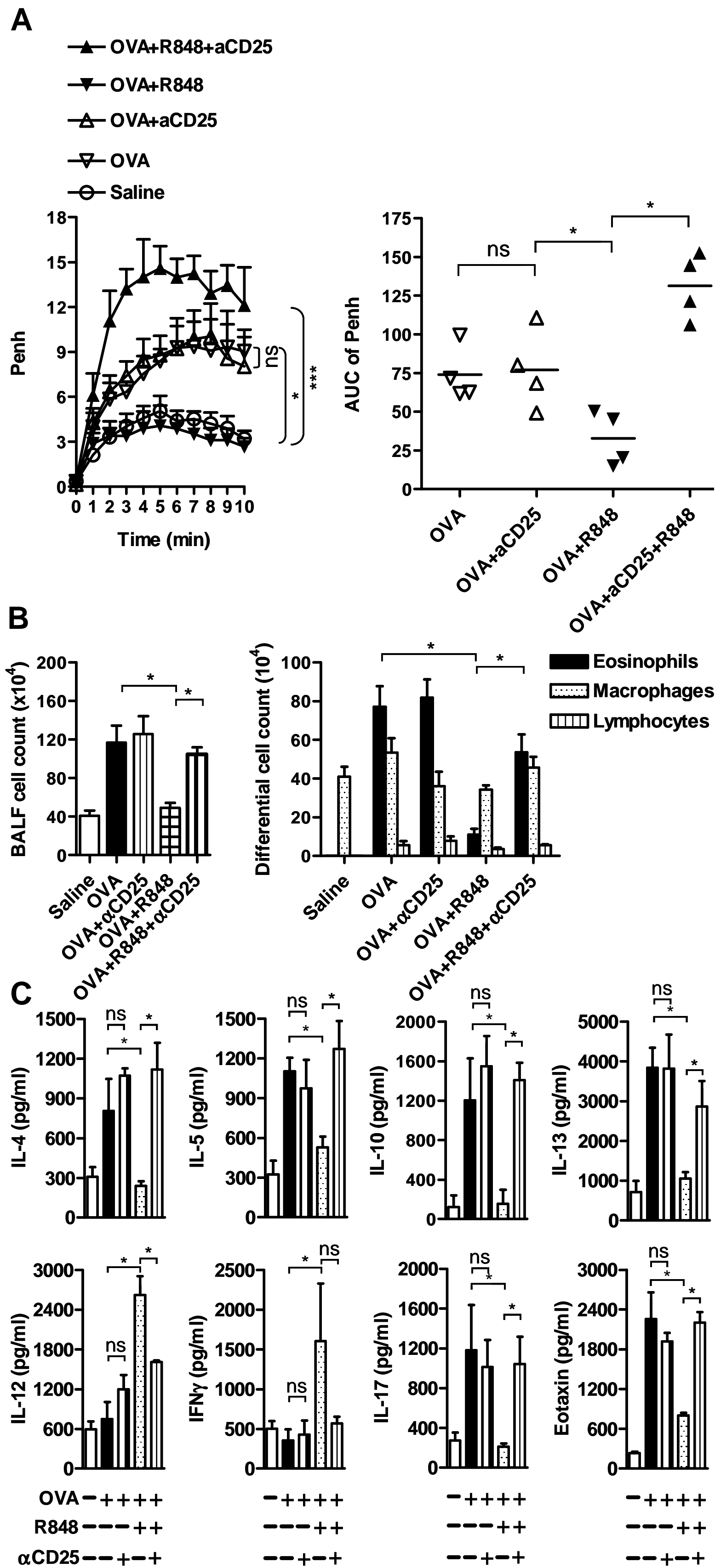
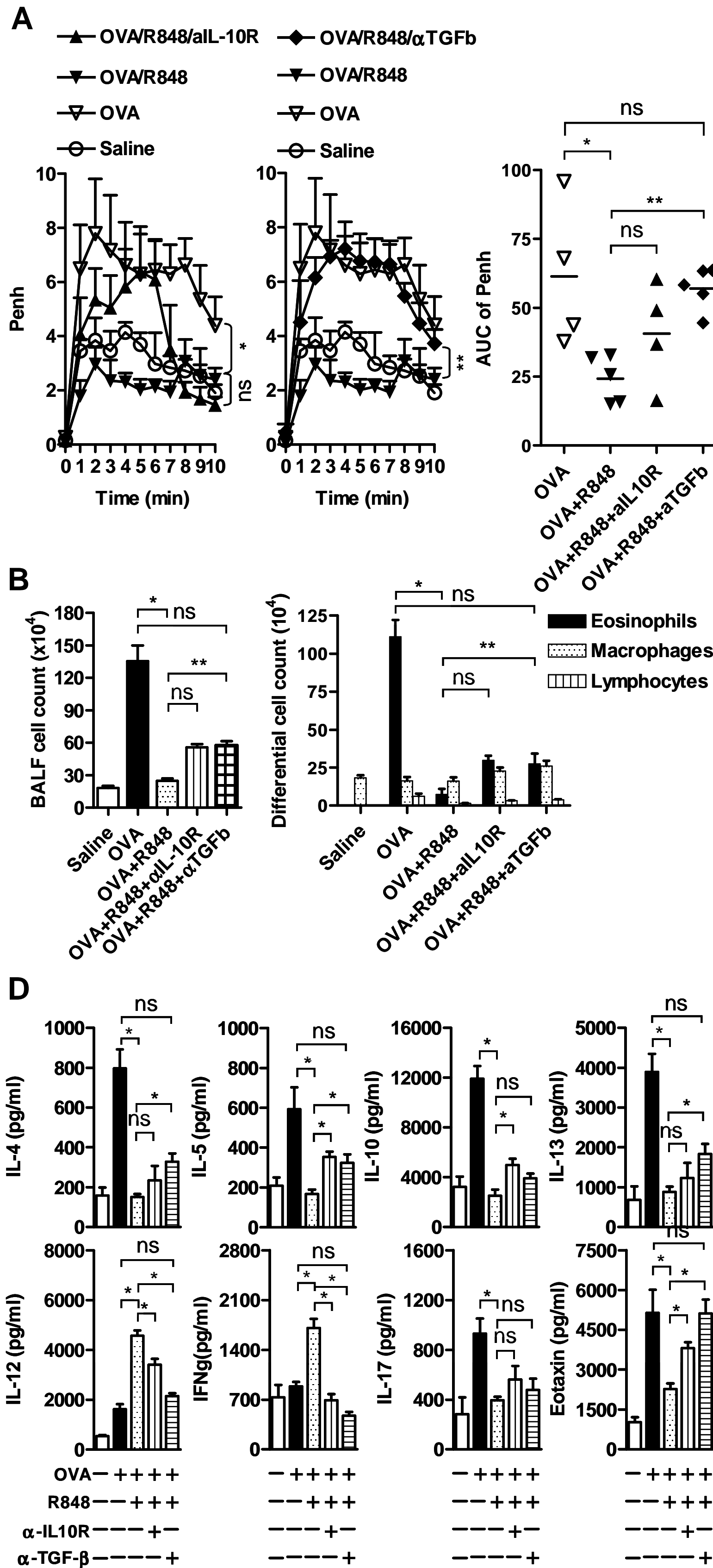


Figure 5



DISCUSSION-CONCLUSION

Nous avons choisi de focaliser l'ensemble de cette discussion sur l'immunorégulation de l'asthme. La difficulté majeure pour concilier et discuter les différents composants du système immunitaire impliqués dans cette pathologie est liée aux observations expérimentales qui paraissent souvent contradictoires. Les résultats présentés au cours de cette thèse n'échappent pas à ces contradictions.

Les cellules iNKT et leur rôle effecteur/régulateur dans l'asthme

La population de lymphocytes iNKT est impliquée dans les réponses précoces aux agents infectieux et dans l'homéostasie du poumon. Des travaux récents ont révélé leur rôle dans la défense contre certains pathogènes comme E.coli, Samonelles, Sphingomonas. En réponse à ces infections, les cellules iNKT sont activées. Outre le ligand synthétique α-GalCer, le plus souvent utilisé dans les expériences *in vitro*, de nouveaux ligands ont été caractérisés et parmi ceux-ci, le glycosphingolipide endogène iGb3, les glycosylcéramides de la paroi des Sphingomonas, les glycolipides de Borrelia burgdorferi (Kinjo et al., 2005; Mattner et al., 2005; Brigl et al., 2003). Dans le modèle d'asthme expérimental induit par des allergènes protéiques, certaines études décrivent les cellules iNKT comme des effecteurs indispensables au développement de l'asthme alors que d'autres ne confirment pas ce rôle (Akbari et al., 2003; Brown et al., 1996; Korsgren et al., 1999; Lisbonne et al., 2003; Cui et al., 1999). Après activation par leur ligand spécifique l'α-GalCer, il a été montré que les cellules iNKT peuvent prévenir ou supprimer les réactions asthmatiques (Hachem et al., 2005; Cui et al., 1999). A l'opposé, des observations expérimentales ont attribué un rôle délétère aux cellules iNKT dans le développement de l'asthme. Elles ont été confortées par des observations cliniques (Akbari et al., 2006; Hamzaoui et al., 2006; Pham-Thi et al., 2006) qui ont conduit certaines équipes à suggérer que les cellules iNKT pourraient être responsables du développement de

l'asthme (Umetsu and DeKruyff, 2010b). Pourtant ces données et les arguments présentés ne paraissent pas encore suffisants pour confirmer cette hypothèse.

Dans notre première étude, nous avons analysé la réponse fonctionnelle des cellules iNKT après stimulation de la voie IL-1R en utilisant la cytokine IL-33. Nous avons observé qu'*in vivo,* l'injection d'IL-33 stimule la prolifération des cellules iNKT et induit une production simultanée d'IFN-γ et d'IL4. *In vitro*, la stimulation par l'IL-33 induit également une production de cytokines par les cellules iNKT du foie et de la rate, augmente l'expression des marqueurs d'activation et induit une prolifération des cellules iNKT d'origine thymique, confirmant l'aptitude de cette population à répondre à l'IL-33 (*Article n°1*). Compte tenu des données rapportées concernant l'expression et les fonctions de ST2 et le rôle facilitant de l'IL-33 dans l'asthme expérimental (Kondo et al., 2008a; Lohning et al., 1998; Liu et al., 2009), nos résultats suggèrent que les cellules iNKT pourraient moduler la réponse d'asthme induite par l'IL-33. L'IL-33 peut être considérée comme un médiateur qui favorise et/ou exacerbe l'asthme et le fait que les cellules iNKT produisent de l'IFN-γ et de l'IL-4, ne permet pas de conclure quant au rôle effecteur ou régulateur de cette population. Une étude comparative de l'effet global *in vivo* de l'IL-33 sur les souris de type sauvage et des souris dépourvues de cellules iNKT pourrait répondre à cette question (même si nous privilègions l'hypothèse d'un rôle régulateur plutôt que délétère).

Relation entre les cellules iNKT et Th17 dans l'asthme

Les cellules iNKT isolées de l'organisme sont intrinsèquement réactives aux ligands de la molécule CD1d *ex vivo,* indiquant que ces cellules sont préactivées à l'état basal. Compte tenu du rôle régulateur des deux cytokines IL-4 et IFN-γ produites par cette population et des observations cliniques publiées, nous avons postulé que les cellules iNKT pourraient participer à la régulation et/ou au développement de la population effectrice Th17

nouvellement caractérisée. Nos résultats ont confirmé cette hypothèse en révélant que les cytokines produites par les cellules iNKT inhibent le développement de la population Th17 à partir des cellules T naïves mises en présence de l'antigène sensibilisant *(Article n°2)*. Cette modulation du développement des cellules Th17 n’est pas sans conséquence sur la régulation de l'asthme. En effet, il a été établi que la signalisation via le récepteur de l'IL-17 était indispensable pour le développement des réponses asthmatiques (Schnyder-Candrian et al., 2006). La différenciation de la lignée Th17 sous l'effet de IL-6, IL-21, TGF-β, IL-23 (chez la souris) ou IL-1, IL-6, IL-23 (chez l'homme) est en équilibre avec celle d'autres populations T effectrices (Th1 et Th2) et surtout T régulatrices, et se distingue de la production d’IL-17 (Wakashin et al., 2008; Bettelli et al., 2006). Bien que l’on admette que l'IL-17 et d'autres cytokines produites par les cellules Th17 ont des effets régulateurs négatifs sur les réactions asthmatiques, on pourrait fort bien imaginer que la différenciation de la population Th17, même durant la phase effectrice, est délétère par son action inhibitrice vis-à-vis des cellules Th1 et T régulatrices et favorise les réponses des cellules Th2 (Schnyder-Candrian et al., 2006; Korn et al., 2007; Schnyder et al., 2010; Wakashin et al., 2008). Nous reviendrons plus loin sur ce concept après avoir discuté de la régulation de l’asthme en fonction des autres populations cellulaires, notamment des cellules T régulatrices.

Basophiles et exacerbation de l'asthme

Dans notre étude sur l’activation des basophiles murins, nous avons identifié une molécule synthétique, le Poly(A:U), mimique des ARN viraux, qui stimule les basophiles *in vitro* et *in vivo* et induit une production d'IL-4, d'IL-6 et d’histamine *(Article n°3)*. Dans cette étude, la réponse des basophiles stimulés par le poly(A:U) a été associée à une exacerbation

de l'asthme et inversement, l'asthme était diminué après élimination des basophiles en présence de l'anticorps déplétant Ba103. Nos résultats et ceux de la littérature sont en faveur d'un rôle exacerbateur des basophiles dans les réponses d'asthme et ceci coïncide avec la production et la libération d'IL-4 et d'histamine par cette population. La voie de signalisation conduisant à la production d'IL-4 par les basophiles stimulés par le Poly(A:U) n'est pas encore élucidée même si nous avons déjà éliminé plusieurs adaptateurs moléculaires des voies de signalisation les mieux décrites impliqués dans les réponses aux ARN viraux, c'est-à-dire, les molécules MyD88, TRIF et IPS-1.

L'asthme ne s'accompagne pas systématiquement d'une réponse allergique et par conséquent les acteurs cellulaires infiltrant les poumons sont variables. Les observations cliniques associant les cas d'asthme sévères induits durant les épisodes infectieux à un nombre augmenté de basophiles ou de neutrophiles dans les expectorations et dans les parois bronchiques, nous conduisent à proposer l'implication de différents composants cellulaires. Si on se réfère aux résultats essentiellement décevants de la plupart des stratégies ciblant la voie de différenciation et/ou d'activation des réponses Th2 et compte tenu du fait que les réponses d'asthme peuvent s'établir en l'absence de cette cytokine, le rôle présumé de l'IL-4 pourrait avoir été surestimé. Aussi, il est possible d'envisager que des médiateurs humoraux autres que l'IL-4, l'IL-5 ou l'IL-13 soient impliqués de façon critique dans l'exacerbation de l'asthme.

En fonction de l'environnement dans lequel les basophiles ont été générés et/ou conditionnés, ces cellules produisent préférentiellement de l'IL-4, de l'histamine ou de l'IL-6. Dans nos études, nous avons observé que les basophiles, mais aussi de nombreuses cellules innées, telles que les cellules dendritiques ou les macrophages, produisent des cytokines en

réponse à une stimulation par le poly(A:U). Si le rôle des cellules résidant dans les voies respiratoires pourrait apparaître de plus en plus critique, non seulement au cours du développement de l'asthme, mais aussi durant le retour à l'homéostasie spontanée ou induite par un traitement, il est certes évident que les cellules immunitaires jouent un rôle primordial dans ces processus (Saarinen, 1984; Agosti et al., 1988).

Des résultats préliminaires suggèrent que des structures bactériennes peuvent stimuler le basophile telles que les lipoprotéines Pam_3CSK. Cette molécule est connue comme agoniste de TLR2. *In vitro*, le Pam_3CSK induit la production d'IL-4 et d'IL-6 par les basophiles murins (travaux non publiés). *In vivo*, cette molécule stimule les basophiles et induit leur recrutement dans différents organes. Des expériences complémentaires *in vivo* devront être réalisées pour confirmer cette activation et analyser ses conséquences dans le modèle d'asthme.

Les données cliniques et/ou expérimentales sont contradictoires puisque certains virus ou bactéries sont considérés comme agents déclenchants ou exacerbateurs de l'asthme alors que d'autres pathogènes ont un effet protecteur (Gershwin, 2006; Tregoning and Schwarze, 2010). Il est très difficile d'examiner les réponses induites par un pathogène entier compte tenu de la variété des molécules exprimées et de la diversité des réponses immunitaires de l'hôte. Aussi, nous avons postulé que l'utilisation d'agonistes TLR d'origine microbienne ou de molécules synthétiques mimant certaines des réponses de pathogènes pourrrait spécifiquement cibler des cellules immunorégulatrices et induire un effet protecteur voire même suppresseur dans l'asthme. C'est ce que nous avons testé expérimentalement.

Protection de l'asthme par la stimulation des TLR

Les travaux de notre équipe ont démontré les effets protecteurs potentiels des agents infectieux vis-à-vis du développement des réponses d'asthme induites expérimentalement. Nous avons testé différents agonistes naturels ou synthétiques des TLR. Les résultats

montrent que plusieurs agonistes TLR protègent les animaux de l'asthme *(Articles n°4, 5 et 6)*. Les conditions expérimentales du modèle varient selon les études. Les agonistes TLR2, 3, 4 et 7, administrés après l'immunisation et avant les stimulations avec l'allergène, protégent les animaux du développement des réponses d'asthme *(Article n°4)*. Les résultats sur les effets protecteurs des agonistes TLR sont en accord avec de nombreux travaux sur la protection induite par des extraits microbiens, des microorganismes entiers morts ou vivants (Braga et al., 2003; Hopfenspirger and Agrawal, 2002; Lagranderie et al., 2008; Lagranderie et al., 2010; Major et al., 2002; Han et al., 2004). L'administration des agonistes TLR avant l'induction des réponses d'asthme pourrait avoir des effets "conditionnants", par le profil des cytokines inflammatoires produites (par exemple IFN-γ, IL-12, IFN de type I pour le cas de R848, agoniste TLR7) qui vont contrecarrer les effets inducteurs de l'asthme. Etant donné le profil de cytokines induites par les agonistes TLR utilisés, il est essentiel d'identifier les cellules cibles à l'origine de ces effets protecteurs. Sur le plan thérapeutique, il serait également intéressant de confirmer si ces effets bénéfiques peuvent persister et si ces molécules peuvent avoir, en plus de leurs effets protecteurs préventifs, un effet thérapeutique, c'est-à-dire moduler les réponses d'asthme déjà établies. Ces questions ont été étudiées pour l'agoniste TLR7.

Effet protecteur de R848

L'effet protecteur du développement des réponses d'asthme induit par l'administration de R848 avant la première stimulation, a été testé et confirmé dans plusieurs conditions d'administration. Concernant le mécanisme d'action, l'agoniste de TLR7 stimule les cellules dendritiques et les cellules NK. Nous avons postulé que les cellules iNKT pourraient aussi être impliquées dans cette protection et avons testé leur rôle dans un modèle de transfert. Les résultats ont montré que R848, en ciblant les cellules iNKT, induisait la production d'IFN-γ, responsable de la protection induite par l'agoniste TLR7 chez les animaux receveurs *(Article 5)*.

Dans cette étude, les cellules du receveur ne sont pas en contact direct avec R848 et par conséquent, ce travail ne confirme pas que les cellules iNKT sont responsables de la protection observée chez une souris ayant reçu directement l'agoniste mais confirme leur participation dans la protection induite. Une seconde étude a été effectuée pour définir l'efficacité suppressive d'un traitement par R848 dans l'asthme.

Pour le patient asthmatique, le moment de sensibilisation et de contact avec l'allergène est indéterminé et imprévisible. Ainsi, nous avons testé la capacité préventive d'un traitement par R848 introduit avant tout contact sensibilisant et, à l'inverse, la capacité suppressive d'un traitement par R848 effectué après l'apparition de la pathologie. Dans la première condition expérimentale, R848 a été administré avant la phase de sensibilisation des animaux à l'allergène. Les résultats observés étaient similaires en présence ou en absence de l'agoniste TLR7 *(données non publiées)*. Ces résultats démontrent que le moment du traitement est critique pour obtenir un effet protecteur.

Pour tester la seconde condition expérimentale, nous avons augmenté le nombre de stimulations dans notre protocole afin de caractériser un éventuel effet suppresseur de R848. Les résultats validant cette hypothèse (*Article n°6*), nous avons étudié les mécanismes responsables de cet effet thérapeutique. Nous avons observé que l'effet suppressif induit par R848 était accompagné d'une induction significative des Treg (en tant que cellules $CD4^+CD25^+$ ou $CD4^+FoxP3^+$) dans les poumons. Pour tester si cette augmentation de Treg était associée avec la suppression, nous avons déplété les animaux en cellules Treg par administration de l'anticorps anti-CD25 (PC61) durant le protocole d'asthme et avant l'administration de R848. Les résultats observés ont montré que la déplétion en Treg corrèle

avec la perte de la suppression induite par R848. Quant aux cytokines potentiellement impliquées, des expériences utilisant des anticorps neutralisant le TGF-β ou des anticorps bloquant le récepteur de l'IL-10 ont montré que cette suppression était partiellement dépendante du TGF-β et, à un degré moindre, de l'IL-10.

En dehors de l'information concernant l'implication des Treg dans l'effet suppressif du traitement de R848, les modulations des Treg observées d'une part dans le groupe traité par R848 et absentes dans le groupe contrôle et les variations des réponses asthmatiques d'autre part dans les groupes d'animaux appauvris en Treg (traités ou non par R848) montrent que le groupe de souris contrôles (non traité par R848) asthmatiques ne présente aucune augmentation du nombre et/ou de l'activité des Treg pour contrecarrer le développement des réponses d'asthme. La déplétion en Treg n'a pas majoré les manifestations dans le groupe contrôle alors que dans le groupe traité par R848 elle a exacerbé les réponses asthmatiques par rapport à leur contrôle. Cette observation suggère que le développement des réponses d'asthme met en jeu des processus qui inhibent le développement et les fonctions suppressives des Treg. Cette constatation coïncide avec certaines observations cliniques suggérant un déficit numérique et/ou fonctionnel des Treg chez les asthmatiques (Mamessier et al., 2005; Robinson, 2009).

D'un point de vue immunologique, cette observation pourrait focaliser notre attention sur les interactions entre les différentes populations T effectrices et T régulatrices. La caractérisation des cellules Treg chez la souris est moins développée que celle décrite chez l'homme et il n'y a pas encore de consensus général strict pour identifier ces cellules. Le traitement par R848 induit une augmentation rapide (1 à 3 jours) des cellules $CD4^{+}CD25^{+}$ supérieure à celle des cellules $CD4^{+}FoxP3^{+}$ suggérant une augmentation d'une population essentiellement "induite" en périphérie. Le résultat concernant l'implication du TGF-β apparait cohérent avec la

suppression compte tenu du vaste éventail de fonctions associées à cette cytokine. Chez la souris, la population produisant de l'IL-10 (dénommée Tr1) semble ne pas avoir une expression stable et forte de CD25 et une expression encore plus faible de FoxP3. Chez l'homme, il existe une sous population de Treg naturels qui produisent essentiellement de l'IL-10 et qui ont aussi une expression faible (ou même nulle) du marqueur CD25. Des cellules analogues chez la souris n'ont pas encore décrites mais on pourrait spéculer sur leur existence. Dans notre étude, l'induction de Treg n'était pas accompagnée d'une augmentation d'IL-10 mais d'une diminution, suggérant que les Treg induits par le traitement R848 ne produisent pas une quantité significative d'IL-10. La diminution de l'IL-10 dans le groupe traité suggère même une disparition des ces cellules sous l'action de R848. En accord avec cette hypothèse, une forte sensibilité à l'apoptose de cette sous-population Treg produisant de l'IL-10 a été rapportée chez l'homme, lorsque ces cellules se développent dans un environnement inflammatoire (Ito et al., 2008).

Le protocole d'asthme, immunisation par l'OVA absorbé sur alun, induit une réponse immunitaire de type Th2 et s'accompagne d'un éventail de réponses provenant des cellules résidant dans la muqueuse bronchique telles que les cellules épithéliales, les fibroblastes et les cellules musculaires lisses. Les cellules immunitaires innées et notamment les mastocytes et basophiles pourraient assurer par eux-mêmes la réponse d'asthme avant que les réponses immunitaires adaptatives de type Th2 soient mises en place. Ce mécanisme pourrait être responsable de l'asthme observé dans les protocoles d'induction très courts de type Umetsu dans lesquels les réponses de type Th2 sont à peine développées. L'utilisation de l'alun dans le protocole d'asthme, bien qu'il soit parfois critiqué, apparait fort utile et nécessaire. En effet, l'alun se comporte comme un adjuvant pour amplifier les réponses Th2. Les cellules résidantes des voies respiratoires et les cellules immunologiques infiltrées ou dans les tissus lymphoides pulmonaires interagissent via la production de nombreuses cytokines, dont l'IL-1,

l'IL-6 et de cytokines et chimiokines de type pro-Th2 comme le TSLP, l'IL-25, l'IL-33, l'IL-3, l'IL-4, l'IL-5, le GM-CSF, l'éotaxine, et TARC.

Historiquement, l'IL-6 a été découverte dans le milieu de culture de plusieurs types de cellules immunitaires et non immunitaires après stimulation par de l'ARN double brin. Cette cytokine pléiotropique, qui est produite en grande quantité durant la phase aigüe de l'inflammation, est considérée comme un biomarqueur de l'inflammation. Les données de la littérature affirment de plus en plus le rôle de cette cytokine dans le développement des cellules Th17 et des Treg à la fois *in vitro* et *in vivo* chez la souris (Bettelli et al., 2006; Veldhoen et al., 2006) et chez l'homme (Acosta-Rodriguez et al., 2007). L'IL-6 exerce des effets synergiques avec le TGF-β dans l'induction des cellules Th17 chez la souris (Acosta-Rodriguez et al., 2007). De nombreux travaux ont démontré que différentes cytokines peuvent participer à l'amplification de cette réponse Th17 et notamment des cytokines de la famille IL-1R telles que l'IL-1 chez l'homme et chez la souris (Lacey et al., 1993; Mantovani et al., 2008; Lust et al., 2009).

Figure 15: Interprétation schématique des interactions cellulaires et humorales participant au développement de l'asthme.

Dans notre modèle expérimental d'asthme, les acteurs cellulaires essentiels concernent les populations de lymphocytes T effecteurs et régulateurs impliquées dans l'asthme comme les cellules Th2, Treg, NKT, Th17, ainsi que les cellules immunitaires innées comme les mastocytes, basophiles, éosinophiles et les cellules résidant dans les voies respiratoires. Si le rôle initiateur du développement de l'asthme est attribué aux cytokines produites précocément dans la muqueuse bronchique notamment l'IL-4, l'IL-6 et le TSLP, l'IL-6 pourrait être impliquée dans le maintien ou l'exacerbation de la pathologie en assumant un rôle critique dans l'équilibre des lymphocytes Th17-Treg-Th2-NKT *(schéma dans la figure 15).*

Plusieurs arguments sont en faveur de cette interprétation: *(1)* Des observations cliniques suggérent un déficit numérique et fonctionnel des Treg chez les patients asthmatiques associé à une augmentation de l'IL-17 dans les poumons et le sérum (Wong et al., 2001b; Molet et al., 2001b; Laan et al., 2002; Barczyk et al., 2003b; Mamessier et al., 2005; Robinson, 2009; Wong et al., 2009). Cette relation a été observée expérimentalement chez la souris (Wakashin et al.,2008; Bettelli et al., 2006; Zhou et al., 2008). *(2)* L'induction expérimentale de la population de Treg est associée à une diminution ou une protection de l'asthme : exemple de notre étude avec l'agoniste TLR7. *(3)* Dans nos études d'asthme expérimental, nous avons mesuré la production de cytokines et chimiokines pro-Th2 telles que l'IL-4, l'IL5, l'IL-13 et l'éotaxine, mais aussi d'autres cytokines telles que l'IL-6 et l'IL-17 dans les poumons. D'une manière générale, nous avons observé une association positive entre la production accrue d'IL-6, d'IL-17, de cytokines et chimiokines pro-Th2 et le développement de l'asthme ou l'exacerbation de celui-ci. Inversement, cette production de cytokines était très faible lorsque les réponses d'asthme étaient inhibées. *(4)* Il a été décrit quel les cellules Th17 se développent préférentiellement sous l'effet de l'IL-6 et de l'IL-23 produites par les cellules épithéliales et d'autres cellules résidant dans les poumons (Wakashin et al.,2008). *(5)* Dans l'étude du rôle

exacerbateur des basophiles, la modulation de l'asthme était associée au nombre de basophiles présents chez l'animal. Il est intéressant de noter qu'après stimulation, cette population augmente numériquement et produit non seulement de l'IL-4 et de l'histamine, mais aussi de l'IL-6 *in vitro* et *in vivo*. Nos résultats et ceux d'autres équipes ont montré que les cellules mononuclées humaines du sang périphérique stimulées par un ARN double-brin tel que le poly(A:U) produisent également de l'IL-6. Ces observations suggèrent que le poly(A:U), qui mime l'ARN double-brin viral, active les cellules innées, et que l'IL-6 produite dans les poumons par de nombreuses populations comme les macrophages alvéolaires, les cellules dendritiques, les cellules épithéliales et les basophiles pourrait jouer un rôle de pivot dans l'exacerbation de l'asthme. *(6)* Nous avons évoqué le rôle potentiellement régulateur des iNKT dans l'asthme qui, après stimulation, peuvent rapidement et en grande quantité de l'IFNγ et/ou de l'IL-4. Dans le cas de l'IL-33, cytokine fortement pro-Th2, la production d'IFN-γ et d'IL-4 peut avoir un effet régulateur négatif sur le dévelopement des cellules Th17. Dans un modèle de transfert, l'implication des cellules iNKT stimulées par R848 est une bonne illustration de l'action régulatrice des cellules iNKT au cours du développement des réponses d'asthme, via la production de leurs cytokines (IFN-γ essentiellement dans notre cas). *(7)* Les résultats obtenus dans l'article 6 ont illustré la participation des populations T régulatrices dans l'effet suppressif induit par R848 et des cytokines immunorégulatrices telles que le TGF-β et l'IL-10. Les cellules Tregs et Th17 ont des fonctions diamétralement opposées, la première induisant une suppression alors que la seconde favorise l'inflammation (Zhou et al., 2008). Différents travaux suggèrent que les cellules Tregs pourraient se convertir en cellules Th17 dans des conditions inflammatoires appropriées. A partir de ces observations, il serait intéressant de tester dans notre protocole, l'effet inhibiteur de R848 sur le développement et/ou la fonctionnalité des Th17.

PERSPECTIVES

Afin de confirmer les hypothèses soulevées au cours de ce travail, plusieurs perspectives sont envisageables:

1. *L'IL-33 et les cellules iNKT*

    - Etudier le rôle des cellules iNKT après traitement par l'IL-33 *in vivo* dans le protocole d'asthme. Pour cela, une étude comparative de l'effet de l'IL-33 *in vivo* sur les souris de type sauvage et dépourvues de cellules iNKT sera envisagée.
    - Dans le modèle d'exacerbation de l'asthme par le poly(A:U), mesurer la production d'IL-33 et évaluer une association éventuelle entre sa libération et la pathologie.

2. *Les iNKT et les lymphocytes Th17 dans l'asthme*

    - Analyser l'induction et l'activation des cellules iNKT, Th17 et des cellules T régulatrices dans le développement expérimental des réponses d'asthme dans un mode dynamique.

3. *Les Basophiles et l'asthme*

    - Valider le rôle exacerbateur des basophiles chez des souris traités par le Poly(A:U) dans l'asthme en étudiant la pathologie chez les souris déficientes en basophiles.
    - Tester le rôle éventuellement initiateur des basophiles dans le développement de la maladie
    - Tester l'effet *in vivo* de PamCSK dans le modèle d'asthme murin.
    - Etudier l'effet in vivo de Poly(A:U) sur les souris déficientes en IL-4 pour documenter le rôle de l'IL-4 dans l'effet induit par le Poly(A:U) sur les souris de type sauvage.
    - Tester l'effet du Poly(A:U) sur les basophiles humains

- Poursuivre l'étude de la signalisation de l'effet du Poly(A:U) sur les basophiles murins *in vitro* en l'orientant vers les molécules de la famille NLR

4. *L'effet des agonistes TLR et le rôle des cellules NKT, Treg et Th17 dans l'asthme.*

- Analyser l'induction et l'activation des cellules iNKT, Th17 et T régulatrices une fois les réponses d'asthme établies et après le traitement par les agonistes TLR.
- Documenter d'autres aspects sur l'effet thérapeutique de R848, sur les souris déficientes en cellules NKT ou en IFN-γ; le maintien de l'effet à long terme; le caractère spécifique de l'antigène; adapter le protocole utilisant des allergènes standardisés.
- Caractériser les mécanismes impliqués dans l'effet suppresseur des cellules T régulatrices dans le protocole utilisant R848.
- Caractériser les cellules T régulatrices induites dans le protocole de traitement par R848. Une étude comparative de l'induction *in vivo* sur les souris immunisées et remises en contact avec l'allergène et sur les souris naïves sera envisagée.
- Etudier les mécanismes de la protection induite par l'immunothérapie spécifique chez l'homme dans le protocoles utilisant des agonistes TLR comme adjuvants.

BIBLIOGRAPHIE

Aaby,P., Shaheen,S.O., Heyes,C.B., Goudiaby,A., Hall,A.J., Shiell,A.W., Jensen,H., and Marchant,A. (2000). Early BCG vaccination and reduction in atopy in Guinea-Bissau. Clin. Exp. Allergy *30*, 644-650.

Abel,B., Thieblemont,N., Quesniaux,V.J., Brown,N., Mpagi,J., Miyake,K., Bihl,F., and Ryffel,B. (2002). Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. J. Immunol. *169*, 3155-3162.

Adcock,I.M., Caramori,G., and Chung,K.F. (2008). New targets for drug development in asthma. Lancet *372*, 1073-1087.

Akbari,O., Faul,J.L., Hoyte,E.G., Berry,G.J., Wahlstrom,J., Kronenberg,M., DeKruyff,R.H., and Umetsu,D.T. (2006). CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. N. Engl. J. Med. *354*, 1117-1129.

Akbari,O., Stock,P., Meyer,E., Kronenberg,M., Sidobre,S., Nakayama,T., Taniguchi,M., Grusby,M.J., DeKruyff,R.H., and Umetsu,D.T. (2003). Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat. Med. *9*, 582-588.

Akdis,C.A. and Akdis,M. (2009). Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. J. Allergy Clin. Immunol. *123*, 735-746.

Allakhverdi,Z., Comeau,M.R., Jessup,H.K., Yoon,B.R., Brewer,A., Chartier,S., Paquette,N., Ziegler,S.F., Sarfati,M., and Delespesse,G. (2007a). Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J. Exp. Med. *204*, 253-258.

Allakhverdi,Z., Comeau,M.R., Jessup,H.K., Yoon,B.R., Brewer,A., Chartier,S., Paquette,N., Ziegler,S.F., Sarfati,M., and Delespesse,G. (2007b). Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J. Exp. Med. *204*, 253-258.

Almqvist,C., Egmar,A.C., van Hage-Hamsten,M., Berglind,N., Pershagen,G., Nordvall,S.L., Svartengren,M., Hedlin,G., and Wickman,M. (2003). Heredity, pet ownership, and confounding control in a population-based birth cohort. J. Allergy Clin. Immunol. *111*, 800-806.

American Thoracic Society. (2000). What constitutes an adverse health effect of air pollution? Official statement of the American Thoracic Society. Am. J. Respir. Crit Care Med. *161*, 665-673.

Anderson,H.R. (2005). Prevalence of asthma. BMJ *330*, 1037-1038.

Apostolou,I., Takahama,Y., Belmant,C., Kawano,T., Huerre,M., Marchal,G., Cui,J., Taniguchi,M., Nakauchi,H., Fournie,J.J., Kourilsky,P., and Gachelin,G. (1999). Murine natural killer T(NKT) cells [correction of natural killer cells] contribute to the granulomatous reaction caused by mycobacterial cell walls. Proc. Natl. Acad. Sci. U. S. A *96*, 5141-5146.

Athie-Morales,V., O'Connor,G.M., and Gardiner,C.M. (2008). Activation of human NK cells by the bacterial pathogen-associated molecular pattern muramyl dipeptide. J. Immunol. *180*, 4082-4089.

Bach,J.F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. N. Engl. J. Med. *347*, 911-920.

Baldini,M., Lohman,I.C., Halonen,M., Erickson,R.P., Holt,P.G., and Martinez,F.D. (1999). A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am. J. Respir. Cell Mol. Biol. *20*, 976-983.

Barbato,A., Turato,G., Baraldo,S., Bazzan,E., Calabrese,F., Panizzolo,C., Zanin,M.E., Zuin,R., Maestrelli,P., Fabbri,L.M., and Saetta,M. (2006). Epithelial damage and angiogenesis in the airways of children with asthma. Am. J. Respir. Crit Care Med. *174*, 975-981.

Barczyk,A., Pierzchala,W., and Sozanska,E. (2003). Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir. Med. *97*, 726-733.

Barnes,P.J. (2004). New drugs for asthma. Nat. Rev. Drug Discov. *3*, 831-844.

Barnes,P.J. (2008a). Immunology of asthma and chronic obstructive pulmonary disease. Nat. Rev. Immunol. *8*, 183-192.

Barnes,P.J. (2008b). Role of GATA-3 in allergic diseases. Curr. Mol. Med. *8*, 330-334.

Barnes,P.J. and Kharitonov,S.A. (1996). Exhaled nitric oxide: a new lung function test. Thorax *51*, 233-237.

Barrett,N.A. and Austen,K.F. (2009). Innate cells and T helper 2 cell immunity in airway inflammation. Immunity. *31*, 425-437.

Basehore,M.J., Howard,T.D., Lange,L.A., Moore,W.C., Hawkins,G.A., Marshik,P.L., Harkins,M.S., Meyers,D.A., and Bleecker,E.R. (2004). A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. J. Allergy Clin. Immunol. *114*, 80-87.

Bashir,M.E., Louie,S., Shi,H.N., and Nagler-Anderson,C. (2004). Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. J. Immunol. *172*, 6978-6987.

Benbernou,N., Esnault,S., Shin,H.C., Fekkar,H., and Guenounou,M. (1997a). Differential regulation of IFN-gamma, IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway. Immunology *91*, 361-368.

Benbernou,N., Esnault,S., Shin,H.C., Fekkar,H., and Guenounou,M. (1997b). Differential regulation of IFN-gamma, IL-10 and inducible nitric oxide synthase in human T cells by cyclic AMP-dependent signal transduction pathway. Immunology *91*, 361-368.

Berger,P., Perng,D.W., Thabrew,H., Compton,S.J., Cairns,J.A., McEuen,A.R., Marthan,R., Tunon De Lara,J.M., and Walls,A.F. (2001). Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. J. Appl. Physiol *91*, 1372-1379.

Berntman,E., Rolf,J., Johansson,C., Anderson,P., and Cardell,S.L. (2005). The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with Salmonella typhimurium. Eur. J. Immunol. *35*, 2100-2109.

Bettelli,E., Korn,T., and Kuchroo,V.K. (2007). Th17: the third member of the effector T cell trilogy. Curr. Opin. Immunol. *19*, 652-657.

Bilenki,L., Yang,J., Fan,Y., Wang,S., and Yang,X. (2004). Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production. Eur. J. Immunol. *34*, 345-354.

Bleck,B., Tse,D.B., Jaspers,I., Curotto de Lafaille,M.A., and Reibman,J. (2006). Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation. J. Immunol. *176*, 7431-7437.

Bluestone,J.A. and Abbas,A.K. (2003). Natural versus adaptive regulatory T cells. Nat. Rev. Immunol. *3*, 253-257.

Bochner,B.S. and Busse,W.W. (2005a). Allergy and asthma. J. Allergy Clin. Immunol. *115*, 953-959.

Bochner,B.S. and Busse,W.W. (2005b). Allergy and asthma. J. Allergy Clin. Immunol. *115*, 953-959.

Bohle,B., Kinaciyan,T., Gerstmayr,M., Radakovics,A., Jahn-Schmid,B., and Ebner,C. (2007). Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J. Allergy Clin. Immunol. *120*, 707-713.

Bourdin et al (2006). Asthme bronchique. EMC (Elsevier SAS, Paris), Pneumologie, 6-039-A-20, 2006.

Braga,E.G., Ananias,R.Z., Mussalem,J.S., Squaiella,C.C., Longhini,A.L., Mariano,M., Travassos,L.R., and Longo-Maugeri,I.M. (2003). Treatment with Propionibacterium acnes modulates the late phase reaction of immediate hypersensitivity in mice. Immunol. Lett. *88*, 163-169.

Braun-Fahrlander,C. (2003). Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. Curr. Opin. Allergy Clin. Immunol. *3*, 325-329.

Brown,D.R., Fowell,D.J., Corry,D.B., Wynn,T.A., Moskowitz,N.H., Cheever,A.W., Locksley,R.M., and Reiner,S.L. (1996). Beta 2-microglobulin-dependent NK1.1+ T cells are not essential for T helper cell 2 immune responses. J. Exp. Med. *184*, 1295-1304.

Burrows,B., Martinez,F.D., Cline,M.G., and Lebowitz,M.D. (1995). The relationship between parental and children's serum IgE and asthma. Am. J. Respir. Crit Care Med. *152*, 1497-1500.

Busse,W.W. (1993). Role and contribution of viral respiratory infections to asthma. Allergy *48*, 57-61.

Caramalho,I., Lopes-Carvalho,T., Ostler,D., Zelenay,S., Haury,M., and Demengeot,J. (2003). Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J. Exp. Med. *197*, 403-411.

Celedon,J.C., Litonjua,A.A., Ryan,L., Platts-Mills,T., Weiss,S.T., and Gold,D.R. (2002). Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. Lancet *360*, 781-782.

Chae,J.J., Komarow,H.D., Cheng,J., Wood,G., Raben,N., Liu,P.P., and Kastner,D.L. (2003). Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. Mol. Cell *11*, 591-604.

Chalmers,G.W., Macleod,K.J., Little,S.A., Thomson,L.J., McSharry,C.P., and Thomson,N.C. (2002). Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. Thorax *57*, 226-230.

Chang,R.H., Feng,M.H., Liu,W.H., and Lai,M.Z. (1997). Nitric oxide increased interleukin-4 expression in T lymphocytes. Immunology *90*, 364-369.

Charpin,D., Birnbaum,J., Haddi,E., Genard,G., Lanteaume,A., Toumi,M., Faraj,F., Van,d.B., X, and Vervloet,D. (1991). Altitude and allergy to house-dust mites. A paradigm of the influence of environmental exposure on allergic sensitization. Am. Rev. Respir. Dis. *143*, 983-986.

Chaudhuri,R., Livingston,E., McMahon,A.D., Thomson,L., Borland,W., and Thomson,N.C. (2003). Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. Am. J. Respir. Crit Care Med. *168*, 1308-1311.

Chavanas,S., Bodemer,C., Rochat,A., Hamel-Teillac,D., Ali,M., Irvine,A.D., Bonafe,J.L., Wilkinson,J., Taieb,A., Barrandon,Y., Harper,J.I., de,P.Y., and Hovnanian,A. (2000). Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. Nat. Genet. *25*, 141-142.

Cheung,P.F., Wong,C.K., and Lam,C.W. (2008). Molecular mechanisms of cytokine and chemokine release from eosinophils activated by IL-17A, IL-17F, and IL-23: implication for Th17 lymphocytes-mediated allergic inflammation. J. Immunol. *180*, 5625-5635.

Chung,K.F. (2006). Intrinsic differences of the airway epithelium in childhood allergic asthma. Am. J. Respir. Crit Care Med. *174*, 1066-1067.

Chvatchko,Y., Proudfoot,A.E., Buser,R., Juillard,P., Alouani,S., Kosco-Vilbois,M., Coyle,A.J., Nibbs,R.J., Graham,G., Offord,R.E., and Wells,T.N. (2003). Inhibition of airway inflammation by amino-terminally modified RANTES/CC chemokine ligand 5 analogues is not mediated through CCR3. J. Immunol. *171*, 5498-5506.

Collison,L.W., Workman,C.J., Kuo,T.T., Boyd,K., Wang,Y., Vignali,K.M., Cross,R., Sehy,D., Blumberg,R.S., and Vignali,D.A. (2007a). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature *450*, 566-569.

Collison,L.W., Workman,C.J., Kuo,T.T., Boyd,K., Wang,Y., Vignali,K.M., Cross,R., Sehy,D., Blumberg,R.S., and Vignali,D.A. (2007b). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature *450*, 566-569.

Conroy,D.M., Jopling,L.A., Lloyd,C.M., Hodge,M.R., Andrew,D.P., Williams,T.J., Pease,J.E., and Sabroe,I. (2003). CCR4 blockade does not inhibit allergic airways inflammation. J. Leukoc. Biol. *74*, 558-563.

Crellin,N.K., Garcia,R.V., Hadisfar,O., Allan,S.E., Steiner,T.S., and Levings,M.K. (2005). Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. J. Immunol. *175*, 8051-8059.

CSGA (1997). A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma. Nat. Genet. *15*, 389-392.

Dahlen,S.E. (2006). Treatment of asthma with antileukotrienes: first line or last resort therapy?
1. Eur. J. Pharmacol. *533*, 40-56.

Daniels,S.E., Bhattacharrya,S., James,A., Leaves,N.I., Young,A., Hill,M.R., Faux,J.A., Ryan,G.F., le Souef,P.N., Lathrop,G.M., Musk,A.W., and Cookson,W.O. (1996). A genome-wide search for quantitative trait loci underlying asthma. Nature *383*, 247-250.

Davies,R.J., Wang,J.H., Trigg,C.J., and Devalia,J.L. (1995). Expression of granulocyte/macrophage-colony-stimulating factor, interleukin-8 and RANTES in the bronchial epithelium of mild asthmatics is down-regulated by inhaled beclomethasone dipropionate. Int. Arch. Allergy Immunol. *107*, 428-429.

de Jong,E.C., Vieira,P.L., Kalinski,P., Schuitemaker,J.H., Tanaka,Y., Wierenga,E.A., Yazdanbakhsh,M., and Kapsenberg,M.L. (2002). Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J. Immunol. *168*, 1704-1709.

DeFranco (2009). Immunité deboeck Edition.

den Haan,J.M., Kraal,G., and Bevan,M.J. (2007). Cutting edge: Lipopolysaccharide induces IL-10-producing regulatory CD4+ T cells that suppress the CD8+ T cell response. J. Immunol. *178*, 5429-5433.

Denning,D.W., O'Driscoll,B.R., Hogaboam,C.M., Bowyer,P., and Niven,R.M. (2006). The link between fungi and severe asthma: a summary of the evidence. Eur. Respir. J. *27*, 615-626.

Diebold,S.S., Kaisho,T., Hemmi,H., Akira,S., and Reis e Sousa (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science *303*, 1529-1531.

Diebold,S.S., Montoya,M., Unger,H., Alexopoulou,L., Roy,P., Haswell,L.E., Al-Shamkhani,A., Flavell,R., Borrow,P., and Reis e Sousa (2003). Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature *424*, 324-328.

Dizier,M.H., Besse-Schmittler,C., Guilloud-Bataille,M., Annesi-Maesano,I., Boussaha,M., Bousquet,J., Charpin,D., Degioanni,A., Gormand,F., Grimfeld,A., Hochez,J., Hyne,G., Lockhart,A., Luillier-Lacombe,M., Matran,R., Meunier,F., Neukirch,F., Pacheco,Y., Parent,V., Paty,E., Pin,I., Pison,C., Scheinmann,P., Thobie,N., Vervloet,D., Kauffmann,F., Feingold,J., Lathrop,M., and Demenais,F. (2000). Genome screen for asthma and related phenotypes in the French EGEA study. Am. J. Respir. Crit Care Med. *162*, 1812-1818.

Donnadieu,E., Jouvin,M.H., Rana,S., Moffatt,M.F., Mockford,E.H., Cookson,W.O., and Kinet,J.P. (2003). Competing functions encoded in the allergy-associated F(c)epsilonRIbeta gene. Immunity. *18*, 665-674.

Drazen,J.M., Yandava,C.N., Dube,L., Szczerback,N., Hippensteel,R., Pillari,A., Israel,E., Schork,N., Silverman,E.S., Katz,D.A., and Drajesk,J. (1999). Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. Nat. Genet. *22*, 168-170.

Drysdale,C.M., McGraw,D.W., Stack,C.B., Stephens,J.C., Judson,R.S., Nandabalan,K., Arnold,K., Ruano,G., and Liggett,S.B. (2000). Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. Proc. Natl. Acad. Sci. U. S. A *97*, 10483-10488.

ECRHS (1996). Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey. Eur. Respir. J. *9*, 687-695.

Eder,W., Ege,M.J., and von,M.E. (2006). The asthma epidemic. N. Engl. J. Med. *355*, 2226-2235.

Eder,W., Klimecki,W., Yu,L., von,M.E., Riedler,J., Braun-Fahrlander,C., Nowak,D., and Martinez,F.D. (2004a). Toll-like receptor 2 as a major gene for asthma in children of European farmers. J. Allergy Clin. Immunol. *113*, 482-488.

Eder,W., Klimecki,W., Yu,L., von,M.E., Riedler,J., Braun-Fahrlander,C., Nowak,D., and Martinez,F.D. (2004b). Toll-like receptor 2 as a major gene for asthma in children of European farmers. J. Allergy Clin. Immunol. *113*, 482-488.

Eder,W., Klimecki,W., Yu,L., von,M.E., Riedler,J., Braun-Fahrlander,C., Nowak,D., and Martinez,F.D. (2005). Opposite effects of CD 14/-260 on serum IgE levels in children raised in different environments. J. Allergy Clin. Immunol. *116*, 601-607.

Esnault,S., Rosenthal,L.A., Wang,D.S., and Malter,J.S. (2008). Thymic stromal lymphopoietin (TSLP) as a bridge between infection and atopy. Int. J. Clin. Exp. Pathol. *1*, 325-330.

Evans,R., III, Mullally,D.I., Wilson,R.W., Gergen,P.J., Rosenberg,H.M., Grauman,J.S., Chevarley,F.M., and Feinleib,M. (1987). National trends in the morbidity and mortality of asthma in the US. Prevalence, hospitalization and death from asthma over two decades: 1965-1984. Chest *91*, 65S-74S.

Fagan,J.K., Scheff,P.A., Hryhorczuk,D., Ramakrishnan,V., Ross,M., and Persky,V. (2001). Prevalence of asthma and other allergic diseases in an adolescent population: association with gender and race. Ann. Allergy Asthma Immunol. *86*, 177-184.

Fageras,B.M., Hmani-Aifa,M., Lindstrom,A., Jenmalm,M.C., Mai,X.M., Nilsson,L., Zdolsek,H.A., Bjorksten,B., Soderkvist,P., and Vaarala,O. (2004). A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. J. Allergy Clin. Immunol. *114*, 561-567.

Falcone,F.H., Zillikens,D., and Gibbs,B.F. (2006). The 21st century renaissance of the basophil? Current insights into its role in allergic responses and innate immunity. Exp. Dermatol. *15*, 855-864.

Fehervari,Z. and Sakaguchi,S. (2004). A paragon of self-tolerance: CD25+CD4+ regulatory T cells and the control of immune responses. Arthritis Res. Ther. *6*, 19-25.

Fischer,K., Scotet,E., Niemeyer,M., Koebernick,H., Zerrahn,J., Maillet,S., Hurwitz,R., Kursar,M., Bonneville,M., Kaufmann,S.H., and Schaible,U.E. (2004). Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. Proc. Natl. Acad. Sci. U. S. A *101*, 10685-10690.

Fitzgerald,K.A., Rowe,D.C., Barnes,B.J., Caffrey,D.R., Visintin,A., Latz,E., Monks,B., Pitha,P.M., and Golenbock,D.T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. *198*, 1043-1055.

Fontenot,J.D., Gavin,M.A., and Rudensky,A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330-336.

Francis,J.N., James,L.K., Paraskevopoulos,G., Wong,C., Calderon,M.A., Durham,S.R., and Till,S.J. (2008). Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. J. Allergy Clin. Immunol. *121*, 1120-1125.

Fujishima,H., Fukagawa,K., Okada,N., Takano,Y., Tsubota,K., Hirai,H., Nagata,K., Matsumoto,K., and Saito,H. (2005). Prostaglandin D2 induces chemotaxis in eosinophils via its receptor CRTH2 and eosinophils may cause severe ocular inflammation in patients with allergic conjunctivitis. Cornea *24*, S66-S70.

Gao,P.S., Kawada,H., Kasamatsu,T., Mao,X.Q., Roberts,M.H., Miyamoto,Y., Yoshimura,M., Saitoh,Y., Yasue,H., Nakao,K., Adra,C.N., Kun,J.F., Moro-oka,S., Inoko,H., Ho,L.P., Shirakawa,T., and Hopkin,J.M. (2000). Variants of NOS1, NOS2, and NOS3 genes in asthmatics. Biochem. Biophys. Res. Commun. *267*, 761-763.

Gauderman,W.J., Avol,E., Gilliland,F., Vora,H., Thomas,D., Berhane,K., McConnell,R., Kuenzli,N., Lurmann,F., Rappaport,E., Margolis,H., Bates,D., and Peters,J. (2004). The effect of air pollution on lung development from 10 to 18 years of age. N. Engl. J. Med. *351*, 1057-1067.

Genestier,L., Taillardet,M., Mondiere,P., Gheit,H., Bella,C., and Defrance,T. (2007). TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J. Immunol. *178*, 7779-7786.

Gern,J.E., Reardon,C.L., Hoffjan,S., Nicolae,D., Li,Z., Roberg,K.A., Neaville,W.A., Carlson-Dakes,K., Adler,K., Hamilton,R., Anderson,E., Gilbertson-White,S., Tisler,C., Dasilva,D., Anklam,K., Mikus,L.D., Rosenthal,L.A., Ober,C., Gangnon,R., and Lemanske,R.F., Jr. (2004). Effects of dog ownership and genotype on immune development and atopy in infancy. J. Allergy Clin. Immunol. *113*, 307-314.

Gershwin (2006). Bronchial Asthma, Humana Press Incs.

Ghosh,S., Elder,A., Guo,J., Mani,U., Patane,M., Carson,K., Ye,Q., Bennett,R., Chi,S., Jenkins,T., Guan,B., Kolbeck,R., Smith,S., Zhang,C., LaRosa,G., Jaffee,B., Yang,H., Eddy,P., Lu,C., Uttamsingh,V., Horlick,R., Harriman,G., and Flynn,D. (2006). Design, synthesis, and progress toward

optimization of potent small molecule antagonists of CC chemokine receptor 8 (CCR8). J. Med. Chem. *49*, 2669-2672.

Gould,H.J. and Sutton,B.J. (2008). IgE in allergy and asthma today. Nat. Rev. Immunol. *8*, 205-217.

Gri,G., Piconese,S., Frossi,B., Manfroi,V., Merluzzi,S., Tripodo,C., Viola,A., Odom,S., Rivera,J., Colombo,M.P., and Pucillo,C.E. (2008). CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. Immunity. *29*, 771-781.

Grunig,G., Warnock,M., Wakil,A.E., Venkayya,R., Brombacher,F., Rennick,D.M., Sheppard,D., Mohrs,M., Donaldson,D.D., Locksley,R.M., and Corry,D.B. (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. Science *282*, 2261-2263.

Guerra,S., Wright,A.L., Morgan,W.J., Sherrill,D.L., Holberg,C.J., and Martinez,F.D. (2004). Persistence of asthma symptoms during adolescence: role of obesity and age at the onset of puberty. Am. J. Respir. Crit Care Med. *170*, 78-85.

Hachem,P., Lisbonne,M., Michel,M.L., Diem,S., Roongapinun,S., Lefort,J., Marchal,G., Herbelin,A., Askenase,P.W., Dy,M., and Leite-de-Moraes,M.C. (2005). Alpha-galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-gamma. Eur. J. Immunol. *35*, 2793-2802.

Hacker,H., Redecke,V., Blagoev,B., Kratchmarova,I., Hsu,L.C., Wang,G.G., Kamps,M.P., Raz,E., Wagner,H., Hacker,G., Mann,M., and Karin,M. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature *439*, 204-207.

Hammad,H. and Lambrecht,B.N. (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat. Rev. Immunol. *8*, 193-204.

Hamzaoui,A., Cheik,R.S., Grairi,H., Abid,H., Ammar,J., Chelbi,H., and Hamzaoui,K. (2006). NKT cells in the induced sputum of severe asthmatics. Mediators. Inflamm. *2006*, 71214.

Hansbro,P.M., Beagley,K.W., Horvat,J.C., and Gibson,P.G. (2004). Role of atypical bacterial infection of the lung in predisposition/protection of asthma. Pharmacol. Ther. *101*, 193-210.

Hart,O.M., Athie-Morales,V., O'Connor,G.M., and Gardiner,C.M. (2005). TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. J. Immunol. *175*, 1636-1642.

Hayakawa,M., Hayakawa,H., Matsuyama,Y., Tamemoto,H., Okazaki,H., and Tominaga,S. (2009). Mature interleukin-33 is produced by calpain-mediated cleavage in vivo. Biochem. Biophys. Res. Commun. *387*, 218-222.

Hayashi,F., Smith,K.D., Ozinsky,A., Hawn,T.R., Yi,E.C., Goodlett,D.R., Eng,J.K., Akira,S., Underhill,D.M., and Aderem,A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099-1103.

He,J.Q., Chan-Yeung,M., Becker,A.B., Dimich-Ward,H., Ferguson,A.C., Manfreda,J., Watson,W.T., and Sandford,A.J. (2003). Genetic variants of the IL13 and IL4 genes and atopic diseases in at-risk children. Genes Immun. *4*, 385-389.

Heil,F., Hemmi,H., Hochrein,H., Ampenberger,F., Kirschning,C., Akira,S., Lipford,G., Wagner,H., and Bauer,S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Heinzmann,A., Mao,X.Q., Akaiwa,M., Kreomer,R.T., Gao,P.S., Ohshima,K., Umeshita,R., Abe,Y., Braun,S., Yamashita,T., Roberts,M.H., Sugimoto,R., Arima,K., Arinobu,Y., Yu,B., Kruse,S., Enomoto,T., Dake,Y., Kawai,M., Shimazu,S., Sasaki,S., Adra,C.N., Kitaichi,M., Inoue,H., Yamauchi,K., Tomichi,N., Kurimoto,F., Hamasaki,N., Hopkin,J.M., Izuhara,K., Shirakawa,T., and Deichmann,K.A. (2000). Genetic variants of IL-13 signalling and human asthma and atopy. Hum. Mol. Genet. *9*, 549-559.

Hemmi,H., Takeuchi,O., Kawai,T., Kaisho,T., Sato,S., Sanjo,H., Matsumoto,M., Hoshino,K., Wagner,H., Takeda,K., and Akira,S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Herz,U., Lacy,P., Renz,H., and Erb,K. (2000). The influence of infections on the development and severity of allergic disorders. Curr. Opin. Immunol. *12*, 632-640.

Hogaboam,C.M., Carpenter,K.J., Schuh,J.M., and Buckland,K.F. (2005). Aspergillus and asthma--any link? Med. Mycol. *43 Suppl 1*, S197-S202.

Holgate,S.T. (2008). The airway epithelium is central to the pathogenesis of asthma. Allergol. Int. *57*, 1-10.

Holgate,S.T. and Polosa,R. (2006). The mechanisms, diagnosis, and management of severe asthma in adults. Lancet *368*, 780-793.

Holgate,S.T. and Polosa,R. (2008). Treatment strategies for allergy and asthma. Nat. Rev. Immunol. *8*, 218-230.

Hopfenspirger,M.T. and Agrawal,D.K. (2002). Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. J. Immunol. *168*, 2516-2522.

Hoshino,K., Kashiwamura,S., Kuribayashi,K., Kodama,T., Tsujimura,T., Nakanishi,K., Matsuyama,T., Takeda,K., and Akira,S. (1999a). The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. J. Exp. Med. *190*, 1541-1548.

Hoshino,K., Takeuchi,O., Kawai,T., Sanjo,H., Ogawa,T., Takeda,Y., Takeda,K., and Akira,S. (1999b). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. *162*, 3749-3752.

Hummelshoj,T., Bodtger,U., Datta,P., Malling,H.J., Oturai,A., Poulsen,L.K., Ryder,L.P., Sorensen,P.S., Svejgaard,E., and Svejgaard,A. (2003). Association between an interleukin-13 promoter polymorphism and atopy. Eur. J. Immunogenet. *30*, 355-359.

Huss,K., Adkinson,N.F., Jr., Eggleston,P.A., Dawson,C., Van Natta,M.L., and Hamilton,R.G. (2001). House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the Childhood Asthma Management Program. J. Allergy Clin. Immunol. *107*, 48-54.

Hwang,E.S., Szabo,S.J., Schwartzberg,P.L., and Glimcher,L.H. (2005). T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science *307*, 430-433.

Hysi,P., Kabesch,M., Moffatt,M.F., Schedel,M., Carr,D., Zhang,Y., Boardman,B., von,M.E., Weiland,S.K., Leupold,W., Fritzsch,C., Klopp,N., Musk,A.W., James,A., Nunez,G., Inohara,N., and Cookson,W.O. (2005). NOD1 variation, immunoglobulin E and asthma. Hum. Mol. Genet. *14*, 935-941.

Infante-Duarte,C., Horton,H.F., Byrne,M.C., and Kamradt,T. (2000). Microbial lipopeptides induce the production of IL-17 in Th cells. J. Immunol. *165*, 6107-6115.

Ito,K., Lim,S., Caramori,G., Cosio,B., Chung,K.F., Adcock,I.M., and Barnes,P.J. (2002). A molecular mechanism of action of theophylline: Induction of histone deacetylase activity to decrease inflammatory gene expression. Proc. Natl. Acad. Sci. U. S. A *99*, 8921-8926.

Ivanov,I.I., McKenzie,B.S., Zhou,L., Tadokoro,C.E., Lepelley,A., Lafaille,J.J., Cua,D.J., and Littman,D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

Kabesch,M., Depner,M., Dahmen,I., Weiland,S.K., Vogelberg,C., Niggemann,B., Lau,S., Illig,T., Klopp,N., Wahn,U., Reinhardt,D., von,M.E., and Nickel,R. (2007). Polymorphisms in eosinophil pathway genes, asthma and atopy. Allergy *62*, 423-428.

Kabesch,M., Peters,W., Carr,D., Leupold,W., Weiland,S.K., and von,M.E. (2003a). Association between polymorphisms in caspase recruitment domain containing protein 15 and allergy in two German populations. J. Allergy Clin. Immunol. *111*, 813-817.

Kabesch,M., Tzotcheva,I., Carr,D., Hofler,C., Weiland,S.K., Fritzsch,C., von,M.E., and Martinez,F.D. (2003b). A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J. Allergy Clin. Immunol. *112*, 893-898.

Kahlenberg,J.M., Lundberg,K.C., Kertesy,S.B., Qu,Y., and Dubyak,G.R. (2005). Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-kappaB-driven protein synthesis. J. Immunol. *175*, 7611-7622.

Kallinich,T., Schmidt,S., Hamelmann,E., Fischer,A., Qin,S., Luttmann,W., Virchow,J.C., and Kroczek,R.A. (2005). Chemokine-receptor expression on T cells in lung compartments of challenged asthmatic patients. Clin. Exp. Allergy *35*, 26-33.

Kameyoshi,Y., Dorschner,A., Mallet,A.I., Christophers,E., and Schroder,J.M. (1992). Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. J. Exp. Med. *176*, 587-592.

Kang,D.C., Gopalkrishnan,R.V., Wu,Q., Jankowsky,E., Pyle,A.M., and Fisher,P.B. (2002). mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc. Natl. Acad. Sci. U. S. A *99*, 637-642.

Karagiannis,S.N., Warrack,J.K., Jennings,K.H., Murdock,P.R., Christie,G., Moulder,K., Sutton,B.J., and Gould,H.J. (2001). Endocytosis and recycling of the complex between CD23 and HLA-DR in human B cells. Immunology *103*, 319-331.

Kato,A., Favoreto S Jr, Avila,P.C., and Schleimer,R.P. (2007). TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. J. Immunol. *179*, 1080-1087.

Kaur,D., Saunders,R., Berger,P., Siddiqui,S., Woodman,L., Wardlaw,A., Bradding,P., and Brightling,C.E. (2006). Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. Am. J. Respir. Crit Care Med. *174*, 1179-1188.

Kawai,T. and Akira,S. (2005). Toll-like receptor downstream signaling. Arthritis Res. Ther. *7*, 12-19.

Kawai,T. and Akira,S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. *11*, 373-384.

Kawai,T., Sato,S., Ishii,K.J., Coban,C., Hemmi,H., Yamamoto,M., Terai,K., Matsuda,M., Inoue,J., Uematsu,S., Takeuchi,O., and Akira,S. (2004). Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nat. Immunol. *5*, 1061-1068.

Kepley,C.L., McFeeley,P.J., Oliver,J.M., and Lipscomb,M.F. (2001). Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. Am. J. Respir. Crit Care Med. *164*, 1053-1058.

Kharitonov,S.A., Yates,D., Robbins,R.A., Logan-Sinclair,R., Shinebourne,E.A., and Barnes,P.J. (1994). Increased nitric oxide in exhaled air of asthmatic patients. Lancet *343*, 133-135.

Kicic,A., Sutanto,E.N., Stevens,P.T., Knight,D.A., and Stick,S.M. (2006). Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. Am. J. Respir. Crit Care Med. *174*, 1110-1118.

Kim,B.S., Kim,I.K., Park,Y.J., Kim,Y.S., Kim,Y.J., Chang,W.S., Lee,Y.S., Kweon,M.N., Chung,Y., and Kang,C.Y. (2010). Conversion of Th2 memory cells into Foxp3+ regulatory T cells suppressing Th2-mediated allergic asthma. Proc. Natl. Acad. Sci. U. S. A *107*, 8742-8747.

Kim,Y.S., Ko,H.M., Kang,N.I., Song,C.H., Zhang,X., Chung,W.C., Kim,J.H., Choi,I.H., Park,Y.M., Kim,G.Y., Im,S.Y., and Lee,H.K. (2007). Mast cells play a key role in the development of late airway hyperresponsiveness through TNF-alpha in a murine model of asthma. Eur. J. Immunol. *37*, 1107-1115.

Kinjo,Y., Tupin,E., Wu,D., Fujio,M., Garcia-Navarro,R., Benhnia,M.R., Zajonc,D.M., Ben-Menachem,G., Ainge,G.D., Painter,G.F., Khurana,A., Hoebe,K., Behar,S.M., Beutler,B., Wilson,I.A., Tsuji,M., Sellati,T.J., Wong,C.H., and Kronenberg,M. (2006). Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat. Immunol. *7*, 978-986.

Kinjo,Y., Wu,D., Kim,G., Xing,G.W., Poles,M.A., Ho,D.D., Tsuji,M., Kawahara,K., Wong,C.H., and Kronenberg,M. (2005). Recognition of bacterial glycosphingolipids by natural killer T cells. Nature *434*, 520-525.

Kiss,A., Montes,M., Susarla,S., Jaensson,E.A., Drouin,S.M., Wetsel,R.A., Yao,Z., Martin,R., Hamzeh,N., Adelagun,R., Amar,S., Kheradmand,F., and Corry,D.B. (2007). A new mechanism regulating the initiation of allergic airway inflammation. J. Allergy Clin. Immunol. *120*, 334-342.

Kling,S., Donninger,H., Williams,Z., Vermeulen,J., Weinberg,E., Latiff,K., Ghildyal,R., and Bardin,P. (2005). Persistence of rhinovirus RNA after asthma exacerbation in children. Clin. Exp. Allergy *35*, 672-678.

Knight,D.A. and Holgate,S.T. (2003). The airway epithelium: structural and functional properties in health and disease. Respirology. *8*, 432-446.

Kondo,Y., Yoshimoto,T., Yasuda,K., Futatsugi-Yumikura,S., Morimoto,M., Hayashi,N., Hoshino,T., Fujimoto,J., and Nakanishi,K. (2008a). Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int. Immunol. *20*, 791-800.

Kondo,Y., Yoshimoto,T., Yasuda,K., Futatsugi-Yumikura,S., Morimoto,M., Hayashi,N., Hoshino,T., Fujimoto,J., and Nakanishi,K. (2008b). Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int. Immunol. *20*, 791-800.

Korsgren,M., Persson,C.G., Sundler,F., Bjerke,T., Hansson,T., Chambers,B.J., Hong,S., Van,K.L., Ljunggren,H.G., and Korsgren,O. (1999). Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. J. Exp. Med. *189*, 553-562.

Kouzaki,H., O'Grady,S.M., Lawrence,C.B., and Kita,H. (2009). Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J. Immunol. *183*, 1427-1434.

Kraft,M. (2000). The role of bacterial infections in asthma. Clin. Chest Med. *21*, 301-313.

Kraneveld,A.D., Kool,M., van Houwelingen,A.H., Roholl,P., Solomon,A., Postma,D.S., Nijkamp,F.P., and Redegeld,F.A. (2005). Elicitation of allergic asthma by immunoglobulin free light chains. Proc. Natl. Acad. Sci. U. S. A *102*, 1578-1583.

Krause,T.G., Hviid,A., Koch,A., Friborg,J., Hjuler,T., Wohlfahrt,J., Olsen,O.R., Kristensen,B., and Melbye,M. (2003). BCG vaccination and risk of atopy. JAMA *289*, 1012-1015.

Kubo,T., Hatton,R.D., Oliver,J., Liu,X., Elson,C.O., and Weaver,C.T. (2004). Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines produced by TLR-activated dendritic cells. J. Immunol. *173*, 7249-7258.

Kufer,T.A., Fritz,J.H., and Philpott,D.J. (2005). NACHT-LRR proteins (NLRs) in bacterial infection and immunity. Trends Microbiol. *13*, 381-388.

Kurowska-Stolarska,M., Kewin,P., Murphy,G., Russo,R.C., Stolarski,B., Garcia,C.C., Komai-Koma,M., Pitman,N., Li,Y., Niedbala,W., McKenzie,A.N., Teixeira,M.M., Liew,F.Y., and Xu,D. (2008). IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. J. Immunol. *181*, 4780-4790.

Kurt-Jones,E.A., Popova,L., Kwinn,L., Haynes,L.M., Jones,L.P., Tripp,R.A., Walsh,E.E., Freeman,M.W., Golenbock,D.T., Anderson,L.J., and Finberg,R.W. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. *1*, 398-401.

Kusel,M.M., de Klerk,N.H., Kebadze,T., Vohma,V., Holt,P.G., Johnston,S.L., and Sly,P.D. (2007). Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J. Allergy Clin. Immunol. *119*, 1105-1110.

Lagranderie,M., Abolhassani,M., Vanoirbeek,J., Lefort,J., Nahori,M.A., Lapa E Silva JR, Huerre,M., Vargaftig,B., and Marchal,G. (2008). Mycobacterium bovis BCG killed by extended freeze-drying reduces airway hyperresponsiveness in 2 animal models. J. Allergy Clin. Immunol. *121*, 471-478.

Lagranderie,M., Abolhassani,M., Vanoirbeek,J.A., Lima,C., Balazuc,A.M., Vargaftig,B.B., and Marchal,G. (2010). Mycobacterium bovis bacillus Calmette-Guerin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. J. Immunol. *184*, 1062-1070.

Lai,C.K., Beasley,R., Crane,J., Foliaki,S., Shah,J., and Weiland,S. (2009). Global variation in the prevalence and severity of asthma symptoms: phase three of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax *64*, 476-483.

Lambrecht,B.N., De,V.M., Coyle,A.J., Gutierrez-Ramos,J.C., Thielemans,K., and Pauwels,R.A. (2000). Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. J. Clin. Invest *106*, 551-559.

Lamers,M.C. and Yu,P. (1995). Regulation of IgE synthesis. Lessons from the study of IgE transgenic and CD23-deficient mice. Immunol. Rev. *148*, 71-95.

Larche,M. (2007). Regulatory T cells in allergy and asthma. Chest *132*, 1007-1014.

Latvala,J., von,H.L., Lindholm,H., and Haahtela,T. (2005). Trends in prevalence of asthma and allergy in Finnish young men: nationwide study, 1966-2003. BMJ *330*, 1186-1187.

Lauener,R.P., Birchler,T., Adamski,J., Braun-Fahrlander,C., Bufe,A., Herz,U., von,M.E., Nowak,D., Riedler,J., Waser,M., and Sennhauser,F.H. (2002). Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet *360*, 465-466.

Lazarus,R., Klimecki,W.T., Raby,B.A., Vercelli,D., Palmer,L.J., Kwiatkowski,D.J., Silverman,E.K., Martinez,F., and Weiss,S.T. (2003). Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics *81*, 85-91.

Lazarus,R., Raby,B.A., Lange,C., Silverman,E.K., Kwiatkowski,D.J., Vercelli,D., Klimecki,W.J., Martinez,F.D., and Weiss,S.T. (2004). TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. Am. J. Respir. Crit Care Med. *170*, 594-600.

Lazarus,S.C., Chinchilli,V.M., Rollings,N.J., Boushey,H.A., Cherniack,R., Craig,T.J., Deykin,A., DiMango,E., Fish,J.E., Ford,J.G., Israel,E., Kiley,J., Kraft,M., Lemanske,R.F., Jr., Leone,F.T., Martin,R.J., Pesola,G.R., Peters,S.P., Sorkness,C.A., Szefler,S.J., Wechsler,M.E., and Fahy,J.V. (2007). Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. Am. J. Respir. Crit Care Med. *175*, 783-790.

Lee,H.C. and Ziegler,S.F. (2007). Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF-kappaB. Proc. Natl. Acad. Sci. U. S. A *104*, 914-919.

Leff,A.R. (2001). The Antileukotriene Working Group consensus conference overview. Ann. Allergy Asthma Immunol. *86*, 1-3.

Lehner,M., Bailo,M., Stachel,D., Roesler,W., Parolini,O., and Holter,W. (2007). Caspase-8 dependent apoptosis induction in malignant myeloid cells by TLR stimulation in the presence of IFN-alpha. Leuk. Res. *31*, 1729-1735.

Leibowitz,U., Antonovsky,A., Medalie,J.M., Smith,H.A., Halpern,L., and Alter,M. (1966). Epidemiological study of multiple sclerosis in Israel. II. Multiple sclerosis and level of sanitation. J. Neurol. Neurosurg. Psychiatry *29*, 60-68.

Lemanske,R.F., Jr., Jackson,D.J., Gangnon,R.E., Evans,M.D., Li,Z., Shult,P.A., Kirk,C.J., Reisdorf,E., Roberg,K.A., Anderson,E.L., Carlson-Dakes,K.T., Adler,K.J., Gilbertson-White,S., Pappas,T.E., Dasilva,D.F., Tisler,C.J., and Gern,J.E. (2005). Rhinovirus illnesses during infancy predict subsequent childhood wheezing. J. Allergy Clin. Immunol. *116*, 571-577.

Leonardi-Bee,J., Pritchard,D., and Britton,J. (2006). Asthma and current intestinal parasite infection: systematic review and meta-analysis. Am. J. Respir. Crit Care Med. *174*, 514-523.

Lewkowicz,P., Lewkowicz,N., Sasiak,A., and Tchorzewski,H. (2006). Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. J. Immunol. *177*, 7155-7163.

Lisbonne,M., Diem,S., de Castro,K.A., Lefort,J., Araujo,L.M., Hachem,P., Fourneau,J.M., Sidobre,S., Kronenberg,M., Taniguchi,M., Van,E.P., Dy,M., Askenase,P., Russo,M., Vargaftig,B.B., Herbelin,A., and Leite-de-Moraes,M.C. (2003). Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. J. Immunol. *171*, 1637-1641.

Liu,H., Komai-Koma,M., Xu,D., and Liew,F.Y. (2006). Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. Proc. Natl. Acad. Sci. U. S. A *103*, 7048-7053.

Liu,J.B., Zhong,N.S., Li,S.N., Li,X.B., Liang,Z.P., and Wang,G.F. (2003). [The effect of BCG on airway inflammatory reaction of experimental asthma mice]. Xi. Bao. Yu Fen. Zi. Mian. Yi. Xue. Za Zhi. *19*, 346-348.

Liu,Y.J., Soumelis,V., Watanabe,N., Ito,T., Wang,Y.H., Malefyt,R.W., Omori,M., Zhou,B., and Ziegler,S.F. (2007). TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu. Rev. Immunol. *25*, 193-219.

Loke,W.M., Proudfoot,J.M., McKinley,A.J., and Croft,K.D. (2006). Augmentation of monocyte intracellular ascorbate in vitro protects cells from oxidative damage and inflammatory responses. Biochem. Biophys. Res. Commun. *345*, 1039-1043.

Lordan,J.L., Bucchieri,F., Richter,A., Konstantinidis,A., Holloway,J.W., Thornber,M., Puddicombe,S.M., Buchanan,D., Wilson,S.J., Djukanovic,R., Holgate,S.T., and Davies,D.E. (2002). Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. J. Immunol. *169*, 407-414.

Loza,M.J. and Chang,B.L. (2007). Association between Q551R IL4R genetic variants and atopic asthma risk demonstrated by meta-analysis. J. Allergy Clin. Immunol. *120*, 578-585.

Lund,J., Sato,A., Akira,S., Medzhitov,R., and Iwasaki,A. (2003). Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. *198*, 513-520.

Ly,T.W. and Bacon,K.B. (2005). Small-molecule CRTH2 antagonists for the treatment of allergic inflammation: an overview. Expert. Opin. Investig. Drugs *14*, 769-773.

Ma,Y., Zhao,J., Han,Z.R., Chen,Y., Leung,T.F., and Wong,G.W. (2009). Very low prevalence of asthma and allergies in schoolchildren from rural Beijing, China. Pediatr. Pulmonol. *44*, 793-799.

Maddox,L. and Schwartz,D.A. (2002). The pathophysiology of asthma. Annu. Rev. Med. *53*, 477-498.

Major,T., Wohlleben,G., Reibetanz,B., and Erb,K.J. (2002). Application of heat killed Mycobacterium bovis-BCG into the lung inhibits the development of allergen-induced Th2 responses. Vaccine *20*, 1532-1540.

Mallia,P. and Johnston,S.L. (2006). How viral infections cause exacerbation of airway diseases. Chest *130*, 1203-1210.

Malo,J.L., Lemiere,C., Gautrin,D., and Labrecque,M. (2004). Occupational asthma. Curr. Opin. Pulm. Med. *10*, 57-61.

Mamessier,E., Botturi,K., Vervloet,D., and Magnan,A. (2005). [T regulatory lymphocytes, atopy and asthma: a new concept in three dimensions]. Rev. Mal Respir. *22*, 305-311.

Mantel,P.Y., Kuipers,H., Boyman,O., Rhyner,C., Ouaked,N., Ruckert,B., Karagiannidis,C., Lambrecht,B.N., Hendriks,R.W., Crameri,R., Akdis,C.A., Blaser,K., and Schmidt-Weber,C.B. (2007). GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS. Biol. *5*, e329.

Maratheftis,C.I., Andreakos,E., Moutsopoulos,H.M., and Voulgarelis,M. (2007). Toll-like receptor-4 is up-regulated in hematopoietic progenitor cells and contributes to increased apoptosis in myelodysplastic syndromes. Clin. Cancer Res. *13*, 1154-1160.

Marsh,D.G., Neely,J.D., Breazeale,D.R., Ghosh,B., Freidhoff,L.R., Ehrlich-Kautzky,E., Schou,C., Krishnaswamy,G., and Beaty,T.H. (1994). Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science *264*, 1152-1156.

Martin,A.J., Campbell,D.A., Gluyas,P.A., Coates,J.R., Ruffin,R.E., Roder,D.M., Latimer,K.M., Luke,C.G., Frith,P.A., Yellowlees,P.M., and . (1995). Characteristics of near-fatal asthma in childhood. Pediatr. Pulmonol. *20*, 1-8.

Martinon,F. and Tschopp,J. (2005). NLRs join TLRs as innate sensors of pathogens. Trends Immunol. *26*, 447-454.

Matsuda,H., Suda,T., Sato,J., Nagata,T., Koide,Y., Chida,K., and Nakamura,H. (2005). alpha-Galactosylceramide, a ligand of natural killer T cells, inhibits allergic airway inflammation. Am. J. Respir. Cell Mol. Biol. *33*, 22-31.

Mattner,J., Debord,K.L., Ismail,N., Goff,R.D., Cantu,C., III, Zhou,D., Saint-Mezard,P., Wang,V., Gao,Y., Yin,N., Hoebe,K., Schneewind,O., Walker,D., Beutler,B., Teyton,L., Savage,P.B., and Bendelac,A. (2005). Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature *434*, 525-529.

McGeachy,M.J. and Cua,D.J. (2007). The link between IL-23 and Th17 cell-mediated immune pathologies. Semin. Immunol. *19*, 372-376.

Medina-Tato,D.A., Watson,M.L., and Ward,S.G. (2006). Leukocyte navigation mechanisms as targets in airway diseases. Drug Discov. Today *11*, 866-879.

Megyeri,K., Au,W.C., Rosztoczy,I., Raj,N.B., Miller,R.L., Tomai,M.A., and Pitha,P.M. (1995). Stimulation of interferon and cytokine gene expression by imiquimod and stimulation by Sendai virus utilize similar signal transduction pathways. Mol. Cell Biol. *15*, 2207-2218.

Melen,E., Wickman,M., Nordvall,S.L., van Hage-Hamsten,M., and Lindfors,A. (2001). Influence of early and current environmental exposure factors on sensitization and outcome of asthma in pre-school children. Allergy *56*, 646-652.

Meylan,E., Burns,K., Hofmann,K., Blancheteau,V., Martinon,F., Kelliher,M., and Tschopp,J. (2004). RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat. Immunol. *5*, 503-507.

Min,B. and Paul,W.E. (2008). Basophils: in the spotlight at last. Nat. Immunol. *9*, 223-225.

Miyara,M., Yoshioka,Y., Kitoh,A., Shima,T., Wing,K., Niwa,A., Parizot,C., Taflin,C., Heike,T., Valeyre,D., Mathian,A., Nakahata,T., Yamaguchi,T., Nomura,T., Ono,M., Amoura,Z., Gorochov,G., and Sakaguchi,S. (2009). Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. *30*, 899-911.

Mobs,C., Slotosch,C., Loffler,H., Jakob,T., Hertl,M., and Pfutzner,W. (2010). Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. J. Immunol. *184*, 2194-2203.

Moffatt,M.F. and Cookson,W.O. (1997). Tumour necrosis factor haplotypes and asthma. Hum. Mol. Genet. *6*, 551-554.

Molet,S., Hamid,Q., Davoine,F., Nutku,E., Taha,R., Page,N., Olivenstein,R., Elias,J., and Chakir,J. (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J. Allergy Clin. Immunol. *108*, 430-438.

Molimard,M. and Marthan,R. (2000). [Genetic polymorphism and asthma] Rev. Mal Respir. *17*, 564-568.

Moller-Larsen,S., Nyegaard,M., Haagerup,A., Vestbo,J., Kruse,T.A., and Borglum,A.D. (2008). Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax *63*, 1064-1069.

Montoya,C.J., Jie,H.B., Al-Harthi,L., Mulder,C., Patino,P.J., Rugeles,M.T., Krieg,A.M., Landay,A.L., and Wilson,S.B. (2006). Activation of plasmacytoid dendritic cells with TLR9 agonists initiates invariant NKT cell-mediated cross-talk with myeloid dendritic cells. J. Immunol. *177*, 1028-1039.

Morahan,G., Huang,D., Wu,M., Holt,B.J., White,G.P., Kendall,G.E., Sly,P.D., and Holt,P.G. (2002). Association of IL12B promoter polymorphism with severity of atopic and non-atopic asthma in children. Lancet *360*, 455-459.

Morishima,Y., Ishii,Y., Kimura,T., Shibuya,A., Shibuya,K., Hegab,A.E., Iizuka,T., Kiwamoto,T., Matsuno,Y., Sakamoto,T., Nomura,A., Taniguchi,M., and Sekizawa,K. (2005). Suppression of eosinophilic airway inflammation by treatment with alpha-galactosylceramide. Eur. J. Immunol. *35*, 2803-2814.

Moussion,C., Ortega,N., and Girard,J.P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'?. PLoS. One. *3*, e3331.

Mullen,M., Mullen,B., and Carey,M. (1993). The association between beta-agonist use and death from asthma. A meta-analytic integration of case-control studies. JAMA *270*, 1842-1845.

Murawski,M.R., Bowen,G.N., Cerny,A.M., Anderson,L.J., Haynes,L.M., Tripp,R.A., Kurt-Jones,E.A., and Finberg,R.W. (2009). Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J. Virol. *83*, 1492-1500.

Muzio,M., Bosisio,D., Polentarutti,N., D'amico,G., Stoppacciaro,A., Mancinelli,R., van't Veer,C., Penton-Rol,G., Ruco,L.P., Allavena,P., and Mantovani,A. (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. *164*, 5998-6004.

Nagarajan,N.A. and Kronenberg,M. (2007). Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J. Immunol. *178*, 2706-2713.

Nagata,M. and Nakagome,K. (2010). Allergen immunotherapy in asthma: current status and future perspectives. Allergol. Int. *59*, 15-19.

Nakamura,Y., Azuma,M., Okano,Y., Sano,T., Takahashi,T., Ohmoto,Y., and Sone,S. (1996). Upregulatory effects of interleukin-4 and interleukin-13 but not interleukin-10 on granulocyte/macrophage colony-stimulating factor production by human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. *15*, 680-687.

Namkung,J.H., Lee,J.E., Kim,E., Cho,H.J., Kim,S., Shin,E.S., Cho,E.Y., and Yang,J.M. (2007). IL-5 and IL-5 receptor alpha polymorphisms are associated with atopic dermatitis in Koreans. Allergy *62*, 934-942.

Negishi,H., Fujita,Y., Yanai,H., Sakaguchi,S., Ouyang,X., Shinohara,M., Takayanagi,H., Ohba,Y., Taniguchi,T., and Honda,K. (2006). Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. Proc. Natl. Acad. Sci. U. S. A *103*, 15136-15141.

Neukirch,F., Pin,I., Knani,J., Henry,C., Pison,C., Liard,R., Romazzini,S., and Bousquet,J. (1995). Prevalence of asthma and asthma-like symptoms in three French cities. Respir. Med. *89*, 685-692.

Newcomb,D.C. and Peebles,R.S., Jr. (2009). Bugs and asthma: a different disease?. Proc. Am. Thorac. Soc. *6*, 266-271.

Nga,N.N., Chai,S.K., Bihn,T.T., Redding,G., Takaro,T., Checkoway,H., Son,P.H., Van,D.K., Keifer,M., Trung,l., V, and Barnhart,S. (2003). ISAAC-based asthma and atopic symptoms among Ha Noi school children. Pediatr. Allergy Immunol. *14*, 272-279.

Nicholson,P.J., Cullinan,P., Taylor,A.J., Burge,P.S., and Boyle,C. (2005). Evidence based guidelines for the prevention, identification, and management of occupational asthma. Occup. Environ. Med. *62*, 290-299.

Niedbala,W., Wei,X.Q., Cai,B., Hueber,A.J., Leung,B.P., McInnes,I.B., and Liew,F.Y. (2007). IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. Eur. J. Immunol. *37*, 3021-3029.

Noguchi,E., Nishimura,F., Fukai,H., Kim,J., Ichikawa,K., Shibasaki,M., and Arinami,T. (2004). An association study of asthma and total serum immunoglobin E levels for Toll-like receptor polymorphisms in a Japanese population. Clin. Exp. Allergy *34*, 177-183.

Nomura,T. and Sakaguchi,S. (2007). Foxp3 and Aire in thymus-generated Treg cells: a link in self-tolerance. Nat. Immunol. *8*, 333-334.

Nouri-Aria,K.T., Irani,A.M., Jacobson,M.R., O'brien,F., Varga,E.M., Till,S.J., Durham,S.R., and Schwartz,L.B. (2001). Basophil recruitment and IL-4 production during human allergen-induced late asthma. J. Allergy Clin. Immunol. *108*, 205-211.

O'Neill,L.A., Brown,Z., and Ward,S.G. (2003). Toll-like receptors in the spotlight1. Nat. Immunol. *4*, 299.

Oboki,K., Ohno,T., Saito,H., and Nakae,S. (2008). Th17 and allergy. Allergol. Int. *57*, 121-134.

Oganesyan,G., Saha,S.K., Guo,B., He,J.Q., Shahangian,A., Zarnegar,B., Perry,A., and Cheng,G. (2006). Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature *439*, 208-211.

Ogra,P.L. (2004). Respiratory syncytial virus: the virus, the disease and the immune response. Paediatr. Respir. Rev. *5 Suppl A*, S119-S126.

Okayama,Y., Tkaczyk,C., Metcalfe,D.D., and Gilfillan,A.M. (2003). Comparison of Fc epsilon RI- and Fc gamma RI-mediated degranulation and TNF-alpha synthesis in human mast cells: selective utilization of phosphatidylinositol-3-kinase for Fc gamma RI-induced degranulation. Eur. J. Immunol. *33*, 1450-1459.

Omori,M. and Ziegler,S. (2007). Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. J. Immunol. *178*, 1396-1404.

Ong,Y.E., Menzies-Gow,A., Barkans,J., Benyahia,F., Ou,T.T., Ying,S., and Kay,A.B. (2005). Anti-IgE (omalizumab) inhibits late-phase reactions and inflammatory cells after repeat skin allergen challenge. J. Allergy Clin. Immunol. *116*, 558-564.

Oppmann,B., Lesley,R., Blom,B., Timans,J.C., Xu,Y., Hunte,B., Vega,F., Yu,N., Wang,J., Singh,K., Zonin,F., Vaisberg,E., Churakova,T., Liu,M., Gorman,D., Wagner,J., Zurawski,S., Liu,Y., Abrams,J.S., Moore,K.W., Rennick,D., de Waal-Malefyt,R., Hannum,C., Bazan,J.F., and Kastelein,R.A. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. *13*, 715-725.

Osna,N., Elliott,K., and Khan,M.M. (2001). Regulation of interleukin-10 secretion by histamine in TH2 cells and splenocytes. Int. Immunopharmacol. *1*, 85-96.

Ota,M.O., van der Sande,M.A., Walraven,G.E., Jeffries,D., Nyan,O.A., Marchant,A., and McAdam,K.P. (2003). Absence of association between delayed type hypersensitivity to tuberculin and atopy in children in The Gambia. Clin. Exp. Allergy *33*, 731-736.

Ownby,D.R., Johnson,C.C., and Peterson,E.L. (2002). Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. JAMA *288*, 963-972.

Ozdemir,C., Akkoc,T., Bahceciler,N.N., Kucukercan,D., Barlan,I.B., and Basaran,M.M. (2003). Impact of Mycobacterium vaccae immunization on lung histopathology in a murine model of chronic asthma. Clin. Exp. Allergy *33*, 266-270.

Ozinsky,A., Smith,K.D., Hume,D., and Underhill,D.M. (2000). Co-operative induction of pro-inflammatory signaling by Toll-like receptors. J. Endotoxin. Res. *6*, 393-396.

Panina-Bordignon,P., Papi,A., Mariani,M., Di,L.P., Casoni,G., Bellettato,C., Buonsanti,C., Miotto,D., Mapp,C., Villa,A., Arrigoni,G., Fabbri,L.M., and Sinigaglia,F. (2001). The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. J. Clin. Invest *107*, 1357-1364.

Park,S.H., Benlagha,K., Lee,D., Balish,E., and Bendelac,A. (2000). Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. Eur. J. Immunol. *30*, 620-625.

Pasare,C. and Medzhitov,R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science *299*, 1033-1036.

Pasare,C. and Medzhitov,R. (2005a). Control of B-cell responses by Toll-like receptors. Nature *438*, 364-368.

Pasare,C. and Medzhitov,R. (2005b). Toll-like receptors: linking innate and adaptive immunity. Adv. Exp. Med. Biol. *560*, 11-18.

Paul,W.E. and Zhu,J. (2010). How are T(H)2-type immune responses initiated and amplified? Nat. Rev. Immunol. *10*, 225-235.

Pearce,N., Ait-Khaled,N., Beasley,R., Mallol,J., Keil,U., Mitchell,E., and Robertson,C. (2007). Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax *62*, 758-766.

Peat,J.K., Haby,M., Spijker,J., Berry,G., and Woolcock,A.J. (1992). Prevalence of asthma in adults in Busselton, Western Australia. BMJ *305*, 1326-1329.

Pelosi,U., Porcedda,G., Tiddia,F., Tripodi,S., Tozzi,A.E., Panetta,V., Pintor,C., and Matricardi,P.M. (2005). The inverse association of salmonellosis in infancy with allergic rhinoconjunctivitis and asthma at school-age: a longitudinal study. Allergy *60*, 626-630.

Peng,G., Guo,Z., Kiniwa,Y., Voo,K.S., Peng,W., Fu,T., Wang,D.Y., Li,Y., Wang,H.Y., and Wang,R.F. (2005). Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science *309*, 1380-1384.

Pettipher,R., Hansel,T.T., and Armer,R. (2007). Antagonism of the prostaglandin D2 receptors DP1 and CRTH2 as an approach to treat allergic diseases. Nat. Rev. Drug Discov. *6*, 313-325.

Pham-Thi,N., de,B.J., and Leite-de-Moraes,M.C. (2006). Invariant natural killer T cells in bronchial asthma. N. Engl. J. Med. *354*, 2613-2616.

Piliponsky,A.M., Pickholtz,D., Gleich,G.J., and Levi-Schaffer,F. (2001). Human eosinophils induce histamine release from antigen-activated rat peritoneal mast cells: a possible role for mast cells in late-phase allergic reactions. J. Allergy Clin. Immunol. *107*, 993-1000.

Platts-Mills,T., Vaughan,J., Squillace,S., Woodfolk,J., and Sporik,R. (2001). Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet *357*, 752-756.

Porubsky,S., Speak,A.O., Luckow,B., Cerundolo,V., Platt,F.M., and Grone,H.J. (2007). Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. Proc. Natl. Acad. Sci. U. S. A *104*, 5977-5982.

Provoost,S., Maes,T., van Durme,Y.M., Gevaert,P., Bachert,C., Schmidt-Weber,C.B., Brusselle,G.G., Joos,G.F., and Tournoy,K.G. (2009). Decreased FOXP3 protein expression in patients with asthma. Allergy *64*, 1539-1546.

Puddicombe,S.M., Torres-Lozano,C., Richter,A., Bucchieri,F., Lordan,J.L., Howarth,P.H., Vrugt,B., Albers,R., Djukanovic,R., Holgate,S.T., Wilson,S.J., and Davies,D.E. (2003). Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium. Am. J. Respir. Cell Mol. Biol. *28*, 61-68.

Pykalainen,M., Kinos,R., Valkonen,S., Rydman,P., Kilpelainen,M., Laitinen,L.A., Karjalainen,J., Nieminen,M., Hurme,M., Kere,J., Laitinen,T., and Lahesmaa,R. (2005). Association analysis of common variants of STAT6, GATA3, and STAT4 to asthma and high serum IgE phenotypes. J. Allergy Clin. Immunol. *115*, 80-87.

Qiu,Y., Zhu,J., Bandi,V., Guntupalli,K.K., and Jeffery,P.K. (2007). Bronchial mucosal inflammation and upregulation of CXC chemoattractants and receptors in severe exacerbations of asthma. Thorax *62*, 475-482.

Quesniaux,V., Fremond,C., Jacobs,M., Parida,S., Nicolle,D., Yeremeev,V., Bihl,F., Erard,F., Botha,T., Drennan,M., Soler,M.N., Le,B.M., Schnyder,B., and Ryffel,B. (2004). Toll-like receptor pathways in the immune responses to mycobacteria. Microbes. Infect. *6*, 946-959.

Quinnell,R.J., Pritchard,D.I., Raiko,A., Brown,A.P., and Shaw,M.A. (2004). Immune responses in human necatoriasis: association between interleukin-5 responses and resistance to reinfection. J. Infect. Dis. *190*, 430-438.

Radulovic,S., Jacobson,M.R., Durham,S.R., and Nouri-Aria,K.T. (2008). Grass pollen immunotherapy induces Foxp3-expressing CD4+ CD25+ cells in the nasal mucosa. J. Allergy Clin. Immunol. *121*, 1467-72, 1472.

Raghuraman,G., Geng,Y., and Wang,C.R. (2006). IFN-beta-mediated up-regulation of CD1d in bacteria-infected APCs. J. Immunol. *177*, 7841-7848.

Rank,M.A., Kobayashi,T., Kozaki,H., Bartemes,K.R., Squillace,D.L., and Kita,H. (2009). IL-33-activated dendritic cells induce an atypical TH2-type response. J. Allergy Clin. Immunol. *123*, 1047-1054.

Robertson,C.F., Heycock,E., Bishop,J., Nolan,T., Olinsky,A., and Phelan,P.D. (1991). Prevalence of asthma in Melbourne schoolchildren: changes over 26 years. BMJ *302*, 1116-1118.

Robinson,D.S. (2009). Regulatory T cells and asthma. Clin. Exp. Allergy *39*, 1314-1323.

Rosenberg,H.F., Phipps,S., and Foster,P.S. (2007). Eosinophil trafficking in allergy and asthma. J. Allergy Clin. Immunol. *119*, 1303-1310.

Rosenwasser,L.J., Klemm,D.J., Dresback,J.K., Inamura,H., Mascali,J.J., Klinnert,M., and Borish,L. (1995). Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin. Exp. Allergy *25 Suppl 2*, 74-78.

Rothenberg,M.E. (1998). Eosinophilia. N. Engl. J. Med. *338*, 1592-1600.

Ruprecht,C.R. and Lanzavecchia,A. (2006). Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur. J. Immunol. *36*, 810-816.

Sakaguchi,S. and Powrie,F. (2007). Emerging challenges in regulatory T cell function and biology. Science *317*, 627-629.

Salaun,B., Coste,I., Rissoan,M.C., Lebecque,S.J., and Renno,T. (2006). TLR3 can directly trigger apoptosis in human cancer cells. J. Immunol. *176*, 4894-4901.

Sanders,S.P., Proud,D., Permutt,S., Siekierski,E.S., Yachechko,R., and Liu,M.C. (2004). Role of nasal nitric oxide in the resolution of experimental rhinovirus infection. J. Allergy Clin. Immunol. *113*, 697-702.

Sandor,F. and Buc,M. (2005a). Toll-like receptors. I. Structure, function and their ligands. Folia Biol. (Praha) *51*, 148-157.

Sandor,F. and Buc,M. (2005b). Toll-like receptors. II. Distribution and pathways involved in TLR signalling. Folia Biol. (Praha) *51*, 188-197.

Sandor,F. and Buc,M. (2005c). Toll-like receptors. III. Biological significance and impact for human medicine. Folia Biol. (Praha) *51*, 198-203.

Sayers,I., Barton,S., Rorke,S., Beghe,B., Hayward,B., Van,E.P., Keith,T., Clough,J.B., Ye,S., Holloway,J.W., Sampson,A.P., and Holgate,S.T. (2003). Allelic association and functional studies of promoter polymorphism in the leukotriene C4 synthase gene (LTC4S) in asthma. Thorax *58*, 417-424.

Schedel,M., Carr,D., Klopp,N., Woitsch,B., Illig,T., Stachel,D., Schmid,I., Fritzsch,C., Weiland,S.K., von,M.E., and Kabesch,M. (2004). A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels. J. Allergy Clin. Immunol. *114*, 1100-1105.

Schildgen,O., Geikowski,T., Glatzel,T., Simon,A., Wilkesmann,A., Roggendorf,M., Viazov,S., and Matz,B. (2004). New variant of the human metapneumovirus (HMPV) associated with an acute and severe exacerbation of asthma bronchiale. J. Clin. Virol. *31*, 283-288.

Schmitz,J., Owyang,A., Oldham,E., Song,Y., Murphy,E., McClanahan,T.K., Zurawski,G., Moshrefi,M., Qin,J., Li,X., Gorman,D.M., Bazan,J.F., and Kastelein,R.A. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. *23*, 479-490.

Schneider,E., Machavoine,F., Pleau,J.M., Bertron,A.F., Thurmond,R.L., Ohtsu,H., Watanabe,T., Schinkel,A.H., and Dy,M. (2005). Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. J. Exp. Med. *202*, 387-393.

Schneider,E., Petit-Bertron,A.F., Bricard,R., Levasseur,M., Ramadan,A., Girard,J.P., Herbelin,A., and Dy,M. (2009a). IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. J. Immunol. *183*, 3591-3597.

Schneider,E., Petit-Bertron,A.F., Bricard,R., Levasseur,M., Ramadan,A., Girard,J.P., Herbelin,A., and Dy,M. (2009b). IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. J. Immunol. *183*, 3591-3597.

Schneider,E., Tonanny,M.B., Lisbonne,M., Leite-de-Moraes,M., and Dy,M. (2004). Pro-Th1 cytokines promote Fas-dependent apoptosis of immature peripheral basophils. J. Immunol. *172*, 5262-5268.

Schnyder-Candrian,S., Togbe,D., Couillin,I., Mercier,I., Brombacher,F., Quesniaux,V., Fossiez,F., Ryffel,B., and Schnyder,B. (2006). Interleukin-17 is a negative regulator of established allergic asthma. J. Exp. Med. *203*, 2715-2725.

Schroth,M.K., Grimm,E., Frindt,P., Galagan,D.M., Konno,S.I., Love,R., and Gern,J.E. (1999). Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. *20*, 1220-1228.

Schulz,O., Sewell,H.F., and Shakib,F. (1998). Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. J. Exp. Med. *187*, 271-275.

Sears,M.R., Greene,J.M., Willan,A.R., Wiecek,E.M., Taylor,D.R., Flannery,E.M., Cowan,J.O., Herbison,G.P., Silva,P.A., and Poulton,R. (2003). A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N. Engl. J. Med. *349*, 1414-1422.

Sehmi,R., Dorman,S., Baatjes,A., Watson,R., Foley,R., Ying,S., Robinson,D.S., Kay,A.B., O'Byrne,P.M., and Denburg,J.A. (2003). Allergen-induced fluctuation in CC chemokine receptor 3 expression on bone marrow CD34+ cells from asthmatic subjects: significance for mobilization of haemopoietic progenitor cells in allergic inflammation. Immunology *109*, 536-546.

Shigeoka,A.A., Kambo,A., Mathison,J.C., King,A.J., Hall,W.F., da Silva,C.J., Ulevitch,R.J., and McKay,D.B. (2010). Nod1 and nod2 are expressed in human and murine renal tubular epithelial cells and participate in renal ischemia reperfusion injury. J. Immunol. *184*, 2297-2304.

Shin,H.D., Park,B.L., Jung,J.H., Wang,H.J., Park,H.S., Choi,B.W., Hong,S.J., Lee,Y.M., Kim,Y.H., and Park,C.S. (2003). Association of thromboxane A2 receptor (TBXA2R) with atopy and asthma. J. Allergy Clin. Immunol. *112*, 454-457.

Shirakawa,T., Enomoto,T., Shimazu,S., and Hopkin,J.M. (1997). The inverse association between tuberculin responses and atopic disorder. Science *275*, 77-79.

Shirakawa,T., Li,A., Dubowitz,M., Dekker,J.W., Shaw,A.E., Faux,J.A., Ra,C., Cookson,W.O., and Hopkin,J.M. (1994). Association between atopy and variants of the beta subunit of the high-affinity immunoglobulin E receptor. Nat. Genet. *7*, 125-129.

Silverman,E.S., Palmer,L.J., Subramaniam,V., Hallock,A., Mathew,S., Vallone,J., Faffe,D.S., Shikanai,T., Raby,B.A., Weiss,S.T., and Shore,S.A. (2004). Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am. J. Respir. Crit Care Med. *169*, 214-219.

Simpson,A., John,S.L., Jury,F., Niven,R., Woodcock,A., Ollier,W.E., and Custovic,A. (2006). Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. Am. J. Respir. Crit Care Med. *174*, 386-392.

Simpson,A. and Martinez,F.D. (2010). The role of lipopolysaccharide in the development of atopy in humans. Clin. Exp. Allergy *40*, 209-223.

Singh,S. and Evans,T.W. (1997). Nitric oxide, the biological mediator of the decade: fact or fiction?. Eur. Respir. J. *10*, 699-707.

Sirois,J., Menard,G., Moses,A.S., and Bissonnette,E.Y. (2000). Importance of histamine in the cytokine network in the lung through H2 and H3 receptors: stimulation of IL-10 production. J. Immunol. *164*, 2964-2970.

Skold,M., Xiong,X., Illarionov,P.A., Besra,G.S., and Behar,S.M. (2005). Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. J. Immunol. *175*, 3584-3593.

Smithgall,M.D., Comeau,M.R., Yoon,B.R., Kaufman,D., Armitage,R., and Smith,D.E. (2008). IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. Int. Immunol. *20*, 1019-1030.

Sokol,C.L., Barton,G.M., Farr,A.G., and Medzhitov,R. (2008). A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat. Immunol. *9*, 310-318.

Sporik,R., Holgate,S.T., Platts-Mills,T.A., and Cogswell,J.J. (1990). Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study. N. Engl. J. Med. *323*, 502-507.

Sriram,V., Du,W., Gervay-Hague,J., and Brutkiewicz,R.R. (2005). Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. Eur. J. Immunol. *35*, 1692-1701.

Stein,R.T., Sherrill,D., Morgan,W.J., Holberg,C.J., Halonen,M., Taussig,L.M., Wright,A.L., and Martinez,F.D. (1999). Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet *354*, 541-545.

Stock,P., Lombardi,V., Kohlrautz,V., and Akbari,O. (2009). Induction of airway hyperreactivity by IL-25 is dependent on a subset of invariant NKT cells expressing IL-17RB. J. Immunol. *182*, 5116-5122.

Strachan,D.P. (1989). Hay fever, hygiene, and household size. BMJ *299*, 1259-1260.

Suarez,C.J., Parker,N.J., and Finn,P.W. (2008). Innate immune mechanism in allergic asthma. Curr. Allergy Asthma Rep. *8*, 451-459.

Sutmuller,R.P., den Brok,M.H., Kramer,M., Bennink,E.J., Toonen,L.W., Kullberg,B.J., Joosten,L.A., Akira,S., Netea,M.G., and Adema,G.J. (2006). Toll-like receptor 2 controls expansion and function of regulatory T cells. J. Clin. Invest *116*, 485-494.

Tantisira,K., Klimecki,W.T., Lazarus,R., Palmer,L.J., Raby,B.A., Kwiatkowski,D.J., Silverman,E., Vercelli,D., Martinez,F.D., and Weiss,S.T. (2004a). Toll-like receptor 6 gene (TLR6): single-nucleotide polymorphism frequencies and preliminary association with the diagnosis of asthma. Genes Immun. *5*, 343-346.

Tantisira,K.G., Hwang,E.S., Raby,B.A., Silverman,E.S., Lake,S.L., Richter,B.G., Peng,S.L., Drazen,J.M., Glimcher,L.H., and Weiss,S.T. (2004b). TBX21: a functional variant predicts improvement in asthma with the use of inhaled corticosteroids. Proc. Natl. Acad. Sci. U. S. A *101*, 18099-18104.

Tantisira,K.G., Lake,S., Silverman,E.S., Palmer,L.J., Lazarus,R., Silverman,E.K., Liggett,S.B., Gelfand,E.W., Rosenwasser,L.J., Richter,B., Israel,E., Wechsler,M., Gabriel,S., Altshuler,D., Lander,E., Drazen,J., and Weiss,S.T. (2004c). Corticosteroid pharmacogenetics: association of sequence variants in CRHR1 with improved lung function in asthmatics treated with inhaled corticosteroids. Hum. Mol. Genet. *13*, 1353-1359.

Taube,C., Wei,X., Swasey,C.H., Joetham,A., Zarini,S., Lively,T., Takeda,K., Loader,J., Miyahara,N., Kodama,T., Shultz,L.D., Donaldson,D.D., Hamelmann,E.H., Dakhama,A., and Gelfand,E.W. (2004).

Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. J. Immunol. *172*, 6398-6406.

Taylor-Robinson,A.W., Liew,F.Y., Severn,A., Xu,D., McSorley,S.J., Garside,P., Padron,J., and Phillips,R.S. (1994). Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. Eur. J. Immunol. *24*, 980-984.

Teran,L.M. (2000). CCL chemokines and asthma. Immunol. Today *21*, 235-242.

Tewfik,M.A., Bosse,Y., Lemire,M., Hudson,T.J., Vallee-Smejda,S., Al-Shemari,H., Laprise,C., and Desrosiers,M. (2009). Polymorphisms in interleukin-1 receptor-associated kinase 4 are associated with total serum IgE. Allergy *64*, 746-753.

Thomas,S.Y., Hou,R., Boyson,J.E., Means,T.K., Hess,C., Olson,D.P., Strominger,J.L., Brenner,M.B., Gumperz,J.E., Wilson,S.B., and Luster,A.D. (2003). CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. J. Immunol. *171*, 2571-2580.

Ting,J.P., Lovering,R.C., Alnemri,E.S., Bertin,J., Boss,J.M., Davis,B.K., Flavell,R.A., Girardin,S.E., Godzik,A., Harton,J.A., Hoffman,H.M., Hugot,J.P., Inohara,N., Mackenzie,A., Maltais,L.J., Nunez,G., Ogura,Y., Otten,L.A., Philpott,D., Reed,J.C., Reith,W., Schreiber,S., Steimle,V., and Ward,P.A. (2008). The NLR gene family: a standard nomenclature. Immunity. *28*, 285-287.

Toelle,B.G., Ng,K., Belousova,E., Salome,C.M., Peat,J.K., and Marks,G.B. (2004). Prevalence of asthma and allergy in schoolchildren in Belmont, Australia: three cross sectional surveys over 20 years. BMJ *328*, 386-387.

Tregoning,J.S. and Schwarze,J. (2010). Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin. Microbiol. Rev. *23*, 74-98.

Tukenmez,F., Bahceciler,N.N., Barlan,I.B., and Basaran,M.M. (1999). Effect of pre-immunization by killed Mycobacterium bovis and vaccae on immunoglobulin E response in ovalbumin-sensitized newborn mice. Pediatr. Allergy Immunol. *10*, 107-111.

Umetsu,D.T. and DeKruyff,R.H. (2010). Natural killer T cells are important in the pathogenesis of asthma: The many pathways to asthma. J. Allergy Clin. Immunol.

van der Pouw Kraan TC, van,V.A., Boeije,L.C., van Tuyl,S.A., de Groot,E.R., Stapel,S.O., Bakker,A., Verweij,C.L., Aarden,L.A., and van der Zee,J.S. (1999). An IL-13 promoter polymorphism associated with increased risk of allergic asthma. Genes Immun. *1*, 61-65.

Vanhoutte,F., Paget,C., Breuilh,L., Fontaine,J., Vendeville,C., Goriely,S., Ryffel,B., Faveeuw,C., and Trottein,F. (2008). Toll-like receptor (TLR)2 and TLR3 synergy and cross-inhibition in murine myeloid dendritic cells. Immunol. Lett. *116*, 86-94.

Varadaradjalou,S., Feger,F., Thieblemont,N., Hamouda,N.B., Pleau,J.M., Dy,M., and Arock,M. (2003). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur. J. Immunol. *33*, 899-906.

Vasilakos,J.P., Smith,R.M., Gibson,S.J., Lindh,J.M., Pederson,L.K., Reiter,M.J., Smith,M.H., and Tomai,M.A. (2000). Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. Cell Immunol. *204*, 64-74.

Vercelli,D. (2008). Discovering susceptibility genes for asthma and allergy. Nat. Rev. Immunol. *8*, 169-182.

Vladich,F.D., Brazille,S.M., Stern,D., Peck,M.L., Ghittoni,R., and Vercelli,D. (2005). IL-13 R130Q, a common variant associated with allergy and asthma, enhances effector mechanisms essential for human allergic inflammation. J. Clin. Invest *115*, 747-754.

Von Ehrenstein,O.S., von,M.E., Illi,S., Baumann,L., Bohm,O., and von,K.R. (2000). Reduced risk of hay fever and asthma among children of farmers. Clin. Exp. Allergy *30*, 187-193.

Wahn,U., Lau,S., Bergmann,R., Kulig,M., Forster,J., Bergmann,K., Bauer,C.P., and Guggenmoos-Holzmann,I. (1997). Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J. Allergy Clin. Immunol. *99*, 763-769.

Walley,A.J., Chavanas,S., Moffatt,M.F., Esnouf,R.M., Ubhi,B., Lawrence,R., Wong,K., Abecasis,G.R., Jones,E.Y., Harper,J.I., Hovnanian,A., and Cookson,W.O. (2001). Gene polymorphism in Netherton and common atopic disease. Nat. Genet. *29*, 175-178.

Wang,C., Deng,L., Hong,M., Akkaraju,G.R., Inoue,J., and Chen,Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346-351.

Wang,C.C. and Rook,G.A. (1998). Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed Mycobacterium vaccae. Immunology *93*, 307-313.

Wang,H., Su,Z., and Schwarze,J. (2009). Healthy but not RSV-infected lung epithelial cells profoundly inhibit T cell activation. Thorax *64*, 283-290.

Wang,T., Town,T., Alexopoulou,L., Anderson,J.F., Fikrig,E., and Flavell,R.A. (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat. Med. *10*, 1366-1373.

Wang,X., Moser,C., Louboutin,J.P., Lysenko,E.S., Weiner,D.J., Weiser,J.N., and Wilson,J.M. (2002). Toll-like receptor 4 mediates innate immune responses to Haemophilus influenzae infection in mouse lung. J. Immunol. *168*, 810-815.

Wang,Y.H., Ito,T., Wang,Y.H., Homey,B., Watanabe,N., Martin,R., Barnes,C.J., McIntyre,B.W., Gilliet,M., Kumar,R., Yao,Z., and Liu,Y.J. (2006). Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. Immunity. *24*, 827-838.

Ward,J.K., Belvisi,M.G., Fox,A.J., Miura,M., Tadjkarimi,S., Yacoub,M.H., and Barnes,P.J. (1993). Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. J. Clin. Invest *92*, 736-742.

Wei,J., Duramad,O., Perng,O.A., Reiner,S.L., Liu,Y.J., and Qin,F.X. (2007). Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc. Natl. Acad. Sci. U. S. A *104*, 18169-18174.

Wenzel,S.E., Balzar,S., Cundall,M., and Chu,H.W. (2003). Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J. Allergy Clin. Immunol. *111*, 1345-1352.

Werner,M., Topp,R., Wimmer,K., Richter,K., Bischof,W., Wjst,M., and Heinrich,J. (2003). TLR4 gene variants modify endotoxin effects on asthma. J. Allergy Clin. Immunol. *112*, 323-330.

Weskamp,G., Ford,J.W., Sturgill,J., Martin,S., Docherty,A.J., Swendeman,S., Broadway,N., Hartmann,D., Saftig,P., Umland,S., Sehara-Fujisawa,A., Black,R.A., Ludwig,A., Becherer,J.D., Conrad,D.H., and Blobel,C.P. (2006). ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. Nat. Immunol. *7*, 1293-1298.

WHO/NHLBI workshop report (2009). Global strategy for asthma management and prevention. Available from URL: **http://www.ginasthma.com**.

Williams,L.K., McPhee,R.A., Ownby,D.R., Peterson,E.L., James,M., Zoratti,E.M., and Johnson,C.C. (2006). Gene-environment interactions with CD14 C-260T and their relationship to total serum IgE levels in adults. J. Allergy Clin. Immunol. *118*, 851-857.

Williams,L.K., Oliver,J., Peterson,E.L., Bobbitt,K.R., McCabe,M.J., Jr., Smolarek,D., Havstad,S.L., Wegienka,G., Burchard,E.G., Ownby,D.R., and Johnson,C.C. (2008). Gene-environment interactions between CD14 C-260T and endotoxin exposure on Foxp3+ and Foxp3- CD4+ lymphocyte numbers and total serum IgE levels in early childhood. Ann. Allergy Asthma Immunol. *100*, 128-136.

Williams,T.J. and Jose,P.J. (2000). Role of eotaxin and related CC chemokines in allergy and asthma. Chem. Immunol. *78*, 166-177.

Wills-Karp,M., Luyimbazi,J., Xu,X., Schofield,B., Neben,T.Y., Karp,C.L., and Donaldson,D.D. (1998). Interleukin-13: central mediator of allergic asthma. Science *282*, 2258-2261.

Wjst,M., Fischer,G., Immervoll,T., Jung,M., Saar,K., Rueschendorf,F., Reis,A., Ulbrecht,M., Gomolka,M., Weiss,E.H., Jaeger,L., Nickel,R., Richter,K., Kjellman,N.I., Griese,M., von,B.A., Gappa,M., Riedel,F., Boehle,M., van,K.S., Schoberth,P., Szczepanski,R., Dorsch,W., Silbermann,M., Wichmann,H.E., and . (1999). A genome-wide search for linkage to asthma. German Asthma Genetics Group. Genomics *58*, 1-8.

Woltmann,G., McNulty,C.A., Dewson,G., Symon,F.A., and Wardlaw,A.J. (2000). Interleukin-13 induces PSGL-1/P-selectin-dependent adhesion of eosinophils, but not neutrophils, to human umbilical vein endothelial cells under flow. Blood *95*, 3146-3152.

Wong,C.K., Ho,C.Y., Ko,F.W., Chan,C.H., Ho,A.S., Hui,D.S., and Lam,C.W. (2001). Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin. Exp. Immunol. *125*, 177-183.

Wynn,T.A. (2009). Basophils trump dendritic cells as APCs for T(H)2 responses. Nat. Immunol. *10*, 679-681.

Yamamoto,M., Sato,S., Hemmi,H., Hoshino,K., Kaisho,T., Sanjo,H., Takeuchi,O., Sugiyama,M., Okabe,M., Takeda,K., and Akira,S. (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yamamoto,M., Sato,S., Hemmi,H., Uematsu,S., Hoshino,K., Kaisho,T., Takeuchi,O., Takeda,K., and Akira,S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. *4*, 1144-1150.

Yang,D., Chen,Q., Su,S.B., Zhang,P., Kurosaka,K., Caspi,R.R., Michalek,S.M., Rosenberg,H.F., Zhang,N., and Oppenheim,J.J. (2008). Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses. J. Exp. Med. *205*, 79-90.

Yang,Y., Huang,C.T., Huang,X., and Pardoll,D.M. (2004). Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. Nat. Immunol. *5*, 508-515.

Yates,D.H., Kharitonov,S.A., Robbins,R.A., Thomas,P.S., and Barnes,P.J. (1995). Effect of a nitric oxide synthase inhibitor and a glucocorticosteroid on exhaled nitric oxide. Am. J. Respir. Crit Care Med. *152*, 892-896.

Yazdanbakhsh,M. and Matricardi,P.M. (2004). Parasites and the hygiene hypothesis: regulating the immune system?. Clin. Rev. Allergy Immunol. *26*, 15-24.

Ying,S., Meng,Q., Corrigan,C.J., and Lee,T.H. (2006a). Lack of filaggrin expression in the human bronchial mucosa. J. Allergy Clin. Immunol. *118*, 1386-1388.

Ying,S., Zhang,G., Gu,S., and Zhao,J. (2006b). How much do we know about atopic asthma: where are we now?. Cell Mol. Immunol. *3*, 321-332.

Yoneyama,M. and Fujita,T. (2004). [RIG-I: critical regulator for virus-induced innate immunity]. Tanpakushitsu Kakusan Koso *49*, 2571-2578.

Yoneyama,M., Kikuchi,M., Natsukawa,T., Shinobu,N., Imaizumi,T., Miyagishi,M., Taira,K., Akira,S., and Fujita,T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. *5*, 730-737.

Yu,M., Tsai,M., Tam,S.Y., Jones,C., Zehnder,J., and Galli,S.J. (2006). Mast cells can promote the development of multiple features of chronic asthma in mice. J. Clin. Invest *116*, 1633-1641.

Zambelli-Weiner,A., Ehrlich,E., Stockton,M.L., Grant,A.V., Zhang,S., Levett,P.N., Beaty,T.H., and Barnes,K.C. (2005). Evaluation of the CD14/-260 polymorphism and house dust endotoxin exposure in the Barbados Asthma Genetics Study. J. Allergy Clin. Immunol. *115*, 1203-1209.

Zanin-Zhorov,A., Cahalon,L., Tal,G., Margalit,R., Lider,O., and Cohen,I.R. (2006). Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. J. Clin. Invest *116*, 2022-2032.

Zhang,D., Zhang,G., Hayden,M.S., Greenblatt,M.B., Bussey,C., Flavell,R.A., and Ghosh,S. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. Science *303*, 1522-1526.

Zhang,Y., Rogers,K.H., and Lewis,D.B. (1996). Beta 2-microglobulin-dependent T cells are dispensable for allergen-induced T helper 2 responses. J. Exp. Med. *184*, 1507-1512.

Zhou,D., Mattner,J., Cantu,C., III, Schrantz,N., Yin,N., Gao,Y., Sagiv,Y., Hudspeth,K., Wu,Y.P., Yamashita,T., Teneberg,S., Wang,D., Proia,R.L., Levery,S.B., Savage,P.B., Teyton,L., and Bendelac,A. (2004). Lysosomal glycosphingolipid recognition by NKT cells. Science *306*, 1786-1789.

Zhou,L., Lopes,J.E., Chong,M.M., Ivanov,I.I., Min,R., Victora,G.D., Shen,Y., Du,J., Rubtsov,Y.P., Rudensky,A.Y., Ziegler,S.F., and Littman,D.R. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature *453*, 236-240.

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Ginger prevents Th2-mediated immune responses in a mouse model of airway inflammation

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ABSTRACT

It is well documented that compounds from rhizomes of *Zingiber officinale*, commonly called ginger, have anti-inflammatory properties. Here, we show that ginger can exert such functions *in vivo*, namely in a mouse model of Th2-mediated pulmonary inflammation. The preparation of ginger aqueous extract (Zo.Aq) was characterized by mass spectrometry as an enriched fraction of *n*-gingerols. Intraperitoneal injections of this extract before airway challenge of ovalbumin (OVA)-sensitized mice resulted in a marked decrease in the recruitment of eosinophils to the lungs as attested by cell counts in bronchoalveolar lavage (BAL) fluids and histological examination. Resolution of airway inflammation induced by Zo.Aq was accompanied by a suppression of the Th2 cell-driven response to allergen *in vivo*. Thus, IL-4, IL-5 and eotaxin levels in the lungs as well as specific IgE titres in serum were clearly diminished in ginger-treated mice relative to their controls after allergen sensitization and challenge. Finally, we found that [6]-gingerol, a major constituent of ginger, was sufficient to suppress eosinophilia in our model of inflammation. This is the first evidence that ginger can suppress Th2-mediated immune responses and might thus provide a possible therapeutic application in allergic asthma.

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1. Introduction

Ginger (or Zo for *Zingiber officinale* Roscoe, Zingiberaceae) has a long history of medical use [1,2]. As a dietary supplement or herbal medicinal product, it is widely used to treat pregnancy-related nausea [3–5] as well as others forms of nausea such as motion sickness [6]. Ginger has also been valued for centuries for its anti-inflammatory properties. Thus, in traditional medicine, ginger has been used to treat a wide range of ailments including stomachaches, diarrhoea, toothache, gingivitis arthritis and asthmatic respiratory disorders [7]. During the past 35 years, many laboratories have renewed their interest in ginger as a treatment of chronic inflammatory conditions. Ginger was found to inhibit arachidonic acid metabolism via the cyclooxygenase and lipooxygenase pathways [8,9]. More recently, research in this field entered a new phase with the discovery that ginger inhibited the induction of genes encoding cytokines and chemokines that are synthesized and secreted at sites of inflammation [10–12].

Asthma is characterized by a chronic inflammatory reaction in the airways. Numerous humoral and cellular immunological abnormalities have been reported in this disease [13–16]. It is well known that T-helper (Th)-lymphocytes play a key role in the regulation of immune and inflammatory reactions through the release of cytokines [17–19]. Th-lymphocytes can be divided into at least two subsets based on their cytokine secretion profiles. Th1-lymphocytes are characterized by the production of predominantly interleukin (IL)-2, interferon (IFN)-γ and tumour-necrosis factor (TNF)-α whereas Th2-lymphocytes are characterized by the production of predominantly IL-4, IL-5 and IL-6 that favour the synthesis of IgE. It is now well recognized that asthma is caused by Th2-driven inflammatory responses, which enhances airway eosinophilia and mucus production in the lungs [20].

Ginger is well known for its ability to inhibit the synthesis of several pro-inflammatory cytokines including IL-1, TNF-α and IL-8 by variable types of cells *in vitro* [21], and to affect Th1-derived responses *in vivo* [22]. However, whether ginger can also affect Th2-derived inflammatory responses *in vivo* has not been evaluated so far. Here, we provide the first evidence that ginger can suppress Th2-mediated immune responses in a mouse model of pulmonary inflammation.

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2. Materials and methods

2.1. Sample preparations of ginger

The rhizoma of *Zo* were collected from the market of Abidjan (Ivory Coast) and dried at 70 °C. The plant was authenticated at the National herbarium in Abidjan (CNF) where a voucher specimen was deposited. It was ground into a fine powder using a pestle and mortar and the powder was soaked in distilled water for 24 h at room temperature. The resulting solution was filtered and evaporated to remove excess water and the concentrated solution was freeze dried. From a 50 g sample of dried rhizoma, 5 g of solid materiel was obtained with a yield of 10%. Extract solutions were stored at 4 °C until use. The solid material was subsequently reconstituted in a known volume of distilled water and then serially diluted. In order to prepare the methanolic (MeOH) total extract, the powder of *Zo* (30 g) was refluxed in methanol (600 ml) in a Soxhlet apparatus for 2 days. MeOH was evaporated under reduced pressure, to give a brown extract (yield: 11%). The material was subsequently reconstituted in a known volume of sunflower oil. [6]-gingerol was purchased from VWR international company (Fontenay-sous-bois, France) and was reconstituted in distilled PBS 1X containing MeOH 1%. The preparation was sonicated just before use.

2.2. HPLC/ESI-MS/MS analysis of ginger extracts

High performance liquid chromatography–tandem mass spectrometry (HPLC/MS/MS) analysis of aqueous (Zo.Aq) and MeOH total (Zo. Me) extracts was conducted at 30 °C with a C18 Sunfire column (WATERS, 5 µm, 4.6× 150 mm) equipped with a C18 Sunfire pre-column (5 µm), on a HPLC Agilent system 1100 series coupled to an electrospray (ESI)-quadripole-time-of-flight (TOF) spectrometer (PE SCIEX QSTAR PULSAR, PE Sciex Instruments). A gradient of H_2O (A) and CH_3CN (B) was used (1 → 40 min: 20% B → 100% B, 40 → 55 min: 100% B), at a flow rate of 1 ml/min. The acquisition parameters for positive and negative modes were respectively: GS1 (nebulizing gas): 50 psi, GS2 (drying gas): 55 psi, curtain gas: 35 psi, ionspray voltage: 5500 V or −4500 V, DP (declustering potentiel): 30 V or −30 V, FP (focalising potential): 180 V or −180 V, DP2: 20 V or −20 V, CAD (collision gaz pressure): 2 mTorr (N_2). For MS/MS experiments, collision energy was set at 35 eV or −35 eV. Mass range measured was *m/z* 100–800. These were the optimized parameters for the maximum transmission of the gingerol-derived ions. The identity of [6]-gingerol was assessed by co-injection with a commercial standard.

2.3. Quantification of gingerols by HPLC-DAD

To determine the amounts of gingerols injected to animals, -diode array detection (HPLC-DAD) studies of Zo.Aq and MeOH total (Zo.Me) extracts were conducted. For quantifications, a commercial [6]-gingerol standard and the extracts were diluted extemporaneously in MeOH. UV quantification was conducted using a WATERS (St Quentin en Yvelines, France) chromatographic system (717 autosampler, 600 pump, photodiode array detector 996 equipped with an X-ACT in-line degazer, Jour Research) under the same chromatographic conditions as above. Full spectral scanning was performed from 210–400 nm, and DAD was set at 280 nm and 230 nm for analysis. Study was conducted at 280 nm, the limit of quantification being more satisfactory than at 230 nm (10^{-9} g *versus* $2.5.10^{-8}$ g injected). The straight-line regression equation was obtained with 11 dilutions ranging from 0 to 0.075 mg/ml (injections 10 µl) by plotting peak area against concentration, with excellent correlation (R^2 > 0.9997). Precision was tested for three concentrations of the standard corresponding to the low, medium and high ranges of the calibration curve, and was comprised between 0.4 and 2.5% (relative standard deviation).

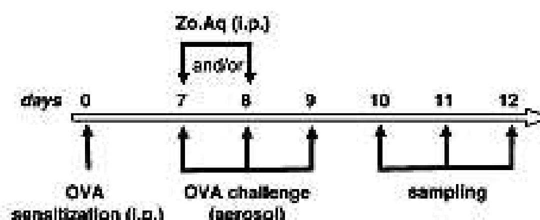

Fig. 1. Schematic diagram illustrating the protocol for OVA sensitization, challenges, and ginger treatment. NOD and C57BL/6 mice were sensitized with OVA and alum (day 0) and challenged by aerosol exposure on days 7, 8 and 9 with OVA or saline. Unless stated, mice were treated on days 7 and 8 1 h before the challenge via i.p. administration of ginger (Zo.Aq) at a dose of 360 mg/kg or PBS, and 1 day after the last challenge, the absolute number of eosinophils in the bronchoalveolar lavage fluid (BALF) was measured. In the same mice, cytokine protein levels were determined in lungs, sera and/or BALF. In a separate set of experiments, lungs were collected for tissue histopathology analysis.

2.4. Animals

NOD mice were bred and maintained in our animal facility under specific pathogen-free conditions whereas C57BL/6 mice were obtained from the Laboratory Animal Center Janvier (CERJ; le Genest-Isle, France). For experiments, 6–8 week old males weighing 20–25 g were used. Housing conditions and all *in vivo* experiments were performed according to the institutional ethical committee of France and the guidelines established by the European Union on Animal Care (CEE Council 86/609).

2.5. Sensitization, airway challenge, and Zo treatment

Mice were sensitized by intraperitonal injection (i.p.) of 100 µg of ovalbumin (OVA, INC Biomedicals, Inc, OH) emulsified in 1.6 mg of alum hydroxide (Merck, Darmstadt, Germany) in a total volume of 400 µl on day 1. Sensitized and naïve (NaCl) mice received aerosolized allergen challenge (50 mg OVA for 20 min) on 3 consecutive days (days 7, 8, and 9) using an ultrasonic nebulizer (Ultra-Neb99, Devilbiss). An hour before the first and/or the second challenge (days 7 and 8), OVA-sensitized mice were injected i.p. with Zo at variable doses comprised between 45 to 720 mg/kg b.w. (Fig. 1).

2.6. Bronchoalveolar lavage (BAL) and lung homogenates

Animals were anesthetized by the intraperitoneal injection of urethane (ethylcarbamate, 2 g/kg b.m., Sigma Aldrich, Stonheim, Germany). After intubation, lungs were lavaged, and BAL fluid was recovered (0.5 ml of PBS; 5 times) through the trachea. Total leukocyte numbers were measured with a hemocytometer using Turk dye exclusion method. Differential cell counts were performed under light microscopy in a blinded manner, counting at least 200 cells according to the standard morphologic criteria on cytocentrifuged preparations (Shandon Cytospin 4, Thermo Electron Corporation) with a fast staining procedure (HAEME-Schnellfärbung, Labor+Technik Eberhard Lehmann, Berlin, Germany). Homogenates were prepared after cannulating the trachea and perfusing the airway. The lungs were then removed and vigorously vortexed. The resulting cell suspension was homogenized and centrifuged for 10 min at 1600 g at 4 °C. Supernatants were removed and stored at −20 °C until cytokine measurement.

2.7. Determination of cytokines and OVA-specific IgE titres by ELISA

The levels of eotaxin, IL-4, IL-5 and IFN-γ in BALF and lung homogenate were assessed by sandwich ELISA (R&D Systems Europe) according to the manufacturer's instructions. The levels of OVA-specific IgE were determined by sandwich ELISA, as previously described [8,9]. Levels of samples were related to an internal standard from pooled sera of hyperimmunized NOD mice. Data were expressed as IgE index

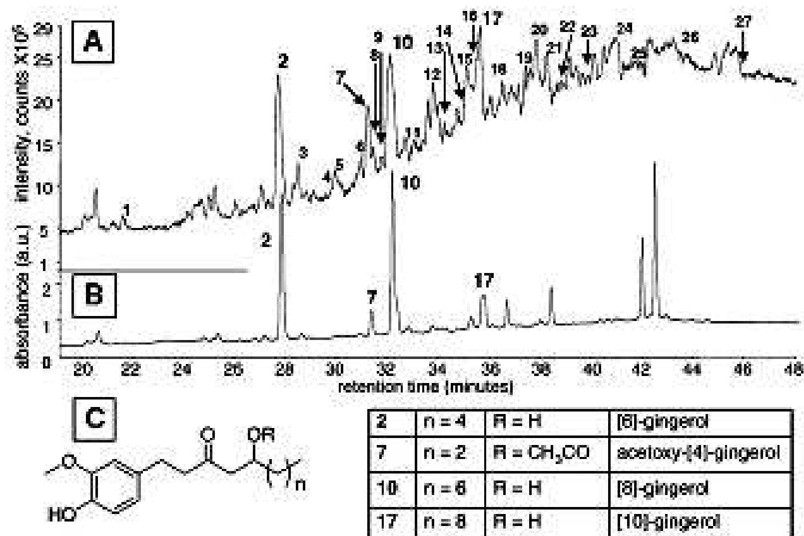

Fig. 2. LC/MS analysis of ginger rhizome extract Zo.Me. (A) Total ion current (TIC) chromatogram from positive ion ESI-HPLC-MS; (B) HPLC-DAD chromatogram set at 230 nm of the crude ginger rhizome extract. (C) Chemical structures of major gingerol-related compounds identified in extracts from ginger rhizome.

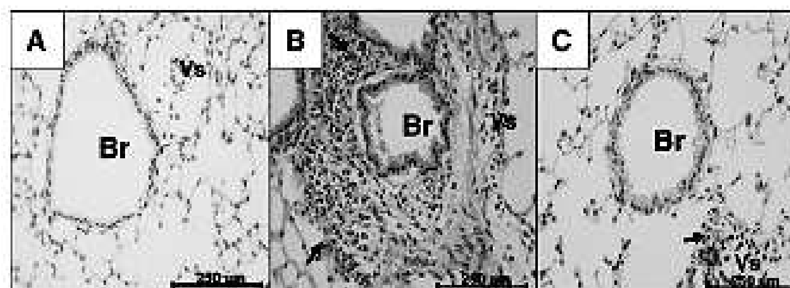

Fig. 3. An aqueous extract of ginger inhibits OVA-induced airway cell infiltration and mucus production in NOD mice. NOD mice were sensitized with OVA and challenged with OVA (B,C) or saline (A) as described in Fig. 1, and received (C) or not an aqueous extract of ginger (Zo.Aq; 360 mg/kg) 1 h before the first and second challenges. One day after the last challenge, lungs were collected, fixed and stained with hematoxylin and eosin (A–C; magnification ×400). Representative sections are shown for each treatment group. Dense perivascular and peribronchiolar infiltrates mainly composed of eosinophils are present in lung of OVA-challenged mice while they are dramatically decreased in lung of OVA-challenged mice treated with Zo.Aq and absent of their saline-challenged controls. Mucin is increased in the bronchiolar lining cells in OVA-challenged mice while it is absent from the epithelium of mice challenged with saline and present at low levels in mice treated with Zo.Aq. Arrows indicate locations of cell infiltration (B,C). Br: bronchiols; Vs: vessels.

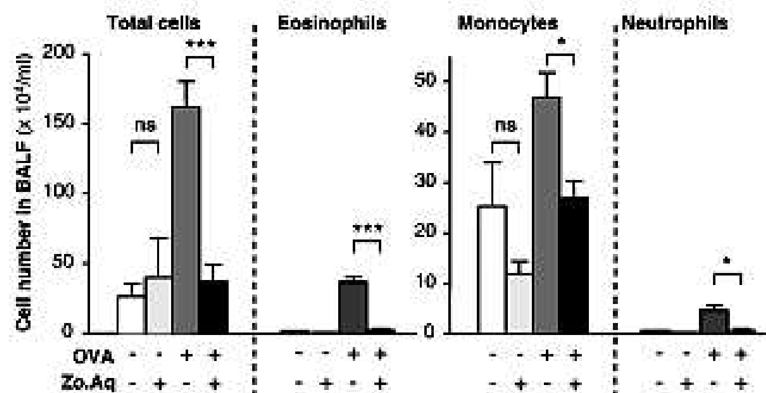

Fig. 4. Zo.Aq inhibits OVA-induced airway eosinophilia in NOD mice. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not Zo.Aq 1 h before the first and second challenges. One day after the last challenge, the absolute number of total cells, eosinophils, monocytes and neutrophils in the BALF was measured. Data shown are means±S.E.M. of 3 to 12 mice per group. *p<0.05; ***p<0.001.

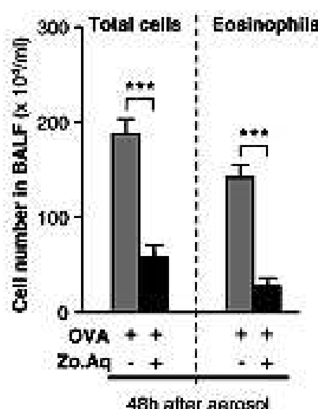

Fig. 5. The inhibitory effect of Zo.Aq on airway inflammation is maintained two days after the last challenge. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not Zo.Aq (360 mg/kg) 1 h before the first and second challenges. One day or two days after the last challenge, the absolute number of total cells and eosinophils in BALF was measured. Data shown are mean±S.E.M. of 3 to 12 mice per group. ***$p<0.001$.

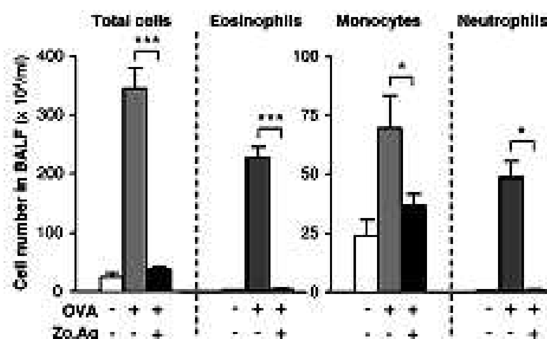

Fig. 7. Inhibition of OVA-induced airway inflammation by Zo.Aq applies to C57BL/6 mice. C57BL/6 mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not Zo.Aq (360 mg/kg) 1 h before the first and the second challenges. One day after the last challenge, the absolute number of total cells, eosinophils, monocytes and neutrophils in the BALF was measured. Data shown are mean±S.E.M. of 3 to 6 mice per group. *$p<0.05$; ***$p<0.001$.

calculated as follows: IgE index=|OD sample−OD buffer only|/|OD positive control−OD buffer only|.

2.8. Determination of lung histology

Lungs were perfused to eliminate residual blood, removed and fixed in paraformaldehyde. Paraffin-embedded tissues were sliced and 5 μm thick paraffin sections were stained with hematoxylin and eosin (H&E) or with periodic acid Schiff (PAS) for determination of cell infiltration and mucus-producing cells. Histological examination was evaluated by light microscopy in a blinded fashion by a histopathologist (D.D.).

2.9. Statistical analysis

Statistical analysis was performed by using the Graph Pad Prism Software (version 3.03) to test whether treatment with ginger extracts leads to a significant reduction in several parameters associated with allergic disease. Results are expressed as means ±standard error of the mean (SEM). Student's *t*-test was used for analysis of significance among the groups. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Characterization of the different constituents in the ginger extracts

Both Zo.Aq and Zo.Me total extracts were analysed by HPLC/DAD (230, 280 nm) and HPLC ESI-MS in positive and negative modes. The study of retention times, UV spectra, molecular ions and fragments [23,24] led to the identification of 27 gingerol-related compounds, such as gingerols, shogaols and paradols, as well as their acetylated and methylated derivatives. The major ones were [6]-gingerol (peak 2), acetoxy-[4]-gingerol (7), [8]-gingerol (10), and [10]-gingerol (17) (Fig. 2). [8]-gingerol and [6]-gingerol appeared to be the major constituents in the Zo.Aq extract (19.2% and 5.8%, w/w, respectively, as determined by HPLC-DAD quantitation). Similar values were found for the Zo.Me total extract. Their concentration in the rhizoma used for this study was thus calculated as being 30.3 and 9.2 mg/kg, respectively.

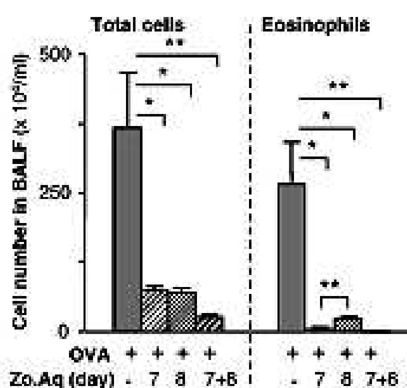

Fig. 6. Single dose of Zo.Aq is sufficient to inhibit airway inflammation in NOD mice. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received Zo.Aq (360 mg/kg) on day 7, day 8, or on days 7+8 1 h before the challenge. One day after the last challenge, the absolute number of total cells and eosinophils in BALF was measured. Data shown are mean±S.E.M. of 3 to 5 mice per group. *$p<0.05$; **$p<0.01$.

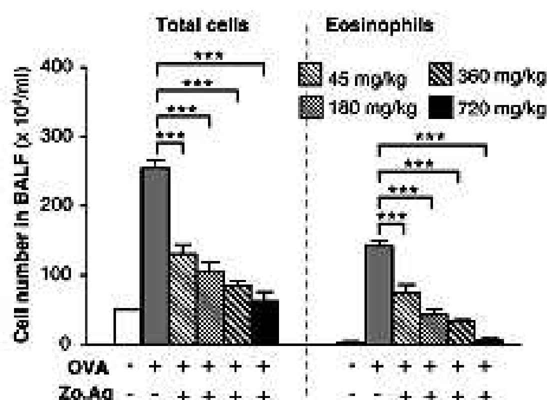

Fig. 8. Inhibition of OVA-induced airway inflammation by Zo.Aq is dose-dependent. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not variable doses (45, 180, 360 and 720 mg/kg) of Zo.Aq on days 7 and 8 1 h before the challenge. One day after the last challenge, the absolute number of total cells and eosinophils in the BALF was measured. Data shown are mean±S.E.M. of 3 to 10 mice per group. ***$p<0.001$.

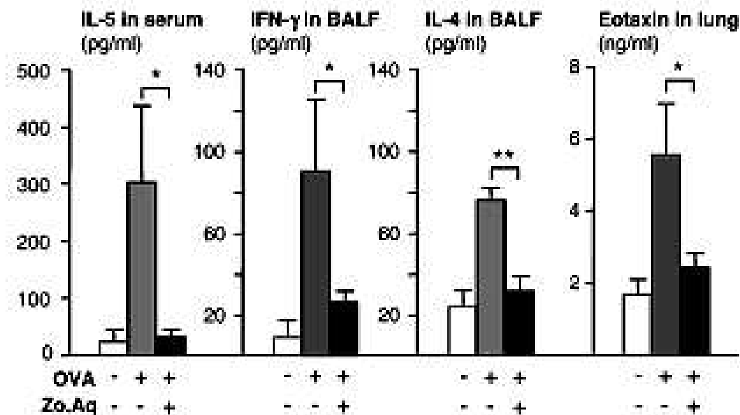

Fig. 9. Zo.Aq inhibits OVA-induced cytokine production in NOD mice. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not Zo.Aq (360 mg/kg) 1 h before the first and second challenges. One day after the last challenge, BALF and lungs were collected. IL-4, IFN-γ, IL-5, and eotaxin protein levels were measured in BALF, serum and lung tissue homogenate supernatants by ELISA. Data shown are mean±S.E.M. of 3 to 12 mice per group. $^{*}p<0.05$; $^{**}p<0.01$.

3.2. Inhibition of airway inflammation in OVA-immunized and -challenged mice treated with aqueous ginger extract

Experimental OVA-dependent allergic asthma is characterized by airway eosinophilia and mucus production [25]. We have previously reported that the NOD mouse is a relevant model of experimental allergic asthma [26]. Thus, after sensitization and aerosol challenges with OVA, NOD mice develop eosinophilic airway inflammation (Fig. 3B versus Fig. 3A; Fig. 4) and intense mucus production (data not shown) in the lungs. These two typical features of Th2-mediated pulmonary inflammation were clearly reduced in NOD mice treated with Zo.Aq used at the dose of 360 mg/kg. Indeed, airway eosinophilia (Fig. 3C) and mucosal cells (data not shown) in the bronchiolar lining cells were virtually absent in OVA-immunized and -challenged animals receiving Zo.Aq 1 h before the first and the second challenges, relative to their OVA-immunized and -challenged controls (Fig. 3A). The effect of Zo.Aq on the recruitment of inflammatory cells into BALF in OVA-challenged mice is shown in Fig. 4. In OVA-immunized and -challenged mice used as positive controls, total cell numbers 1 day after the last challenge were significantly increased. The frequency of inflammatory cells in decreasing order was: eosinophils, monocytes and neutrophils. The administration of Zo.Aq dramatically reduced the number of cells recruited, including eosinophils, monocytes and neutrophils. In a single series of follow-up experiments we have extended observations up to 2 days after the last challenge, again demonstrating a comparable suppressive effect of Zo.Aq on eosinophilic recruitment in BALF (Fig. 5) and in the lungs (data not shown). We next addressed the question whether the inhibitory effect required repeated Zo.Aq exposures. To this end, we compared groups of OVA-sensitized animals that were exposed to single injection of Zo.Aq (1 h before the first or the second challenge) versus two successive injections of Zo.Aq (1 h before the first and the second challenge). Fig. 6 shows that a single Zo.Aq injection was sufficient to reduce markedly the number of eosinophils in BALF, although the two successive injections of the product resulted in a more complete suppressive effect. These data indicate that the inhibitory effect of Zo.Aq is robust and persistent.

Another important point was to examine whether the inhibitory effect of Zo.Aq was dependent on the NOD background or conversely if it applies to other strains susceptible to experimental allergic asthma. To address this issue, we used C57BL/6 mice, a strain that also develop mucus production and eosinophilic inflammation in the lungs after OVA challenge [27,28]. Fig. 7 shows that both total cell and eosinophil counts were dramatically decreased in the BALF of C57BL/6 after OVA challenge and treatment with Zo.Aq (360 mg/kg), relative to controls challenged with OVA.

We next examined whether the inhibition of airway inflammation obtained with Zo.Aq was dose-dependent. As shown in Fig. 8, treatment with increased doses of Zo.Aq comprised between 45 and 720 mg/kg showed that the efficiency of the product in terms of reduction of the number of eosinophils in BALF was closely dependent on the dose used. Statistical analysis indicates that the inhibition effect of Zo.Aq at the doses of 45 mg/kg, 180 mg/kg, 360 mg/kg, and 720 mg/kg are significantly different from control groups. Zo.Aq exhibited anti-inflammatory activity with a half-response value around 45 mg/kg. Altogether, these findings clearly show that treatment with Zo.Aq inhibits both eosinophilia and airway inflammation in OVA-immunized and -challenged mice.

3.3. Production of both Th1 and Th2 cytokines and eotaxin after OVA challenge is affected in Zo.Aq-treated NOD mice

Allergic airway inflammation is accompanied by the generation of IL-4, IL-5 and IL-13 as well as chemokines, such as eotaxin [29]. As shown in Fig. 9, levels of IL-4, eotaxin and IL-5 in BALF, lungs and sera, respectively, were significantly reduced in OVA-immunized and -challenged NOD mice treated with Zo.Aq (360 mg/kg). However, no significant difference was noted for IL-13 in lungs (data not shown). It should be noticed that the pro-Th1 cytokine IFN-γ that we were able to

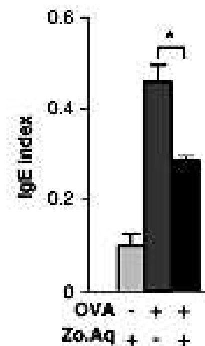

Fig. 10. Zo.Aq inhibits OVA-induced IgE production in NOD mice. NOD mice were sensitized with OVA and challenged with OVA or saline as described in Fig. 1, and received or not Zo.Aq (360 mg/kg) 1 h before the first and second challenges. Three day after the last challenge, blood was collected and anti-OVA-specific IgE were measured in serum by ELISA. Data shown are mean±S.E.M. of 3 to 12 mice per group. $^{*}p<0.05$.

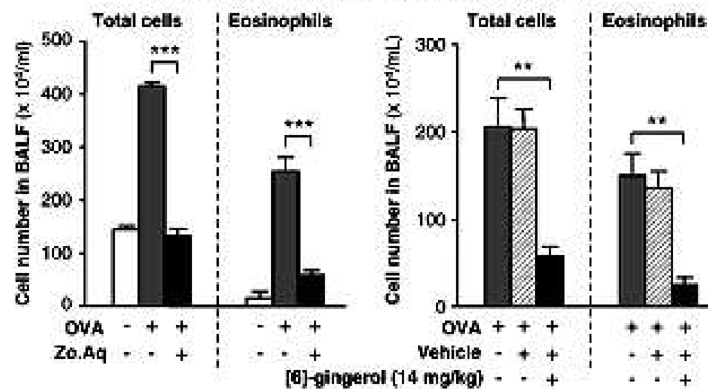

Fig. 11. [6]-gingerol inhibits OVA-induced airway inflammation. NOD mice were sensitized and challenged with OVA or saline as described in Fig. 1, and received or not the aqueous extract (Zo.Aq; 360 mg/kg) or the [6]-gingerol solution (14 mg/kg) only 1 h before the first challenge. One or two days after the last challenge, the absolute number of total cells and eosinophils in the BALF of Zo.Aq- or [6]-gingerol-treated mice was measured. Data shown are mean±S.E.M. of 3 to 6 mice per group. $**p<0.01$; $***p<0.001$.

detect after OVA challenge was also significantly diminished in BALF of Zo.Aq-treated mice.

3.4. *Production of OVA-specific IgE is affected in Zo.Aq-treated NOD mice*

IL-4 is required for Th2 cell differentiation and isotype switching in B cells from IgM to IgE [30,31]. Sensitization and challenge with OVA caused significant increase in OVA-specific IgE in the serum 72 h after the last challenge, which were significantly reduced in mice treated with Zo.Aq (360 mg/kg) (Fig. 10).

3.5. *[6]-Gingerol inhibits OVA-induced airway inflammation*

[6]-Gingerol is a constituent of ginger which is well documented for its anti-inflammatory activities [32]. For this reason, we decided to examine whether [6]-gingerol shares with the Zo.Aq extract the capacity to suppress airway inflammation in our model. This was the case as attested by the capacity of [6]-gingerol at the dose of 14 mg/kg to suppress eosinophilia in BALF when administered 1–3 h before the first and the second challenges (Fig. 11). These findings suggest that the anti-inflammatory effect of ginger in our model might be mediated by [6]-gingerol.

4. Discussion

Ginger, the powdered rhizome of the herb *Zingiber officinale*, is widely used in traditional medicine as a treatment for several diseases, including chronic inflammatory diseases [1]. Although the anti-inflammatory properties of ginger are well documented, only a few studies have evaluated the effects of this natural product on the immune responses *in vivo* [22]. The objective of this study was to evaluate the effects of ginger on airway inflammation *in vivo* in a mouse model of allergen-induced allergic asthma.

Following sensitization, inhalation of OVA induces allergic inflammatory changes characterized by infiltration of inflammatory cells, mainly eosinophils, into regions around the airways and pulmonary blood vessels and by increasing in number of mucus-producing cells within the airways. The main finding in the present study was that these OVA-induced inflammatory changes in lung tissues were suppressed by ginger extracts.

Allergic reactions in the airways are well known to be mediated *via* two pathways. The first pathway involves induction of IgE by B cells *via* IL-4 released from Th2 cells and mast cells [33–37]). In the present study, ginger treatment led to a decreased production of IL-4 in lung tissues of OVA-sensitized and -challenged mice, a phenomenon which was accompanied by a significant reduction of IgE antibody titres in the serum. Thus, the inhibitory effects of ginger on airway inflammation may be due in part to the decrease in IL-4 levels. The other pathway involves recruitment of eosinophils *via* release of IL-5 and eotaxin from Th2 cells or mast cells. Our results showed that the decrease in the number of eosinophils in BAL fluid and lung tissue, and the number of mucus-producing goblet cells in the airway mucosa were concomitant with the decrease in IL-5 and eotaxin. Therefore, from all these data, it can be assumed that the inhibitory effects of ginger extracts on airway inflammation are due to the decrease in Th2 cytokines and eotaxin.

An important point is to know whether the inhibitory effects of ginger apply to other cell types and molecules than those implicated in Th2-mediated inflammation. Although the predominant inflammatory cells recruited into asthmatic lung tissues are eosinophils, neutrophils and macrophages have been also found to be elevated in the BALF and lung tissues of OVA-sensitized and -challenged mice ([38], our data). Here, we showed that ginger inhibits the recruitment of neutrophils and macrophages too. Furthermore, we found reduced levels of the pro-Th1 cytokine IFN-γ in BALF of the sensitized- and challenged-mice treated with ginger. This latter finding is in agreement with previous studies showing that ginger extracts can inhibit the synthesis of several pro-Th1 cytokines [39,40].

In our study, 27 gingerol-related compounds were identified in the aqueous extract, [6] and [8]-gingerol being the principal compounds, as described in the literature [23,24]. It should be mentioned that most of the activities of ginger have been attributed to [6]-gingerol. Indeed, [6]-gingerol has been shown to have antioxidant activities, to promote angiogenesis, and to suppress the production of pro-inflammatory cytokines (TNF-α, IL-1 and IL-12) from macrophages [40]. From our study, two lines of evidence led us to assume that gingerol components are responsible for the inhibition of airway inflammation obtained with our ginger aqueous extracts. Firstly, the Zo.Me total extract which retains the gingerol constituents, shares with the Zo.Aq extract the capacity to suppress airway inflammation in our model (data not shown). Secondly, we found that a purified fraction of [6]-gingerol used alone has the same anti-inflammatory activity than the total extract. Although we cannot exclude that some of the effects of ginger extracts observed here are caused by synergistic action of several chemically homogeneous molecules, our findings suggest that the anti-inflammatory effect of ginger in our model is at least in part mediated by [6]-gingerol.

Ginger's rhizoma is used in several pharmacopoeias (African, Chinese, European and Indian) for his anti-inflammatory activities [41]. It is therefore expected that ginger extracts are particularly safe. Accordingly, neither side effects nor toxicological changes were observed in our hands (data not shown) as well as in several other studies [42].

In our study, ginger was in most cases administered before the induction of airway inflammation with allergen challenge and was effective in preventing its development. However, it should be mentioned that its administration only after the induction of the challenge phase remained efficient to inhibit eosinophilia (see Fig. 6). These results are important and useful for a future exploitation of ginger to develop natural substances not only to prevent allergic asthma but also to alleviate the established disease.

To summarize, our study provides the first evidence that ginger inhibits allergen-induced lung inflammation by reducing airway eosinophilia, Th2 cytokines, and allergen-specific antibodies, thus supporting its anti-inflammatory role during the allergic response in the lung. Ginger is therefore a promising new therapeutic approach for the preventive treatment of allergy.

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References

[1] Afzal M, Al-Hadidi D, Menon M, Pesek MM, Dhami MSI. Ginger: an ethnomedical, chemical and pharmacological review. Drug Metab Drug Interact 2001;18:159–90.

[2] Awang DVC. Ginger. Can Pharmaceut J 1992;125:309–11.

[3] Vutyavanich T, Kraisarin T, Ruangsri RA. Ginger for nausea and vomiting in pregnancy: randomized, double-masked, placebo-controlled trial. Obstet Gynecol 2001;97:577–82.

[4] Boone SA, Shields KM. Treating pregnancy-related nausea and vomiting with ginger. Ann Pharmacother 2005;39:1710–3.

[5] Borrelli F, Capasso R, Aviello G, Pittler MH, Izzo AA. Effectiveness and safety of ginger in the treatment of pregnancy-induced nausea and vomiting. Obstet Gynecol 2005;105:849–56.

[6] Chrubasik S, Pittler MH, Roufogalis BD. Zingiberis rhizoma: a comprehensive review on the effect and efficacy profiles. Phytomedicine 2005;12:684–701.

[7] Dedov VN, Tran VH, Duke CC, Connor M, Christie MJ, Mandadi S, et al. Gingerols: a novel class of vanilloid receptor (VR1) agonists. Br J Pharmacol 2002;137:793–8.

[8] Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol 1971;231:232–5.

[9] Kiuchi F, Shibuya M, Sankawa U. Inhibitors of prostaglandin biosynthesis from ginger. Chem Pharm Bull Tokyo 1982;30:754–7.

[10] Frondoza CG, Frazier C, Polotsky A, Lahiji K, Hungerford DS. TNF-alpha expression of synoviocyte cultures is inhibited by hydroxyl-alkoxy-phenyl compounds (HAPC) from ginger. Trans Orthop Res Soc 2000;46:1103.

[11] Frondoza CG, Sohrabi A, Polotsky A, Phan PV, Hungerford DS, Lindmark L. An in vitro screening assay for inhibitors of proinflammatory mediators in herbal extracts using human synoviocyte cultures. In Vitro Cell Dev Biol Anim 2004;40:95–101.

[12] Phan PV, Sohrabi A, Polotsky A, Hungerford DS, Lindmark L, Frondoza CG. Ginger extract components suppress induction of chemokine expression in human synoviocytes. J Altern Complement Med 2005;11:149–54.

[13] Ray A, Cohn L. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. J Clin Invest 1999;104:985–93.

[14] Viegi G, Annesi I, Matteelli G. Epidemiology of asthma. In: Chung F, Fabbri LM, editors. Asthma, European respiratory monograph no. 23. Sheffield, UK: European Respiratory Journals Ltd.; 2003. p. 1–25.

[15] Busse WW, Lemanske RF. Asthma. N Engl J Med 2001;344:350–62.

[16] Larché M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol 2003;111:450–63.

[17] Barnes PJ. Pathophysiology of asthma. In: Barnes PJ, Rodger IW, Thomson NC, editors. Asthma: basic mechanisms and clinical management. London: Academic Press; 1998. p. 487–506.

[18] Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348–57.

[19] Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17:138–46.

[20] Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–73.

[21] Grzanna R, Lindmark L, Frondoza CG. Ginger—an herbal medicinal product with broad anti-inflammatory actions. J Med Food 2005;8:125–32.

[22] Shen CL, Hong KJ, Kim SW. Comparative effects of ginger root (Zingiber officinale Rosc.) on the production of inflammatory mediators in normal and osteoarthrotic sow chondrocytes. J Med Food 2005;8:149–53.

[23] Jiang H, Solyom AM, Timmermann BN, Gang DR. Characterization of gingerol-related compounds in ginger rhizome (Zingiber officinale Rosc.) by high performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 2005;19:2957–64.

[24] He X, Bernart MW, Lian L, Lin L. High-performance liquid chromatography–electrospray mass spectrometric analysis of pungent constituents of ginger. J Chromatogr B 1998;796:327–34.

[25] Hailé S, Lefort J, Joseph D, Gounon P, Huerre M, Vargaftig BB. Mucous-cell metaplasia and inflammatory-cell recruitment are dissociated in allergic mice after antibody- and drug-dependent cell depletion in a murine model of asthma. Am J Resp Cell Mol Biol 1999;20:891–902.

[26] Araujo LM, Lefort J, Nahori MA, Diem S, Zhu R, Dy M, et al. Exacerbated Th2-mediated airway inflammation and hyperresponsiveness in autoimmune diabetes-prone NOD mice: a critical role for CD1d-dependent NKT cells. Eur J Immunol 2004;34:327–35.

[27] Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, Hachem P, et al. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. J Immunol 2003;171:1637–41.

[28] Hachem P, Lisbonne M, Michel ML, Diem S, Roongapinun S, Lefort J, et al. Alpha-galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN gamma. Eur J Immunol 2005;35:2793–802.

[29] Barnes PJ. Cytokine modulators as novel therapies for airway disease. Eur Respir J Suppl 2001;34:67–77.

[30] Kopf M, Brombacher F, Köhler G, Kienzle G, Widmann KH, Lefrang K, et al. IL-4-deficient Balb/c mice resist infection with Leishmania major. J Exp Med 1996;184:1127–36.

[31] Holgate ST. Cytokine and anti-cytokine therapy for the treatment of asthma and allergic disease. Cytokine 2004;28:152–7.

[32] Young HY, Luo YL, Cheng HY, Hsieh WC, Liao JC, Peng WH. Analgesic and anti-inflammatory activities of [6]-gingerol. J Ethnopharmacol 2005;96:207–10.

[33] Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999;103:779–88.

[34] Romagnani S. T-cell responses in allergy and asthma. Curr Opin Allergy Clin Immunol 2001;1:73–8.

[35] Renauld JC. New insights into the role of cytokines in asthma. J Clin Pathol 2001;54:577–89.

[36] Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. Respir Res 2001;2:66–70.

[37] Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. Chemokines in asthma: cooperative interaction between chemokines and IL-13. J Allergy Clin Immunol 2003;111:227–42.

[38] McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. J Immunol 2004;172:2903–8.

[39] Shen CL, Hong KJ, Kim SW. Effects of ginger (Zingiber officinale Rosc.) on decreasing the production of inflammatory mediators in sow osteoarthrotic cartilage explants. J Med Food 2003;6:323–8.

[40] Williams AS, Richards PJ, Thomas E, Carty S, Nowell MA, Goodfellow RM, et al. Interferon-gamma protects against the development of structural damage in experimental arthritis by regulating polymorphonuclear neutrophil influx into diseased joints. Arthritis Rheum 2007;56:2244–54.

[41] Kati-Coulibaly S, Lyoussi A, Ahui LB, Cossou V. Traditional therapeutical indication, pharmacological properties of zingiber. Med Nutr 2003;39:127–36.

[42] Argento A, Tiraferri E, Marzaloni M. Oral anticoagulants and medicinal plants. An emerging interaction. Ann Ital Med Int 2000;15:139–43.