University of Liege

Faculty of Medecine

Department of Clinical Sciences

Pneumology-Allergology

GIGA I^3-Research Unit

Immuno-inflammatory mechanisms in refractory asthma

Maïté Manise

Promoter : Prof. Dr. Renaud Louis

Thesis submitted to fulfill the requirements for the degree of
doctor in medical science
2012

A ceux que j'aime,

« Le bonheur le plus doux est celui qu'on partage »

Jacques Delille

REMERCIEMENTS

Je souhaite tout d'abord remercier mon promoteur, le Professeur Renaud Louis, pour m'avoir accueilli au sein de son laboratoire et m'avoir accordé sa confiance. Je lui suis également reconnaissante pour sa grande disponibilité, son pédagogisme ainsi que ses qualités scientifiques et humaines. J'ai appris énormément à ses côtés et je lui adresse toute ma gratitude.

J'exprime toute ma reconnaissance à l'ensemble de mon jury de thèse, le Professeur Edouard Louis, président de mon comité, ainsi qu'aux Professeurs Didier Cataldo, Jean-Louis Corhay, Michel Moutschen et Clio Ribbens pour leurs précieux conseils et le temps consacré à l'évaluation de mon travail.

Je voudrais également remercier chaleureusement le Professeur Guy Joos de l'Université de Gand ainsi que le Professeur Juanita Vernooy de l'Université de Maastricht, membres extérieurs du jury, pour leur aimable participation.

Merci au Professeur Pierrette Melin, chef de service de microbiologie, de me permettre de continuer mes recherches au CHU après ma thèse grâce à un nouveau projet. Je me réjouis à l'idée d'entamer cette future collaboration.

Dans le cadre du PAI, nous avons eu l'occasion de collaborer avec le Pr. Claus Bachert ainsi que Mme Gabriele Holtappels. Leurs conseils judicieux ainsi que leur enthousiasme m'ont grandement aidé dans l'élaboration de cette thèse. Qu'ils en soient remerciés.

Le Docteur Florence Schleich a apporté au sein de notre équipe dynamisme ainsi que compétences médicales et scientifiques. Merci à elle.

Je tiens également à remercier l'ensemble de mes collègues vétérinaires et biologistes et toute l'équipe technique du laboratoire et des EFR pour leur aimable contribution et, en particulier, Mme Jocelyne Sele, pour sa disponibilité et son investissement. Merci aussi à Mme Claude Fouyn pour son excellent travail à la Clinique de l'Asthme.

Je voudrais également remercier les secrétaires de pneumologie pour leur formidable travail administratif. Un tout grand merci à Mme Mady Moor pour ses qualités organisationnelles et sa gentillesse.

Merci à M. Augustin Godet et M. Cédric Graas ainsi qu'à toute l'équipe du SEGI pour le support informatique. Leurs connaissances sans faille m'ont été d'une grande aide à chaque étape de ce doctorat.

Mes remerciements vont également à la firme pharmaceutique Novartis avec qui nous avons eu l'occasion de collaborer dans le cadre d'études cliniques et qui a gracieusement accepté de prendre en charge l'organisation de la réception.

Mes pensées et ma reconnaissance vont bien sûr à l'ensemble des patients qui ont accepté de donner un peu de leur temps afin de rendre ces recherches possibles.

Du fond du coeur, je tiens à remercier ma famille pour leur soutien au cours de ces longues années d'études.

Maman, je voulais te remercier pour ta gentillesse et toute l'attention que tu portes à mon travail. Tu as toujours été présente pour moi à chaque fois que j'en avais besoin. Ton soutien et tes précieux conseils m'ont guidé et me guideront encore tout au long de la vie.

Mamy et papy, je n'ai assez de mots pour vous remercier. Dès mon plus jeune âge, vous m'avez appris le sens des vraies valeurs. Vous m'avez entouré de tout votre amour et de votre tendresse et sans vous, je n'en serais pas là aujourd'hui. Merci pour votre présence, votre affection et vos encouragements. Papy, de là-haut, j'espère que tu es fier de moi et de ce que j'ai accompli. J'aurais tellement aimé que tu sois encore parmi nous pour partager ce moment avec toi. Saches que tu es dans mes pensées, à chaque étape importante, et que je ne t'oublie pas...

Enfin, je voudrais remercier l’homme exceptionnel qui partage ma vie, mon compagnon Allan, pour sa patience et sa compréhension. Il est l’épaule solide sur laquelle je peux toujours me reposer. Son amour, sa gentillesse, et ses bons petits plats m’ont apporté soutien et réconfort durant la réalisation de cette thèse.

TABLE OF CONTENTS

PUBLICATIONS

First author

- **Manise M**, Louis R. Asthme réfractaire : mécanismes sous-jacents, diagnostic et nouvelles approches thérapeutiques. Rev Med Liège 2008 ; 63, 494-99

- **Manise M**, Schleich F, Gusbin N et al. Cytokine production from sputum cells and blood leukocytes in asthmatics according to disease severity. Allergy 2010 ; 65, 889-96

- **Manise M**, Schleich F, Quaedvlieg V et al. Disturbed cytokine production at the systemic level in difficult to control atopic asthma. Evidence for raised IL-4 and decreased IFN-γ release following LPS stimulation. Int. Arch. Allergy Immunol. 2012 ; 158, 1-8

- **Manise M**, Holtappels G, Schleich F et al. Sputum IgE and cytokines in asthma: relationship with disease severity and sputum cellular profile (submitted)

Co-author

- Schleich F, **Manise M**, Louis R. L'omalizumab (Xolair) dans le traitement de l'asthme allergique persistant sévère. Rev Med Liège 2009 ; 64, 313-7

- Schleich F, Seidel L, Sele J, **Manise M** et al. Exhaled nitric oxyde tresholds associated with a sputum eosinophil count ≥ 3% in a cohort of unselected patients with asthma. Thorax 2010 ; 65, 889-96

- Louis R, **Manise M**, Sele J et al. Inertie et observance thérapeutiques en tant que facteurs influencant le contrôle de l'asthme. Rev Med Liège 2010 ; 65, 338-42

- Moermans C, Heinen V, Nguyen M, Henket M, Sele J, **Manise M** et al. Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease. Cytokine 2011 ; 56, 298-304

- Schleich F, Asandei R, **Manise M** et al. Is $FeNO_{50}$ a useful diagnostic tool in suspected asthma? Int. J Clin Pract. (accepted in October 2011)

LIST OF ABBREVIATIONS

- ACQ : asthma control questionnaire
- ADAM 33 : disintegrin and metalloproteinase domain-containing protein 3
- AHR : airway hyperresponsiveness
- APC : antigen presenting cells
- ATS : American Thoracic Society
- BAL : brochoalveolar lavage
- BDP : beclometasone dipropionate
- BHR : bronchial hyper-responsiveness
- CD : cluster of differentiation
- COPD : chronic obstructive pulmonary disease
- CSF : colony stimulating factor
- CXCR : chemokine receptor type
- DAMP : damage-associated molecular pattern
- Der p : Dermatophagoides pteronyssus
- ECP : eosinophilic cationic protein
- EGF : epidermal growth factor
- EMTU : epithelial mesenchymal trophic unit
- ERS : European Respiratory Society
- FEV1 : forced expiratory volume in 1 seconde
- Foxp3 : forkhead box protein 3
- GATA : trans-acting T-cell specific transcription factor
- GERD : gastroesophageal reflux disease
- GINA : Global Initiative for Asthma
- Gly : glycine
- GM-CSF : Granulocyte macrophage colony stimulating factor
- ICS : inhaled corticosteroids
- IFN : interferon
- IL : interleukin
- JAK : janus kinase

- LABA : long-acting β2 agonists
- LPS : lipopolysaccharide
- MAP : multianalyte profiling
- MDI : metered dose inhaled
- MHC : major histocompatibility complex
- NADPH : nicotinamide adenine dinucleotide phosphate
- NK : natural killer
- NO : nitric oxyde
- OSA : obstructive sleep apnea
- PAMP : pathogen-associated molecular pattern
- PAR : protease-activated receptor
- PBS : phosphate buffered saline
- PHA : phytohemagglutinin
- PRR : poliovirus receiver-related protein
- RPMI : royal park memorial institute
- RNA : ribonucleic acid
- STAT : signal transducer and activator of transcription
- TCR : T cell receptor
- TGF : transforming growth factor
- tIgE : total immunoglobulin E
- TIMP : tissue inhibitor of metalloproteinase
- TLR : toll-like receptor
- TNF : tumor necrosis factor
- Treg : T regulator lymphocyte
- VCAM : vascular cell adhesion molecule

SUMMARY

The majority of asthmatics can be well controlled with reasonable doses of inhaled corticosteroids and/or long-acting β2-agonists. However, a subset of patients called « refractory asthmatics » remained uncontrolled despite high doses of inhaled and sometimes also oral corticosteroids. Although mild-to-moderate asthma is known to be Th2 driven, we need a better understanding of the immunological mechanisms leading to refractory asthma.

In the first part of this work, we have assessed cytokine production (IL-4, IL-6, IL-10, IFN-γ and TNF-α) from sputum and blood cell culture in refractory asthmatics defined according to the ATS criteria and compared them with mild untreated and moderate treated asthmatics and non-atopic healthy subjects. The majority of refractory asthmatics still exhibited intense eosinophilic airway inflammation despite heavy treatment with corticosteroids. We found that moderate and refractory asthmatics were characterized by a lower IL-6 production from sputum cells. At the systemic level, the three groups of asthmatics exhibited raised IL-4 production from peripheral blood leucocytes when compared to healthy subjects. Moreover, moderate asthmatics displayed raised IL-10 production when compared to healthy subjects and refractory asthmatics.

In the second part, we compared the stimulated cytokine production from peripheral blood leucocytes in allergic asthmatics classified according to their level of asthma control (ACQ 7 Juniper). We showed that both controlled and uncontrolled asthmatics as well as atopic non-asthmatics spontaneously produced more IL-4 than healthy subjects. IL-4 release induced by LPS was greater in both asthma groups compared to atopic non-asthmatics and non-atopic healthy subjects. By contrast, IFN-γ release induced by LPS was lower in uncontrolled asthmatics than in controlled asthmatics and non-atopic healthy subjects.

Finally, we have assessed sputum total IgE and cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ and TNF-α) in a large group of asthmatics classified according to disease severity, sputum cellular phenotype and atopy. Total IgE (tIgE) were detectable in sputum supernatant from the majority of subjects. We found a strong correlation between total sputum and serum

IgE. The three groups of asthmatics exhibited higher tIgE levels than healthy subjects without any significant difference between the groups of asthmatics. By contrast, when classifying the patients according to cellular phenotype, eosinophilic asthmatics were characterized by raised sputum IgE, IL-5 and IL-13 compared to healthy subjects and pauci-granulocytic asthmatics. Atopic asthmatics also distinguished from non-atopic asthmatics and healthy subjects by raised sputum tIgE levels without any significant difference regarding sputum cytokine levels.

Conclusion

Refractory asthmatics keep, for the majority of them, the eosinophilic asthma phenotype with persistence of raised IL-4 production at systemic level. While the increased production of IL-4 in response to LPS distinguishes asthma from atopy, a diminished release of interferon-γ in response to LPS seems to be a feature that distinguishes refractory asthma from milder forms of the disease. Finally, sputum IgE which was raised in all groups of asthmatics irrespective of disease severity, is strongly associated with sputum eosinophilia and a Th2 cytokine pattern.

RESUME

La majorité des patients asthmatiques peuvent être bien contrôlés grâce à des doses raisonnables de corticoïdes inhalés et de β2 agonistes à longue durée d'action. Cependant, un sous-groupe de patients appelés « asthmatiques réfractaires » demeurent non contrôlés en dépit de l'utilisation de hautes doses de corticoïdes inhalés et parfois aussi de corticoïdes oraux. Bien que l'asthme léger à modéré soit associé à la voie Th2, nous avons besoin de mieux comprendre les mécanismes immunologiques impliqués dans l'asthme réfractaire.

Dans la première partie de ce travail, nous avons étudié la production de cytokines (IL-4, IL-6, IL-10, IFN-γ et TNF-α) à partir de cultures cellulaires d'expectorations et de sang chez des asthmatiques réfractaires définis selon les critères de l'ATS et nous les avons comparés à des asthmatiques légers et modérés et à des sujets sains. Les asthmatiques réfractaires restaient en majorité éosinophiliques en dépit d'un traitement intensif par corticoïdes. Nous avons trouvé que les asthmatiques modérés et réfractaires étaient caractérisés par une diminution locale de la production d'IL-6. Au niveau systémique, les 3 groupes d'asthmatiques produisaient plus d'IL-4 que les sujets sains. De plus, les asthmatiques modérés se distinguaient des sujets sains et des asthmatiques réfractaires par une production accrue d'IL-10.

Dans la seconde partie, nous avons comparé la production spontanée et stimulée de cytokines à partir des leucocytes du sang périphérique chez des asthmatiques classés en fonction du niveau de contrôle de leur asthme (ACQ 7 Juniper). Nous avons montré que les asthmatiques contrôlés et non contrôlés ainsi que les atopiques non asthmatiques produisaient de façon spontanée plus d'IL-4 que les sujets sains. La libération d'IL-4 induite par le LPS était plus élevée chez les asthmatiques que chez les atopiques non-asthmatiques et chez les sujets sains non-atopiques. A l'opposé, la libération d'IFN-γ induite par le LPS était moindre chez les asthmatiques non contrôlés en comparaison avec les asthmatiques contrôlés et les sujets sains non-atopiques.

Enfin dans la troisième partie, nous avons dosé les IgE totales et les cytokines au niveau des surnageants d'expectorations (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ et TNF-α) dans un large groupe de patients asthmatiques classés en fonction du degré de sévérité, du phénotype

cellulaire des expectorations et de l'atopie. Les IgE totales sont détectables dans les surnageants des expectorations de la majorité des sujets. Nous avons trouvé une forte corrélation entre les IgE totales des expectorations et les IgE sériques. Les 3 groupes d'asthmatiques ont des taux d'IgE totales plus élevés que les sujets sains sans qu'il n'y ait de différence significative entre les groupes d'asthmatiques. Alors que le degré de sévérité ne fait pas apparaître de différence, les asthmatiques éosinophiliques se distinguent des sujets sains et des asthmatiques pauci-granulocytiques par des taux accrus d'IgE, d'IL-5 et d'IL-13 dans les expectorations. Les asthmatiques atopiques présentent également des taux plus élevés d'IgE au niveau des expectorations en comparaison aux sujets sains et aux asthmatiques non-atopiques sans qu'il n'y ait toutefois de différences concernant les taux de cytokines.

Conclusion

L'asthme réfractaire reste en majorité éosinophilique avec persistance d'une production accrue d'interleukine-4 au niveau systémique. Alors que la production accrue d'interleukine-4 par les leucocytes du sang périphérique en réponse au LPS distingue l'asthme de l'atopie, la carence de production d'interferon-γ après stimulation par le LPS est une caractéristique qui distingue l'asthme réfractaire des formes plus modérées. Enfin le taux d'IgE totales des voies aériennes, accru chez tous les asthmatiques, est davantage associé à l'éosinophilie dans les voies aériennes et à un profil en cytokine Th2 qu'à la sévérité de la maladie.

SAMENVATTING

Bij de meeste astmapatiënten is het mogelijk de aandoening onder controle te houden door middel van redelijke dosissen inhalatiecorticosteroïden en langwerkende β2- agonisten. Een subgroep van patiënten, ook wel «refractaire astmapatiënten » genoemd, blijft echter ongecontroleerd en dat ondanks het gebruik van hoge dosissen inhalatiecorticosteroïden of soms zelfs orale corticosteroïden. Alhoewel licht tot matig astma wordt geassocieerd met de Th2 pathway, is het toch noodzakelijk de onderliggende immunologische mechanismen betrokken bij refractair astma beter te begrijpen.

Het eerste deel van dit onderzoek bestond eruit de productie van cytokines (IL-4, IL-6, IL-10, IFN-γ en TNF-α) te bestuderen aan de hand van expectoratie celculturen en bloed van refractaire astmapatiënten, gedefinieerd volgens de ATS criteria. Vervolgens hebben we deze gegevens vergeleken met de gegevens van patiënten met licht tot matig astma en gezonde personen. Bij de meeste refractaire astmapatiënten blijft de eosinofilie bestaan ondanks een intensieve corticosteroïdenbehandeling. We hebben vastgesteld dat matig en refractair astma gekarakteriseerd wordt door een lokale vermindering van de IL-6 productie. De 3 groepen astmapatiënten maakten meer systemisch IL-4 aan dan de gezonde personen. Bovendien onderscheidden de patiënten met matig astma zich van de gezonde personen en refractaire astmapatiënten door een verhoogde productie van IL-10.

In het tweede deel van het onderzoek hebben we de spontane en gestimuleerde productie van cytokines door leukocyten uit perifeer bloed bij astmapatiënten, met elkaar vergeleken in functie van het niveau van hun astmacontrole (ACQ 7 Juniper). Hierbij werd aangetoond dat astmapatiënten, al dan niet onder controle alsook atopische patiënten zonder astma, spontaan meer IL-4 produceerden dan de gezonde personen. Het vrijkomen van IL-4 geïnduceerd door LPS was hoger bij astmapatiënten dan bij atopische patiënten zonder astma en gezonde personen zonder atopie. Daarentegen kwam IFN-γ, geïnduceerd door LPS in mindere mate vrij bij patiënten met ongecontroleerd astma dan bij patiënten met gecontroleerd astma en gezonde niet-atopische personen.

In het derde deel tenslotte, hebben we bij een grote groep van astmapatiënten, onderverdeeld in functie van de ernst van de aandoening, het cellulaire fenotype van de expectoraties en de atopie, de IgE totalen en de cytokines gedoseerd op het supernatant van de expectoraties (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ et TNF-α). De IgE totalen zijn detecteerbaar in het supernatant van de expectoraties bij het grootste deel van de personen. We hebben een sterke correlatie vastgesteld tussen de IgE totalen van de expectoraties en de serum IgE. De 3 groepen astmapatiënten hadden hogere waarden voor de IgE totalen in vergelijking met de gezonde personen, zonder dat er een significant verschil bestond tussen de 3 groepen astmapatiënten. Alhoewel de graad van de ernst van de aandoening geen verschillen aan het licht brengt, onderscheiden de patiënten met eosinofiel astma zich van de gezonde personen en van de patiënten met paucigranulocyten astma door hoge IgE, IL-5 en IL-13 waarden in de expectoraties. De atopische astmapatiënten vertonen eveneens verhoogde IgE waarden in de expectoraties in vergelijking met de gezonde personen en de niet-atopische astmapatiënten, zonder dat er echter een verschil is met betrekking tot de cytokinewaarden.

Besluit

Refractair astma blijft voornamelijk eosinofiel met een aanhoudende verhoogde productie van serum interleukine-4. Terwijl de perifere bloed leukocyten als reactie op LPS meer interleukine-4 aanmaken en hiermee astma van atopie kan onderscheiden worden, is het tekort aan de productie van interferon-g na LPS inductie kenmerkend voor refractair astma en onderscheidt zich hierdoor van de meer matige vormen van de aandoening. Tenslotte wordt de waarde van de IgE totalen van de luchtwegen, verhoogd bij alle astmapatiënten, meer met de eosinofilie in de luchtwegen en met het cytokine Th2 profiel geassocieerd dan met de ernst van de aandoening.

I. INTRODUCTION

1. Definition of refractory asthma

In the first versions of Global Initiative for Asthma [1], patients were classified into intermittent, mild persistent, moderate persistent and severe persistent based on symptoms, short-acting β2-agonist use, night-time awakenings and the percentage predicted forced expiratory volume in 1s (FEV1) or the peak expiratory flow. This initial classification did not include disease responsiveness to treatment. That's the reason why the update 2002 of GINA took into account the fact that treatment required to control asthma was part of the disease severity.

Most patients with asthma have mild to moderate disease that can be well controlled with long-acting β2-agonists and/or low doses of inhaled corticosteroids [2].

However, a subset of patients with asthma exists, in whom even high doses of these drugs fail to control the disease. Despite emphasis placed on this asthma phenotype since 10 years, refractory asthma remains poorly understood clinically and immunologically and represents a challenge for doctors and scientists involved in the respiratory field. In 1999, the ERS Task Force defined difficult asthmatics as patients with poorly controlled asthma despite prescription of a reasonable dose of ICS (defined as ≥ 2000µg beclomethasone dipropionate (BDP) or equivalents in adults) [3].

In 2000, an ATS workshop adopted the term "refractory asthma". The definition included one of two major criteria (continuous high-dose ICS or oral CS for >50% of the time during the previous year) with two out of seven additional minor criteria: requirement of additional controller medications, aspects of disease stability, exacerbations and lung function [4] .

The diagnosis of severe asthma should be reserved for patients who have refractory asthma after an extensive re-evaluation and an appropriate observation period of at least 6 months[5].

Major criteria	Minor criteria
Use of oral corticosteroids (≥50% of the time).	Requirement for daily treatment with long-acting β2-agonists, theophylline or leucotriene antagonists.
Continuous use of high doses inhaled corticosteroids (≥ 1200 eq bude/day)	Daily asthma symptoms requiring rescue medication
	Persistent airway obstruction (FEV1<80% predicted); diurnal PEF variability 20%
	≥ 1 urgent care visit for asthma per year
	≥ 3 courses of oral steroid bursts in the last year
	Prompt deterioration with ≤25% reduction in oral or inhaled steroid dose
	Near fatal asthma event in the past

Table 1. Definition of refractory asthma according to the ATS criteria

2. Epidemiology

It is estimated that 300 million people suffer from asthma worldwide and an additional 100 million people are predicted to be affected by 2025. Studies of both children and adults have revealed low prevalence rate (2%-4%) in Asian countries and high rates (15%-20%) in the United Kingdom, Canada, Australia, New Zealand and other developed countries [6]. Despite receiving high doses of inhaled and sometimes also oral corticosteroids, 5-10% of adult patients remain difficult to control with persistent symptoms and frequent exacerbations [7]. Among those patients, a significant proportion remained non-adherent to their treatment as it has been shown that up to 88% of patients admitted poor adherence to inhaled combination of corticoids and LABA [8]. There is, however, a fraction of patients (5-10%) which shows real resistance to corticoids [9].

3. Economic burden

The total annual cost of illness related to asthma ranges between 6 and 10 billion US dollars [10]. In United Kingdom, asthma cost is estimated to be greater than that of diabetes [11]. Drug costs increase with disease severity. In addition to long-acting β2-agonists (LABA) and high doses of inhaled corticosteroids, refractory asthmatics often require additional controller medications such as anti-leucotrienes, theophylline, oral corticosteroids and anti-IgE therapy. The anti-IgE monoclonal antibody omalizumab is the most recent therapeutic option for a limited portion of refractory asthmatics sensitised to a perennial aeroallergen and has been shown to reduce exacerbations and emergency visits, improve lung function, symptom scores and quality of life [12]. The clinical efficacy is, however, obtained at the expense of high cost. It ranges between $15.000 and $44.000 depending on the dose received by the patient [13;14]. More than 40% of its economic impact is believed to be associated with emergency room use, hospitalizations and death which occurs essentially in uncontrolled asthmatics. In addition to drug and hospitalisation costs, economic impact of severe asthma is largely dependent on indirect costs including absenteeism and sick pay.
A study from Europe estimated that the average cost per patient with severe asthma is nearly 6 times the cost of care for a patient with mild asthma [15].

4. Factors contributing to the severity of asthma

4.1. Genetics

Family and twin studies have indicated that genetics plays an important role in the development of asthma and allergy [16]. Epidemiological studies revealed that children with a familial history of asthma, especially those who have an asthmatic parent, are more at risk to have asthma [17].
Genome-wide linkages studies and case-control studies have identified 18 genomic regions and more than 100 genes associated with asthma and allergy in 11 different populations.
In particular, there are consistently replicated regions on the long arms of chromosomes 2, 5, 6, 12 and 13 [18].
Some studies suggest that there are susceptibility genes for disease severity in addition to those that predict asthma itself. Several genes have been associated with severe disease,

including the Gly 16 allele of the β_2-adrenoreceptor gene [19] the C-509 T allele of TGF-β, [20], a tissue inhibitor of metalloproteinase 1(TIMP-1) [21] and also IL-4 and IL-4RA [22]. ADAM33 is the first novel gene that appears to be closely associated with both BHR and accelerated decrease in baseline lung function [23;24]. The results of a recent study indicate that genetic variants regulating *ORMDL3* expression are determinants of susceptibility to childhood asthma [25]. Another recent genome-wide association study has identified a highly significant association between asthma and ORMDL3 [26]. It is the third member of a novel class of genes that encode transmembrane proteins anchored in the endoplasmic reticulum.

4.2 Atopy

Although atopy is less common in patients with severe asthma than in mild to moderate asthma [27], it is found in approximately 60% of refractory patients in whom perennial allergens like house dust mite, moulds or cockroaches can contribute to disease severity. Zureik et al have also shown that sensitization to moulds is a powerful risk factor for severe asthma in adults. This should be taken into account in primary prevention, management, and patients' education [28]. Moreover, the mould species Aspergillus can contribute to an allergic bronchopulmonary aspergillosis, an uncommon form of severe asthma which can lead to proximal bronchiectasis in the absence of treatment [29].

In the USA and English severe asthma registries, atopy was found in 71% and 57% of patients respectively. In our national data base of refractory asthma the proportion of atopic patients is 69%.

4.3. Tobacco

Environmental factors like atmospheric pollutants can also play an important role. Tobacco smoke mainly contributes to the disease severity. Cigarette smoke is a highly complex mixture, and many of its components are known to be carcinogens and mutagens. Cigarette smoke has been linked to many chronic lung disorders and is a major cause of lung cancer. Acute exposure to cigarette smoke is related to airway and systemic inflammation and oxidative stress [30]. Although symptoms improve after the cessation of smoking, the oxidant and protease burden in the airways continues for months leading to an intense neutrophilic response and increasing resistance to corticosteroids [31;32]. Cigarette smoke induces the

release of the neutrophil chemoattractant IL-8 from cultured human bronchial epithelial cells. BAL samples from non asthmatic smokers have greater concentrations of neutrophils, macrophages, and a number of cytokines, including IL-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 than non smokers, with a cigarette dose-related relationship for some of these factors [33]. Cigarette smoke has the capacity to damage the bronchi in a number of ways, including direct toxicity to the bronchial epithelium, oxidative damage, recruitment of inflammatory cells, and increased epithelial permeability, and is also associated with the development of irreversible airflow limitation which can lead to an accelerated lung function decline in susceptible subjects [34].
In our severe asthma registry, 12% of patients were current smokers at time of investigations while, in comparison, they were only 6% in the UK registry.

	Belgium	UK	USA
Age (years)	48±19	50±14	41±13
Sex (% female)	54%	63%	64%
Tobacco status (NS/FS/CS)	126/70/22 (57%/31%/12%)	233/114/22 (61%/30%/6%)	-
BMI	27 (16-41)	28 (14-32)	-
Atopy (%)	69%	57%	71%

Table 2. Demographic characteristics of refractory asthmatics (data from the Belgian Severe Asthma Registry compared with UK and USA).

4.4. Occupational asthma

Occupational exposure (painter, hairdresser, baker) also plays a role in the severity of the disease. In occupational asthma caused by irritant chemicals, pathological changes consisting of fibrosis of the bronchial wall and epithelial denudation with a fibrinohaemorrhagic exsudate in the submucosa have been observed without eosinophilic inflammation.
Once the process is established, immunological phenotypes can continue irrespective of exposure and leads to airway remodelling and permanent loss of lung function [35].

Other types of occupational asthma caused by macromolecules like that of baker, can however generate a classic eosinophilic inflammation [36].

4.5. Viral and bacterial infections

Respiratory viral infections are a common cause of asthma exacerbations [37;38] with rhinovirus being the most frequently virus detected in both children[39] and adults [40;41]. In asthmatic patients, both innate and adaptive antiviral immunity may be impaired. Viral infections contribute to airway inflammation and exacerbations. Impaired interferon response following a rhinovirus infection may allow the virus to continue to replicate and damage the airway epithelium [42].
Mycoplasma pneumoniae and *Chlamydia pneumoniae* have also been involved as bacterial agents playing a role in asthma exacerbations [43].

4.6. Drugs

Some medications like β-blockers are not indicated in asthmatic patients as they have the potential to cause bronchospasm [11]. Aspirin sensitivity in adults is associated with severe asthma, increasing leukotriene production, blood eosinophilia, rhinosinusitis most often associated with nasal polyposis and a poor response to corticosteroids [44].

4.7. Psychological troubles

Psychological disorders and stressful conditions have often been shown to influence asthma control and management, although how psychological disturbances can influence the clinical expression of asthma is still uncertain [45].
Anxiety, depression and panic disorders are more frequent in asthmatic patients than in the general population [46].
Depression may affect control of asthma in reducing adherence to the treatment and follow-up. Poor compliance or adherence to the medications, in particular inhaled corticosteroids, is certainly a major factor in the increasing severity of the disease [47;48].

4.8. Gender

Severe asthma is more common in woman than in man as it is the case for asthma in adult in general. In the Belgian severe asthma registry, there was 54% of female while they were 63% and 64% in the UK and USA registries. During childhood, boys are more affected than girls but there is a change in teenagers which persist in adults. Endocrine factors may contribute to the disease as suggested by the fluctuations seen during the menses in women [49]. In the same idea, pregnancy has an impact on the clinical expression of the disease (especially during the two last quarters) although it may either worsen or improve asthma control [50].

4.9. Obesity

Obesity has been recently shown to have an impact on the severity of asthma, especially in women [51]. Loss of weight is associated with a better control of the disease without modification of the treatment. Asthma symptomatology is probably aggravated by the negative mechanical effect of obesity on the diaphragm function. However there is a possibility that obesity may worsen asthma control through other mechanisms. It is known that endocrine factors linked to obesity like leptin or other adipokines (adiponectin, resistin) have an action on immune and inflammatory cells [52]. Consequently, we cannot exclude that obesity could modify the severity of asthma by an immuno-inflammatory mechanism.

4.10. Rhinosinusopathy

Nasal polyps and chronic rhino-sinusitis are known to be aggravating factors playing a role in the severity of the disease [53]. In particular, severe sinus pathology is an important factor contributing to recurrent exacerbations in difficult to control asthma. Interestingly, refractory asthmatics with severe rhino-sinusitis and polyposis display heavy eosinophilic inflammation [54].

4.11. Gastro-oesophageal reflux

Gastro-oesophageal reflux is often associated with asthma in adults and children [55]. Gastro-oesophageal reflux disease could worsen asthma either by direct effects on airway responsiveness or via aspiration-induced inflammation [56]. In some cases, proton pump inhibitors are able to improve asthma control [57]. However, it seems that overall the effects of this class of drug is rather disappointing in improving asthma control casting doubt on a major role for GERD in asthma pathophysiology [58].

4.12. Obstructive sleep apnea

Obstructive sleep apnea is associated with obesity and could possibly influence asthma by promoting GERD [59]. OSA is associated with an upper airway inflammatory process that has the potential to influence lower airways and could cause oxidative stress and inflammation in these airways [60].

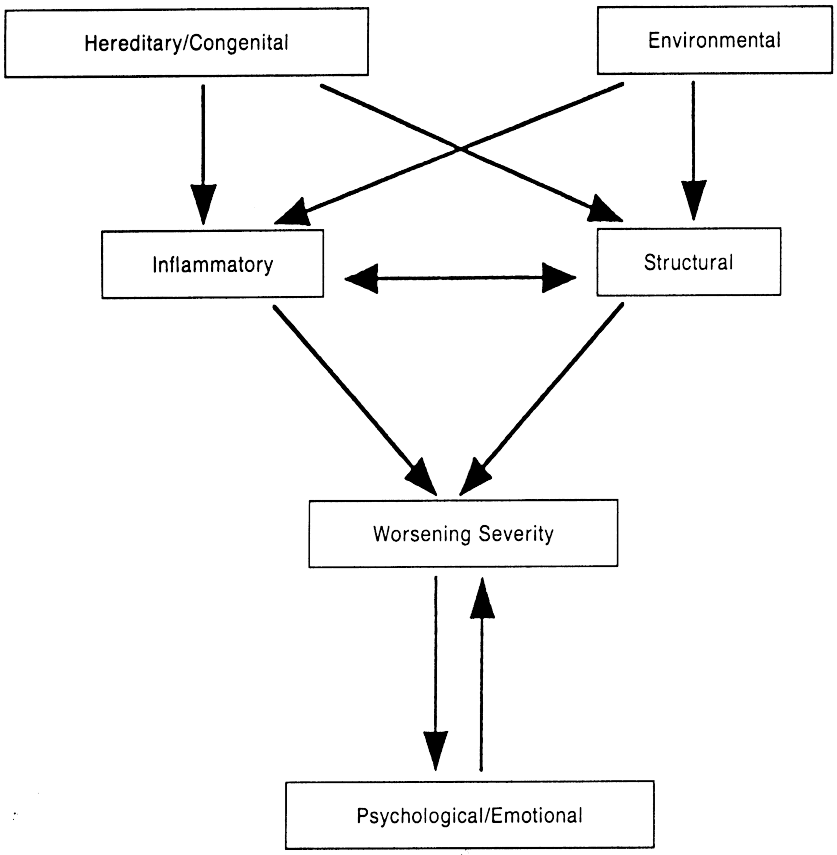


Fig 1. The complex interactions contributing to asthma severity (from Wenzel S.E.,Clin Exp Allergy, 1998).

5. Asthma diagnosis

Diagnosis of refractory asthma is not easy and requires careful investigation and follow-up. It is not so rare that patients considered as refractory asthmatics may in fact suffer from other disease such as vocal cord dysfunction, bronchiectasis or COPD [61]. Before defining a patient as a refractory asthmatic, the patient must be followed by a respiratory specialist for an observational period of at least 6 months, the asthma diagnosis must be confirmed and other pathology like vocal cords dysfunction excluded. Adherence to medications must also be checked. The demonstration of increased airway variability is necessary to ascertain the asthma diagnosis. The methacholine provocation test is an interesting tool to confirm bronchial hyper-reactivity. Unfortunately, in many refractory asthmatics, FEV1 baseline is often tool low (<65% predicted) to use this approach. A significant reversibility (≥ 12%) to short-acting β2-agonists can help to make the diagnosis but a number of refractory asthmatics have fixed airway obstruction as a consequence of airway remodelling with poor reversibility to inhaled b2- agonists [62]. In some patients, the airway reversibility can only be detected by a treatment with inhaled corticoids or a short course of oral corticoids. Airway inflammation, which is another important facet of the disease, can be measured non-invasively by measuring the sputum cell count. Severe asthma has been associated with both eosinophilic [63;64] and neutrophilic airway inflammation [64;65] Exhaled NO is a good marker of eosinophilic airway inflammation [66;67].

6. Cellular and molecular mechanisms of severe asthma

6.1. Inflammatory cells

6.1.1. Lymphocytes

Lymphocytes play a major role in the immune system. They can be differentiated in three subtypes: natural killer (NK) cells, T and B lymphocytes.

Natural killer cells are able to recognize infected cells and tumours by the changes on the surface of major histocompatibility complex (MHC) class 1. NK cells can be activated by the family of interferon [68]. Once they are activated, they can release granules which can destroy the altered cells.

T lymphocytes migrate from bone marrow to thymus where they finish their maturation, they are involved in cell-mediated immunity. They possess a specific receptor on their surface called TCR. There are several subtypes of T cells. T helper cells are also known as $CD4^+$ T cells because they express the CD4 protein on their surface. They become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of Antigen Presenting Cells (APCs). Once they are activated, they divide rapidly and secrete cytokines which play a major role in the regulation of the immune system. These cells can differentiate into one of several subtypes, including Th1, Th2, Th17 or Treg, which secrete different cytokines to facilitate a different type of immune response [69].

Cytotoxic cells are also known as $CD8^+$ T cells since they express the CD8 glycoprotein on their surface. These cells recognize their targets by binding to antigen associated with MHC class I. They destroy infected cells and tumour cells and are implicated in transplant rejection.

Memory cells are able to "remember" each specific pathogen encountered and to provide a strong and fast response if the pathogen is detected again.

T regulatory cells (T_{reg}) suppress activation of the immune system and thereby maintain tolerance to self-antigens. They are crucial for the maintenance of "immunological tolerance".

T_{reg} cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3 [70].
It has recently been shown that regulatory T lymphocytes were defective in allergic patients[71].

B lymphocytes end their maturation in the bone marrow in mammalian (or in the bursa of Fabricius in birds) and are responsible for humoral immunity. The principal functions of B cells are to synthesize antibodies against antigens, to perform the role of antigen-presenting cells (APCs) and eventually develop into memory B cells after activation by antigen interaction. Once a B cell encounters its cognate antigen and receives an additional signal from a T helper cell, it can further differentiate into one of the two types of B cells: plasma B cells and memory B cells [72].

The percentage of CD4+ T lymphocytes correlated with disease severity and the number of eosinophils in bronchial biopsy specimens. Glucocorticoid treatment may improve lung function and reduce the percentage of CD4+ T cells expressing mRNA encoding IL-3, IL-5 and GM-CSF [73].
Although further studies are still needed, it has been suggested that there may be an inverse correlation between T_{reg} activity and clinical manifestations of allergic diseases[74;75]. However, another recent study has shown that CD4+FoxP3+ T_{reg} cells were increased in BAL of patients with moderate to severe asthma compared with patients with mild asthma and healthy subjects [76].
CD8+ lymphocytes may have, as previously suggested in patients with COPD [77], a significant role in the clinical course of asthma. Van Rensen E et al have shown that outcome of asthma, as determined by the annual decline in FEV1 can be predicted by bronchial CD8+ lymphocytes infiltrate [78].

6.1.2 Eosinophils

Eosinophils are effector cells previously described as having a natural role in the defence against parasites [79]. Their role in allergic diseases and asthma is now well established [80]. Inhalation of allergen in a sensitised subject initially led to a decrease in the numbers of circulating eosinophils, followed by a rebound increase corresponding to the release from the

bone marrow in response to increased local production of IL-5 [81]. At the airway level allergen challenge causes a marked increase in sputum eosinophils after a few hours [82]. Eosinophils play a major role in promoting allergic inflammation through the release of pro-inflammatory mediators like cyteinyl-leucotrienes. Eosinophil is made of large secondary granules, each containing four basic proteins. Among those proteins, the best known is the eosinophilic cationic protein (ECP) released during degranulation of eosinophils. Levels of ECP are elevated in Th2 atopic diseases such as allergic asthma and the protein has the ability to bind lipopolysaccharide (LPS) and other bacteria cell wall components [83] which might have a priming influence on the immune system. Serum ECP levels are thought to reflect the activation state of eosinophils [84]. ECP has also been detected in other physiological fluids like BAL or sputum [85] and was shown to be proportional to disease severity in asthmatics [63;86].

Eosinophils are recognized to be a major source of cytokines. In addition to IL-4 and IL-13, human eosinophils can also produce, store, and secrete over 30 cytokines, including Th1-associated IL-12 [87] and IFN-γ [88]. The roles of those cytokines are described in section 1.6.4.

Our experience with induced sputum showed that refractory asthmatics still exhibited, for the majority of them, an eosinophilic pattern despite heavy treatment with corticoids (see fig 2 below). Therefore, the reduction of eosinophilic inflammation in those severe patients represents a real challenge for the clinician. Majoring the dose of inhaled corticosteroids or introducing a course of oral corticosteroids can be useful when the eosinophilic inflammation is still present despite medications. Indeed, corticosteroids are known to be more efficient in eosinophilic asthma when compared to neutrophilic asthma [89].

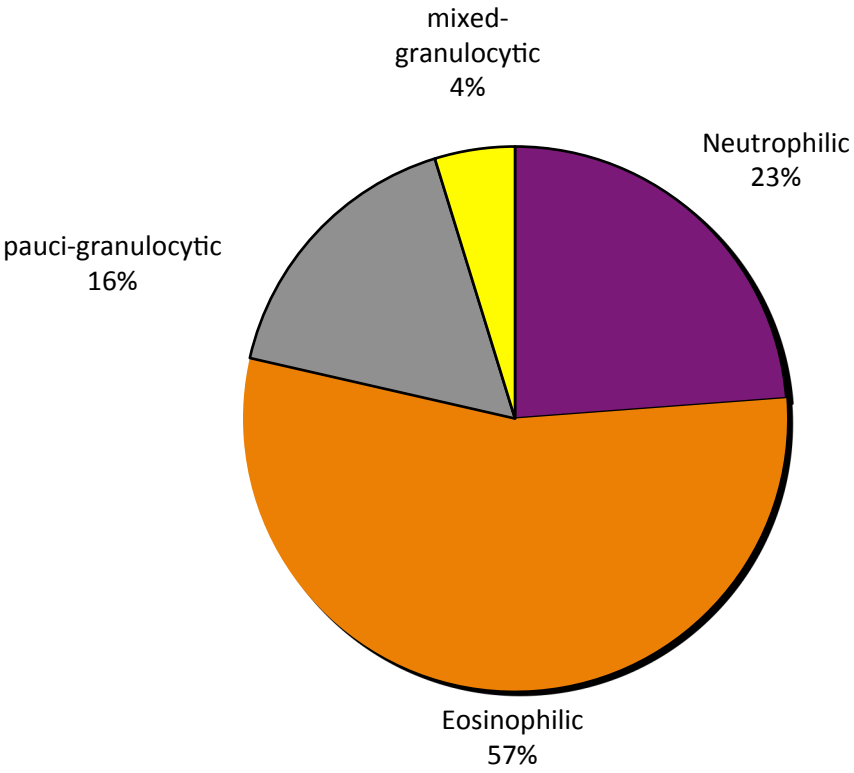


Fig 2. Sputum cellular characteristics in refractory asthma (N=42, data from the Belgian Severe Asthma Registry).

6.1.3. Neutrophils

Neutrophils play an important role in the processus of phagocytosis. They possess a unique capacity to engulf and thereby eliminate pathogens and cell debris. They are equipped with specialized receptors to recognize their targets. This complex machinery mediates internalization and initiates an assortment of degradative mechanisms that culminate in killing and disposal of the engulfed particles [90].

They have a bactericidal action by production of superoxide that can react to form hydrogen peroxide, which participates in bacterial killing. The enzyme which catalyzes this reaction is the NADPH oxydase [91].

Neutrophils are key anti-infectious actors, they can be quickly and efficiently mobilized and they constitute the first line of defence against the pathogens [92].

Some studies showed that neutrophils were present in increased quantities in refractory patients compared with patients with milder disease [64;65].

It was suggested that the increased neutrophilic inflammation might explain the poor clinical response to corticosteroids [93]. Potential explanation for the poor response to CS in neutrophilic asthmatics is inflammation-induced changes in the binding affinity of the glucocorticoid receptor and alteration in CS suppression of transcription factor binding [94].

The mechanisms by which neutrophils accumulate in the airways of asthmatics remain to be elucidated, however, chemoattractants for neutrophils such as CXC chemokines may affect either the accumulation or functional status of neutrophils in such patients [95].

It seems that neutrophilic inflammation in refractory asthmatics may be partially attenuated by chronic treatment with clarithromycin [96].

6.1.4. Basophils

Basophils appear in many specific kinds of inflammatory reactions, particularly those that cause allergic symptoms. Basophils contain anticoagulant heparin, which prevents blood from clotting too quickly. They also contain the vasodilator histamine, which promotes blood flow to tissues. Together with eosinophils, they play a role both in parasitic infections and in allergy [97]. Basophils are important effector cells in IgE-mediated allergic inflammation [98]. Normally, basophils are rare circulating granulocytes and constitute less than 1% of blood leucocytes and are not present in tissues in normal conditions. However, they can be

recruited at sites where an antigen is present and contribute to immediate hypersensitivity reactions [99]. Interestingly, basophils were shown to be potent source of IL-4 [100].

6.1.5. Macrophages

They are produced by the differentiation of monocytes in tissues. Macrophages function in both innate and adaptative immunity. Their main role is the phagocytosis of cellular debris and pathogens. After digesting a pathogen, the macrophage will present the antigen of the pathogen to the corresponding helper T cell. The presentation is done by integrating it into the cell membrane and displaying it attached to an MHC class II molecule. They also provide a line of defence against tumor cells or cells infected by fungus or parasites. Currently, it is a major opinion that there are several activated forms of macrophages [101].
The macrophage does not generate a specific response for an antigen, but attacks the cells present in the local area in which it was activated.
Macrophages are important immune and inflammatory effectors cells in asthma. The alveolar airway macrophage is in a greater state of activation in patients with asthma than in healthy subjects [102]. In asthmatics, they are able to release amounts of pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ or GM-CSF. Bhavsar P et al have shown that alveolar macrophages from patients with severe asthma demonstrate corticosteroid insensitivity compared with those of patients with non-severe asthma [103].

6.1.6. Epithelial cells

The respiratory epithelium acts as a physical barrier that separates the external environment from the pulmonary internal environment. Epithelial cells connected by tight junctions contribute to the barrier function of the airways. They express a poliovirus receiver-related protein (PRR), toll like receptors (TLRs) and protease-activated receptors (PARs), which recognize bacterial agents and allergens. The interaction between the epithelium and other bronchial wall elements generate a specific structure called EMTU (epithelial mesenchymal trophic unit). It is considered today that the epithelial cell plays a central role in the chronic inflammation and the airway remodelling seen in the asthmatic process [104].

6.2. EMTU

In severe asthmatics, the epithelial mesenchymal trophic unit (EMTU) which controls the local tissue microenvironment and maintains tissue homeostasis is dysregulated [105]. Environmental agents are able to cause epithelial damages resulting in production of signals that act on the underlying mesenchyme to propagate and amplify inflammation and remodelling in the submucosa. The most frequent risk factors for developing and amplifying asthma act through the EMTU. Defective epithelial tight junction associated with impaired barrier function [106] could explain the susceptibility of asthmatics to respiratory viruses, air pollutant or tobacco smoke. Furthermore, the demonstration of defective epithelial repair in asthma comes from the observation of overexpression of the epidermal growth factor (EGF) receptor in proportion to disease severity [107]. Activation of EGF receptors in the presence of an oxidative stimulus leads to the secretion of pro-inflammatory cytokines such as CXCL8, which is chemoattractant for neutrophils. Through such amplification mechanisms, delayed and incomplete epithelial repair both promotes ongoing chronic inflammation and initiates remodelling in an attempt to limit penetration of the airway by damaging inhaled toxicants or microorganisms. In this modified environment, recruitment and activation of inflammatory cells like monocytes, mastocytes and neutrophils change. Moreover, despite high doses of inhaled or oral corticosteroids, there is persistence or increase of mastocytes, especially those near smooth muscles cells in which there are high concentrations in TNF-α [108]. Mastocytes are also an important source of interleukin 13, a mediator implicated in inflammatory and remodelling processes [109].

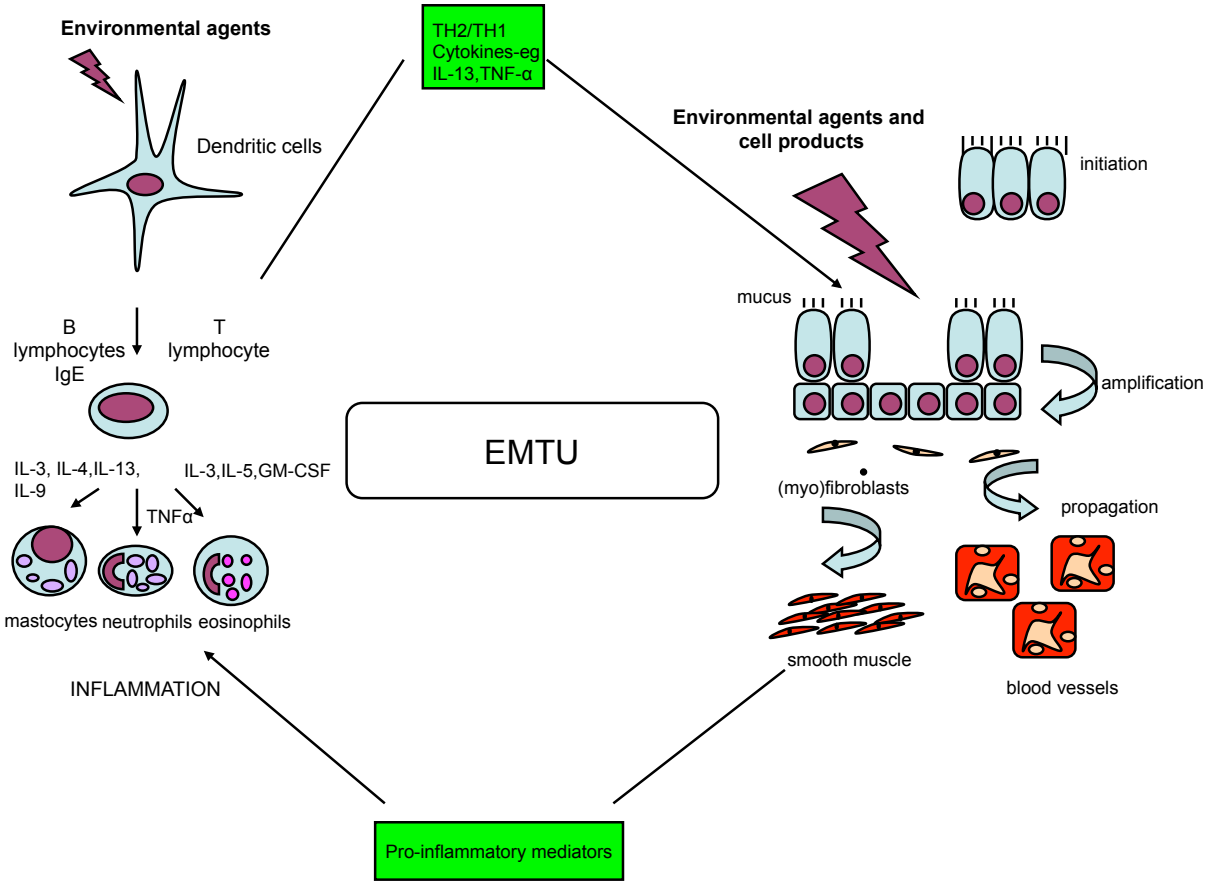


Fig 3. Inflammatory and remodelling responses in asthma with activation of the epithelial mesenchymal trophic unit (EMTU) (adapted from Holgate,S.T., Lancet 2006).

6.3. Immunological pathways

6.3.1. Th1/Th2 paradigm

Mild to moderate asthma is characterized by involvement of T helper type 2 (Th2) mediated inflammation. Strong associations with allergic disease have also provided a strong case for a primary role of Th2 cells in the development of asthma. Th2 cytokines (IL-4, IL-5, IL-13 and IL-9) may have a role in the expression and development of airways inflammation and hyper-reactivity and atopic individuals have higher Th2 responses than non atopic individuals [110]. In a recent study Woodruff and al showed that a gene signature for Th2-driven inflammation in airway epithelial cells was only prominent in half of patients with asthma while non-Th2-mechanisms operate in the remaining half [111]. Thus, asthma can be divided broadly into Th2-high and Th2-low molecular phenotypes. Interestingly, they found that airway obstruction was improved with inhaled steroids in the Th2-high subgroup but not in the Th2-low subgroup. This study also suggests that Th2-driven inflammation is the molecular mechanism underlying the cellular phenotype of asthma known as “eosinophilic asthma”. They demonstrate that Th2-high asthmatics were characterized by airway eosinophilia and subepithelial fibrosis [64].

The Th2 immune process alone is no sufficient to explain the persistence of refractory asthma in adults. Moreover, the Th2 hypothesis can not explain why airway hyper-responsiveness and tissue remodelling are not clearly linked to inflammation or why some patients have severe asthma despite optimal medications and it doesn’t take into account the heterogeneity of the disease. Immunological data on severe asthma are much controversial. There is report of neutrophilic Th1 and IL-8 driven inflammation in some severe patients [65]. Until recently, innate immunity was thought to be only a rapid front-line defender against infections. It works by triggering host defence after recognition of pathogen associated molecular patterns (PAMPs) on invading pathogens. However, endogenous ligands released from damage associated molecular patterns (DAMPs) are important activators of innate immunity. Like PAMPs, DAMPs acts through a toll-like receptor system and its MyD88 transduction pathway to directly promote inflammation. It is now thought that neutrophilic asthma may be driven by epithelial activation with PAMPS and DAMPS [112]. Because innate immunity is intrinsically resistant to corticosteroids, it could explain that why asthma severity worsens, corticosteroids sensitivity decreases [113].

On the other hand numerous studies have also shown persistence of intense eosinophilic inflammation in severe asthma [7;63] despite heavy treatment with corticoids. Furthermore a long-term follow-up study showed that severe atopic asthmatics had reduced Th1 (IFN-γ) responses to house dust mite compared to adults with resolved asthma [114]. These studies would point a persistent imbalance towards Th2 profile in refractory asthma.
These observations suggest that asthma is indeed heterogeneous, with different phenotypes and immunological mechanisms, some dependent and some independent of Th2 cells and requiring different therapeutic approaches.

6.3.2. The T regulator

The "hygiene hypothesis" was initially explained in terms of a shift in favour of Th2 responses because of reduced pro-Th1-inducing microbial exposure in the western world [115]. However this does not explain that Th1 diseases such as type I diabetes have increased in the same period. This paradox has been solved by the demonstration of a deficient T regulator pathway in both allergic Th2 and Th1 diseases [75] (See above). Therefore reduced T_{reg} activity has been put forward as the unifying mechanism that would explain the concomitant rise in both Th1 and Th2 diseases. The way by which the western living style could decrease T_{reg} activity remains, however, unclear.

6.3.3. Th17

Despite the focus on a predominant Th2 pattern in asthma, studies have shown that 30- 50% of asthmatics were characterized by nonatopic, non IgE-dependent and non-eosinophilic inflammation [112]. Th17 cells produce a number of cytokines, but in particular IL-17A and IL-17F. The main role of Th17 cells is their faculty to recruit and activate neutrophils, either directly through IL-8 production or indirectly by inducing the production of colony stimulating factors (CSF) and CXCL8 by tissue resident cells [116]. These cells have been implicated in the pathogenesis of a number of autoimmune diseases, including psoriasis and rheumatoid arthritis [117].
It has been reported that increased AHR in response to methacholine in asthmatics positively correlates with IL-17A levels in the sputum [118]. The Th17 pathway seems to be resistive to the action of corticoids. This mechanism could explain the increase in neutrophilia seen in

some severe and steroid-resistant asthmatics [119]. Bullens D et al found that sputum IL-17A and IL-8 mRNA levels are significantly elevated in asthma patients compared to healthy controls which suggests that Th17 cell infiltration in asthmatic airways links T cell activity with neutrophilic inflammation in asthma [120].

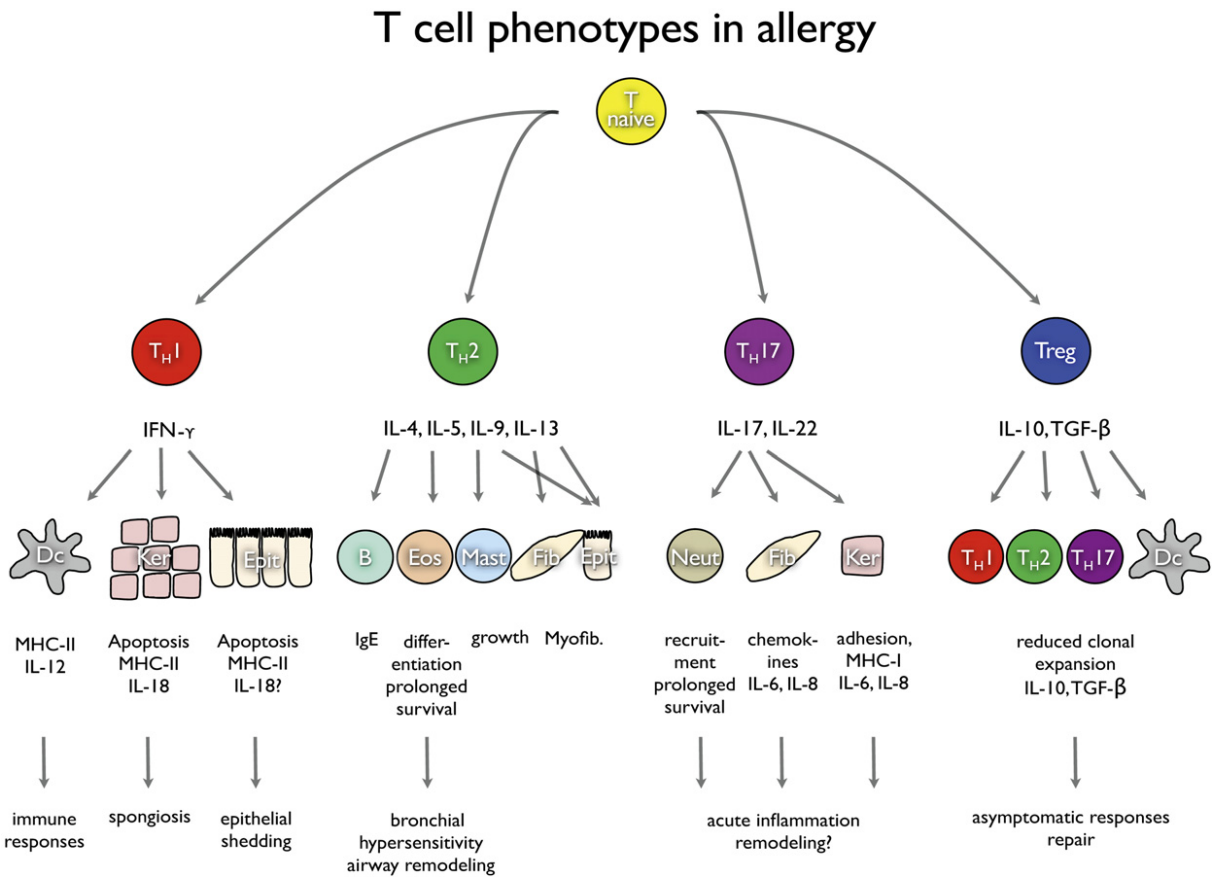


Fig 4. Schematic overview of T-cell phenotypes in allergic asthma. (from Schmidt-Weber, C.B., JACI, 2007)

6.4. Cytokines

6.4.1. Interleukin 4

IL-4 is derived from Th2 lymphocytes but also natural killer (NK) cells, basophils, eosinophils and mast cells [121]. IL-4 is a key cytokine in the development of allergic inflammation. The production of IL-4 is mediated by the transcription factor GATA-3 [122]. STAT-6 also plays an important role in IL-4 mediated biological responses [123].
There are two types of complex receptors systems for IL-4. The type I receptor made of the IL-4Rα and the γ chain only allows the binding of IL-4 while the type II receptor made of the IL-4Rα and the IL-13Rα1 chain is a common receptor for IL-4 (by binding to the IL-4Rα chain) and IL-13 (by binding to the IL-13Rα1 chain). This shared use of the IL-4Rα chain by both IL-4 and IL-13 could explain many of the common biological activities of these cytokines [124]. IL-4 is able to stimulate MHC class II molecules, CD40, surface IgM and low-affinity IgE receptor (CD23) expression by B cells and enhances the antigen-presenting capacity of B cells. However, one of the major roles of IL-4 is the induction of the immunoglobulin isotype switch from IgM to IgE [125].
In addition to the effects on B cells, IL-4 can also act on T cells by driving the initial differentiation of naïve T-helper type 0 (Th0) lymphocytes toward a Th2 phenotype. This biological activity is restricted to IL-4 because only IL-4 receptors and not IL-13 receptors are expressed on T cells. IL-4 also plays an important role in allergic immune responses because of its ability to prevent apoptosis of T lymphocytes. Corticosteroids are known to cause apoptosis in mature T-helper cell lines. IL-4 and IL-2 have a synergistic action that makes lymphocytes refractory to the anti-inflammatory effects of corticosteroids [126].
Another important activity of IL-4 in promoting cellular inflammation in the asthmatic lung is to induce expression of VCAM-1.
IL-4 also interacts with mast cells to stimulate IgE receptor expression and regulates expression of leucotriene C4 synthase, thereby determining their capacity to produce cysteinyl-leucotrienes [127].
IL-4 has also been shown to regulate airway eosinophilia but the mechanisms need to be specified. We have previously showed that production of IL-4 from airways cells was increased in atopic asthma and correlated with the magnitude of eosinophilic inflammation after allergenic challenge [82] IL-4 increases the expression of eotaxin and other

inflammatory cytokines from fibroblasts and contributes to inflammation and lung remodelling in chronic asthma [128].

6.4.2. Interleukin 13

IL-13 is produced by activated T cells, basophils, eosinophils, and mast cells and is thought to be a central mediator of inflammation in asthma.
The functions of IL-13 widely overlap with those of IL-4. IL-13 is also able to induce the IgE isotype switch and VCAM-1 expression. IL-13Rα1 expression is more limited than IL-4 receptors explaining the unique ability of IL-4 to induce Th2 lymphocyte differentiation and mast cell activation. However, IL-13 is more widely produced than IL-4, including by Th1-like lymphocytes and is more readily identified in allergic inflammatory tissue. Many of the effects of IL-13 are mediated via the transcription factor STAT-6 [129]. This cytokine has been found to be increased in the airways of patients with asthma and is thought to mediate several features of asthma, including airway hyper-responsiveness, inflammation, mucus metaplasia, and activation and proliferation of airway fibroblasts, which contribute to adverse airway remodelling [130].

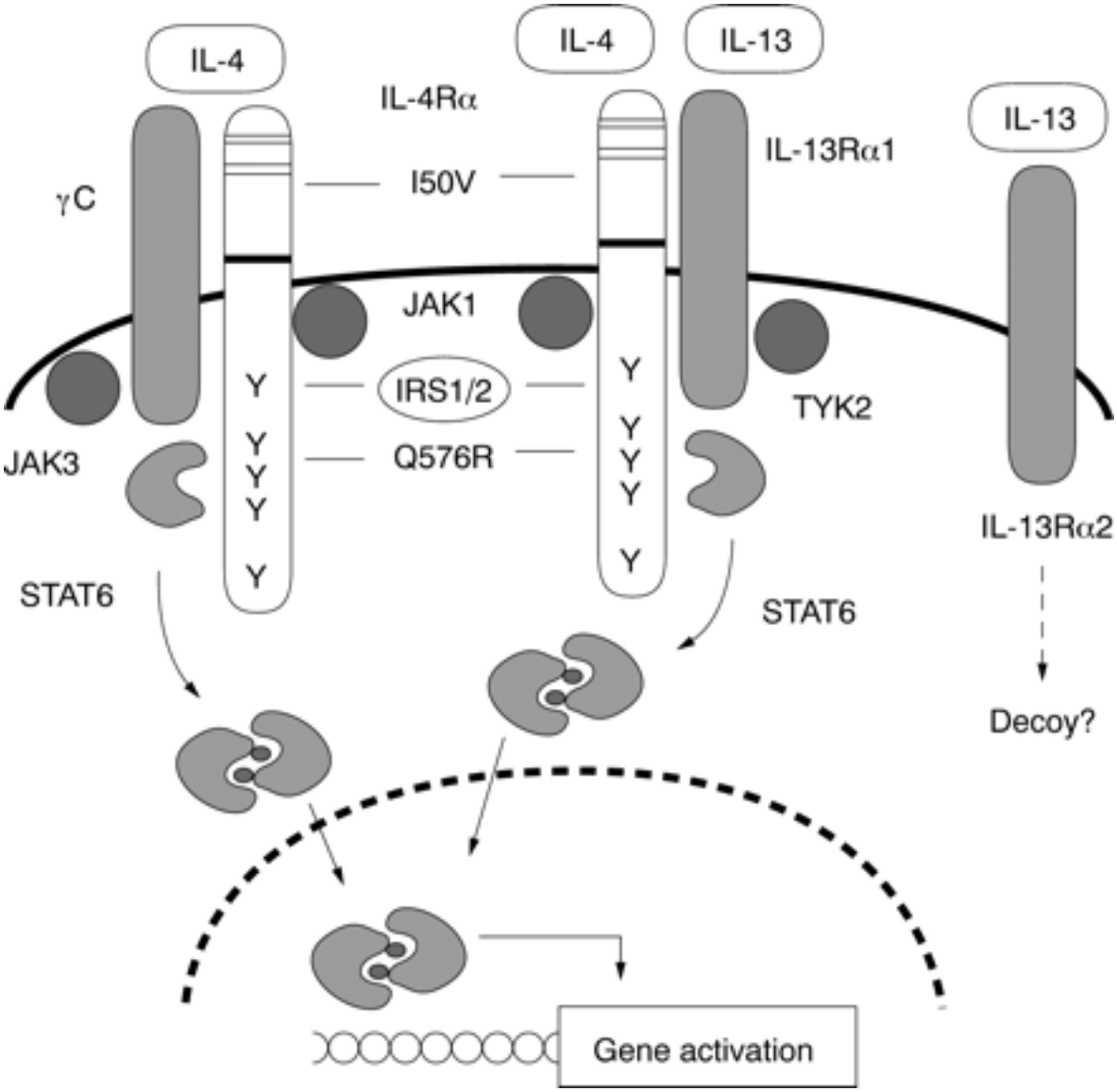


Fig 5. Schematic representation of the IL-4/IL-13 signalling pathway (from Renauld,J.C., J Clin Pathol, 2001)

6.4.3. Interleukin 5

IL-5 is produced by Th2 cells and mast cells and is the most important eosinophilic cytokine. IL-5 is able to stimulate eosinophil production and release from the bone marrow, has a chemotactic effect on eosinophils, activates mature eosinophils, induces eosinophil secretion and enhances cytotoxicity [131]. Several studies have linked atopic asthma and eosinophilia with increased IL-5 production [132;133]. Other activities of IL-5 include basophil differentiation and maturation of cytotoxic T lymphocytes. Like for IL-4, the transcription factor GATA-3 is involved in IL-5 gene transcription in human peripheral CD4+ T cells. GATA-3 was found to be increased during Th2 phenotype activation [134].

The importance of interleukin-5 in asthma pathophysiology has been recently underscored by the demonstration of the efficacy of mepolizumab, an anti-IL-5 monoclonal antibody, in refractory eosinophilic asthma (see below).

6.4.4 Interferon-γ

Interferon-γ is a key cytokine produced by Th1 cells, but also derived from NK cells and cytotoxic T cells. It is the most important cytokine for cell-mediated immunity. Interferon-γ is a type 2 interferon involved in host defence against microorganisms [135]. The cellular responses are activated through its interaction with a heterodimeric receptor consisting of Interferon gamma receptor 1 (IFNGR1) and Interferon gamma receptor 2 (IFNGR2). IFN-γ binding to the receptor activates the JAK-STAT pathway. IFN-γ mediates increased MHC class I and II expressions and stimulates antigen presentation and cytokine production by APCs. IFN-γ stimulates mononuclear phagocytic functions like adherence, secretion, phagocytosis, respiratory burst and nitric oxide production. At the site of cellular immune responses, there is accumulation of macrophages and activation that leads to the death of intracellular pathogens.
The major role of this cytokine is the defence against infections [136]. IL-12 and IFN-γ coordinate the link between pathogen recognition by innate immune cells and the induction of specific immunity, by mediating a positive feedback loop to amplify the Th1 response. IFN-γ also plays a role in delayed-type hypersensitivity cutaneous reaction, first defined in the context of the immune response to mycobacteria. Those reactions are crucial for defence against intracellular pathogens, and also represent the cellular mechanisms underlying

pathologic responses to allergens. IFN-γ inhibits viral replication. Although all types of IFN are crucial in the immediate cellular response to viral infection, the immunomodulatory activities of IFN-γ are particularly important for long-term control of viral infection [137]. We previously found that IFN-γ in the whole blood from atopic asthmatics but not from intrinsic asthmatics was decreased as compared to healthy subjects [82]. It is believed that some difficult-to-control asthma may be linked to persistent infection [138]. A recent study looking at bronchial biopsies has shown that subepithelial IFN-γ was increased in severe asthmatics when compared to moderate asthmatics [139]. Using the whole blood model, Magnan et al found an overproduction of IFN-γ in relationship with an increased capacity of CD8+ T cells to produce IFN-γ. The number of IFN-γ-producing CD8+ T cells was also related to asthma severity [140].

6.4.5. Interleukin 6

The most important source of IL-6 is mononuclear phagocytic cells but IL-6 is also produced by many other cells like T and B lymphocytes, endothelial cells, fibroblasts, hepatocytes, bone marrow cells [121].
IL-6 favours the differentiation of B lymphocytes into mature plasma cells and their ability to secrete immunoglobulin. The role of IL-6 is not limited to B cells as it can also promote T cell subsets. IL-6 is a potent regulatory factor playing a role in the switch of the immune response from the induction of Foxp3 regulatory T cells to pathogenic Th17 cells [141].
Classically IL-6 has been viewed as a pro- inflammatory cytokine, rather than as a regulatory cytokine. Recent advances have documented a series of IL-6 activities that are critical for resolving innate immunity and promoting acquired immune responses [142]. IL-6 signals through a receptor complex consisting of the subunit gp 130 that is classically activated through IL-6 binding to a membrane-bound cognate receptor IL-6R. However, many biological activities of IL-6 are mediated via a naturally occulting soluble receptor (IL-6 R) [143] which forms an agonistic complex with IL-6 that binds gp130 to trigger cellular responses. Regulation of this activity is called “IL-6 *trans*-signaling” and affords IL-6 to trigger responses in cell types that would remain unresponsive to IL-6 itself. Some studies showed that IL-6 was elevated in sputum [144] and serum [145] from asthmatic patients. However, its role in asthma remains unclear because of its dual pro-and anti-inflammatory roles and further studies are still needed.

6.4.6. Interleukin 10

IL-10 is produced by many cells including Th1 and Th2 lymphocytes, cytotoxic T cells, B lymphocytes, mast cells, and mononuclear phagocytic cells. IL-10 is a potent regulatory cytokine that decreases inflammatory responses and protects airways from developing inflammatory responses to inhaled allergens [146]. IL-10 activity is mediated by its specific cell surface receptor complex, which is expressed on a variety of cells and particularly on immune cells. This receptor is composed of two chains, α and β, both members of the class II cytokine family. They form a homodimer (IL-10R1 and IL10-R2) by which IL-10 can exert its biological activities. Some studies showed that JAK 1 and STAT 3 are required for IL-10 signalling [147].

IL-10 can suppress pro-inflammatory cytokine production and the antigen presenting capacity of macrophages, monocytes and dendritic cells. IL-10 also down regulates expression of TLR4, the signal-transducing receptor for LPS [148].

IL-10 strongly inhibited cytokine production and proliferation of CD4+ T cells via its down regulatory effects on APC function [149]. By contrast, IL-10 has stimulatory effects on CD8+ T cells and induces their recruitment, proliferation and cytotoxic activity [150].

Expression of IL-10 by APCs in the respiratory tract of healthy subjects has a critical role in the induction and maintenance of tolerance to allergens. In contrast, asthma and allergic rhinitis are often associated with a diminution of IL-10 expression in the allergic airways which contribute to the development of inflammation. Takanashi et al showed that IL-10 levels in sputum supernatant were decreased in asthma and COPD [151]. By contrast, other studies have shown an increased in IL-10 mRNA positive cells [152] and secretion of IL-10 by macrophages from BAL fluids of asthmatic patients when compared to healthy subjects [153].

The fact that IL-10 can inhibit eosinophil survival and IL-4 induced IgE synthesis supports the modulating role of IL-10 in human allergic diseases [154]. There is an antagonism between these inhibitory effects and the activating effects on B lymphocytes which stimulate immunoglobulin secretion and cell proliferation. Recombinant human IL-10 has been developed and is currently being tested in inflammatory bowel disease, rheumatoid arthritis, psoriasis, organ transplantation and chronic hepatitis C but its effect in asthma has yet to be studied [155].

6.4.7. Tumor necrosis factor alpha

TNF-α is mainly produced by mononuclear phagocytes (as opposed to TNF-β which is derived from lymphocytes) but can also be produced by many other cells like neutrophils, lymphocytes, natural killer cells, endothelial cells or mast cells [121]. TNF-α and TNF-β bind to the same two distinct cell-surface receptors, TNF-receptor I (p75) and TNF-receptor II (p55) with similar affinity and produce similar but not identical effects. The most important inducer of TNF by monocytes is LPS which acts through toll-like receptor 2 (TLR2) and TLR4.

TNF-α is an important cytokine in the innate immune response and provides immediate host defence against invading organisms before activation of the adaptive immune system [156]. TNF-α is a potent activator of neutrophils, mediating adherence, chemotaxis, degranulation and the respiratory burst. It has also been implicated in the initiation, maintenance and propagation of airway inflammation in asthma. Moreover, TNF-α has several properties that might be relevant to refractory asthma including recruitment of neutrophils [157], induction of glucocorticoid resistance [158], myocytes proliferation [159], and stimulation of fibroblast growth and maturation into myofibroblasts by promoting transforming growth factor-α expression [160].

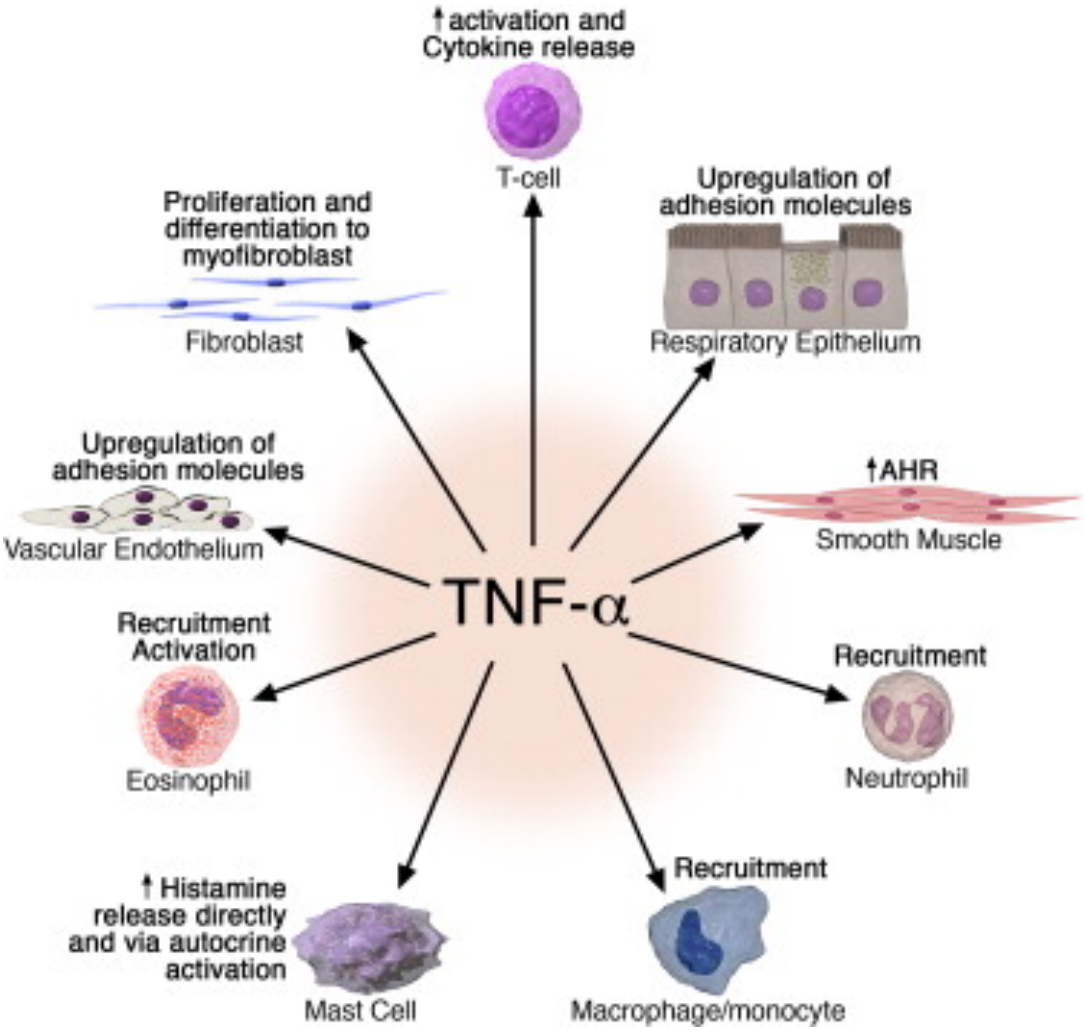


Fig 6. Role of TNF-α in the pathogenesis of asthma (from Brightling,C., JACI, 2008)

6.4.8. Interleukin 17

The IL-17 family is composed from five members, designated IL17A-F. IL-17 is produced by CD4+ T cells which constitute a distinct lineage, termed Th17 cells. In addition to Th17 cells, other cell types have also been described to produce IL-17 including natural killer cells, CD8+ T cells and lymphoid tissue inducer-like cells [161]. IL-17 is able to induce expression of a variety of cytokines and chemokines including IL-6, IL-11, GM-CSF, CXCL8, CXCL10 and TGF-β.

The main role of IL-17 is the recruitment and activation of neutrophils, either directly through IL-8 production [116] or indirectly by inducing the production of colony stimulating factor (CSF) and CXCL8 by tissue resident cells [162].

IL-17 measured by flow cytometry is upregulated in PBMC from allergic asthmatics and the level of the cytokine measured by ELISA is increased in serum and PBMC culture from the same patients [163]. Both in plasma and in activated blood mononuclear cells from allergic asthmatics, the increase of IL-17 is accompanied with higher levels of IL-23 that is a critical regulator of IL-17 [163].

Furthermore, Th17 cells and levels of IL-17 in airway and blood tend to increase with the magnitude of bronchial hyper-responsiveness and the disease severity in asthmatic patients [118]. In human subjects, hyper-IgE syndrome is associated with a genetic deficiency in Th17 cell differentiation [164]. The increasing susceptibility of these patients to infections with candida species and staphylococcus aureus is consistent with the role of Th17 cells in immunity against these pathogens [165].

Different strategies to down-regulate IL-17 responses by inhibition of molecules involved in IL-17 signaling or by blocking IL-17 itself by regulating the differentiation and activation of Th17 cells have been applied as a therapeutic approach for many inflammatory disorders [166]. As IL-23 is required for IL-17 from T cells, blocking IL-23 may be a promising therapeutic approach to reduce Il-17 production [167].

6.5. IgE

IgE plays an important role in allergy and is associated with type I hypersensitivity. An allergic reaction is initiated when an antigen crosslinks immunoglobulin E (IgE) antibodies bound to their high-affinity receptor [168]. There are two types of receptors FCε. The FCεRI which is the high-affinity IgE receptor is found on the surface of mast cells and basophils while FCεRII which is the low-affinity receptor (also known as CD23) is expressed on the surface of macrophages, eosinophils, platelets and some T cells. CD23 may also facilitate antigen presentation, an IgE-dependent mechanism whereby B cells expressing CD23 are able to present an allergen to specific T helper cells leading to a Th2 response with the production of antibodies [169].

Serum IgE levels were shown to be associated with asthma in population based studies irrespective of atopic status [170]. Local IgE production may also play a role in the pathogenesis of asthma. To our knowledge, very few studies have investigated the airway levels of sputum IgE in asthmatic disease. More than 10 years ago, Nahm et al validated the induced sputum model as a non-invasive method for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients. They found that house dust mite specific IgE were detectable in induced sputum supernatant from 7 of 10 house dust mite sensitive asthmatics based on skin prick tests [171]. Margarit et al showed, in a small group of asthmatics, that total IgE can be measured in induced sputum and was increased as compared to healthy subjects. Although they found sputum and serum IgE to be related, they did not find a correlation between sputum total IgE and albumin suggesting that sputum IgE could be, at least in part, locally produced [172]. It is assumed that IgE production is tightly regulated by the balance between Th1 and Th2 cytokines, interleukin-4 and 13 being involved in the immunological switch towards IgE [173]. A very recent study conducted by Mouthuy et al has shown that total IgE and specific IgE may be measurable in sputum from asthmatics irrespective of their atopic status even if their ability to prime local mast cells is still unclear in non atopic subjects [174].

7. Complementary treatment to inhaled corticosteroids in refractory asthma

The aims and objectives of a good treatment is to minimise or eliminate asthma symptoms, to achieve the best possible lung function, to prevent asthma exacerbations, to the above with the fewest drugs, to keep short-term and long-term adverse effects to a minimum and to educate the patient about the disease and goals of management. One other important objective should be prevention of decline in lung function and development of fixed airflow obstruction, which happens in some patients with severe asthma. Although a combination of inhaled corticoids and long-acting β2-agonist has proved to be very efficient in the large majority of asthmatic patients [175], a small group of asthmatics may remain uncontrolled.

Therapy-resistant asthma has been defined as persisting symptoms despite high-dose inhaled steroids plus long-acting β2-agonist, with the requirement for either maintenance systemic steroids or at least two rescue courses of steroids over 12 months despite additional medications like theophylline or leucotriene-receptor antagonist. For those patients with refractory asthma, new treatment have recently been validated which are often based on the inhibition of a mediator thought to be key in the pathophysiology. The particularity of those treatments is that they only apply to a targeted group of patients.

Among the new emerging treatment, omalizumab is certainly the one which has been the most convincingly validated so far [176] and the drug is currently used in clinical practice as a complementary treatment to combination therapy in severe refractory allergic asthma. Omalizumab is a recombinant humanized IgG1 monoclonal antibody which binds to human IgE at the same epitope as that of the high affinity IgE receptor (FceRI) on mast cells and basophils. Therefore omalizumab blocks IgE from binding to mast cells and basophils. Patients who can benefit from the use of omalizumab in Belgium are those with total serum IgE between 70 and 700 KU/l, a sensitization to a perennial aeroallergen including house dust mite, cats, dogs and/or moulds, at least 2 severe exacerbations in the last year and those who failed to be controlled despite high doses of inhaled and/or oral corticosteroids and long-acting b2-agonist. The drug is administered subcutaneously once every 2 or 4 weeks depending on the dose based on serum total IgE and weight. Omalizumab produces an improvement of symptoms and quality of life and a reduction of exacerbations [177-179]. Secondary effects are rare and consist of diarrhea, vomiting, headache or reactions at the injection site [178;180]. However, we need further studies to exclude long-term deleterious effect. On the other hand, omalizumab is considerably more expansive than conventional

asthma therapy. The cost of treatment may range from $4,000 to $20,000 per year, depending on the dose with an average of approximately $12,000 per year [181].

Identification of the inflammatory phenotype in refractory asthma seems to be of great interest as the demonstration of a persistent eosinophilic inflammation may allow the patient to benefit from monoclonal antibodies directed against interleukine-5. Mepolizumab is a humanized monoclonal antibody that recognizes interleukin-5, a cytokine known to stimulate the growth and tissular survival of eosinophils. Pilot monocentric studies have shown that, when administrated subcutaneously in severe refractory asthmatics, mepolizumab not only reduces blood and sputum eosinophilia but also, more importantly, asthma exacerbations [182;183]. The drug has, however, no effect on airway hyperresponsiveness (AHR), baseline airway calibre or symptoms. Large-scale multicentric studies are currenly ongoing in order to further validate this new treatment.

Among other potentially interesting new treatment are two other biologicals targeted against other Th2 cytokines. Pitrakinra is a recombinant human interleukin-4 variant that inhibits the interleukin-4Rα receptor complex and interferes with the actions of both interleukin 4 and interleukin 13 [184]. The data showed that the decrease in FEV1 after allergen challenge was significantly attenuated when compared to placebo after 4 weeks of inhalation of pitrakinra leading to the hypothesis that dual inhibition of IL-4 and IL-13 can affect the course of the late asthmatic response after experimental allergen challenge.

A very recent study using lebrikizumab, a monoclonal antibody to interleukin 13, in patients uncontrolled despite inhaled corticoids has found a small but significant improvement in airway calibre as measured by expiratory flow rates when compared to placebo. The lebrikizumab effect was, however, essentially seen in those patients with high pretreatment levels of serum periostin and high exhaled nitric oxide [185]. There is now a need to confirm results obtained with pitrakinra and lebrikizumab in large scale and long-term studies.

In contrast to what has been shown for Crohn disease and rheumatoid arthritis, the efficacy of anti-TNF therapies in subjects with moderate to severe asthma has been disappointing so far. Although an early pilot study brought great promise [186], the most recent studies investigating several treatment against TNF-α did not report significant clinical improvement in moderate and severe asthmatics. Infliximab failed to demonstrate clinical efficacy in subjects with moderate asthma [187].

Similarly etanercept failed to improve asthma control, lung function, bronchial hyper responsiveness and to reduce asthma exacerbation in severe asthmatics [188].

Interleukine-2 is a key cytokine in lymphocyte biological function. Daclizumab is a humanized IgG1 monoclonal antibody against the IL-2Rα chain (CD25) of activated lymphocyte. When administered intravenously every 2 weeks, Daclizumab improved pulmonary function and asthma control in patients with moderate to severe chronic asthma inadequately controlled on ICS [189].

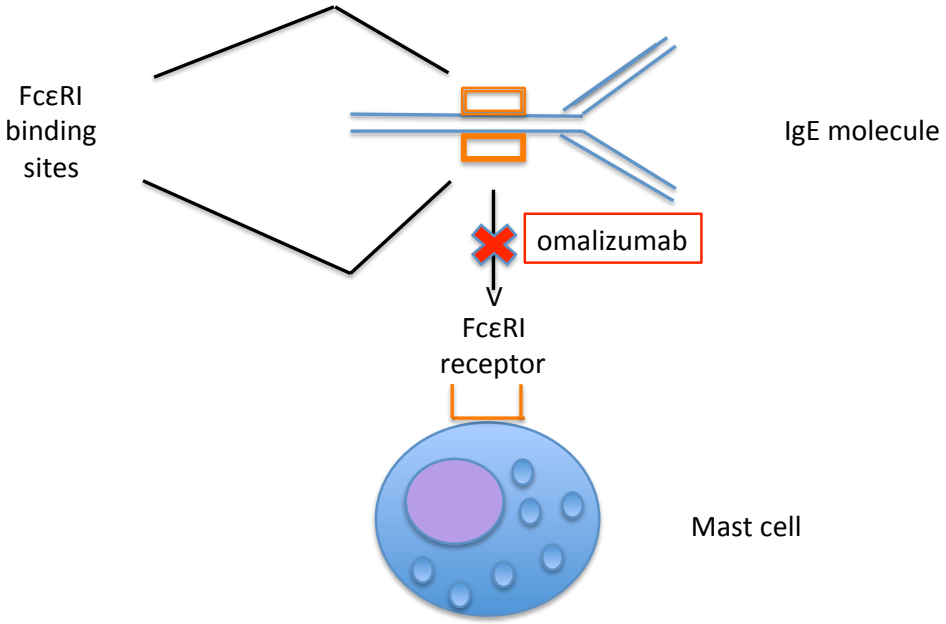


Fig 7. Omalizumab (Anti-IgE): mechanism of action.

References

1. Global Initiative for Asthma. Global strategy for asthma management and prevention NHLBI/WHO workshop report March 1993. National Institutes of Health. National Heart, Lung and Blood Institute. Publication Number 95-3659. January 1995. 1995. Ref Type: Generic

2. O'Byrne PM, Parameswaran K. Pharmacological management of mild or moderate persistent asthma. Lancet 2006; 368:794-803.

3. Chung KF, Godard P, Adelroth E, Ayres J, Barnes N, Barnes P, Bel E, Burney P, Chanez P, Connett G, Corrigan C, de Blic J, Fabbri L, Holgate ST, Ind P, Joos G, Kerstjens H, Leuenberger P, Lofdahl CG, McKenzie S, Magnussen H, Postma D, Saetta M, Salmeron S, Sterk P. Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. Eur Respir J 1999; 13:1198-208.

4. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000; 162:2341-51.

5. Chanez P, Wenzel SE, Anderson GP, Anto JM, Bel EH, Boulet LP, Brightling CE, Busse WW, Castro M, Dahlen B, Dahlen SE, Fabbri LM, Holgate ST, Humbert M, Gaga M, Joos GF, Levy B, Rabe KF, Sterk PJ, Wilson SJ, Vachier I. Severe asthma in adults: what are the important questions? J Allergy Clin Immunol 2007; 119:1337-48.

6. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy 2004; 59:469-78.

7. Wenzel S. Severe asthma in adults. Am J Respir Crit Care Med 2005; 172:149-60.

8. Gamble J, Stevenson M, McClean E, Heaney LG. The prevalence of nonadherence in difficult asthma. Am J Respir Crit Care Med 2009; 180:817-22.

9. Durham A, Adcock IM, Tliba O. Steroid resistance in severe asthma: current mechanisms and future treatment. Curr Pharm Des 2011; 17:674-84.

10. Smith DH, Malone DC, Lawson KA, Okamoto LJ, Battista C, Saunders WB. A national estimate of the economic costs of asthma. Am J Respir Crit Care Med 1997; 156:787-93.

11. Barnes PJ, Woolcock AJ. Difficult asthma. Eur Respir J 1998; 12:1209-18.

12. Molimard M, de Blay F, Didier A, Le G, V. Effectiveness of omalizumab (Xolair) in the first patients treated in real-life practice in France. Respir Med 2008; 102:71-6.

13. Schleich F, Manise M, Louis R. [Omralizumab (Xolair) in severe persistent allergic asthma]. Rev Med Liege 2009; 64:313-7.

14. Wu AC, Paltiel AD, Kuntz KM, Weiss ST, Fuhlbrigge AL. Cost-effectiveness of omalizumab in adults with severe asthma: results from the Asthma Policy Model. J Allergy Clin Immunol 2007; 120:1146-52.

15. Serra-Batlles J, Plaza V, Morejon E, Comella A, Brugues J. Costs of asthma according to the degree of severity. Eur Respir J 1998; 12:1322-6.

16. Willemsen G, van Beijsterveldt TC, van Baal CG, Postma D, Boomsma DI. Heritability of self-reported asthma and allergy: a study in adult Dutch twins, siblings and parents. Twin Res Hum Genet 2008; 11:132-42.

17. Holgate ST. Genetic and environmental interaction in allergy and asthma. J Allergy Clin Immunol 1999; 104:1139-46.

18. Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun 2006; 7:95-100.

19. Contopoulos-Ioannidis DG, Manoli EN, Ioannidis JP. Meta-analysis of the association of beta2-adrenergic receptor polymorphisms with asthma phenotypes. J Allergy Clin Immunol 2005; 115:963-72.

20. Silverman ES, Palmer LJ, Subramaniam V, Hallock A, Mathew S, Vallone J, Faffe DS, Shikanai T, Raby BA, Weiss ST, Shore SA. Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am J Respir Crit Care Med 2004; 169:214-9.

21. Lose F, Thompson PJ, Duffy D, Stewart GA, Kedda MA. A novel tissue inhibitor of metalloproteinase-1 (TIMP-1) polymorphism associated with asthma in Australian women. Thorax 2005; 60:623-8.

22. Sandford AJ, Chagani T, Zhu S, Weir TD, Bai TR, Spinelli JJ, Fitzgerald JM, Behbehani NA, Tan WC, Pare PD. Polymorphisms in the IL4, IL4RA, and FCERIB genes and asthma severity. J Allergy Clin Immunol 2000; 106:135-40.

23. Holgate ST, Davies DE, Powell RM, Holloway JW. ADAM33: a newly identified gene in the pathogenesis of asthma. Immunol Allergy Clin North Am 2005; 25:655-68.

24. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu Z, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Benchekroun Y, Capparell N, Wang M, Adair R, Feng Y, Dubois J, FitzGerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST, Keith TP. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature 2002; 418:426-30.

25. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, Depner M, von Berg A, Bufe A, Rietschel E, Heinzmann A, Simma B, Frischer T, Willis-Owen SA,

Wong KC, Illig T, Vogelberg C, Weiland SK, von Mutius E, Abecasis GR, Farrall M, Gut IG, Lathrop GM, Cookson WO. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007; 448:470-3.

26. Galanter J, Choudhry S, Eng C, Nazario S, Rodriguez-Santana JR, Casal J, Torres-Palacios A, Salas J, Chapela R, Watson HG, Meade K, LeNoir M, Rodriguez-Cintron W, Avila PC, Burchard EG. ORMDL3 gene is associated with asthma in three ethnically diverse populations. Am J Respir Crit Care Med 2008; 177:1194-200.

27. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. European Network for Understanding Mechanisms of Severe Asthma. Eur Respir J 2003; 22:470-7.

28. Zureik M, Neukirch C, Leynaert B, Liard R, Bousquet J, Neukirch F. Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey. BMJ 2002; 325:411-4.

29. Gibson PG. Allergic bronchopulmonary aspergillosis. Semin Respir Crit Care Med 2006; 27:185-91.

30. Louhelainen N, Rytila P, Haahtela T, Kinnula VL, Djukanovic R. Persistence of oxidant and protease burden in the airways after smoking cessation. BMC Pulm Med 2009; 9:25.

31. Chaudhuri R, Livingston E, McMahon AD, Thomson L, Borland W, Thomson NC. Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. Am J Respir Crit Care Med 2003; 168:1308-11.

32. Gusbin N, Garzaniti N, Louis R. [Asthma and tobacco]. Rev Med Liege 2006; 61:81-6.

33. Kuschner WG, D'Alessandro A, Wong H, Blanc PD. Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. Eur Respir J 1996; 9:1989-94.

34. James AL, Palmer LJ, Kicic E, Maxwell PS, Lagan SE, Ryan GF, Musk AW. Decline in lung function in the Busselton Health Study: the effects of asthma and cigarette smoking. Am J Respir Crit Care Med 2005; 171:109-14.

35. Mapp CE, Boschetto P, Maestrelli P, Fabbri LM. Occupational asthma. Am J Respir Crit Care Med 2005; 172:280-305.

36. Brisman J. Baker's asthma. Occup Environ Med 2002; 59:498-502.

37. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, Slater L, Lewis-Antes A, Kon OM, Holgate ST, Davies DE, Kotenko SV, Papi A, Johnston SL. Role of deficient type III interferon-lambda production in asthma exacerbations. Nat Med 2006; 12:1023-6.

38. Johnston NW, Sears MR. Asthma exacerbations . 1: epidemiology. Thorax 2006; 61:722-8.

39. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, Printz MC, Lee WM, Shult PA, Reisdorf E, Carlson-Dakes KT, Salazar LP, DaSilva DF, Tisler CJ, Gern JE, Lemanske RF, Jr. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med 2008; 178:667-72.

40. Nicholson KG, Kent J, Hammersley V, Cancio E. Risk factors for lower respiratory complications of rhinovirus infections in elderly people living in the community: prospective cohort study. BMJ 1996; 313:1119-23.

41. Papadopoulos NG, Christodoulou I, Rohde G, Agache I, Almqvist C, Bruno A, Bonini S, Bont L, Bossios A, Bousquet J, Braido F, Brusselle G, Canonica GW, Carlsen KH, Chanez P, Fokkens WJ, Garcia-Garcia M, Gjomarkaj M, Haahtela T, Holgate ST, Johnston SL, Konstantinou G, Kowalski M, Lewandowska-Polak A, Lodrup-Carlsen K, Makela M, Malkusova I, Mullol J, Nieto A, Eller E, Ozdemir C, Panzner P, Popov T, Psarras S, Roumpedaki E, Rukhadze M, Stipic-Markovic A, Todo BA, Toskala E, van Cauwenberge P, van Drunen C, Watelet JB, Xatzipsalti M, Xepapadaki P, Zuberbier T. Viruses and bacteria in acute asthma exacerbations--a GA(2) LEN-DARE systematic review. Allergy 2011; 66:458-68.

42. Mallia P, Johnston SL. How viral infections cause exacerbation of airway diseases. Chest 2006; 130:1203-10.

43. Richeldi L, Ferrara G, Fabbri LM, Lasserson TJ, Gibson PG. Macrolides for chronic asthma. Cochrane Database Syst Rev 2005;CD002997.

44. Morwood K, Gillis D, Smith W, Kette F. Aspirin-sensitive asthma. Intern Med J 2005; 35:240-6.

45. Wright RJ, Rodriguez M, Cohen S. Review of psychosocial stress and asthma: an integrated biopsychosocial approach. Thorax 1998; 53:1066-74.

46. Lehrer P, Feldman J, Giardino N, Song HS, Schmaling K. Psychological aspects of asthma. J Consult Clin Psychol 2002; 70:691-711.

47. Opolski M, Wilson I. Asthma and depression: a pragmatic review of the literature and recommendations for future research. Clin Pract Epidemiol Ment Health 2005; 1:18.

48. Rubin NJ. Severe asthma and depression. Arch Fam Med 1993; 2:433-40.

49. Skobeloff EM, Spivey WH, Silverman R, Eskin BA, Harchelroad F, Alessi TV. The effect of the menstrual cycle on asthma presentations in the emergency department. Arch Intern Med 1996; 156:1837-40.

50. Murphy VE, Gibson PG, Smith R, Clifton VL. Asthma during pregnancy: mechanisms and treatment implications. Eur Respir J 2005; 25:731-50.

51. Shore SA. Obesity and asthma: cause for concern. Curr Opin Pharmacol 2006; 6:230-6.

52. Sood A, Ford ES, Camargo CA, Jr. Association between leptin and asthma in adults. Thorax 2006; 61:300-5.

53. Bresciani M, Paradis L, Des RA, Vernhet H, Vachier I, Godard P, Bousquet J, Chanez P. Rhinosinusitis in severe asthma. J Allergy Clin Immunol 2001; 107:73-80.

54. ten Brinke A, Grootendorst DC, Schmidt JT, De Bruine FT, van Buchem MA, Sterk PJ, Rabe KF, Bel EH. Chronic sinusitis in severe asthma is related to sputum eosinophilia. J Allergy Clin Immunol 2002; 109:621-6.

55. Gibson PG, Henry RL, Coughlan JL. Gastro-oesophageal reflux treatment for asthma in adults and children. Cochrane Database Syst Rev 2003;CD001496.

56. McCallister JW, Parsons JP, Mastronarde JG. The relationship between gastroesophageal reflux and asthma: an update. Ther Adv Respir Dis 2011; 5:143-50.

57. Kiljander TO, Harding SM, Field SK, Stein MR, Nelson HS, Ekelund J, Illueca M, Beckman O, Sostek MB. Effects of esomeprazole 40 mg twice daily on asthma: a randomized placebo-controlled trial. Am J Respir Crit Care Med 2006; 173:1091-7.

58. Hsu CS, Kao JH. Esomeprazole for asthma. N Engl J Med 2009; 361:206-8.

59. Kahrilas PJ. Obstructive sleep apnea and reflux disease: bedfellows at best. Chest 2010; 137:747-8.

60. Mehra R, Redline S. Sleep apnea: a proinflammatory disorder that coaggregates with obesity. J Allergy Clin Immunol 2008; 121:1096-102.

61. Heaney LG, Robinson DS. Severe asthma treatment: need for characterising patients. Lancet 2005; 365:974-6.

62. Fabbri LM, Romagnoli M, Corbetta L, Casoni G, Busljetic K, Turato G, Ligabue G, Ciaccia A, Saetta M, Papi A. Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2003; 167:418-24.

63. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. Am J Respir Crit Care Med 2000; 161:9-16.

64. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. Am J Respir Crit Care Med 1999; 160:1001-8.

65. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. Am J Respir Crit Care Med 1999; 160:1532-9.

66. Berry MA, Shaw DE, Green RH, Brightling CE, Wardlaw AJ, Pavord ID. The use of exhaled nitric oxide concentration to identify eosinophilic airway inflammation: an observational study in adults with asthma. Clin Exp Allergy 2005; 35:1175-9.

67. Schleich FN, Seidel L, Sele J, Manise M, Quaedvlieg V, Michils A, Louis R. Exhaled nitric oxide thresholds associated with a sputum eosinophil count >/=3% in a cohort of unselected patients with asthma. Thorax 2010; 65:1039-44.

68. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999; 17:189-220.

69. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol 2002; 2:933-44.

70. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005; 6:345-52.

71. Mamessier E, Botturi K, Vervloet D, Magnan A. [T regulatory lymphocytes, atopy and asthma: a new concept in three dimensions]. Rev Mal Respir 2005; 22:305-11.

72. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood 2008; 112:1570-80.

73. Corrigan CJ, Hamid Q, North J, Barkans J, Moqbel R, Durham S, Gemou-Engesaeth V, Kay AB. Peripheral blood CD4 but not CD8 t-lymphocytes in patients with exacerbation of asthma transcribe and translate messenger RNA encoding cytokines which prolong eosinophil survival in the context of a Th2-type pattern: effect of glucocorticoid therapy. Am J Respir Cell Mol Biol 1995; 12:567-78.

74. Provoost S, Maes T, van Durme YM, Gevaert P, Bachert C, Schmidt-Weber CB, Brusselle GG, Joos GF, Tournoy KG. Decreased FOXP3 protein expression in patients with asthma. Allergy 2009; 64:1539-46.

75. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004; 22:531-62.

76. Smyth LJ, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. Chest 2010; 138:905-12.

77. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallesco G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1999; 160:711-7.

78. van Rensen EL, Sont JK, Evertse CE, Willems LN, Mauad T, Hiemstra PS, Sterk PJ. Bronchial CD8 cell infiltrate and lung function decline in asthma. Am J Respir Crit Care Med 2005; 172:837-41.

79. Klion AD, Nutman TB. The role of eosinophils in host defense against helminth parasites. J Allergy Clin Immunol 2004; 113:30-7.

80. Bochner BS. Systemic activation of basophils and eosinophils: markers and consequences. J Allergy Clin Immunol 2000; 106:S292-S302.

81. Wood LJ, Inman MD, Denburg JA, O'Byrne PM. Allergen challenge increases cell traffic between bone marrow and lung. Am J Respir Cell Mol Biol 1998; 18:759-67.

82. Bettiol J, Sele J, Henket M, Louis E, Malaise M, Bartsch P, Louis R. Cytokine production from sputum cells after allergenic challenge in IgE-mediated asthma. Allergy 2002; 57:1145-50.

83. Torrent M, Navarro S, Moussaoui M, Nogues MV, Boix E. Eosinophil cationic protein high-affinity binding to bacteria-wall lipopolysaccharides and peptidoglycans. Biochemistry 2008; 47:3544-55.

84. Fujimoto K, Kubo K, Matsuzawa Y, Sekiguchi M. Eosinophil cationic protein levels in induced sputum correlate with the severity of bronchial asthma. Chest 1997; 112:1241-7.

85. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. Thorax 1998; 53:91-5.

86. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simony-Lafontaine J, Godard P, . Eosinophilic inflammation in asthma. N Engl J Med 1990; 323:1033-9.

87. Grewe M, Czech W, Morita A, Werfel T, Klammer M, Kapp A, Ruzicka T, Schopf E, Krutmann J. Human eosinophils produce biologically active IL-12: implications for control of T cell responses. J Immunol 1998; 161:415-20.

88. Woerly G, Roger N, Loiseau S, Dombrowicz D, Capron A, Capron M. Expression of CD28 and CD86 by human eosinophils and role in the secretion of type 1 cytokines (interleukin 2 and interferon gamma): inhibition by immunoglobulin a complexes. J Exp Med 1999; 190:487-95.

89. Dente FL, Bacci E, Bartoli ML, Cianchetti S, Costa F, Di Franco A, Malagrino L, Vagaggini B, Paggiaro P. Effects of oral prednisone on sputum eosinophils and cytokines in patients with severe refractory asthma. Ann Allergy Asthma Immunol 2010; 104:464-70.

90. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. Microbes Infect 2003; 5:1299-306.

91. Babior BM. The leukocyte NADPH oxidase. Isr Med Assoc J 2002; 4:1023-4.

92. Kantari C, Pederzoli-Ribeil M, Witko-Sarsat V. The role of neutrophils and monocytes in innate immunity. Contrib Microbiol 2008; 15:118-46.

93. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax 2002; 57:875-9.

94. Adcock IM, Lane SJ, Brown CR, Lee TH, Barnes PJ. Abnormal glucocorticoid receptor-activator protein 1 interaction in steroid-resistant asthma. J Exp Med 1995; 182:1951-8.

95. Kikuchi S, Kikuchi I, Takaku Y, Kobayashi T, Hagiwara K, Kanazawa M, Nagata M. Neutrophilic inflammation and CXC chemokines in patients with refractory asthma. Int Arch Allergy Immunol 2009; 149 Suppl 1:87-93.

96. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. Am J Respir Crit Care Med 2008; 177:148-55.

97. Voehringer D. The role of basophils in helminth infection. Trends Parasitol 2009; 25:551-6.

98. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu Rev Immunol 2005; 23:749-86.

99. Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q, Humbert M, Barnes NC, Robinson DS, Ying S, Kay AB. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol 2000; 105:99-107.

100. MacGlashan D, Jr. Granulocytes: new roles for basophils. Immunol Cell Biol 2008; 86:637-8.

101. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8:958-69.

102. Viksman MY, Liu MC, Bickel CA, Schleimer RP, Bochner BS. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. I. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. Am J Respir Crit Care Med 1997; 155:858-63.

103. Bhavsar P, Hew M, Khorasani N, Torrego A, Barnes PJ, Adcock I, Chung KF. Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. Thorax 2008; 63:784-90.

104. Mota PA, Todo-Bom A. [The role of the epithelial cell in asthma]. Rev Port Pneumol 2009; 15:461-72.

105. Holgate ST. Asthma: more than an inflammatory disease. Curr Opin Allergy Clin Immunol 2002; 2:27-9.

106. Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol 2007; 120:1233-44.

107. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. FASEB J 2000; 14:1362-74.

108. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. Am J Respir Crit Care Med 2005; 171:431-9.

109. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jasmin C, Canonica GW, Azzarone B. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. Int Immunol 1998; 10:1421-33.

110. Kay AB. Origin of type 2 helper T cells. N Engl J Med 1994; 330:567-9.

111. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009; 180:388-95.

112. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 2002; 57:643-8.

113. Zhang N, Truong-Tran QA, Tancowny B, Harris KE, Schleimer RP. Glucocorticoids enhance or spare innate immunity: effects in airway epithelium are mediated by CCAAT/enhancer binding proteins. J Immunol 2007; 179:578-89.

114. Smart JM, Horak E, Kemp AS, Robertson CF, Tang ML. Polyclonal and allergen-induced cytokine responses in adults with asthma: resolution of asthma is associated with normalization of IFN-gamma responses. J Allergy Clin Immunol 2002; 110:450-6.

115. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002; 347:869-77.

116. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, Cosmi L, Lunardi C, Annunziato F, Romagnani S, Cassatella MA. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood 2010; 115:335-43.

117. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells-origins, features and possible roles in rheumatic disease. Nat Rev Rheumatol 2009; 5:325-31.

118. Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med 2003; 97:726-33.

119. Foley SC, Hamid Q. Images in allergy and immunology: neutrophils in asthma. J Allergy Clin Immunol 2007; 119:1282-6.

120. Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, Ceuppens JL. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respir Res 2006; 7:135.

121. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol 2010; 125:S53-S72.

122. Kaminuma O, Mori A, Kitamura N, Hashimoto T, Kitamura F, Inokuma S, Miyatake S. Role of GATA-3 in IL-5 gene transcription by CD4+ T cells of asthmatic patients. Int Arch Allergy Immunol 2005; 137 Suppl 1:55-9.

123. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T, Akira S. Essential role of Stat6 in IL-4 signalling. Nature 1996; 380:627-30.

124. Oh CK, Geba GP, Molfino N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. Eur Respir Rev 2010; 19:46-54.

125. Coffman RL, Ohara J, Bond MW, Carty J, Zlotnik A, Paul WE. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. J Immunol 1986; 136:4538-41.

126. 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.

127. Hsieh FH, Lam BK, Penrose JF, Austen KF, Boyce JA. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C(4) synthase expression by interleukin 4. J Exp Med 2001; 193:123-33.

128. Bergeron C, Page N, Barbeau B, Chakir J. Interleukin-4 promotes airway remodeling in asthma: regulation of procollagen I (alpha1) gene by interleukin-4. Chest 2003; 123:424S.

129. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. J Exp Med 1998; 187:939-48.

130. Saha SK, Berry MA, Parker D, Siddiqui S, Morgan A, May R, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Increased sputum and bronchial biopsy IL-13 expression in severe asthma. J Allergy Clin Immunol 2008; 121:685-91.

131. Renauld JC. New insights into the role of cytokines in asthma. J Clin Pathol 2001; 54:577-89.

132. Motojima S, Akutsu I, Fukuda T, Makino S, Takatsu K. Clinical significance of measuring levels of sputum and serum ECP and serum IL-5 in bronchial asthma. Allergy 1993; 48:98-106.

133. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326:298-304.

134. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997; 89:587-96.

135. Billiau A, Matthys P. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev 2009; 20:97-113.

136. Nakane A, Minagawa T, Yasuda I, Yu C, Kato K. Prevention by gamma interferon of fatal infection with Listeria monocytogenes in mice treated with cyclosporin A. Infect Immun 1988; 56:2011-5.

137. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M. Immune response in mice that lack the interferon-gamma receptor. Science 1993; 259:1742-5.

138. Cosentini R, Tarsia P, Canetta C, Graziadei G, Brambilla AM, Aliberti S, Pappalettera M, Tantardini F, Blasi F. Severe asthma exacerbation: role of acute Chlamydophila pneumoniae and Mycoplasma pneumoniae infection. Respir Res 2008; 9:48.

139. Shannon J, Ernst P, Yamauchi Y, Olivenstein R, Lemiere C, Foley S, Cicora L, Ludwig M, Hamid Q, Martin JG. Differences in airway cytokine profile in severe asthma compared to moderate asthma. Chest 2008; 133:420-6.

140. Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM, Casano BB, Prato SJ, Fert V, Bongrand P, Vervloet D. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. Am J Respir Crit Care Med 2000; 161:1790-6.

141. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, Vollmar P, Stritesky GL, Kaplan MH, Waisman A, Kuchroo VK, Oukka M. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A 2008; 105:18460-5.

142. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. Nat Immunol 2004; 5:971-4.

143. Jones SA, Rose-John S. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. Biochim Biophys Acta 2002; 1592:251-63.

144. Neveu WA, Allard JL, Raymond DM, Bourassa LM, Burns SM, Bunn JY, Irvin CG, Kaminsky DA, Rincon M. Elevation of IL-6 in the allergic asthmatic airway is independent of inflammation but associates with loss of central airway function. Respir Res 2010; 11:28.

145. Yokoyama A, Kohno N, Fujino S, Hamada H, Inoue Y, Fujioka S, Ishida S, Hiwada K. Circulating interleukin-6 levels in patients with bronchial asthma. Am J Respir Crit Care Med 1995; 151:1354-8.

146. Moore KW, O'Garra A, de Waal MR, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993; 11:165-90.

147. Finbloom DS, Larner AC. Regulation of the Jak/STAT signalling pathway. Cell Signal 1995; 7:739-45.

148. Muzio M, Bosisio D, Polentarutti N, D'amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, Mantovani A. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 2000; 164:5998-6004.

149. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991; 147:3815-22.

150. Santin AD, Hermonat PL, Ravaggi A, Bellone S, Pecorelli S, Roman JJ, Parham GP, Cannon MJ. Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8(+) cytotoxic T lymphocytes. J Virol 2000; 74:4729-37.

151. Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, Okamura K. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. Eur Respir J 1999; 14:309-14.

152. Robinson DS, Tsicopoulos A, Meng Q, Durham S, Kay AB, Hamid Q. Increased interleukin-10 messenger RNA expression in atopic allergy and asthma. Am J Respir Cell Mol Biol 1996; 14:113-7.

153. Magnan A, van Pee D, Bongrand P, Vervloet D. Alveolar macrophage interleukin (IL)-10 and IL-12 production in atopic asthma. Allergy 1998; 53:1092-5.

154. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol 2003; 111:S460-S475.

155. Desai D, Brightling C. Cytokine and anti-cytokine therapy in asthma: ready for the clinic? Clin Exp Immunol 2009; 158:10-9.

156. Medzhitov R, Janeway C, Jr. Innate immunity. N Engl J Med 2000; 343:338-44.

157. Thomas PS, Yates DH, Barnes PJ. Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. Am J Respir Crit Care Med 1995; 152:76-80.

158. Franchimont D, Martens H, Hagelstein MT, Louis E, Dewe W, Chrousos GP, Belaiche J, Geenen V. Tumor necrosis factor alpha decreases, and interleukin-10 increases, the sensitivity of human monocytes to dexamethasone: potential regulation of the glucocorticoid receptor. J Clin Endocrinol Metab 1999; 84:2834-9.

159. Amrani Y, Panettieri RA, Jr., Frossard N, Bronner C. Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. Am J Respir Cell Mol Biol 1996; 15:55-63.

160. Sullivan DE, Ferris M, Pociask D, Brody AR. Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway. Am J Respir Cell Mol Biol 2005; 32:342-9.

161. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, Littman DR, O'Shea JJ. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009; 206:35-41.

162. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008; 28:454-67.

163. Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. Int Arch Allergy Immunol 2010; 151:297-307.

164. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J Exp Med 2008; 205:1551-7.

165. Minegishi Y, Saito M, Nagasawa M, Takada H, Hara T, Tsuchiya S, Agematsu K, Yamada M, Kawamura N, Ariga T, Tsuge I, Karasuyama H. Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome. J Exp Med 2009; 206:1291-301.

166. Ivanov S, Linden A. Interleukin-17 as a drug target in human disease. Trends Pharmacol Sci 2009; 30:95-103.

167. Wakashin H, Hirose K, Iwamoto I, Nakajima H. Role of IL-23-Th17 cell axis in allergic airway inflammation. Int Arch Allergy Immunol 2009; 149 Suppl 1:108-12.

168. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker HA, Fear D, Smurthwaite L. The biology of IGE and the basis of allergic disease. Annu Rev Immunol 2003; 21:579-628.

169. Poole JA, Matangkasombut P, Rosenwasser LJ. Targeting the IgE molecule in allergic and asthmatic diseases: review of the IgE molecule and clinical efficacy. J Allergy Clin Immunol 2005; 115:S376-S385.

170. Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N Engl J Med 1989; 320:271-7.

171. Nahm DH, Park HS. Analysis of induced sputum for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients. Clin Exp Allergy 1998; 28:686-93.

172. Margarit G, Belda J, Juarez C, Martinez C, Ramos A, Torrejon M, Granel C, Casan P, Sanchis J. [Total IgE in the sputum and serum of patients with asthma]. Allergol Immunopathol (Madr) 2005; 33:48-53.

173. Levy F, Kristofic C, Heusser C, Brinkmann V. Role of IL-13 in CD4 T cell-dependent IgE production in atopy. Int Arch Allergy Immunol 1997; 112:49-58.

174. Mouthuy J, Detry B, Sohy C, Pirson F, Pilette C. Presence in sputum of functional dust mite-specific IgE antibodies in intrinsic asthma. Am J Respir Crit Care Med 2011; 184:206-14.

175. Bateman ED, Boushey HA, Bousquet J, Busse WW, Clark TJ, Pauwels RA, Pedersen SE. Can guideline-defined asthma control be achieved? The Gaining Optimal Asthma ControL study. Am J Respir Crit Care Med 2004; 170:836-44.

176. Holgate S, Casale T, Wenzel S, Bousquet J, Deniz Y, Reisner C. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. J Allergy Clin Immunol 2005; 115:459-65.

177. Busse WW, Massanari M, Kianifard F, Geba GP. Effect of omalizumab on the need for rescue systemic corticosteroid treatment in patients with moderate-to-severe persistent IgE-mediated allergic asthma: a pooled analysis. Curr Med Res Opin 2007; 23:2379-86.

178. Humbert M, Berger W, Rapatz G, Turk F. Add-on omalizumab improves day-to-day symptoms in inadequately controlled severe persistent allergic asthma. Allergy 2008; 63:592-6.

179. Molimard M, de Blay F, Didier A, Le G, V. Effectiveness of omalizumab (Xolair) in the first patients treated in real-life practice in France. Respir Med 2008; 102:71-6.

180. Lanier BQ, Corren J, Lumry W, Liu J, Fowler-Taylor A, Gupta N. Omalizumab is effective in the long-term control of severe allergic asthma. Ann Allergy Asthma Immunol 2003; 91:154-9.

181. Strunk RC, Bloomberg GR. Omalizumab for asthma. N Engl J Med 2006; 354:2689-95.

182. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, Marshall RP, Bradding P, Green RH, Wardlaw AJ, Pavord ID. Mepolizumab and exacerbations of refractory eosinophilic asthma. N Engl J Med 2009; 360:973-84.

183. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, Hargreave FE, O'Byrne PM. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med 2009; 360:985-93.

184. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. Lancet 2007; 370:1422-31.

185. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, Mosesova S, Eisner MD, Bohen SP, Matthews JG. Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365:1088-98.

186. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, Bradding P, Brightling CE, Wardlaw AJ, Pavord ID. Evidence of a role of tumor necrosis factor alpha in refractory asthma. N Engl J Med 2006; 354:697-708.

187. Erin EM, Leaker BR, Nicholson GC, Tan AJ, Green LM, Neighbour H, Zacharasiewicz AS, Turner J, Barnathan ES, Kon OM, Barnes PJ, Hansel TT. The effects of a monoclonal antibody directed against tumor necrosis factor-alpha in asthma. Am J Respir Crit Care Med 2006; 174:753-62.

188. Holgate ST, Noonan M, Chanez P, Busse W, Dupont L, Pavord I, Hakulinen A, Paolozzi L, Wajdula J, Zang C, Nelson H, Raible D. Efficacy and safety of etanercept in moderate-to-severe asthma: a randomised, controlled trial. Eur Respir J 2011; 37:1352-9.

189. Busse WW, Israel E, Nelson HS, Baker JW, Charous BL, Young DY, Vexler V, Shames RS. Daclizumab improves asthma control in patients with moderate to severe persistent asthma: a randomized, controlled trial. Am J Respir Crit Care Med 2008; 178:1002-8.

II. Publication 1

Asthme réfractaire: mécanismes sous-jacents, diagnostic et nouvelles approches thérapeutiques

M. Manise, R. Louis

Rev Med Liege 2008; 63, 494-99

ASTHME RÉFRACTAIRE : mécanismes sous-jacents, diagnostics et nouvelles approches thérapeutiques

M. Manise (1), R. Louis (2)

RÉSUMÉ : La prévalence de l'asthme a récemment augmenté dans le monde entier. La plupart des cas peuvent être pris en charge efficacement par l'administration de médicaments anti-inflammatoires et de bronchodilatateurs. Toutefois, environ 10% des patients demeurent insuffisamment contrôlés en dépit de l'utilisation de hautes doses de corticostéroïdes inhalés et de β-2 agonistes à longue durée d'action. Ces personnes constituent un groupe hétérogène englobant des patients soit sous-traités, soit réellement réfractaires aux traitements actuels.

Mots-clés : *Asthme réfractaire - Phénotypes - Mécanismes - Traitement*

Refractory asthma : underlying mechanisms, diagnosis and new therapeutic approach

SUMMARY : There has been a recent increase in the prevalence of asthma worldwide. Most cases can be satisfactorily managed with a combination of inhaled corticoids and bronchodilators. However, some 10% of patients remain symptomatic despite high doses of inhaled corticosteroids and long-acting β-2 agonists. They represent a heterogeneous group consisting of those who are either undertreated, or really refractory to current available treatment.

Keywords : *Refractory asthma - Phenotypes - Mechanisms - Treatment*

Introduction

Au cours des dernières années, la littérature a insisté sur le caractère hétérogène de la maladie asthmatique en identifiant plusieurs phénotypes (1). La connaissance du phénotype d'un patient asthmatique pourrait constituer une avancée sérieuse dans la prise en charge de la pathologie. Il existe 3 grandes catégories de phénotypes : les phénotypes définis par des critères cliniques/physiologiques, les phénotypes en relation avec des facteurs environnementaux et les phénotypes inflammatoires.

Cependant, des interactions potentielles existent entre ces groupes. Ainsi, l'exposition à certains agents environnementaux détermine le profil inflammatoire bronchique. Par ailleurs, un phénotype donné n'est pas fixé de façon permanente; il peut en effet varier en fonction des interactions avec l'environnement (Tableau I) (2).

L'asthme réfractaire constitue un phénotype clinique d'asthme caractérisé par une résistance aux effets combinés de hautes doses de corticoïdes inhalés et de β-2 mimétiques à longue durée d'action. Bien que de nombreux facteurs environnementaux comme les allergènes, la fumée de cigarette, la pollution atmosphérique, les infections, les hormones et certains médicaments spécifiques puissent contribuer à ce phénotype, d'autres facteurs associés à des modifications de la réponse inflammatoire au niveau des voies respiratoires doivent être pris en compte (3).

Selon le consensus de l'American Thoracic Society (ATS), l'asthme sévère/réfractaire se définit par la présence d'au moins 1 critère majeur et 2 critères mineurs repris dans le Tableau II (4).

Les patients avec asthme réfractaire sont ceux qui présentent le plus d'altérations au niveau de leur mode de vie avec de nombreuses visites non programmées chez le médecin, des recours aux services d'urgence et des hospitalisations fréquentes (5, 6). Une certaine proportion d'entre eux pourraient cependant être contrôlés efficacement s'ils n'avaient été mal diagnostiqués, sous-diagnostiqués ou bien sous-traités. La sévérité de l'asthme peut aussi résulter d'une mauvaise adhérence au traitement prescrit ou bien de difficultés à utiliser correctement les systèmes d'inhalation, d'où l'importance de l'éducation du patient à sa maladie et à son traitement (7). Par asthme réfractaire véritable, on entend les patients dont la difficulté de contrôle de la maladie n'est pas liée à des erreurs de diagnostic ou un manque d'observance thérapeutique (8).

Les facteurs contribuant à la sévérité de l'asthme

Atopie

Bien que l'atopie soit moins fréquente chez les patients atteints d'asthme sévère que chez ceux souffrant d'un asthme léger à modéré (9), elle

Tableau 1. Catégories phénotypiques associées à l'asthme

Phénotypes cliniques ou physiologiques	• Age lors de la première crise • Tendance aux exacerbations • Limitation chronique des débits aériens • Susceptibilité et résistance aux traitements
Phénotypes en relation avec des facteurs environnementaux	• Aspirine ou médicaments anti-inflammatoires non stéroïdiens • Allergènes environnementaux • Allergènes occupationnels ou irritants • Cycle menstruel • Exercice physique
Phénotypes inflammatoires	• Eosinophilique • Neutrophilique • Pauci-granulocytique

(1) Doctorante, (2) Professeur, Chef de Service, Service de Pneumologie, CHU Sart Tilman, Liège.

Tableau II. Critères définissant l'asthme sévère/réfractaire selon l'American Thoracic Society (ATS)

Critères majeurs	Critères mineurs
Corticothérapie orale continue ou presque continue (>50% de l'année).	Besoin d'un traitement additionnel journalier avec une médication de contrôle (β2-agonistes longue action, théophylline, antagonistes des leucotriènes).
Utilisation de hautes doses de corticostéroïdes inhalés.	Utilisation journalière (ou presque) des β-2 agonistes courte action.
	Obstruction persistante des voies respiratoires (avec un VEMS < 80% des valeurs prédites).
	Au moins une visite en urgence par an.
	3 cures ou plus de corticostéroïdes oraux par année.
	Un épisode asthmatique grave dans le passé ayant conduit le patient aux soins intensifs.
	Une détérioration clinique rapide lors d'une réduction ≤ 25% de la dose de corticoïdes oraux ou inhalés.

est présente chez environ 60% des patients atteints d'asthme réfractaire. Chez ceux-ci, des allergènes perannuels tels que les acariens, les moisissures et les blattes peuvent contribuer à aggraver la pathologie. Parmi les allergènes, l'aspergillus mérite d'être singularisé. En effet, une sensibilisation à son égard peut conduire à l'aspergillose broncho-pulmonaire allergique, une maladie rare mais qui constitue un phénotype important car mal traitée, elle peut conduire à des bronchiectasies (10).

Tabac et polluants atmosphériques

D'autres facteurs environnementaux comme les infections ou l'exposition à des polluants atmosphériques jouent un rôle important. Parmi les polluants, la fumée de cigarette est un facteur important qui contribue à la sévérité de l'asthme en provoquant une réponse neutrophilique intense et en augmentant la résistance aux corticostéroïdes (11-13).

Agents professionnels

Les expositions professionnelles (peintres, coiffeurs, boulangers) sont également incriminées.

Médicaments

L'utilisation de certains médicaments (β-bloquants) est particulièrement contre-indiquée chez les patients asthmatiques. L'asthme induit par l'aspirine, essentiellement rencontré chez les adultes, constitue un phénotype caractérisé par un asthme sévère, une augmentation de la production de leucotriènes, une intense éosinophilie sanguine, une sévère rhinosinusite souvent associée à des polypes nasaux et une réponse médiocre aux corticoïdes.

Troubles psychologiques

L'asthme sévère peut également être associé à des désordres psychologiques ou psychiatriques (dépression, anxiété, peur, panique, problèmes comportementaux) (14).

Sexe

L'asthme sévère est deux à trois fois plus commun chez les femmes que chez les hommes.

Quand il survient durant l'enfance, il est plus fréquent chez les garçons, mais la tendance s'inverse pendant l'adolescence et persiste à l'âge adulte.

Ceci résulte probablement de facteurs endocriniens comme le suggèrent aussi les fluctuations de sévérité de l'asthme en fonction du cycle menstruel chez la femme. Dans le même ordre d'idée, la grossesse a souvent un impact sur l'expression clinique de l'asthme (surtout pendant les deux derniers trimestres).

Obésité

L'obésité est un nouveau facteur de risque reconnu ayant un impact sur l'asthme et sa sévérité, surtout chez la femme (15). On a observé qu'une perte de poids s'accompagnait d'un meilleur contrôle de la pathologie à traitement pharmacologique inchangé. C'est certainement par un effet néfaste sur la mécanique diaphragmatique que l'obésité majore la symptomatologie asthmatique. Néanmoins, il est prouvé que des facteurs endocrines liés à l'obésité, comme la leptine ou d'autres adipokines (adiponectine, résistine), ont aussi une action sur les cellules immunes et inflammatoires (16). Par conséquent, on ne peut exclure que l'obésité module aussi la sévérité de la maladie asthmatique par un mécanisme immuno-inflammatoire.

Rhinosinusopathie

La coexistence de rhinite chronique sévère, de polypes nasaux et de sinusites contribue

aussi à aggraver la sévérité de la maladie et, ce, indépendamment d'un possible contexte d'hypersensibilité à l'aspirine (17). En particulier, une pathologie sinusale sévère s'avère être un facteur important contribuant aux exacerbations récurrentes dans les asthmes difficiles (18).

Reflux gastro-oesophagien

Le reflux gastro-oesophagien est souvent associé à l'asthme chronique chez les adultes et les enfants (19). Son rôle moteur, comme facteur contribuant à la sévérité, est encore débattu. Dans certains cas, les inhibiteurs de la pompe à protons permettent d'améliorer le contrôle de l'asthme (20).

Mécanismes de l'asthme sévère

Dans certains asthmes sévères à composante allergique, on observe les mécanismes inflammatoires prévalant dans les formes légères à modérées, mais selon un processus amplifié. On y retrouve des cellules Th2-like jouant un rôle déterminant par la production de cytokines Th2 et de chémokines impliquées dans la régulation des IgE ainsi que dans la maturation, le recrutement, l'activation des mastocytes, basophiles et éosinophiles (21) (Fig 1).

De plus, lors d'asthme sévère, on observe le plus souvent une infiltration importante de neutrophiles, une destruction tissulaire et un remodelage profond des voies aériennes (22). Certaines données récentes font état d'une activation de la voie Th1 dans certains asthmes réfractaires (23).

Les voies respiratoires de ces patients présentent les caractéristiques du processus continu de dégradation et de réparation tissulaire. La réponse à ces atteintes crée les stimuli favorables au recrutement du mésenchyme sous-jacent et à sa participation dans les processus de réparation via la libération de facteurs de croissance comme l'Epidermal Growth Factor (EGF), les Transforming Growth Factor α et β, l'Insulin-like Growth Factor (IGF), le Fibroblast Growth Factor (β-FGF) qui, ensemble, assurent le remodelage et la vasculogenèse.

La fonction épithéliale perturbée et l'augmentation de la réponse mésenchymateuse amplifient le rôle potentiel de l'EMTU (Epithelial-Mesenchymal Trophic Unit) qui est associé à une perte de la fonction pulmonaire, des désordres chroniques et un remodelage des voies respiratoires (24). Ce processus entraîne l'accumulation de dépôts de collagènes et de protéoglycans au niveau de la lamina reticularis de la membrane basale avec, pour conséquence, un épaississement et une augmentation de la rigidité des voies respiratoires (25).

Au sein de ce micro-environnement modifié, la capacité à recruter, retenir et activer des cellules inflammatoires sélectives comme les monocytes, les mastocytes et les neutrophiles change. De ce fait, en dépit de hautes doses de corticostéroïdes inhalés ou oraux, les mastocytes persistent ou bien augmentent en nombre, surtout ceux situés à proximité des amas de muscle

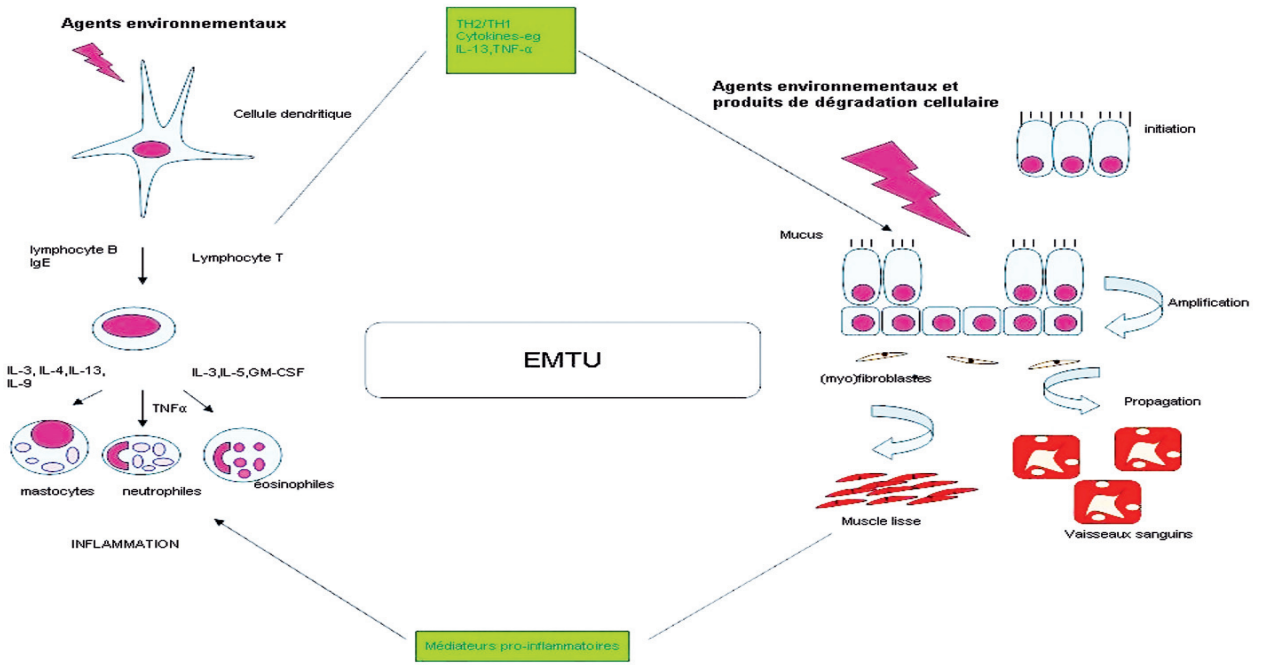


Figure 1. Réponse inflammatoire et remodelage dans l'asthme avec activation de l'EMTU (Epithelial Mesenchymal Trophic Unit).

lisse et on retrouve de hautes concentrations en TNF-α dans ces cellules (26). Les mastocytes sont également une source importante d'interleukine13, un médiateur important dans les processus inflammatoires et de remodelage.

Les facteurs qui maintiennent l'activation de l'EMTU incluent les allergènes (surtout les acariens et les moisissures), les infections virales répétées et les polluants atmosphériques (y compris la fumée de cigarette).

Diagnostic et évaluation de l'asthme sévère

Lorsqu'on est confronté à un patient suspect d'asthme réfractaire, il convient tout d'abord de confirmer le diagnostic d'asthme et d'exclure des pathologies autres qui pourraient mimer un asthme telle une dysfonction des cordes vocales. Ensuite il faudra procéder à l'évaluation des facteurs sous-jacents (y compris un sous-traitement ou bien une faible adhérence au traitement) et la détermination du phénotype.

La présence du phénotype caractérisé par un certain degré d'obstruction permanente, une réduction de la variabilité diurne des débits aériens et une extension du processus pathologique aux petites voies aériennes peut être problématique pour poser le diagnostic différentiel avec une bronchiolite oblitérante (27). Dans ce cas, une spirométrie, une radiographie et un scanner thoracique et, si le VEMS est > à 60% des valeurs prédites, un test de provocation bronchique à la métacholine peuvent être utiles.

A l'opposé de ce phénotype, on retrouve le «britlle asthma» qui traduit un asthme éminemment instable aux fluctuations intenses et rapides des débits aériens qui peut être diagnostiqué sur base d'une surveillance du débit expiratoire de pointe (28).

L'évaluation du degré d'inflammation bronchique est aujourd'hui considéré comme une étape essentielle dans la détermination du phénotype. L'analyse cytologique du sputum constitue un examen intéressant en portant une attention particulière sur les taux d'éosinophiles et de neutrophiles. L'oxyde nitrique exhalé (NO exhalé) est un indicateur utile et non invasif de l'inflammation éosinophilique, mais la prise de corticoïdes et le tabagisme actif chez certains patients peuvent fausser les résultats (29).

Traitement de l'asthme sévère

On a observé que la suppression des facteurs prédisposants n'avait pas toujours l'effet escompté sur le contrôle de l'asthme. A titre d'exemple, bien que les allergènes inhalés représentent un facteur connu participant au processus inflammatoire, les stratégies d'éviction ont souvent donné des résultats décevants (30). Le sevrage tabagique, quand il est applicable, est essentiel. Non seulement le tabac accroît la morbidité de l'asthme, mais il induit aussi une résistance aux corticoïdes (31). L'arrêt du tabagisme s'accompagne d'un accroissement rapide de la valeur du VEMS dans les semaines qui suivent (32).

Le traitement pharmacologique standard de l'asthme sévère inclut de hautes doses de corticostéroïdes inhalés combinés à un β-2 agoniste à longue durée d'action, souvent administrés dans un système d'inhalation unique (33). Les corticoïdes inhalés voient, en général, leur efficacité plafonner aux alentours de 800-1000 µg de béclométasone dipropionate équivalents par jour. Par ailleurs, en raison des effets secondaires locaux et systémiques (ostéoporose, cataracte, fragilité cutanée), il vaut mieux éviter d'administrer des doses dépassant 2000 µg/jour pendant des périodes prolongées. Un nouveau corticoïde, le ciclésonide, semble avoir un meilleur index thérapeutique permettant l'administration de doses plus faibles. Cependant, il n'a pas encore été approuvé pour être utilisé dans le cadre de l'asthme sévère.

S'il est fermement établi que les corticoïdes inhalés sont essentiels dans la réduction de la morbidité et de la mortalité liées à l'asthme, un vif débat s'est récemment engagé dans la littérature concernant la sécurité d'emploi des β-2 mimétiques à longue durée d'action (SMART). Lors d'une étude de 28 semaines concernant l'usage du salmeterol chez des patients souffrant d'asthme modéré à sévère, l'utilisation de ce dernier a dû être interrompu prématurément à cause d'une augmentation significative de la mortalité dans le groupe traité. Notre conviction, basée sur l'analyse des données de la littérature, est que les β-2 mimétiques sont sûrs lorsqu'ils sont associés à des corticoïdes inhalés.

Les antagonistes des récepteurs aux cystéinyl-leucotriènes (34) et la théophylline (35) représentent des thérapies complémentaires souvent utilisées en pratique clinique. Néanmoins, aucune étude contrôlée n'a encore validé ces médicaments dans les asthmes réfractaires. Dans certains cas d'asthme réfractaire, une thérapie orale aux corticoïdes doit être prescrite en veillant à utiliser la plus petite dose efficace possible en l'administrant selon un schéma «jours alternés».

Quand *Chlamydia pneumoniae* est suspecté comme facteur contribuant à l'obstruction bronchique persistante (36) ou bien aux exacerbations (37), l'addition de macrolides peut se révéler efficace.

Dans l'aspergillose bronchopulmonaire allergique (ABPA), l'adjonction d'itraconazole au traitement par corticoïdes semble apporter un bénéfice (38).

De hautes doses d'immunoglobulines humaines en IV se sont avérées efficaces chez certains patients asthmatiques sévères avec dépendance aux corticostéroïdes oraux (39). Chez ces derniers, on a observé des effets suppressifs sur l'inflammation persistante, mais le coût et les inconvénients de ce type de traitement doivent faire réfléchir quant à son utilisation et les dernières recommAndations du GINA ne le conseillent pas.

Il est aussi important de traiter des comorbidités dont on sait qu'elles aggravent la pathologie asthmatique. L'évaluation et le traitement des formes de rhinosinusite représentent une part importante du plan de traitement lors d'un asthme sévère qui se traduit par un meilleur contrôle de la maladie asthmatique (40). De même, le reflux gastro-oesophagien doit être traité avec un inhibiteur de la pompe à protons à hautes doses tel que l'ésoméprazole, ou bien le lansoprazole (41).

La perte de poids, une régulation hormonale chez les femmes chez qui un lien a été établi entre l'asthme et le cycle menstruel sont des éléments essentiels à prendre en compte dans la prise en charge.

Une éosinophilie persistante accompagnée d'une atteinte systémique comme la mononeuropathie multiple (42) en présence d'asthme sévère doit faire penser à un Churg-Strauss Syndrome, qui, en plus des corticoïdes oraux, nécessite un traitement au cyclophosphamide.

Dans certaines circonstances extrêmes, la transplantation pulmonaire bilatérale connaît également un certain succès (43)

Nouvelles approches thérapeutiques

L'immunoglobuline humanisée monoclonale dirigée contre les IgE humaines, l'omalizumab, représente une avancée dans le traitement de l'asthme allergique sévère quand les symptômes persistent en dépit d'une combinaison thérapeutique optimale (44). L'omalizumab qui se fixe sur l'IgE empêche celle-ci de se lier aux cellules inflammatoires portant à leur surface les récepteurs de forte affinité pour les IgE, à savoir les mastocytes et les basophiles qui jouent un rôle essentiel dans le déclenchement de la réaction allergique. Dans une population sélectionnée d'asthmatiques allergiques sévères dont les taux d'IgE sériques étaient situés entre 30 et 700 Ku/l, l'application de ce traitement permet une amélioration des symptômes et de la qualité de vie des asthmatiques et, surtout, réduit clairement la fréquence des exacerbations sévères. Les effets secondaires sont peu fréquents et consistent le plus souvent en céphalées, troubles digestifs (diarrhée, nausées,…) ou bien réactions au niveau du site d'injection. Néanmoins, nous manquons encore de recul pour écarter toute possibilité d'effets délétères au cours de traitements prolongés sur plusieurs années.

Dans les formes sévères de la maladie asthmatique, on a pu observer une surexpression de cytokines classiquement associées au pattern dit Th1. Dans ces cas, le blocage des cytokines Th1 présente un certain intérêt lorsque les corticostéroïdes inhalés se sont révélés inefficaces. Suite à l'augmentation de l'expression du TNF-α dans les voies respiratoires des patients souffrant d'asthme sévère réfractaire (45), des études ont investigué l'effet thérapeutique de l'étanercept, un inhibiteur du TNF-α, dans ce phénotype asthmatique. L'étanercept s'est révélé capable de réduire la symptomatologie, d'améliorer la qualité de vie et de réduire de façon substantielle le niveau d'hyperréactivité bronchique (46). Ces données devront cependant être confirmées dans des études portant sur des cohortes plus larges dans le cadre d'essais multicentriques. Par ailleurs, la sécurité d'utilisation au long cours du TNF-α dans les maladies respiratoires chroniques doit encore être établie.

Conclusion

L'asthme réfractaire est aujourd'hui reconnu comme un phénotype asthmatique particulier. Sa base physiopathologique ne se résume pas à un seul processus inflammatoire Th2. Après s'être assuré du diagnostic, il est essentiel de démasquer certaines comorbidités jouant le rôle de facteurs aggravants. Dans ces formes d'asthme, des thérapeutiques alternatives aux corticoïdes sont nécessaires à une meilleure maîtrise de la maladie. Parmi celles-ci, l'anti-IgE apporte un bénéfice aux patients à composante allergique avec sensibilisation à l'égard d'un allergène perannuel. Il est vraisemblable que d'autres traitements issus des progrès de la biotechnologie verront le jour dans les années à venir.

Bibliographie

1. Wenzel S.— Asthma : defining of the persistent adult phenotypes. *Lancet*, 2006, **368**, 804-813.
2. The Encarta World Dictionary, 1st edn.New York: St Martin's Press, 1999.
3. Holgate ST, Polosa R.— The mechanisms, diagnosis, and management of severe asthma in adults. *Lancet*, 2006, **368**, 780-793.
4. Fahy J, Irvin C, et al.— Proceedings of the ATS Workshop on Refractory asthma. *Am J Respir Crit Care Med*, 2000, **162**, 2341-2351.

5. Goddard P, Chanez P, Siraudin L, et al.— Costs of asthma are correlated with severity, a year prospective study. *Eur Respir J*, 2002, **19**, 61-67.

6. Dolan CM, Fraher K, Bleecker E, et al.— Design and baseline characteristics of the epidemiology and natural history of asthma : a large cohort of patients with severe or difficult-to-treat asthma. *Ann Allergy Asthma Immunol*, 2004, **92**, 32-39.

7. Chanez P, Wenzel S, et al.— Severe asthma in adults: What are the important questions? *J Allergy Clin Immunol*, 2007, **119**, 1337-1348.

8. Harrison B.— Difficult asthma in adults : recognition and approaches to management. *Intern Med J*, 2005, **35**, 543-547.

9. European Network for Understanding Mechanisms of Severe Asthma.The ENFUMOSA cross-sectionnal European multicentre study of the clinical phenotype of chronic severe asthma. *Eur Respir J*, 2003, **22**, 470-477.

10. Gibson P.— Allergic bronchopulmonary aspergillosis.*Semin Respir Crit care Med*, 2006, **27**, 185-191.

11. Silverman R, Boudreaux E, Woodruff P, et al.— Cigarette smoking among asthmatic adults presenting to 64 emergency departments. *Chest*, 2003, **123**, 1472-1479.

12. Sturdy P, Butland B, Anderson H, et al.— Deaths certified as asthma and use of medical services : a national case-control study. *Thorax*, 2005, **60**, 909-915.

13. James A, Palmer L, Kicic E, et al.— Decline in lung function in the Busselton Health Study : the effects of asthma and cigarette smoking. *Am J Respir Crit Care Med*, 2005, **171**, 109-114.

14. Leher P, Feldman J, Giardino N, et al.— Psychological aspects of asthma. *J Consult Clin Psychol*, 2002, **70**, 691-711.

15. Shore S, Johnston R.— Obesity and asthma. *Pharmacol Ther*, 2006, **110**, 83-102.

16. Sood A, Ford E, Camargo C.— Association between leptin and asthma in adults.*Thorax*, 2006, **61**, 300-305.

17. Breciani M, Paradis L, Des Roches A, et al.— Rhinosinusitis in severe asthma. *J Allergy Clin Immunol*, 2001, **107**, 73-80.

18. Ten Brinke A, Sterk P, Spinhoven P.— Risk factors of frequent exacerbations in difficult-to-treat asthma.*Eur Respir J*, 2005, **26**, 812-818.

19. Gibson P, Henry R, Coughlan J.— Gastro-oesophageal reflux treatment for asthma in adults and children.*Cochrane Database Syst Rev*, 2003, **2**, CD001496.

20. Kiljander T, Harding S, Field S, et al.— Effects of esomeprazole 40mg twice daily on asthma : a randomized placebo-controlled trial. *Am J Resp Crit Care Med*, 2006, **173**, 1007-1091.

21. Borish L, Steinke J.— Cytokines and chemokines. *J All Clin Immunol*, 2003, **111**, 460-475.

22. Jatakanon A, Uasuf C, Maziak W, et al.— Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med*, 1999, **160**, 1532-1539.

23. Magnan A, Mély L, Camilla C, et al.— Assessment of the Th1/Th2 Paradigm in whole blood in atopy and asthma.Increased IFN-γ-producing CD8+ T Cells in asthma. *Am J Respir Crit Care Med*, 2003, **161**, 1790-1796.

24. Knight D, Holgate S.— The airway epithelium: structural and functional properties in health and disease. *Respirology*, 2003, **8**, 432-436.

25. Bumbacea D, Campbell D, Nguyen L, et al.— Parameters associated with persistant airflow obstruction in chronic severe asthma. *Eur Resp J*, 2004, **24**, 122-128.

26. Balzar S, Strand M, Wenzel S, et al.— Relationship of small airway chymase-positive mast cells and lung function in severe asthma.Am J Respir Crit Care Med, 2005, **171**, 431-439.

27. Miller M, Johnson C, Miller D, et al.— Severity assessment in asthma : an evolving concept. *J Allergy Clin Immunol*, 2005, **116**, 990-995.

28. Ayres J, Miles J, Barnes P.— Brittle asthma. *Thorax* 1998, **53**, 315-321.

29. Brightling C.— Clinical applications of induced sputum. *Chest*, 2006, **129**, 1344-1348.

30. Woodcock A,Forster L, Matthews E, et al.— Control of exposure to mite allergen-impermeable bed covers for adults with asthma. *N Engl J Med*, 2003, **349**, 225-236.

31 N Gusbin, N Garzaniti, R Louis.— Asthme et tabac. *Rev Méd de Liège*, 2006, **61**, 81-86.

(32) Chaudhuri R, Livingston E, Lafferty J. et al.Effects of smoking cessation on lung function and airway inflammation in smokers with asthma. *Am J Respir Crit Care Med*, 2006, **174**, 127-133.

33. Global Initiative for asthma.— Global strategy for asthma management and prevention. A six part management program. NIH publication number 02-3659.http://www.ginasthma.org.

34. Currie P, Lee D, Srivastava P.— Long-acting bronchodilator or leukotriene modifier as add-on therapy to inhaled corticosteroids in persistent asthma? *Chest*, 2005, **128**, 2954-2962.

35. Shah L, Wilson A, Gibson P, et al.— Long acting beta2-agonists versus theophylline for maintenance treatment of asthma. *Cochrane Database Syst Rev*, 2003, 3, CD001281.

36. Richeldi L, Ferrara G, Fabri L, et al.— Macrolides for chronic asthma. *Cochrane Database Syst Rev*, 2005, 4, CD002997.

37. Johnston S, Blasi F, Black P, et al.— The effect of telithromycin in acute exacerbations of asthma. *N Engl J Med*, 2006, **354**, 1589-600.

38. Berry A, Hargadon B, Shelley M. et al.— Evidence of a role of tumor necrosis factor α in refractory asthma. *New Eng J Med*, 2006, **354**, 697-708.

39. Haque S, Boyce N, Thien F, et al.— Role of intraveinous immunoglobulin in severe steroid –dependant asthma. *Intern Med J*, 2003, **33**, 341-44.

40. Bachert C, Patou J, Van Cauwenberge P.— The role of sinus disease in asthma. *Curr Opin Allergy Clin Immunol*, 2006, **6**, 29-36.

41. Littner M, et al.— Effects of 24 weeks of lansoprazole therapy on asthma symptoms, exacerbations, quality of life and pulmonary function in adult asthmatic patients with acid reflux symptoms. *Chest*, 2005, **128**, 1128-1135.

42. Kawakami T, Soma Y, Kawasaki K, et al.— Initial cutaneous manifestations consistent with mononeuropathy multiplex in Churg-Strauss syndrome. *Arch Dermatol*, 2005, **141**, 873-878.

43. Wirtz H, Kroegel C, Caffier P, et al.— Bilateral lung transplantation for severe persistant and difficult asthma. *J Heart Lung Transplant*, 2005, **24**, 1700-1703.

44. Holgate S, Casale T, Wenzel S, et al.— The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunology*, 2005, **115**, 459-465.

45. Howarth P, Babu K, Arshad H, et al.— Tumour necrosis factor(TNF-α) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax*, 2005, **60**, 1012-1018.

46. Amrani Y, Chen H, Panettieri R.— Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyperresponsiveness in asthma? *Respir Res*, 2000, **1**, 49-53.

Les demandes de tirés à part sont à adresser au Pr. R. Louis, Chef de service, Service de Pneumologie, CHU Sart Tilman, 4000 Liège, Belgique.

III. PURPOSE OF THE STUDY

There is a need for a better characterisation and a better understanding of the inflammatory process and the molecular mechanisms involved in refractory asthma. The aim of this work was to compare cytokine production and IgE amount at systemic and airway level in refractory asthmatics compared with mild-to-moderate asthmatics and healthy subjects. We have also sought to determine whether the cytokine release and the IgE levels were influenced by the sputum cellular phenotype.

1. **In the first part of this project, we have assessed the spontaneous cytokine release from blood leucocytes and sputum cells.**

a) Blood and sputum cytokine assay

In order to assess the systemic cytokine production we have used the whole blood model. Peripheral blood samples were collected in apyrogenic and heparinized tubes and cultured for 24h without any stimulation. After the 24h of culture, cytokines (IL-4, IL-6, IL-10, IFN-γ, TNF-α) were measured by a classical two-step sandwich type immunoassay (ELISA).

In order to assess the airway cytokine release, we have used the sputum cell culture model. For sputum induction, patients were first premedicated with 400µg inhaled salbutamol administered by MDI (+ Spacer) and sputum was induced by inhalation of hypertonic saline (NaCl 5%) when FEV1 post salbutamol was ≥ 65% predicted and isotonic saline (NaCl 0.9%) when FEV1 was < 65% predicted. Saline was combined with additional salbutamol delivered by an ultrasonic nebuliser (Ultra-Neb 2000, Devilbiss). Each subject inhaled the aerosol for three consecutive periods of 5 min for a total time of 15 min. For safety reasons, FEV1 was monitored every 5 min and the induction stopped when FEV1 fell by more than 20% from post-bronchodilatation values. The whole sputum was collected in a plastic container, weighted and homogenized by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 sec and centrifuged at 800 *g* for 10 min at 4°C. Supernatant was separated from cell pellet which was suspended in RPMI 1640 supplemented with 100 U penicillin/ml,

100µg streptomycin/ml and centrifuged at 400g for 10 min at 4°C. Cells were washed once more with RPMI 1640 + antibiotics. Squamous cells, total cell counts and cell viability checked by trypan blue exclusion were performed with a manual haemocytometer. The differential cell count was performed on cytospins stained with Diff-Quick after counting 400 cells. A determined volume of RPMI + antibiotics was then added to the cell suspension to obtain a concentration of 2.10^6 non squamous cells/ml.

b) Effect of prednisolone on cytokine release in vitro

In order to evaluate the impact of corticoids on cytokine production "in vitro", effect of prednisolone on IL-6 (sputum), IL-4 and IL-10 (blood) was assessed in healthy subjects and asthmatics from the different asthma groups. Cells were cultured for 24h with or without prednisolone at the concentrations of 10^{-7}, 10^{-8} and 10^{-9}M.

2. In the second part of the project we have assessed the stimulated cytokine release from blood leucocytes and sputum cells in response to phytohaemagglutinin and endotoxin (LPS).

The procedures were similar to that described in part 1 apart the fact that cytokine release was assessed after cell stimulation for 24H with phytohaemagglutinin (PHA 1 µg/ml) which is a polyclonal activator of T lymphocyte, a key cell in adaptative immunity or with lipopolysaccharide (1ng/ml), a PAMP signalling through TLR and activating innate immunity.

3. In the third part we have measured total IgE (tIgE) and cytokines in sputum supernatant.

a) Sputum supernatant collection

Sputum was induced and processed similarly to what was described in part 1. Once supernatant was collected it was placed at -80°C until assays.

b) Measurement of cytokines

All induced sputum samples were concentrated by use of centrifugal evaporator. 1 ml of induced sputum was entirely airdried in a SpeedVac SC 100 centrifuge (Savant, Thermo Scientific). Afterwards, the pellet was resuspended in 100 µl distilled water and mixed. All samples were assayed for IL-4, IL-6, IL-10, IL-5, IL-17, IL-13, IFN-γ and TNF-α with the Luminex xMAP Technology by using commercially available Fluorokine MAP Kits (R&D Systems Europe Ltd, Abingdon, United Kingdom) following to the manufacturer´s guidelines and measured on a Bio-Plex 200 Platform (Bio-Rad Laboratories S.A.-N.V, Nazareth Eke, Belgium).

c) Measurement of total IgE

Total IgE were measured with ImmunoCAP system with a detection limit of 0.1 kU/l (Phadia AB, Uppsala; Sweden).

IV. Publication 2

Cytokine production from sputum cells and blood leukocytes in asthmatics according to disease severity

M. Manise, F. Schleich, N. Gusbin, L. Godinas, M. Henket,
N. Antoine, J.L. Corhay, R. Louis

Allergy 2010 ; 65, 889-96

ORIGINAL ARTICLE AIRWAY DISEASES

Cytokine production from sputum cells and blood leukocytes in asthmatics according to disease severity

M. Manise, F. Schleich, N. Gusbin, L. Godinas, M. Henket, N. Antoine, J. L. Corhay & R. Louis

Department of Respiratory Medicine, CHU Sart-Tilman, University of Liege, GIGA, Research Group i[3], Liège, Belgium

To cite this article: Manise M, Schleich F, Gusbin N, Godinas L, Henket M, Antoine N, Corhay JL, Louis R. Cytokine production from sputum cells and blood leukocytes in asthmatics according to disease severity. *Allergy* 2010; **65**: 889–896.

Keywords
corticoids; interleukin-10; interleukin-4; interleukin-6; refractory asthma.

Correspondence
Maïté Manise, Pneumology-Allergology, Bât B35, CHU Sart-Tilman, Liège, Belgium.
Tel.: 0032 43668568
Fax: 0032 43613732
E-mail: mmanise@student.ulg.ac.be

Accepted for publication 13 November 2009

DOI:10.1111/j.1398-9995.2009.02296.x

Edited by: Marc Humbert

Abstract

Background: Although mild to moderate asthma is known to be Th2 driven, cytokines produced in refractory asthma might not fit the classical Th2 pattern.
Methods: The aim of our study was to assess the cytokine production by sputum and blood cells from 15 refractory asthmatics (American Thoracic Society Criteria) compared to 15 mild untreated and 17 moderate treated asthmatics and 22 healthy subjects. Spontaneous production of interleukin (IL)-4, IL-6, IL-10, interferon-γ, and tumor necrosis factor α was measured by immunotrapping after 24 h sputum or blood cell culture.
Results: Moderate and refractory asthmatics were both characterized by a lower production of IL-6 from their airway cells compared to healthy subjects. However, the difference was no longer significant when expressing the results per gram of sputum. No significant difference between the three groups was found regarding other cytokines. As for cytokine production from blood, the three groups of asthmatics exhibited raised production of IL-4 when compared to healthy subjects, and this was true when results were expressed per blood volume or after normalization for total leukocyte cell count. Moderate asthmatics exhibited greater production of IL-10 when compared to refractory asthmatics and healthy subjects when results were normalized for total leukocyte cell count.
Conclusions: Sputum cells from moderate and refractory asthmatics release less IL-6. While the systemic overproduction of IL-4 was observed through the all spectrum of asthma severity, moderate asthmatics exhibited greater systemic IL-10 production compared to refractory asthmatics.

A subgroup of patients with asthma, called refractory asthmatics, may have persistent symptoms, airflow obstruction, and asthma exacerbation despite high medication use including high dosage of inhaled or even oral steroids (1). In addition to impairing quality of life and sometimes placing the patient at risk of life-threatening exacerbations, refractory asthma represents a burden for health costs (2).

While mild to moderate asthma has been extensively studied and is now considered as Th2 immunological disturbance leading to eosinophilic inflammation (3), the studies dedicated to cellular and molecular mechanisms in refractory asthma have been more limited and have cast some doubts about the Th2 paradigm (4).

Although a fraction of refractory asthmatics still display eosinophilic inflammation (5), neutrophil may become a pivotal cell in a substantial part of them (6). Whether Th2 mechanisms are still prevailing in refractory asthmatics have not been extensively studied. Recent works have indicated that refractory asthma might deviate from the classic Th2 pattern and suggested that tumor necrosis factor α (TNF-α) may play a pivotal role (7, 8). On the other hand, some authors have put forward the hypothesis that refractory severe asthma might result from a deficiency in anti-inflammatory agent such as lipoxins (9).

We have previously validated the model of sputum cell culture to investigate the ability of airway cells to generate cytokines in mild to moderate asthma (10). The purpose of our study was to extent our research to refractory asthmatics. To this end, we have assessed the spontaneous cytokine production from sputum and blood cell culture in refractory asthmatics relative to that seen into mild untreated asthmatics, moderate asthmatics receiving low to moderate dose of

inhaled corticoids and healthy subjects. Interleukin-4 (IL-4) and interferon-γ (IFN-γ) were chosen as markers of the Th2/Th1 balance, TNF-α and IL-10 as pro- and anti-inflammatory cytokines, respectively (10), and IL-6 as a cytokine playing a role in the transition from innate toward adaptive immunity (11).

Materials and methods

Study design and subject characteristics

Patient demographic, functional, and treatment characteristics are given in Table 1. We studied 15 subjects (nine females) with refractory asthma and compared them with 22 healthy subjects, 15 mild untreated asthmatics, and 17 moderate asthmatics receiving low to moderate dose of inhaled corticoids. Our refractory asthmatics were defined according to the ATS workshop criteria (1). They were all well-known asthmatics diagnosed on the basis of significant forced expiratory volume in 1 s (FEV1) reversibility (≥12% from baseline) to β2 agonists or bronchial hyperresponsiveness to methacholine (PC20M < 16 mg/ml). All of them had been followed for more than 6 months in our asthma clinic at CHU Liege Sart-Tilman, between January 2006 and June 2009, had received detailed education about their disease (12) including allergen avoidance when appropriate and were thought to be compliant with their treatment. They all had uncontrolled disease as reflected by an asthma control questionnaire (ACQ) >1.5 despite receiving high doses of inhaled corticosteroids (>880 μg/day fluticasone and 1200 μg/day budesonide) and long-acting β2 agonists. Significant pulmonary co-morbidities like bronchiectasis, fibrosis, and emphysema had been excluded on the basis of high resolution chest computed tomography. Moderate asthmatics, also recruited from our asthma clinic, were characterized by a better control of asthma than refractory asthmatics and lower doses of inhaled corticosteroids. Mild asthmatics were newly diagnosed asthmatics either recruited from our asthma clinic or coming from the hospital staff members. None of them had been taking inhaled corticoids, leukotrienes receptor antagonists, or theophylline over the last 2 months. Healthy subjects were recruited by advertising among the hospital and laboratory staff members. They all had normal spirometry and PC20M > 16 mg/ml. Both mild asthmatics and healthy subjects denied respiratory tract infection in the past 4 weeks prior to sputum sampling.

The protocol had been approved by the local ethics committee, and every subject gave his written informed consent.

Table 1 Demographic and functional characteristics

	Healthy subjects (N = 22)	Mild asthmatics (N = 15)	Moderate asthmatics (N = 17)	Refractory asthmatics (N = 15)
Age	42 ± 13	36 ± 16	50 ± 15†	36 ± 17
Sex (m/f)	13/9	8/7	11/6	6/9
Tobacco status (ns/es/cs)	12/5/5	13/0/2	14/2/1	6/3/6
BMI	24.5 ± 3.23	22.2 ± 2.02	25.17 ± 4.2	29.13 ± 7.9††
Prick test+	0	14	10	14
NO (ppb)	–	75 (0–235)	66 (10–161)	27 (4–165)
IgE	–	61 (15–349)	132 (62–1283)	190 (1–2532)
FEV1 (%)	108 ± 16	94.5 ± 17	85 ± 16**	60 ± 16***†
FVC (%)	110 ± 17	97 ± 16	90 ± 18*	80.5 ± 12***
FEV1/FVC (%)	83 ± 7.7	83 ± 8	78 ± 11‡	63 ± 13***†††
Reversibility (%)	–	–	–	21 ± 20
ACQ	–	1.14 (0.49–1.5)	1.86 (0.71–3.5)‡‡	3.6 (1.83–5.57)†††
PC20M	–	2.9 (0.2–16)	0.73 (0.64–2)	3.4§
Oral CS	0	0	2	6
Inhaled CS (eq budesonide/day)	–		800 (200–2000)‡‡‡	2000 (1000–6000)
LABA	–	–	12	15
LTRA	–	–	4	9
Theophylline	–	–	2	4
Exacerbation rate in the previous year	–	–	–	0.6 ± 0.7
Minor criteria (ATS)	–	–	–	3.6 ± 1.4

Results are expressed as mean ± SD, except PC20M expressed as geometric mean (range) and NO, and IgE expressed as median (range). FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; BMI, body mass index; ACQ, Juniper asthma control questionnaire; CS, corticosteroids; LABA, long acting beta 2 agonist; LTRA, leucotriene receptor antagonist.

*P < 0.05, **P < 0.01, ***P < 0.001 *vs* healthy subjects; †P < 0.05, ††P < 0.01, †††P < 0.001 *vs* mild asthmatics; ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 *vs* refractory asthmatics.

§Only one patient tested.

Peripheral blood sampling and cell count

Peripheral blood samples were collected in apyrogenic, heparinized tubes (Venosafe; TERUMO®, Leuven, Belgium). The total and differential blood cell counts were obtained with an Advia 120 automatic counter (Siemens, Erlangen, Germany). Counting and cell typing were based on flow cytometry with bidimensional volume distribution, peroxydase concentration, and lobularity of leukocytes as parameters.

Sputum induction and processing

After premedication with 400 μg inhaled salbutamol administered by metered dose inhaler (MDI) (+ Spacer), sputum was induced by inhalation of hypertonic saline (NaCl 5%) when FEV1 postsalbutamol was ≥65% predicted and isotonic saline (NaCl 0.9%) when FEV1 was <65% predicted. Saline was combined with additional salbutamol delivered by an ultrasonic nebulizer (Ultra-Neb 2000; Devilbiss, Somerset, PA, USA) with an output set at 0.9 ml/min as previously described (13). Each subject inhaled the aerosol for three consecutive periods of 5 min for a total time of 15 min. For safety reasons, FEV1 was monitored every 5 min, and the induction stopped when FEV1 fell by more than 20% from postbronchodilatation values.

The whole sputum was collected in a plastic container, weighed, and homogenized by adding three volumes of phosphate-buffered saline, vortexed for 30 s, and centrifuged at 800 ***g*** for 10 min at 4°C. Supernatant was separated from cell pellet, which was suspended in Roswell park memorial institute medium (RPMI) 1640 supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml, and centrifuged at 400 ***g*** for 10 min at 4°C. Cells were washed once more with RPMI 1640 + antibiotics. Squamous cells, total cell counts, and cell viability checked by trypan blue exclusion were performed

Table 2 Sputum and blood cell counts

	Healthy subjects	Mild asthmatics	Moderate asthmatics	Refractory asthmatics
Sputum				
Sputum weight (g)	4.6 (1.89–9.52)	4 (0.88–12.57)	4.12 (0.88–6.57)	3.46 (0.58–10.85)
Total nonsquamous cells (10^6 cells/g)	0.53 (0.05–2.4)	1 (0.15–2.12)	0.75 (0.11–22.6)	2 (0.45–12.59)**
Squamous cells (%)	18 (0–46)	20 (0–53)	16 (0–55)	7.5 (0–29)
Viability (%)	58 (29–77)	47 (29–85)	59 (37–85)	69 (30–93)
Macrophages				
(%)	34 (8–96)	58 (11–92)	36 (0.8–54)	16.5 (1–72)†
10^3/g	240 (10–1800)	320 (30–1170)	240 (10–2500)	250 (130–1770)
Lymphocytes				
(%)	2.6 (0.2–10)	1.6 (0–14)	0.8 (0–11.4)	1.2 (0.6.4)
10^3/g	10 (0.7–240)	20 (2.4–210)	30 (0–570)	10 (0–110)
Neutrophils				
(%)	26 (0–87)	28 (0–84)	45 (4–96)	50 (0–99)
10^3/g	210 (0–1150)	340 (15–2640)	440 (50–21 700)*	900 (0–12 500)*
Eosinophils				
(%)	0 (0–3.6)	3 (0–23.4)*	0.8 (0–88)	9 (0–80)**
10^3/g	0 (0–20)	60 (2–330)**	65 (0–3900)**	100 (0–2600)***
Epithelial cells				
(%)	13 (1.6–66)	5 (1–48)	4.2 (0.2–25)	5 (0–26)
10^3/g	70 (7–350)	30 (10–1020)	35 (0–530)	100 (0–700)
Blood				
Leukocytes (10^3/μl)	6.4 (4.2–12.2)	7.5 (4.4–12.2)	6.3 (4–13.7)	9.4 (7.2–18)***†
Neutrophils				
(%)	51.8 (40.9–72.8)	53 (40.1–65.3)	55.5 (38.2–76.2)	58.2 (33.1–84)
(number/μl)	3520 (0–6190)	3820 (1960–6200)	3600 (1730–10 500)	4940 (2380–15 200)**
Lymphocytes				
(%)	35.4 (18.8–46.9)	34.2 (22.2–41.8)	28.1 (16–42.6)	25.4 (9.7–38.7)*
(number/μl)	1900 (0–4770)	2200 (1700–5900)	1960 (1260–2300)‡	2580 (1760–21 000)
Monocytes				
(%)	6.1 (4.7–11.5)	5.2 (4–7.6)	6 (3.5–9.6)	5.8 (4.8–9)
(number/μl)	400 (0–1000)	400 (220–590)	430 (210–700)	580 (360–12 700)*†
Eosinophils				
(%)	1.7 (0.7–6.3)	5.1 (1.6–10)*	4.5 (0.4–7.5)	4.4 (0.1–20.9)
(number/μl)	120 (0–370)	360 (100–810)**	300 (30–480)	480 (20–54 000)**

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* healthy subjects; †$P < 0.05$, *vs* mild asthmatics; ‡$P < 0.05$, *vs* refractory asthmatics.

with a manual hemocytometer. The differential cell count was performed on cytospins stained with Diff-Quick after counting 400 hundreds cells. A determined volume of RPMI + antibiotics was then added to the cell suspension to obtain a concentration of 2×10^6 nonsquamous cells/ml.

Blood and sputum cell culture and cytokine assay

Cytokines (IL-4, IL-6, IL-10, TNF-α, IFN-γ) were measured by a two-step sandwich type immunoassay. The antibodies and standards were purchased from Biosource (Cytosets; Biosource, Invitrogen, Merelbeke, Belgium). Fifty microliters from standards or whole blood (diluted twice) or sputum cell suspension (2×10^6 cells/ml) was incubated at 37°C with 200 µl RPMI 1640 supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml (Cambrex, Verviers, Belgium), and 2% of inactivated fetal calf serum (Cambrex) in apyrogen microwells, which were coated previously with specific antibodies directed toward the chosen cytokines. After 24 h, the wells were washed, and 150 µl of a solution containing biotinylated detection antibodies specific to the cytokines was added for 2 h at room temperature. The wells were washed again and filled with a solution containing streptavidin horseradish peroxidase for 45 min at room temperature. Then, 100 µl tetramethylbenzidine chromogen solution was added for 10–20 min in the dark. The reaction was stopped by adding 50 µl H_2SO_4 1 M. The amount of substrate converted to products was thereafter detected as optical densities at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan Ascent; Thermo Labsystems, Helsinki, Finland).

To evaluate the impact of corticoids on cytokine production '*in vitro*', effect of prednisolone on IL-6 (sputum) and IL-4 and IL-10 (blood) was assessed in healthy subjects and asthmatics representing the different asthma groups. Sputum cells were cultured for 24 h with or without prednisolone (10^{-7}, 10^{-8}, 10^{-9} M), and each well was filled with 200 µl RPMI + 50 µl sputum cell suspension or 100 µl RPMI + 50 µl sputum cell suspension + 100 µl prednisolone at the chosen concentration. As for blood cells, 50 µl of whole blood diluted twice was added in the presence of 200 µl RPMI or 100 µl RPMI + 100 µl prednisolone at the chosen concentration.

Cytokine production was than assessed by immunotrapping as previously described. The sensitivities of our assays were 6 pg/ml for IL-4, 6 pg/ml for IL-6, 4 pg/ml for IL-10, 6 pg/ml for TNF-α, and 7 pg/ml for IFN-γ.

Statistical analysis

Blood and sputum cell counts as well as cytokine levels were expressed as median (range). Comparisons between the four groups were performed by Kruskall–Wallis test (nonparametric ANOVA) followed, in case of significance, by Dunn's multiple comparisons test. For *in vitro* experiments, statistical analysis was performed using a one-sample '*t*'

Table 3 Cytokine production from sputum and blood cells

	Healthy subjects	Intermittent asthmatics	Moderate asthmatics	Refractory asthmatics
Sputum (10^5 cells/well)				
IL-4 (pg/ml)	0 (0–20)	0 (0–10)	0 (0–80)	0 (0–78)
IL-6 (pg/ml)	31 (0–396)	38 (0–1281)	10 (0–130)**†	12 (0–419)*
IL-10 (pg/ml)	53 (4–913)	93 (2–1364)	29 (2.5–154)	68 (3–1087)
IFN-γ (pg/ml)	7 (0–579)	0 (0–130)	0 (0–494)	0 (0–3020)
TNF-α (pg/ml)	2034 (49–4000)	1594 (0–4000)	1463 (80–3711)	2107 (0–3639)
Sputum (amount/g of sputum)				
IL-4 (pg/g Sl)	0 (0–0.47)	0 (0–28)	0 (0–1002)	0 (0–416)
IL-6 (pg/g Sl)	13 (0–148)	22 (0–446)	3 (0–32.5)	8 (0–2765)
IL-10 (pg/g Sl)	14 (0–194)	44 (0–627)	34 (0–21 175)	56 (0–4864)
IFN-γ (pg/g Sl)	0 (0–1.2)	0 (0–44)	0 (0–801)	0 (0–19 932)
TNF-α (pg/g Sl)	486 (0–5685)	1234 (0–7604)	781 (24–49 745)	1219 (0–63 107)
Blood (50 µl diluted twice)				
IL-4 (pg/ml)	0 (0–9)	20 (1–41)***	30 (0–284)***	13 (0–349)***
IL-6 (pg/ml)	0 (0–152)	0 (0–264)	0 (0–29)	0 (0–193)
IL-10 (pg/ml)	0 (0–6)	0 (0–45)	7 (0–150)	0 (0–430)
IFN-γ (pg/ml)	0 (0–239)	0 (0–57)	0 (0–97)	7 (0–89)
TNF-α (pg/ml)	45 (0–793)	104 (9–1403)	109 (32–261)	43 (0–2733)
Blood (10^5 cells/well)				
IL-4 (pg/5×10^5 cell)	0 (0–1.6)	2.4 (0–5.3)*	3.5 (0–49)***	1.3 (0–213.5)**
IL-6 (pg/5×10^5 cell)	0 (0–20)	0 (0–24.4)	5.4 (0–2.9)	0 (0–23)
IL-10 (pg/5×10^5 cell)	0 (0–1)	0 (0–5.6)	1.2 (0–16.5)**‡	0 (0–45.5)
IFN-γ (pg/5×10^5 cell)	0 (0–27)	0 (0–5.3)	0 (0–5.2)	0.48 (0–10.5)
TNF-α (pg/5×10^5 cell)	6.6 (0–112.8)	14 (1.1–177)	12.7 (3.8–41)	4 (0–289)

TNF-α, tumor necrosis factor α; IL, interleukin.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* healthy subjects; †$P < 0.05$ *vs* mild asthmatics; ‡$P < 0.05$ *vs* refractory.

test or a paired '*t*' test. A *P*-value <0.05 was considered as statistically significant.

Results

Demographics, lung function, and asthma control

The subjects were well matched for their age and tobacco consumption. As opposed to healthy subjects, most of asthmatics were atopic. Body mass index values were significantly greater in refractory (29.13 ± 8) than in mild (22.2 ± 2) asthmatics ($P < 0.01$). The proportion of smokers was similar between the groups (Table 1).

As expected, FEV1 values in refractory asthmatics were clearly altered (60 ± 16%) when compared to moderate asthmatics (85 ± 16%), intermittent asthmatics (95 ± 17%), and healthy subjects (108 ± 16%) (Table 1). Similarly, forced vital capacity (FVC) was also significantly decreased in refractory asthmatics when compared to the three other groups but to a lesser extent than FEV1 so that the ratio FEV1/FVC was significantly lower in refractory asthma (63 ± 13%) than in moderate asthma (78 ± 11) ($P < 0.05$), mild asthma (83 ± 8%), and healthy (83 ± 7) ($P < 0.001$ for both). Reflecting poor asthma control, ACQ score was higher in refractory asthma than in mild and moderate asthma ($P < 0.001$ and $P < 0.01$, respectively).

Sputum and blood cell counts

Both mild and refractory asthmatics displayed a raised sputum eosinophil count when compared to healthy subjects ($P < 0.05$ and $P < 0.01$, respectively) (Table 2). Refractory asthmatics had a greater total sputum cell count compared to healthy subjects ($P < 0.01$).The three groups of asthmatics had a raised absolute sputum eosinophil counts when compared to healthy subjects ($P < 0.01$ for mild and moderate asthmatics and $P < 0.001$ for refractory asthmatics) while moderate and refractory asthmatics exhibited a raised absolute neutrophil count when compared to healthy subjects ($P < 0.05$ for both) (Table 2).

In the blood, refractory asthmatics were also characterized by a greater total circulating leukocyte count than healthy subjects ($P < 0.001$) and mild asthmatics ($P < 0.05$) and had raised absolute neutrophil and eosinophil counts when compared to healthy subjects ($P < 0.01$ for both) (Table 2).

Cytokine production from sputum and blood cell culture

The results regarding cytokine production from sputum and blood cells are given in Table 3. Sputum cells from moderate and refractory asthmatics displayed a significantly decreased production of IL-6 when compared to healthy subjects ($P < 0.01$ and $P < 0.05$, respectively) while moderate asthmatics also produced significantly less IL-6 than mild asthmatics ($P < 0.05$) (Fig. 1A). However, this difference disappeared when normalizing the results per gram of sputum because of the clearly greater total cell counts per volume (or gram) of sputum in refractory asthmatics. There was no significant difference between the groups for IL-4, IL-10, IFN-γ, and TNF-α.

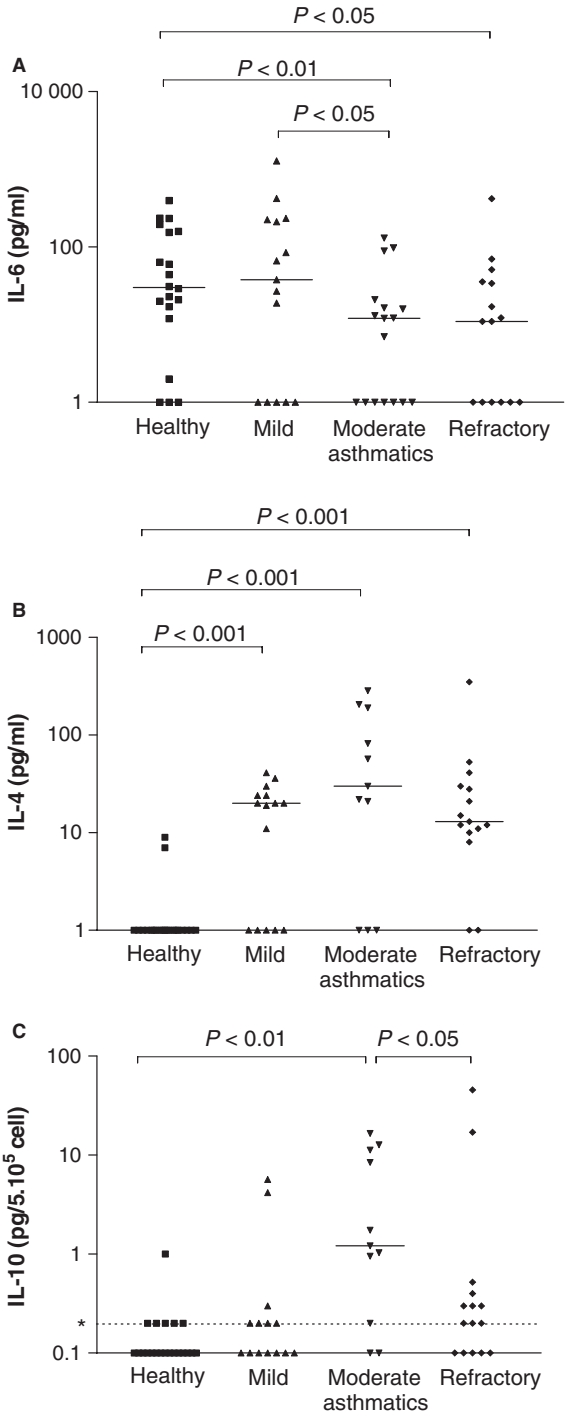


Figure 1 Spontaneous production of IL-6 by sputum cells (A), IL-4 by blood cells (B), and IL-10 by blood cells. Values of IL-10 were normalized for one hundred thousand cells. (C) Lines represent median values, dashed line represents the detection threshold. IL, interleukin.

As far as blood cells are concerned mild, moderate, and refractory asthmatics displayed raised IL-4 production when compared to healthy subjects ($P < 0.001$ for both) (Fig. 1B). This difference held true when normalizing the results per leukocyte cell count (Table 3). There was a strong trend to have greater IL-10 production in moderate asthmatics ($P = 0.07$) when results were expressed as cytokine produced per blood volume, and the difference between moderate asthmatics and refractory asthmatics and healthy subjects became significant when normalizing the cytokine levels per leukocyte cell count (Fig. 1C). No difference was found regarding IL-6, IFN-γ, and TNF-α.

The relationship between the production of cytokines and the sputum and blood cellular pattern was given in Table 4. In blood, the magnitude of IFN-γ release was proportional to the percentage of eosinophils ($P < 0.05$). No other significant correlation was noticed even though sputum IL-4 levels tended to relate to sputum eosinophils and inversely to sputum neutrophils.

In subjects not treated with inhaled corticoids (five healthy subjects and four mild asthmatics), prednisolone from 10^{-9} to 10^{-7} induced a dose dependent inhibition of IL-6 reaching 17 ± 31% not significant (NS), 12 ± 16% (NS), and 42 ± 10%, respectively ($P < 0.01$) (Fig. 2A). Similar experiments were performed for evaluating the impact of prednisolone on the IL-4 production by blood cells in asthmatics. There was also a significant dose dependent inhibition from 10^{-9} to 10^{-7} reaching 10 ± 15% (NS), 37 ± 16% ($P < 0.05$), and 41 ± 12% ($P < 0.01$), respectively (Fig. 2B). Prednisolone effect on IL-10 production from blood cells was assessed in healthy subjects and mild asthmatics not treated with inhaled corticoids. While prednisolone tended to amplify IL-10 production at 10^{-9} (34 ± 20%, $P = 0.13$), it produced a significant inhibition at 10^{-8} and 10^{-7} that reached 29 ± 11% ($P < 0.05$) and 31 ± 11% ($P < 0.05$), respectively (Fig. 2C).

Discussion

Our study shows that airways cells from moderate and refractory asthmatics, as opposed to those of mild asthmatics, produce less IL-6 than those of healthy subjects. At the systemic level, circulating leukocytes from both mild, moderate, and refractory asthmatics produce more IL-4 when compared to healthy subjects.

Our finding that moderate and refractory asthmatics display an impaired production of IL-6 from their sputum cell culture may appear somewhat surprising as asthma severity is thought to be partly related to uncontrolled airway inflammation (14). Indeed, IL-6 has been viewed as a pro-inflammatory cytokine playing a pivotal role in lymphocyte activation (15). The detrimental consequences of IL-6 bioactivity in chronic disease must be balanced by its capacity to protect against septic shock and to direct resolution of acute inflammation. Recent advances have documented a series of IL-6 activities that are critical for resolving innate immunity and promoting acquired immune response (11). In this view, a lack of IL-6 production might favor intense airway inflammation following natural lipopolysaccharide (LPS) exposure. It is of interest to note that neutrophilic airway inflammation was more pronounced in our moderate and severe asthmatics, which might be in line with this hypothesis. It should be kept in mind, however, that the lack of IL-6 production by sputum cells seen *ex vivo* in moderate and refractory asthmatics might be counterbalanced *in vivo* by the greater number of cells present in the sputum from these patients.

One potential explanation for the decreased production of IL-6 seen in refractory asthmatics might be the high dose of inhaled or oral corticoids received by the patients. It is well known that corticoids are able to repress IL-6 production (16). Our *in vitro* experiments clearly show that prednisolone can reduce production of IL-6 from sputum cell culture in patients not receiving inhaled corticoids. This gives credits to the hypothesis that reduced production in IL-6 in moderate and severe asthmatics is depending on the treatment rather than reflecting an intrinsic property of the disease itself.

It has also been shown that β2 agonists may have the reverse effect by enhancing the IL-6 production (16). If β2 agonists used by the patients had to be responsible for the difference between the groups, we would have expected a difference in the opposite direction that is a raised production

Table 4 Correlation between cytokines and sputum/blood cell count

	IL-4 (pg/10^5 cells)	IL-6 (pg/10^5 cells)	IL-10 (pg/10^5 cells)	IFN-γ (pg/10^5 cells)	TNF-α (pg/10^5 cells)
Sputum					
Neutro (%)	−0.35	−0.24	−0.01	−0.27	−0.01
Lympho (%)	−0.14	0.34	0.02	0.02	0.02
Eosino (%)	0.36	−0.09	0.1	0.3	−0.22
Macro (%)	−0.01	0.19	−0.03	0.12	0.12
Epith cells (%)	0.02	0.23	0.02	−0.12	0.27
Blood					
Neutro (%)	−0.03	0.02	−0.02	−0.4	−0.12
Lympho (%)	−0.05	−0.06	0	0.25	0.11
Eosino (%)	0.26	0.16	0.18	0.35*	0.27
Mono (%)	−0.04	0.05	−0.19	−0.24	0
Baso (%)	0.12	0.16	0.12	0.17	0

TNF-α, tumor necrosis factor α; IFN-γ, interferon-γ; IL, interleukin.
*$P < 0.05$ after Bonferroni correction for multiple correlations.

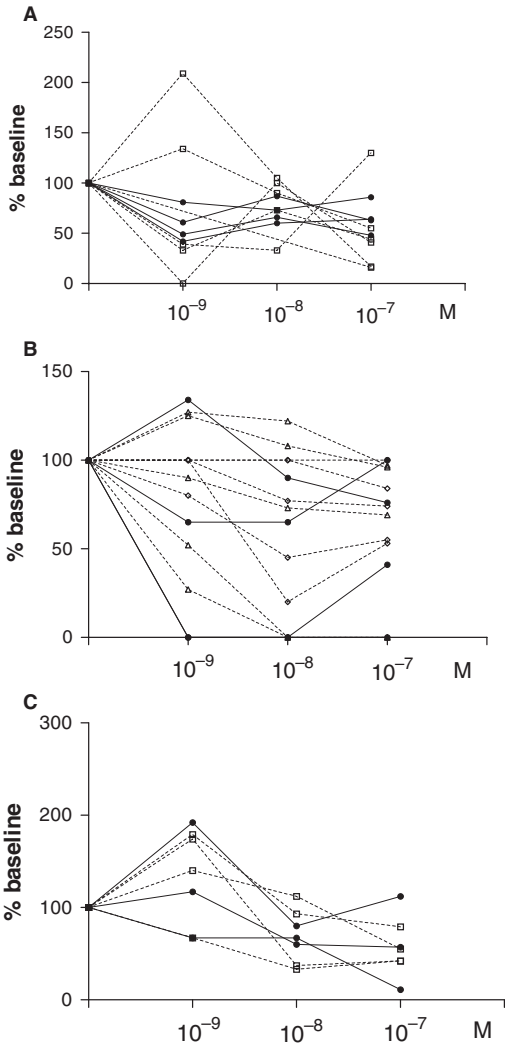


Figure 2 Effect of prednisolone on IL-6 from sputum cells (A), IL-4 from blood (B), and IL-10 from blood (C). Results are expressed as percentage of baseline. One line represents one patient; healthy subjects are represented by squares and discontinued lines, intermittent asthmatics by full circles and continued lines, moderate asthmatics by triangles and discontinued lines, and refractory asthmatics by diamonds and discontinued lines. IL, interleukin.

of IL-6 in moderate and severe asthma. Furthermore, in our experimental setting, all the subjects inhaled high dose of β2 agonist during the procedure of sputum induction, which would tend to neutralize the impact of β2 agonists on the different groups.

When considering cytokine production from blood leukocytes, all groups of asthmatics exhibited a raised production of IL-4 when compared to healthy subjects. This might reflect the atopic status of our patients as the majority of our asthmatics were atopic. Our finding of increased production of IL-4 in mild asthmatics is keeping with the classical Th2 pattern found in mild atopic asthma (17) and confirms our previous data (18). IL-4 is thought to have a role in the physiological response to allergen challenge. A recent study has demonstrated that a local treatment with Pitrakinra (an IL-4 variant inhibiting the binding of IL-4 to IL-4Rα receptor complex) can substantially reduce the late phase reaction following allergen exposure (19). We found that this Th2 pattern was still present in refractory asthma but not enhanced when compared to mild asthma. However, the raised IL-4 production persisted despite the use of high dose of inhaled corticoids and in some of them oral corticoids, a drug that was found to inhibit IL-4 production from blood cells in our model. For the whole group of refractory asthmatics, the raised production of IL-4 appears to be confined to the blood compartment and failed to emerge in the airways. In refractory asthmatics, it is conceivable that high doses of inhaled corticoids have impeded the overproduction of IL-4 from airway cells (20) but have not been able to do the same at the systemic level. Interestingly, while no increased production at the airway levels was observed for the whole group of refractory asthmatics, those refractory asthmatics with high sputum eosinophil counts (>3%) display a significant rise in IL-4 production when compared to healthy subjects. This indicates that IL-4 may still be operating in the airways of those patients in whom corticoids are unable to control eosinophilic inflammation. The role of IL-4 in those patients may be significant in driving airway remodelling (21).

The stimulus driving intense eosinophilic inflammation together with raised IL-4 in our refractory asthmatics despite heavy treatment is unknown. As the majority of asthmatics were atopic, it would be conceivable that allergen exposure may be responsible for that inflammatory pattern. However, our previous data showed that experimental allergen challenge resulted in a sharp rise in IL-4, IL-6, and IL-10 production from sputum cells (22), a profile of cytokine response not found here in our refractory asthmatics.

Of interest is the observation that blood IL-10 production was found to be increased in moderate asthmatics when compared to refractory asthmatics when results were normalized per leukocytes. This might be related to the dose of corticoids received by the patients as our *in vitro* data seem to indicate a dual effect of prednisolone on IL-10 production from leukocytes with a low concentration enhancing IL-10 release while higher concentrations causing a clear inhibition. The raised production of IL-10 in moderate asthmatics might be a mechanism that limits inflammation and prevent these patients to become more severe.

The correlation analysis between the cytokine production and the cellular profile of blood and sputum reveals that a high fraction of eosinophil in blood cell count was associated with greater IFN-γ release. This confirms previous data and highlights once more that eosinophilic inflammation may also be linked to Th1 inflammation (23, 24).

A finding from our study is that cytokine mainly produced by the mononuclear phagocytic cells like TNF-α (25), IL-6, and IL-10 are produced at a much greater level from sputum than from blood cells whereas the reverse was observed for IL-4. This may reflect the difference in the cellular composi-

tion between sputum and blood leukocytes. Sputum contains a greater proportion of mononuclear phagocytic cells while blood contains a greater percentage of lymphocytes. Here, we did not try to identify, which cell is the source of cytokines nor did we try to perform selection of a particular cell type. However, we believe our model of mixed cell culture preserving potential cell interaction is meaningful in reflecting what happens *in vivo*.

We conclude that moderate and refractory asthmatics exhibit impaired IL-6 production in the airways more likely to be because of treatment by inhaled corticoids. At the systemic level, refractory asthmatics do not distinguish from mild to moderate asthmatics in producing raised amount of IL-4 although it is noticeable that this raised production persists despite heavy treatment with inhaled and sometimes oral corticoids. The raised IL-10 production seen in moderate asthmatics might be because of the low dose of corticoids received by these patients and could be considered as a protective mechanism against more severe disease. Further studies looking in details at the *ex vivo* regulation of cytokine production by corticoids in refractory asthmatics are warranted.

Acknowledgments

This work was supported by pôle d'attraction interuniversitaire (PAI) grant P6/35: Belgian Air$_e$way study consortium and unrestricted research grants from GSK, Astrazeneca and Novartis. We also thank J. Sele for excellent technical assistance.

References

1. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;**162**:2341–2351.
2. Laforest L, Com-Ruelle L, Devouassoux G, Pison C, Van Ganse E. [Economic aspects of severe asthma]. *Presse Med* 2008;**37**:117–128.
3. Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med* 2001;**344**:30–37.
4. Chanez P, Wenzel SE, Anderson GP, Anto JM, Bel EH, Boulet LP et al. Severe asthma in adults: what are the important questions? *J Allergy Clin Immunol* 2007;**119**:1337–1348.
5. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;**161**:9–16.
6. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;**160**:1532–1539.
7. Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W et al. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005;**60**:1012–1018.
8. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH et al. Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N Engl J Med* 2006;**354**:697–708.
9. Bonnans C, Vachier I, Chavis C, Godard P, Bousquet J, Chanez P. Lipoxins are potential endogenous antiinflammatory mediators in asthma. *Am J Respir Crit Care Med* 2002;**165**:1531–1535.
10. Quaedvlieg V, Henket M, Sele J, Louis R. Cytokine production from sputum cells in eosinophilic versus non-eosinophilic asthmatics. *Clin Exp Immunol* 2006;**143**:161–166.
11. Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 2005;**175**:3463–3468.
12. Lavorini F, Magnan A, Dubus JC, Voshaar T, Corbetta L, Broeders M et al. Effect of incorrect use of dry powder inhalers on management of patients with asthma and COPD. *Respir Med* 2008;**102**:593–604.
13. Delvaux M, Henket M, Lau L, Kange P, Bartsch P, Djukanovic R et al. Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma. *Thorax* 2004;**59**:111–115.
14. Bai TR, Knight DA. Structural changes in the airways in asthma: observations and consequences. *Clin Sci (Lond)* 2005;**108**:463–477.
15. Hillion S, Dueymes M, Youinou P, Jamin C. IL-6 contributes to the expression of RAGs in human mature B cells. *J Immunol* 2007;**179**:6790–6798.
16. Johnston SL, Martin RJ. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*: a role in asthma pathogenesis? *Am J Respir Crit Care Med* 2005;**172**: 1078–1089.
17. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;**326**:298–304.
18. Bettiol J, Bartsch P, Louis R, De Groote D, Gevaerts Y, Louis E et al. Cytokine production from peripheral whole blood in atopic and nonatopic asthmatics: relationship with blood and sputum eosinophilia and serum IgE levels. *Allergy* 2000;**55**:1134–1141.
19. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007;**370**:1422–1431.
20. Wallin A, Sandstrom T, Cioppa GD, Holgate S, Wilson S. The effects of regular inhaled formoterol and budesonide on preformed Th-2 cytokines in mild asthmatics. *Respir Med* 2002;**96**:1021–1025.
21. Bergeron C, Page N, Barbeau B, Chakir J. Interleukin-4 promotes airway remodeling in asthma: regulation of procollagen I (alpha1) gene by interleukin-4. *Chest* 2003;**123**:424S.
22. Bettiol J, Sele J, Henket M, Louis E, Malaise M, Bartsch P et al. Cytokine production from sputum cells after allergenic challenge in IgE-mediated asthma. *Allergy* 2002;**57**:1145–1150.
23. Shirai T, Inui N, Suda T, Chida K. Correlation between peripheral blood T-cell profiles and airway inflammation in atopic asthma. *J Allergy Clin Immunol* 2006;**118**:622–626.
24. Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma – increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000;**161**:1790–1796.
25. Borish LC, Steinke JW. 2. Cytokines and chemokines. *J Allergy Clin Immunol* 2003;**111**:S460–S475.

IV. Publication 3

Disturbed cytokine production at the systemic level in difficult to control atopic asthma. Evidence for raised IL-4 and decreased IFN-γ release following LPS stimulation

M. Manise, F. Schleich, V. Quaedvlieg, C. Moermans, M. Henket, J. Sele, J.L. Corhay, R. Louis

Int. Arch. Allergy Immunol. 2012 ; 158, 1-8

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2012;158:1–8
DOI: 10.1159/000329858

Received: November 23, 2010
Accepted after revision: May 30, 2011
Published online: December 28, 2011

Disturbed Cytokine Production at the Systemic Level in Difficult-to-Control Atopic Asthma: Evidence for Raised Interleukin-4 and Decreased Interferon-γ Release following Lipopolysaccharide Stimulation

M. Manise F. Schleich V. Quaedvlieg C. Moermans M. Henket J. Sele
J.L. Corhay R. Louis

Department of Respiratory Medicine, CHU Sart-Tilman, GIGA Research Group i[3], Liège, Belgium

Key Words
Interleukin-4 · Interferon-γ · Endotoxin · Asthma control

Abstract
Background: Disturbed cytokine production is thought to govern inflammation in asthma, which, in its turn, may lead to uncontrolled disease. The aim of this study was to assess the relationship between cytokine production from blood leucocytes and the level of asthma control. ***Methods:*** We compared the production of interleukin (IL)-4, IL-6, IL-10, interferon (IFN)-γ and tumour necrosis factor-α from peripheral blood leucocytes in non-atopic healthy subjects (n = 22), atopic non-asthmatics (n = 10), well-controlled asthmatics [Juniper asthma control questionnaire (ACQ) score <1.5; n = 20] and patients with uncontrolled asthma despite inhaled or oral corticoids (ACQ score ≥1.5; n = 20). Fifty microlitres of peripheral blood was incubated for 24 h with RPMIc, lipopolysaccharide (LPS; 1 ng/ml) or phytohaemagglutinin (1 μg/ml), and cytokines were measured by immunotrapping (ELISA). ***Results:*** Both controlled and uncontrolled asthmatics as well as atopic non-asthmatics spontaneously produced more IL-4 than non-atopic healthy subjects ($p < 0.001$). IL-4 production induced by LPS was significantly greater ($p < 0.05$) in both asthma groups compared to atopic non-asthmatics and non-atopic healthy subjects. By contrast, IFN-γ release induced by LPS was lower in uncontrolled asthmatics than in non-atopic healthy subjects ($p < 0.05$) and controlled asthmatics ($p < 0.05$). IL-10 release after LPS was greater in uncontrolled asthmatics than in atopic non-asthmatics ($p < 0.05$). No difference was observed regarding other cytokines. ***Conclusion:*** Blood cells from patients with difficult-to-control atopic asthma display highly skewed Th2 cytokine release following LPS stimulation.

Introduction

Human studies suggest that exposure to lipopolysaccharide (LPS) can influence the development and severity of asthma. Endotoxin is considered to have a dual role in asthma. While it may prevent the development of atopy when exposure occurs in early life [1], this bacterial product may worsen asthma control when inhaled by adult asthmatics in whom the disease is already well established [2].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/12/1581–0001$38.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Maïté Manise
Pneumology-Allergology, Bât B35, CHU Sart-Tilman
Avenue de l'hôpital B35
BE–4000 Liège (Belgium)
Tel. +32 4366 8568, E-Mail mmanise@student.ulg.ac.be

Airway exposure to endotoxin is known to promote airway [3] and systemic [4] neutrophilic inflammation, but this bacterial compound has a broad range of activities in vitro. Endotoxin is a potent stimulus for the innate immune system and is able to activate both the mononuclear [5] and granulocyte fraction from blood leucocytes [6, 7]. Some studies have suggested that persistent, difficult-to-treat asthma may be linked to an impaired innate immunity favouring chronic infection [8].

Although pathological heterogeneity of the disease has been highlighted over the past years [9], asthma often features an airway eosinophilic inflammation [10, 11] orchestrated by Th2 cytokines [12, 13]. Our recent study showed that uncontrolled asthma encountered in daily practice is associated with increased airway eosinophilic inflammation as compared to well-controlled asthma [14]. Whether this relationship is also observed at the systemic level has not been investigated.

The purpose of our study was to determine if there was any relationship between asthma control and cytokine production from blood leucocytes in response to endotoxin. Interleukin (IL)-4 and interferon (IFN)-γ were chosen as markers of the Th2/Th1 balance, tumour necrosis factor (TNF)-α and IL-10 as pro- and anti-inflammatory cytokines, respectively [15], and IL-6 as a cytokine playing a role in the transition from innate towards adaptive immunity [16].

The present study was performed on atopic asthmatics recruited from our asthma clinic and classified into two subgroups according to their level of asthma control [controlled asthma, i.e. asthma control questionnaire (ACQ) score <1.5, and uncontrolled asthma, i.e. ACQ score ≥1.5].

In order to clarify the role of asthma versus atopy in cytokine production, asthmatics were compared to atopic non-asthmatics and non-atopic healthy subjects.

Materials and Methods

Study Design and Subject Characteristics

Patient demographics and functional and treatment characteristics are given in table 1. The Juniper ACQ is known to have strong evaluative and discriminative properties and can be used with confidence to measure asthma control [17].

In this study, 20 controlled atopic asthmatics (ACQ score <1.5) were compared with 20 uncontrolled atopic asthmatics (ACQ score ≥1.5), 10 atopic non-asthmatics [grass pollen rhinitis studied out of season with a provocative concentration of methacholine producing a 20% fall in forced expiratory volume in 1 s (FEV_1) (PC20M) >16 mg/ml] and 22 non-atopic healthy subjects. Patients were recruited from our asthma clinic at CHU Liege Sart-Tilman between January 2006 and June 2009, and the group of atopic non-asthmatics comprised subjects with asymptomatic rhinitis recruited from a database for a clinical trial on immunotherapy.

All asthmatics were diagnosed on the basis of significant FEV_1 reversibility (≥12% from baseline) with β_2-agonists or bronchial hyperresponsiveness to methacholine (PC20M <16 mg/ml). Atopy was defined as a positive skin prick test reaction (wheal ≥3 mm compared with control) to common aeroallergens, including house dust mites, cat and dog dander, grass, tree, pollen and moulds.

The protocol was approved by the local ethics committee, and every subject gave written informed consent.

Peripheral Blood Sampling and Cell Count

Peripheral blood samples were collected in apyrogenic, heparinized tubes (Venosafe, Terumo®, Belgium). Total and differential blood cell counts were obtained with an Advia 210 automatic counter (USA). Counting and cell typing were based on flow cytometry with bidimensional volume distribution, peroxidase concentration and lobularity of leucocytes as parameters.

Blood Cell Culture and Cytokine Assay

Cytokines (IL-4, IL-6, IL-10, TNF-α, IFN-γ) were measured by a two-step sandwich-type immunoassay. The antibodies and standards were purchased from Biosource (Cytosets, Biosource, Invitrogen, Belgium). Fifty microlitres of standards or whole blood (diluted twice) was incubated at 37°C with 200 μl of Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Cambrex, Verviers, Belgium) and 2% of inactivated fetal calf serum (Cambrex), or LPS (*Salmonella enteridis*, Sigma, St. Louis, Mo., USA; 1 ng/ml) or phytohaemagglutinin (PHA; Biochrom AG, Berlin, Germany; 1 μg/ml) in apyrogen microwells which had previously been coated with specific antibodies directed towards the chosen cytokines.

After 24 h, the wells were washed and 100 μl of a solution containing biotinylated detection antibodies specific to the cytokines was added for 2 h at room temperature. The wells were washed again and filled with a solution containing streptavidin horseradish peroxidase for 45 min at room temperature. Then, 100 μl of tetramethylbenzidine chromogen solution was added for 10–20 min in the dark. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The amount of substrate converted to products was thereafter detected as the optical density at 450 nm in an ELISA reader (Multiscan Ascent, Thermo Labsystems, Helsinki, Finland). The sensitivities of our assays were 6 pg/ml for IL-4, 6 pg/ml for IL-6, 4 pg/ml for IL-10, 6 pg/ml for TNF-α and 7 pg/ml for IFN-γ.

Statistical Analysis

Blood cell counts as well as cytokine levels were expressed as medians (range), unless otherwise stated. Comparisons between the four groups were performed by Kruskal-Wallis test (nonparametric ANOVA) followed, in the case of significance, by Dunn's multiple-comparison test. A p value <0.05 was considered statistically significant.

Table 1. Demographic, functional, airway inflammatory and treatment characteristics according to ACQ scores

	Non-atopic healthy subjects (n = 22)	Atopic non-asthmatics (n = 10)	Asthmatics with ACQ <1.5 (n = 20)	Asthmatics with ACQ ≥1.5 (n = 20)
Age, years	42 ± 13	33 ± 10	43 ± 19	40 ± 16
Males/females	13/9	5/5	13/7	7/13
Tobacco status				
Never smoked	12	1	17	12
Ex-smoker	5	6	2	2
Current smoker	5	3	1	6
BMI	24 ± 3	23 ± 3	25 ± 5	28 ± 7[c]
Positive skin prick test	0	10	20	20
NO, ppb	–	11 (6–27)	20 (5–222)	55 (8–165)[c]
IgE, kU/l	–	63 (21–303)	331 (56–1,670)	356 (37–2,532)[c]
Sputum eosinophils, %	0 (0–3.6)	0 (0–4)	2.2 (0.4–19.4)[a]	8.8 (0–80.4)[b, c]
Sputum neutrophils, %	38 (0–87)	23 (2–52)	40 (3–93)	40 (0–99)
FEV_1, %	108 ± 16	103 ± 12	87 ± 19[a]	65 ± 17[b, e, f]
FVC, %	111 ± 17	103 ± 12	97 ± 18	85 ± 16[b]
FEV_1/FVC, %	83 ± 8	85 ± 8	76 ± 15	65 ± 13[b, d]
Reversibility, %	–	–	6.8 ± 4.5	21 ± 18[d]
ACQ score	–	–	0.86 (0.49–1.28)	3.21 (2.07–4.58)[e]
PC20M, mg/ml	–	–	3.09 (0.62–16)	0.83 (0.2–3.4)
Oral CS	0	0	0	6
Inhaled CS	0/22	0/10	9/20	19/20
Inhaled CS, eq budesonide/day	–	–	0 (0–1,600)	2,000 (1,600–2,800)[e]
LABA	–	–	9/20	17/20
LTRA	–	–	3	11
Theophylline	–	–	–	6

Results are expressed as means ± SD or numbers of patients, except PC20M, which is expressed as the geometric mean (range), and NO, IgE, ACQ score, inhaled corticosteroids and sputum neutrophils and eosinophils, which are expressed as medians (range). FVC = Forced vital capacity; BMI = body mass index; LABA = long acting β_2-agonist; LTRA = leucotriene receptor antagonist; CS = corticosteroids. [a] $p < 0.01$, [b] $p < 0.001$ versus non-atopic healthy subjects; [c] $p < 0.05$, [d] $p < 0.01$, [e] $p < 0.001$ versus atopic non-asthmatics; [f] $p < 0.05$ versus asthmatics with ACQ <1.5.

Results

Demographic, Lung Function, Airway Inflammation and Treatment Characteristics according to Asthma Control

The subjects were well matched for their age and tobacco consumption. As expected, FEV_1 values were clearly different (65 ± 17% pred.) in the group with ACQ score ≥1.5 when compared to the group with ACQ score <1.5 (87 ± 19% pred.; $p < 0.05$) and to atopic non-asthmatics and non-atopic healthy subjects ($p < 0.001$ for both). Similarly, forced vital capacity was also significantly decreased in the uncontrolled asthma group as compared to non-atopic healthy subjects, and the ratio of FEV_1 to forced vital capacity was also significantly lower in uncontrolled asthmatics (65 ± 13%) than in non-atopic healthy subjects (83 ± 8%; $p < 0.001$) and atopic non-asthmatics (85 ± 8%; $p < 0.01$).

It is also of interest to note that patients with an ACQ score ≥1.5 were taking higher doses of inhaled corticosteroids (2,000 eq budesonide/day, range 1,600–2,800) in comparison with patients with an ACQ score <1.5 (0 eq budesonide/day, range 0–1,600). Nine out of 20 controlled asthmatics and 17 out of 20 uncontrolled asthmatics were receiving inhaled long-acting β_2-agonists. Some uncontrolled asthmatics were also taking oral corticosteroids (6/20), leucotriene receptor antagonists (11/20) or theophylline (6/20).

Controlled and uncontrolled asthmatics exhibited higher sputum eosinophil counts than non-atopic healthy subjects ($p < 0.01$ and $p < 0.001$, respectively), while uncontrolled patients also had a greater sputum

Table 2. Blood cell counts

	Non-atopic healthy subjects	Atopic non-asthmatics	Asthmatics with ACQ <1.5	Asthmatics with ACQ ≥1.5
Leucocytes, /μl	6,410 (4,200–12,200)	6,390 (4,000–7,490)	7,770 (5,430–13,280)[d]	8,690 (5,860–18,130)[b, f]
Neutrophils, %	52 (41–73)	49 (42–75)	55 (41–84)	58 (33–90)
Neutrophils, /μl	3,564 (2,088–5,914)	3,300 (1,760–5,230)	4,025 (2,850–11,100)	4,720 (2,380–15,190)[b, d]
Lymphocytes, %	35 (19–47)	40 (19–47)	33 (9–41)[c, f]	26 (9–42)[g]
Lymphocytes, /μl	1,918 (598–3,546)	1,940 (1,270–2,930)	2,250 (1,230–3,950)	2,360 (810–3,670)
Monocytes, %	6.1 (4.7–11.5)	8.5 (4.3–11.2)	5.6 (3.9–10.6)	5.7 (0.7–9)
Monocytes, /μl	404 (256–1,014)	460 (300–810)	430 (300–790)	445 (60–1,190)
Eosinophils, %	1.7 (0.7–6.3)	1.7 (0.4–4.3)	4.3 (0.6–8.8)	3.8 (0.1–21)
Eosinophils, /μl	120 (30–370)	90 (30–190)	315 (70–650)[a, d]	350 (9–1,350)[a, d]
Basophils, %	0.7 (0.4–1.5)	0.5 (0.1–0.9)	0.7 (0.2–1.6)	0.6 (0–1.9)
Basophils, μl	45 (21–98)	30 (10–60)	55 (10–150)	75 (20–150)[e]

Results are expressed as medians (range). [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus non-atopic healthy subjects; [d] $p < 0.05$, [e] $p < 0.01$, [f] $p < 0.001$ versus atopic non-asthmatics; [g] $p < 0.001$ versus asthmatics with ACQ <1.5.

Table 3. Cytokine production from a standardized blood volume

Cytokine	Stimulant	Non-atopic healthy subjects	Atopic non-asthmatics	Asthmatics with ACQ <1.5	Asthmatics with ACQ ≥1.5
IL-4, pg/ml	RPMI	0 (0–9)	32 (0–169)[b]	16 (0–57)[b]	12 (0–349)[b]
	PHA	61 (6–176)	172 (11–276)[a]	70 (21–171)	120 (7–761)
	LPS	0 (0–24)	17 (0–171)[a]	28 (0–98)[b]	43 (0–541)[b]
IL-6, pg/ml	RPMI	0 (0–152)	0 (0–24)	0 (0–264)	0 (0–1,421)
	PHA	81 (0–492)	36 (0–471)	86 (0–2,362)	58 (0–1,754)
	LPS	307 (14–1,472)	184 (96–649)	311 (20–1,123)	167 (1–1,760)
IL-10, pg/ml	RPMI	0 (0–6)	9 (0–138)	1 (0–45)	0 (0–430)
	PHA	321 (72–696)	172 (90–404)	212 (57–657)	328 (35–1,681)
	LPS	487 (83–832)	289 (98–842)	470 (187–1,346)	684 (14–2,388)[c]
IFN-γ, pg/ml	RPMI	0 (0–239)	0 (0–39)	0 (0–57)	7 (0–89)
	PHA	684 (25–2,972)	1,001 (124–2,503)	413 (31–1,630)	398 (123–1,407)
	LPS	54 (0–936)	60 (0–1,145)	53 (7–398)	31 (0–238)[a, d]
TNF-α, pg/ml	RPMI	42 (0–793)	0 (0–110)	64 (0–1,403)	50 (0–2,733)
	PHA	1,993 (666–7,552)	1,160 (739–2,338)	1,610 (928–7,536)	2,269 (267–4,654)
	LPS	2,350 (1,140–5,755)	1,649 (1,370–2,754)	2,509 (592–5,995)	2,571 (80–5,052)

Results are expressed as medians (range). Values for LPS and PHA represent the raw data as they were measured from ELISA without subtracting RPMI results. [a] $p < 0.05$, [b] $p < 0.001$ versus non-atopic healthy subjects; [c] $p < 0.05$ versus atopic non-asthmatics; [d] $p = 0.06$.

eosinophil count than atopic non-asthmatics ($p < 0.05$; table 1).

Blood Cell Counts

Asthmatics with an ACQ score ≥1.5 had a greater total blood cell count compared to non-atopic healthy subjects ($p < 0.01$) and atopic non-asthmatics ($p < 0.001$).

Both groups of asthmatics exhibited significantly raised systemic absolute eosinophil counts when compared to atopic non-asthmatics and non-atopic healthy subjects ($p < 0.05$ for both).

The absolute neutrophil count was significantly increased in the uncontrolled group when compared to non-atopic healthy subjects and atopic non-asthmatics ($p < 0.01$ and $p < 0.05$, respectively).

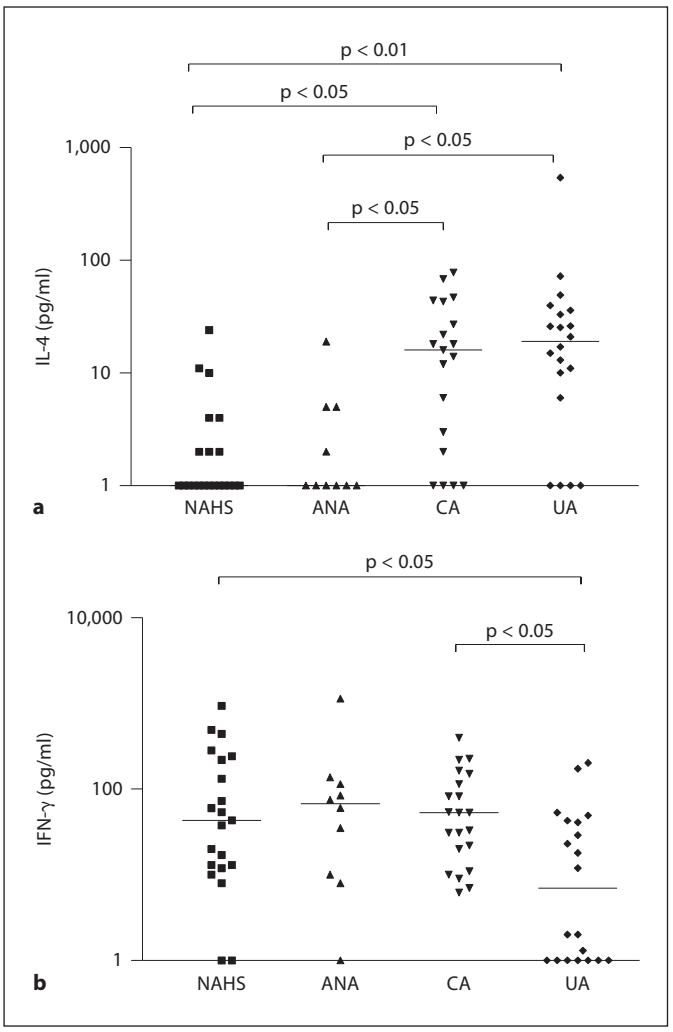


Fig. 1. LPS-induced IL-4 (**a**) and IFN-γ (**b**) production from blood leucocytes in non-atopic healthy subjects (NAHS), atopic non-asthmatics (ANA), controlled asthmatics (CA; ACQ score <1.5) and uncontrolled asthmatics (UA; ACQ score ≥1.5). Each point represents the subtraction of LPS – RPMI for IL-4 and IFN-γ. Zero values were transformed to 1 for graphic representation. The bars represent the median.

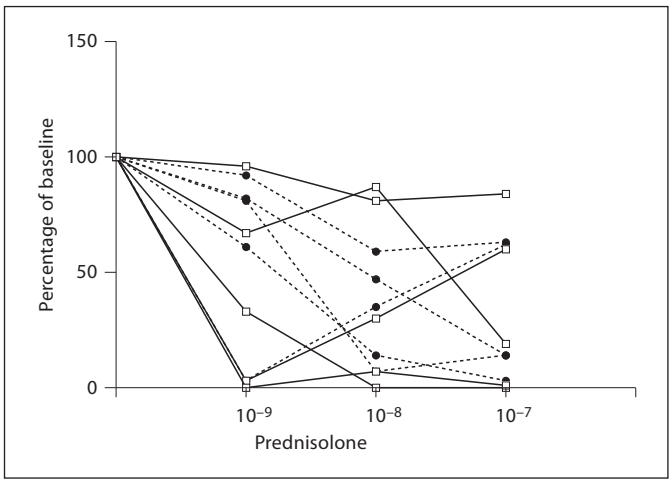


Fig. 2. Effect of prednisolone (in M) on IFN-γ from blood leucocytes in healthy subjects and patients with difficult-to-control asthma. Each line represents 1 patient; healthy subjects are represented by squares and continuous lines and patients with difficult-to-control asthma by circles and dashed lines. Results are expressed as a percentage of baseline.

There was no difference between controlled and uncontrolled asthmatics apart from the percentage of lymphocytes, which was lower in uncontrolled asthmatics ($p < 0.001$; table 2).

Cytokine Production from Blood Cell Culture

The results regarding cytokine production from blood cells are given in table 3.

Both groups of asthmatics and the atopic non-asthmatics were characterized by a significantly raised spontaneous IL-4 production ($p < 0.001$ for the three groups). Likewise, controlled and uncontrolled asthmatics also exhibited raised IL-4 production after stimulation by LPS when compared to atopic non-asthmatics ($p < 0.05$ for both) and non-atopic healthy subjects ($p < 0.05$ and $p < 0.01$, respectively; fig. 1).

IL-4 measured after LPS stimulation in atopic non-asthmatics was also greater than in non-atopic healthy subjects ($p < 0.05$), although there was no evidence of a real response to LPS in this case, the production of IL-4 being quite similar to that seen with RPMI alone (table 3). After stimulation by PHA, only atopic non-asthmatics exhibited raised IL-4 production when compared to non-atopic healthy subjects ($p < 0.05$).

The group with uncontrolled asthma showed lower release of IFN-γ following LPS exposure when compared to non-atopic healthy subjects ($p < 0.05$; fig. 1) and controlled asthmatics ($p < 0.05$). Uncontrolled asthmatics also differed from atopic healthy subjects in terms of increased IL-10 production (table 3). There was no significant difference between the groups with regard to the production of other cytokines.

In the controlled asthma group, there were no significant differences regarding cytokine production between those who were steroid naïve (n = 12) and those regularly receiving inhaled corticoids (n = 8). We did not find any relationship between the dose of inhaled corticosteroids

received by the patients and the level of IFN-γ production ($r = -0.2$, $p = 0.21$).

The effect of prednisolone on IFN-γ production from blood cells was assessed in vitro in a pool of healthy subjects and patients with difficult-to-control asthma (n = 10). At 10^{-9}, 10^{-8} and 10^{-7} M, it produced significant inhibition of 48 ± 12% ($p < 0.01$), 63 ± 10% ($p < 0.001$) and 68 ± 10% ($p < 0.0001$), respectively (fig. 2).

Discussion

Our study shows that endotoxin-induced cytokine release from blood leucocytes reveals a clear Th2 pattern in asthmatics and atopic non-asthmatics as compared to non-atopic healthy subjects. In particular, LPS-induced IL-4 release was clearly increased in controlled and uncontrolled asthmatics when compared to non-atopic healthy subjects. By contrast, uncontrolled asthmatics displayed a strikingly decreased production of IFN-γ in response to endotoxin as compared to non-atopic healthy subjects.

Although greater in asthmatics than in healthy subjects, the blood eosinophil count was not associated with uncontrolled asthma, which is different from results we recently reported for sputum eosinophilia [12]. Blood neutrophilia was raised in uncontrolled asthmatics as compared to atopic non-asthmatics and non-atopic healthy subjects but did not distinguish controlled from uncontrolled asthmatics.

The raised spontaneous production of IL-4 seen in asthmatics and atopic non-asthmatics supports the pivotal role of Th2-driven inflammation in atopic diseases [18]. It has been clearly demonstrated that stimulation of peripheral blood monuclear cells (PBMC) in vitro with an allergen resulted in greater release of IL-4 in sensitized subjects [19]. Our study expands this finding by using endotoxin as another type of environmental stimulus. Although endotoxin is rather considered to favour a Th1 pathway accompanied by neutrophilic inflammation [7], our results show that endotoxin enhances IL-4 release from circulating leucocytes in asthmatics but not in atopic non-asthmatics nor in non-atopic healthy subjects.

This finding indicates that amplification of Th2 cytokine release from leucocytes following endotoxin exposure is restricted to atopic asthmatics but did not distinguish controlled from uncontrolled asthmatics. Remarkably, there was no relationship between the level of asthma control and spontaneous IL-4, which is a hallmark of atopy rather than of asthma.

Our results are in keeping with those of Magnan et al. [20], who, using the same whole-blood model, found that IL-4 release was more dependent on atopy than on asthma. Interestingly, circulating leucocytes from non-atopic healthy subjects, the large majority of whom failed to spontaneously release IL-4, were also largely unable to release this cytokine after stimulation with LPS. In this respect, IL-4 behaves differently from other cytokines like IL-6, IL-10 or IFN-γ, which, although not spontaneously produced by the majority of healthy subjects, are clearly released following exposure to LPS. In contrast to LPS, PHA induces IL-4 production by leucocytes from healthy subjects. This shows that healthy subjects are perfectly capable of producing this Th2 cytokine under certain circumstances. In our study, atopic non-asthmatics were particularly prompt in releasing IL-4 in response to PHA.

As we worked on a whole-blood model including all types of leucocytes, cells involved in IL-4 release may differ according to the type of stimulus. While T lymphocytes are recognized to be strongly activated by the polyclonal activator PHA and are probably the main source of IL-4 after PHA stimulation [21], release of IL-4 following LPS is perhaps more dependent on the granulocyte fraction, as eosinophils [22] and basophils [23] are also able to release this cytokine. Whichever the mechanisms, it is clear that asthmatics, and in particular patients with difficult-to-control asthma, still exhibit raised IL-4 release despite heavy treatment with inhaled and sometimes oral corticoids, a class of drug that shows a convincing inhibitory effect on IL-4 production both in vitro [24, 25] and in vivo [24, 26].

In contrast to what was found for IL-4, uncontrolled asthmatics differed from non-atopic healthy subjects in terms of a diminution of IFN-γ production following LPS exposure, which points to a deficiency of the Th1 pathway in response to this bacterial product in the more severe types of asthma. Treatment with a high dose of inhaled corticoids or oral corticoids may play a role in this reduction of IFN-γ release, as we found that prednisolone inhibited IFN-γ release from blood leucocytes in vitro in a similar way to that reported by Braun et al. [24] from PBMC. However, the impact of treatment with corticoids on IFN-γ production in vivo is highly controversial [26, 27]. In addition, the fact that, in the group with well-controlled asthma, patients taking inhaled corticoids failed to differ from their steroid-naïve counterparts suggests that inhaled corticoids might not be the main reason for the reduced production of IFN-γ seen in our patients with difficult-to-control asthma. Moreover we did not find a relationship between the dose of inhaled corticoids

and the level of IFN-γ produced in asthmatics. Our finding is in keeping with the literature, although the methodology used may differ between studies. Peripheral blood cells from children with both mild and moderate-to-severe atopic asthma were found to release less IFN-γ than those of healthy children when stimulated by lectins like concanavalin A or PHA [28, 29]. Furthermore, Leonard et al. [19] found that, in adult subjects, IFN-γ release from PBMC following allergen stimulation in vitro was lower than in atopic non-asthmatics and healthy subjects. Additionally, in that study, IFN-γ release was inversely related to symptom score in asthmatics [19]. As for the consequences of impaired IFN-γ production, it is important to mention that IFN-γ is a type 2 IFN involved in host defence against micro-organisms [30]. It is believed that some difficult-to-control asthma may be linked to persistent infection [31]. In view of this, impaired IFN-γ production in response to LPS may be an immunological feature that can make asthmatics prone to chronic infection [8, 32].

In conclusion, stimulation of blood leucocytes by endotoxin enhances IL-4 release in controlled and uncontrolled atopic asthmatics, which differentiates atopic asthmatics from atopic non-asthmatics and non-atopic healthy subjects. In addition, it reveals an impairment of IFN-γ production selectively observed in uncontrolled asthmatics. This impairment of IFN-γ release combined with increased secretion of IL-4 highlights the strongly skewed immune response towards the Th2 pattern following endotoxin stimulation in difficult-to-control asthma.

Acknowledgments

This work was supported by Pôle d'Attraction Interuniversitaire grant P6/35, the Belgian Air$_e$way study consortium and unrestricted research grants from GSK, Astrazeneca and Novartis.

References

1 Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E: Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869–877.
2 Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R, Sergysels R: Severity of asthma is related to endotoxin in house dust. Am J Respir Crit Care Med 1996; 154:1641–1646.
3 Nightingale JA, Rogers DF, Hart LA, Kharitonov SA, Chung KF, Barnes PJ: Effect of inhaled endotoxin on induced sputum in normal, atopic, and atopic asthmatic subjects. Thorax 1998;53:563–571.
4 Michel O, Dentener M, Corazza F, Buurman W, Rylander R: Healthy subjects express differences in clinical responses to inhaled lipopolysaccharide that are related with inflammation and with atopy. J Allergy Clin Immunol 2001;107:797–804.
5 Liu AH: Innate microbial sensors and their relevance to allergy. J Allergy Clin Immunol 2008;122:846–858.
6 Meerschaert J, Busse WW, Bertics PJ, Mosher DF: CD14(+) cells are necessary for increased survival of eosinophils in response to lipopolysaccharide. Am J Respir Cell Mol Biol 2000;23:780–787.
7 Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN , Dower SK, Whyte MK: Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol 2003;170:5268–5275.
8 Black PN, Scicchitano R, Jenkins CR, Blasi F, Allegra L, Wlodarczyk J, Cooper BC: Serological evidence of infection with *Chlamydia pneumoniae* is related to the severity of asthma. Eur Respir J 2000;15:254–259.
9 Wenzel SE: Asthma: defining of the persistent adult phenotypes. Lancet 2006;368: 804–813.
10 Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID: Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax 2002;57:875–879.
11 Louis R, Sele J, Henket M, Cataldo D, Bettiol J, Seiden L, Bartsch P: Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness. Allergy 2002;57:907–912.
12 Quaedvlieg V, Henket M, Sele J, Louis R: Cytokine production from sputum cells in eosinophilic versus non-eosinophilic asthmatics. Clin Exp Immunol 2006;143:161–166.
13 Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV: T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388–395.
14 Quaedvlieg V, Sele J, Henket M, Louis R: Association between asthma control and bronchial hyperresponsiveness and airways inflammation: a cross-sectional study in daily practice. Clin Exp Allergy 2009;39:1822–1829.
15 Borish LC, Steinke JW: 2. Cytokines and chemokines. J Allergy Clin Immunol 2003; 111:S460–S475.
16 Jones SA: Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 2005;175:3463–3468.
17 Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR: Development and validation of a questionnaire to measure asthma control. Eur Respir J 1999;14:902–907.
18 Durham SR: Allergic inflammation. Pediatr Allergy Immunol 1993;4:7–12.
19 Leonard C, Tormey V, Burke C, Poulter LW: Allergen-induced cytokine production in atopic disease and its relationship to disease severity. Am J Respir Cell Mol Biol 1997;17: 368–375.

20 Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM, Casano BB, Prato SJ, Fert V, Bongrand P, Vervloet D: Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. Am J Respir Crit Care Med 2000;161:1790–1796.
21 Nowell PC: Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. Cancer Res 1960;20:462–466.
22 Hoffmann HJ, Dahl C, Schiotz PO, Berglund L, Dahl R: Lectins interact differentially with purified human eosinophils, cultured cord blood-derived mast cells and the myeloid leukaemic cell line AML14.3D10: induction of interleukin-4 secretion is conserved among granulocytes, but is not proportional to agglutination or lectin-glycoprotein interaction. Clin Exp Allergy 2003;33:930–935.
23 MacGlashan D Jr: Granulocytes: new roles for basophils. Immunol Cell Biol 2008;86:637–638.
24 Braun CM, Huang SK, Bashian GG, Kagey-Sobotka A, Lichtenstein LM, Essayan DM: Corticosteroid modulation of human, antigen-specific Th1 and Th2 responses. J Allergy Clin Immunol 1997;100:400–407.
25 Manise M, Schleich F, Gusbin N, Godinas L, Henket M, Antoine N, Corhay JL, Louis R: Cytokine production from sputum cells and blood leukocytes in asthmatics according to disease severity. Allergy 2010;65:889–896.
26 Bentley AM, Hamid Q, Robinson DS, Schotman E, Meng Q, Assoufi B, Kay AB, Durham SR: Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa. Am J Respir Crit Care Med 1996;153:551–556.
27 John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, Barnes PJ, Chung KF: Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. Am J Respir Crit Care Med 1998;157:256–262.
28 Hoekstra MO, Hoekstra Y, De Reus D, Rutgers B, Gerritsen J, Kauffman HF: Interleukin-4, interferon-gamma and interleukin-5 in peripheral blood of children with moderate atopic asthma. Clin Exp Allergy 1997;27:1254–1260.
29 Nurse B, Haus M, Puterman AS, Weinberg EG, Potter PC: Reduced interferon-gamma but normal IL-4 and IL-5 release by peripheral blood mononuclear cells from Xhosa children with atopic asthma. J Allergy Clin Immunol 1997;100:662–668.
30 Billiau A, Matthys P: Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev 2009;20:97–113.
31 Cosentini R, Tarsia P, Canetta C, Graziadei G, Brambilla AM, Aliberti S, Pappalettera M, Tantardini F, Blasi F: Severe asthma exacerbation: role of acute *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* infection. Respir Res 2008;9:48.
32 Johnston SL, Martin RJ: *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae:* a role in asthma pathogenesis? Am J Respir Crit Care Med 2005;172:1078–1089.

Supplement to: Disturbed Cytokine Production at the Systemic Level in Difficult-to-Control Atopic Asthma: Evidence for Raised Interleukin-4 and Decreased Interferon-γ Release following Lipopolysaccharide Stimulation

Methods

As for blood leucocytes, we have assessed the effect of phytohaemagglutinin and lipopolysaccharide on sputum cells in a small group of 6 asthmatics encompassing the all disease severity spectrum. Cytokines (IL-4, IL-6, IL-10, IFN-γ, TNF-α) were cultured for 24h. Fifty microlitres of standards or sputum was incubated at 37°C with 200 μl of Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Cambrex, Verviers, Belgium) and 2% of inactivated fetal calf serum (Cambrex), or LPS (Salmonella *enteridis,* Sigma, St. Louis, Mo., USA; 1 ng/ml) or phytohaemagglutinin (PHA; Biochrom AG, Berlin, Germany; 1 μg/ml) in apyrogen microwells which had previously been coated with specific antibodies directed towards the chosen cytokines. After 24h culture, cytokines were measured by a two-step sandwich-type immunoassay as described previously.

Results

There was no significant difference between spontaneous and stimulated cytokine production from sputum cells either by PHA or LPS (Table 1).

Table 1. Cytokine release from sputum cells after stimulation with phytohaemagglutinin (PHA) and lipopolysaccharide (LPS).

	RPMI	PHA	LPS
IL-4 (pg/ml)	0 (0-96)	0 (0-86)	0 (0-0)
IL-6 (pg/ml)	605 (134-1473)	443 (57-1393)	474 (29-1201)
IL-10 (pg/ml)	380 (152-594)	274 (34-512)	397 (166-591)
IFN-γ (pg/ml)	2 (0-7)	0 (0-9)	0 (0-17)
TNF-α (pg/ml)	1584 (912-5000)	1309 (549-5000)	1589 (31-5000)

Results are expressed as median (range)

Discussion

In sharp contrast with what we observed with blood leucocytes, sputum cells were found to be resistant to the stimulation by a lectin or endotoxin in terms of cytokine release. However, the spontaneous production of IL-6, IL-10 and TNF-α were much greater in sputum cells when compared to the spontaneous production from blood leucocytes. This probably reflects a natural activation of airway cells by the innate immune system in response to environmental triggers (polluting particles) which makes those cells unresponsive to further stimulation. Our data are in line with those reported by Cho et al who show that PMA and ionomycin were unable to stimulate intracellular cytokine in sputum lymphocytes while having a marked effect on blood lymphocytes [1]. Likewise, Dentemer et al as well as Scheicher et al, failed to show any upregulation of TNF-α release from sputum cells after endotoxin [2;3]. By contrast Liu et al found that stimulation with PHA was necessary to detect INF-γ and IL-5 from sputum cell culture whereas these cytokines were undetectable when cells were let to spontaneously release cytokines[4].

References

1. Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. Am J Respir Crit Care Med 2005; 171:224-30.

2. Dentener MA, Louis R, Cloots RH, Henket M, Wouters EF. Differences in local versus systemic TNFalpha production in COPD: inhibitory effect of hyaluronan on LPS induced blood cell TNFalpha release. Thorax 2006; 61:478-84.

3. Scheicher ME, Teixeira MM, Cunha FQ, Teixeira AL, Jr., Filho JT, Vianna EO. Eotaxin-2 in sputum cell culture to evaluate asthma inflammation. Eur Respir J 2007; 29:489-95.

4. Liu LY, Swensen CA, Kelly EA, Kita H, Busse WW. The relationship of sputum eosinophilia and sputum cell generation of IL-5. J Allergy Clin Immunol 2000; 106:1063-9.

VI. Publication 4

Sputum IgE and cytokines in asthma: relationship with disease severity and sputum cellular profile

M. Manise, G. Holtappels, F. Schleich, C. Bachert, R. Louis

Submitted

Sputum IgE and cytokines in asthma: relationship with disease severity and sputum cellular profile

M. Manise[1], G. Holtappels[2], F. Schleich[1], C Bachert[2], R. Louis[1]

1 Department of Pneumology, CHU Sart-Tilman, Liege, Belgium
2 Upper Airway Research Laboratory, ENT Department, Ghent University Hospital, Ghent, Belgium

Address for correspondence: Maïté Manise
Pneumology-Allergology
Bât B35, CHU Sart-Tilman
Liège, Belgium
E-mail: mmanise@student.ulg.ac.be
Phone: 0032 43668568
Fax: 0032 436137

Short title: Sputum IgE and cytokines in asthma

Abstract

Background:

Local IgE production may play a role in asthma pathogenesis. The aim of the study was to assess sputum total IgE and cytokines in asthmatics according to disease severity and sputum cellular phenotype.

Methods:

We studied 143 subjects including 22 non atopic healthy subjects, 39 mild-to-moderate untreated, 47 mild-to-moderate treated and 35 refractory asthmatics (American Thoracic Society criteria) recruited from our asthma clinic at CHU Liege.
Sputum supernatant total IgE (tIgE) were measured by ImmunoCAP and sputum supernatant cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ and TNF-α) were measured with the Luminex xMAP Technology by using commercially available Fluorokine MAP kits.

Results:

After concentrating sputum samples, total IgE was detectable in the majority of subjects. Mild-to-moderate untreated and treated asthmatics and refractory asthmatics had higher tIgE levels than healthy subjects ($p<0.05$, $p< 0.05$ and $p<0.01$ respectively). In asthmatics, sputum tIgE levels were higher in atopic than in non atopic and healthy subjects and were strongly correlated to serum total IgE ($r=0.72$, $p<0.0001$). Additionally, sputum eosinophils correlated with sputum tIgE ($r=0.43$, $p<0.0001$) and with serum IgE ($r=0.35$, $p<0.001$).Sputum cytokine levels were not different between healthy subjects and asthmatics when classified according to disease severity. However, the eosinophilic asthma phenotype (sputum eosinophil count > 3%) was characterised by raised sputum tIgE, IL-5 and IL-13 compared to healthy subjects ($p<0.001$, $p< 0.001$ and $p<0.05$ respectively) and pauci-granulocytic asthma ($p<0.01$, $p<0.001$ and $p<0.05$ respectively).

Conclusion:
Asthmatics distinguish from healthy subjects by higher sputum tIgE which correlates with serum IgE. Total sputum IgE was associated with the eosinophilic asthma phenotype and, to a lesser extent, with atopic status, irrespective of disease severity.

Introduction

A significant fraction of asthmatic patients, defined as refractory asthmatics, remain poorly controlled with chronic symptoms, exacerbations and airflow obstruction despite receiving high doses of inhaled and sometimes also oral corticosteroids [1].Those patients have a poor quality of life and represent an important economic burden for the society because of sick leave, hospitalizations or emergency room visits and medication costs [2].

Thus, we need a better understanding of the pathogenesis of refractory asthma [3;4].

A new class of drug based on neutralisation of serum IgE by monoclonal antibody has proved to be useful in severe atopic refractory asthmatics [5].Local production of IgE might not be reflected by serum IgE or atopic status. Very recent data have shown that tIgE and specific IgE may be measurable in sputum from asthmatics irrespective of their atopic status even if their ability to prime local mast cells is still unclear [6].However, it has been demonstrated that local IgE in nasal polyp samples is functional [7]. IgE binding to its high affinity receptor FcεRI results in cell activation independent of the presence of any allergen. This makes of local IgE an important mediator in the mast cell activation pathway. Severe asthma was shown to be associated with intense mast cell infiltration and signs of mast cell degranulation. The relationship between the severity of asthma and the local IgE production has not been investigated so far. However, there is evidence that total IgE and especially specific IgE to staphylococcal superantigens are correlated to lung function parameters in refractory asthmatics [8].

It is now recognised that asthma actually comprises several inflammatory phenotypes and Simpson has proposed to break down asthma according to the granulocyte fraction contained in sputum cells [9]. How local IgE production is related to the airway cellular inflammatory profile remains poorly studied. It is assumed that IgE production is tightly regulated by the balance between Th1 and Th2 cytokines, interleukin-4 and 13 being involved in the immunological switch towards IgE [10]. Beside classical Th2 profile there has been recent interest for the IL-17 pathway in asthma and in particular in severe neutrophilic asthma [11]. Whether IL-17 pathway and neutrophilic asthma are related to disease severity and local IgE synthesis has not been studied so far. In human subjects hyper-IgE syndrome is associated with a genetic deficiency in Th17 cell

differentiation [12]. The increasing susceptibility of these patients to infections with candida species and staphylococcus aureus is consistent with the role of Th17 cells in immunity against these pathogens [13].

The purpose of our study was to assess total sputum and serum IgE and sputum cytokines in a large sample of asthmatics encompassing the whole disease severity spectrum. We also aimed to determine whether IgE and cytokines were related to a particular airway cellular phenotype.

Material and methods

Study design and subjects characteristics

Patient demographic, functional and treatment characteristics are given in table 1. In this study we enrolled 121 subjects consecutively recruited from our asthma clinic at CHU Liege (39 mild to moderate untreated, 47 mild to moderate treated and 35 refractory). All asthmatics were diagnosed on the basis of significant FEV1 reversibility (≥ 12% from baseline) to β2-agonists or bronchial hyperresponsiveness to methacholine (PC20M < 16 mg/ml). Atopy was defined as a positive skin prick test reaction (weal ≥ 3mm compared with control) to common aeroallergens including house dust mites, cat and dog dander, grass, tree, pollen and moulds. Refractory asthmatics (N=35) were defined according to the ATS criteria. Refractory asthmatics had been followed for at least 6 months in our department and received education about their disease before entering this study. Different groups of asthmatics were compared to 22 non atopic healthy subjects. The eosinophilic asthma phenotype was defined by a sputum eosinophil count ≥3% while sputum was considered to be neutrophilic when neutrophil count exceeded 76% (> 1.7 SD of mean neutrophil count derived from our own normal reference values[14]. Those who had less than 3% eosinophil count and less than 76% neutrophil count were considered as pauci-granulocytic. Those with eosinophil count > 3% and neutrophil count > 76% were considered as mixed granulocytic but discarded from further analysis because only 3 patients satisfied these criteria.
The protocol had been approved by the local ethic committee and every subject gave his written informed consent.

Peripheral blood sampling, serum IgE and cell count measurement

Peripheral blood samples were collected in serum tubes with gel (Venosafe, TERUMO®, Belgium). Tubes were centrifuged at 800*g* for 10 min at 4°C and sera were conserved into aliquots at -80°C until assay. The total and differential blood cell counts were obtained with an Advia 210 automatic counter (USA). Counting and cell typing were

based on flow cytometry with bidimensionnal volume distribution, peroxydase concentration and lobularity of leukocytes as parameters. Serum total IgE were measured with the ImmunoCAP system with a detection limit of 2 kU/l (Phadia AB, Uppsala; Sweden).

Sputum induction and processing

After premedication with 400µg inhaled salbutamol administered by MDI (+ Spacer), sputum was induced by inhalation of hypertonic saline (NaCl 5%) when FEV1 post salbutamol was $\geq$ 65% predicted and isotonic saline (NaCl 0.9%) when FEV1 was < 65% predicted. Saline was combined with additional salbutamol delivered by an ultrasonic nebuliser (Ultra-Neb 2000, Devilbiss) with an output set at 0.9ml/min as previously described[15]. Each subject inhaled the aerosol for three consecutive periods of 5 min and for a total time of 15 min. For safety reasons, FEV1 was monitored every 5 min and the induction stopped when FEV1 fell by more than 20% from post-bronchodilatation values.
The whole sputum was collected in a plastic container, weighted and homogenized by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 sec and centrifuged at 800 *g* for 10 min at 4°C. Supernatant was separated from cell pellet. We added DTT (dithiotreitol) to the cells which were agitated for 20min. Cells were washed once more with PBS and resuspended in 1ml. Squamous cells, total cell counts and cell viability checked by trypan blue exclusion were performed with a manual haemocytometer. The differential cell count was performed on cytospins stained with Diff-Quick after counting 400 cells.

Cytokines and sputum IgE measurement

All induced sputum samples were concentrated by use of centrifugal evaporator. 1 ml of induced sputum was entirely airdried in a SpeedVac SC 100 centrifuge (Savant, Thermo Scientific). Afterwards the pellet was resuspended in 100 µl distilled water and mixed. All samples were assayed for IL-4, IL-6, IL-10, IL-5, IL-17, IL-13, IFN-γ and TNF-α with the Luminex xMAP Technology by using commercially available Fluorokine MAP Kits (R&D Systems Europe Ltd, Abingdon, United Kingdom) following to the manufacturer´s

guidelines and measured on a Bio-Plex 200 Platform (Bio-Rad Laboratories S.A.-N.V, Nazareth Eke, Belgium). The detection limits were 3 pg/ml for IL-17, 1.5pg/ml for IL-5, 4 pg/ml for IFN-γ, 4 pg/ml for TNF-α, 2 pg/ml for IL-6, 1 pg/ml for IL-4, 11 pg/ml for IL-13 and 0.5 pg/ml for IL-10. Total IgE were measured with ImmunoCAP system with a detection limit of 0.1 kU/l (Phadia AB, Uppsala; Sweden).

Statistical analysis

Results were expressed as median (range) unless otherwise stated. Comparisons between the four groups were performed by Kruskall-Wallis Test (non parametric ANOVA) followed, in case of significance, by Dunn's multiple comparisons Test. Correlations were performed by calculating the Spearman coefficient. A P value < 0.05 was considered as statistically significant.

Results

Patient characteristics

Demographic, lung function, airway inflammation according to disease severity are given in table 1. The subjects were well matched for their age and tobacco consumption. Exhaled nitric oxide azote ($FeNO_{50}$) was higher in mild-to-moderate untreated asthmatics compared to healthy subjects ($p<0.05$). As expected, FEV1 values were clearly altered in the refractory group when compared to healthy subjects, mild-to-moderate untreated and to mild-to-moderate treated asthmatics ($p<0.001$ for both). Similarly FVC and the ratio FEV1/FVC were also significantly decreased in the refractory group as compared to other groups ($p<0.01$). As expected ACQ score was higher in refractory patients reflecting a poor asthma control ($p<0.001$). All groups of asthmatics exhibited higher sputum eosinophil counts than non atopic healthy subjects ($p<0.01$ for mild-to-moderate treated and untreated and $p<0.001$ for refractory asthmatics) without any difference between the asthmatic groups. There was no difference between groups regarding the percentage of sputum neutrophil counts (Table 1).

Treatment characteristics are given in table 2. Refractory patients were taking higher doses of inhaled corticosteroids in comparison with mild-to-moderate treated asthmatics. All refractory asthmatics and 32 out of 47 mild-to-moderate treated asthmatics were receiving inhaled LABA. Some refractory asthmatics were also taking oral corticosteroids, LTRA or theophylline.

Sputum and serum IgE and sputum cytokine levels according to disease severity

Results are given in table 3. Total IgE (tIgE) was detectable in the sputum supernatant from the majority of subjects. Mild-to-moderate treated and untreated asthmatics as well as refractory asthmatics had higher tIgE than healthy subjects ($p<0.05$ and $p<0.01$ for refractory) but groups of asthmatics did not differ from each other (Fig.1A). There was a strong correlation between sputum and serum IgE in asthmatics ($r=0.72$, $p<0.0001$) (Fig.2). Similar to what was seen in sputum, total serum IgE were not different between the asthmatic groups but clearly higher than in non atopic healthy subjects.

Sputum and serum IgE and cytokine levels according to sputum cellular phenotypes

When patients were classified according to their sputum cellularity, there were 40 eosinophilic (≥3%), 15 neutrophilic (>76%), 41 pauci-granulocytic and 3 mixed granulocytic. Sputum tIgE, but not serum tIgE, were increased in eosinophilic asthmatics when compared to healthy subjects ($p<0.001$) and pauci-granulocytic asthmatics ($p<0.01$) (Fig.1B) (Table 5). Sputum IgE were detectable in 88 % of eosinophilic asthmatics and in only 56% of pauci-granulocytic asthmatics ($p<0.01$) and 59% of healthy subjects ($p<0.05$) (Table 4). Serum tIgE were lower in neutrophilic than in eosinophilic asthmatics ($p<0.05$).

As far as cytokines are concerned, IL-5 was increased in eosinophilic asthmatics when compared to healthy subjects ($p<0.001$), neutrophilic ($p<0.01$) and pauci-granulocytic ($p<0.001$). Eosinophilic asthmatics were also characterized by greater IL-13 levels when compared with healthy subjects and pauci-granulocytic patients ($p<0.05$ for both). No difference was found regarding other tested cytokines.

Sputum and serum IgE and sputum cytokine levels according to atopy

Atopic asthmatics (N=70) distinguished from healthy subjects and non atopic asthmatics (N=51) by raised sputum IgE levels {0.36 Ku/l (0-31.2) vs 0.1 Ku/L (0-5.4) ($p<0.001$) and vs 0.16Ku/L (0-12.1) ($p<0.05$) respectively} (Fig1C). IgE was detectable in 84% of atopic asthmatics but in only 56% of patients with non atopic asthma and 59% of healthy subjects ($p<0.01$ and $p<0.05$ respectively) (Table 4). However a few non atopic asthmatics exhibited high sputum IgE levels (Fig.1C). No difference was observed regarding sputum cytokine levels between atopic asthmatics and non atopic asthmatics and healthy subjects (Data not shown).

Correlation between cells, IgE and cytokines

In asthmatics, we found a positive correlation between IL-5/IL-13 and sputum eosinophils expressed either as a percentage ($r=0.49$, $p<0.0001$ and $r=0.32$, $p<0.01$ respectively) or as absolute values ($r=0.5$, $p<0.0001$ and $r= 0.4$, $p<0.001$ respectively). Total sputum IgE correlated with sputum IL-5 ($r=0.59$, $p<0.0001$) and TNF-α ($r=0.4$, $p<0.0001$).

Discussion

Our study shows that asthmatics have higher total IgE concentrations in the sputum as compared to healthy subjects. Our data are in keeping with the recent finding of Mouthuy et al and extend our knowledge in the field by showing that sputum IgE levels are not related to disease severity but clearly increased in those exhibiting airway eosinophilic inflammation.

Likewise there was no difference regarding cytokine production when patients were classified according to disease severity but eosinophilic asthmatics exhibited a peculiar cytokine profile featuring raised Th2 IL-5 and IL-13 levels.

The role of IgE has been traditionally assigned to allergic reaction towards an aeroallergen in sensitized patients. In the nineties, Humbert et al have drawn attention to the potential role of IgE in non atopic asthma by showing increased expression of the receptor FcεRI in the bronchial mucosa in asthmatics irrespective of the atopic status [16]. Mast cells are major effector cells in IgE dependent immediate hypersensitivity reactions and in IgE associated immune responses against certain parasites [17;18]. The liaison of an allergen to IgE bound at the mast cell surface is a powerful event leading to mast cell degranulation [19].

However, it is now admitted that the binding of IgE itself to its high affinity receptor at cell surface is an event sufficient to trigger cell activation[16]. To our knowledge, very few studies have investigated the levels of sputum IgE in asthmatic disease. More than 10 years ago, Park et al validated the induced sputum model as a non-invasive method for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients [20]. They found that house dust mite specific IgE were detected in induced sputum supernatant from 7 of 10 house dust mite sensitive asthmatics based on skin prick tests. Margarit et al showed, in a small group of asthmatics, that total IgE can be measured in induced sputum and was increased as compared to healthy subjects. Although they found sputum and serum IgE to be related, they did not find a correlation between sputum total IgE and albumin suggesting that sputum IgE could be, at least in part, locally produced [21]. Moreover, a very recent study has shown that IgE production occurs both in atopic and in intrinsic asthma and that part of this IgE recognizes Der p antigens [6]. Here, in a larger group of asthmatics encompassing all disease severity

spectrum, we have assessed whether sputum IgE may be somehow related to asthma severity. We confirm the raised sputum IgE levels in asthmatics but also show that sputum IgE was not related to asthma severity. It is noteworthy that the level of local IgE was still clearly increased in refractory asthmatics which points out the inability of corticosteroids to control the immunological pathway leading to IgE synthesis. This makes understandable why omalizumab may be affective in refractory atopic asthmatics. In contrast to what Mouthuy et al reported, we found that sputum IgE levels were higher in atopic than in non atopic asthmatics and that, overall, non atopic asthmatics did not distinguish from non atopic healthy subjects [6]. This, however, does not preclude the possibility that non atopic asthmatics still have greater levels of sputum IgE directed towards common aeroallergens as shown by Mouthuy et al with respect to IgE against house dust mites. Moreover we found a convincing relationship between sputum and serum IgE in our group of asthmatics suggesting that part of the sputum IgE may be related to plasma exsudation. Alternatively this might reflect a global predisposition to produce IgE in several compartments of the body.

Like for sputum IgE, serum total IgE were not related to disease severity. This does not preclude the possibility that IgE specifically directed towards aeroallergens [22]or bacterial components [8]play a role in disease severity.

Rearranging the asthmatics according to sputum cellular profile rather than according to clinical disease severity results in an interesting finding. Eosinophilic asthmatics clearly distinguished from healthy subjects and pauci-granulocytic asthmatics by raised sputum IgE but not raised serum IgE. Our study reveals, but not explores, the mechanisms underlying this strong relationship. It is well known from bronchial allergenic challenge experiments that mast cell activation by an allergen exposure is a powerful event to stimulate eosinophil tissular recruitment. By contrast to what is seen in eosinophilic asthmatics, neutrophilic asthmatics were characterised by low IgE production both at the airway and at the systemic level. This is in agreement with the view that neutrophilic asthma is less dependent on IgE mediated reaction but rather related to pollutant exposure or infections [23].

When patients were classified according to disease severity, we did not find any significant difference regarding cytokine production between groups. However, eosinophilic asthmatics display raised IL-5 and IL-13 sputum supernatant levels when compared with healthy subjects, neutrophilic and pauci-granulocytic asthmatics. IL-5 is

a Th2 cytokine known to be able to promote eosinophil differentiation and release from the bone marrow into the blood stream. Moreover this cytokine has also a chemotactic effect on eosinophils and enhances secretion and cytotoxicity [24]. Therefore, it is not surprising that IL-5 appears to be strikingly linked to the eosinophilic pathway [25]. IL-5 has modulatory effects on IgE synthesis and, together with IL-6, increases IL-4-dependent IgE synthesis [26]. IL-13 is another Th2 cytokine thought to be a central mediator of inflammation in asthma. It has pleiotropic effects that mimics key features of asthma like increased smooth muscle contractility [27] or mucus secretion [28] and shares the same heterodimer receptor as IL-4 by binding to the α chain [29]. Berry M et al have investigated whether IL-13 expression and production was increased in asthma. They found greater IL-13 protein expression in bronchial biopsies by immunohistochemistry with eosinophils being the major source of IL-13 within the bronchial mucosa. Furthermore levels of IL-13 measured by ELISA were also raised in asthmatics [30]. Those findings are in keeping with our demonstration that raised sputum IL-13 levels were only found in eosinophilic asthmatics. However the fact that IL-13, like IL-5, is not increased in non eosinophilic asthma indicates that these Th2 cytokines are essentially related to a peculiar inflammatory profile rather than to asthma itself. This is in keeping with the study of Erin et al who showed that IL-5 and IL-13 were elevated in patients with severe eosinophilic asthma although we did not find an increase of IL-4 in our study [31]. Nevertheless eosinophilic bronchitis, while showing high IL-5 production, fails to discriminate from healthy subjects by increased IL-13 production [30;32].

It is common belief that a Th2 microenvironment is crucial in underlying atopy, this inherited predisposition to mount an IgE response towards common aeroallergens. Our data show, however, that Th2 profile is rather associated with eosinophilic inflammation than with atopy by itself. It is well recognised that eosinophilic inflammation may develop in asthma irrespective of the atopic status [33].

Other cytokines did not show any relationship with disease severity nor with inflammatory cellular phenotype. In particular it is noteworthy that sputum TNF-α level did not increase in refractory asthmatics, which is in keeping with the overall negative effect of golimumab in refractory asthma [34]. TNF-α levels were not related neither to any cellular phenotype. Likewise IL-17 was associated neither with asthma severity nor with neutrophilic inflammation. This may appear somewhat surprising as IL-17 has

been shown to promote neutrophil recruitment and activation [35]. Our finding also contrast to what Bullens et al reported using sputum mRNA but mRNA and proteins levels are not necessarily tightly related [36].

Cytokines like IL-4, IFN-γ and IL-10 were undetectable in the majority of patients. Sputum processing may influence the level of cytokines measured in the supernatant [37]. In our study we cannot, however, incriminate the use of DTT as the supernatant was only diluted with PBS, the mucolytic agent being reserved to the cellular part for improving the quality of cytopsins.

Conclusion

Our study shows that asthmatics have raised sputum IgE levels irrespective of disease severity but associated with the eosinophilic phenotype and, to a lesser extent, atopic status. Our results suggest that the use of anti-IgE in asthma may be particularly beneficial in the eosinophilic asthma phenotype.

Acknowledgment: This work was supported by PAI (pôle d'attraction interuniversitaire) grant P6/35: Belgian Air$_e$way study consortium and unrestricted research grants from GSK, Astrazeneca and Novartis.

Table 1. Demographic, functional and airway inflammatory characteristics according to disease severity

	Healthy subjects (N=22)	Mild-to-moderate untreated (N=39)	Mild-to-moderate treated (N=47)	Refractory asthmatics (N=35)
Age (years)	42±13	46±16	48±17	48±12
Sex (m/f)	14/8	21/18	22/25	16/19
Tobacco status (ns/es/cs)	13/3/6	26/9/4	23/16/8	15/12/8
BMI	25±6	27±5	25±5	27±5
Atopy	0	27	26	17
$FENO_{50}$ (ppb)	21 (6-48)	42 (8-222)*	21 (4-222)	23 (10-141)
FEV1 (%)	103±16	96±13	87±20*	62±24***†††‡‡‡
FVC (%)	108±13	103±13	96±15	80±23***†††‡‡
FEV1/FVC (%)	81±7	78±7	73±12	62±13***†††‡‡
Reversibility (%)	-	9±9	8±5	16±20
PC20M (mg/ml)	> 16 mg/ml	3.02 (0.44-14.24)	2.29 (0.13-14)	ND
ACQ	ND	1.1 (0-3)	1.2 (0-4.2)	3.2 (0.9-5.2)††† ‡‡‡
Blood eosinophils (%)	1.7 (0.7-6.3)	3.5 (0.2-9)	2.9 (0.3-12.3)	3.2 (0.4-24)
Blood neutrophils (%)	53 (47-69)	52 (43-71)	53 (40-72)	60 (42-85)
Sputum eosinophils (%)	0 (0-11)	2 (0-7)**	2 (0-67)**	3 (0-89)***
Sputum neutrophils (%)	35 (0-88)	50 (3-220)	47 (5-99)	52 (0-100)

*Age, BMI and lung function are expressed as mean ± SD, PC20M as geometric mean and other paramaters as median (range) * p<0.05, ** p<0.01, *** p<0.001 vs healthy subjects; † p<0.05, †† p<0.01, ††† p<0.001 vs mild-to-moderate untreated; ‡ p<0.05, ‡‡ p<0.01, ‡‡‡ p<0.001 vs mild-to-moderate treated. ND=not defined*

Table 2. Treatment characteristics according to disease severity

	Healthy subjects (N=22)	Mild-to-moderate untreated (N=39)	Mild-to-moderate treated (N=47)	Refractory asthmatics (N=35)
Inhaled CS	0	0	47	35
Inhaled CS (eq bud/day)	0	0	800 (0-2400)	2000 (1600-4400) ‡‡‡
LABA	0	0	32	35
LTRA	0	2	12	12
Theophylline	0	0	1	7
Hospi/patient/year	0	0	0	0.4
Exacerbation/patient/year	0	0	0	1
Oral CS≥50% time	0	0	0	5

‡‡‡ p<0.001 vs mild-to-moderate treated

Table 3. Total sputum and serum IgE and sputum cytokine levels according to disease severity

	Healthy subjects (N=22)	Mild-to-moderate untreated (N=39)	Mild-to-moderate treated (N=47)	Refractory asthmatics (N=35)
Sputum IgE (kU/l)	0.1 (0-5.4)	0.29 (0.02-6.4)*	0.19 (0.02-31.2)*	0.44 (0.02-12.72)**
Serum IgE (kU/l)	72 (5-195)	195 (7-1223)**	184 (11-9235)*	185 (7-1845)**
IL-17 (pg/ml)	0 (0-11)	0 (0-51)	0 (0-14)	3 (0-99)
IL-5 (pg/ml)	0 (0-27)	0 (0-59)	0 (0-34)	0 (0-125)
IFN-γ (pg/ml)	0 (0-0)	0 (0-0)	0 (0-193)	0 (0-13)
TNF-α (pg/ml)	8 (0-146)	4 (0-93)	4 (0-194)	6 (0-830)
IL-6 (pg/ml)	70 (12-158)	46 (0-554)	81 (2-1051)	63 (0-1183)
IL-4 (pg/ml)	0 (0-0)	0 (0-19)	0 (0-15)	0 (0-2)
IL-13 (pg/ml)	0 (0-18)	0 (0-189)	0 (0-43)	0 (0-117)
IL-10 (pg/ml)	ND	0 (0-0)	0 (0-21)	0 (0-3)

**p<0.05, ** p<0.01, *** p<0.001 vs healthy subjects, ND=not done*

Table 4. Number of patients with detectable sputum IgE levels

Phenotypes	Fraction of patients	Percentage of patients
Healthy subjects	13/22	59%
MTM untreated	26/39	67%
MTM treated	27/39	69%
Refractory	20/29	69%
Eosinophilic	36/41* φφ	88%
Neutrophilic	10/16	62%
Pauci-granulocytic	24/43	56%
Atopic	49/58 ϕ ΔΔ	84%
Non-atopic	22/39	56%

*MTM= mild-to-moderate, * p<0.05 vs healthy subjects, φφ p<0.01 vs pauci-granulocytic asthmatics, ϕ p<0.05 vs healthy subjects, ΔΔ p<0.01 vs non atopic asthmatics*

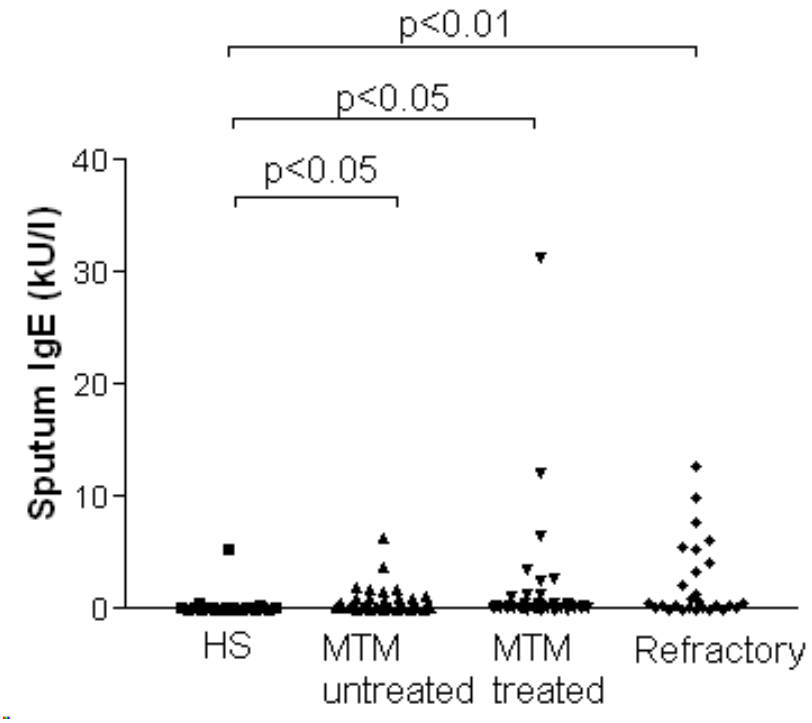
Table 5. Total sputum and serum IgE and sputum cytokine levels according to sputum cellular phenotype

	Healthy subjects (N=22)	Eosinophilic (N=40)	Neutrophilic (N=15)	Pauci-granulocytic (N=41)
Sputum IgE (kU/l)	0.1 (0-5.4)	0.6 (0.02-31)***	0.2 (0.02-8)	0.2 (0.02-6)††
Serum IgE (kU/l)	72 (5-195)	222 (9-9235)***	44 (7-1670)†*	125 (7-2177)*
IL-17 (pg/ml)	0 (0-11)	0 (0-51)	0 (0-99)	0 (0-17)
IL-5 (pg/ml)	0 (0-27)	6 (0-125)***	0 (0-15) ††	0 (0-40)†††
IFN-γ (pg/ml)	0 (0-0)	0 (0-13)	0 (0-0)	0 (0-192)
TNF-α (pg/ml)	8 (0-146)	5 (0-54)	7 (0-830)	4 (0-194)
IL-6 (pg/ml)	70 (12-158)	59 (0-487)	35 (2-1183)	83 (5-1002)
IL-4 (pg/ml)	0 (0-0)	0 (0-19)	0 (0-0)	0 (0-15)
IL-13 (pg/ml)	0 (0-18)	11 (0-189)*	0 (0-26)	0 (0-75)†
IL-10 (pg/ml)	ND	0 (0-3)	0 (0-0)	0 (0-21)

** p<0.05, **p<0.01, *** p<0.001 vs healthy subjects, † p<0.05, †† p<0.01, ††† p<0.001 vs eosinophilic asthmatics, ND=not done*

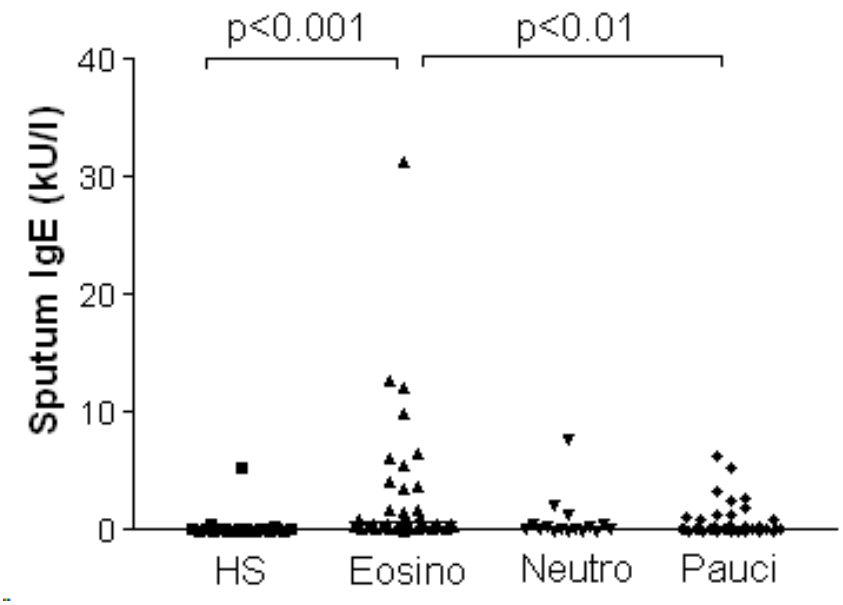
Fig 1. Sputum total IgE in asthmatics according to disease severity (A), sputum cellular profile (B) and atopy (C).

A.



HS= healthy subjects, MTM=mild-to-moderate

B.


p<0.001
p<0.01
Sputum IgE (kU/l)
40
30
20
10
0
HS
Eosino
Neutro
Pauci

C.

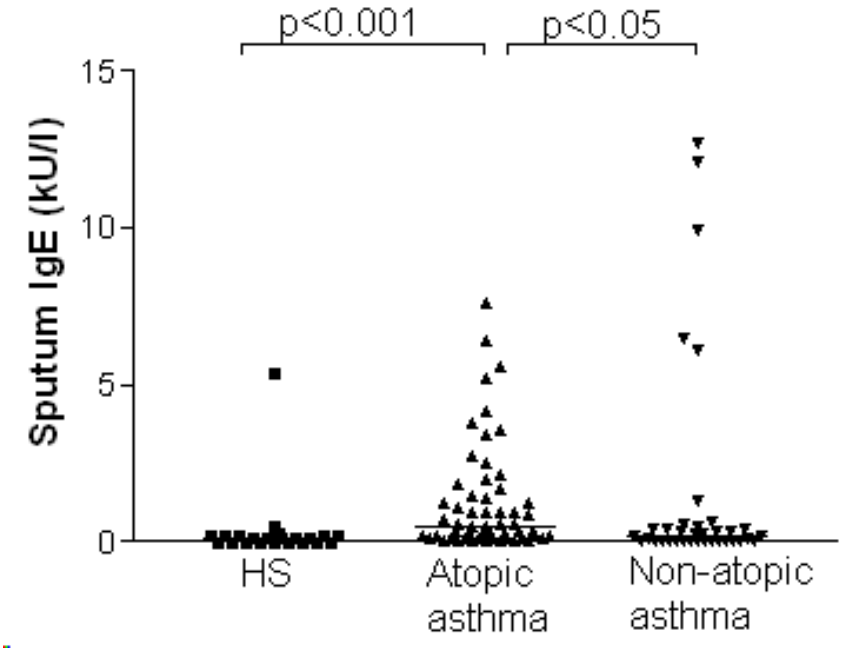
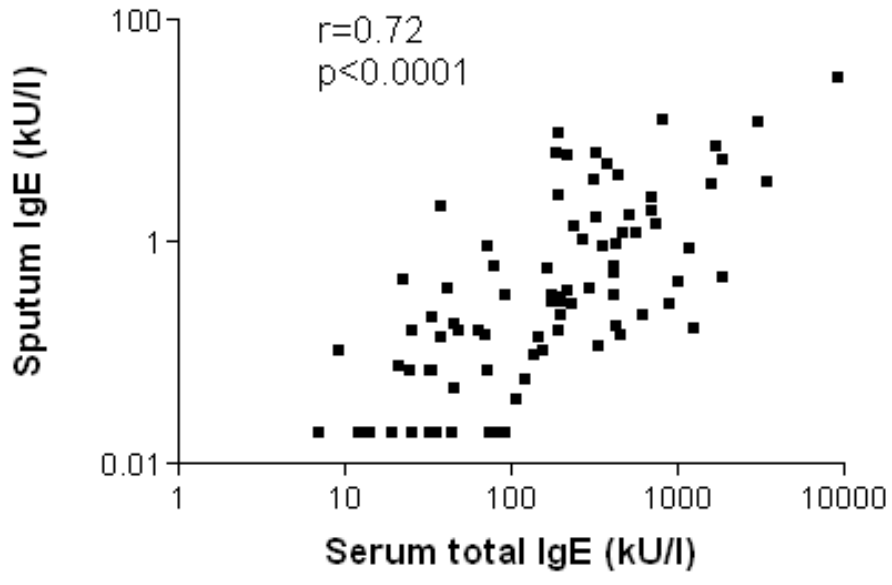

p<0.001
p<0.05
Sputum IgE (kU/l)
15
10
5
0
HS
Atopic asthma
Non-atopic asthma

Fig 2. Correlation between sputum IgE and serum total IgE in asthmatics



References

1. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000; 162:2341-51.

2. Godard P, Chanez P, Siraudin L, Nicoloyannis N, Duru G. Costs of asthma are correlated with severity: a 1-yr prospective study. Eur Respir J 2002; 19:61-7.

3. Chanez P, Wenzel SE, Anderson GP, Anto JM, Bel EH, Boulet LP, Brightling CE, Busse WW, Castro M, Dahlen B, Dahlen SE, Fabbri LM, Holgate ST, Humbert M, Gaga M, Joos GF, Levy B, Rabe KF, Sterk PJ, Wilson SJ, Vachier I. Severe asthma in adults: what are the important questions? J Allergy Clin Immunol 2007; 119:1337-48.

4. Holgate ST, Holloway J, Wilson S, Howarth PH, Haitchi HM, Babu S, Davies DE. Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. J Allergy Clin Immunol 2006; 117:496-506.

5. Holgate ST, Djukanovic R, Casale T, Bousquet J. Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy. Clin Exp Allergy 2005; 35:408-16.

6. Mouthuy J, Detry B, Sohy C, Pirson F, Pilette C. Presence in sputum of functional dust mite-specific IgE antibodies in intrinsic asthma. Am J Respir Crit Care Med 2011; 184:206-14.

7. Zhang N, Holtappels G, Gevaert P, Patou J, Dhaliwal B, Gould H, Bachert C. Mucosal tissue polyclonal IgE is functional in response to allergen and SEB. Allergy 2011; 66:141-8.

8. Kowalski ML, Cieslak M, Perez-Novo CA, Makowska JS, Bachert C. Clinical and immunological determinants of severe/refractory asthma (SRA): association with Staphylococcal superantigen-specific IgE antibodies. Allergy 2011; 66:32-8.

9. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology 2006; 11:54-61.

10. Levy F, Kristofic C, Heusser C, Brinkmann V. Role of IL-13 in CD4 T cell-dependent IgE production in atopy. Int Arch Allergy Immunol 1997; 112:49-58.

11. Kamath AV, Pavord ID, Ruparelia PR, Chilvers ER. Is the neutrophil the key effector cell in severe asthma? Thorax 2005; 60:529-30.

12. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J Exp Med 2008; 205:1551-7.

13. Minegishi Y, Saito M, Nagasawa M, Takada H, Hara T, Tsuchiya S, Agematsu K, Yamada M, Kawamura N, Ariga T, Tsuge I, Karasuyama H. Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome. J Exp Med 2009; 206:1291-301.

14. Godinas L, Schleich F, Louis R. Induced sputum towards normal values. Loukides, S, Kostikas, K., and Barnes, P. Non-invasive assessment of airways inflammation in asthma and COPD. 113-24. 2011. Paschalidis. Ref Type: Generic

15. Delvaux M, Henket M, Lau L, Kange P, Bartsch P, Djukanovic R, Louis R. Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma. Thorax 2004; 59:111-5.

16. Humbert M, Grant JA, Taborda-Barata L, Durham SR, Pfister R, Menz G, Barkans J, Ying S, Kay AB. High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. Am J Respir Crit Care Med 1996; 153:1931-7.

17. Galli SJ, Maurer M, Lantz CS. Mast cells as sentinels of innate immunity. Curr Opin Immunol 1999; 11:53-9.

18. Galli SJ. Mast cells and basophils. Curr Opin Hematol 2000; 7:32-9.

19. Asai K, Kitaura J, Kawakami Y, Yamagata N, Tsai M, Carbone DP, Liu FT, Galli SJ, Kawakami T. Regulation of mast cell survival by IgE. Immunity 2001; 14:791-800.

20. Nahm DH, Park HS. Analysis of induced sputum for studying allergen-specific IgE antibodies in airway secretion from asthmatic patients. Clin Exp Allergy 1998; 28:686-93.

21. Margarit G, Belda J, Juarez C, Martinez C, Ramos A, Torrejon M, Granel C, Casan P, Sanchis J. [Total IgE in the sputum and serum of patients with asthma]. Allergol Immunopathol (Madr) 2005; 33:48-53.

22. Zureik M, Neukirch C, Leynaert B, Liard R, Bousquet J, Neukirch F. Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey. BMJ 2002; 325:411-4.

23. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 2002; 57:643-8.

24. Renauld JC. New insights into the role of cytokines in asthma. J Clin Pathol 2001; 54:577-89.

25. Takatsu K, Nakajima H. IL-5 and eosinophilia. Curr Opin Immunol 2008; 20:288-94.

26. Bacharier LB, Geha RS. Molecular mechanisms of IgE regulation. J Allergy Clin Immunol 2000; 105:S547-S558.

27. Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. Am J Respir Cell Mol Biol 2009; 40:159-67.

28. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol 2010; 125:S53-S72.

29. Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK, von Mutius E. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol 2006; 117:269-74.

30. Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. J Allergy Clin Immunol 2004; 114:1106-9.

31. Erin EM, Jenkins GR, Kon OM, Zacharasiewicz AS, Nicholson GC, Neighbour H, Tennant RC, Tan AJ, Leaker BR, Bush A, Jose PJ, Barnes PJ, Hansel TT. Optimized dialysis and protease inhibition of sputum dithiothreitol supernatants. Am J Respir Crit Care Med 2008; 177:132-41.

32. Park SW, Jangm HK, An MH, Min JW, Jang AS, Lee JH, Park CS. Interleukin-13 and interleukin-5 in induced sputum of eosinophilic bronchitis: comparison with asthma. Chest 2005; 128:1921-7.

33. Barnes PJ. Intrinsic asthma: not so different from allergic asthma but driven by superantigens? Clin Exp Allergy 2009; 39:1145-51.

34. Wenzel SE, Barnes PJ, Bleecker ER, Bousquet J, Busse W, Dahlen SE, Holgate ST, Meyers DA, Rabe KF, Antczak A, Baker J, Horvath I, Mark Z, Bernstein D, Kerwin E, Schlenker-Herceg R, Lo KH, Watt R, Barnathan ES, Chanez P. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. Am J Respir Crit Care Med 2009; 179:549-58.

35. Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. J Leukoc Biol 2010; 88:1163-70.

36. Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, Ceuppens JL. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respir Res 2006; 7:135.

37. Kelly MM, Keatings V, Leigh R, Peterson C, Shute J, Venge P, Djukanovic R. Analysis of fluid-phase mediators. Eur Respir J Suppl 2002; 37:24s-39s.

PART VII. GENERAL DISCUSSION AND PERSPECTIVES

Although affecting a limited number of patients, refractory asthma, defined by persistent symptoms, airflow obstruction and exacerbations despite the use of high doses of inhaled corticosteroids combined with LABA and sometimes also oral corticosteroids, remains poorly understood, often frustrating to treat and account for a large part of financial burden linked to asthma [1]. Beyond the classical adaptive Th2 immunity, there is growing evidence that innate immunity driven by PAMP or DAMP may play a role in asthma and particularly in refractory asthma [2]. Thanks to the development of induced sputum, the concept of cellular inflammatory phenotype has emerged in asthma over the last ten years. A better understanding of the molecular mechanisms pertaining to refractory asthma may lead to new therapeutic development including monoclonal antibodies directed against cytokines[3]. Omalizumab, a monoclonal antibody directed against human IgE, is now an established treatment strategy in severe persistent allergic asthma [4]. In this work, we have studied the immuno-inflammatory mechanisms of refractory asthma, especially the cytokine production from sputum and blood cell culture either spontaneously or after phytohaemagglutinin or lipopolysaccharide exposure. We have also measured cytokines in sputum supernatant and sputum IgE levels in asthmatics classified according to their disease severity or their sputum cellular profile.

In the first part of this project, we have assessed spontaneous cytokine production by sputum and blood cells in refractory asthmatics and compared them with mild untreated and moderate treated asthmatics and with healthy subjects.

We found that moderate and refractory asthmatics were both characterized by a lower production of IL-6 from their airway cells when compared with mild untreated asthmatics and healthy subjects. This finding may appears somewhat surprising as asthma severity is thought to be partly related to uncontrolled airway inflammation. One potential explanation for the IL-6 decrease might be the treatment with corticosteroids in both moderate and refractory asthmatics. Our *in vitro* experiments confirm this possibility as we have shown that prednisolone was able to reduce IL-6 production from sputum cells in patients not receiving

inhaled corticosteroids. The consequence of reduced IL-6 in asthma remains uncertain but lack of IL-6 could make more difficult airway inflammation to resolve following stimulation of innate immunity by infectious agents or particles [5]. However, it should be kept in mind that this deficiency in *ex vivo* IL-6 production by sputum cells in refractory asthmatics might be counterbalanced *in vivo* by the greater number of cells present in the sputum from refractory asthmatics. Therefore the total amount of interleukine-6 present in the airways *in vivo* might not be so different between severe asthmatics as compared to healthy subjects and mild intermittent asthmatics.

Interleukine-4 is a key Th2 cytokine mainly produced by T cells which is thought to be involved in asthma and allergy [6;7]. When considering cytokine production from blood leucocytes, all groups of asthmatics exhibited a raised IL-4 production when compared to healthy subjects. We think that this is largely due to the atopic status of our asthmatic patients as the majority of them were atopic. The role of atopy in the raised spontaneous production was confirmed in the second part of our work (see below). It is interesting to note that the raised IL-4 production persisted in refractory asthmatics despite the use of inhaled corticoids and, in some of them, oral corticoids, a drug which was found to inhibit IL-4 production from blood leucocytes *in vitro*. Interestingly, in refractory asthmatics with a high sputum eosinophil count, there was a significant raised of IL-4 production at the airway level when compared to healthy subjects. This means that IL-4 may still be operating in the airways of those patients in whom corticoids are unable to control eosinophilic inflammation. This confirms our previous study conducted in mild to moderate asthmatics where we found that IL-4 production from sputum cells was essentially observed in those patients with sputum eosinophil count greater than 3% [8]. Therefore there might be a potential interest for treating these patients with biological targeting IL-4[9]. It is noteworthy that COPD, which shares intense airway remodelling and persistent airway obstruction with refractory asthma, actually expresses very different cytokine profile. Indeed, using the same experimental models, we found that COPD were characterised by a raised spontaneous production of IFN-γ both at the systemic and the airway level while there was no IL-4 production [10].

Another cytokine supposed to play a key role in the fine-tuning of inflammation is interleukine-10. Previous works on IL-10 in severe asthma have led to contrasting results [11-13]. Although we did not find difference between any groups of asthmatics and healthy subjects at the airway level, IL-10 production from blood leucocytes was found to be raised in moderate asthmatics. Our *in vitro* experiment actually showed a dual effect of corticosteroids on IL-10 release from human leucocytes with the low concentration enhancing IL-10 while

higher concentrations causing a clear inhibition. It could therefore be speculated that high doses of corticosteroids received by refractory asthmatics could explain the lower production of IL-10 seen in those patients as compared to moderate asthmatics.

In the second part of this project, we have assessed the relationship between the stimulated cytokine production from blood leucocytes and sputum cells and asthma severity. As stimuli, we have chosen phytohaemagglutinin and endotoxin as agents driving T lymphocyte response and innate immunity respectively. We have compared the production of IL-4, IL-6, IL-10, IFN-γ and TNF-α from peripheral blood leucocytes in atopic uncontrolled and well-controlled asthmatics (according to the asthma control questionnaire Juniper), atopic non-asthmatics and non-atopic healthy subjects. Our uncontrolled asthmatics were considered as severe/ refractory based on the high dose of inhaled corticosteroids they were receiving.
We found that both controlled and uncontrolled asthmatics as well as atopic non-asthmatics spontaneously produced more IL-4 than non-atopic healthy subjects. The raised spontaneous production of IL-4 seen in asthmatics and atopic non-asthmatics supports the pivotal role of Th2-driven inflammation in atopic diseases. However there was no relationship between spontaneous IL-4 and the level of asthma control which means that this cytokine is a hallmark of atopy rather than of asthma.
Interestingly, IL-4 production was induced by LPS only in asthmatics while the response to this PAMP was virtually absent in non-atopic healthy subjects and atopic non-asthmatics. This may appear somewhat surprising as endotoxin is rather considered to favour a Th1 pathway accompanied by neutrophilic inflammation [14]. Our results indicate that endotoxin exposure actually amplifies Th2 cytokine release from blood leucocytes in atopic asthmatics but did not allow us to distinguish between the mild and the severe form of the disease.
By contrast, IFN-γ release induced by LPS was found to be specifically decreased in difficult-to-control asthmatics. We suggest that there is a deficiency of the Th1 pathway in response to this environmental stimulus in the more severe types of asthma. The treatment with high doses of inhaled corticosteroids and sometimes also oral corticoids may be responsible for this reduction of IFN-γ release as we have shown with our *in vitro* experiments that prednisolone inhibited IFN-γ release from blood leucocytes. However, the fact that, in the group with well-controlled asthma, patients taking inhaled corticoids failed to differ from their steroid-naïve counterparts suggests that the inhaled corticoids might not be the main

reason for the reduced production of IFN-γ seen in difficult-to-control asthma. Moreover we did not find any significant correlation between the dose of inhaled corticosteroids and the levels of IFN-γ following stimulation by LPS in asthmatics. It has been suggested that chronic infection may be a factor driving disease severity [15]. It could be speculated that this reduced release of IFN-γ in difficult-to-control asthmatics may make them more prone to persistent infections as this type of interferon is involved in host defence against micro-organisms.
As we worked on a whole blood model we can only speculate on the blood cell type accounting for the observed differences. Experiments selectively focusing on mononuclear cells or granulocytes could cast light on cellular mechanisms involved in the disturbed cytokine release seen in asthmatics and in refractory asthmatics in particular.

In the third part of this project, we have assessed sputum total IgE and cytokines in asthmatics according to disease severity and sputum cellular phenotype. As opposed to the two previous works, we have here investigated IgE and cytokines contained in the sputum supernatant rather than in sputum and blood cell culture. Our study shows that asthmatics have higher total IgE in their sputum as compared to healthy subjects. Although it is well known that asthmatics have generally higher serum IgE than healthy subjects, to our knowledge, very few studies have investigated the levels of sputum IgE in asthmatic disease. A very recent study has shown that IgE production occurs both in atopic and in intrinsic asthma and that part of this IgE recognizes Der p antigens [16]. Here, we confirmed the raised sputum IgE levels in asthmatics but also show that sputum IgE was not related to asthma severity. It is noteworthy that the level of local IgE was still clearly increased in refractory asthmatics which points out the inhability of corticosteroids to control the immunological pathway leading to IgE synthesis. This is why omalizumab may be effective in refractory atopic asthmatics[17]. When patients were classified according to their sputum cellular profile, we found that eosinophilic asthmatics distinguished from healthy subjects and pauci-granulocytic asthmatics by raised sputum IgE but not raised serum IgE. By contrast, neutrophilic asthmatics were characterized by low IgE production both at the airway and at the systemic level. The selection of patients for treatment with omalizumab has essentially been based on serum IgE. Although generally providing satisfactory results in refractory allergic asthma, the response to the treatment has shown variability according to the subjects. So far, no clear predicting factor of a good response has been identified [18]. Our results

suggest that the use of omalizumab in asthma may be particularly beneficial in the eosinophilic asthma phenotype.

When patients were classified according to disease severity, we did not find any significant difference regarding cytokine production between groups. However, as for IgE, the reclassification according to the cellular phenotype brings interesting finding. The eosinophilic asthmatics display raised IL-5 and IL-13 sputum supernatant levels when compared with healthy subjects, neutrophilic and pauci-granulocytic asthmatics. The fact that these two cytokines are not increased in non eosinophilic asthma confirms that the Th2 profile is related to a peculiar inflammation and not to asthma itself. It also underscores the importance of an adequate selection of the patients when considering treatment with monoclonal antibodies against cytokines. The fact that IL-5 is specifically raised in those patients with intense eosinophilic inflammation explain why anti-IL-5 proved to be effective in this type of asthma [19;20] and not in all type of severe asthma[21].

Other cytokines did not show any relationship with disease severity or with inflammatory cellular phenotype. It was anticipated that the levels of IL-17 or those of TNF-α could have been increased in severe neutrophilic asthmatics. However, we did not find any evidence of raised levels of these cytokines in severe asthma or in those with a prominent neutrophilic inflammation. The lack of evidence for raised TNF-α fits with the overall disappointing results of anti TNF-α in asthma and in particular in refractory asthma [22].

In conclusion,

Our work has shown that the group of refractory asthmatics is heterogeneous in terms of cellular airway inflammation. The majority of refractory asthmatics still exhibit intense eosinophilic inflammation featuring Th2 cytokines. However, there is a group of neutrophilic and even pauci-granulocytic asthma. One of the interesting finding of our work is the demonstration of impaired IFN-γ release from blood leucocytes in refractory asthmatics in response to endotoxin. This would perhaps call for an experimental study using IFN-γ supplementation in refractory asthma. Our data also indicate that molecular mechanisms in asthma are more convincingly related to the type of cellular inflammation than to asthma severity itself, which has important consequences in terms of treatment. Different molecular mechanisms call on different treatment approaches and support the emerging concept of treatment tailoring in asthma.

References

1. Global Initiative for Asthma. Global strategy for asthma management and prevention NHLBI/WHO workshop report March 1993. National Institutes of Health. National Heart, Lung and Blood Institute. Publication Number 95-3659. January 1995. 1995.

2. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet 2008; 372:1107-19.

3. Barnes PJ. New therapies for asthma: is there any progress? Trends Pharmacol Sci 2010; 31:335-43.

4. Humbert M, Berger W, Rapatz G, Turk F. Add-on omalizumab improves day-to-day symptoms in inadequately controlled severe persistent allergic asthma. Allergy 2008; 63:592-6.

5. Jones SA, Rose-John S. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. Biochim Biophys Acta 2002; 1592:251-63.

6. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326:298-304.

7. 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.

8. Quaedvlieg V, Henket M, Sele J, Louis R. Cytokine production from sputum cells in eosinophilic versus non-eosinophilic asthmatics. Clin Exp Immunol 2006; 143:161-6.

9. Oh CK, Geba GP, Molfino N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. Eur Respir Rev 2010; 19:46-54.

10. Moermans C, Heinen V, Nguyen M, Henket M, Sele J, Manise M, Corhay JL, Louis R. Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease. Cytokine 2011; 56:298-304.

11. Magnan A, van Pee D, Bongrand P, Vervloet D. Alveolar macrophage interleukin (IL)-10 and IL-12 production in atopic asthma. Allergy 1998; 53:1092-5.

12. Robinson DS, Tsicopoulos A, Meng Q, Durham S, Kay AB, Hamid Q. Increased interleukin-10 messenger RNA expression in atopic allergy and asthma. Am J Respir Cell Mol Biol 1996; 14:113-7.

13. Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, Okamura K. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. Eur Respir J 1999; 14:309-14.

14. Nightingale JA, Rogers DF, Hart LA, Kharitonov SA, Chung KF, Barnes PJ. Effect of inhaled endotoxin on induced sputum in normal, atopic, and atopic asthmatic subjects. Thorax 1998; 53:563-71.

15. Kroegel C, Mock B, Reissig A. Interferons and their application in lung diseases. Chest 2003; 124:2406-7.

16. Mouthuy J, Detry B, Sohy C, Pirson F, Pilette C. Presence in sputum of functional dust mite-specific IgE antibodies in intrinsic asthma. Am J Respir Crit Care Med 2011; 184:206-14.

17. Molimard M, de Blay F, Didier A, Le G, V. Effectiveness of omalizumab (Xolair) in the first patients treated in real-life practice in France. Respir Med 2008; 102:71-6.

18. Massanari M, Holgate ST, Busse WW, Jimenez P, Kianifard F, Zeldin R. Effect of omalizumab on peripheral blood eosinophilia in allergic asthma. Respir Med 2010; 104:188-96.

19. Castro M, Mathur S, Hargreave F, Boulet LP, Xie F, Young J, Wilkins HJ, Henkel T, Nair P. Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. Am J Respir Crit Care Med 2011; 184:1125-32.

20. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, Marshall RP, Bradding P, Green RH, Wardlaw AJ, Pavord ID. Mepolizumab and exacerbations of refractory eosinophilic asthma. N Engl J Med 2009; 360:973-84.

21. Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS, Danzig M, Cuss F, Pauwels RA. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. Am J Respir Crit Care Med 2003; 167:1655-9.

22. Holgate ST, Noonan M, Chanez P, Busse W, Dupont L, Pavord I, Hakulinen A, Paolozzi L, Wajdula J, Zang C, Nelson H, Raible D. Efficacy and safety of etanercept in moderate-to-severe asthma: a randomised, controlled trial. Eur Respir J 2011; 37:1352-9.