Systemic inflammatory responses in asthma

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Systemische ontstekingsresponsen in astma

(met een samenvatting in het Nederlands)

Proefschrift

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We weten niet hoe we overkomen, maar we staan altijd aan de overkant

(Willem, Gerjanne & Esmée)

Contents

Abbreviations used in this thesis

Ab	Antibody
AHR	Airway hyperresponsiveness
AMP	Adenosine-5'-monophosphate
AP-1	Activator protein-1
APC	Antigen presenting cell
ATCC	American type culture collection
AU	Arbitrary units
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CaMKI	Calcium-calmodulin-dependent kinase I
CKLiK	CaMKI-like kinase
cPLA2	Cytosolic phospholipase A2
CREB	CyclicAMP response element binding protein
COPD	Chronic obstructive pulmonary disease
Dex	Dexamethasone
EAR	Early asthmatic response
ECP	Eosinophilic cationic protein
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ERK	Extracellular regulatory kinase
FADD	Fas-associated death domain
FcR	Fc-receptor
FEV_1	Forced expiratory volume in one second
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GC	Glucocorticoid
GCR	Glucocorticoid receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
GRE	GC-sensitive responsive element
HSA	Human serum albumin
Hsp90	Heat shock protein 90
ICS	Inhaled corticosteroids
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
JNK	c-Jun amino-terminal kinase
JNKK	Jun N-terminal kinase kinase
LAR	Late asthmatic response
MAPK	Mitogen-activated protein kinase
MAPKAP-K2	Mitogen-activated protein kinase-activated protein kinase 2
MAPKKK	MAPK kinase kinase
MBP	Major basic protein
MCF	Median channel fluorescence
MCP-1	Monocyte chemotactic protein-1
MES	2-(N-morpholino)ethanesulfonate
memTNF	Membrane-expressed TNF
MIP-1β	Macrophage inflammatory protein-1-beta
MK2	See MAPKAP-K2
MKK	MAPK kinase
MMP	Matrix metalloproteinase
MoAb	Monoclonal antibody
MoPhab	Monoclonal phage antibody
NF-κB	Nuclear factor κb
PBS	Phosphate buffered saline
PC_{20}	Pharmacological concentration of inhaled compound that is needed to cause a fall of 20% in FEV_1
PECAM	Platelet-endothelial cell adhesion molecule
PI3K	Phophatidylinoisitol 3'-kinase
PKA	Protein kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
ROS	Reactive oxygen species
RT	Room temperature
SAPK	Stress-activated protein kinase
SH2	Src-homology-2
sIgA	Secretory IgA
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
sTNF	Soluble TNF
TAB1	TAK1-binding protein-1
TAK1	TGFβ-activated kinase-1
TARC	Thymus and activation-regulated chemokine
Tf	Transcription factor
TGFβ	Transforming growth factor-β
Th1	T-helper 1
Th2	T-helper 2
TNFα	Tumor necrosis factor-alpha
TNFAIP6	Tumor necrosis factor-alpha-induced protein 6
TNF-R1	TNF-receptor type 1
TNF-R2	TNF-receptor type 2
TRADD	TNF-R-associated death domain
Treg	Regulatory T-cell

Chapter 1

General Introduction

General Introduction

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1. Introduction - Asthma

Allergic asthma is characterized by a reversible airway dysfunction and hyper-responsiveness for different stimuli in terms of bronchoconstriction. This increased responsiveness of the tracheo-bronchial tree can be triggered by inhalation of both specific stimuli such as allergens and aspecific stimuli such as cold air and exercise. Allergen exposures cause an inflammatory process in asthmatics, which can lead to chronic inflammatory responses found in both the airways as well as in the systemic compartment. Asthma is often part of an atopic syndrome characterized by raised levels of allergen specific Immunoglobulin(Ig)-E in peripheral blood. Environmental factors, the genetic background and the involvement of inflammatory cells contribute to the development of several clinical phenotypes of asthma. Treatment of asthmatics is in most of these phenotypes based on bronchodilation by β2-agonists and inhibition of the inflammatory process by corticosteroids.

2. Epidemiology of Asthma: Prevalence, risk factors and economic burden

Burden of asthma - Asthma is a common chronic disease affecting an estimated 300 million people worldwide[1] with a prevalence ranging from 2 till 18% between countries[1,2]. The incidence of asthma is increasing[1,3]. Main contributors to this increase include urbanization and adoption of modern lifestyles by communities[1,2,4].

Environmental factors - Changes in environmental factors are considered to be important determinants of the increase in the prevalence of asthma[5]. Air pollution is a minor environmental factor causing the increase in asthma prevalence, because prevalence rates of asthma can be relatively low in regions with the highest levels of air pollution[5-9]. It also seems unlikely that people are becoming more exposed to allergens as there is no substantial evidence that allergen exposure has significantly increased in the world during the rise in the prevalence of asthma[5]. In addition, a strong correlation between allergen exposure intensity and development of allergic diseases has not been found[5-7,10]. Nonetheless, both enhanced air pollution and the increase in allergen exposure can worsen existing asthma[11].

A more accepted hypothesis, concerning the influence of environmental factors, is

the so-called 'hygiene hypothesis'. This paradigm explains the increase in allergic diseases by decreased exposures to endotoxins and/or infections in early life due to increased hygiene levels and use of antibiotics[5,12-14]. Support for this hypothesis comes from (animal) studies showing that bacterial and viral infections can direct the immune system from an atopy- and allergy-related T-Helper (Th)2-response to a Th1-response (see also Section 3). Loss or prevention of exposure to these infectious agents prevents the normal skewing of the immune system to a (more) Th1-type and, thereby, potentiates Th2-related reactions and an increase in atopy-related diseases. However, this theory still needs to be proven and is currently debated[12,15,16]. The theory is now being "modernized" by inclusion of regulatory T-cells (Tregs) in the pathogenesis of both allergic and autoimmune diseases and is focused more on an overall hyperreactivity of the immune system in people in westernized countries rather than an increase in only Th-2 responses (see ref.[17] for e.g. the 'counter-regulatory' model).

Next to a cleaner environment, changes in lifestyle and in diet patterns may influence the risk of developing allergic diseases[5,6,18,19]. Another environmental risk factor that can increase the risk of developing asthma is (prenatal[20]) exposure to tobacco smoke[8,21,22]. Most likely a combination of several environmental factors in a unique interaction with the genetic make-up (see below) drives the increase in asthma prevalence.

Host factors - Next to environmental risk factors of asthma, also host-related factors such as genetic factors influence the type, onset, severity and persistence of asthma[5,6]. Many twin and family studies have shown strong influences of the genetic make-up on the expression of atopy-related complaints. In a study of over 11,000 Danish twin pairs it has been suggested that genetic factors are responsible for 73% of asthma susceptibility[23]. Multiple chromosomal regions, single nucleotide polymorphisms (SNPs) and gene products have been implicated in the pathogenesis of asthma and allergic inflammation. In this respect atopy, which is an important risk factor of asthma[24], and airway hyperresponsiveness were linked to genes found on chromosome 5[25,26]. Many of these linkages have been summarized in recent reviews and studies (see refs.[25-33]). A consensus is present that many of these linkages are associated with susceptibility of the disease rather than causing the disease itself[31,34]. Besides predisposition to the development of atopy and asthma, variations in genes (or expression of genes) can cause differences in sensitivity to therapy. Much emphasis is directed to this pharmacogenomics in asthma therapy in order to improve and individualize

pharmacotherapy in this disease.

Mortality - Besides the morbidity of asthma, worldwide about 1 in every 250 deaths died of asthma[1] with large differences in case fatality rates between countries[2]. In many countries, better management of asthma has resulted in a declining mortality rate[2]. However, morbidity and mortality of asthma increase in populations characterized by socioeconomic deprivation and certain ethnicities[35].

Economic burden - The economic burden of asthma is considerable and takes about 1-3% of the medical expenditures for direct medical costs such as hospitalisations and pharmaceuticals[1,2,36]. Besides these costs, indirect costs such as time lost from work and premature death increase the total economic burden of asthma[1]. The costs of asthma are influenced by many factors[2] such as severity[37,38] and the extent to which exacerbations are controlled. Especially patients with severe asthma and/or difficult-to-threat asthma contribute disproportionately to the overall costs of asthma[39,40].

Despite the fact that epidemiological data is increasing and helping to characterize the onset, prevalence and development of asthma, well-defined methods to characterize asthma phenotypes are lacking[6,41]. With respect to these limitations, epidemiology of asthma is in many cases measured via surveys and symptom questionnaires[6] albeit with their own limitations[42].

3. Inflammatory processes involved in the pathogenesis of allergic asthma

Inflammation in the airways of allergic asthma patients is characterized by involvement of T-helper 2 cells - Both inflammatory and airway cells, including eosinophils, T-cells, macrophages, mast cells and epithelial cells are involved in a complex network that regulates the onset and progression of the inflammation in the airways of asthmatics[43,44]. These cells produce and are responsive to a multitude of inflammatory mediators such as cytokines and chemokines. Especially, Th2-type cytokines including Interleukin (IL)-3, IL-4, IL-5, IL-6, IL-9, IL-13 and granulocyte macrophage-colony stimulating factor (GM-CSF) are associated with the regulation of inflammatory cell responses in allergic diseases[45-48]. These cytokines stimulate e.g. growth, differentiation and functionality of pro- and anti-inflammatory cells[47,48].

By switching off Th2 responses via Th1 cytokines, such as IFN-y and IL-12, a proper balance is established to prevent aberrant inflammatory responses in the

normal situation[49]. In asthma an imbalance in these control mechanisms exists leading to a persistent inflammatory process that is skewed towards Th2-related responses[49]. Another control on the regulation of the cytokine profile in asthmatics involves T-regulatory (Treg) cells, which can suppress both the Th1- and Th2-responses by secretion of inhibitory cytokines with among them IL-10[49] and transforming growth factor-β (TGFβ)[50,51].

Early asthmatic response (EAR) - The cascade of the immune reaction in the airways of allergic asthma patients starts with the sensitisation phase. In short, the initiation of the immune response against allergen starts with antigen presentation to Th2 cells by antigen presenting cells (APC) such as macrophages and dendritic cells. In the atopic immune responses Th2 cells become activated and secrete IL-4 and IL-13, which in conjunction with contact signals involving CD40L on T-cells and CD40 on B cells[52,53], leads to activation of B-cells to produce allergen-specific IgE. This IgE plays a central role in Type I allergic inflammation[54,55]. IgE binds to the FcεRI receptor on mast cells causing these cells to release vaso-constrictive histamine and inflammatory mediators such as leukotrienes, prostaglandins, cytokines and chemokines[45,54,56-58]. This release of mediators leads to smooth muscle contraction in the airways, mucous secretion, and vasodilatation in a so called early asthmatic response (EAR)[54,56,58].

Late asthmatic response (LAR) - Next to the inflammatory cells mediating the Type I hyperreactivity during an EAR, other inflammatory and stromal (e.g. epithelial cells) cells contribute to chronic inflammation in asthmatics by secretion of (pro-)inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNFα), GM-CSF, thymus and activation-regulated chemokine (TARC), RANTES, IL-8 and monocyte chemotactic protein (MCP-1)[45,59-62]. The release of these mediators contributes to both T-cell mediated responses as well as granulocyte/monocyte innate immune responses. This results in additional recruitment of inflammatory cells such as lymphocytes, eosinophils, neutrophils, basophils and macrophages, from peripheral blood to the lung starting approximately 3h after allergen exposure[56,63-68]. This “late” recruitment of inflammatory cells and the subsequent inflammatory response combined with an increased bronchoconstriction is characterized as the late asthmatic response (LAR), which can occur within 3h, is maximal at 6-8h and can persist for 24h after allergen exposure[56,69]. Remarkably, only around 50% of all asthmatics develop a LAR after allergen exposure and it is currently unclear why the other 50% does not experience this response.

4. Contribution of differential activation of phagocytes to asthma phenotypes

A complex interplay between inflammatory cells, genetic background and environmental factors lead to the development of chronic inflammation and different clinical phenotypes in asthmatics. In Section 2 epidemiological, clinical and experimental data concerning the environmental and genetic factors that contribute to the development of clinical phenotypes of asthmatics are discussed. In this section the involvement of phagocytes is described in the context of the development of several phenotypes of asthma.

Phagocytes such as granulocytes, macrophages and monocytes play an important role in the pathogenesis of asthma[70-72]. After allergen exposure, these cells are recruited to and activated in the lung[73].

Eosinophils - In asthmatics, eosinophilia is found in peripheral blood, airways and bone marrow after allergen exposure[74-81]. Especially IL-5 plays a prominent role in the regulation of eosinophil differentiation, recruitment from the bone marrow, recruitment to the lung, activation and survival[81-83]. However, eosinophilia in the lung tissue of asthmatics is still poorly understood in terms of responsible cytokines. Eosinophils contribute to airway inflammation by degranulation, which causes these cells to release their cytotoxic substances such as eosinophilic cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin and eosinophil peroxidase[84,85]. Among these substances, MBP is able to cause airway constriction and hyperresponsiveness[86-88]. In addition, eosinophils can produce toxic reactive oxygen radicals after proper activation of a membrane bound NADPH oxidase[89,90]. Besides the release of toxic mediators, eosinophils produce and release pro-inflammatory cytokines, chemokines and other inflammatory mediators[84,85]. The recruitment and activation of eosinophils is, therefore, thought to be involved in the propagation of chronic inflammation in the airways of asthmatics.

However, a recent study denied the central role of eosinophils in allergic airway responses[91]. In that study Leckie *et al*. described the effects of a humanized (blocking) IL-5 antibody in allergic asthmatics and did not show a change in airway hyperresponsiveness nor effects on allergen-induced late allergen responses, while levels of eosinophils were decreased in peripheral blood[91]. Therefore, the role of eosinophils in the pathogenesis in asthma is still elusive but the suggested dissociation between eosinophils, airway hyperresponsiveness, lung function and symptoms of asthma may be explained by 1) the multitude of involved

(inflammatory) cells and mediators[92] and/or 2) the fact that tissue eosinophils are not fully responsive to anti-IL-5 therapy[93]. However, the development of mice without eosinophils at least provided evidence that eosinophils do play a role in the pathogenesis of allergic asthma in murine models[94,95].

In many cases, inflammatory cells such as granulocytes are not directly activated but first undergo a (reversible[96]) process referred to as priming[97-99]. During priming responses, inflammatory cells are not directly activated by inflammatory stimuli, but acquire increased responsiveness towards heterologous stimuli[97,98]. Mechanisms of eosinophil priming *in vivo* are poorly understood, but priming *in vitro* can be achieved by Th2 cell-derived cytokines such as IL-5, chemokines and bioactive lipids and peptides[100,101]. These *in vitro* priming responses modulate eosinophil migration, adhesion, degranulation, immunoglobulin receptor activity and respiratory burst[102-106]. In addition, priming of these cells can result in responsiveness towards factors such as chemokine IL-8 which do not function on unprimed cells[102]. Overall, most of the research involved in priming is done using *in vitro* studies but several studies have shown enhanced eosinophil priming in (allergic) asthmatics using priming associated epitopes[107] or investigations of functional responses of isolated cells[108-112].

Priming of eosinophils *in vivo* is particularly associated with an enhanced functionality in adhesion responses[112]. This phenotype likely facilitates recruitment of these cells from the blood to the airways. This hypothesis is supported by demonstration of increased chemotactic responses and transendothelial movement of primed eosinophils[109-111,113] that might be mediated by regulation of their adhesion molecules[114].

Neutrophils - Besides eosinophils, neutrophils have also been implicated in the pathogenesis of asthma[56,115-119]. Neutrophils are particularly associated with inflammation in more severe asthma, which is characterized by elevated levels of these cells in tissue, sputum and bronchoalveolar lavage (BAL)-fluid in patients with this disease phenotype[116,118,120]. However, Douwes *et al.* argued that neutrophil responses occur both in severe and mild-to-moderate asthmatics[117]. These neutrophil associated phenotypes can exist either alone or in conjunction with eosinophils. The presence of neutrophils in asthma phenotypes is associated with an increase in production and release of the proteolytic enzyme matrix metalloproteinase (MMP)-9, reactive oxygen species (ROS) and inflammatory cytokines[115,121-124]. These findings suggest that neutrophils participate in the irreversible loss of lung function and the remodelling process in the airways of

asthmatics and, thereby, contribute to the development of chronic inflammation[56,125]. However, priming of neutrophils in peripheral blood or the airways of allergic asthmatics has not been clearly described. Suggestive for both the neutrophil priming in the lung and their recruitment from peripheral blood to this compartment is the association between enhanced neutrophil numbers in the airways and increased IL-8 levels in sputum[116,126].

In severe asthma, patients are treated with corticosteroids (see Section 5). Although corticosteroids are known to reduce eosinophilic inflammation and are suggested to inhibit their survival, the effect of this treatment on neutrophils is less clear[127,128]. Corticosteroid treatment can increase the lifespan of neutrophils by inhibiting apoptosis[129-134]. However, neutrophil inflammation in asthmatics can not simply be ascribed by delay of neutrophil apoptosis and increased eosinophil apoptosis by steroid treatment, because eosinophilic inflammation is also found in severe and well-controlled asthmatics despite their use of corticosteroids[135,136].

Basophils - Also basophilic granulocytes are linked with allergic inflammation[137]. These cells are the least abundant leukocytes in peripheral blood. However, these cells contain biogenic amines, such as histamine, in their cytoplasmic granules that are released in analogy to mast cells by cross-linking of IgE receptors[138]. Basophils can, therefore, be an active participant in the pathogenesis of asthma as an important cellular source of pro-inflammatory mediators[139].

5. Treatment of the inflammatory processes in asthmatics: pharmacotherapy using glucocorticosteroids

Treatment - First line in asthma pharmacotherapy consists of β2-agonists and glucocorticosteroids[140,141]. β2-agonists are used by inhalation to induce relaxation of smooth muscle and, thereby, reduce airway bronchoconstriction. For inhibition of the chronic inflammatory responses in asthma corticosteroids are the most effective anti-inflammatory medication. In patients with mild-to-moderate asthma and those with persistent symptoms corticosteroids can be applied by inhalation or systemically[140,142,143]. Different inhaled corticosteroids (ICS) vary in their potencies and topical activities[144], although it has been suggested that delivery devices and pharmacokinetic differences influence clinical efficacy more than the different potencies of corticosteroids[145-147].

(Non-)responders - Despite regular use of corticosteroids, a small proportion of asthmatics fail to respond satisfactorily to this treatment[143,148,149]. In asthmatics who are normally responsive to steroids, these compounds inhibit inflammation at many levels. They reduce cytokine production, differentiation, recruitment and activation of inflammatory cells. In addition, they improve pulmonary function by reducing airway hyperresponsiveness, exacerbations and symptoms[140,142-144,150].

Effects of corticosteroids - Most of the actions of corticosteroids occur after prolonged incubation and are mediated by genomic processes (at least several hours; for mechanisms see Section 6.2.2). However, a rapid effect of the steroid budenoside was identified in an animal model of asthma[151]. Budenoside inhibited allergic asthma reactions in the guinea pig within 10 min, most likely via a non-genomic mechanism. In asthmatics, rapid effects of fluticasone on lung function were demonstrated by Luijk *et al*[152]. In addition, acute nongenomic vasoconstriction by corticosteroids[153,154] is considered as an important anti-inflammatory mechanism in the airways of asthmatics[155]. Although there are obviously rapid effects of corticosteroids in many cellular systems, their contributions to the control of inflammation in asthma are poorly defined. In Chapter 5 we show for the first time a rapid, most likely non-genomic, priming effect of corticosteroids on eosinophil effector functions. The working mechanisms of corticosteroids behind both the genomic and nongenomic level are described in Section 6.2.2.

Adverse events - Next to the beneficial anti-inflammatory effects of corticosteroids, adverse events can occur during treatment with ICS or oral corticosteroids. In several pharmaco-epidemiological studies adverse effects of corticosteroids have been found[156]. Examples of steroid-induced adverse effects include growth reduction and adverse ocular effects of which glaucoma and cataract are most common (reviewed in ref.[156]). Besides these adverse effects, corticosteroids also affect bone density[156]. Especially oral corticosteroids induce bone loss by decreasing the bone mineral density[157-160]. These factors significantly increase the risk of osteoporosis and osteoporotic fractures. Pharmaco-epidemiological and clinical studies concerning these effects on bone loss by ICS are conflicting[156,160-164]. Therefore, more research is needed to establish the risk of ICS on bone density and bone fractures. In any case, it is important to use the lowest effective dose of ICS to limit the effects on bone metabolism[156].

Compliance to therapy - Another very important issue in pharmaco-epidemiological research of corticosteroids includes compliance to steroid

therapy[156,165,166]. Poor compliance is a major barrier to treatment of asthma. This can be caused by many factors such as safety concerns[167], simplicity of the device, dosing regimens and unpleasant tastes[168,169]. But also the onset of clinical effectiveness of ICS after several days can cause patients to discontinue their chronic therapy in daily routine[166,170]. Poor compliance to asthma pharmacotherapy and asthma regiments can influence the severity and clinical phenotypes of asthma.

6. Regulation of eosinophil and neutrophil effector functions

Granulocytes express several membrane receptors that are important in regulation of their priming and activation. The following section focuses on signalling pathways induced by granulocyte stimulating mediators that are relevant to this thesis.

6.1. Membrane receptors on granulocytes

Granulocyte functions are (in part) regulated by the functionality of their membrane receptors, which can be modulated upon interaction with environmental factors. Activated receptors can initiate signal transduction pathways involved in cellular activation. The following groups of receptors will be discussed: cytokine receptors, G-protein coupled receptors (GPCRs), Fc-receptors and receptors involved in granulocyte adhesion and migration.

6.1.1. Cytokine receptors

Cytokine receptors that are involved in hematopoietic functions can be divided into three subfamilies based upon homologies in their molecular structure of which at least one common subunit is shared within a subfamily of receptors[171]. Many of these receptors elicit different functions on distinct cell types although redundancy in signal transduction from the receptors occurs mostly with receptors that share the same common subunit. This subunit is Gp130 for the receptors of the IL-6 type of cytokines[172], while the IL-2R gamma-chain is shared between the receptors for

IL-2, IL-4, IL-7 and IL-15[172]. The third class shares the common β-chain (CD131) and includes the GM-CSF, IL-5 and IL-3 receptors[171].

6.1.2. Common β-chain (CD131) containing receptors

The β-chain (CD131) containing receptors are a group of receptors that share a common β-subunit and include the GM-CSF, IL-5 and IL-3 receptor[171]. The β-chain of these receptors is not able to bind cytokines by itself but is essential in formation of a high affinity receptor after association with the cytokine-specific α-chains[171]. Upon ligand binding to the specific α-chains hetero-dimerization with β-chains occurs and the formation of the trimeric complex (cytokine/α-chain/β-chain) leads to phosphorylation of multiple tyrosine residues in the β-chain[171,173-176]. This phosphorylation is initiated by Janus kinase 2 (JAK2) that is constitutively bound to the β-chain[177,178]. Dimerization of receptors brings two JAKs in close proximity causing them to cross-phosphorylate each other and the tails of the β-chain. The phosphorylated tyrosines serve as docking sites for proteins containing Src-homology-2 (SH2) domains. Signal transducer and activator of transcription (STAT) family members are well known proteins that can be activated by the phosphorylated tyrosines in the β-chain, although STATs were also shown to be activated via JAKs[174-176,179]. Upon activation, STATs dimerize and translocate to the nucleus and subsequently regulate target genes. No clear preference for certain phosphorylated Y-residues in the β-chain has been identified in the activation of STAT's, such as STAT 5[180,181].

Besides activation of STATs, phosphorylated tyrosine residues in the β-chain can also bind kinases that initiate different signal transduction pathways. For example, tyrosines Y577 and Y612 have been shown to be critical mediators of the phophatidylinoisitol 3'-kinase (PI3K)/Protein Kinase B (PKB) and the Ras/Raf/ extracellular regulatory kinase (ERK) pathways from the β-chain[182]. Other kinases, such as Src kinases, adaptor proteins such as Shc and phosphatases such as SHP2 can also bind to the β-chain. These effector molecules transduce signals downstream from the receptor to activate cellular functions like proliferation, priming, activation and differentiation[179,182-185]. Next to mitogen-activated protein kinase (MAPK) ERK activation, also P38 MAPK can be activated by β-chain containing receptors[103] although the exact residues in the receptor that cause P38 MAPK activation are currently unknown.

6.1.3. Tumour necrosis receptor family

Within the family of TNF-receptors two members are known to bind TNFα: TNF-receptor type 1 (TNF-R1/ p55) and TNF-receptor type 2 (TNF-R2/ p75)[186,187]. TNF-R1 is constitutively expressed in most tissues whereas expression of TNF-R2 is highly regulated and particularly found on cells of the immune system[187]. In general, the role of TNF-R2 is likely underestimated because this receptor can only be fully activated by membrane-expressed TNF (memTNF) and not by soluble TNF (sTNF)[187,188].

TNF-R1 contains domains in the intracellular part of the receptor that can signal for death as well as survival[189]. Upon activation several signalling molecules that are associated with apoptosis, such as TNF-R-associated death domain (TRADD) and Fas-associated death domain (FADD), are recruited to the cytoplasmic tail of TNF-R1[189]. These intermediary signalling molecules lead to activation of the effectors of apoptosis namely the caspases[189].

Apart from regulation of apoptosis, a complex array of other signalling pathways leads eventually to activation of nuclear factor κb (NF-κB), transcription factor (Tf) Ap-1 and mitogen-activated protein kinase kinases (MKKs) with downstream activation of MAPKs P38 and JNK[187,189]. From these signalling pathways is activation of NF-κB particularly associated with cellular survival[190-192].

The fact that TNF-RI can initiate both apoptosis and survival pathways within the same cell type shows that the dominance of either pathway is likely determined by several mechanisms, which might include 1) co-signals emanating from other receptors[193], 2) the dose of TNFα[194] and/or 3) environmental factors such as adhesion[195] and cellular stress[196,197].

6.1.4. G protein-coupled receptors

Many receptors of inflammatory mediators on granulocytes belong to the family of serpentine receptors, which are also called G-protein coupled seven-transmembrane receptors (GPCRs). Chemoattractants, including chemokines, mediate their signals through this group of receptors[198]. Serpentine receptors couple to heterotrimeric G-proteins consisting of an α and βγ-subunit[199-201]. Ligand binding to the receptor results in activation of the G-protein by the exchange of GDP for GTP in the Gα subunit, causing this subunit to dissociate from the βγ-

subunit. This dissociation leads to activation of a variety of intracellular signal transduction pathways with among them PI3K, Protein Kinase A (PKA), GTPases, MAPKs and phospholipase C (PLC)[200-202] of which the latter results in signalling based on increases in cytosolic free Ca^{2+} and activation of Protein Kinase C (PKC)[203]. Changes in intracellular Ca^{2+} are associated with induction of multiple functions, such as activation of cellular kinases and phosphatases, degranulation, phagosome-lysosome fusion, regulation of cytoskeletal binding proteins, transcriptional control and modulation of surface receptors[204].

6.1.5. Immunoglobulin receptors

Next to cytokines, antibodies or immunoglobulins (Igs) are involved in the regulation of granulocyte activation. Igs consist of a 'Y'-shaped structure with a variable region (Fab fragment) on top of the v-shaped structure that specifically interacts with an antigen. The other part of this structure is the constant Fc-region, which binds to single transmembrane Fc-receptors (FcRs) on cells involved in the immune system. Based on differences in the Fc-region five subclasses of Igs are described: IgA, the previously mentioned IgE, IgG, IgM and IgD. Each subclass of Igs has its specific receptor[205-210]: FcαR, FcεR, FcγR, FcμR[205], FcδR[206,207], respectively.

Fc-receptors - Next to the FcRs that are specific for the different subclasses of immunoglobulins (see above), one receptor can bind multiple subclasses and is involved in translocation of plasma Ig across epithelial surfaces (poly-Ig receptor, FcRn). The different FcRs regulate leukocyte activation upon Ig-binding to the receptors. This results in Ig-mediated cellular functions such as degranulation, cytokine production, phagocytosis and respiratory burst[208,209]. Many of the leukocyte FcRs are complexes of a ligand-binding α-chain and accessory subunits (β-, γ- or ζ-subuntis). These subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) which are important for stabilisation of FcR expression, ligand affinity and play an important role in signal transduction[211-213]. The focus in this thesis is directed to the functions of the receptors for IgA and IgG on granulocytes with special attention to eosinophils.

IgA/FcαR - IgA binds to its specific receptor FcαRI (CD89), which is expressed by all cells of the innate immune system[211]. CD89 can bind both IgA subclasses IgA1 and IgA2, which are present in serum and mucosal secretions[211].

Serum IgA is mainly monomeric[211] but in (mucosal) secretions it exists as secretory IgA (sIgA). The latter form is a dimer, in which the monomers are joined by a polypeptide termed J-chain and this dimer is linked to a 'secretory component'[211]. This type of IgA is involved in first-line defense of mucosal surfaces[211] and is thought to play an important role in the regulation of granulocyte functions in the airways of asthmatics[214]. In addition, IgAs that are specific to allergens are present in patients with allergic diseases such as asthma[214,215].

IgG/FcγR - IgG can bind to three classes of receptors, which are FcγRI, FcγRII and FcγRIII[216]. These receptors regulate several effects that are important in the cross-talk between humoral and cellular immune reactions. Signal transduction by these receptors results in cellular activation (signalling through e.g. ITAMs) or negative regulation of cellular responses (signalling through immunoreceptor tyrosine-based inhibition motifs (ITIMs)). Cells involved in immune responses such as monocytes, macrophages, NK cells, mast cells, eosinophils, neutrophils, and platelets express the activating FcγRs[216]. However, several of these cells co-express inhibitory FcγRs which indicates that physiological thresholds of activating/inhibiting stimuli determine whether cells are activated or inhibited[216]. It seems, therefore, likely that differential expression of these receptors on effector cells during differentiation will determine whether effector cells will have a potent cytotoxic phenotype or a more suppressive phenotype[216].

6.1.6. Receptors involved in cellular adhesion, diapedesis and migration

Granulocytes are known to extravasate from peripheral blood to the tissue at inflammatory loci. This extravasation is preceded by priming of these cells in peripheral blood, rolling along and attachment to endothelial cells, trans-endothelial diapedesis and migration into the inflammatory site (see Fig. 1)[217-220]. Rolling of granulocytes at the endothelial cell surface involves low-affinity and reversible glue-like interactions mediated by selectins and their ligands. Selectins are a family of glycoprotein surface adhesion molecules, including L-selectin (leukocytes), E-selectin (endothelial cells), and P-selectin (platelets and endothelial cells)[219]. After rolling, the granulocytes can adhere to endothelium via a stationary high-affinity adhesive interaction. This interaction is mediated mainly by integrins and their ligands, such as ICAMs[219]. Integrins are heterodimeric proteins consisting

of an α-subunit and β-subunit. Among the family of integrins, the β2-integrins are present on leukocytes and are essential for normal leukocyte trafficking and migration[219]. In this class of integrins, four different α-subunits (CD11a, CD11b, CD11c and CD11d) are known to bind to a common β-subunit (CD18). The relative contribution of each of the α-subunits to leukocyte adherence and transmigration can vary depending on the cell-type and stimulus[219,221].

The integrins and selectins participate in leukocyte diapedesis, but also other adhesion molecules such as platelet-endothelial cell adhesion molecule (PECAM)-1 might be involved in this process[219]. After diapedesis, leukocytes migrate towards their destination after proper stimulation by chemokines and/or chemoattractants.

Cells migrate with a typical teardrop resembling shape. Its broad and flat edge is called the lamellipodium and is the leading edge of the cells. The narrow end at the rear of the cells is referred to as uropod[222]. Protrusion or extension of the lamellipodium is mediated via cellular activation by chemoattractants and crosslinking of integrins.

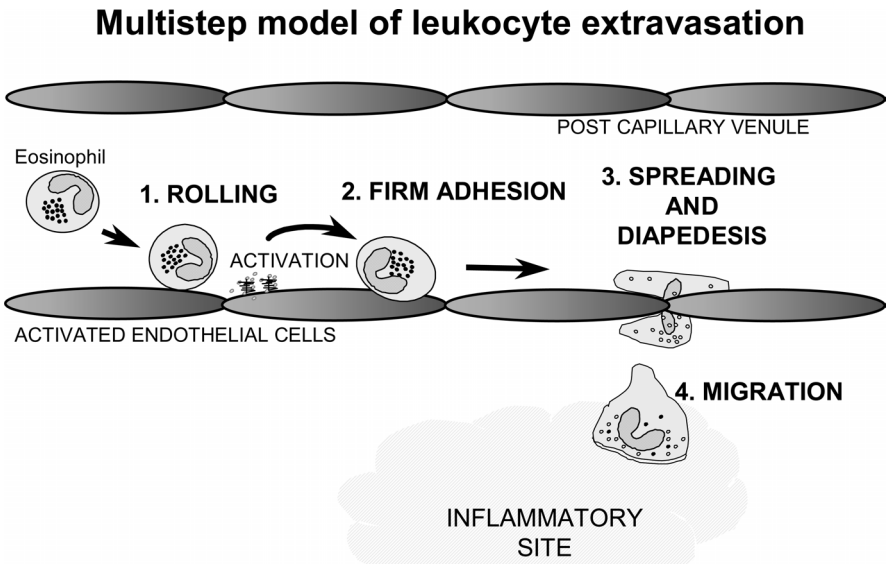

Figure 1. Multistep paradigm for leukocyte emigration: 1. Free flowing eosinophils can tether and role along the endothelial wall provided that the proper selectins and ligands are expressed. This first step in the process is not sensitive for inside-out signaling (see Section 6.1.7). 2. Upon stimulation by chemokines or other chemotactic factors presented by endothelial cells, eosinophils activate their integrins by inside-out signaling (see Section 6.1.7) and the cells arrest on the surface. 3. In response to gradients of chemotactic factors the cells move to the intercellular junctions and diapedese through the endothelium in an integrin and PECAM-1 dependent manner. 4. After diapedesis the leukocytes migrate to the site of inflammation in an integrin dependent fashion.

6.1.7. Inside-out and outside-in regulation of Fc-receptors and integrins

One of the first processes that occur after cellular (pre)activation by cytokines is the functional modulation of immune receptors by a process generally termed inside-out signalling. Inside-out regulation of granulocytes is also a control mechanism for the establishment of a correct immune homeostasis to prevent aspecific activation of these potentially cytotoxic cells.

Inside-out control is a rather unique control system as this mechanism is responsible for the (continuous negative) signaling that actively keep receptors in a non-functional state or switch the receptors to a fully functional state[223]. This is in contrast to other signaling modules that are present in a default inactive configuration, such as kinase/protease cascades and production of second messengers.

Fc-receptors and integrins are regulated by inside-out control in inflammatory cells[223-226]. These receptors are generally present in a non-functional form on the cell membranes of unprimed phagocytes, which means that the receptors have a low affinity and avidity for their ligand. Upon cellular interactions with cytokines these receptors quickly gain functionality, which is mediated via intracellular signaling from the cytokine receptors towards cytoplasmic domains in the Fc-receptors and integrins. Functional Fc-receptors and integrins bind ligand with high affinity and subsequently with high avidity (Fig. 2).

Several mechanisms that are regulating the inside-out control of these receptors are subject to this thesis. Previous work of our group and studies of others have shown that several intracellular signaling pathways and proteins are instrumental in these inside-out processes, such as the PI3K- and P38 MAPK-pathway, small-GTPases and a recently cloned kinase that has homology to calcium-calmodulin-dependent kinase I (CaMKI): CaMKI-like kinase (CKLiK)[103,227-230]. In addition, the spatial organization of parts of the membranes into specific areas, such as lipid rafts and adhesiosomes, contribute importantly to the optimal functionality of both Fc-receptors and integrins.

Besides their regulation via inside-out signaling, these Fc-receptors and integrins can also function as signaling receptors that transduce signals from the receptor into the cell[211,226,231]. This process is termed ‘outside-in’ signal transduction. Fc-receptors and integrins might even influence each other’s functionality using both outside-in and inside-out regulation[232].

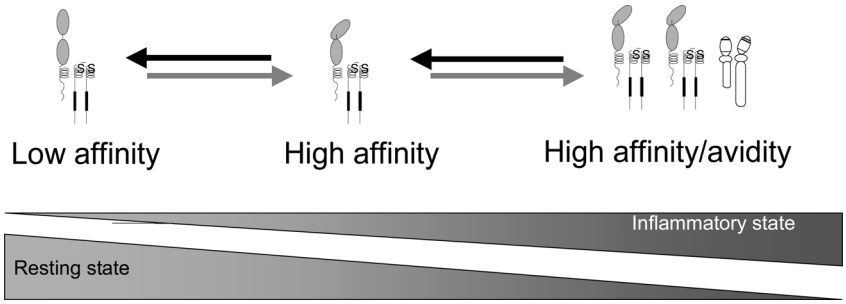

Figure 2. Functional state of Fc-receptors and integrins in the inflammatory process.

6.2. Signal transduction

From cellular receptors numerous intracellular signalling pathways link receptor activation with cellular functions. Also corticosteroids use intracellular signalling components to direct their actions after passage of the lipophilic steroid through the cellular membrane. Describing all signalling pathways in granulocytes is beyond the scope of this introduction but in the studies that are described in this thesis the focus was mainly directed towards the following signalling pathways: the P38 MAPK pathway and the signalling through steroid receptors.

6.2.1. The P38 MAPK signalling pathway

MAPK family - Among the signalling pathways, the MAPK pathways contribute to many functional outcomes of granulocytes after activation by inflammatory mediators. In general three different classes of MAPKs are described, depending on the level of signal transduction. MAPK kinase kinases (MAPKKKs) constitute the first level that is activated by an activated receptor. These MAPKKKs transmit their signals to MAPK kinases (MAPKKs or MKKs) that subsequently activate MAPKs. The MAPK family is subdivided into three major members[233,234]: 1) ERK, 2) c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and 3) P38 MAPK.

P38 MAPK - P38 MAPK is phosphorylated by MKK3 and MKK6. In addition, P38 MAPK can be activated by the Rho-family of small GTPases (e.g. Rac and Cdc42)[235-237] and even by members of the MAPK JNK-family including the Jun N-terminal kinase kinase (JNKK), which is also termed MKK4[238,239]. P38 MAPK can be present in both cytosolic and nuclear compartments where it

phosphorylates and activates transcription factors or other protein kinases. P38 MAPK is activated by dual phosphorylation on a conserved Thr180-X-Tyr182 motif.

P38 Autophosphorylation - Next to activation of P38 MAPK by phosphorylation via an upstream MKK, recently two other pathways have been described that involve induction of P38 MAPK autophosphorylation[240-242]. One of these pathways is regulated via TAK1-binding protein-1 (TAB1)[240]. After proper cellular activation TAB1 binds to P38 MAPK causing P38 MAPK autophosphorylation. The second pathway includes the stimulus-dependent phosphorylation of another phosphorylation site on P38 MAPK, Tyrosine 323 (Y323)[242]. The association of P38 MAPK with TAB1 and Y323 phosphorylation both result in dual phosphorylation of P38 MAPK on its conserved Thr-X-Tyr motif. P38 MAPK dual phosphorylation mediated via upstream MKKs is insensitive to SB203580. SB203580 inhibits P38 MAPK via occupation of the ATP-binding pocket within the P38 MAPK kinase cleft without influencing the Thr-X-Tyr phosphorylation sites[243-246]. However, P38 MAPK phosphorylation under conditions of an association with TAB1 or Y323 phosphorylation is sensitive to SB203580 indicating that these P38 MAPK activations occur via a process in which P38 MAPK autophosphorylation is involved[240,242,247,248].

P38 MAPK and inflammation - Activation of P38 MAPK via one of the three mentioned pathways can be associated with inflammation and tissue remodeling and might, therefore, be involved in a variety of pathophysiological responses[249]. Recently performed inhibitor studies using small molecule inhibitors against P38 MAPK have indicated that P38 MAPK is a central mediator in pulmonary inflammatory diseases[250-254] such as chronic obstructive disease (COPD) and asthma. These studies showed that P38 MAPK is involved in e.g. granulocyte infiltration, inflammatory mediator production and regulation of inflammatory cell apoptosis. Although the regulation of P38 MAPK in pulmonary diseases is not very well described with regard to individual cellular processes, P38 MAPK activation induces the expression of several granulocyte stimulating inflammatory cytokines and chemoattractants in pulmonary diseases[250,251,254].

6.2.2. Glucocorticosteroid-induced signalling

The effects of a glucocorticoid (GC) start with binding of the steroid to the intracellular glucocorticoid receptor (GCR) after the lipophilic steroid has passed

the plasma membrane[143,144]. GCs can bind to two isoforms of the GCR, GCRα and GCRβ. The latter is a silent decoy receptor and raised levels of this splice variant are associated with steroid-insensitive asthma[144,255,256], but its importance therein is debated[143]. Other mechanisms of steroid resistance in asthma include aberrant P38 MAPK activation, defective inhibition of Tfs and/or defective histone acetylation (for a review see ref.[143]).

Binding of the GCR to GCs causes the GCR to dissociate from Heat shock protein 90 (Hsp90), to which it is bound in the absence of ligand. Hsp90 keeps the GCR in the cytosol in the absence of a ligand. After ligand induced dissociation from Hsp90 the GC-GCR complex translocates to the nucleus and dimerizes with another GC-GCR complex to form a signalling competent dimer. This dimer can interact with a GC-sensitive responsive element (GRE) on DNA causing either trans-activation or trans-repression of transcription of genes. Besides interactions with GREs, the GC-GCR dimer can also bind to Tfs to inhibit or enhance transcription of genes. This latter mechanism of steroid actions is most likely involved in reducing inflammatory responses[144].

Transcription factors such as activator protein-1 (AP-1), NF-κB and cyclicAMP response element binding protein (CREB) are involved in the inflammatory process in the airways of asthmatics[142-144]. Especially AP-1 and NF-κB mediate the transcription of genes encoding chemoattractants, cytokines, cytokine receptors and cell adhesion molecules in this inflammatory process[144]. GC-GCR dimers can bind to a GRE to prevent AP-1 binding to DNA and interact directly with AP-1 or NF-κB to inhibit their binding to DNA. In this way transcription of inflammatory genes is repressed in the airways of asthmatics. NF-κB can also be inhibited by a second mechanism, which involves the steroid-induced enhancement of the expression of the NF-κB inhibitory protein IκBα[144,257].

These genomic actions require prolonged incubation times (>30 min) with corticosteroids[258,259]. Besides long-term incubation with corticosteroids, recent literature have shown rapid effects of corticosteroids on cellular systems and several diseases, which most obviously do not occur via genomic actions of the GC-GCR complex[258-262]. The exact mechanisms of these rapid effects of steroids are poorly understood. In recent reviews[258,259] three mechanisms were summarized by which corticosteroids can induce rapid effects: 1) by specific interaction with the cytosolic corticosteroid receptor resulting in fast but poorly defined mechanisms[262-264], 2) by specific interaction with membrane bound steroid receptors, which are poorly characterized[258,259,265] and 3) as a result of non-receptor mediated

physicochemical interactions with cellular membranes[266].

7. Aims of the studies

Granulocytes and monocytes are important mediators in the onset, maintenance and progression of inflammation in allergic asthma. Inflammatory responses of these cells are, next to the environmental and genetic factors (see Section 2), contributing to the development of clinical phenotypes of asthma. In these inflammatory responses, the priming, recruitment and activation of granulocytes and monocytes are highly regulated and complex processes.
The focus of the studies presented in this thesis was directed towards the regulation and outcome of priming of granulocytes and monocytes in several phenotypes of asthmatics. As priming and activation of inflammatory cells might be influenced by steroid treatment, the effects of corticosteroids were studied on several effector functions of granulocytes. To understand the regulation of priming and activation in granulocytes, components of intracellular signalling pathways were studied. The following research questions were addressed, concerning leukocyte functions *in vivo* and *in vitro*:

1. Are leukocytes distinctly primed in different phenotypes of asthma?
2. Do allergen provocations affect the priming of granulocytes in asthmatics?
3. What are the effects of treatment with glucocorticoids on leukocyte priming responses in asthmatics?
4. What are the effects of glucocorticoids upon eosinophil effector functions?

8. References

1. Masoli M, Fabian D, Holt S, Beasley R: The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy 59: 469-478, 2004
2. Bousquet J, Bousquet PJ, Godard P, Daures JP: The public health implications of asthma. Bull.World Health Organ 83: 548-554, 2005
3. Ring J, Kramer U, Schafer T, Behrendt H: Why are allergies increasing? Curr.Opin.Immunol. 13: 701-708, 2001
4. Bousquet J, Ndiaye M, Ait-Khaled N, Annesi-Maesano I, Vignola AM: Management of chronic respiratory and allergic diseases in developing countries. Focus on sub-Saharan Africa. Allergy 58: 265-283, 2003
5. Barnes PJ: Update on asthma. Isr.Med.Assoc.J. 5: 68-72, 2003
6. Keller MB, Lowenstein SR: Epidemiology of asthma. Semin.Respir.Crit. Care Med. 23: 317-329, 2002

7. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet 351: 1225-1232, 1998
8. Von Mutius E: The environmental predictors of allergic disease. J.Allergy Clin.Immunol. 105: 9-19, 2000
9. Von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Roell G, Thiemann HH: Prevalence of asthma and atopy in two areas of West and East Germany. Am.J.Respir.Crit Care Med. 149: 358-364, 1994
10. Crater SE, Platts-Mills TA: Searching for the cause of the increase in asthma. Curr.Opin.Pediatr. 10: 594-599, 1998
11. Koenig JQ: Air pollution and asthma. J.Allergy Clin.Immunol. 104: 717-722, 1999
12. Liu AH, Murphy JR: Hygiene hypothesis: fact or fiction? J.Allergy Clin.Immunol. 111: 471-478, 2003
13. Mattes J, Karmaus W: The use of antibiotics in the first year of life and development of asthma: which comes first? Clin.Exp.Allergy 29: 729-732, 1999
14. Von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S, Waser M, Nowak D: Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin.Exp.Allergy 30: 1230-1234, 2000
15. Elston DM: The hygiene hypothesis and atopy: bring back the parasites? J.Am.Acad.Dermatol. 54: 172-179, 2006
16. Yazdanbakhsh M, Kremsner PG, van Ree R: Allergy, parasites, and the hygiene hypothesis. Science 296: 490-494, 2002
17. Wills-Karp M, Santeliz J, Karp CL: The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat.Rev.Immunol. 1: 69-75, 2001
18. Smit HA: Chronic obstructive pulmonary disease, asthma and protective effects of food intake: from hypothesis to evidence? Respir.Res. 2: 261-264, 2001
19. Weiss ST: Diet as a risk factor for asthma. Ciba Found.Symp. 206: 244-257, 1997
20. Martinez FD, Cline M, Burrows B: Increased incidence of asthma in children of smoking mothers. Pediatrics 89: 21-26, 1992
21. Bjorksten B: Risk factors in early childhood for the development of atopic diseases. Allergy 49: 400-407, 1994
22. Martinez FD, Antognoni G, Macri F, Bonci E, Midulla F, De Castro G, Ronchetti R: Parental smoking enhances bronchial responsiveness in nine-year-old children. Am.Rev.Respir.Dis. 138: 518-523, 1988
23. Skadhauge LR, Christensen K, Kyvik KO, Sigsgaard T: Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. Eur.Respir.J. 13: 8-14, 1999
24. Pearce N, Pekkanen J, Beasley R: How much asthma is really attributable to atopy? Thorax 54: 268-272, 1999
25. Postma DS, Koppelman GH, Meyers DA: The genetics of atopy and airway hyperresponsiveness. Am.J.Respir.Crit Care Med. 162: S118-S123, 2000
26. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CI, Meyers DA, Levitt RC: Genetic susceptibility to asthma-bronchial hyperresponsiveness coinherited with a major gene for atopy. N.Engl.J.Med. 333: 894-900, 1995
27. Lilly CM: Diversity of asthma: evolving concepts of pathophysiology and lessons from genetics. J.Allergy Clin.Immunol. 115: S526-S531, 2005
28. Ober C, Hoffjan S: Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun. 2006
29. Halapi E, Hakonarson H: Recent development in genomic and proteomic research for asthma. Curr.Opin.Pulm.Med. 10: 22-30, 2004
30. Cookson W: Genetics and genomics of asthma and allergic diseases. Immunol.Rev. 190: 195-206, 2002
31. Malerba G, Pignatti PF: A review of asthma genetics: gene expression studies and recent candidates. J.Appl.Genet. 46: 93-104, 2005
32. Cookson WO, Moffatt MF: Genetics of asthma and allergic disease. Hum.Mol.Genet. 9: 2359-2364, 2000
33. Shapiro SD, Owen CA: ADAM-33 surfaces as an asthma gene. N.Engl.J.Med. 347: 936-938, 2002
34. D'Amato G, Liccardi G, D'Amato M, Holgate S: Environmental risk factors and allergic bronchial asthma.

Clin.Exp.Allergy 35: 1113-1124, 2005
35. McFadden ER, Jr.: Acute severe asthma. Am.J.Respir.Crit Care Med. 168: 740-759, 2003
36. Weiss KB, Sullivan SD: The health economics of asthma and rhinitis. I. Assessing the economic impact. J.Allergy Clin.Immunol. 107: 3-8, 2001
37. Van Ganse E, Laforest L, Pietri G, Boissel JP, Gormand F, Ben Joseph R, Ernst P: Persistent asthma: disease control, resource utilisation and direct costs. Eur.Respir.J. 20: 260-267, 2002
38. 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. 19: 61-67, 2002
39. Bootman JL, Crown WH, Luskin AT: Clinical and economic effects of suboptimally controlled asthma. Manag.Care Interface 17: 31-36, 2004
40. Wenzel S: Severe asthma: epidemiology, pathophysiology and treatment. Mt.Sinai J.Med. 70: 185-190, 2003
41. Burney P: Interpretation of epidemiological surveys of asthma. Ciba Found.Symp. 206: 111-118, 1997
42. Peat JK, Toelle BG, Marks GB, Mellis CM: Continuing the debate about measuring asthma in population studies. Thorax 56: 406-411, 2001
43. Holgate ST: The cellular and mediator basis of asthma in relation to natural history. Lancet 350 Suppl 2: SII5-SII9, 1997
44. Busse WW, Lemanske RF, Jr.: Asthma. N.Engl.J.Med. 344: 350-362, 2001
45. Renauld JC: New insights into the role of cytokines in asthma. J.Clin.Pathol. 54: 577-589, 2001
46. Georas SN, Guo J, De Fanis U, Casolaro V: T-helper cell type-2 regulation in allergic disease. Eur.Respir.J. 26: 1119-1137, 2005
47. Ritz SA, Stampfli MR, Davies DE, Holgate ST, Jordana M: On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends Immunol. 23: 396-402, 2002
48. Holgate ST: Cytokine and anti-cytokine therapy for the treatment of asthma and allergic disease. Cytokine 28: 152-157, 2004
49. Chung F: Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma. Mediators.Inflamm. 10: 51-59, 2001
50. Robinson DS, Larche M, Durham SR: Tregs and allergic disease. J.Clin.Invest 114: 1389-1397, 2004
51. Hawrylowicz CM, O'Garra A: Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat.Rev.Immunol. 5: 271-283, 2005
52. Karnowski A, Yu P, Achatz G, Lamers MC: The road to the production of IgE is long and winding. Am.J.Respir.Crit Care Med. 162: S71-S75, 2000
53. Oettgen HC, Geha RS: IgE regulation and roles in asthma pathogenesis. J.Allergy Clin.Immunol. 107: 429-440, 2001
54. Milgrom H: Anti-IgE therapy in children with asthma. Minerva Pediatr. 56: 469-479, 2004
55. Walker S, Monteil M, Phelan K, Lasserson TJ, Walters EH: Anti-IgE for chronic asthma in adults and children. Cochrane.Database.Syst.Rev. CD003559, 2004
56. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM: Asthma. From bronchoconstriction to airways inflammation and remodeling. Am.J.Respir.Crit Care Med. 161: 1720-1745, 2000
57. Bradding P: The role of the mast cell in asthma: a reassessment. Curr.Opin.Allergy Clin.Immunol. 3: 45-50, 2003
58. Brightling CE, Bradding P, Pavord ID, Wardlaw AJ: New insights into the role of the mast cell in asthma. Clin.Exp.Allergy 33: 550-556, 2003
59. Takizawa H: Bronchial epithelial cells in allergic reactions. Curr.Drug Targets Inflamm.Allergy 4: 305-311, 2005
60. Cromwell O, Hamid Q, Corrigan CJ, Barkans J, Meng Q, Collins PD, Kay AB: Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. Immunology 77: 330-337, 1992
61. Marini M, Vittori E, Hollemborg J, Mattoli S: Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. J.Allergy Clin.Immunol. 89: 1001-1009, 1992

62. Stellato C, Beck LA, Gorgone GA, Proud D, Schall TJ, Ono SJ, Lichtenstein LM, Schleimer RP: Expression of the chemokine RANTES by a human bronchial epithelial cell line. Modulation by cytokines and glucocorticoids. J.Immunol. 155: 410-418, 1995
63. Nocker RE, Out TA, Weller FR, Mul EP, Jansen HM, Van der Zee JS: Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. Int.Arch.Allergy Immunol. 119: 45-53, 1999
64. Aalbers R, Kauffman HF, Vrugt B, Koeter GH, de Monchy JG: Allergen-induced recruitment of inflammatory cells in lavage 3 and 24 h after challenge in allergic asthmatic lungs. Chest 103: 1178-1184, 1993
65. Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR: Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J.Allergy Clin.Immunol. 92: 313-324, 1993
66. Montefort S, Gratziou C, Goulding D, Polosa R, Haskard DO, Howarth PH, Holgate ST, Carroll MP: Bronchial biopsy evidence for leukocyte infiltration and upregulation of leukocyte-endothelial cell adhesion molecules 6 hours after local allergen challenge of sensitized asthmatic airways. J.Clin.Invest 93: 1411-1421, 1994
67. Bentley AM, Kay AB, Durham SR: Human late asthmatic reactions. Clin.Exp.Allergy 27 Suppl 1: 71-86, 1997
68. Aalbers R, De Monchy JG, Kauffman HF, Smith M, Hoekstra Y, Vrugt B, Timens W: Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. Eur.Respir.J. 6: 840-847, 1993
69. Cockcroft DW, Murdock KY: Changes in bronchial responsiveness to histamine at intervals after allergen challenge. Thorax 42: 302-308, 1987
70. Tomita K, Tanigawa T, Yajima H, Fukutani K, Matsumoto Y, Tanaka Y, Sasaki T: Identification and characterization of monocyte subpopulations from patients with bronchial asthma. J.Allergy Clin.Immunol. 96: 230-238, 1995
71. Kimpen JL: Respiratory syncytial virus and asthma. The role of monocytes. Am.J.Respir.Crit Care Med. 163: S7-S9, 2001
72. Hallsworth MP, Soh CP, Lane SJ, Arm JP, Lee TH: Selective enhancement of GM-CSF, TNF-alpha, IL-1 beta and IL-8 production by monocytes and macrophages of asthmatic subjects. Eur.Respir.J. 7: 1096-1102, 1994
73. Larsen GL, Holt PG: The concept of airway inflammation. Am.J.Respir.Crit Care Med. 162: S2-S6, 2000
74. Diaz P, Gonzalez MC, Galleguillos FR, Ancic P, Cromwell O, Shepherd D, Durham SR, Gleich GJ, Kay AB: Leukocytes and mediators in bronchoalveolar lavage during allergen-induced late-phase asthmatic reactions. Am.Rev.Respir.Dis. 139: 1383-1389, 1989
75. O'Byrne P: Asthma pathogenesis and allergen-induced late responses. J.Allergy Clin.Immunol. 102: S85-S89, 1998
76. Gleich GJ: Why do allergic individuals accumulate more eosinophils at the site of an IgE-mediated reaction than do normal subjects? Clin.Exp.Allergy 20: 245-246, 1990
77. Gleich GJ: The eosinophil and bronchial asthma: current understanding. J.Allergy Clin.Immunol. 85: 422-436, 1990
78. Woolley KL, Adelroth E, Woolley MJ, Ellis R, Jordana M, O'Byrne PM: Effects of allergen challenge on eosinophils, eosinophil cationic protein, and granulocyte-macrophage colony-stimulating factor in mild asthma. Am.J.Respir.Crit Care Med. 151: 1915-1924, 1995
79. Saracli T, Scott RB: Comparative study of simultaneous blood and nasal secretion eosinophilia in children with allergic diseases. J.Asthma Res. 4: 219-227, 1967
80. De Monchy JG, Kauffman HF, Venge P, Koeter GH, Jansen HM, Sluiter HJ, De Vries K: Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am.Rev.Respir.Dis. 131: 373-376, 1985
81. Hogan SP, Koskinen A, Matthaei KI, Young IG, Foster PS: Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. Am.J.Respir.Crit Care Med. 157: 210-218, 1998
82. Walker C, Virchow JC, Jr., Bruijnzeel PL, Blaser K: T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. J.Immunol. 146: 1829-1835, 1991

83. Rothenberg ME, Hogan SP: The eosinophil. Annu.Rev.Immunol. 24: 147-174, 2006
84. Thomas LH, Warner JA: The eosinophil and its role in asthma. Gen.Pharmacol. 27: 593-597, 1996
85. Kay AB, Phipps S, Robinson DS: A role for eosinophils in airway remodelling in asthma. Trends Immunol. 25: 477-482, 2004
86. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D: Cytotoxic properties of the eosinophil major basic protein. J.Immunol. 123: 2925-2927, 1979
87. Motojima S, Frigas E, Loegering DA, Gleich GJ: Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. Am.Rev.Respir.Dis. 139: 801-805, 1989
88. Gundel RH, Letts LG, Gleich GJ: Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. J.Clin.Invest 87: 1470-1473, 1991
89. Bankers-Fulbright JL, Kita H, Gleich GJ, O'Grady SM: Regulation of human eosinophil NADPH oxidase activity: a central role for PKCdelta. J.Cell Physiol 189: 306-315, 2001
90. Yagisawa M, Yuo A, Yonemaru M, Imajoh-Ohmi S, Kanegasaki S, Yazaki Y, Takaku F: Superoxide release and NADPH oxidase components in mature human phagocytes: correlation between functional capacity and amount of functional proteins. Biochem.Biophys.Res.Commun. 228: 510-516, 1996
91. Leckie MJ, Ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, Hansel TT, Holgate ST, Sterk PJ, Barnes PJ: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 356: 2144-2148, 2000
92. Leckie MJ: Anti-interleukin-5 monoclonal antibodies: preclinical and clinical evidence in asthma models. Am.J.Respir.Med. 2: 245-259, 2003
93. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB: Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. J.Clin.Invest 112: 1029-1036, 2003
94. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C: A critical role for eosinophils in allergic airways remodeling. Science 305: 1776-1779, 2004
95. Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, Lenkiewicz E, Colbert D, Rinaldi L, Ackerman SJ, Irvin CG, Lee NA: Defining a link with asthma in mice congenitally deficient in eosinophils. Science 305: 1773-1776, 2004
96. Kitchen E, Rossi AG, Condliffe AM, Haslett C, Chilvers ER: Demonstration of reversible priming of human neutrophils using platelet-activating factor. Blood 88: 4330-4337, 1996
97. Coffer PJ, Koenderman L: Granulocyte signal transduction and priming: cause without effect? Immunol.Lett. 57: 27-31, 1997
98. Koenderman L, Coffer PJ: Controlling allergic inflammation by signaling regulation of eosinophils. Allergy 56: 204-214, 2001
99. Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, De Kruif J, Logtenberg T: Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. J.Leukoc.Biol. 68: 58-64, 2000
100. Koenderman L, Van der Bruggen T, Schweizer RC, Warringa RA, Coffer P, Caldenhoven E, Lammers JW, Raaijmakers JA: Eosinophil priming by cytokines: from cellular signal to in vivo modulation. Eur.Respir.J.Suppl 22: 119s-125s, 1996
101. Lampinen M, Carlson M, Hakansson LD, Venge P: Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy 59: 793-805, 2004
102. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L: RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. Blood 83: 3697-3704, 1994
103. Bracke M, Coffer PJ, Lammers JW, Koenderman L: Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. J.Immunol. 161: 6768-6774, 1998
104. Sanmugalingham D, De Vries E, Gauntlett R, Symon FA, Bradding P, Wardlaw AJ: Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. Clin.Exp.Allergy 30: 255-263, 2000

105. Carlson M, Peterson C, Venge P: The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation. Allergy 48: 437-442, 1993
106. Van der Bruggen T, Kok PT, Raaijmakers JA, Verhoeven AJ, Kessels RG, Lammers JW, Koenderman L: Cytokine priming of the respiratory burst in human eosinophils is Ca2+ independent and accompanied by induction of tyrosine kinase activity. J.Leukoc.Biol. 53: 347-353, 1993
107. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L: Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005
108. Bracke M, Van de Graaf E, Lammers JW, Coffer PJ, Koenderman L: In vivo priming of FcalphaR functioning on eosinophils of allergic asthmatics. J.Leukoc.Biol. 68: 655-661, 2000
109. Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koenderman L: Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. J.Allergy Clin.Immunol. 91: 1198-1205, 1993
110. Moser R, Fehr J, Olgiati L, Bruijnzeel PL: Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. Blood 79: 2937-2945, 1992
111. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L: In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 79: 1836-1841, 1992
112. Hakansson L, Bjornsson E, Janson C, Schmekel B: Increased adhesion to vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 of eosinophils from patients with asthma. J.Allergy Clin.Immunol. 96: 941-950, 1995
113. Bruijnzeel PL, Kuijper PH, Rihs S, Betz S, Warringa RA, Koenderman L: Eosinophil migration in atopic dermatitis. I: Increased migratory responses to N-formyl-methionyl-leucyl-phenylalanine, neutrophil-activating factor, platelet-activating factor, and platelet factor 4. J.Invest Dermatol. 100: 137-142, 1993
114. Mengelers HJ, Maikoe T, Brinkman L, Hooibrink B, Lammers JW, Koenderman L: Immunophenotyping of eosinophils recovered from blood and BAL of allergic asthmatics. Am.J.Respir.Crit Care Med. 149: 345-351, 1994
115. Wenzel SE, Balzar S, Cundall M, Chu HW: Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J.Allergy Clin.Immunol. 111: 1345-1352, 2003
116. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ: Neutrophilic inflammation in severe persistent asthma. Am.J.Respir.Crit Care Med. 160: 1532-1539, 1999
117. Douwes J, Gibson P, Pekkanen J, Pearce N: Non-eosinophilic asthma: importance and possible mechanisms. Thorax 57: 643-648, 2002
118. 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. 22: 470-477, 2003
119. Gibson PG, Simpson JL, Saltos N: Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119: 1329-1336, 2001
120. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ: Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am.J.Respir.Crit Care Med. 156: 737-743, 1997
121. Kelly EA, Busse WW, Jarjour NN: Increased matrix metalloproteinase-9 in the airway after allergen challenge. Am.J.Respir.Crit Care Med. 162: 1157-1161, 2000
122. Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidart JM, Louis R: Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. Chest 122: 1553-1559, 2002
123. Teramoto S, Shu CY, Ouchi Y, Fukuchi Y: Increased spontaneous production and generation of superoxide anion by blood neutrophils in patients with asthma. J.Asthma 33: 149-155, 1996
124. Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ: Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. Chest 122: 1543-1552,

2002
125. Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST: Airway remodeling in asthma: new insights. J.Allergy Clin.Immunol. 111: 215-225, 2003
126. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG: Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. Am.J.Respir.Crit Care Med. 161: 769-774, 2000
127. O'Donnell RA, Frew AJ: Is there more than one inflammatory phenotype in asthma? Thorax 57: 566-568, 2002
128. Belvisi MG: Regulation of inflammatory cell function by corticosteroids. Proc.Am.Thorac.Soc. 1: 207-214, 2004
129. Haslett C: Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am.J.Respir.Crit Care Med. 160: S5-11, 1999
130. Zhang X, Moilanen E, Adcock IM, Lindsay MA, Kankaanranta H: Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. Life Sci. 71: 1523-1534, 2002
131. Zhang X, Moilanen E, Kankaanranta H: Beclomethasone, budesonide and fluticasone propionate inhibit human neutrophil apoptosis. Eur.J.Pharmacol. 431: 365-371, 2001
132. Liles WC, Dale DC, Klebanoff SJ: Glucocorticoids inhibit apoptosis of human neutrophils. Blood 86: 3181-3188, 1995
133. Cox G: Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. J.Immunol. 154: 4719-4725, 1995
134. Meagher LC, Cousin JM, Seckl JR, Haslett C: Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J.Immunol. 156: 4422-4428, 1996
135. Giannini D, Di Franco A, Cianchetti S, Bacci E, Dente FL, Vagaggini B, Paggiaro PL: Analysis of induced sputum before and after withdrawal of treatment with inhaled corticosteroids in asthmatic patients. Clin.Exp.Allergy 30: 1777-1784, 2000
136. 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. 161: 9-16, 2000
137. Knol EF, Mul FP, Lie WJ, Verhoeven AJ, Roos D: The role of basophils in allergic disease. Eur.Respir.J.Suppl 22: 126s-131s, 1996
138. Bochner BS: Systemic activation of basophils and eosinophils: markers and consequences. J.Allergy Clin.Immunol. 106: S292-S302, 2000
139. Costa JJ, Weller PF, Galli SJ: The cells of the allergic response: mast cells, basophils, and eosinophils. JAMA 278: 1815-1822, 1997
140. Barnes PJ: Drugs for asthma. Br.J.Pharmacol. 147 Suppl 1: S297-S303, 2006
141. Barnes PJ: Efficacy of inhaled corticosteroids in asthma. J.Allergy Clin.Immunol. 102: 531-538, 1998
142. Barnes PJ, Pedersen S, Busse WW: Efficacy and safety of inhaled corticosteroids. New developments. Am.J.Respir.Crit Care Med. 157: S1-53, 1998
143. Barnes PJ: Corticosteroid effects on cell signalling. Eur.Respir.J. 27: 413-426, 2006
144. Umland SP, Schleimer RP, Johnston SL: Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. Pulm.Pharmacol.Ther. 15: 35-50, 2002
145. Kelly HW: Pharmaceutical characteristics that influence the clinical efficacy of inhaled corticosteroids. Ann.Allergy Asthma Immunol. 91: 326-334, 2003
146. Kelly HW: Comparison of inhaled corticosteroids. Ann.Pharmacother. 32: 220-232, 1998
147. Kelly HW: Comparative potency and clinical efficacy of inhaled corticosteroids. Respir.Care Clin.N.Am. 5: 537-553, 1999
148. Szefler SJ, Leung DY: Glucocorticoid-resistant asthma: pathogenesis and clinical implications for management. Eur.Respir.J. 10: 1640-1647, 1997
149. Adcock IM, Lane SJ: Corticosteroid-insensitive asthma: molecular mechanisms. J.Endocrinol. 178: 347-355, 2003
150. Boushey HA: Effects of inhaled corticosteroids on the consequences of asthma. J.Allergy Clin.Immunol. 102: S5-16, 1998

151. Zhou J, Kang ZM, Xie QM, Liu C, Lou SJ, Chen YZ, Jiang CL: Rapid nongenomic effects of glucocorticoids on allergic asthma reaction in the guinea pig. J.Endocrinol. 177: R1-R4, 2003
152. Luijk B, Kempsford RD, Wright AM, Zanen P, Lammers JW: Duration of effect of single-dose inhaled fluticasone propionate on AMP-induced bronchoconstriction. Eur.Respir.J. 23: 559-564, 2004
153. Kumar SD, Brieva JL, Danta I, Wanner A: Transient effect of inhaled fluticasone on airway mucosal blood flow in subjects with and without asthma. Am.J.Respir.Crit Care Med. 161: 918-921, 2000
154. Mendes ES, Pereira A, Danta I, Duncan RC, Wanner A: Comparative bronchial vasoconstrictive efficacy of inhaled glucocorticosteroids. Eur.Respir.J. 21: 989-993, 2003
155. Wanner A, Horvath G, Brieva JL, Kumar SD, Mendes ES: Nongenomic actions of glucocorticosteroids on the airway vasculature in asthma. Proc.Am.Thorac.Soc. 1: 235-238, 2004
156. Allen DB, Bielory L, Derendorf H, Dluhy R, Colice GL, Szefler SJ: Inhaled corticosteroids: past lessons and future issues. J.Allergy Clin.Immunol. 112: S1-40, 2003
157. Van Staa TP, Leufkens HG, Abenhaim L, Zhang B, Cooper C: Use of oral corticosteroids and risk of fractures. J.Bone Miner.Res. 15: 993-1000, 2000
158. Van Staa TP, Leufkens HG, Abenhaim L, Zhang B, Cooper C: Oral corticosteroids and fracture risk: relationship to daily and cumulative doses. Rheumatology.(Oxford) 39: 1383-1389, 2000
159. Lane NE, Lukert B: The science and therapy of glucocorticoid-induced bone loss. Endocrinol.Metab Clin.North Am. 27: 465-483, 1998
160. Niewoehner CB, Niewoehner DE: Steroid-induced osteoporosis. Are your asthmatic patients at risk? Postgrad.Med. 105: 79-8, 91, 1999
161. Langhammer A, Norjavaara E, de Verdier MG, Johnsen R, Bjermer L: Use of inhaled corticosteroids and bone mineral density in a population based study: the Nord-Trondelag Health Study (the HUNT Study). Pharmacoepidemiol.Drug Saf 13: 569-579, 2004
162. Efthimiou J, Barnes PJ: Effect of inhaled corticosteroids on bones and growth. Eur.Respir.J. 11: 1167-1177, 1998
163. Egan JJ, Maden C, Kalra S, Adams JE, Eastell R, Woodcock AA: A randomized, double-blind study comparing the effects of beclomethasone and fluticasone on bone density over two years. Eur.Respir.J. 13: 1267-1275, 1999
164. Wong CA, Subakumar G, Casey PM: Effects of asthma and asthma therapies on bone mineral density. Curr.Opin.Pulm.Med. 8: 39-44, 2002
165. Gillissen A: Managing asthma in the real world. Int.J.Clin.Pract. 58: 592-603, 2004
166. O'Connell EJ: Optimizing inhaled corticosteroid therapy in children with chronic asthma. Pediatr.Pulmonol. 39: 74-83, 2005
167. Spector S: Noncompliance with asthma therapy-are there solutions? J.Asthma 37: 381-388, 2000
168. Silvers WS, Cohen R, D'Alonzo G, Fishken D, Lim JC, Stokes AL, McNally CF: Comparative taste evaluation of aerosolized formulations of triamcinolone acetonide, flunisolide, and flunisolide with menthol. Clin.Ther. 15: 988-993, 1993
169. Cochrane MG, Bala MV, Downs KE, Mauskopf J, Ben Joseph RH: Inhaled corticosteroids for asthma therapy: patient compliance, devices, and inhalation technique. Chest 117: 542-550, 2000
170. Boulet LP: Perception of the role and potential side effects of inhaled corticosteroids among asthmatic patients. Chest 113: 587-592, 1998
171. Geijsen N, Koenderman L, Coffer PJ: Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. Cytokine Growth Factor Rev. 12: 19-25, 2001
172. Grotzinger J: Molecular mechanisms of cytokine receptor activation. Biochim.Biophys.Acta 1592: 215-223, 2002
173. Itoh T, Liu R, Yokota T, Arai KI, Watanabe S: Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. Mol.Cell Biol. 18: 742-752, 1998
174. Van Dijk TB, Caldenhoven E, Raaijmakers JA, Lammers JW, Koenderman L, De Groot RP: Multiple tyrosine residues in the intracellular domain of the common beta subunit of the interleukin 5 receptor are involved in activation of STAT5. FEBS Lett. 412: 161-164, 1997

175. De Groot RP, Coffer PJ, Koenderman L: Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. Cell Signal. 10: 619-628, 1998
176. Duronio V, Clark-Lewis I, Federsppiel B, Wieler JS, Schrader JW: Tyrosine phosphorylation of receptor beta subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. J.Biol.Chem. 267: 21856-21863, 1992
177. Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD, Ihle JN: JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol.Cell Biol. 14: 4335-4341, 1994
178. Ogata N, Kouro T, Yamada A, Koike M, Hanai N, Ishikawa T, Takatsu K: JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor alpha and betac subunit, respectively, and are activated upon IL-5 stimulation. Blood 91: 2264-2271, 1998
179. Itoh T, Liu R, Arai K, Watanabe S: Differential influence of tyrosine residues of the common receptor beta subunit on multiple signals induced by human GM-CSF. J.Allergy Clin.Immunol. 103: S462-S470, 1999
180. Itoh T, Liu R, Arai K, Watanabe S: Differential influence of tyrosine residues of the common receptor beta subunit on multiple signals induced by human GM-CSF. J.Allergy Clin.Immunol. 103: S462-S470, 1999
181. Van Dijk TB, Caldenhoven E, Raaijmakers JA, Lammers JW, Koenderman L, de Groot RP: Multiple tyrosine residues in the intracellular domain of the common beta subunit of the interleukin 5 receptor are involved in activation of STAT5. FEBS Lett. 412: 161-164, 1997
182. Dijkers PF, van Dijk TB, de Groot RP, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ: Regulation and function of protein kinase B and MAP kinase activation by the IL-5/GM-CSF/IL-3 receptor. Oncogene 18: 3334-3342, 1999
183. Lanfrancone L, Pelicci G, Brizzi MF, Aronica MG, Casciari C, Giuli S, Pegoraro L, Pawson T, Pelicci PG, Arouica MG: Overexpression of Shc proteins potentiates the proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of Grb2/SoS and Grb2/p140 complexes to the beta receptor subunit. Oncogene 10: 907-917, 1995
184. Pratt JC, Weiss M, Sieff CA, Shoelson SE, Burakoff SJ, Ravichandran KS: Evidence for a physical association between the Shc-PTB domain and the beta c chain of the granulocyte-macrophage colony-stimulating factor receptor. J.Biol.Chem. 271: 12137-12140, 1996
185. Okuda K, Foster R, Griffin JD: Signaling domains of the beta c chain of the GM-CSF/IL-3/IL-5 receptor. Ann.N.Y.Acad.Sci. 872: 305-312, 1999
186. Bazzoni F, Beutler B: The tumor necrosis factor ligand and receptor families. N.Engl.J.Med. 334: 1717-1725, 1996
187. Wajant H, Pfizenmaier K, Scheurich P: Tumor necrosis factor signaling. Cell Death.Differ. 10: 45-65, 2003
188. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P: The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 83: 793-802, 1995
189. Gupta S: A decision between life and death during TNF-alpha-induced signaling. J.Clin.Immunol. 22: 185-194, 2002
190. Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C, Rossi AG: NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. J.Biol.Chem. 274: 4309-4318, 1999
191. Karin M, Lin A: NF-kappaB at the crossroads of life and death. Nat.Immunol. 3: 221-227, 2002
192. Li Q, Verma IM: NF-kappaB regulation in the immune system. Nat.Rev.Immunol. 2: 725-734, 2002
193. Salamone G, Giordano M, Trevani AS, Gamberale R, Vermeulen M, Schettinni J, Geffner JR: Promotion of neutrophil apoptosis by TNF-alpha. J.Immunol. 166: 3476-3483, 2001
194. Van den Berg JM, Weyer S, Weening JJ, Roos D, Kuijpers TW: Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. J.Leukoc.Biol. 69: 467-473, 2001
195. Mayadas TN, Cullere X: Neutrophil beta2 integrins: moderators of life or death decisions. Trends Immunol. 26: 388-395, 2005
196. Pantano C, Shrivastava P, McElhinney B, Janssen-Heininger Y: Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-Jun N-terminal kinase. J.Biol.Chem. 278: 44091-44096, 2003

197. Schett G, Steiner CW, Xu Q, Smolen JS, Steiner G: TNFalpha mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response. Cell Death.Differ. 10: 1126-1136, 2003
198. Baggiolini M: Chemokines and leukocyte traffic. Nature 392: 565-568, 1998
199. Bourne HR: How receptors talk to trimeric G proteins. Curr.Opin.Cell Biol. 9: 134-142, 1997
200. Patel TB: Single transmembrane spanning heterotrimeric g protein-coupled receptors and their signaling cascades. Pharmacol.Rev. 56: 371-385, 2004
201. McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS: G-protein signaling: back to the future. Cell Mol.Life Sci. 62: 551-577, 2005
202. M'Rabet L, Coffer P, Zwartkruis F, Franke B, Segal AW, Koenderman L, Bos JL: Activation of the small GTPase rap1 in human neutrophils. Blood 92: 2133-2140, 1998
203. Hubbard KB, Hepler JR: Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cell Signal. 18: 135-150, 2006
204. Mandeville JT, Maxfield FR: Calcium and signal transduction in granulocytes. Curr.Opin.Hematol. 3: 63-70, 1996
205. Ohno T, Kubagawa H, Sanders SK, Cooper MD: Biochemical nature of an Fc mu receptor on human B-lineage cells. J.Exp.Med. 172: 1165-1175, 1990
206. Coico RF, Xue B, Wallace D, Pernis B, Siskind GW, Thorbecke GJ: T cells with receptors for IgD. Nature 316: 744-746, 1985
207. Adachi M, Ishizaka K: IgD-binding factors from mouse T lymphocytes. Proc.Natl.Acad.Sci.U.S.A 83: 7003-7007, 1986
208. Hulett MD, Hogarth PM: Molecular basis of Fc receptor function. Adv.Immunol. 57: 1-127, 1994
209. Ravetch JV, Kinet JP: Fc receptors. Annu.Rev.Immunol. 9: 457-492, 1991
210. Takai T: Fc receptors and their role in immune regulation and autoimmunity. J.Clin.Immunol. 25: 1-18, 2005
211. Monteiro RC, Van De Winkel JG: IgA Fc receptors. Annu.Rev.Immunol. 21: 177-204, 2003
212. Daeron M: Structural bases of Fc gamma R functions. Int.Rev.Immunol. 16: 1-27, 1997
213. Fodor S, Jakus Z, Mocsai A: ITAM-based signaling beyond the adaptive immune response. Immunol.Lett. 2005
214. Pilette C, Durham SR, Vaerman JP, Sibille Y: Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A? Proc.Am.Thorac.Soc. 1: 125-135, 2004
215. Delacroix DL, Dive C, Rambaud JC, Vaerman JP: IgA subclasses in various secretions and in serum. Immunology 47: 383-385, 1982
216. Ravetch JV, Bolland S: IgG Fc receptors. Annu.Rev.Immunol. 19: 275-290, 2001
217. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314, 1994
218. Resnick MB, Weller PF: Mechanisms of eosinophil recruitment. Am.J.Respir.Cell Mol.Biol. 8: 349-355, 1993
219. Seely AJ, Pascual JL, Christou NV: Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Crit Care 7: 291-307, 2003
220. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314, 1994
221. Granger DN, Kubes P: The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. J.Leukoc.Biol. 55: 662-675, 1994
222. Lauffenburger DA, Horwitz AF: Cell migration: a physically integrated molecular process. Cell 84: 359-369, 1996
223. Kinashi T: Intracellular signalling controlling integrin activation in lymphocytes. Nat.Rev.Immunol. 5: 546-559, 2005
224. Bracke M, Lammers JW, Coffer PJ, Koenderman L: Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. Blood 97: 3478-3483, 2001
225. Nagarajan S, Fifadara NH, Selvaraj P: Signal-specific activation and regulation of human neutrophil Fc

gamma receptors. J.Immunol. 174: 5423-5432, 2005
226. Ginsberg MH, Partridge A, Shattil SJ: Integrin regulation. Curr.Opin.Cell Biol. 17: 509-516, 2005
227. De Bruyn KM, Rangarajan S, Reedquist KA, Figdor CG, Bos JL: The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. J.Biol.Chem. 277: 29468-29476, 2002
228. Bracke M, Nijhuis E, Lammers JW, Coffer PJ, Koenderman L: A critical role for PI 3-kinase in cytokine-induced Fcalpha-receptor activation. Blood 95: 2037-2043, 2000
229. Kinashi T, Asaoka T, Setoguchi R, Takatsu K: Affinity modulation of very late antigen-5 through phosphatidylinositol 3-kinase in mast cells. J.Immunol. 162: 2850-2857, 1999
230. Verploegen S, Ulfman L, van Deutekom HW, Van Aalst C, Honing H, Lammers JW, Koenderman L, Coffer PJ: Characterization of the role of CaMKI-like kinase (CKLiK) in human granulocyte function. Blood 106: 1076-1083, 2005
231. Garcia-Garcia E, Rosales C: Signal transduction during Fc receptor-mediated phagocytosis. J.Leukoc.Biol. 72: 1092-1108, 2002
232. Ortiz-Stern A, Rosales C: Cross-talk between Fc receptors and integrins. Immunol.Lett. 90: 137-143, 2003
233. Cobb MH, Goldsmith EJ: How MAP kinases are regulated. J.Biol.Chem. 270: 14843-14846, 1995
234. Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. Curr.Opin.Cell Biol. 9: 180-186, 1997
235. Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM: Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J.Biol.Chem. 270: 23934-23936, 1995
236. Whitmarsh AJ, Yang SH, Su MS, Sharrocks AD, Davis RJ: Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. Mol.Cell Biol. 17: 2360-2371, 1997
237. Bagrodia S, Derijard B, Davis RJ, Cerione RA: Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J.Biol.Chem. 270: 27995-27998, 1995
238. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, Karin M: Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268: 286-290, 1995
239. Ganiatsas S, Kwee L, Fujiwara Y, Perkins A, Ikeda T, Labow MA, Zon LI: SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proc.Natl.Acad.Sci.U.S.A 95: 6881-6886, 1998
240. Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J: MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295: 1291-1294, 2002
241. Mittelstadt PR, Salvador JM, Fornace AJ, Jr., Ashwell JD: Activating p38 MAPK: new tricks for an old kinase. Cell Cycle 4: 1189-1192, 2005
242. Salvador JM, Mittelstadt PR, Guszczynski T, Copeland TD, Yamaguchi H, Appella E, Fornace AJ, Jr., Ashwell JD: Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. Nat.Immunol. 6: 390-395, 2005
243. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL: Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201, 2000
244. Kumar S, Jiang MS, Adams JL, Lee JC: Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem.Biophys.Res.Commun. 263: 825-831, 1999
245. Gum RJ, McLaughlin MM, Kumar S, Wang Z, Bower MJ, Lee JC, Adams JL, Livi GP, Goldsmith EJ, Young PR: Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. J.Biol.Chem. 273: 15605-15610, 1998
246. Frantz B, Klatt T, Pang M, Parsons J, Rolando A, Williams H, Tocci MJ, O'Keefe SJ, O'Neill EA: The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. Biochemistry 37: 13846-13853, 1998
247. Kim L, Del Rio L, Butcher BA, Mogensen TH, Paludan SR, Flavell RA, Denkers EY: p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. J.Immunol. 174: 4178-4184, 2005

248. Tanno M, Bassi R, Gorog DA, Saurin AT, Jiang J, Heads RJ, Martin JL, Davis RJ, Flavell RA, Marber MS: Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. Circ.Res. 93: 254-261, 2003
249. Schieven GL: The biology of p38 kinase: a central role in inflammation. Curr.Top.Med.Chem. 5: 921-928, 2005
250. Barnes PJ: Novel signal transduction modulators for the treatment of airway diseases. Pharmacol.Ther. 109: 238-245, 2006
251. Underwood DC, Osborn RR, Bochnowicz S, Webb EF, Rieman DJ, Lee JC, Romanic AM, Adams JL, Hay DW, Griswold DE: SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am.J.Physiol Lung Cell Mol.Physiol 279: L895-L902, 2000
252. Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW, Griswold DE: SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. J.Pharmacol.Exp.Ther. 293: 281-288, 2000
253. Wong WS: Inhibitors of the tyrosine kinase signaling cascade for asthma. Curr.Opin.Pharmacol. 5: 264-271, 2005
254. Foltz IN, Lee JC, Young PR, Schrader JW: Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. J.Biol.Chem. 272: 3296-3301, 1997
255. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA: The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. J.Biol.Chem. 274: 27857-27866, 1999
256. Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, Chrousos GP, Klemm DJ: Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. J.Exp.Med. 186: 1567-1574, 1997
257. Wissink S, Van Heerde EC, Van der Burg B, Van der Saag PT: A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. Mol.Endocrinol. 12: 355-363, 1998
258. Goulding NJ: The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus. Curr.Opin.Pharmacol. 4: 629-636, 2004
259. Buttgereit F, Scheffold A: Rapid glucocorticoid effects on immune cells. Steroids 67: 529-534, 2002
260. Qi AQ, Qiu J, Xiao L, Chen YZ: Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. J.Neurosci.Res. 80: 510-517, 2005
261. Schmidt BM, Gerdes D, Feuring M, Falkenstein E, Christ M, Wehling M: Rapid, nongenomic steroid actions: A new age? Front Neuroendocrinol. 21: 57-94, 2000
262. Croxtall JD, Choudhury Q, Flower RJ: Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. Br.J.Pharmacol. 130: 289-298, 2000
263. Croxtall JD, van Hal PT, Choudhury Q, Gilroy DW, Flower RJ: Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br.J.Pharmacol. 135: 511-519, 2002
264. Limbourg FP, Huang Z, Plumier JC, Simoncini T, Fujioka M, Tuckermann J, Schutz G, Moskowitz MA, Liao JK: Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. J.Clin.Invest 110: 1729-1738, 2002
265. Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A, Buttgereit F: Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. FASEB J. 18: 70-80, 2004
266. Schmid D, Burmester GR, Tripmacher R, Kuhnke A, Buttgereit F: Bioenergetics of human peripheral blood mononuclear cell metabolism in quiescent, activated, and glucocorticoid-treated states. Biosci.Rep. 20: 289-302, 2000

Chapter 2

Multiple granulocyte priming phenotypes in peripheral blood of allergic asthmatics

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Submitted

Abstract

Background: Allergic asthma is associated with chronic airway and systemic immune responses. Systemic responses include priming of peripheral blood eosinophils, which is enhanced after allergen challenge. In a subpopulation of asthmatics neutrophils are associated with bronchial inflammation.
Objective: To monitor granulocyte priming in allergic asthmatics as consequence of chronic and acute inflammatory signals initiated by allergen challenge.
Methods: Blood was taken at baseline and 6-72h after allergen challenge in asthmatics with and without late asthmatic responses (LARs). Systemic granulocyte priming was studied using 1) expression of cellular markers such as αm (CD11b), L-selectin and a priming epitope recognized by monoclonal phage antibody A17 and 2) gene expression profiles in neutrophils.
Results: Eosinophils of asthmatics have a primed phenotype identified using cellular surface markers. Neutrophils of asthmatics were subtle primed, which was mainly identified using gene expression profiling. After allergen challenge, an acute increase in eosinophil and neutrophil priming was found only in patients experiencing a LAR using priming markers and gene expression analysis, respectively. In contrast, granulocyte αm (CD11b) and L-selectin levels were not different between controls and asthmatics and not affected by allergen challenge. Interestingly, expression of both adhesion molecules was positively correlated and αm expression on eosinophils and neutrophils correlated positively with bronchial hyperresponsiveness.
Conclusion: Different phases and/or phenotypes of allergic asthma are associated with distinct priming profiles of inflammatory cells in peripheral blood.
Clinical Implication: Insight in differences of systemic innate immune responses will lead to better definitions of asthma-subtypes and to better designs of new therapeutic options.

Introduction

Allergic asthma is accompanied by a chronic inflammation in the airways[1]. This inflammation is characterized by the presence of T-helper 2 (Th2)-type cytokines including interleukin (IL)-3, IL-4, IL-5, IL-13 and GM-CSF. These cytokines stimulate growth, differentiation and functionality of inflammatory cells that have been implicated in asthma, such as B/T-cells, mast cells, basophils and eosinophils[2,3]. Besides these typical Th2-cytokines, increasing evidence indicates the involvement of other cytokines, such as TNFα, in chronic inflammation of asthma[3,4].

Next to the importance of eosinophils, neutrophils are also implicated in the pathogenesis of asthma[5-10]. The presence of neutrophils is associated with an increase in the concentration of the proteolytic enzyme matrix metalloproteinase (MMP)-9 in BAL-fluid, tissue and sputum[5,11-13] suggesting that neutrophils participate in the remodelling process that occurs in the airways of asthmatics.

In several studies, eosinophils of asthma patients have been reported to exhibit a pre-activated or 'primed' phenotype in peripheral blood. This was demonstrated by an enhanced expression of priming associated epitopes[14,15]. Priming of these inflammatory cells is particularly associated with an enhanced functionality of adhesion associated responses and points, therefore, to a phenotype that facilitates the recruitment of eosinophils from the blood to the airways. This was shown by increased chemotactic responses and transendothelial movement of these (primed) cells, which were in part mediated by upregulation of adhesion molecules[16-20].

In contrast, priming of neutrophils in peripheral blood of allergic asthmatics has not been described as clearly as for eosinophils. In comparison with healthy donors, neutrophils of asthmatics are not characterized by differences in expression of (pre-)activation markers such as αm (CD11b) and L-selectin (CD62L)[15,21]. However, we have previously shown that pre-activation of peripheral blood neutrophils can be measured by gene expression analysis in asthma[22] and chronic obstructive pulmonary disease[23]. The determination of priming of neutrophils probably needs these very sensitive measures as (primed) neutrophils are thought to quickly leave the peripheral blood in response to inflammatory reactions that are induced after e.g. exposure to allergens[24-26].

Little is known regarding priming phenotypes of inflammatory cells in the context of different phases and/or phenotypes of allergic asthma. Our first study on the

kinetics of priming of inflammatory cells showed that allergen challenges lead to a short burst of priming of eosinophils, but not of neutrophils, in peripheral blood after 6h[15]. The data corroborated other data in which differential activation of inflammatory cells in response to allergen challenges in allergic asthmatics are shown[27,28]. Reactions to allergen exposures are characterized by two phenomena in the airways of allergic asthma patients, the early asthmatic response (EAR) and the late asthmatic response (LAR). These responses are associated with airflow limitation and an increase in airway hyperresponsiveness (AHR)[28]. The LAR, which is maximal after 6-8h[29], exhibits characteristics that resemble the chronic inflammatory phase of allergic asthma in the bronchial tissue: e.g. infiltration of inflammatory cells starting as early as 3h after allergen challenge[27,30]. Systemic inflammatory responses during the LAR have been described in several studies and were associated with an increase in blood eosinophils that likely had characteristics of primed cells[30-32].

In this study, the mechanisms involved in the systemic innate immune responses of allergic asthmatics were investigated before and after allergen challenge. The allergen-induced priming of peripheral blood eosinophils and neutrophils were measured in mild atopic asthmatics with and without allergen induced LARs.

Materials and methods

Patients and healthy controls

We included 45 non-smoking, steroid naïve patients with a diagnosis of asthma according to the GINA guidelines (Table 1)[33]. All patients had stable asthma without a respiratory tract infection in the last 4 weeks before entering the study. They had positive allergen skin prick tests[34] with at least one positive reaction to a common inhaled allergen and they all had documented airway hyperresponsiveness (AHR; PC_{20}-methacholine (< 8 mg/ml)). When patients used low doses of inhaled or nasal corticosteroids, this medication was stopped at least 4 weeks before they entered the study. No other anti-asthma drugs were allowed during the study except short acting β_2-agonists. Patients receiving an inhalation challenge had a baseline FEV_1 of >70% of the predicted values after withholding β_2-agonists for 8h at both the control visit and on the day of the allergen challenge.

Healthy subjects without asthma symptoms or presence of atopy (Table 1), were selected from the laboratory and clinical staff. The medical ethics committee of the

Table 1. Characteristics (mean(±SEM)) of controls and mild asthmatics.

	Controls	Asthmatics
Number	26	45
Age (yrs)	30.1(3.0)	24.5(1.1)
Gender (M/F)	14/12	33/12
Atopy	-	+
Baseline FEV_1 (% pred.)	-	93.9(1.8)
Methacholine-PC_{20} (mg/ml)	-	1.4(0.3)

University Medical Center Utrecht (Utrecht, The Netherlands) approved the study and all patients gave their written informed consent.

Study design for allergen provocation studies

We investigated leukocyte priming in peripheral blood before and 6, 24 and 72h after allergen challenge (see below)[15]. Patient characteristics were assessed within one week prior to the allergen challenge. Therefore, physical examination and measurement of vital parameters were performed together with measurement of baseline FEV_1, methacholine challenge (see below) and skin prick tests. Furthermore, a sham allergen challenge using saline inhalation was performed. Blood samples were taken to measure leukocyte priming at baseline (15 min before start) and 6h after sham challenge. In addition, FEV_1 was measured at the same time points. AHR assessed by a methacholine challenge test was measured at the end of this clinical assessment day (7h after sham challenge). On a second admission day, within a week after the assessment day, an allergen challenge was performed with repeated FEV_1 measurements. Blood samples were taken at the same time points as during sham challenge. At the third day of admission, 24h after allergen challenge, patients returned to the hospital for assessment of symptoms and a methacholine challenge was performed. FEV_1 and peripheral blood leukocyte priming measurements were performed 24 and 72h after allergen challenge.

Inhalation challenges

a) Methacholine challenge

AHR was measured using an inhalation provocation test with methacholine. To determine the methacholine-PC_{20}, methacholine was inhaled in doubling concentrations at 5 minutes intervals in a range starting from 0.038 mg/ml to a

maximum of 8 mg/ml according to a standardized challenge protocol[35]. FEV_1 was measured 30 and 90 sec after two min tidal breathing from a calibrated nebuliser (model 646, Devilbiss Inc., Somerset, PA; 0.13 ml/min) while the nose was clipped. All FEV_1 measurements were performed with a digital spirometer (Sensorloop, SensorMedics Corporation, Yorba Linda, CA). The challenge with inhaled methacholine was performed until the FEV_1 fell by at least 20% from baseline FEV_1 to determine the PC_{20}-values of asthmatics. Methacholine (Sigma-Aldrich; St. Louis, MO, USA) was dissolved in saline (0.9%) solution.

b) Allergen challenge

All patients were admitted to the hospital in the morning. After a short rest period they underwent an allergen challenge according to a standardised challenge procedure[35]. Subject's specific skin sensitivity together with the measured baseline PC_{20}-methacholine were used in the Cockcroft formula to calculate the lowest dose of allergen to start with during the allergen challenge[34]. FEV_1 was measured 10 sec after two min tidal breathing in the same calibrated nebuliser as was used for measurement of the PC_{20}-methacholine while the nose was clipped.

For safety reasons we started two concentrations below the calculated PC_{20}-allergen. Thereafter, the patients inhaled doubling concentrations of allergen with 10 min intervals till the FEV_1 dropped ≥15 % from the baseline FEV_1. When a fall in FEV_1 of ≥15 % was reached this was recorded as the early asthmatic response (EAR), FEV_1 measurements were continued every 10 min for the first hour, then every 30 min till 2h and subsequently every hour until 7h after the allergen challenge. Then all patients inhaled salbutamol and, when they were stable, they were dismissed from the hospital with instructions to record symptoms and their peak flow at regular intervals. The LAR was defined as a decrease in FEV_1 of more than or equal to 15% from baseline[35]. Subjects with both an EAR and a LAR were defined as dual responders and the subjects with only an EAR as single responders.

Neutrophil isolation and stimulation

Neutrophils were isolated from 30-100 ml blood anti-coagulated with sodium heparin and the isolation was performed as described previously[36]. In short, blood was diluted 2.5:1 with PBS containing 0.32% (wt/vol) sodium citrate and human pasteurized plasma-protein solution (4 g/L). Granulocytes and erythrocytes were isolated by centrifugation over Ficoll-Pague (Pharmacia, Uppsala, Sweden).

Erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. After isolation, granulocytes were resuspended in PBS containing 0.32% sodium citrate and human pasteurized plasma-protein solution (4 g/L).
Granulocytes from 100 ml whole blood of healthy donors (less then 4 % eosinophils) were resuspended per 25 million cells ($5x10^6$/ml) in HEPES buffered RPMI 1640 medium from Life Technologies (Breda, The Netherlands) supplemented with L-glutamine and 0.5% human serum albumin (HSA; Sanquin, Amsterdam, The Netherlands). The cells were preincubated for 15 min at 37°C. Subsequently, cells were mock-stimulated or stimulated with TNFα (100 U/ml) from Boehringer Mannheim (Germany) or GM-CSF (10^{-10}M) from Genzyme (Boston, MA) for 3h at 37°C.

RNA isolation and cDNA synthesis
After stimulation or directly after isolation, neutrophils were washed once with ice-cold PBS and pellets (of $10x10^6$ cells each) were dissolved in 1 ml Trizol (Invitrogen, Carlsbad, CA) and subsequently frozen for at least 12h at -80°C. Total mRNA was extracted according to manufacturer's instructions with the exception that all centrifugation was done at 4°C. cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers. Samples containing 1 µg of total RNA were heated for 3 min at 65°C and quickly chilled on ice. A mixture of 25 µl (total volume) containing the RNA, 20 µg/ml oligo(dT) primers, 5 µl of 5x RT buffer, 20 mM DTT, 2 mM each deoxynucleoside triphosphate (dNTP), 20 units of RNase inhibitor and 400 units of MMLV reverse transcriptase was incubated at 37°C for 90 min, followed by MMLV reverse transcriptase inactivation for 10 min at 65°C. cDNA was stored at -20°C before further use. All reagents used for cDNA synthesis, except dNTPs (Amersham, UK), were obtained from Invitrogen (Carlsbad, CA).

Microarray Labelling
Per sample, 2 µl external controls (kindly provided by Genomics Laboratory, Utrecht, The Netherlands[37]) and 2 µl $oligo(dT)_{12-18}$ primers (0.5 µg/ml; Amersham, UK) were added to three aliquots of 5 µg mRNA from stimulated neutrophils in a total volume of 15 µl each. Aliquots were heated at 70°C for 15 min. Complementary DNA synthesis was performed in 30 µl using the aliquots and a mixture of 1 µl Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA), 6

µl 5x RT buffer, 3 µl DTT (100mM), 2 µl dGAC-mix (1mM each dNTP; Amersham, UK), 0.5 µl dTTP (1mM; Amersham, UK), 1 µl H_2O and 1.5 µl 2-aminoallyl-dUTP (1mM; Sigma, St. Louis, MO). Contaminating RNA was removed by adding 10 µl 1M NaOH and 10 µl 500mM EDTA for 15 min at 65°C and subsequently 25 µl HEPES buffer (1M, pH 7.5) was added. Per sample, three cDNA aliquots were put together and purified using Microcon-30 columns (Millipore, Bedford, MA). Purified cDNA was concentrated to 8 µl (about 250-400 ng) and 1 µl sodiumbicarbonate (500mM, pH9) was added. Dyes Cy3 and Cy5 (1.25 µl; Amersham, UK), dissolved in DMSO, were coupled to cDNA for 1h at room temperature. The dyes were subsequently quenched with 4.5 µl 4M hydroxylamine (Sigma, St. Louis, MO) for 15 min at room temperature. Free dyes were removed using Chromaspin 30 columns (Clontech, Mountain View, CA). The efficiency of RNA isolation, cDNA synthesis and dye incorporation was measured with a spectrofotometer (UV 1240mini, Shimadzu, Columbia, MD).

Hybridisation and scanning of micorarrays

Per experiment, two slides were hybridized with 200-300 ng labelled cDNA per sample (with 2-6 % dye-labeled nucleosides). Microarray slides included 19200 70-mer oligos (16659 genes of *H. sapiens* and 2541 control spots) and were produced in the Genomics Laboratory (University Medical Center, Utrecht, The Netherlands[37]). The first slide was hybridized using the sample of cDNA resulting from 3h mock-stimulated neutrophils labeled to Cy3 and the sample of cDNA resulting from 3h cytokine-stimulated neutrophils labeled with Cy5. In the second slide, the dyes were swapped between the samples. Slides were prehybridized for 45 min at 42 °C with a solution of 25ml SSC (20x; Invitrogen, Carlsbad, CA), 25 ml formamide, 49 ml ddH_20, 1 ml SDS (10%) and 1 g bovine serum albumin (Sigma, St. Louis, MO). Cy3 and Cy5 labeled samples were put together in a total volume of 80 µl and mixed with 80 µl of preheated (42°C) hybridization solution (125 µl formamide, 125 µl SSC (20x), 5 µl SDS (10%), 5 µl Herring Sperm DNA (10 µg/µl, Invitrogen, Carlsbad, CA) and 5 µl tRNA (10 µg/µl, Roche, Indianapolis, IN)). Before preheating at 42°C the total mix was heated at 95°C for 5 min. Slides were washed in H_2O and subsequently in isopropanol and dried by centrifugation at 900 rpm for 2 min. Labeled samples were put on the slides and coverslips were put over the slides. Hybridization was performed for 16-20h at 42°C in a Corning Hybridization Chamber (Corning, NY). Slides were washed for 4 min with 100 ml 1x

SSC and 0.2% SDS, subsequently with 100 ml 0.1x SSC and 0.2% SDS and the last wash solution 100ml 0.1x SSC. Slides were dried by centrifugation at 1000 rpm for 2 min. Slides were scanned in an Agilent DNA Microarray Scanner (G2566BA; Agilent Technologies, Palo Alto, CA). Image analysis was performed using Imagene 4.0 (Biodiscovery, Marina Del Rey, CA). Normalisation of the Imagene data was performed as described before[37]. Data were normalised based on the Lowess print-tip normalisation on genes. For calculations, the mean values were extracted from the Imagene files. The number of differentially expressed genes in TNFα- or GM-CSF-stimulated neutrophils in at least 3 arrays were for TNFα: 141 genes upregulated and 173 genes downregulated, and for GM-CSF: 223 genes upregulated and 51 genes downregulated.

Real time PCR

All primers used for real time PCR were from Sigma-Genosys (The Woodlands, TX). For β-actin, a 174 bp fragment was amplified using primers as described by Kruezer *et al*.[38] All other primers were designed using Primer 3 software of the whitehead institute/MIT center for genome research[39]. Sequences of the primers are listed in Table 2. Real-time PCR was performed using 0.1 µl of cDNA (see section: RNA isolation and cDNA synthesis), 12.5 µl of SYBR green I PCR mastermix (Biorad, Hercules, CA) and 400 nM of each primer (total volume: 25 µl). Amplification and detection were performed with a MyiQ Real-Time PCR detection system (Biorad, Hercules, CA) under the following conditions: 3 min at 95°C to activate DNA polymerase and 45 cycles of 10 seconds at 95°C, 20 sec at 61°C and 25 sec at 72°C. The reporter dye signal was measured against the passive signal of the internal reference dye (fluorescein) to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Results were normalized for the housekeeping genes β-actin and GAPDH. After each PCR run, primers were

Table 2. Sequences of primers used for Real Time RT PCR

Target Sequence	Forward primer	Reverse primer
β-Actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
GAPDH	AGAAGGCTGGGGCTCATTT	GAGGCATTGCTGATGATCTTG
MIP-1β	CCATGAAGCTCTGCGTGACT	AGCCCATTGGTGCTGAGAG
TNFAIP6	AGCACGGTCTGGCAAATACA	CCGCCTTCAAATTCACACAC
CD83	TCCTGAGCTGCGCCTACAG	AAGTCCACATCTTCGGAGCAA

checked for a-specific products using dissociation curves and PCR efficiency was checked by standard dilution curves. Results were calculated as described before[23] according to the $2^{-\Delta\Delta CT}$ method[40] and expressed as fold induction (^{2}LOG).
Average gene expression of mock-stimulated neutrophils or neutrophils derived from healthy controls was set at zero on the ^{2}LOG-scale. Analysis of gene expression levels of asthmatics before and after allergen challenge was done by comparing their gene expression levels with the average gene expression levels of healthy controls.
The contaminating eosinophils in the neutrophil/granulocyte-fraction did not influence gene expressions as none of the individual expression levels of the genes correlated with the individual percentage of eosinophils in the granulocyte fraction of the blood.

Procedure for staining granulocytes with monoclonal phage antibody (MoPhab) A17, CD11b and CD62L

Blood was collected in tubes containing sodium heparin as anti-coagulant and was immediately put on ice after venapuncture. From each tube two samples of 50 µl were taken per FACS analysis (i.e. staining with antibodies against integrin subunit αm (CD11b) or L-selectin (CD62L) or staining with MoPhab A17). The first sample was immediately put on ice until further processing. The second sample was stimulated with 10^{-6}M N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma, St. Louis, MO) for 10 min at 37ºC in order to determine the expression of epitopes after maximal *in vitro* stimulation. Hereafter, the cells were kept on ice during the remainder of the analysis.
For staining of MoPhab A17 a directly FITC-labeled MoPhab A17 was used as described before[15,41]. In short, MoPhab A17 was diluted 1/10 with PBS containing 4% milk powder. A 100 µl of this mix was added to whole blood samples of 50 µl each and cells with the mix were incubated for 60 min on ice. For staining of αm and L-selectin, 1 µl CD11b antibody (clone 44A, 10 µg/ml) and 1 µl CD62L antibody (clone dreg56[42], 10 µg/ml) were respectively added to 50 µl blood samples and cells with the antibodies were incubated for 30 min on ice. These monoclonal antibodies were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection (ATCC; Rockville, MD).
After incubation with the different antibodies, erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4˚C. In the case of anti-αm (CD11b) and anti-L-selectin (CD62L) antibodies, cells were counter-stained with 1

µl GAM-FITC (Becton Dickinson, Mountain View, CA). For all conditions, cells were washed at 4˚C and resuspended in ice-cold PBS containing 1% HSA. Cells were analyzed using a FACSvantage flowcytometer (Becton Dickinson, Mountain View, CA). Neutrophils and eosinophils were identified according to their specific side and forward scatter signals[15,21,41]. Data are reported as median fluorescence intensity (MCF) in arbitrary units (AU).

To confirm that, during allergen exposures, effects on granulocyte expression levels of MoPhab A17, αm and L-selectin were induced by allergens instead of its solvent saline, changes in unstimulated and fMLP-stimulated neutrophil and eosinophil responses were evaluated after saline inhalation at the first assessment day (see above), which did not show significant changes (data not shown).

Statistics

The results are expressed as means ± SEMs. Differences between study groups were compared using Mann-Whitney U tests or Wilcoxon signed rank tests. We used repeated measurement analysis of variance for statistical evaluation of gene expression data. Repeated measurement analysis showed that the regulation of the genes TNFAIP6, MIP-1β and CD83 correlated statistically to each other under each condition used in this study. This indicates that stimuli (e.g. TNFα or GM-CSF) or allergen exposure (baseline or 6h and 24h after allergen challenge) had similar effects on each gene (TNFAIP6, MIP-1β and CD83). The repeated measurement analysis delivered an estimate of the difference in response due to stimuli (e.g. TNFα or GM-CSF) or allergen exposure (baseline or 6h and 24h after allergen challenge). Correlation studies were performed using Spearman's correlation tests. All statistical tests were performed using the statistical software package SPSS version 13.0 (Chicago, Ill). P values <.05 were considered statistically significant (*P<0.05; **P<0.005; ***P<0.0005; ****P<0.00005; *****P<0.000005; ******P<at least 0.0000005).

Results

Priming responses of eosinophils and neutrophils are associated with differential expression of activation epitopes.

Eosinophils from allergic asthma patients exhibited higher expression levels of epitopes recognized by MoPhab A17[41] compared to cells from normal donors (Fig.

1A and 1B)[15]. In marked contrast to eosinophils, neutrophil expression levels of MoPhab A17 did not differ between healthy controls and asthmatics (Fig. 1A and 1C). Further characterization of granulocyte priming was performed by staining for the integrin-subunit αm (CD11b) and L-selectin (CD62L). Both markers on eosinophils and neutrophils did not differ significantly between healthy controls and asthmatics (Fig. 1D and 1E, respectively), making these markers less useful for the determination of granulocyte priming in the systemic compartment in allergic asthma. Interestingly, a clear positive correlation was found between the expressions of αm (CD11b) and L-selectin (CD62L) on both eosinophils and neutrophils from asthmatics (Fig. 2).

Eosinophils of asthmatics had an enhanced responsiveness to the innate immune stimulus fMLP in the context of MoPhab A17 expression compared to these cells of controls (Fig. 1B). Interestingly, also neutrophils of asthmatics had an enhanced responsiveness to fMLP (Fig. 1C). Similar to earlier studies[43,44] activation of eosinophils and neutrophils by fMLP *in vitro* increased the expression of αm (CD11b) and decreased the expression of L-selectin (CD62L) on granulocytes of both asthmatics (Fig. 1F) and controls (data not shown). In agreement with the unstimulated samples, also the fMLP-stimulated granulocytes did not show differences in CD11b or L-selectin expression between controls and asthmatics (data not shown).

Phenotype of neutrophils of mild asthmatics identified by a gene expression reference profile.

The data in Fig. 1 suggest that neutrophil priming in asthmatics is subtle and can only be detected in the context of responsiveness towards innate stimuli such as fMLP. To gain more insight into the priming of neutrophils we focused on gene expression profiles that are induced by pro-inflammatory cytokines such as TNFα and GM-CSF. Gene-array analysis provided us with cytokine-induced mRNA profiles of neutrophils activated by TNFα (100 U/ml) and GM-CSF (10^{-10}M). Three genes were shown to be differentially expressed in such a way that their regulation could be validated by Real-Time RT PCR with sufficient window to be applied for phenotyping of neutrophils from asthmatic donors (Fig. 3). The expression of these three genes, tumor necrosis factor alpha-induced protein 6 (TNFAIP6), macrophage inflammatory protein 1-beta (MIP-1β) and CD83, were upregulated by TNFα and downregulated by GM-CSF in neutrophils of healthy donors (Fig. 3A).

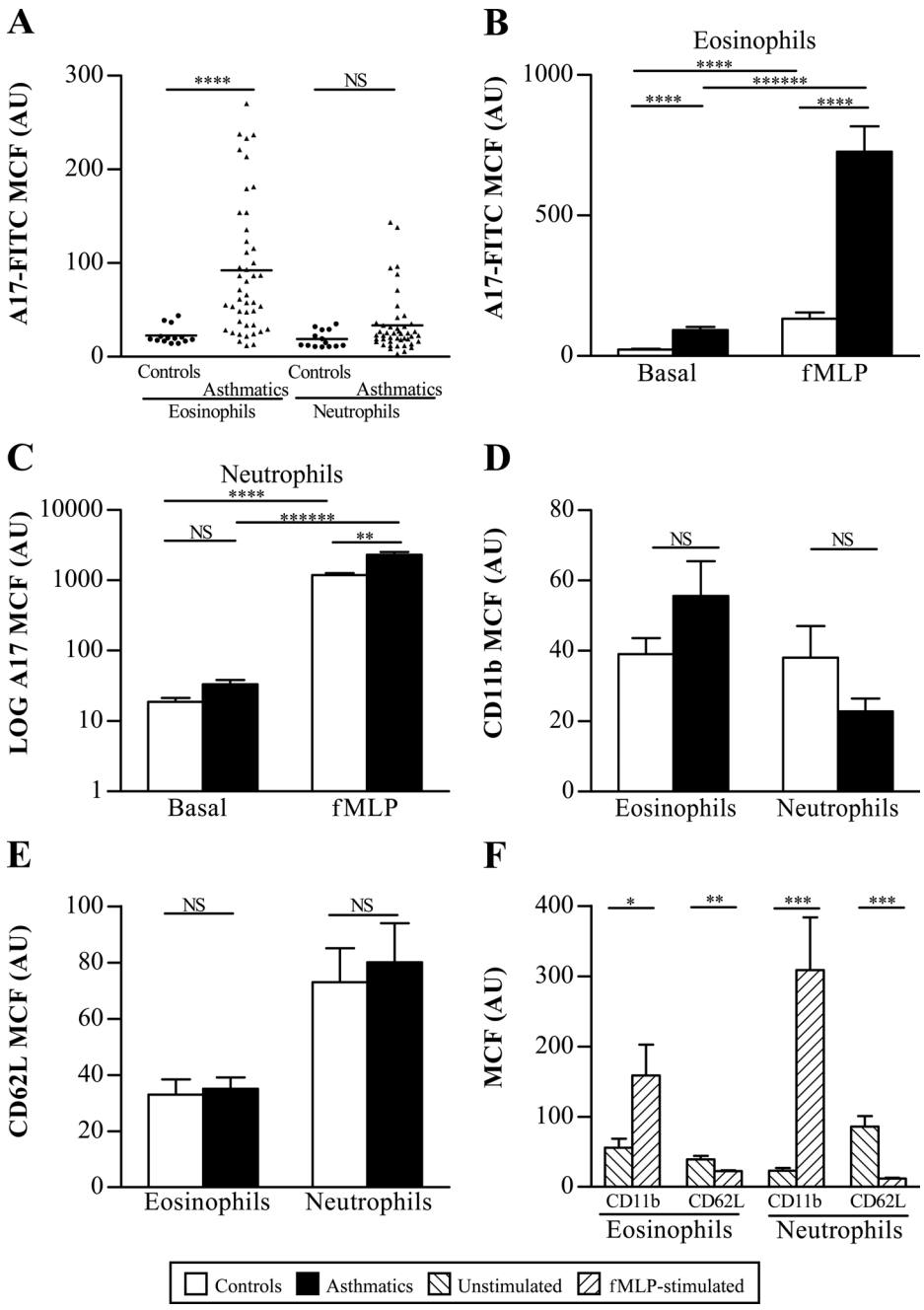

Figure 1. *In vivo* priming of eosinophils (**A**, **B**, **D**-**F**) and neutrophils (**A**, **C**-**F**) in asthmatics compared to cells from controls. Priming was measured by determination of expression of MoPhab A17 (**A**-**C**), αm (CD11b; **D** and **F**) or L-selectin (CD62L; **E** and **F**). Statistics was performed using Mann-Whitney U tests. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU).

Statistical analysis showed that this small gene profile was able to identify primed cells and to distinguish between a TNFα-type and a GM-CSF-type of priming.

After the identification of the differentially expressed genes (Fig. 3A) it was tested whether the subtle neutrophil priming of asthmatics was reflected by modulation of

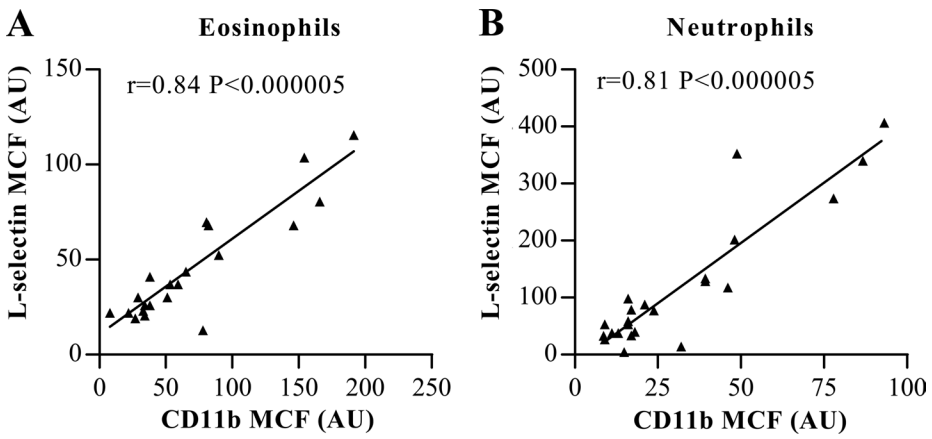

Figure 2. Integrin-subunit αm (CD11b) expression correlates positively with L-selectin (CD62L) expression on eosinophils (**A**; n=25) and neutrophils (**B**; n=23) in peripheral blood of asthmatics. Data regarding αm (CD11b) expression levels are plotted against the L-selectin (CD62L) expression values within the same patient. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU).

this profile. Therefore, gene expression levels of these genes in granulocytes of 14 asthmatics were compared to that of cells of healthy controls (Fig. 3B). A significant downregulation of these genes was found for asthmatics compared to controls.

Different priming phenotypes of granulocytes are found in single and dual responders after allergen challenge.

We next investigated the effect of bronchial allergen challenge on the systemic inflammatory responsiveness of granulocytes. This was investigated in allergic patients who developed both an EAR and a LAR and in patients who only developed an EAR (Table 3). No differences were found on basal MoPhab A17

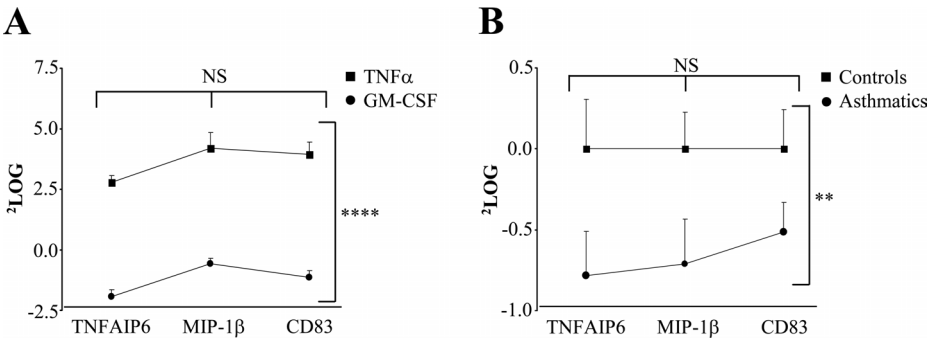

Figure 3. Neutrophil priming in asthmatics is visualized by changes in gene expression and resembles a GM-CSF stimulated gene-expression profile. Three genes (TNFAIP6, MIP-1β and CD83) were grouped, because they were differentially regulated in neutrophils activated with TNFα and GM-CSF (n=8; panel **A**). In asthmatics (n=14, panel **B**) the grouped gene expression was significantly downregulated compared to controls (n=14, panel **B**).

Table 2. Characteristics (mean(±SEM)) of single and dual responders.

	Single Responders	Dual responders
Number	7	10
Age (yrs)	23.6(3.4)	22.7(0.9)
Gender (M/F)	7/0	4/6
Baseline FEV_1 (% pred.)	94.1(3.8)	87.9(2.8)
Methacholine-PC_{20} (mg/ml)*	0.79(0.08-5.22)	0.66(0.08-4.90)
Allergen challenge with HDM~, cat or grass	2/3/2	8/2/0
Early asthmatic response (% fall in FEV_1 from baseline)	19.8(2.3)	24.9(2.5)
Late asthmatic response (% fall in FEV_1 from baseline)	1.8(1.1)	30.0(4.1)
Average onset late asthmatic response	-	5h

* Geometric mean of baseline Methacholine-PC_{20} (range)
~ HDM: House dust mite

expression of neutrophils and eosinophils between dual and single responders (Fig. 4). Six hours after allergen challenge, eosinophils showed an increased expression of MoPhab A17 in dual responders, whereas no effect was seen in single responders. Interestingly, allergen challenges had no effect on A17 expression on neutrophils in both single and dual responders (Fig. 4).

As expression of A17 on neutrophils was not affected at basal levels and 6h after allergen challenge, we investigated the responsiveness of the cells to fMLP. As shown in Fig. 5, the responsiveness for fMLP of neutrophils and eosinophils was differentially modulated by allergen challenge in dual and single responders. In dual responders, eosinophils did not show an enhanced responsiveness to fMLP for MoPhab A17 after allergen challenge (Fig. 5A). In contrast, upon allergen

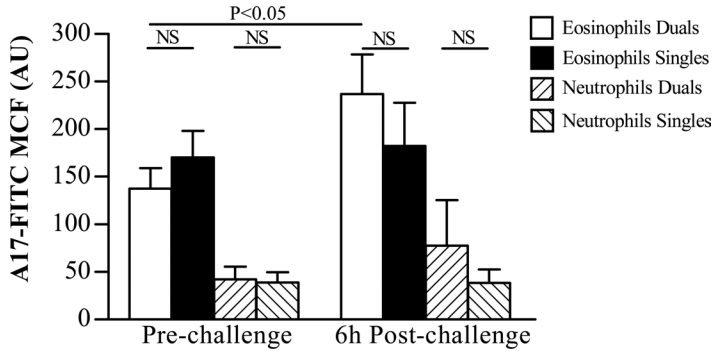

Figure 4. Differential MoPhab A17 expression before and after allergen challenge on eosinophils and neutrophils between dual (n=10) and single responders (n=7). The differences in priming between dual and single responders were analyzed using Mann-Whitney U tests. Differences between pre- and post-challenge MoPhab A17 expressions were analyzed using Wilcoxon signed-rank tests. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU).

challenge neutrophils of these dual responders showed an enhanced responsiveness to *in vitro* stimulation with fMLP (Fig. 5B). In single responders on the other hand, eosinophils showed a decreased responsiveness to fMLP stimulation after allergen challenge (Fig. 5C), whereas neutrophils were not affected in these patients (Fig. 5D). In contrast to MoPhab A17, no differences in basal or fMLP-stimulated expression of CD11b or L-selectin were found before and 6h after allergen challenge in dual nor single responders (data not shown).

To study the effect of allergen exposure on gene expression in neutrophils (Fig. 3), we included seven additional dual responders. These patients had the following characteristics:

- 7 male;
- mean age(±SEM): 21.9±1.1;
- mean PC_{20}-methacholine(±SEM): 2.0±0.7 mg/ml;
- mean percentage FEV_1-predicted(±SEM): 89.2±1.0%;
- mean percentage fall(±SEM) in FEV_1 during EAR and LAR were 24.6±3.5% and 29.0±2.7%, respectively.

Six hours after allergen challenge a significant downregulation of the genes

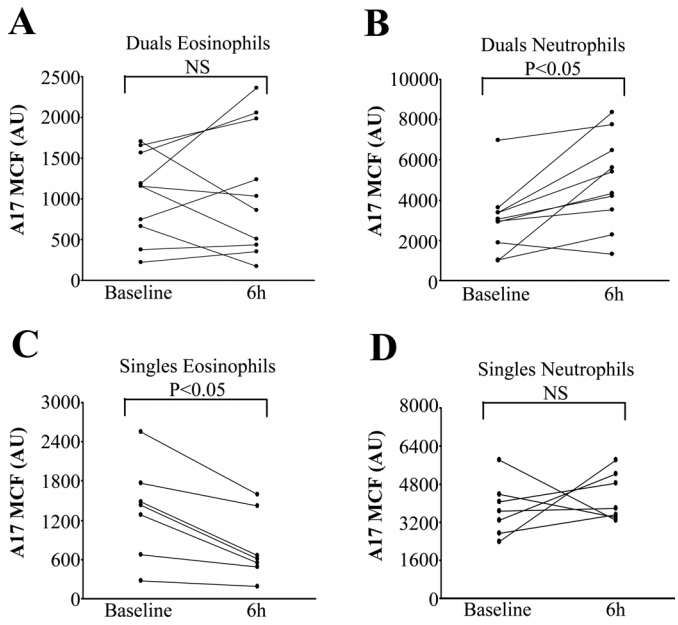

Figure 5. Differential responsiveness for fMLP in peripheral blood neutrophils and eosinophils between dual and single responders. Priming was measured by determination of expression levels of MoPhab A17 on eosinophils and neutrophils of dual responders (**A** and **B**, respectively) and single responders (**C** and **D**, respectively) before and 6h after allergen challenge. Data were analyzed using Wilcoxon signed-rank tests. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU).

TNFAIP6, MIP-1β and CD83 was found in neutrophils of these 7 patients compared to baseline values (repeated measurement analysis: P<0.05; data not shown).
Granulocyte priming responses that were induced by allergen-exposures were normalized to baseline 24h after allergen challenge (data not shown).

Different inflammatory responses are reflected by differences in granulocyte priming phenotypes.
To determine if any of the priming markers reflected chronic inflammation, the correlation between these markers with long-term characteristics of the asthma phenotype, such as enhanced airway hyperresponsiveness, was studied.
Previous studies have shown that αm (CD11b) expression on eosinophils in peripheral blood correlated inversely with low levels of AHR to histamine[21]. We could confirm the correlation between CD11b and eosinophil AHR in our patients (Fig. 6A; data shown are from dual responders; Table 3). In addition to the correlation in eosinophils we also identified a strong correlation between AHR and CD11b expression levels on neutrophils (Fig. 6B; data shown are from dual responders; Table 3). In the same patient group, no correlation was found between AHR and L-selectin levels on eosinophils (r=-0.50, P=0.22) and neutrophils (r=-0.67, P=0.06), but this might be caused by the low power of the study. In addition, no correlation was found between AHR and MoPhab A17 expression levels on eosinophils (r=-0.32, P=0.41) and neutrophils (r=-0.30, P=0.44).

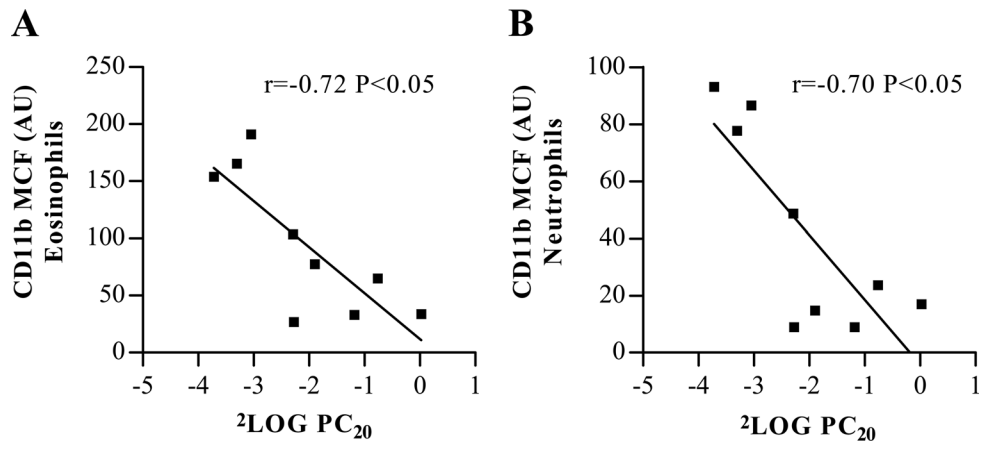

Figure 6. Low baseline (prior to allergen challenge) ^{2}LOG PC_{20}-methacholine values (<1.5 mg/ml) correlate with αm (CD11b) expression on peripheral blood eosinophils (**A**) and neutrophils (**B**) of dual responders (n=9). Data regarding αm (CD11b) expression levels are plotted against the baseline PC_{20}-methacholine in the same patient. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU).

Discussion

Priming of inflammatory cells in peripheral blood is a general process in chronic inflammatory diseases, such as allergic asthma[15,16,41,45]. Important inflammatory processes such as chemotaxis, adhesion and transendothelial migration can all be pre-activated *in vivo* in peripheral blood cells. This process can be mimicked by adding (Th2-)cytokines to non-primed granulocytes from healthy individuals *in vitro*[16,45,46]. Therefore, priming of peripheral blood granulocytes reflects part of the systemic inflammatory responses in allergic asthma[16,47]. We used this priming response to study the systemic innate immune responses in different phenotypes and phases of allergic asthma with focus on modulation of both eosinophils and neutrophils.

The expression of the integrin-subunit αm (CD11b) and/or L-selectin (CD62L) is modulated by activation *in vitro* and is, therefore, used as marker of leukocyte activation[43,44,48]. Previous studies have shown that no differences in expression of αm or L-selectin were found on granulocytes of mild(-to-moderate) asthmatics (with or without treatment) compared to cells of controls[21,44]. To compare and validate the kinetics of the priming measured by MoPhab A17, we compared its expression with αm and L-selectin expressions. The data in Fig. 1 indicates that MoPhab A17 is more sensitive to priming stimuli compared to αm (CD11b), as MoPhab A17 could recognize eosinophil priming in asthmatics even without allergen challenge[15] whereas αm (CD11b) and L-selectin (CD62L) expression levels were similar on eosinophils or neutrophils of both asthmatics and controls.

Much to our surprise strong positive correlations were found between L-selectin (CD62L) and αm (CD11b) expression levels on both eosinophils and neutrophils from asthmatics (Fig. 2A and 2B). These data suggest that the expressions of αm (CD11b) and L-selectin (CD62L) are controlled differently as compared to the modulated expression levels of these markers on cells activated *in vitro* (Fig. 1F)[43,44] or in the lung tissue *in vivo*[49]. Our data are consistent with a model that predicts the cellular expression of αm^{bright}/L-$selectin^{bright}$ to comprise a phenotype that facilitates cellular adhesion and homing in allergic asthmatics. During subsequent extravasation from the blood to the lung L-selectin is shed from the surface whereas αm is even more increased[49].

In marked contrast to eosinophils no differences were found between asthmatics and healthy controls in basal expression of MoPhab A17 on peripheral blood neutrophils[15]. However, these cells exhibited an enhanced responsiveness towards

the chemoattractant fMLP (Fig. 1C) demonstrating that neutrophils in these patients were subtly primed *in vivo*.

We examined gene expression levels in neutrophils of mild asthmatics and healthy controls to define the priming phenotype of neutrophils in more detail. A cytokine-induced mRNA reference profile was established using three genes that were differentially controlled by GM-CSF and TNFα (Fig. 3). The gene expression profile found in neutrophils of asthmatics resembled a GM-CSF gene expression profile, which is in agreement with the hypothesis that Th2-cytokines, such as GM-CSF, dominate the inflammatory processes in asthma.

We next addressed the question whether differences in asthma phenotypes were associated with differences in systemic innate immune responses. Therefore, priming was measured before and after allergen challenges in two types of mild allergic asthmatics: single and dual responders. These two groups of patients did not distinguish themselves at baseline in terms of disease severity, baseline FEV_1 or AHR. The only apparent difference was the presence of an allergen-induced LAR in dual responders. No consensus is present why dual responders experience a LAR, whereas single responders do not. This study sheds more light on the differences in immune mechanisms between both patient groups. In marked contrast to single responders the LAR in dual responders was characterised by an increased expression of MoPhab A17 on peripheral blood eosinophils 6h after allergen challenge. In the same patients the neutrophil compartment was not changed in this context.

It was surprising that the neutrophil compartment did not seem to be targeted during the allergen induced systemic activation of the innate immune system, as neutrophils are recruited to the lung after allergen provocation[24,26] and these cells are involved in the persistent inflammation in asthmatics[5,6,8,9]. Therefore, we decided to study the priming of the neutrophil compartment in more detail. We showed that neutrophils were more sensitive for fMLP-stimulation after allergen challenge in dual responders, whereas neutrophils in the blood of single responders were not influenced (Fig. 5). Also enhanced neutrophil priming was identified by analysis of gene expression levels in dual responders 6h after allergen challenge. This priming phenotype after allergen challenge reflected a GM-CSF induced pattern, suggesting again that predominantly Th2-cytokines participate in the systemic compartment after allergen exposure in asthmatics. Furthermore, these data indicate that analysis of gene expression can be used to study subtle neutrophil priming in asthmatics.

The present study cannot explain which mechanisms are involved in the differences in fMLP responsiveness of granulocytes between single and dual responders. It is, however, tempting to speculate that asthma patients only develop a late phase asthmatic reaction when both eosinophil and neutrophil compartments are functionally upregulated.

In marked contrast to the expression of MoPhab A17, which is rapidly upregulated during acute responses (e.g. allergen challenge), αm (CD11b) expression levels on eosinophils and neutrophils nicely correlated with low levels of bronchial hyperresponsiveness of asthmatics (Fig. 6) as was also seen by In 't Veen *et al*.[21] These findings are in line with the hypothesis that the expression of αm (CD11b) and L-selectin (CD62L) on eosinophils and neutrophils better reflect a more continuous inflammatory tone caused by a chronic tissue inflammation.

In conclusion, multiple priming phenotypes of peripheral blood granulocytes can be identified using cell-epitope expression analysis and gene expression profiling. Different types of allergic asthma (e.g. single EAR or dual EAR/LAR after allergen challenge) are characterized by distinct priming phenotypes of eosinophils and neutrophils. The data underline the presence of differences between persistent chronic inflammation and acute allergen induced inflammation. These differences in innate responses will have consequences for the diagnosis of multiple disease phenotypes and also for the design of new therapeutic options for the distinct classes of patients.

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References

1. Busse WW, Lemanske RF, Jr.: Asthma. N.Engl.J.Med. 344: 350-362, 2001
2. Ritz SA, Stampfli MR, Davies DE, Holgate ST, Jordana M: On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends Immunol. 23: 396-402, 2002
3. Holgate ST: Cytokine and anti-cytokine therapy for the treatment of asthma and allergic disease. Cytokine 28: 152-157, 2004

4. Thomas PS: Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol.Cell Biol. 79: 132-140, 2001
5. Wenzel SE, Balzar S, Cundall M, Chu HW: Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J.Allergy Clin.Immunol. 111: 1345-1352, 2003
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. 160: 1532-1539, 1999
7. Douwes J, Gibson P, Pekkanen J, Pearce N: Non-eosinophilic asthma: importance and possible mechanisms. Thorax 57: 643-648, 2002
8. 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. 22: 470-477, 2003
9. Gibson PG, Simpson JL, Saltos N: Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119: 1329-1336, 2001
10. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ: Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am.J.Respir.Crit Care Med. 156: 737-743, 1997
11. Kelly EA, Busse WW, Jarjour NN: Increased matrix metalloproteinase-9 in the airway after allergen challenge. Am.J.Respir.Crit Care Med. 162: 1157-1161, 2000
12. Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidart JM, Louis R: Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. Chest 122: 1553-1559, 2002
13. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE: Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. J.Allergy Clin.Immunol. 112: 1064-1071, 2003
14. Koenderman L, Van der Bruggen T, Schweizer RC, Warringa RA, Coffer P, Caldenhoven E, Lammers JW, Raaijmakers JA: Eosinophil priming by cytokines: from cellular signal to in vivo modulation. Eur.Respir.J.Suppl 22: 119s-125s, 1996
15. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L: Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005
16. Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koenderman L: Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. J.Allergy Clin.Immunol. 91: 1198-1205, 1993
17. Moser R, Fehr J, Olgiati L, Bruijnzeel PL: Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. Blood 79: 2937-2945, 1992
18. Ebisawa M, Liu MC, Yamada T, Kato M, Lichtenstein LM, Bochner BS, Schleimer RP: Eosinophil transendothelial migration induced by cytokines. II. Potentiation of eosinophil transendothelial migration by eosinophil-active cytokines. J.Immunol. 152: 4590-4596, 1994
19. Lantero S, Alessandri G, Spallarossa D, Scarso L, Rossi GA: Stimulation of eosinophil IgE low-affinity receptor leads to increased adhesion molecule expression and cell migration. Eur.Respir.J. 16: 940-946, 2000
20. Mengelers HJ, Maikoe T, Brinkman L, Hooibrink B, Lammers JW, Koenderman L: Immunophenotyping of eosinophils recovered from blood and BAL of allergic asthmatics. Am.J.Respir.Crit Care Med. 149: 345-351, 1994
21. In 't Veen JC, Grootendorst DC, Bel EH, Smits HH, Van Der Keur M, Sterk PJ, Hiemstra PS: CD11b and L-selectin expression on eosinophils and neutrophils in blood and induced sputum of patients with asthma compared with normal subjects. Clin.Exp.Allergy 28: 606-615, 1998
22. Pals CM, Verploegen SA, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ: Identification of cytokine-regulated genes in human leukocytes in vivo. J.Allergy Clin.Immunol. 105: 760-768, 2000
23. Oudijk EJ, Nijhuis EH, Zwank MD, Van de Graaf EA, Mager HJ, Coffer PJ, Lammers JW, Koenderman L: Systemic inflammation in COPD visualised by gene profiling in peripheral blood neutrophils. Thorax 60: 538-

544, 2005
24. Nocker RE, Out TA, Weller FR, Mul EP, Jansen HM, Van der Zee JS: Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. Int.Arch.Allergy Immunol. 119: 45-53, 1999
25. Koh YY, Dupuis R, Pollice M, Albertine KH, Fish JE, Peters SP: Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. Am.J.Respir.Cell Mol.Biol. 8: 493-499, 1993
26. Taube C, Dakhama A, Rha YH, Takeda K, Joetham A, Park JW, Balhorn A, Takai T, Poch KR, Nick JA, Gelfand EW: Transient neutrophil infiltration after allergen challenge is dependent on specific antibodies and Fc gamma III receptors. J.Immunol. 170: 4301-4309, 2003
27. Aalbers R, Kauffman HF, Vrugt B, Koeter GH, De Monchy JG: Allergen-induced recruitment of inflammatory cells in lavage 3 and 24 h after challenge in allergic asthmatic lungs. Chest 103: 1178-1184, 1993
28. Weersink EJ, Postma DS, Aalbers R, De Monchy JG: Early and late asthmatic reaction after allergen challenge. Respir.Med. 88: 103-114, 1994
29. Cockcroft DW, Murdock KY: Changes in bronchial responsiveness to histamine at intervals after allergen challenge. Thorax 42: 302-308, 1987
30. Bentley AM, Kay AB, Durham SR: Human late asthmatic reactions. Clin.Exp.Allergy 27 Suppl 1: 71-86, 1997
31. Grutters JC, Brinkman L, Aslander MM, Van den Bosch JM, Koenderman L, Lammers JW: Asthma therapy modulates priming-associated blood eosinophil responsiveness in allergic asthmatics. Eur.Respir.J. 14: 915-922, 1999
32. Bancalari L, Dente FL, Cianchetti S, Prontera C, Taccola M, Bacci E, Carletti A, Di Franco A, Giannini D, Vagaggini B, Ferdeghini M, Paggiaro PL: Blood markers of early and late airway responses to allergen in asthmatic subjects. Relationship with functional findings. Allergy 52: 32-40, 1997
33. National Heart, Lung, and Blood Institute, Global strategy for asthma management and prevention NHLBI/WHO Workshop Report, National Institutes of Health, Bethesda (MD) (1995) updated April 2002. Publication number 95–3659.
34. Cockcroft DW, Murdock KY, Kirby J, Hargreave F: Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. Am.Rev.Respir.Dis. 135: 264-267, 1987
35. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, Juniper EF, Malo JL: Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur.Respir.J.Suppl 16: 53-83, 1993
36. Verploegen S, Van Leeuwen CM, Van Deutekom HW, Lammers JW, Koenderman L, Coffer PJ: Role of Ca2+/calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphotidylinositol-3 kinase. Eur.J.Biochem. 269: 4625-4634, 2002
37. Van de Peppel J, Kemmeren P, Van Bakel H, Radonjic M, Van Leenen D, Holstege FC: Monitoring global messenger RNA changes in externally controlled microarray experiments. EMBO Rep. 4: 387-393, 2003
38. Kreuzer KA, Lass U, Landt O, Nitsche A, Laser J, Ellerbrok H, Pauli G, Huhn D, Schmidt CA: Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. Clin.Chem. 45: 297-300, 1999
39. Rozen S, Skaletsky H: Primer3 on the WWW for general users and for biologist programmers. Methods Mol.Biol. 132: 365-386, 2000
40. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25: 402-408, 2001
41. Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, de Kruif J, Logtenberg T: Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. J.Leukoc.Biol. 68: 58-64, 2000
42. Ulfman LH, Kuijper PH, van der Linden JA, Lammers JW, Zwaginga JJ, Koenderman L: Characterization of eosinophil adhesion to TNF-alpha-activated endothelium under flow conditions: alpha 4 integrins mediate initial attachment, and E-selectin mediates rolling. J.Immunol. 163: 343-350, 1999
43. Neeley SP, Hamann KJ, White SR, Baranowski SL, Burch RA, Leff AR: Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils.

Am.J.Respir.Cell Mol.Biol. 8: 633-639, 1993

44. Chung KF, Mann BS: Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. Respir.Res. 7: 59, 2006
45. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L: In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 79: 1836-1841, 1992
46. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L: RANTES- and interleukin-8-induced responses in normal eosinophils: effect of priming with interleukin-5. Blood 83: 3697-3704, 1994
47. Bochner BS: Systemic activation of basophils and eosinophils: markers and consequences. J.Allergy Clin.Immunol. 106: S292-S302, 2000
48. Thorne KJ, Richardson BA, Mazza G, Butterworth AE: A new method for measuring eosinophil activating factors, based on the increased expression of CR3 alpha chain (CD11b) on the surface of activated eosinophils. J.Immunol.Methods 133: 47-54, 1990
49. Mengelers HJ, Maikoe T, Hooibrink B, Kuypers TW, Kreukniet J, Lammers JW, Koenderman L: Down modulation of L-Selectin expression on eosinophils recovered from bronchoalveolar lavage fluid after allergen provocation. Clin.Exp.Allergy 23: 196-204, 1993

Chapter 3

Identification of distinct asthma phenotypes by priming associated markers on peripheral blood leukocytes

Blood samples as tool to identify asthma subphenotypes

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In preparation

Abstract

Background: Allergic asthma is associated with systemic immune responses, which involve enhanced priming of peripheral blood eosinophils in patients with a mild and stable phenotype. Besides eosinophils, other leukocytes such as neutrophils and monocytes are associated with peripheral inflammation and development of asthma phenotypes.

Objective: This study was performed to monitor leukocyte priming in allergic asthmatics who had distinct characteristics based upon their pharmacotherapies.

Methods: Blood was taken from allergic asthmatics who did not use medication and from patients who used relatively high doses of inhaled corticosteroids (difficult-to-treat asthmatics). Systemic priming of leukocytes was studied using 1) expression of the cellular marker αm (CD11b), and 2) priming epitopes recognized by monoclonal phage antibodies A17 and A27.

Results: Eosinophils from patients with mild stable asthma were characterised by a priming phenotype consisting of $A17^{bright}$, $A27^{bright}$ and $CD11b^{dim}$ expressions. This priming phenotype was not found for monocytes and neutrophils in the same patients. Interestingly, difficult-to-treat asthmatics had a systemic innate immune response with primed neutrophils and monocytes for all three investigated priming markers without signs of (pre-)activation of eosinophils.

Conclusion: Different phenotypes of allergic asthma are associated with distinct priming profiles of inflammatory cells in peripheral blood.

Clinical Implication: Priming profiles of inflammatory cells in blood samples are a straightforward tool to identify different (pharmacotherapeutical) phenotypes of allergic asthmatics and might help to establish decisions on pharmaceutical treatment in asthma patients.

Introduction

Asthma patients are generally phenotyped based upon their clinical characteristics such as lung function parameters, their symptoms, atopy status and/or medication requirements[1,2]. However, within one phenotype of patients several sub-phenotypes can exists as has been demonstrated for mild asthma patients[1], complicating clear-cut definitions of asthma populations. Therefore, the development of diagnostic markers that enable better characterisation of asthma phenotypes will help to improve individual and tailor-made therapies of asthma.

A hallmark of asthma is the chronic airway inflammation. Much emphasis has been given to the role of proinflammatory eosinophils in this inflammation[3-6]. Kinetics of eosinophil inflammation in asthmatics are reflected by increased pre-activation responses of these cells in the systemic compartment[3]. These pre-activation responses are rather subtle and referred to as priming[3,7-9]. In priming responses, inflammatory cells are not activated by inflammatory stimuli, but increase their responsiveness towards distinct stimuli.

Next to the importance of eosinophils, neutrophils are also implicated in the pathogenesis of asthma[10-14]. Neutrophils have particularly been associated with more severe phenotypes of asthma[11,13,15]. Elevated levels of neutrophils in tissue, sputum and bronchoalveolar lavage (BAL)-fluid have been found in severy asthmatics. Recently, neutrophil responses were also linked with non-eosinophilic asthma in mild(-to moderate) patients[12]. On the other hand, priming of neutrophils was not identified in mild(-to-moderate) asthmatics either measured via 1) recently developed monoclonal phage antibodies (MoPhabs) A17 and A27, which are an excellent tool in the measurement of kinetics of acute leukocyte priming and activation responses[3,7], or via 2) expression of other markers associated with cellular activation such as adhesion molecules αm (CD11b) and L-selectin (CD62L)[16-18].

In contrast to neutrophils and eosinophils, hardly any data exists about monocytes, which constitute another cellular compartment that might be involved in the inflammation in asthmatics[19,20].

In this study we used peripheral blood leukocyte expression levels of αm (CD11b) and epitopes recognized by MoPhabs A17 and A27 as biological markers to phenotype two populations of asthmatics. These populations could clinically be characterised as a similar group with respect to their lung function parameters and airway hyperresponsiveness, but were very different regarding their requirements

of pharmacotherapy. We will show evidence, using cellular priming markers, that in asthmatics a systemic immune response involving neutrophils and monocytes is associated with a poor treatment response.

Materials and methods

Patients and healthy controls

We included 30 non-smoking patients with a diagnosis of allergic asthma (Table 1) and categorised these patients according to their requirements of pharmacotherapy. Patients with phenotype 1 (Table 1) were mild asthmatics who did not use treatment with anti-inflammatory agents such as corticosteroids (anti-inflammatory medication was stopped at least 4 weeks prior to entry of the study). These mild patients were included according to the GINA guidelines[2] and were allowed to use short-acting β_2-agonists. Patients with phenotype 2 (Table 1) were asthma patients who received high doses of steroids[21,22]. They were characterized as 'difficult-to-treat' asthma patients based upon their requirements of corticosteroids that they needed to stabilise their lung functions. Difficult-to-treat asthmatics were allowed to use anti-histaminics. Only those difficult-to-treat

Table 1. Characteristics (Mean(±SEM)) of controls and asthmatics.

	Controls	**Asthmatics Phenotype 1~**	**Asthmatics Phenotype 2#**
Number	14	16	14
Age (yrs)	32(3.1)	28(2.6)	36(2.6)
Gender (M/F)	7/7	16/0	6/8
Atopy/Allergy	-	+	+
Baseline FEV_1 (% pred.)	-	93.8(3.7)	103.5(4.4)
PC_{20}	-	1.8(0.4)	3.0(0.8)
Pharmacotherapy (µg)	-	-	4 patients: flixotide 1000 2 patients: flixotide 2000 6 patients: pulmicort 800 1 patient: pulmicort 1000 1 patient: qvar 400

~ PC_{20}-metacholine

PC_{20}-histamine

patients were recruited who were similar to mild stable asthma patients based on their clinical parameters (allergy, FEV1-predicted (>80%) and airway hyperresponsiveness (AHR)). All asthma patients had positive allergen skin prick tests[23], did not have a respiratory tract infection in the last 4 weeks before entering the study and all had documented AHR (phenotype 1: PC_{20}-methacholine and phenotype 2: PC_{20}-histamine).
Healthy subjects without having asthma symptoms or presence of atopy (negative allergen skin prick tests[23]) were selected from the laboratory and clinical staff (n=14: 7 male, 7 female; Table 1). The medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands) approved the study and all patients gave their written informed consent.

Procedure for staining leukocytes with monoclonal phage antibodies (MoPhabs) A17 and A27 and CD11b

Blood was collected in tubes containing sodium heparin as anti-coagulant and was immediately put on ice after venapuncture. From each tube two samples of 50 µl were taken per FACS analysis (i.e. staining with FITC-labelled MoPhab A17, FITC-labelled MoPhab A27 or CD11b-PE). The first sample was immediately put on ice until further processing. The second sample was stimulated with 10^{-6}M N-formyl-methionyl-leucyl-phenylalanine ((fMLP) Sigma; St. Louis, MO) for 10 min at 37ºC and subsequently put on ice. Blood samples were stained with directly FITC-labeled monoclonal phage antibody (MoPhab) A17 or MoPhab A27 as described before[3,7]. In short, MoPhab A17 or A27 was diluted 1/10 with PBS. A 100 µl of this mix was added to whole blood samples of 50 µl each and cells with the mix were incubated for 60 min on ice. For staining of αm, directly labeled CD11b-PE ((1/100) Dako; Glostrup, Denmark) antibody was added to 50 µl whole blood samples and cells with CD11b-PE were incubated for 30 min on ice.
After the staining with FITC-labelled MoPhab A17, FITC-labelled MoPhab A27 or CD11b-PE erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4˚C. Subsequently, the cells were washed at 4˚C and resuspended in ice-cold PBS containing 1% human serum albumin ((HSA); Sanquin, Amsterdam, The Netherlands) for analysis. Cells were analyzed in a FACSvantage flowcytometer (Becton Dickinson, Mountain View, CA). Neutrophils, eosinophils and monocytes were identified according their specific side scatter and forward scatter signals[3,7,16]. Data are reported as median fluorescence intensity (MCF) in arbitrary units (AU).

Statistics

The results are expressed as means ± SEMs. Differences between study groups were compared using Mann-Whitney U tests. Differences between unstimulated and stimulated samples were performed using Wilcoxon signed rank tests. All statistical tests were performed using the statistical software package SPSS version 11.0 (Chicago, Ill). P values <.05 were considered as statistically significant (*P<0.05; **P<0.005; ***P<0.0005).

Results

Different priming phenotypes of leukocytes are found in distinct phenotypes of allergic asthmatics.

In our previous work priming of eosinophils of mild asthmatics was identified using cellular staining with MoPhabs A17 and A27 (Chapter 2 and ref.[3]). In these studies no priming of neutrophils could be identified using these MoPhabs. In Fig. 1 similar results are shown for mild asthmatics classified as phenotype 1, confirming the presence of eosinophil inflammation in peripheral blood of these patients. Next to MoPhabs A17 and A27, the eosinophil and neutrophil expressions of αm (CD11b) were not different between mild asthmatics with phenotype 1 and healthy donors (Fig. 1).

In contrast to asthmatics with phenotype 1, asthmatics classified as phenotype 2 did not show eosinophil priming for any of the three markers compared to these cells of healthy controls, but did show clear neutrophil priming for all three investigated markers (Fig. 1).

In addition to granulocytes we also studied the priming of monocytes without prior isolation of monocytes. Remarkably, peripheral blood monocyte priming was enhanced in asthmatics with phenotype 2 compared to the priming of these cells of healthy controls (Fig. 1). On the other hand, monocyte priming was not enhanced in asthma patients with phenotype 1, but the expression levels of priming epitopes recognized by MoPhab A17 were significantly lower on peripheral blood monocytes of these patients compared to the monocyte expression levels of MoPhab A17 of healthy controls (Fig. 1).

As priming is defined as the process that enhances responses but does not necessarily induce them, we set out experiments to study expression levels of priming epitopes in the context of activated cells. Therefore, we studied the

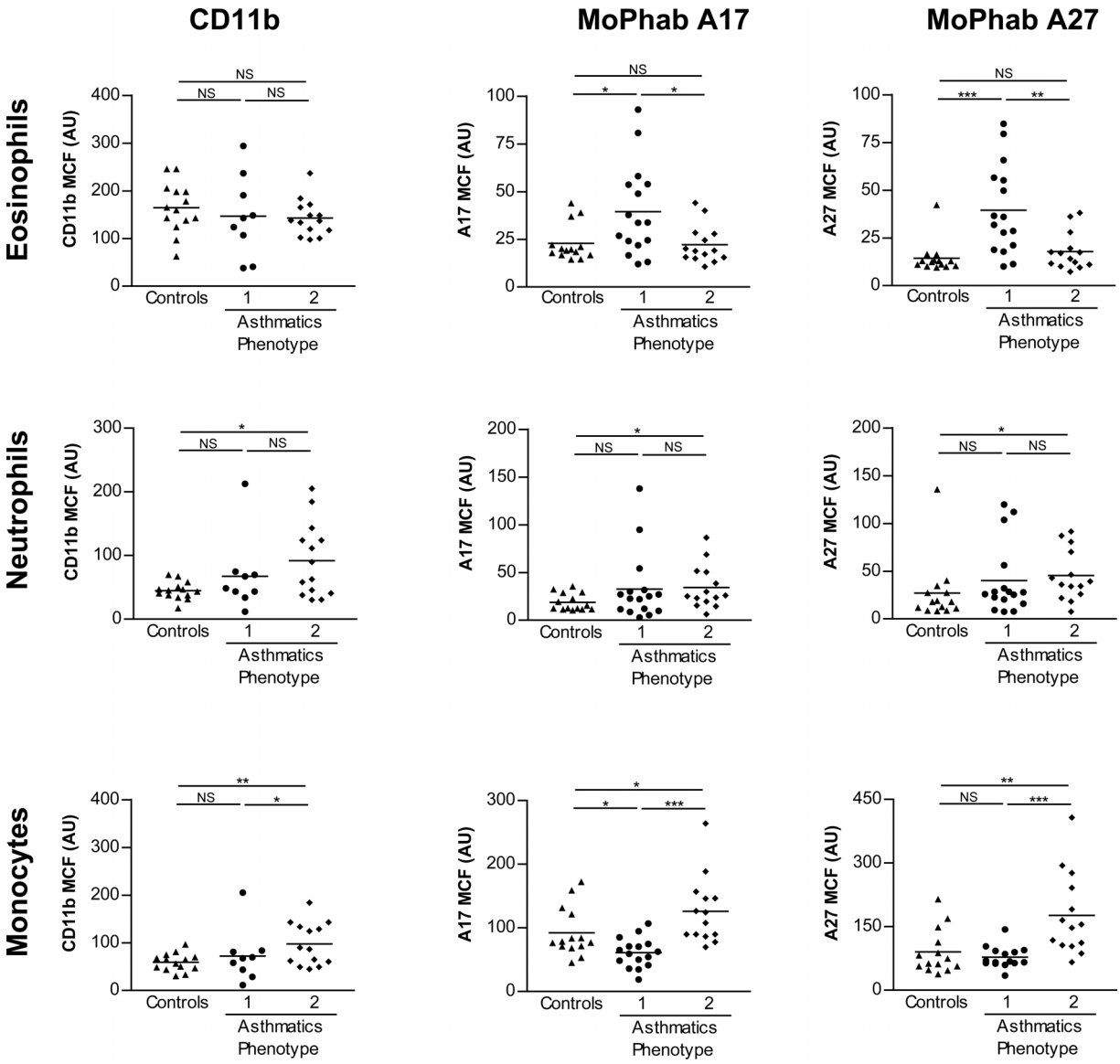

Figure 1. Priming of eosinophils, neutrophils and monocytes in mild stable asthmatics (phenotype 1), difficult-to-treat asthmatics (phenotype 2) and controls measured via cellular expression of αm (CD11b) and epitopes recognized by MoPhabs A17 and A27. Statistics were performed using Mann-Whitney U tests. Fluorescence (MCF) is given in arbitrary units (AU).

responsiveness of leukocytes to a high concentration of stimulus fMLP (10^{-6}M). Stimulation of leukocytes with fMLP resulted in significant induction of all three priming epitopes (Fig. 2). The sensitivity of eosinophils for fMLP was not significantly different between asthmatics with phenotype 1 compared to healthy controls for any of the three markers (Fig. 2). Eosinophils from phenotype 2 were characterised by a complex responsiveness to fMLP. Upon activation eosinophil expression levels of CD11b were lower in asthmatics with phenotype 2 compared

to these cells of controls whereas the expression levels of MoPhab A27 were increased (Fig. 2). For neutrophils no differences were found after activation between controls and asthmatics with phenotypes 1 or 2 in the context of expressions of αm (CD11b), MoPhab A17 and MoPhab A27 (Fig. 2). Monocytes had a significant lower MoPhab A17 expression in asthmatics with phenotype 1 compared to these cells of healthy controls after fMLP-stimulation (Fig. 2). All other monocyte responses to fMLP did not differ significantly between asthmatics (with both phenotypes) and healthy controls.

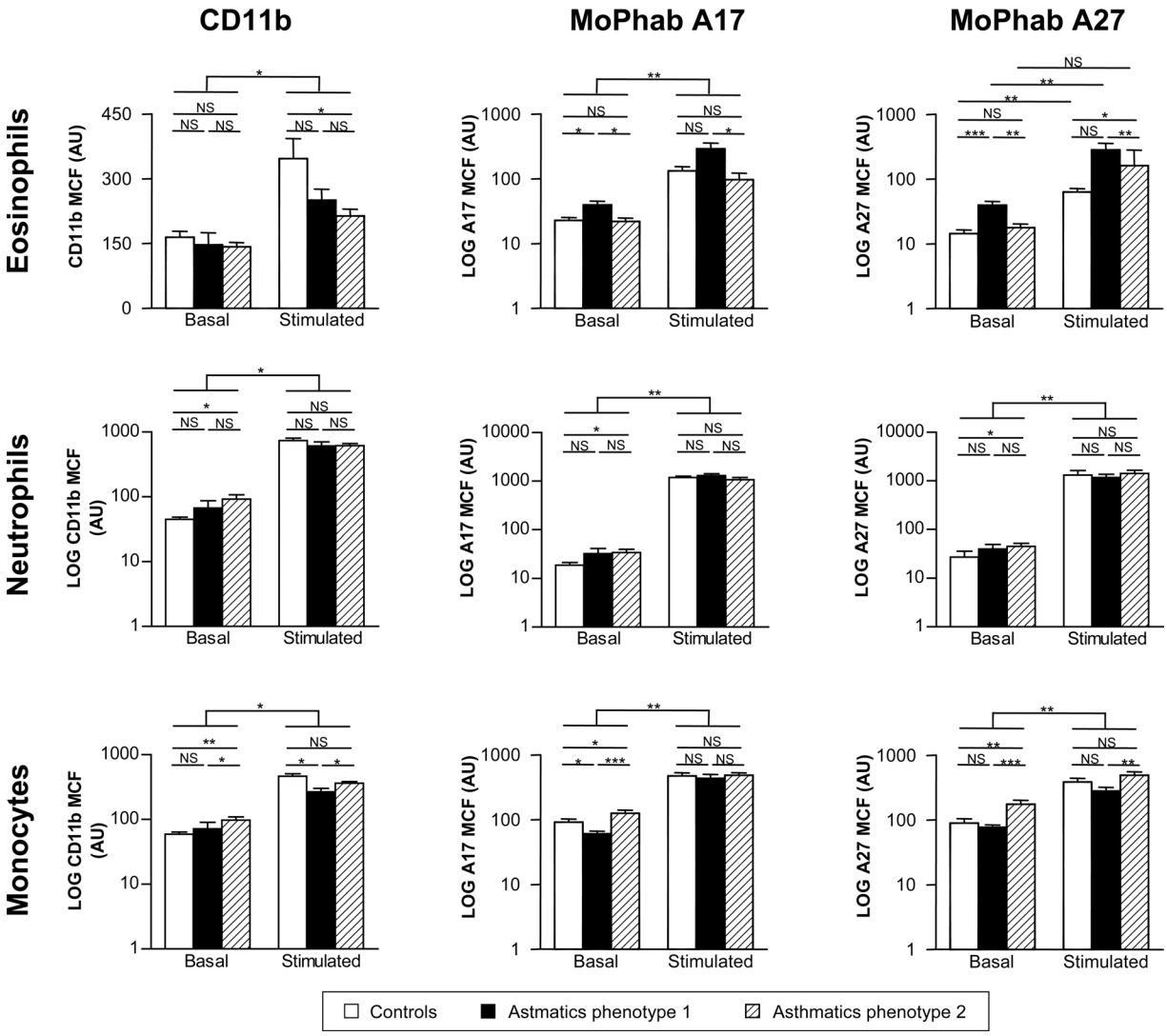

Figure 2. Priming of eosinophils, neutrophils and monocytes in mild stable asthmatics (phenotype 1), difficult-to-treat asthmatics (phenotype 2) and controls measured via cellular expression of αm (CD11b) and epitopes recognized by MoPhabs A17 and A27. Priming was measured with and without fMLP-stimulation (10^{-6}M). Statistics were performed using Mann-Whitney U tests (between groups) or Wilcoxon signed rank tests (between unstimulated and stimulated samples). Fluorescence (MCF) is given in arbitrary units (AU).

Discussion

In this study, the peripheral blood leukocyte priming profile of asthmatics requiring treatment with relative high doses of corticosteroids was compared to that of healthy controls and mild asthmatics who did not use steroids. The group of (high-dose) steroid dependent patients were referred to as difficult-to-treat asthmatics. However, their clinical characteristics were similar to the 'mild' asthmatic population based on several clinical parameters (allergy, FEV1-predicted (>80%) and bronchial hyperresponsiveness (PC_{20} to either methacholine or histamine (<8 mg/ml)). The difficult-to-treat patients are different patients than those defined as 'difficult' asthmatics[24] or steroid resistant[25] as these difficult-to-treat patients did respond to therapy, although less well-controlled than stable patients.

In order to establish phenotypical differences between these patient groups, we investigated pre-activation responses of leukocytes as read-out for the systemic inflammatory reactions in these patients[3,7]. These responses were measured by determination of cellular expression levels of the integrin αm (CD11b)[26-28] and priming associated epitopes recognized by MoPhabs A17 and A27[3,7].

The inflammatory phenotype of the mild asthmatics without treatment (phenotype 1) was characterized by primed eosinophil responses as we have shown before (Chapter 2 and ref.[3]). These responses could only be identified using expression levels of priming epitopes recognized by MoPhabs A17 and A27, but not via expression of the integrin αm (CD11b). Therefore, expressions of MoPhabs A17 and A27 seem to be more sensitive to priming stimuli compared to cellular expression of αm (CD11b). Neutrophils and monocytes were not characterized by a primed phenotype in these mild patients (Fig. 1) as was seen before[16-18,26].

Patients with phenotype 2 had no eosinophil priming for any of the priming markers, but neutrophil and monocyte MoPhab A17 and MoPhab A27 priming were enhanced compared to these cells of healthy controls. These priming responses of neutrophils and monocytes were accompanied by elevated expression levels of the cell-activation marker αm (CD11b). Remarkably, also for severe asthma patients who received corticosteroids, enhanced αm (CD11b) expression without prior stimulation has been shown[18], which is in agreement with data that suggests that neutrophils participate in the chronic inflammation of severe asthmatics[10,11,13,15]. In addition, we have shown in our previous work (Chapter 2) that αm (CD11b) expression levels on granulocytes were associated with bronchial hyperresponsiveness, which is a characteristic of the chronic inflammation in the

airways of asthmatics.

We next studied the responsiveness of leukocytes to a high concentration of the innate immune stimulus fMLP (10^{-6}M) to investigate the priming responses in more detail. For eosinophils no consistent data were acquired among the three priming markers between the asthmatics and controls after activation, while data of neutrophils and monocytes showed hardly any differences of these priming markers on leukocytes between controls and asthmatics with both phenotypes. Therefore, the data presented in this study clearly indicate that leukocyte priming is already (and even more consistent) present among both asthma phenotypes without prior activation of the cells.

Nothing is known whether the phenotype of difficult-to-treat asthmatics was present before hand (disturbed phenotype) or was induced by treatment with high doses of steroids (adaptation phenotype). However, the latter possibility is less likely because we did not find inhibition of eosinophil priming nor any priming effect on neutrophils and monocytes in stable asthma patients that were followed for one year during long-term treatment with the steroid flixotide (500 µg bid; L. Koenderman; unpublished results, data not shown). In addition, in other studies no differences in αm (CD11b) expression levels on leukocytes were found between mild(-to-moderate) asthma patients with or without treatment and healthy controls[16-18]. Further research is also needed to determine whether the basal increased priming responses of neutrophils and monocytes in patients with phenotype 2 reflect increased potencies of these cells to migrate and extravasate to the lung as has been strongly suggested for primed eosinophils in asthmatics[3,16].

We conclude from this study that investigation of priming markers on peripheral blood leukocytes can be used to characterise the phenotype of an asthmatic, whereby the need for invasive, expensive and tedious techniques such as bronchoscopies, bronchoalveolar lavage or sputum induction[29], is circumvented.

References

1. Fabbri LM, Stoloff S: Is mild asthma really 'mild'? Int.J.Clin.Pract. 59: 692-703, 2005
2. National Heart, Lung, and Blood Institute. Global strategy for asthma management and prevention NHLBI/WHO Workshop Report. 1995. National Institutes of Health. Updated April, 2002. National Institutes of Health Bethesda, MD. Publication No. 95-3659.
3. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L: Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in

response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005
4. Thomas LH, Warner JA: The eosinophil and its role in asthma. Gen.Pharmacol. 27: 593-597, 1996
5. Kay AB, Phipps S, Robinson DS: A role for eosinophils in airway remodelling in asthma. Trends Immunol. 25: 477-482, 2004
6. Gleich GJ: Mechanisms of eosinophil-associated inflammation. J.Allergy Clin.Immunol. 105: 651-663, 2000
7. Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, De Kruif J, Logtenberg T: Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. J.Leukoc.Biol. 68: 58-64, 2000
8. Koenderman L, Van der Bruggen T, Schweizer RC, Warringa RA, Coffer P, Caldenhoven E, Lammers JW, Raaijmakers JA: Eosinophil priming by cytokines: from cellular signal to in vivo modulation. Eur.Respir.J.Suppl 22: 119s-125s, 1996
9. Coffer PJ, Koenderman L: Granulocyte signal transduction and priming: cause without effect? Immunol.Lett. 57: 27-31, 1997
10. Wenzel SE, Balzar S, Cundall M, Chu HW: Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J.Allergy Clin.Immunol. 111: 1345-1352, 2003
11. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ: Neutrophilic inflammation in severe persistent asthma. Am.J.Respir.Crit Care Med. 160: 1532-1539, 1999
12. Douwes J, Gibson P, Pekkanen J, Pearce N: Non-eosinophilic asthma: importance and possible mechanisms. Thorax 57: 643-648, 2002
13. 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. 22: 470-477, 2003
14. Gibson PG, Simpson JL, Saltos N: Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119: 1329-1336, 2001
15. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ: Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am.J.Respir.Crit Care Med. 156: 737-743, 1997
16. In ‘t Veen JC, Grootendorst DC, Bel EH, Smits HH, Van Der Keur M, Sterk PJ, Hiemstra PS: CD11b and L-selectin expression on eosinophils and neutrophils in blood and induced sputum of patients with asthma compared with normal subjects. Clin.Exp.Allergy 28: 606-615, 1998
17. Maestrelli P, De Fina O, Bertin T, Papiris S, Ruggieri MP, Saetta M, Mapp CE, Fabbri LM: Integrin expression on neutrophils and mononuclear cells in blood and induced sputum in stable asthma. Allergy 54: 1303-1308, 1999
18. Mann BS, Chung KF: Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. Respir.Res. 7: 59, 2006
19. Tomita K, Tanigawa T, Yajima H, Fukutani K, Matsumoto Y, Tanaka Y, Sasaki T: Identification and characterization of monocyte subpopulations from patients with bronchial asthma. J.Allergy Clin.Immunol. 96: 230-238, 1995
20. Hallsworth MP, Soh CP, Lane SJ, Arm JP, Lee TH: Selective enhancement of GM-CSF, TNF-alpha, IL-1 beta and IL-8 production by monocytes and macrophages of asthmatic subjects. Eur.Respir.J. 7: 1096-1102, 1994
21. Gibson PG, Powell H: Initial corticosteroid therapy for asthma. Curr.Opin.Pulm.Med. 12: 48-53, 2006
22. Powell H, Gibson PG: High dose versus low dose inhaled corticosteroid as initial starting dose for asthma in adults and children. Cochrane.Database.Syst.Rev. CD004109, 2004
23. Cockcroft DW, Murdock KY, Kirby J, Hargreave F: Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. Am.Rev.Respir.Dis. 135: 264-267, 1987
24. Barnes PJ, Woolcock AJ: Difficult asthma. Eur.Respir.J. 12: 1209-1218, 1998
25. Barnes PJ: Corticosteroid resistance in airway disease. Proc.Am.Thorac.Soc. 1: 264-268, 2004
26. Berends C, Hoekstra MO, Dijkhuizen B, e Monchy JG, Gerritsen J, Kauffman HF: Expression of CD35 (CR1) and CD11b (CR3) on circulating neutrophils and eosinophils from allergic asthmatic children. Clin.Exp.Allergy 23: 926-933, 1993

27. Thorne KJ, Richardson BA, Mazza G, Butterworth AE: A new method for measuring eosinophil activating factors, based on the increased expression of CR3 alpha chain (CD11b) on the surface of activated eosinophils. J.Immunol.Methods 133: 47-54, 1990
28. Neeley SP, Hamann KJ, White SR, Baranowski SL, Burch RA, Leff AR: Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. Am.J.Respir.Cell Mol.Biol. 8: 633-639, 1993
29. Jayaram L, Parameswaran K, Sears MR, Hargreave FE: Induced sputum cell counts: their usefulness in clinical practice. Eur.Respir.J. 16: 150-158, 2000

Chapter 4

Glucocorticoid treatment inhibits long-term activation of eosinophils by IL-5 without affecting immediate signals

Distinct effects of glucocorticoids on eosinophil functionality

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Submitted

Abstract

Background: Eosinophils and Th2-derived cytokines, such as IL-5, play an important role in chronic inflammatory responses in allergic asthmatics. Treatment of allergic asthmatics with glucocorticoids reduces symptoms and decreases eosinophil recruitment to inflammatory sites.

Objective: To investigate the effect of dexamethasone (Dex) on eosinophil migratory and adhesive responses *in vitro*.

Methods: Eosinophils were cultured for 16h in the presence or absence of Dex and/or IL-5. The next day, the migratory responses of eosinophils were investigated using a modified Boyden chamber assay. Also, the effects on adhesion, expression of αmβ2 (CD11b/CD18) and IL-5Rα (CD125), and viability were measured by flowcytometry utilizing fluorescent beads coated with Fc-ICAM-1, directly labeled antibodies and Annexin-V/propidium iodide, respectively.

Results: Overnight incubation with Dex dose-dependently inhibited long-term (16h) IL-5 priming of eosinophil chemokinesis and β2-integrin mediated adhesion. In contrast, overnight incubation with Dex in the absence of IL-5 had no effect on eosinophil migration and adhesion responses induced by either cytokines IL-5 and GM-CSF or chemoattractants C5a and PAF. The different effects of Dex on eosinophil migration and adhesion in the presence of IL-5 priming compared with the absence of IL-5 priming could not be attributed to changes in cellular surface expression of integrin αmβ2 (CD11b/CD18) or the IL-5-receptor. The expression of αm (CD11b) was significantly upregulated by long-term IL-5 treatment, whereas the expression of the IL-5 receptor was significantly downregulated under these conditions. In addition, we show that Dex inhibition on long-term IL-5 treatment did not affect intrinsic functionality of αmβ2, since this integrin could still be activated by Mn^{2+} (0.5mM). Control experiments revealed that viability/apoptosis of eosinophils was not affected by Dex.

Conclusion: These data indicate that Dex can inhibit inside-out control of integrin-mediated adhesion functions only in long-term cytokine-primed eosinophils and imply that steroids act primarily on pre-activated eosinophils.

Introduction

Many reports have emphasized the important role of eosinophilic granulocytes in allergic diseases. For instance, eosinophils and their toxic granule products are found in the inflamed bronchial tissue in allergic asthma[1,2]. As eosinophils belong to the most cytotoxic cells in the human body, immune activation of these cells *in vivo* is tightly controlled via interactions with inflammatory cytokines and chemoattractants. By these interactions eosinophils can adapt a primed or pre-activated state, which results in enhanced responsiveness to distinct inflammatory stimuli[3]. Indeed, in several disorders associated with eosinophilia such as allergic asthma and the hypereosinophilic syndrome, evidence has been provided for increased serum levels of eosinophil priming cytokines such as interleukin (IL)-5 and granulocyte macrophage-colony stimulating factor (GM-CSF)[4-6]. In addition, eosinophils derived from peripheral blood of allergic asthmatics are characterized by enhanced migratory responses towards platelet-activating factor (PAF) and other chemoattractants *in vitro*, indicating the existence of primed eosinophils *in vivo*[7,8]. Furthermore, eosinophils derived from normal donors can be primed *in vitro* with picomolar concentrations of cytokines GM-CSF, IL-3, or IL-5. These *in vitro* responses reflect the *in vivo* primed phenotype[7].

In allergic asthma inhaled corticosteroids (ICS) treatment is known to be effective, because it reduces bronchial hyperresponsiveness and other asthma symptoms[9,10]. This treatment also causes a decrease in circulating eosinophils and a reduction in eosinophils infiltrating the inflamed tissue. This leads to decreased numbers of eosinophils in bronchoalveolar lavage (BAL) fluid and sputum[11-13]. ICS treatment additionally leads to decreased levels of eosinophilic cationic protein (ECP) in BAL-fluid, which indicates that corticosteroids can decrease the activity of eosinophils *in vivo*[12].

In this report we investigated the effect of glucocorticoid dexamethasone (Dex) on eosinophil responses involved in cellular recruitment. It was shown that Dex affects eosinophil adhesion functionality after prolonged incubation (16h) provided that the cells were co-cultured with cytokines such as IL-5. These conditions did not affect apoptosis and integrin functionality *per se*. Our data suggest that the capacity of Dex to modulate eosinophil responses requires priming by cytokines. Therefore, priming responses potentiate both activating signals for various functional responses as well as negative signals mediated by e.g. anti-inflammatory corticosteroids.

Materials and methods

Reagents and cytokines

PAF (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphoryl-choline), C5a, propidium iodide, Triton X-100, RU 486, and Dex were purchased from Sigma (St. Louis, MO). Recombinant human GM-CSF was from Genzyme (Boston, MA), and TNFα from Boehringer Mannheim (Mannheim, Germany). Recombinant human IL-5 was a gift from Dr I. Uings (GlaxoWellcome, Stevenage, UK). Ficoll-paque was obtained from Pharmacia (Uppsala, Sweden). Human serum albumin (HSA) was from Sanquin (Amsterdam, the Netherlands). RNase A was from Boehringer Mannheim (Mannheim, Germany). Annexin-V-FITC was obtained from Kordia Life Sciences (Leiden, The Netherlands). All other materials were reagent grade. Experiments were carried out in incubation buffer, containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES, 5 mM glucose, and 1.0 % HSA (wt/vol).

Antibodies

Directly labeled CD11b-PE and CD18-FITC were from Dako (Glostrup, Denmark) and directly labeled IL-5Rα-PE was from Becton Dickinson (San Jose, CA). Functionally blocking monoclonal antibody (MoAb) IB4 (anti β2-integrin) and control MoAb W6/32 (anti-HLA-A, HLA-B, and HLA-C) were isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (ATCC; Rockville, MD).

Cell isolation

Granulocytes were isolated from 100 ml whole blood of healthy donors anti-coagulated with trisodium citrate (0.4% (w/v) (pH 7.4). Blood was diluted 2.5:1 with PBS containing trisodium citrate (0.4% (w/v) (pH 7.4) and human pasteurized plasma-protein solution (4 g/L). Granulocytes and erythrocytes were isolated by centrifugation over Ficoll-Pague. Erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4˚C. After isolation, granulocytes were resuspended in PBS containing trisodium citrate (0.4% (w/v) (pH 7.4) and human pasteurized plasma-protein solution (4 g/L).

Eosinophils were isolated from the granulocyte fraction by negative selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec, Auburn, CA)[14]. In addition, anti-CD3- and anti-CD14-conjugated microbeads (MACS; Miltenyi Biotec,

Auburn, CA) were added to the granulocyte suspension to minimize mononuclear cell contamination. Purity of eosinophils was >95%.

Culture conditions

Isolated cells were cultured overnight (16h) in HEPES buffered RPMI-1640 medium supplemented with L-glutamine (Life Technologies; Breda, The Netherlands), 10% fetal calf serum (FCS), 1 mg/ml penicillin, and 1 mg/ml streptomycin in a CO_2 incubator (5% CO_2, 37 °C). Stimuli (IL-5, Dex or a combination) were added at final concentrations as described in the figure legends. When RU 486 was used, eosinophils were pretreated for 2h with RU 486 before addition of Dex and/or IL-5. The next day, cultured cells were resuspended in incubation buffer and experiments were performed in this buffer. Viability was measured via trypan blue exclusion and varied between 80-85% for eosinophils cultured without IL-5 (no difference between Dex-treated and untreated eosinophils) and between 85-90% for eosinophils cultured in the presence of IL-5. Viability was also measured with propidium iodide staining and flowcytometric analyses (see below), however no differences were found compared to the trypan blue assay.

Apoptosis assay

Apoptosis was measured using a flowcytometric assay as described before[15-17]. In short: eosinophils (10^7/ml) resuspended in incubation buffer were fixed in 70% ethanol (final concentration) at -20°C for at least 2h. The cells were then centrifuged and resuspended in buffer (pH 7.8) containing 45 mM Na_2HPO_4, 2.5 mM citric acid, and 0.1% Triton X-100. After incubation for 20 min at 37°C, 0.2 ml of the cell suspension was added to 1.5 ml staining buffer (pH 6.8) containing 10 mM PIPES, 0.1 M NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 5 units/ml RNase A, and 20 μg/ml propidium iodide. After incubation in staining buffer for 30 min at room temperature, the cells were analyzed in a FACSvantage flowcytometer (Becton Dickinson, San Jose, CA). The percentage of apoptotic cells was analyzed by FACS as the percentage of cells with a DNA content of <2N.

Eosinophil apoptosis was also determined by the binding of Annexin-V. Eosinophils were resuspended in incubation buffer after overnight culture and subsequently labeled with Annexin-V-FITC. This labeling occurred according to the instructions of the manufacturer (Kordia Life Sciences, Leiden, The Netherlands). The percentage of apoptotic cells was determined as the percentage of annexin-V positive cells using flowcytometry (FACScalibur; Becton Dickinson, San Jose, CA).

Migration assay

Eosinophil migration was measured using a modification of the method according to Boyden[18] using a 48-well microchemotaxis chamber (Neuroprobe; Cabin John, MD). Chemokinetic or chemotactic stimuli (cytokines or chemoattractants) or incubation buffer (30 μl) were added to the lower compartments. Two filters were placed between lower and upper compartments. The lower filter had a pore width of 0.45 μm (Millipore; Bedford, MA, type HA) and the upper filter (nitrocellulose) had a pore width of 8 μm (thickness 150 μm; Sartorius, Göttingen, Germany; type SM 113). Before use, the filters were soaked in incubation buffer. Overnight cultured eosinophils were placed in the upper compartments (25 μl of $2x10^6$ cells/ml) and the chambers were incubated for 2.5h at 37°C. The upper filters were removed, fixed in butanol/ethanol (20/80%, v/v) for 10 min and stained with Weigert solution (composition: 1% (v/v) haematoxylin in ethanol mixed with a 70 mM acidic $FeCl_3$ solution at a 1:1 ratio). The filters were dehydrated with ethanol, made transparent with xylene and fixed. All migratory responses of eosinophils were quantified with an image analysis system (Quantimet 570C, Leica Cambridge ltd.) using Quantimet 570 Control Software (QUIC, version 2.02) and a customly made software program. An automated microscope, Leitz DMRXE (Leica; Weitzlar, Germany) was used to step through the filters in the Z direction with 17 intervals of 10 μm. Eosinophils were counted at each level and the total migration at each level was calculated. The results are expressed as migratory index (μm/cell), which is calculated by the cumulative migration of all intervals (μm) divided by the total number of cells. In this index the cells at level 0 μm were not included in the calculation. The mean of four randomly chosen points on each filter was calculated.

Fluorescent bead adhesion assay

The fluorescent bead adhesion assay was performed as described previously[19,20]. Fluorescent beads (TransFluorSpheres, 488/645 nm, 1.0 μm; Molecular Probes) were coated with ICAM-1 Fc fusion protein that was derived from CHO-ICAM-1 Fc producing cells (kindly provided by Prof. Y. van Kooyk, VU University Medical Center Amsterdam, The Netherlands; These cells contained an expression vector that was kindly provided by Prof. C.D. Buckley, University of Birmingham, UK). In short, 20 μl streptavidin (5 mg/ml in 50 mM MES (2-(N-morpholino)ethanesulfonate) buffer) was added to 50 μl TransFluorSpheres, mixed with 30 μl 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC; 1.33 mg/mL),

and incubated at room temperature (RT) for 2h. The reaction was stopped by addition of glycine to a final concentration of 100 mM. Streptavidin-coated beads were washed 3 times with PBS and resuspended in 150 µl PBS, 0.5% bovine serum albumin (BSA) (wt/vol). Then, streptavidin-coated beads (15 µl) were incubated with biotinylated goat anti-human anti-Fc Fab2 fragments (6 µg/ml) in 0.3 ml PBS, 0.5% BSA for 2h at 37°C. The beads were washed once with PBS, 0.5% BSA and incubated with ICAM-1 Fc supernatant overnight at 4°C. The ligand-coated beads were washed; resuspended in 100 µl PBS, 0.5% BSA, 0.01% sodium azide; and stored at 4°C. For the fluorescent bead adhesion assay, eosinophils were resuspended in incubation buffer ($5x10^4$) under identical (stimuli-) conditions as those used during overnight culture. Cells were preincubated with or without control anti-HLA-ABC, MoAb W6/32 (10 µg/ml; ATCC hybridoma, Rockville, MD) or anti-β2-integrin-blocking MoAb IB4 (10 µg/ml; ATCC hybridoma, Rockville, MD) for 10 min at 37°C. The ligand-coated beads (40 beads/cell) and stimuli were added in a 96-well V-shaped-bottom plate. Next, the eosinophils were added and incubated for 15 min at 37°C. The cells were subsequently washed and resuspended in incubation buffer (4°C) and kept on ice until analysis. Binding of the fluorescent beads to the eosinophils was determined by flowcytometry using a FACScalibur flowcytometer (Becton Dickinson, Mountain View, CA). Binding is depicted as the percentage of eosinophils binding to ICAM-1-coated beads.

Immunofluorescent labelling of eosinophils with CD11b-PE, CD18-FITC and IL-5Rα-PE

The expressions of the surface markers αm (CD11b), β2 (CD18) and IL-5R (IL-5Rα) were determined after overnight culture under several conditions as indicated in the experiments. After 16h, the cultured eosinophils were resuspended in incubation buffer and put at 4°C. Eosinophils were stained for 30 min at 4°C with 1/100 (vol/vol) CD11b-PE for αm and 1/100 (vol/vol) CD18-FITC for β2 or 1/50 (vol/vol) IL-5Rα-PE for Il-5Rα. Hereafter, the cells were washed with cold incubation buffer and analyzed using a FACScalibur flowcytometer (Becton Dickinson, San Jose, CA). Data are reported as median channel fluorescence (MCF) in arbitrary units (AU).

Statistical analysis

Results are expressed as means ± SE or SEM. Statistical evaluation of the data was performed using the Student's paired or unpaired t-tests; In migration assays, dose-

response curves were evaluated with Analysis of Variance followed by a Newman-Keuls test. P values <0.05 were considered to be significant. All statistical tests were performed using SPSS vs. 11.0 (Chicago, Ill).

Results

Glucocorticoids inhibit several eosinophil effector functions *in vivo* and *in vitro*[11-13]. In this study the effect of Dex on eosinophil migration and adhesion was studied. The effect of Dex on eosinophil random migration or chemokinesis induced by long-term (overnight) incubation with IL-5 was studied using a Boyden chamber assay[18]. In these experiments, freshly isolated eosinophils were incubated in medium for 16h without or with several concentrations of IL-5 (10^{-13}-10^{-10}M) in the absence or presence of Dex (10^{-6}M) or these cells were cultured without or with different concentrations of Dex (10^{-9}-10^{-6}M) in the absence or presence IL-5 (10^{-10}M). The next day, the cells were washed, resuspended in incubation buffer, and random migration (towards incubation buffer) was measured. As is shown in Fig. 1A, long-term incubation with IL-5 dose-dependently stimulated random eosinophil migration. However, in the presence of Dex (10^{-6}M), chemokinesis was significantly inhibited towards baseline levels for all concentrations of IL-5. Dex induced a dose-dependent inhibition of IL-5-mediated random eosinophil migration (Fig. 1B), with an optimal

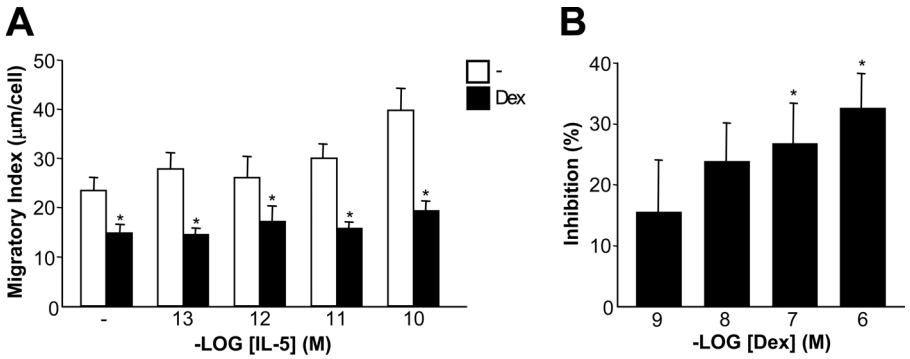

Figure 1. Effect of dexamethasone on eosinophil chemokinesis (random migration). (**A**) Human peripheral blood eosinophils were cultured (10^6 cells/ml) for 16h without or with several concentrations of IL-5 (10^{-13}-10^{-10}M) in the absence (white bars) or presence (black bars) of Dex (10^{-6}M). (**B**) Human peripheral blood eosinophils were cultured (10^6 cells/ml) for 16h in the presence of IL-5 (10^{-10}M) without or with several concentrations of Dex (10^{-9}-10^{-6}M). The next day, migratory responses were measured in a modified Boyden chamber assay towards incubation buffer (**A, B**). Results are expressed as mean migratory index ±SE (µm/cell; n=6) (**A**) or as mean percentage inhibition ±SE of control values (n=6) (**B**).*P<0.05 difference from control values.

inhibitory concentration of 10^{-6}M.

To study whether the effects of Dex on chemokinesis were steroid receptor mediated, we performed similar experiments in the presence of the glucocorticoid receptor (GCR) antagonist RU 486, which has a three times higher affinity for the GCR than Dex[21]. Eosinophils were pre-incubated with RU 486 (10^{-5}M) for 2h at 37°C before culturing the cells overnight in the presence of Dex (10^{-6}M) and/or IL-5 (10^{-10}M). The next day, cultured cells were resuspended in incubation buffer and migration experiments were performed. Mean eosinophil migratory indexes ±SEM (μm/cell; n=5) were: 39.0±3.2 for RU 486 with IL-5, and 19.1±2.8 for Dex with IL-5 and 34.7±2.8 for RU 486 with Dex and IL-5. RU 486 significantly prevented the inhibition of Dex on eosinophil chemokinesis, when eosinophils were co-cultured with IL-5 ($P<0.05$). In contrast, addition of RU 486 and RU 486 with Dex had no effect on eosinophil migratory responsiveness towards buffer and were 23.3±3.3 and 22.0±2.7, respectively.

Next, the effect of Dex (10^{-6}M; 16hr) on eosinophil migration induced by several stimuli was measured. Therefore, migratory responses of long-term (16h) Dex-treated or buffer-treated eosinophils were studied in the presence of optimal concentrations of cytokines IL-5 (10^{-9}M) and GM-CSF (10^{-9}M) and chemoattractants PAF (10^{-6}M) and C5a (10^{-8}M). As is shown in Fig. 2, eosinophil migration induced by these compounds (corrected for altered background migration) was not significantly influenced by overnight exposure to Dex. These data suggest that Dex inhibits eosinophil migratory responses particularly under conditions of long-term priming by cytokines.

As β2-integrins are thought to be important for migration responses of human

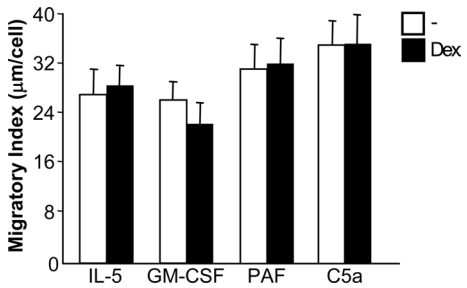

Figure 2. Effect of dexamethasone on cytokine- and chemoattractant-induced eosinophil migration. Human peripheral blood eosinophils were cultured (10^6 cells/ml) for 16h in the absence (white bars) or presence (black bars) of Dex (10^{-6}M). Migratory responses induced either by cytokines IL-5 (10^{-9}M) and GM-CSF (10^{-9}M) or by chemoattractants PAF (10^{-6}M) and C5a (10^{-8}M) were measured in a modified Boyden chamber assay. Results are expressed as mean migratory index ±SE (μm/cell), corrected for their background migration (n=7).

eosinophils, we studied the effect of Dex on the functionality of β2-integrins by measuring the binding of these cells to the αmβ2 ligand ICAM-1. Freshly isolated eosinophils were incubated overnight for 16h in the presence of IL-5 (10^{-10}M) and different concentrations of Dex (10^{-10}-10^{-6}M). As is shown in Fig. 3A, overnight incubation with Dex induced a dose-dependent inhibition of IL-5-mediated binding to ICAM-1 coated beads. This binding was β2-integrin dependent as a blocking antibody against β2-integrins (MoAb clone IB4, 10 µg/ml; data not shown), but not a control antibody (W6/32, 10 µg/ml; data not shown), abrogated the binding of eosinophils to ICAM-1-coated beads. Fig. 3A also shows that the effect of Dex on ICAM-1 binding is steroid receptor mediated, as RU 486 (10^{-5}M) significantly, although not completely, reduced the inhibition seen with Dex (10^{-6}M) in combination with IL-5 (10^{-10}M). Overnight incubation of eosinophils with Dex 10^{-10}M, Dex 10^{-8}M, Dex 10^{-6}M, Dex 10^{-6}M with Ru 486 10^{-5}M or Ru 486 10^{-5}M did not affect background binding (Fig. 3A; white bar) of ICAM-1 coated beads to these cells. The percentage eosinophils bound to beads ±SEM of these conditions were 8.2±4.5, 8.1±5.7, 6.4±4.4, 7.7±2.7 and 7.9±5.6, respectively.

In contrast to the data shown in Fig. 3A, overnight incubation of eosinophils with Dex (10^{-6}M) in the absence of a cytokine did not affect the adhesive responsiveness of eosinophils towards short-term activation of these cells with IL-5 (10^{-10}M; Fig. 3B). This is in agreement with the lack of effect of overnight incubation with Dex in the absence of cytokines on eosinophil chemokinesis (Fig. 2).

Next, the question was addressed whether the inhibitory effect of Dex in the presence of IL-5 was due to blocking of the intrinsic integrin receptor activity (i.e. the receptor is non-functional) or is mediated via inhibitory effects of Dex on the IL-5 induced inside-out regulation towards the functionality of these integrin receptors. In Fig. 3C it is shown that the inhibitory effect of Dex is not mediated via a direct effect on integrin receptor activity as the divalent cation Mn^{2+} could restore ICAM-1-binding.

To exclude the possibility that the inhibitory effect of Dex on the IL-5-induced integrin activation and chemokinesis was mediated either via reduction of expression of αmβ2 (CD11b/CD18) or IL-5 receptor-α (IL-5Rα/CD125) on the cell membrane, we investigated the cellular expression levels of αm (CD11b), β2 (CD18) and IL-5Rα (CD125) after overnight incubation without or with Dex in the presence and absence of IL-5 (10^{-10}M). As shown in Fig. 4, overnight incubation with Dex did not decrease the integrin expression in the presence of IL-5 either measured via staining of the cells with CD11b (Fig. 4A) or CD18 antibodies (Fig.

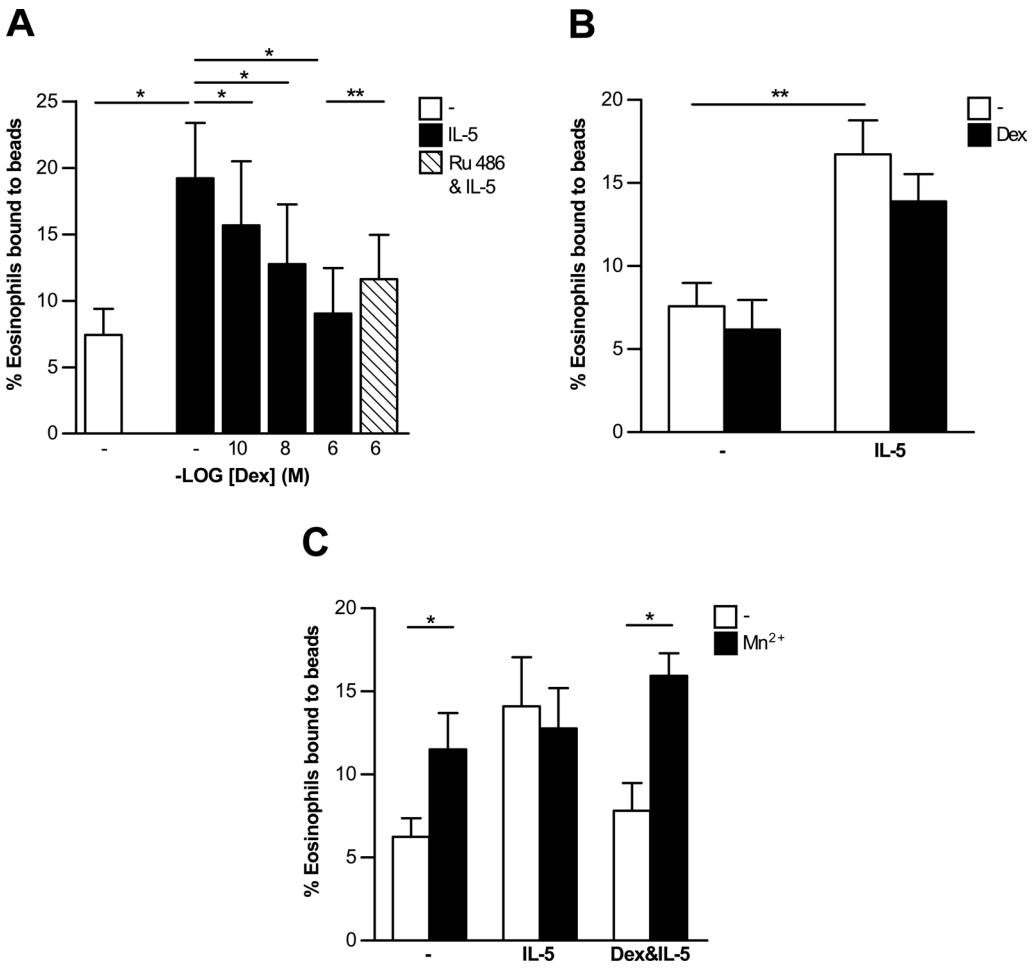

Figure 3. The effect of dexamethasone on long-term IL-5 induced binding of ICAM-1 coated beads to eosinophils. (**A**) Eosinophils were cultured (10^6 cells/ml) for 16h in the absence of stimuli (white bar) or in the presence of IL-5 (10^{-10}M) and different concentrations of Dex (10^{-10}-10^{-6}M; black bars) and/or RU 486 (10^{-5}M; hatched bar). The next day, the cells were added to wells containing ICAM-1-coated beads under identical (stimuli-)conditions as in the overnight culture. (**B**) Eosinophils were cultured (10^6 cells/ml) for 16h in the absence (white bars) or presence (black bars) of Dex (10^{-6}M). The next day, cultured cells were added to wells containing ICAM-1-coated beads and buffer or IL-5 (10^{-10}M). (**C**) Eosinophils were cultured (10^6 cells/ml) for 16h in the absence or presence of IL-5 (10^{-10}M) without or with Dex (10^{-6}M). The next day, the cells were added to wells containing ICAM-1-coated beads under identical (stimuli-)conditions as in the overnight culture (white bars). Also Mn^{2+} (0.5 mM) was added to wells containing these stimuli (black bars). For all conditions (**A-C**): after incubation (30 min at 37°C) the percentage of cells that bound ICAM-1 coated beads was determined by flowcytometry. Mean values of cells that were positive for beads are presented ± SEM. For all conditions at least three independent experiments were performed. A paired Student's t test ((**A**) and (**B**)) or unpaired t test (**C**) was used to perform statistical analysis (* P<0.05 and ** P<0.005).

4B). IL-5 caused a significant induction of CD11b expression after prolonged culture of eosinophils (Fig. 4A), which has also been shown by others before[22]. In contrast, IL-5 significantly reduced the level of its own receptor on eosinophils as compared to untreated cells (Fig. 5)[23]. However, these effects were not modulated by co-incubation with Dex. These data indicate that Dex acts on the IL-5-induced

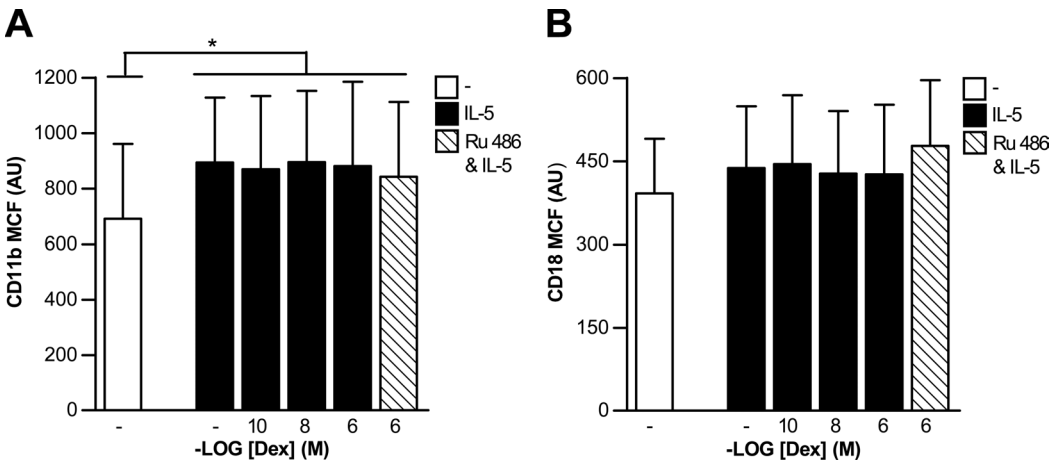

Figure 4. Influence of dexamethasone on integrin expression levels. Human peripheral blood eosinophils were cultured (10^6 cells/ml) for 16h in the presence of buffer (white bars), IL-5 (10^{-10}M) without or with several concentrations Dex (10^{-10}-10^{-6}M; black bars) or IL-5 (10^{-10}M) with Dex (10^{-6}M) and RU 486 (10^{-5}M; hatched bar). Hereafter, cells were washed with cold incubation buffer and incubated for 30 min on ice with directly labeled CD11b-PE (**A**) and CD18-FITC (**B**). Subsequently, cells were washed with cold incubation buffer and analysed by flowcytometry. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU). Mean values are presented ± SEM (n=3). A paired Student's t test was used to perform statistical analysis (* P< at least 0.05).

intracellular signalling in eosinophils without influencing cellular levels of involved receptors in priming of cellular adhesion and migration.

Previous studies have suggested that corticosteroids (including Dex) can

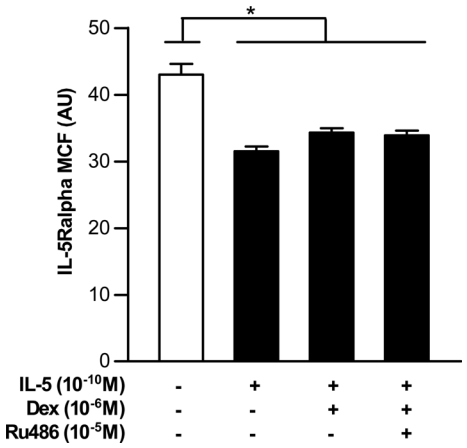

Figure 5. Influence of dexamethasone on IL-5 receptor-α expression level. Human peripheral blood eosinophils were cultured (10^6 cells/ml) for 16h in the presence of buffer, IL-5 (10^{-10}M), IL-5 (10^{-10}M) with Dex (10^{-6}M) or IL-5 (10^{-10}M) with Dex (10^{-6}M) and RU 486 (10^{-5}M). Hereafter, cells were washed with cold incubation buffer and incubated for 30 min on ice with directly labeled IL-5Rα-PE. Subsequently, cells were washed with cold incubation buffer and analysed by flowcytometry. Data are depicted as median channel fluorescence (MCF) and given in arbitrary units (AU). Mean values are presented ± SEM (n=3). A paired Student's t test was used to perform statistical analysis (* P < at least 0.05).

induce apoptosis in eosinophils[24-28]. As apoptosis might influence directly the observed different functional responses of eosinophils, we evaluated whether Dex indeed induced apoptosis under our assay conditions. Eosinophil apoptosis was measured after incubation for 16h in the presence or absence of Dex, using a flowcytometric assay consisting of the smaller than 2N DNA method (Table 1)[15-17]. Table 1 shows that incubation for 16h with different concentrations of Dex (10^{-9}-10^{-6}M) did not induce additional apoptosis in human eosinophils compared to untreated control cells. It also shows that in the presence of IL-5 (10^{-10}M), the percentage of apoptotic cells was decreased but that Dex had no effect on this IL-5-induced survival. In addition to the data presented in Table 1, apoptosis measured via Annexin-V staining also did not show differences between different concentrations of Dex (10^{-9}-10^{-6}M) after 16h in the presence and absence of IL-5 (data not shown).

Discussion

Cytokines produced by Th2 lymphocytes, such as IL-4, IL-5, and GM-CSF are thought to play an important role in allergic diseases[29]. IL-4 induces immunoglobulin isotype switching of B cells to IgE production, whereas IL-5 and GM-CSF are involved in eosinophil differentiation, recruitment, priming and activation[30-32]. Glucocorticoids (GCs) are an important treatment modality for allergic asthma by reducing the inflammatory processes. This modulation of the inflammatory response is induced by other relevant cellular systems[11,33-35]. For instance, GCs are known to

Table 1. Effect of dexamethasone on eosinophil apoptosis[#].

Treatment	n	Apoptotic cells (%)
Buffer	8	15.5 ± 2.5
Dex 10^{-9}M	4	11.0 ± 2.3
Dex 10^{-8}M	4	11.8 ± 2.6
Dex 10^{-7}M	6	15.3 ± 2.2
Dex 10^{-6}M	8	12.9 ± 1.8
IL-5 10^{-10}M	6	6.2 ± 1.2
IL-5 10^{-10}M / Dex 10^{-6}M	6	6.3 ± 0.8

[#] Results are expressed as percent apoptotic cells (mean ± SE of n different experiments).

inhibit the release and production of several proinflammatory and priming cytokines, such as TNFα, IL-1, IL-2, IL-4, IL-6, and the chemokine RANTES, which subsequently leads to a redirection of the allergic inflammatory response[34-39].

In this study we investigated the direct *in vitro* effects of Dex on eosinophil migratory responses and the activation status of the αmβ2-integrin under conditions of IL-5-induced eosinophil priming. In addition, the effects of steroids on the membrane expressions of αmβ2 and IL-5Rα were studied to better define the modulatory mechanism of steroids on eosinophil functionality under priming conditions.

Recent work from our group shows that rapid effects (30 min) of Dex on eosinophils specifically affect the ability of FcαRI (CD89) to bind IgA-coated targets, while adhesion and migration responses remain unaltered (see Chapter 5)[40]. In the work presented here, we show that Dex can interfere with adhesion and migration of human eosinophils after long-term (16hr) incubation. Our data show that these long-term effects of steroids on adhesion-associated processes are evident only in the presence of a cytokine such as IL-5. Hence random eosinophil migration and β2-integrin activity were only significantly inhibited by long-term incubation with Dex when co-cultured with IL-5. In the absence of the cytokine, long-term Dex incubation had no significant inhibitory effect on β2-integrin activity and eosinophil migration induced by optimal concentrations of cytokine IL-5. Similar results were found in migration studies with optimal concentrations of the chemoattractants C5a and PAF or cytokine GM-CSF. These data indicate that steroids can particularly inhibit the locomotion and adhesion of primed cells.

It is tempting to speculate that eosinophils under homeostatic conditions (i.e. in the absence of pro-inflammatory cytokines) are not very sensitive for GCs. The situation changes when pro-inflammatory cytokines interact with the cells and adhesion functions become primed. Under this situation of immunological “stress” the cells become more susceptible for endogenous modulatory signals such as GCs. In agreement with our hypothesis, several studies have provided evidence for elevated levels of Th2-derived cytokines that can prime eosinophils, such as IL-5 and GM-CSF, in peripheral blood and lungs of allergic asthmatics[4,6,41,42]. Also mRNA for IL-5 has been detected in mucosal bronchial biopsies from asthmatics[43,44]. In addition, previous data strongly suggest the inhibition of recruitment of eosinophils by GCs treatment *in vivo*[11,45] in such environments that facilitate priming. Unfortunately, nothing is known regarding homing of eosinophils under normal homeostatic conditions.

The difference between long-term Dex incubation with and without cytokines could not be attributed to reduction in cellular surface expression of αmβ2

(CD11b/CD18) or IL-5R (CD125). In addition, we have shown that Dex inhibition on long-term IL-5 treatment did not decrease the intrinsic activity of integrin-receptors to bind ligand, as they can be activated by a direct stimulus such as Mn^{2+}. These data imply that Dex inhibits long-term IL-5 treatment responses via reduction of IL-5 primed or stimulated intracellular pathways which facilitate inside-out activation of integrins.

Currently, not much is known regarding the molecular mechanisms by which ligand bound steroid receptors inhibit eosinophil activation or priming in the presence of cytokines. Dex-mediated downmodulation of inflammatory responses in inflammatory cells seems primarily mediated via inhibition of transcription of inflammatory genes by interaction of the activated GCR with relevant transcription factors. Indeed, a lot of studies have emphasized the importance of the interaction of the activated GCR with transcription factors involved in inflammation such as AP-1 and NF-*k*B (for reviews see ref.[35,46,47]). However, from the experiments presented here, it is not clear whether Dex inhibits eosinophil functionality in the presence of IL-5 through genomic or non-genomic mechanisms[48]. In addition, further research is needed to identify other factors, beyond the GCR, in GCs-mediated reduction of eosinophil adhesion, because RU 486 could only partly inhibit Dex-mediated repression of eosinophil adhesion under primed conditions (Fig. 3A).

Liu *et al*. showed a central role for annexin-1 expression and cPLA2 translocation in steroid-induced inhibition of eosinophil adhesion[22]. In their model steroids block eosinophil adhesion by upregulation of annexin-1 on the plasma membrane, which subsequently leads to blocking of cPLA2 translocation to the nuclear membrane. However, these effects of steroids occur already in the absence of cytokines. These findings are in contrast to our findings, as we did not observe significant inhibition of steroid pre-treatment without cytokines on IL-5-induced adhesion. The reasons underlying these differences are not clear but may include the use of different steroids (Dex vs. fluticasone propionate) and the use of different techniques to study adhesion. Also the source of eosinophils can be important as eosinophils used in the study of Liu *et al.* were derived from mild atopics who might have exhibited an already cytokine-induced primed phenotype. Eosinophils used in our study were derived from donors with no atopic diseases.

A potential mechanism complicating the interpretation of our data is the possibility of Dex-induced apoptosis in our cells. Several studies have reported that steroids can induce eosinophil apoptosis[24-28]. Although the onset time of induction of eosinophil apoptosis by steroids varies between studies[22,24-27,49], we and others

showed that eosinophils can survive incubation with steroids up to 16h whether or not co-incubated with survival cytokines[22,24,27,49]. To increase the complexity concerning eosinophil apoptosis, Bloom *et al*.[50] have shown that glucocorticoid effects on eosinophil survival are dependent on the concentration of IL-5. Steroids in combination with suboptimal concentrations of IL-5 reduced cellular survival, but enhanced eosinophil survival in the presence of optimal concentrations of IL-5. Therefore, the (onset time of the) effect of steroids on eosinophil apoptosis is most likely dependent on the presence of certain environmental conditions that are not defined yet. Particular emphasis should be given to the source of eosinophils (priming status) and parameters that can influence culturing conditions of these cells, such as the duration of eosinophil incubation with steroids and the composition of serum. Nevertheless, we show that eosinophils survive overnight assay conditions in which relative high concentrations of steroids were continuously present.

In conclusion, our data suggest that an effective regulation of eosinophils by Dex is dependent on the continuous presence of pro-inflammatory cytokines such as IL-5. We hypothesize that *in vivo* GCs may inhibit long-term eosinophil priming and activating signals via acting on inside-out regulation of integrins, thereby modulating the homing potential of eosinophils during the allergic inflammatory response.

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References

1. Gleich GJ: The eosinophil and bronchial asthma: current understanding. J.Allergy Clin.Immunol. 85: 422-436, 1990
2. Durham SR, Loegering DA, Dunnette S, Gleich GJ, Kay AB: Blood eosinophils and eosinophil-derived proteins in allergic asthma. J.Allergy Clin.Immunol. 84: 931-936, 1989
3. Coffer PJ, Koenderman L: Granulocyte signal transduction and priming: cause without effect? Immunol.Lett. 57: 27-31, 1997
4. Walker C, Virchow JC, Jr., Bruijnzeel PL, Blaser K: T cell subsets and their soluble products regulate

eosinophilia in allergic and nonallergic asthma. J.Immunol. 146: 1829-1835, 1991
5. Butterfield JH, Leiferman KM, Abrams J, Silver JE, Bower J, Gonchoroff N, Gleich GJ: Elevated serum levels of interleukin-5 in patients with the syndrome of episodic angioedema and eosinophilia. Blood 79: 688-692, 1992
6. Corrigan CJ, Haczku A, Gemou-Engesaeth V, Doi S, Kikuchi Y, Takatsu K, Durham SR, Kay AB: CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy. Am.Rev.Respir.Dis. 147: 540-547, 1993
7. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L: In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 79: 1836-1841, 1992
8. Warringa RA, Koenderman L, Kok PT, Kreukniet J, Bruijnzeel PL: Modulation and induction of eosinophil chemotaxis by granulocyte-macrophage colony-stimulating factor and interleukin-3. Blood 77: 2694-2700, 1991
9. Reed CE: Aerosol glucocorticoid treatment of asthma. Adults. Am.Rev.Respir.Dis. 141: S82-S88, 1990
10. Barnes PJ: Effect of corticosteroids on airway hyperresponsiveness. Am.Rev.Respir.Dis. 141: S70-S76, 1990
11. Belvisi MG: Regulation of inflammatory cell function by corticosteroids. Proc.Am.Thorac.Soc. 1: 207-214, 2004
12. Adelroth E, Rosenhall L, Johansson SA, Linden M, Venge P: Inflammatory cells and eosinophilic activity in asthmatics investigated by bronchoalveolar lavage. The effects of antiasthmatic treatment with budesonide or terbutaline. Am.Rev.Respir.Dis. 142: 91-99, 1990
13. Baigelman W, Chodosh S, Pizzuto D, Cupples LA: Sputum and blood eosinophils during corticosteroid treatment of acute exacerbations of asthma. Am.J.Med. 75: 929-936, 1983
14. Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C: An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J.Immunol.Methods 145: 105-110, 1991
15. Hotz MA, Gong J, Traganos F, Darzynkiewicz Z: Flow cytometric detection of apoptosis: comparison of the assays of in situ DNA degradation and chromatin changes. Cytometry 15: 237-244, 1994
16. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795-808, 1992
17. Dijkers PF, Van Dijk TB, De Groot RP, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ: Regulation and function of protein kinase B and MAP kinase activation by the IL-5/GM-CSF/IL-3 receptor. Oncogene 18: 3334-3342, 1999
18. Boyden S: The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J.Exp.Med. 115: 453-466, 1962
19. Geijtenbeek TB, Van Kooyk Y, Van Vliet SJ, Renes MH, Raymakers RA, Figdor CG: High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia. Blood 94: 754-764, 1999
20. Verploegen S, Ulfman L, van Deutekom HW, van Aalst C, Honing H, Lammers JW, Koenderman L, Coffer PJ: Characterization of the role of CaMKI-like kinase (CKLiK) in human granulocyte function. Blood 106: 1076-1083, 2005
21. Gagne D, Pons M, Philibert D: RU 38486: a potent antiglucocorticoid in vitro and in vivo. J.Steroid Biochem. 23: 247-251, 1985
22. Liu J, Zhu X, Myo S, Lambertino AT, Xu C, Boetticher E, Munoz NM, Sano M, Cordoba M, Learoyd J, Meliton A, Johnson M, Leff AR: Glucocorticoid-induced surface expression of annexin 1 blocks beta2-integrin adhesion of human eosinophils to intercellular adhesion molecule 1 surrogate protein. J.Allergy Clin.Immunol. 115: 493-500, 2005
23. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H, Jarjour NN, Busse WW, Kelly EA: Decreased expression of membrane IL-5 receptor alpha on human eosinophils: II. IL-5 down-modulates its receptor via a proteinase-mediated process. J Immunol. 169: 6459-6466, 2002
24. Lamas AM, Leon OG, Schleimer RP: Glucocorticoids inhibit eosinophil responses to granulocyte-macrophage colony-stimulating factor. J.Immunol. 147: 254-259, 1991
25. Wallen N, Kita H, Weiler D, Gleich GJ: Glucocorticoids inhibit cytokine-mediated eosinophil survival. J.Immunol. 147: 3490-3495, 1991
26. Zhang X, Moilanen E, Kankaanranta H: Enhancement of human eosinophil apoptosis by fluticasone

propionate, budesonide, and beclomethasone. Eur.J.Pharmacol. 406: 325-332, 2000
27. Meagher LC, Cousin JM, Seckl JR, Haslett C: Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J.Immunol. 156: 4422-4428, 1996
28. Adachi T, Motojima S, Hirata A, Fukuda T, Kihara N, Kosaku A, Ohtake H, Makino S: Eosinophil apoptosis caused by theophylline, glucocorticoids, and macrolides after stimulation with IL-5. J.Allergy Clin.Immunol. 98: S207-S215, 1996
29. 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. 326: 298-304, 1992
30. Pene J, Rousset F, Briere F, Chretien I, Paliard X, Banchereau J, Spits H, De Vries JE: IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN-gamma. J.Immunol. 141: 1218-1224, 1988
31. Kapsenberg ML, Jansen HM, Bos JD, Wierenga EA: Role of type 1 and type 2 T helper cells in allergic diseases. Curr.Opin.Immunol. 4: 788-793, 1992
32. Lampinen M, Carlson M, Hakansson LD, Venge P: Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy 59: 793-805, 2004
33. Schleimer RP, Bochner BS: The effects of glucocorticoids on human eosinophils. J.Allergy Clin.Immunol. 94: 1202-1213, 1994
34. Schleimer RP: Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic applications in asthma. Am.Rev.Respir.Dis. 141: S59-S69, 1990
35. Umland SP, Schleimer RP, Johnston SL: Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. Pulm.Pharmacol.Ther. 15: 35-50, 2002
36. Bochner BS, Rutledge BK, Schleimer RP: Interleukin 1 production by human lung tissue. II. Inhibition by anti-inflammatory steroids. J.Immunol. 139: 2303-2307, 1987
37. Linden M, Brattsand R: Effects of a corticosteroid, budesonide, on alveolar macrophage and blood monocyte secretion of cytokines: differential sensitivity of GM-CSF, IL-1 beta, and IL-6. Pulm.Pharmacol. 7: 43-47, 1994
38. Kwon OJ, Jose PJ, Robbins RA, Schall TJ, Williams TJ, Barnes PJ: Glucocorticoid inhibition of RANTES expression in human lung epithelial cells. Am.J.Respir.Cell Mol.Biol. 12: 488-496, 1995
39. Stellato C, Beck LA, Gorgone GA, Proud D, Schall TJ, Ono SJ, Lichtenstein LM, Schleimer RP: Expression of the chemokine RANTES by a human bronchial epithelial cell line. Modulation by cytokines and glucocorticoids. J.Immunol. 155: 410-418, 1995
40. Ten Hove W, Houben LA, Raaijmakers JAM, Koenderman L, Bracke M: Rapid Selective Priming of FcαR on Eosinophils by Corticosteroids. J Immunol. 177, 2006; In Press.
41. Walker C, Bauer W, Braun RK, Menz G, Braun P, Schwarz F, Hansel TT, Villiger B: Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. Am.J.Respir.Crit Care Med. 150: 1038-1048, 1994
42. Ritz SA, Stampfli MR, Davies DE, Holgate ST, Jordana M: On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends Immunol. 23: 396-402, 2002
43. Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, Bradley B, Durham SR, Collins JV, Jeffery PK: Interleukin-5 mRNA in mucosal bronchial biopsies from asthmatic subjects. Int.Arch.Allergy Appl.Immunol. 94: 169-170, 1991
44. Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR: Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. Am.J.Respir.Cell Mol.Biol. 8: 35-42, 1993
45. Barnes PJ, Adcock IM: How do corticosteroids work in asthma? Ann.Intern.Med. 139: 359-370, 2003
46. Barnes PJ: Molecular mechanisms and cellular effects of glucocorticosteroids. Immunol.Allergy Clin.North Am. 25: 451-468, 2005
47. Jatakanon A, Lim S, Chung KF, Barnes PJ: An inhaled steroid improves markers of airway inflammation in patients with mild asthma. Eur.Respir.J. 12: 1084-1088, 1998
48. Goulding NJ: The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus.

Curr.Opin.Pharmacol. 4: 629-636, 2004

49. Myo S, Zhu X, Myou S, Meliton AY, Liu J, Boetticher E, Lambertino AT, Xu C, Munoz NM, Leff AR: Additive blockade of beta 2-integrin adhesion of eosinophils by salmeterol and fluticasone propionate. Eur.Respir.J. 23: 511-517, 2004
50. Bloom JW, Chacko J, Lohman IC, Halonen M, Martinez FD, Miesfeld RL: Differential control of eosinophil survival by glucocorticoids. Apoptosis. 9: 97-104, 2004

Chapter 5

Rapid selective priming of FcαR on eosinophils by corticosteroids

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Abstract

Pre-activation or priming of eosinophils by (pro-inflammatory) cytokines is an important process in the pathogenesis of allergic diseases. Several priming-dependent eosinophil responses, such as migration and adhesion are reduced by treatment with corticosteroids. Many inhibitory effects of corticosteroids are mediated by the glucocorticoid receptor via genomic mechanisms, which are evident only after prolonged interaction (>30 min). However, also faster actions of corticosteroids have been identified, which occur in a rapid, non-genomic manner. In this study fast effects of corticosteroids were investigated on the function of eosinophil opsonin receptors. Short-term corticosteroid treatment of eosinophils for maximal 30 min with dexamethasone (Dex) did not influence eosinophil cell surface CD11b/CD18 expression, adhesion and/or chemokinesis. In marked contrast, incubation with Dex resulted in a rapid increase in binding of IgA-beads to human eosinophils showing that Dex can upregulate the activation of FcαR (CD89). This priming response by Dex was dose dependent and optimal between 10^{-8}M to 10^{-6}M and was mediated via the glucocorticoid receptor as its selective antagonist RU38486 (10^{-6}M) blocked the priming effect. In contrast to FcαR, eosinophil FcγRII (CD32) was not affected by Dex. Further characterization of the Dex-induced inside-out regulation of FcαR revealed P38 MAPK as central mediator. Dex dose dependently enhanced P38 MAPK phosphorylation and activation *in situ* as measured by phosphorylation of its downstream target MAPKAP-K2. The dose responses of the Dex-induced activation of these kinases were similar as seen for the priming of FcαR. This work demonstrates that corticosteroids selectively activate the FcαR on eosinophils by activation of P38 MAP-kinase.

Introduction

Eosinophilic granulocytes play an important role in the pathogenesis of allergic diseases and the host immune defence against parasites[1]. These cells belong to the most cytotoxic cells in the human body. Immune activation of these cells *in vivo* is, therefore, tightly controlled by interaction with pro- and anti-inflammatory cytokines and chemoattractants. By these mechanisms the cells can adapt a primed or pre-activated state, which results in enhanced responsiveness to distinct stimuli[2].

Eosinophils of asthmatic and allergic patients exhibit a primed phenotype compared with controls, which is characterized by increased adhesion[3] and migratory responsiveness[4,5]. Priming of these and other eosinophil functions such as degranulation and respiratory burst activation can be achieved *in vitro* by Th2 cell-derived cytokines such as Interleukin (IL)-4, IL-5, and GM-CSF[6-9] which are thought to be present in peripheral blood of allergic patients[10]. In addition, priming of eosinophils can result in responsiveness towards factors such as IL-8, which do not function on unprimed cells[6].

Furthermore, priming of these cells by cytokines results in increased functionality of immunoglobulin (Ig)A and IgG receptors[11] and increased degranulation through these receptors *in vitro*[12]. Freshly isolated eosinophils from asthmatic donors exhibit enhanced IgA-binding compared with cells of healthy donors, suggesting that these cells have been primed *in vivo*[13]. This priming of the IgA receptor functionality is thought to be important in asthmatics, since IgA is abundantly present in mucosal tissues and production of allergen specific IgAs occurs in patients with allergic diseases such as asthma[14,15]. In addition, IgA is a major trigger for eosinophil degranulation[16,17].

Standard therapy in asthma aims at inhibiting inflammation by using inhaled corticosteroids (ICS)[18,19]. It is generally agreed that activation and survival of eosinophils can be blocked by ICS although the amount of clear data regarding these issues is remarkably small[18,20-23]. It has also been described that corticosteroid treatment reduced eosinophil migration[24] and adhesion[22] but hardly affected immunoglobulin-induced degranulation in eosinophils[25]. ICS are thought to exert their function by binding to the glucocorticoid receptor (GCR) and subsequent up- and downregulation of anti-inflammatory and/or pro-inflammatory genes[19].

In addition to this well accepted mechanism at the genomic level, recently rapid non-genomic actions of corticosteroids have been described in cellular systems

and several diseases[26-30]. The exact mechanisms for these rapid effects are, however, poorly defined. Several mechanisms have been proposed by which corticosteroids can induce these rapid effects[27,28]: 1) by specific interaction with the cytosolic corticosteroid receptor resulting in fast but poorly defined mechanisms[30-32], 2) by specific interaction with membrane bound corticosteroid receptors, which are not sufficiently characterized[27,28,33] and 3) as a result of non-receptor mediated physicochemical interactions with cellular membranes[34].

In asthma rapid effects of budenoside have been shown in guinea pigs. In these experiments this corticosteroid could inhibit asthma symptoms within 10 minutes[35]. We obtained data showing similar rapid effects in human asthmatics treated with fluticasone[36]. These data underscore the importance of non-genomic mechanisms involved in the effects of corticosteroids during treatment of allergic asthma. In addition, acute non-genomic vasoconstriction in the bronchial vasculature by corticosteroids[37,38] is considered to be an important mechanism in the resolution of acute asthma complaints by corticosteroid treatment[39].

We will show that short-term treatment of human eosinophils with the corticosteroid dexamethasone (Dex) selectively primes the functionality of the FcαR on these cells through activation of the P38 MAP-kinase signalling pathway. This occurs with no effect 1) on expression of adhesion molecules, 2) on adhesion and migration and 3) on respiratory burst. These data demonstrate a complex mechanism by which corticosteroids can specifically modulate eosinophil functions in mucosal immunity.

Material and Methods

Reagents

Ficoll-paque was obtained from Pharmacia (Uppsala, Sweden). Human serum albumin (HSA) was from Sanquin (Amsterdam, the Netherlands). Recombinant human IL-5 was a gift from Dr I. Uings (GlaxoSmithKline, Stevenage, UK). Dexamethasone and RU38486 (Mifepristone) were obtained from Sigma (St. Louis, MO) and diluted in ethanol. Antibodies used were: Antiphospho-P38 MAPK (Thr180/Tyr182), antiphospho-ERK1/2 (Thr202/Tyr204) and antiphospho-MAPKAP-K2 (Thr334) from Cell Signaling (Beverly, MA) and anti-P38 from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Directly labeled CD11b-PE and CD18-FITC and HRP-coupled swine anti-rabbit were from Dako (Denmark). Functionally

blocking monoclonal antibody (MoAb) IB4 (anti β2-integrin) and the control MoAb W6/32 (anti-HLA-A, HLA-B, and HLA-C) were isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockville, MD). The functionally blocking monoclonal antibody My43 (anti-CD89)[40] was a kind gift of L. Shen (Dartmouth Medical School, NH, USA). Pharmacological inhibitor SB203580 was purchased from Kordia Life Sciences (Leiden, The Netherlands) and pharmacological inhibitors LY294002 and SP600125 were from Biomol (Plymouth Meeting, PA).

Granulocyte isolation

Granulocytes were isolated from 100 ml whole blood of healthy donors anti-coagulated with trisodium citrate (0.4% (w/v) (pH 7.4). Blood was diluted 2.5:1 with PBS containing containing trisodium citrate (0.4% (w/v) (pH 7.4) and human pasteurized plasma-protein solution (4 g/L). Granulocytes and erythrocytes were isolated by centrifugation over Ficoll-Pague. Erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4˚C. After isolation, granulocytes were resuspended in PBS containing trisodium citrate (0.4% (w/v) (pH 7.4) and human pasteurized plasma-protein solution (4 g/L).

Eosinophils were isolated from the granulocyte fraction by negative selection using anti-CD16-conjugated microbeads (MACS; Miltenyi Biotec, Auburn, CA)[41]. In addition, CD3- and CD14-conjugated microbeads (MACS; Miltenyi Biotec, Auburn, CA) were added to the granulocyte suspension to minimize mononuclear cell contamination. Purity of eosinophils was >97%.

Eosinophils were resuspended either in HEPES buffered RPMI-1640 medium supplemented with L-glutamine (Life Technologies, Breda, The Netherlands) and 10% FCS or in incubation buffer (20 mM HEPES, 132 mM NaCl, 6.0 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) HSA).

Western blotting

Eosinophils ($5x10^5$ per sample) resuspended in HEPES-buffered RPMI-1640 medium supplemented with L-glutamine and 10% FCS were allowed to recover for 15 min at 37˚C. Subsequently, cells were mock-stimulated or stimulated with Dex (10^{-6}M) for several time points (0, 15, 30, 60 and 120 min). In other western blotting experiments, cells were mock-stimulated or stimulated with several concentrations of Dex (10^{-12}M to 10^{-6}M) or IL-5 (10^{-10}M) for 15 min at 37°C. After stimulation, cells

were washed two times in PBS at 4˚C. Cells were subsequently lysed in sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol and 2% 2-mercaptoethanol) and boiled for 5 min.
Protein samples were analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P (Millipore). The blots were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl, and 0.3% Tween-20) containing 5% bovine serum albumin (BSA) for 1 h followed by incubation with antiphospho-P38 MAPK (1/1000) and antiphospho-MAPKAP-K2 (1/1000), antiphospho-ERK 1/2 (1/1000) or anti-P38 MAPK (1/1000) in hybridization buffer with 5% BSA overnight at 4°C. After incubation with first antibody, the blots were washed six times 4 min in hybridization buffer. Second antibody (HRP-coupled swine anti-rabbit; 1/3000) was incubated in hybridization buffer with 5% BSA for 1 h at RT followed by washing five times 4 min in incubation buffer and a last wash step in PBS. Detection of all Western blots was performed by enhanced chemiluminescence plus (Amersham, UK) using a Typhoon 9410 (Amersham, UK).

Fluorescent bead adhesion assay
The fluorescent bead adhesion assay was performed as described previously[42,43]. Fluorescent beads (TransFluorSpheres, 488/645 nm, 1.0 µm; Molecular Probes) were coated with ICAM-1 Fc fusion protein (derived from CHO-ICAM-1 Fc producing cells kindly provided by Prof. Y. van Kooyk, VU University Medical Center Amsterdam, The Netherlands containing an expression vector kindly provided by Prof. C.D. Buckley, University of Birmingham, UK).
In short, 20 µl streptavidin (5 mg/ml in 50 mM MES (2-(N-morpholino)ethanesulfonate) buffer) was added to 50 µl TransFluorSpheres, mixed with 30 µl 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC; 1.33 mg/mL), and incubated at room temperature (RT) for 2 hours. The reaction was stopped by addition of glycine to a final concentration of 100 mM. Streptavidin-coated beads were washed 3 times with PBS and resuspended in 150 µl PBS, 0.5% bovine serum albumin (BSA) (wt/vol). Then, streptavidin-coated beads (15 µl) were incubated with biotinylated goat anti-human anti-Fc Fab2 fragments (6 µg/ml) in 0.3 ml PBS, 0.5% BSA for 2 hours at 37°C. The beads were washed once with PBS, 0.5% BSA and incubated with ICAM-1 Fc supernatant overnight at 4°C. The fluorescent ligand-coated beads were washed and subsequently resuspended in 100 µl PBS supplemented with 0.5% BSA and 0.01% sodium azide. Ligand-coated beads were stored at 4°C.

For the fluorescent bead adhesion assay, eosinophils were resuspended in incubation buffer ($5x10^4$). Cells were preincubated with or without control anti-HLA-A, HLA-B, and HLA-C MoAb W6/32 (10 µg/ml; ATCC hybridoma, Rockville, MD), anti-β2-integrin-blocking MoAb IB4 (10 µg/ml; ATCC hybridoma, Rockville, MD) or the FcαR blocking MoAb My43 for 10 min at 37°C. The ICAM-1-coated beads (40 beads/cell) were added in a 96-well V-shaped-bottom plate with several concentrations Dex and/or IL-5. Next, the preincubated eosinophils were added and incubated for 15 min at 37°C. After stimulation, the 96-well V-shaped-bottom plate was put on ice. The cells were washed and resuspended in incubation buffer (4°C) and kept on ice until analysis. Binding of the fluorescent ICAM-1-coated beads to the eosinophils was determined by flow cytometry using a FACScalibur (Becton Dickinson, Mountain View, CA). Binding is depicted as the percentage of eosinophils that bind to ICAM-1-coated beads.

Preparation of Ig-coated magnetic dynabeads

Ig-coated magnetic dynabeads were prepared as described before[11]. In short, Serum IgG and serum IgA were coated to uncoated magnetic dynabeads (M-450, Dynal, Norway). Beads were washed twice with PBS (pH 8.5) and brought to a concentration of 45 mg/ml. IgG or IgA proteins were added at a final concentration of 1 mg/ml to the beads and mixed overnight at 4°C. The next day the beads were washed with borate buffer (0.5 M NaCl, 0.2 M H_3BO_3, and 0.02 M NaOH, pH 8.6) and blocked with 0.1 M lysine monohydrochloride (pH 8.6) in borate buffer for 2 h at room temperature. After two washes with 0.1 M acetate buffer (pH 4), beads were washed once with PBS with 1% (w/v) BSA. Until use, the beads were stored at 4°C in PBS/BSA at a concentration of 30 mg/ml ($4x10^8$ beads/ml). Before the rosette assay, beads were resuspended in 20% (w/v) HSA and left for 20 min at room temperature.

Rosette assay

Rosette assays were performed as described before[11]. Purified eosinophils were washed with Ca^{2+}-free incubation buffer containing 0.5 mM EGTA and brought to a concentration of $8x10^6$ cells/ml. A 50 µl cell suspension ($0.4x10^6$ cells) was incubated at 37°C. For priming, several concentrations of IL-5 and Dex were added 1:10. Cells were incubated with IL-5, Dex or IL-5 with Dex for 15 min at 37°C. After priming, the beads were added in a ratio of 3.5 beads/cell. Cells and beads were mixed briefly and pelleted for 15 sec at 100 rpm. Eosinophils were incubated with

beads during 15 min at 37°C. After incubation cells were resuspended vigorously and rosettes were evaluated under a microscope. All cells that bound two beads or more were defined as rosettes. One hundred cells were scored, and the number of beads that were bound to the cells was counted. The amount of beads bound to a total of 100 cells (bound and unbound to beads) was designated as the rosette index.

Previously we have demonstrated that the rosetting method with Ig-coated magnetic beads is very specific since 1) there is no appreciable background binding of eosinophils to control beads that were coated with OVA and 2) relevant blocking MoAbs against Fc receptors inhibit the binding of Ig-coated beads to these receptors[44].

Inhibition of rosette assays with specific GCR and P38 MAPK inhibitors

For priming-inhibition studies, cells were pre-incubated with specific inhibitors before priming with cytokines. Cells were incubated with P38 MAPK inhibitor SB203580 (10^{-6}M) or glucocorticoid receptor antagonist RU38486 (Mifepristone) (10^{-6}M) for 15 min at 37ºC.

Procedure for staining eosinophils with CD11b-PE and CD18-FITC

Blood from healthy donors was collected in tubes containing sodium heparin as anti-coagulant and put at 4°C. Before stimulation whole blood was incubated for 10 min at 37°C. Whole blood was subsequently stimulated with several concentrations of Dex for 15 min or with Dex 10^{-6}M for 5 or 30 min. Hereafter, the blood was put at 4°C and stained CD11b-PE (1/100) and CD18-FITC (1/100) for 30 min at 4°C. Erythrocytes were lysed and leukocytes were resuspended in ice-cold PBS containing trisodium citrate (0.4% (w/v) (pH 7.4) and HSA (4 g/L). Flow cytometric evaluation of eosinophils labelled with CD11b and CD18 was done using a FACSvantage flowcytometer (Becton Dickinson, Mountain View, CA). Eosinophils were identified according their specific side scatter and forward scatter signals[45]. Data of the flow cytometric analyses are reported as median channel fluorescence (MCF) in arbitrary units (AU).

Statistical analysis

The results of the data are expressed as means ± SEMs. Statistical analysis was performed using paired Student t tests (Statistical software package SPSS version 11.0; Chicago, Ill). P values <.05 were considered statistically significant.

Results

Lack of direct effect of dexamethasone on eosinophil adhesion and integrin expression on the cell surface.

We investigated the effect of short-term incubation of corticosteroids on the functionality of β_2-integrins expressed by human eosinophils. This functionality was investigated by measuring the binding of human eosinophils to ICAM-1 coated fluorescent beads[42,43]. As shown in Fig. 1, IL-5 (10^{-10}M) can induce binding of eosinophils to ICAM-1-coated beads whereas unprimed cells are characterized by a very low binding to these beads. Treatment with Dex (10^{-6}M; 15 min at 37ºC) did not influence the binding of eosinophils to ICAM-1 coated beads in the presence or absence of IL-5. This binding was β2-integrin dependent as a blocking antibody against β2-integrins (MoAb clone IB4, 10 µg/ml) completely blocked the binding of eosinophils to ICAM-1-coated beads, whereas a control antibody anti-HLA-A, HLA-B, and HLA-C (MoAb clone W6/32, 10 µg/ml) did not have effect on any condition used in this assay (data not shown).

In addition to integrin activation, priming of eosinophils can lead to increased expression of integrin CD11b/CD18 (MAC-1) on its cellular surface[46]. As shown in Fig. 2 short-term treatment with several doses of Dex for 15 min did not influence

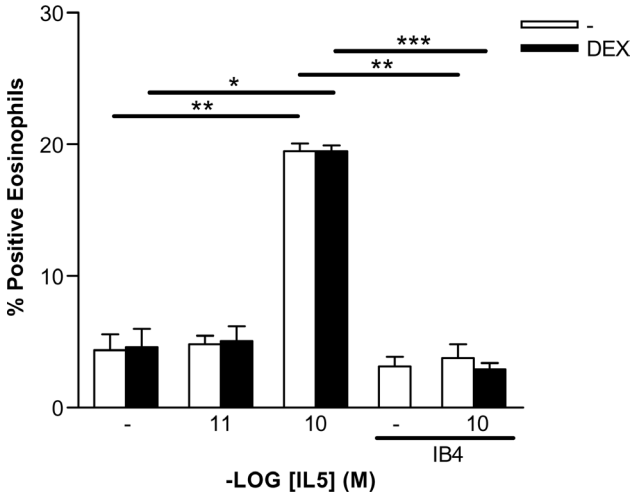

Figure 1. Lack of modulation of eosinophil adhesion by dexamethasone as measured via β2-integrin activation**.** Eosinophils were preincubated for 15 min with buffer or with a blocking antibody against β2-integrins (MoAb clone IB4; 10 µg/ml)) as indicated. Subsequently, these eosinophils were added to wells containing ICAM-1-coated beads and buffer, IL-5 (IL5; 10^{-11}M or 10^{-10}M), Dex (10^{-6}M) or Dex with IL-5. After 15 min at 37°C the cells were washed, and the percentage of cells that were positive for beads was determined by flow cytometry. Mean values are presented ± SEM (n=3). A paired Student's t test was used to perform statistical analyses (* P<0.05, ** P<0.005 and *** P<0.0005).

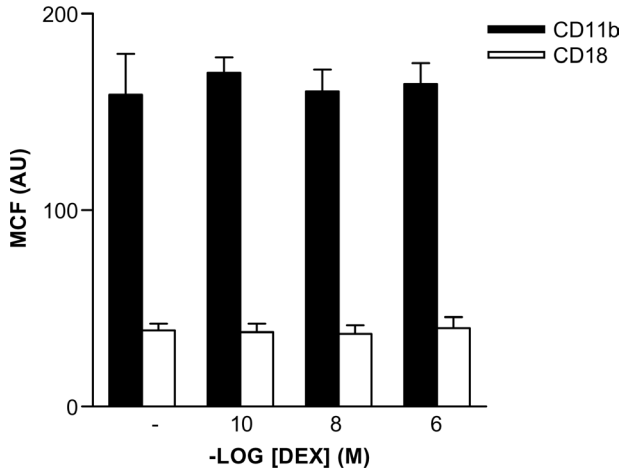

Figure 2. Absence of effect of dexamethasone on the cell surface expression of CD11b/CD18 (MAC-1) on eosinophils. Whole blood was preincubated at 37˚C for 10 min and subsequently stimulated with several doses of Dex (10^{-10}M-10^{-6}M). Whole blood was stained using directly labelled CD11b (black bars) and CD18 (white bars) and fluorescence of cells was measured using FACS-analysis. Eosinophils were identified according to their specific side- and forward scatter signals[45]. Data is expressed as median channel fluorescence (MCF) in arbitrary units (AU) ± SEM (n=3).

CD11b or CD18 expression levels on eosinophils. Detailed time courses with Dex up to 30 min did not reveal a different result (data not shown).

In addition to integrin expression and activation, we also studied chemokinesis of eosinophils during short-term corticosteroid treatment using a modified Boyden chamber assay[47]. Corticosteroids had no rapid effect on eosinophil chemokinesis in the presence or absence of IL-5 (data not shown). Therefore, although corticosteroids are known to inhibit eosinophil adhesion and migration after prolonged incubation, there was no such effect after short-term treatment.

Selective enhancement of FcαR functionality by dexamethasone.

In order to investigate whether other opsonin receptors expressed by eosinophils could be influenced by corticosteroids, the effect of short-term incubation of Dex on immunoglobulin binding was investigated. As shown in Fig. 3A and 3C short-term Dex incubation resulted in increased binding of IgA to both IL-5-primed (Fig. 3A) and, surprisingly, unprimed (Fig. 3A and 3C) eosinophils. This effect was dose dependent as shown for unprimed eosinophils (Fig. 3C) with an optimal corticosteroid concentration between 10^{-8}M and 10^{-6}M. The enhanced binding of IgA-coated beads to Dex- and IL-5-primed eosinophils was mediated by an enhanced functionality of the FcαRI (CD89) as the binding was significantly inhibited by the blocking MoAb My43 (Fig. 3D). Time courses revealed that the rosette formation of Dex-primed eosinophils and IgA-beads was optimal after

incubation for 30-45 min, including 15 min pre-incubation of the cells with Dex prior to adding beads. These kinetics are very similar when compared with cytokine-induced priming of FcαR on eosinophils[44]. In marked contrast, binding of IgG coated beads to resting or pre-activated eosinophils was not affected by corticosteroid treatment (Fig. 3B), indicating that the immunoglobulin receptor FcαR is selectively affected by Dex on eosinophils.

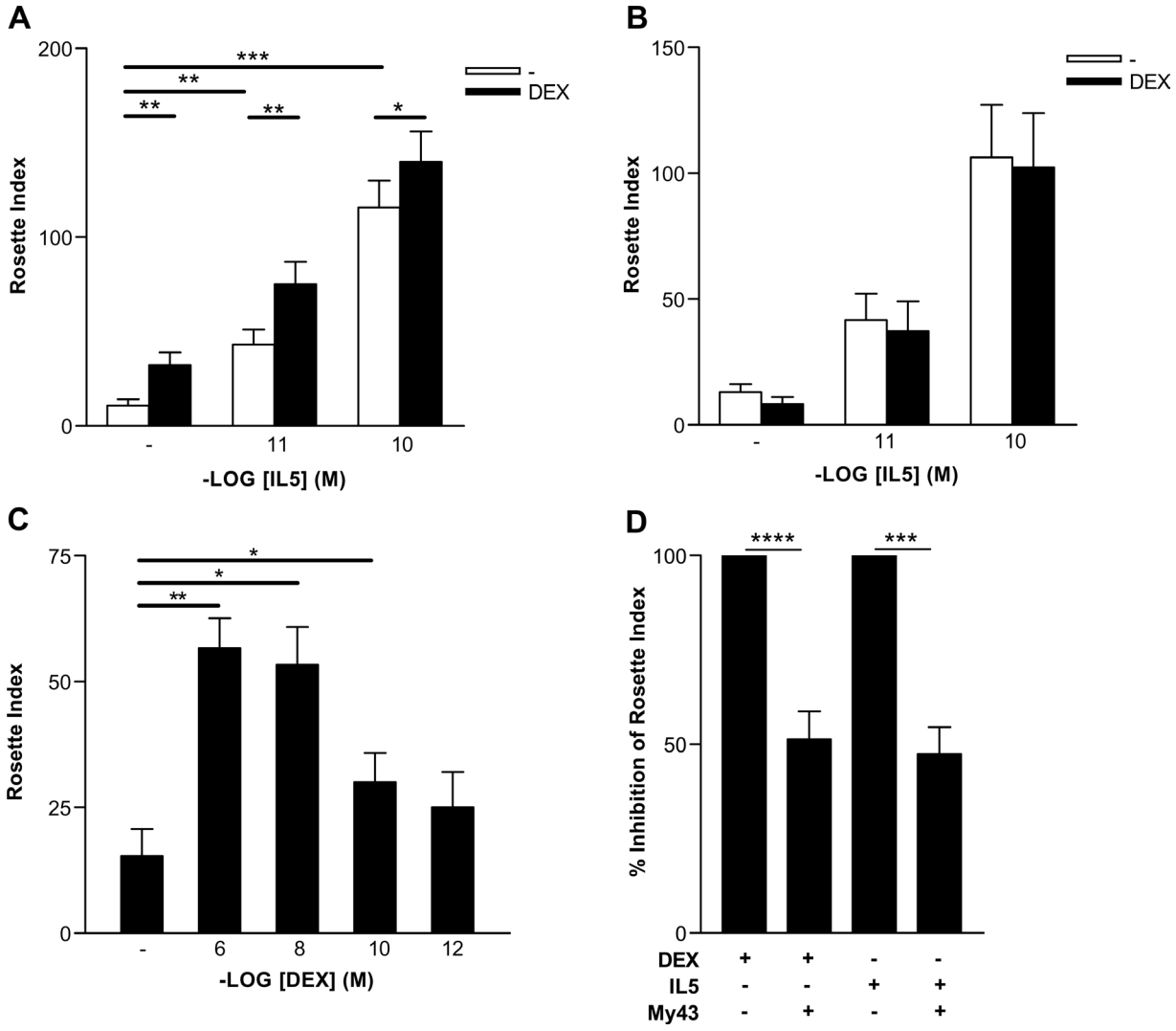

Figure 3. Specific enhancement of FcαR functionality by dexamethasone. Eosinophils were preincubated for 15 min at 37°C with buffer (**A-C**) or MoAb My43 (**D**) and subsequently stimulated at 37°C for 15 min with IL-5 (IL5; 10^{-11}M or 10^{-10}M), Dex (10^{-6}M) or IL-5 together with Dex and incubated with IgA-coated beads (**A**) or IgG-coated beads (**B**) for 15 min. A dose response curve of Dex (10^{-12}M to 10^{-6}M) was performed under the same conditions using IgA-coated beads (**C**). Specificity of the FcαR functionality towards IgA was evaluated using the blocking CD89 MoAb My43 under conditions of IL-5 (10^{-10}M) or Dex (10^{-6}M) treatment (**D**). Binding of beads is expressed as Rosette Index (number of beads/100 cells; **A-C**) or presented as percentage inhibition from Dex- or IL-5-induced rosette index (**D**). Mean values are presented ± SEM (**A** n=8, **B** n = 3, **C** n=3, **D** n=6). A paired Student's t test was used to perform statistical analyses (* P<0.05, ** P<0.005, *** P<0.0005 and **** P<0.00005).

Priming of FcαR by dexamethasone is dependent on the glucocorticoid receptor and is mediated by P38 MAPK and in part by PI3K.

We first addressed the question whether the priming response induced by Dex was mediated by the cytosolic glucocorticoid receptor (GCR). Therefore, IgA binding assays were performed in the absence or presence of the GCR antagonist RU38486. Admission of RU38486 inhibited the Dex-induced IgA-binding, indicating that corticosteroids mediate the rapid effect of FcαR activation via the GCR (Fig. 4).

Previously we have shown that in eosinophils activation of FcαR by cytokines is regulated via inside-out activation[11]. We showed that cytokine-induced activation of FcαR is dependent on intracellular activation of P38 MAPK and phophatidylinoisitol 3'-kinase (PI3K), whereas FcyR regulation depends on activation of the ERK 1/2 pathway. Since Dex only induced IgA binding and did not affect IgG binding, we focused on the PI3K/P38 MAPK pathway. By using the specific P38 MAPK inhibitor SB203580 and PI3K inhibitor LY294002, we show that Dex-induced IgA-binding could be significantly blocked by both inhibitors (Fig. 5A and Fig. 5B, respectively) suggesting that the PI3K/P38 MAPK-pathway is involved in Dex mediated FcαR activation. In agreement with our previous study[11], the binding of IgA to IL-5 primed eosinophils could also be blocked using SB203580 or LY294002 (data not shown). As Jun-N-terminal kinase (JNK) can also be induced by steroids[48], we evaluated

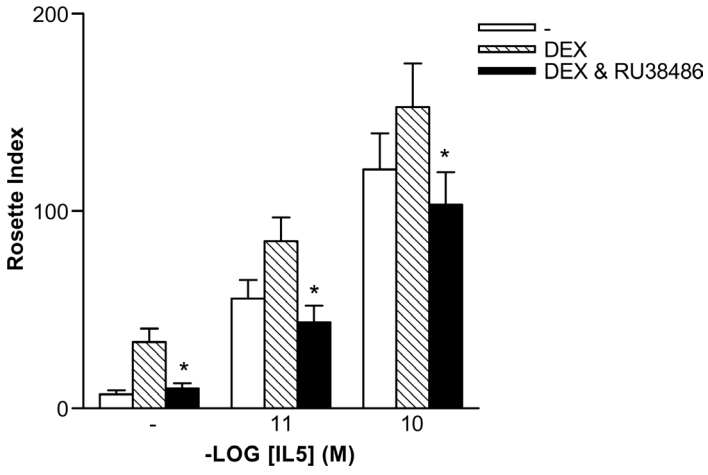

Figure 4. Enhancement of FcαR functionality by dexamethasone is mediated by the glucocorticoid receptor. Eosinophils were preincubated for 15 min at 37°C with buffer or glucocorticoid receptor antagonist RU38486 (10^{-6}M). Subsequently eosinophils were stimulated at 37°C for 15 min with IL-5 (IL5; 10^{-11}M or 10^{-10}M), Dex (10^{-6}M) or IL-5 together with Dex and incubated with IgA-coated beads for 15 min. Binding of beads is expressed as Rosette Index (number of beads/100 cells). Mean values are presented ± SEM (n=4). A paired Student's t test was used to perform statistical analyses (* P<0.05 compared with samples without RU38486).

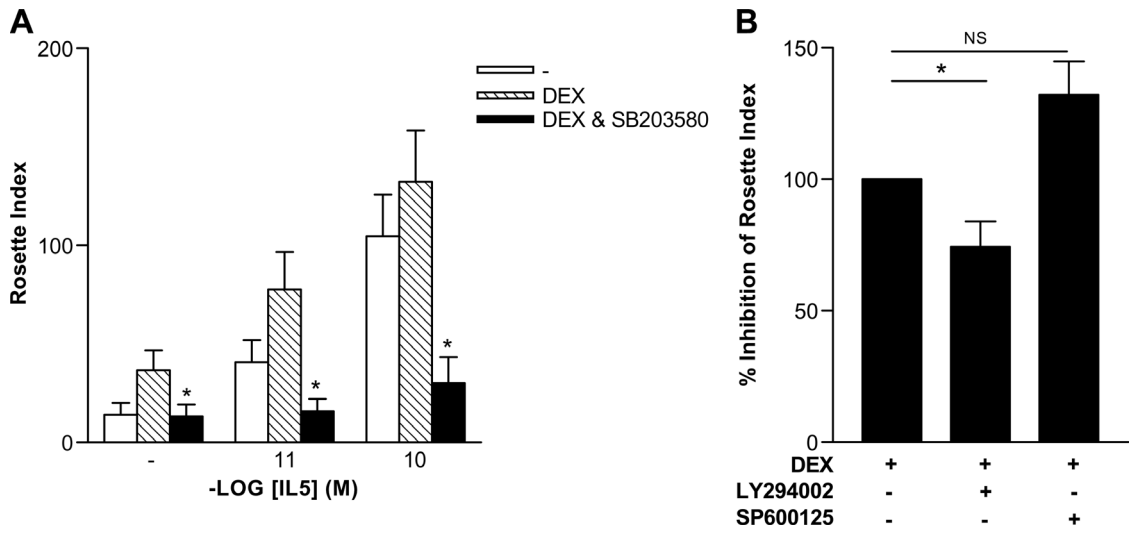

Figure 5. Dexamethasone-induced enhancement of FcαR functionality is mediated by activation of PI3K and P38 MAPK, but not via activation of JNK. Eosinophils were preincubated for 15 min at 37°C with buffer, SB203580 (10^{-6}M; **A**), LY294002 (10^{-6}M; **B**) or SP600125 (10^{-5}M; **B**). Subsequently eosinophils were stimulated at 37°C for 15 min with (**A**) IL-5 (IL5; 10^{-11}M or 10^{-10}M), Dex (10^{-6}M) or IL-5 together with Dex and incubated with IgA-coated beads, or (**B**) stimulated with Dex (10^{-6}M). Binding of beads is expressed as Rosette Index (number of beads/100 cells; **A**) or presented as percentage inhibition from Dex-induced rosette index (**B**). Mean values are presented ± SEM of at least four experiments. Paired Student's t tests were used to perform statistical analyses (* $P<0.05$ compared with samples without inhibitors).

the effect of inhibition of this kinase by the JNK-inhibitor SP600125. As is shown in Fig. 5B this inhibitor did not significantly inhibit Dex-induced formation of IgA-rosettes.

Rapid effect of corticosteroids on P38 MAPK activity in eosinophils.

Inhibition of P38 MAPK by SB203580 results in clear inhibition of Dex-induced IgA-binding by eosinophils, suggesting that the effect of Dex on FcαR is mediated via the P38 MAPK pathway. A short time course of P38 MAPK activation was performed in order to investigate whether P38 MAPK was activated by Dex. Indeed, Dex induced P38 MAPK phosphorylation within 15 min in eosinophils (see Fig. 6A) as was also shown by Zhang *et al*.[48].

We next studied the Dex-induced activation of P38 MAPK *in situ* by evaluation of the phosphorylation of MAPKAP-K2, a specific downstream target for P38 MAPK[49-51]. MAPKAP-K2 phosphorylation was clearly induced by Dex and was maximal after 15 min stimulation (Fig. 6A). Similarly to formation of IgA-rosettes Dex-induced phosphorylation of P38 MAPK was inhibited by the GCR antagonist RU38486 (results not shown). Dex induced phosphorylation of P38 MAPK and

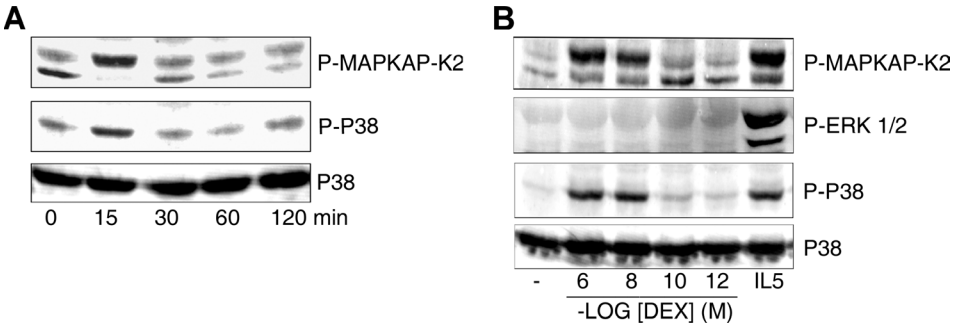

Figure 6. Time and dose-dependent induction of P38 MAPK phosphorylation and activation in eosinophils by dexamethasone. Eosinophils were preincubated for 15 min at 37°C and subsequently stimulated with Dex (10^{-6}M) for 15, 30, 60 or 120 min at 37°C (**A**) or stimulated with several concentrations of Dex (10^{-12}M to 10^{-6}M) or IL-5 (IL5; 10^{-10}M) for 15 min (**B**). After stimulation, cell lysates were prepared in sample buffer. The blots were incubated with Abs to phospho-P38 MAPK, phospho-ERK 1/2 (**B**) and phospho-MAPKAP-K2 and reprobed with an Ab to total P38 MAPK as a loading control. The experiments shown are representative for at least three experiments.

MAPKAP-K2 was dose dependent (range 10^{-12}M to 10^{-6}M) with an optimal dose range of 10^{-8}M -10^{-6}M (Fig. 6B). This dose response curve resembles the dose response curve seen by Dex-induced IgA binding to eosinophils (Fig. 3C). In contrast to IL-5, which induced both the ERK and P38 MAPK pathways, Dex selectively induced P38 MAPK activation.

Discussion

Priming is a process by which cytokines influence the functionality of innate immune cells in normal immunity as well as under pathological conditions e.g. chronic inflammatory diseases such as allergic asthma[2,4,5,13,52]. Priming rapidly upregulates responsiveness towards multiple inflammatory stimuli compared with non-primed cells. These priming responses are involved in the tight control of innate immune cells (such as neutrophils and eosinophils) that belong to the most cytotoxic cells in the body.

In this study we focused on the mechanisms of priming of eosinophil receptors involved in adhesion and in binding to opsonins such as immunoglobulins (Fc-receptors) and complement fragments (MAC-1; CD11b/CD18). These processes were studied in the context of glucocorticoids as these drugs are routinely used to inhibit inflammatory responses in chronic inflammatory diseases such as asthma[18,19]. Previous studies have shown that prolonged (at least several hours) corticosteroid treatment: 1) reduces eosinophil infiltration into the lung and the

residence time at its mucosal surfaces[53] and 2) reduces eosinophil adhesion[22] and migration[24] *in vitro*. Interestingly, corticosteroid treatment hardly affected IgA and IgG mediated degranulation of eosinophils[25].

Our experiments show that in several functional assays, short-term corticosteroid treatment did not influence: 1) expression of CD11b/CD18 on the cell surface of eosinophils, 2) β2-integrin functionality as measured by binding to ICAM-1 coated beads and 3) chemokinesis measured in the absence and presence of IL-5 (Fig. 1 and 2, and data not shown). Also we did not detect a rapid effect of Dex on the respiratory burst of eosinophils (data not shown). These data are in contrast to studies focused on long-term effects of corticosteroids on these cellular responses[22,24].

In marked contrast, a clear upregulation of the functionality of the FcαR on eosinophils was shown during short-term Dex exposure with no effect on FcγRII. The priming of the functionality of FcαR by corticosteroids was additive and not synergistic to the IL-5 induced IgA binding. This process was mediated by FcαRI as blocking this receptor with the MoAb My43[40] significantly inhibited both Dex- as well as IL-5-induced rosette formation (Fig. 3D). Priming of the FcαR by corticosteroids was dependent on P38 MAPK and to a lesser extend on PI3K activation, because this priming response was blocked by the specific P38 inhibitor SB203580 as well as the PI3K inhibitor LY294002 (Fig. 5). This is in line with our previous findings showing the pivotal role of both kinases in cytokine-induced inside-out regulation of FcαR[11]. Zhang *et al*.[48] also showed that Dex can directly activate JNK. However, activation of this kinase does not seem to be essential in signal transduction leading to activation of FcαR (CD89), because the specific inhibitor SP600125 did not significantly inhibit the response. Dex did not influence FcγR, which fits with our previous findings that P38 MAPK is not involved in control of FcγRII[11](see Fig. 3B). Although P38 MAPK was also suggested to be important in eosinophil migration and adhesion[54], our data indicate that short-term activation of P38 MAPK by corticosteroids is not sufficient for modulation of adhesion and/or chemokinesis.

These functional findings prompted us to study the effect of short-term addition of corticosteroids on the P38 MAPK signaling pathway in eosinophils. Addition of corticosteroids to eosinophils led to a fast and transient phosphorylation of P38 MAPK as visualized by western blot analysis with phosphospecific antibodies. This effect was time and dose dependent (Fig. 6) and in line with the data published by Zhang *et al*.[48]. Phosphorylation of P38 MAPK on Thr180 and Tyr182 in a Thr-Gly-

Tyr loop is associated with activation of the kinase[49,50]. A better indication for the activation of P38 MAPK *in situ* is the determination of the phosphorylation of P38 MAPK downstream target kinase MAPKAP-K2[49-51]. Indeed, Dex also induced phosphorylation of MAPKAP-K2 demonstrating that apart from phosphorylation, P38 MAPK is also activated *in situ* by this corticosteroid. Maximal P38 MAPK and MAPKAP-K2 phosphorylation were found with Dex concentrations in the range 10^{-8}M to 10^{-6}M within 15 min after addition of the corticosteroid. This time frame makes it unlikely that genomic actions of the ligand-bound corticosteroid receptor were involved[27,28].

To gain more insight into involvement of the classical glucocorticoid receptor in eosinophils, the different responses were studied in the context of its competitive inhibitor RU38486. Since this inhibitor blocked Dex-induced binding of IgA as well as phosphorylation of P38 MAPK in eosinophils it is likely that the effect of corticosteroids on the FcαR was mediated via the cytosolic glucocorticoid receptor.

In conclusion, glucocorticoids apart from their clear inhibitory effects on various effector mechanisms of innate immune cells can influence these cells in another and unique way. Both eosinophils and IgA are implicated in mucosal immunity and it is striking that corticosteroids specifically prime the interaction between this ligand and these cells *in vitro*. Eosinophils from asthmatic patients even show increased FcαR activity compared with these cells of healthy donors, which is most likely caused by an upregulated PI3K-pathway[13]. By upregulation of this pathway, eosinophils are enhanced sensitive for stimuli that engage P38 MAPK, such as TNFα and corticosteroids. Although the precise mechanisms underlying the inside-out control of FcαR are still poorly understood, these mechanisms converge at the phosphorylation of the C-terminal serine 263 in the cytoplasmic domain of FcαR[55].

In general, glucocorticoids are first choice therapy in treatment of the (mucosal) inflammation in chronic inflammatory diseases such as allergic asthma[56]. It is, however, unclear at this moment whether this short-term corticosteroid induced upregulation of eosinophil functionality is beneficial or detrimental for the natural course of these diseases.

References

1. Gleich GJ, Adolphson CR: The eosinophilic leukocyte: structure and function. Adv.Immunol. 39: 177-253, 1986

2. Coffer PJ, Koenderman L: Granulocyte signal transduction and priming: cause without effect? Immunol.Lett. 57: 27-31, 1997
3. Ulfman LH, Joosten DP, van Aalst CW, Lammers JW, van de Graaf EA, Koenderman L, Zwaginga JJ: Platelets promote eosinophil adhesion of patients with asthma to endothelium under flow conditions. Am.J.Respir.Cell Mol.Biol. 28: 512-519, 2003
4. Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koenderman L: Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. J.Allergy Clin.Immunol. 91: 1198-1205, 1993
5. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L: In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 79: 1836-1841, 1992
6. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L: RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. Blood 83: 3697-3704, 1994
7. Sanmugalingham D, De Vries E, Gauntlett R, Symon FA, Bradding P, Wardlaw AJ: Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. Clin.Exp.Allergy 30: 255-263, 2000
8. Carlson M, Peterson C, Venge P: The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation. Allergy 48: 437-442, 1993
9. Van der Bruggen T, Kok PT, Raaijmakers JA, Verhoeven AJ, Kessels RG, Lammers JW, Koenderman L: Cytokine priming of the respiratory burst in human eosinophils is Ca2+ independent and accompanied by induction of tyrosine kinase activity. J.Leukoc.Biol. 53: 347-353, 1993
10. Walker C, Virchow JC, Jr., Bruijnzeel PL, Blaser K: T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. J.Immunol. 146: 1829-1835, 1991
11. Bracke M, Coffer PJ, Lammers JW, Koenderman L: Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. J.Immunol. 161: 6768-6774, 1998
12. Fujisawa T, Abu-Ghazaleh R, Kita H, Sanderson CJ, Gleich GJ: Regulatory effect of cytokines on eosinophil degranulation. J.Immunol. 144: 642-646, 1990
13. Bracke M, Van de Graaf E, Lammers JW, Coffer PJ, Koenderman L: In vivo priming of FcalphaR functioning on eosinophils of allergic asthmatics. J.Leukoc.Biol. 68: 655-661, 2000
14. Pilette C, Durham SR, Vaerman JP, Sibille Y: Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A? Proc.Am.Thorac.Soc. 1: 125-135, 2004
15. Delacroix DL, Dive C, Rambaud JC, Vaerman JP: IgA subclasses in various secretions and in serum. Immunology 47: 383-385, 1982
16. Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ: IgA-induced eosinophil degranulation. J.Immunol. 142: 2393-2400, 1989
17. Dubucquoi S, Desreumaux P, Janin A, Klein O, Goldman M, Tavernier J, Capron A, Capron M: Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. J.Exp.Med. 179: 703-708, 1994
18. Giembycz MA, Lindsay MA: Pharmacology of the eosinophil. Pharmacol.Rev. 51: 213-340, 1999
19. Umland SP, Schleimer RP, Johnston SL: Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. Pulm.Pharmacol.Ther. 15: 35-50, 2002
20. Zhang X, Moilanen E, Kankaanranta H: Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide, and beclomethasone. Eur.J.Pharmacol. 406: 325-332, 2000
21. Myo S, Zhu X, Myou S, Meliton AY, Liu J, Boetticher E, Lambertino AT, Xu C, Munoz NM, Leff AR: Additive blockade of beta 2-integrin adhesion of eosinophils by salmeterol and fluticasone propionate. Eur.Respir.J. 23: 511-517, 2004
22. Liu J, Zhu X, Myo S, Lambertino AT, Xu C, Boetticher E, Munoz NM, Sano M, Cordoba M, Learoyd J, Meliton A, Johnson M, Leff AR: Glucocorticoid-induced surface expression of annexin 1 blocks beta2-integrin adhesion of human eosinophils to intercellular adhesion molecule 1 surrogate protein. J.Allergy Clin.Immunol. 115: 493-500, 2005
23. Bloom JW, Chacko J, Lohman IC, Halonen M, Martinez FD, Miesfeld RL: Differential control of eosinophil

survival by glucocorticoids. Apoptosis. 9: 97-104, 2004
24. Sale R, Sabatini F, Silvestri M, Serpero L, Petecchia L, Rossi GA: Concentration-dependent activity of mometasone furoate and dexamethasone on blood eosinophils isolated from atopic children: modulation of Mac-1 expression and chemotaxis. Int.Immunopharmacol. 4: 1687-1696, 2004
25. Kita H, Abu-Ghazaleh R, Sanderson CJ, Gleich GJ: Effect of steroids on immunoglobulin-induced eosinophil degranulation. J.Allergy Clin.Immunol. 87: 70-77, 1991
26. Qi AQ, Qiu J, Xiao L, Chen YZ: Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. J.Neurosci.Res. 80: 510-517, 2005
27. Goulding NJ: The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus. Curr.Opin.Pharmacol. 4: 629-636, 2004
28. Buttgereit F, Scheffold A: Rapid glucocorticoid effects on immune cells. Steroids 67: 529-534, 2002
29. Schmidt BM, Gerdes D, Feuring M, Falkenstein E, Christ M, Wehling M: Rapid, nongenomic steroid actions: A new age? Front Neuroendocrinol. 21: 57-94, 2000
30. Croxtall JD, Choudhury Q, Flower RJ: Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. Br.J.Pharmacol. 130: 289-298, 2000
31. Croxtall JD, van Hal PT, Choudhury Q, Gilroy DW, Flower RJ: Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br.J.Pharmacol. 135: 511-519, 2002
32. Limbourg FP, Huang Z, Plumier JC, Simoncini T, Fujioka M, Tuckermann J, Schutz G, Moskowitz MA, Liao JK: Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. J.Clin.Invest 110: 1729-1738, 2002
33. Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A, Buttgereit F: Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. FASEB J. 18: 70-80, 2004
34. Schmid D, Burmester GR, Tripmacher R, Kuhnke A, Buttgereit F: Bioenergetics of human peripheral blood mononuclear cell metabolism in quiescent, activated, and glucocorticoid-treated states. Biosci.Rep. 20: 289-302, 2000
35. Zhou J, Kang ZM, Xie QM, Liu C, Lou SJ, Chen YZ, Jiang CL: Rapid nongenomic effects of glucocorticoids on allergic asthma reaction in the guinea pig. J.Endocrinol. 177: R1-R4, 2003
36. Luijk B, Kempsford RD, Wright AM, Zanen P, Lammers JW: Duration of effect of single-dose inhaled fluticasone propionate on AMP-induced bronchoconstriction. Eur.Respir.J. 23: 559-564, 2004
37. Kumar SD, Brieva JL, Danta I, Wanner A: Transient effect of inhaled fluticasone on airway mucosal blood flow in subjects with and without asthma. Am.J.Respir.Crit Care Med. 161: 918-921, 2000
38. Mendes ES, Pereira A, Danta I, Duncan RC, Wanner A: Comparative bronchial vasoconstrictive efficacy of inhaled glucocorticosteroids. Eur.Respir.J. 21: 989-993, 2003
39. Wanner A, Horvath G, Brieva JL, Kumar SD, Mendes ES: Nongenomic actions of glucocorticosteroids on the airway vasculature in asthma. Proc.Am.Thorac.Soc. 1: 235-238, 2004
40. Shen L: A monoclonal antibody specific for immunoglobulin A receptor triggers polymorphonuclear neutrophil superoxide release. J.Leukoc.Biol. 51: 373-378, 1992
41. Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C: An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J.Immunol.Methods 145: 105-110, 1991
42. Geijtenbeek TB, Van Kooyk Y, Van Vliet SJ, Renes MH, Raymakers RA, Figdor CG: High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia. Blood 94: 754-764, 1999
43. Verploegen S, Ulfman L, van Deutekom HW, van Aalst C, Honing H, Lammers JW, Koenderman L, Coffer PJ: Characterization of the role of CaMKI-like kinase (CKLiK) in human granulocyte function. Blood 106: 1076-1083, 2005
44. Bracke M, Dubois GR, Bolt K, Bruijnzeel PL, Vaerman JP, Lammers JW, Koenderman L: Differential effects of the T helper cell type 2-derived cytokines IL-4 and IL-5 on ligand binding to IgG and IgA receptors expressed by human eosinophils. J.Immunol. 159: 1459-1465, 1997
45. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L:

Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005

46. Liu L, Hakansson L, Ridefelt P, Garcia RC, Venge P: Priming of eosinophil migration across lung epithelial cell monolayers and upregulation of CD11b/CD18 are elicited by extracellular Ca2+. Am.J.Respir.Cell Mol.Biol. 28: 713-721, 2003
47. Boyden S: The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J.Exp.Med. 115: 453-466, 1962
48. Zhang JP, Wong CK, Lam CW: Role of caspases in dexamethasone-induced apoptosis and activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase in human eosinophils. Clin.Exp.Immunol. 122: 20-27, 2000
49. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL: Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201, 2000
50. Kumar S, Jiang MS, Adams JL, Lee JC: Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem.Biophys.Res.Commun. 263: 825-831, 1999
51. Kotlyarov A, Yannoni Y, Fritz S, Laass K, Telliez JB, Pitman D, Lin LL, Gaestel M: Distinct cellular functions of MK2. Mol.Cell Biol. 22: 4827-4835, 2002
52. Koenderman L, Coffer PJ: Controlling allergic inflammation by signaling regulation of eosinophils. Allergy 56: 204-214, 2001
53. Belvisi MG: Regulation of inflammatory cell function by corticosteroids. Proc.Am.Thorac.Soc. 1: 207-214, 2004
54. Ip WK, Wong CK, Wang CB, Tian YP, Lam CW: Interleukin-3, -5, and granulocyte macrophage colony-stimulating factor induce adhesion and chemotaxis of human eosinophils via p38 mitogen-activated protein kinase and nuclear factor kappaB. Immunopharmacol.Immunotoxicol. 27: 371-393, 2005
55. Bracke M, Lammers JW, Coffer PJ, Koenderman L: Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. Blood 97: 3478-3483, 2001
56. Barnes PJ: Efficacy of inhaled corticosteroids in asthma. J.Allergy Clin.Immunol. 102: 531-538, 1998

Chapter 6

Differential regulation of cytokine-induced activation of P38 MAPK in neutrophils and eosinophils

Distinct regulation of P38 MAPK in granulocytes

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Abstract

P38 MAPK is a central mediator in cytokine signalling in human leukocytes. P38 MAPK is activated by phosphorylation of a conserved Thr180-X-Tyr182 motif by dual phosphorylation via the upstream kinases MKK3 and MKK6. Alternatively, P38 MAPK can be activated via autophosphorylation when associated with TAB1. In this study P38 MAPK phosphorylation and activation (measured via phosphorylation of P38 MAPK downstream target MK2) were investigated upon engagement of the GM-CSF- and TNFα-receptors expressed on both eosinophils and neutrophils. The MKK3/MKK6 pathway mediated neutrophil P38 MAPK activation after stimulation with TNFα (100 U/ml) or GM-CSF (10^{-10}M). Under these conditions the activation but not phosphorylation of P38 MAPK could be inhibited by SB203580 (10^{-5}M or 10^{-6}M). In eosinophils SB203580 (10^{-6}M) inhibited both the phosphorylation and activation of P38 MAPK after stimulation with several doses of TNFα (10-10000 U/ml) or GM-CSF (10^{-11}-10^{-9}M), indicating that P38 MAPK activation is mediated via autophosphorylation in eosinophils. This was further supported by the finding that, in marked contrast to neutrophils, MKK3/MKK6 did not show enhanced phosphorylation in eosinophils after cytokine stimulation, but were constitutively phosphorylated. In addition, the involvement of TAB1 was investigated with regard to this cytokine-induced autophosphorylation. Co-immunoprecipitation experiments showed that TAB1 was constitutively associated with P38 MAPK in eosinophils and neutrophils and that cytokine-induced (auto)phosphorylated P38 MAPK was co-precipitated with TAB1. These findings are consistent with the hypothesis that cytokine-induced autophosphorylation of P38 MAPK in primary granulocytes depends on the interaction with TAB1.

Introduction

Aberrant activation of P38 mitogen-activated protein kinase (MAPK) is involved in a variety of pathophysiological responses[1]. Recently, inhibitor studies using small molecule inhibitors against P38 MAPK have indicated that P38 MAPK is a central mediator in pulmonary inflammatory diseases[2-6], such as chronic obstructive pulmonary disease (COPD) and asthma. These studies showed the involvement of P38 MAPK in e.g. granulocyte infiltration into the tissues, production of inflammatory mediators by inflammatory cells and regulation of apoptosis of leukocytes[3,4,7].

Innate immune cells, such as eosinophils and neutrophils, play an important role in the pathogenesis of chronic persistent inflammatory processes that are present in the bronchial tissue of asthma and COPD patients. Elevated numbers, priming and activation of these cells correlate with the type and severity of asthma and COPD[8-12]. Proper regulation of the activation of signal transduction pathways, including the P38 MAPK route, in eosinophils and neutrophils during the inflammatory processes is required to prevent exaggerated damage to host tissue at inflammatory sites. Regulation of P38 MAPK activation occurs via dual phosphorylation on a conserved Thr180-X-Tyr182 motif via the upstream kinases mitogen-activated protein kinase kinase (MKK)3 and MKK6. However, alternative pathways for P38 MAPK activation were recently described. P38 MAPK can be activated independently of MKK3/MKK6 by the association with the scaffold protein TAK1-binding protein 1 (TAB1)[13]. TAB1 binding to P38 MAPK enhances the intramolecular P38 MAPK kinase activity. A second alternative pathway was identified in T-cells and involves the phosphorylation of a previously unappreciated residue on P38 MAPK, Tyr323[14]. In these cells, T-cell receptor (TCR) ligation results in activation of the Zap70 pathway, which subsequently results in the phosphorylation of Tyr323 on P38 MAPK. This phosphorylation also enhances the intramolecular activity of P38 MAPK[14]. In addition, P38 MAPK activation via an association with TAB1 or via phosphorylation of Tyr323 results in dual phosphorylation of P38 MAPK on its conserved Thr-X-Tyr motif[13,14].

The dual phosphorylation of P38 MAPK mediated via MKKs is insensitive to the inhibitor of P38 MAPK activity SB203580, which action lies in occupation of the ATP-binding pocket within the P38 MAPK kinase domain[6,15-17]. SB203580 is a specific inhibitor of the P38α and P38β MAPK isoforms[6,18,19]. However, P38 MAPK phosphorylation under conditions of an association with TAB1 or Tyr323

phosphorylation is sensitive to SB203580[13,14,20,21] indicating that this phosphorylation of P38 MAPK occurs via autophosphorylation of the kinase. It has been shown in neutrophils that the inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNFα) can induce activation of P38 MAPK through activation of the upstream kinases MKK3/MKK6[22]. For eosinophils it is uncertain which upstream kinases are involved in this process. However, a role for phophatidylinoisitol 3'-kinase (PI3K) as upstream activator of P38 MAPK has been implicated in interleukin (IL)-4 and IL-5 induced immunoglobulin-A binding in eosinophils[23].

In this study, the GM-CSF- and TNFα-induced regulation and activation of P38 MAPK in neutrophils and eosinophils were investigated. The cytokines TNFα and GM-CSF were investigated in these processes because they use distinct receptors that are expressed on both cell types. In addition, both cytokines have been associated with chronic inflammation in inflammatory diseases, such as asthma and COPD[8,24-26]. Activity of P38 MAPK *in situ* was investigated by measuring the phosphorylated status of its physiological substrate MAPK-activated protein kinase 2 (MK2)[15,27]. The results show that P38 MAPK is differentially regulated in eosinophils and neutrophils after activation of these cells. After stimulation with TNFα or GM-CSF was neutrophil P38 MAPK mainly activated through phosphorylation by the upstream kinases MKK3/MKK6, whereas in eosinophils engagement of the same receptors led to autophosphorylation of P38 MAPK.

Materials and Methods

Reagents: cytokines, antibodies, and pharmacological inhibitors

Human serum albumin (HSA) was from Sanquin (Amsterdam, the Netherlands). Recombinant human GM-CSF was a gift from Prof. A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia). Recombinant human TNFα was purchased from Roche (Indianapolis, IN). Pharmacological inhibitor SB203580 was purchased from Kordia Life Sciences (Leiden, The Netherlands).

Antibodies used were: antiphospho-P38 MAPK (Thr180/Tyr182), antiphospho-MK2 (Thr334), anti-phospho-MKK3/MKK6 (Ser189/Ser207) and antiphospho-MKK4 (Thr261) from Cell Signaling (Beverly, MA), anti-P38 MAPK (C-20) and anti-TAB1 (N-19) from Santa Cruz (Santa Cruz, CA) and HRP-coupled swine anti-rabbit from Dako (Denmark).

Granulocyte isolation and stimulation

Granulocytes were isolated from 100 ml whole blood of healthy donors anti-coagulated with trisodium citrate (0.4% (w/v) (pH 7.4)). Blood was diluted 2.5:1 with phosphate buffered saline (PBS) containing trisodium citrate (0.4% (w/v) (pH 7.4) and human pasteurized plasma-protein solution (4 g/L). Granulocytes and erythrocytes were isolated by centrifugation over Ficoll-Pague from Pharmacia (Uppsala, Sweden). Erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. After isolation, granulocytes were resuspended in PBS containing trisodium citrate (0.4% (w/v) (pH 7.4)) and human pasteurized plasma-protein solution (4 g/L).

Negative immunomagnetic selection was used to purify eosinophils from granulocytes using anti-CD16-conjugated microbeads (Miltenyi Biotec; Auburn, CA)[28]. In addition, anti-CD3- and CD14-conjugated microbeads (Miltenyi Biotec; Auburn, CA) were added to the granulocyte suspension to avoid mononuclear cell contamination. Purity of eosinophils was >97%.

Neutrophils (less then 3% eosinophils) and eosinophils were resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6.0 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) HSA) and allowed to recover for 15 min at 37°C with or without pharmacological inhibitor SB203580. Subsequently, cells were mock-stimulated or stimulated with TNFα or GM-CSF for 15 min at 37°C. After stimulation, cells were washed two times in PBS at 4°C. Cells were subsequently lysed in sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol and 2% 2-mercaptoethanol) and boiled for 5 min or lysed in lysis buffer and used in immunoprecipitations.

Immunoprecipitation

Immediately after stimulation of $20x10^6$ neutrophils or $2x10^6$ eosinophils, cells were lysed in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM PMSF; to neutrophil samples also 2 mM diisopropyl-phosphorofluoride was added) and samples were vortexed for 10 sec and subsequently agitated for 30 min at 4°C. Thereafter, lysates were centrifuged (14000g, 20 min, 4°C). Supernatants were incubated with 25 µl Protein G Sepharose 4 Fast Flow beads (Sigma; St. Louis, MO) for 1h at 4°C to preclear the samples. After centrifugation (14000g, 30 sec, 4°C), supernatants were incubated with 5 µg of anti-TAB1 antibody (Ab) and agitated for 12h at 4°C.

Subsequently, 25 µl Protein-G sepharose beads were added to the samples and samples were agitated for 8h at 4°C. Then the pellets of the immunoprecipitates were washed three times with lysis buffer and resuspended in sample buffer and boiled for 5 min.

Western Blotting

Protein samples were analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P (Millipore; Bedford, MA) for total cell lysates and to Hybond-LPF (Amersham; UK) for immunoprecipitates. The blots were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl, and 0.3% Tween-20) containing 5% bovine serum albumin (BSA) for 1h at RT followed by incubation with antiphospho-P38 MAPK (1/1000), antiphospho-MK2 (1/1000), antiphospho-MKK4 (1/1000) or anti-P38 MAPK (1/1000) in hybridization buffer with 5% BSA overnight at 4°C. After incubation with the first antibody, the blots were washed six times 4 min in hybridization buffer. Second antibody (HRP-coupled swine anti-rabbit; 1/3000) was incubated in hybridization buffer with 5% BSA for 1h followed by washing five times 4 min in incubation buffer and a last wash step in PBS. Detection of all western blots was performed by enhanced chemiluminescence plus (Amersham; UK) using a Typhoon 9410 (Amersham; UK).

Results

In this study we investigated the mechanisms involved in P38 MAPK phosphorylation and activation in eosinophils and neutrophils upon TNFα and GM-CSF stimulation.

In Fig. 1A and B it is shown that activation of eosinophils with TNFα (10-1000 U/ml) or GM-CSF (10^{-11}-10^{-9}M) for 15 min induced a dose dependent phosphorylation of P38 MAPK. This TNFα- (Fig. 1A) and GM-CSF- (Fig. 1B) induced phosphorylation of P38 MAPK was sensitive to SB203580 (10^{-6}M) indicating that P38 MAPK regulation occurs via autophosphorylation in eosinophils. P38 MAPK activation was measured by investigation of the phosphorylated status of its downstream target MK2. Phosphorylation of MK2 was also dose dependently increased in eosinophils after stimulation with both stimuli and SB203580 inhibited this increase (Fig. 1).

To evaluate the role of MKK3/MKK6 during cytokine-induced P38 MAPK autophosphorylation, we investigated the phosphorylated status of MKK3/MKK6 in

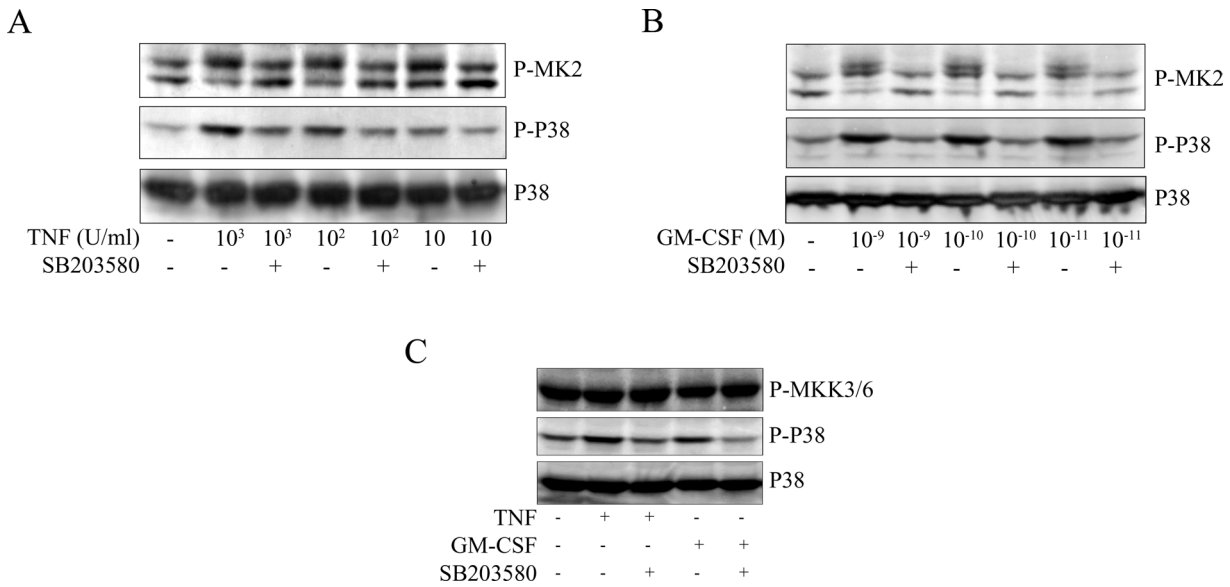

Figure 1. P38 MAPK phosphorylation and activation are mediated via autophosphorylation and not via MKK3/MKK6 in eosinophils. Eosinophils ($0.5x10^6$) were preincubated with or without P38 MAPK catalytic site inhibitor SB203580 (10^{-6}M) for 15 min and stimulated for 15 min with several concentrations of (**A**) TNFα (1000-10 U/ml) or (**B**) GM-CSF (10^{-9}-10^{-11}M) or (**C**) TNFα (100 U/ml) and GM-CSF (10^{-10}M). The experiment shown is representative for at least three experiments.

eosinophils after stimulation of these cells with TNFα (100 U/ml) and GM-CSF (10^{-10}M). TNFα and GM-CSF did not induce activation of the P38 MAPK upstream kinases MKK3/MKK6 in eosinophils, as a constitutively phosphorylated band of MKK3/MKK6 was equally present under all conditions (Fig. 1C).

In marked contrast to eosinophils, SB203580 (10^{-5}M or 10^{-6}M) did not inhibit the TNFα- (100 U/ml) and GM-CSF- (10^{-10}M) induced P38 MAPK phosphorylation in human neutrophils (Fig. 2), but it inhibited P38 MAPK activation, which was measured via the phosphorylated status of MK2 (Fig. 2). Stimulation of P38 MAPK in neutrophils occurs via the activation of upstream kinases MKK3/MKK6 because the phosphorylated levels of MKK3/MKK6 were increased by both TNFα and GM-CSF (Fig. 2).

Besides MKK3/MKK6, also MKK4 is known to be able to phosphorylate and activate P38 MAPK. However, only for neutrophils we could identify a band of phosphorylated MKK4, which was only present 5 min after stimulation (data not shown). We could not identify phosphorylation of MKK4 in stimulated eosinophils (data not shown).

Several studies have suggested that an association of P38 MAPK with TAB1 leads to autophosphorylation of P38 MAPK[13,20,21,29]. Association of P38 MAPK with TAB1

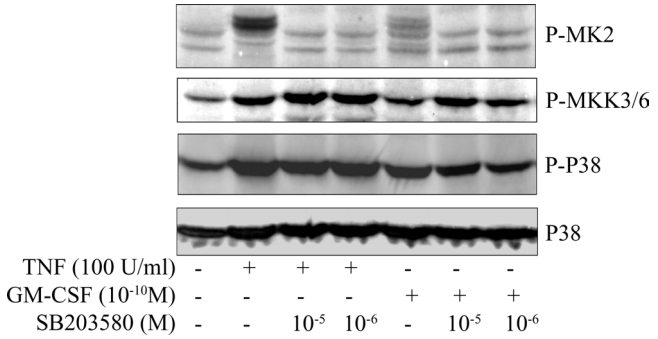

Figure 2. P38 MAPK activation and phosphorylation correlate with phosphorylation of MKK3/MKK6 in human neutrophils. Neutrophils ($2x10^6$) were preincubated with or without P38 MAPK catalytic site inhibitor SB203580 (10^{-5}M or 10^{-6}M) for 15 min and stimulated for 15 min with TNFα (100 U/ml) or GM-CSF (10^{-10}M). The experiment shown is representative for at least three experiments.

in eosinophils was studied by immunoprecipitation using an anti-TAB1 antibody. Subsequently, immunoprecipitates were analyzed by western blotting with anti-phospho-P38 MAPK and anti-P38 MAPK antibodies. Fig. 3 demonstrates that phospho-P38 MAPK co-precipitated with TAB1 in lysates from TNFα- (Fig. 3A; 100 U/ml) and GM-CSF- (Fig. 3B; 10^{-10}M) stimulated eosinophils. Phosphorylation of eosinophil P38 MAPK was inhibited by SB203580 (10^{-6}M) for both stimuli (Fig. 3A and 3B). Inhibition of autophosphorylation by SB203580 (10^{-6}M) did not lead to

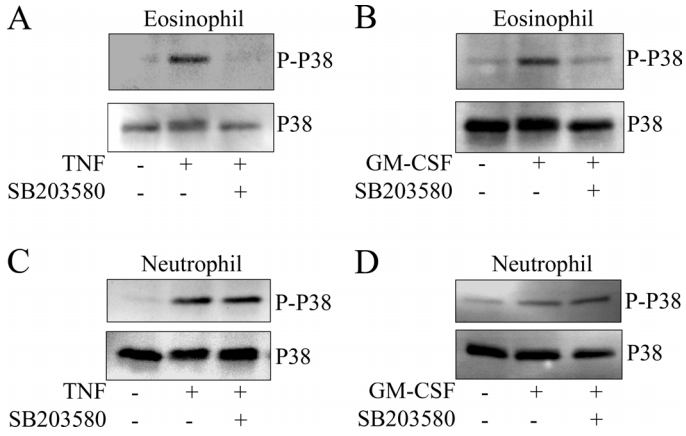

Figure 3. TAB1 is constitutively associated with P38 MAPK in eosinophils and neutrophils and respectively autophosphorylation and phosphorylation of P38 MAPK occur in the P38 MAPK-TAB1 complex. (**A** and **B**) Eosinophils ($2x10^6$) and (**C** and **D**) neutrophils ($20x10^6$) were preincubated with or without P38 MAPK catalytic site inhibitor SB203580 (eosinophils: 10^{-6}M; neutrophils: 10^{-5}M) for 15 min and stimulated for 15 min with (**A** and **C**) TNFα (100 U/ml) or (**B** and **D**) GM-CSF (10^{-10}M). After stimulation, cells were subjected to co-immunoprecipitation with an anti-TAB1 antibody (N-19; Santa Cruz). Precipitates were prepared for immunoblotting on low fluorescent Hybond-LPF membranes (Amersham, UK) and incubated with an anti-phospho-P38 MAPK antibody and reprobed with an antibody that recognized total P38 MAPK. The experiment shown is representative for three experiments.

dissociation of the P38 MAPK-TAB1 complex as equal amounts of co-precipitated total P38 MAPK were present in unstimulated and stimulated samples (Fig. 3A and 3B). This also implies that TAB1 was constitutively associated with P38 MAPK in eosinophils. We next addressed the question whether TAB1 was also constitutively associated with P38 MAPK in neutrophils. As in eosinophils, P38 MAPK was constitutively found in neutrophils in co-immunoprecipitations using anti-TAB1 antibodies (Fig. 3C and 3D). P38 MAPK, which was associated with TAB1, was phosphorylated upon neutrophil activation with TNFα (Fig. 3C; 100 U/ml) and GM-CSF (Fig. 3D; 10^{-10}M). In contrast to eosinophils, SB203580 (10^{-5}M) did not influence the phosphorylation status of TAB1-associated P38 MAPK in neutrophils after cellular activation with TNFα (Fig. 3C) or GM-CSF (Fig. 3D).

Discussion

MAPK kinases play an important role in activation of effector cells in innate and acquired immune responses[30]. Aberrant activation of granulocytes, which are important effector cells in innate immunity, has been associated with pathogenic reactions that might occur in chronic inflammatory diseases such as asthma and COPD.

Activation of P38 MAPK is involved in the regulation of functional responses in these cells and is caused by phosphorylation of a conserved Thr180-X-Tyr182 motif. Phosphorylation of P38 MAPK can be mediated by several mechanisms. One of these mechanisms involves phosphorylation of the Thr-X-Tyr motif by the upstream MAP-kinase kinases, MKK3 and MKK6. MKK3/MKK6-mediated phosphorylation of P38 MAPK is insensitive to the P38 MAPK inhibitor SB203580, due to the fact that SB203580 is a specific inhibitor of P38 MAPK by occupying the ATP-binding pocket without influencing its phosphorylation sites[6,15-17]. Recently, two other mechanisms of P38 MAPK activation have been described in which the phosphorylation of P38 MAPK is sensitive to SB203580, indicating that P38 MAPK mediated autophosphorylation occurs. The first mechanism consists of an association of TAB1 with P38 MAPK[13] and the other involves the phosphorylation of an additional tyrosine (Tyr323) in P38 MAPK[14].

Currently little is known regarding the different routes towards P38 MAPK in primary cells upon engagement of proinflammatory cytokine receptors, such as the TNFα receptor or the receptors for IL-3, IL-5 and GM-CSF, which share a common

β-chain (CD131). The involvement of the MAPKKs MKK3 and MKK6 has been shown in neutrophils in response to GM-CSF or TNFα[22]. In eosinophils, only the upstream activation of PI3K has been implicated in the signal transduction pathway towards P38 MAPK from the β-chain containing IL-5 receptor[23]. Nothing is known regarding the upstream signals that regulate TNFα-induced activation of P38 MAPK in eosinophils. By comparing the mechanisms of P38 MAPK activation in neutrophils and eosinophils stimulated with inflammatory cytokines GM-CSF and TNFα, distinct upstream pathways towards P38 MAPK were found (Fig. 1 and 2). For neutrophils the upstream kinases are most likely MKK3 and MKK6 (Fig. 2) as the kinetics of the phosphorylation of these MKKs after stimulation were similar to the kinetics of P38 MAPK phosphorylation. Furthermore, neutrophil P38 MAPK phosphorylation was insensitive to SB203580 (Fig. 2), which indicates that this MKK-mediated phosphorylation did not occur via P38 MAPK autophosphorylation. Inhibition of phosphorylation of the P38α MAPK downstream target MK2 by SB203580 supports the hypothesis that neutrophil P38 MAPK activity is controlled by signalling pathways that involve MKK3/MKK6.

Besides MKK3 and MKK6, MKK4 can phosphorylate P38 MAPK under certain conditions[31,32]. However, under the conditions used in this study (15 min stimulation), no phosphorylation of MKK4 could be detected in either neutrophils or eosinophils (data not shown). In addition, the kinetics of MKK4 phosphorylation in neutrophils are markedly different from those that are present in the activation of P38 MAPK by MKK3/MKK6. Phosphorylation of MKK4 induced by cytokines such as TNFα is fast (optimal after 5 min) and very transient (hardly detectable after 15 min) in neutrophils (data not shown and ref.[33]). These data make it unlikely that kinase MKK4 plays an important role in the phosphorylation of P38 MAPK in neutrophils.

In marked contrast to neutrophils, phosphorylation and activation of P38 MAPK in eosinophils was sensitive to SB203580 after stimulation with cytokines GM-CSF and TNFα, indicating that P38 MAPK activation occured via autophosphorylation. The kinetics of this autophosphorylation of P38 MAPK in eosinophils were not mirrored by similar kinetics in phosphorylation of MKK3/MKK6 (Fig 1C). The findings suggest that MKK3 and MKK6 are constitutively phosphorylated in eosinophils, while the activation of the MKKs does not lead to P38 MAPK phosphorylation. P38 MAPK is, therefore, activated by an alternative pathway(s) leading to autophosphorylation of the kinase.

Several recent publications have suggested that an association of P38 MAPK with TAB1 is important in the regulation of P38 MAPK autophosphorylation[13,20,21,29]. To evaluate the possibility that autophosphorylation of P38 MAPK in eosinophils was associated with an interaction with TAB1, co-immunoprecipitations were performed. To our surprise, TAB1 was constitutively associated with P38 MAPK in both eosinophils and neutrophils even in the absence of a stimulus (Fig. 3). After stimulation, enhanced phospho-P38 MAPK was present in association with TAB1, which was sensitive to SB203580 in eosinophils but not in neutrophils. In marked contrast to *in vitro* data showing that an interaction of P38 MAPK with TAB1 is responsible for P38 MAPK autophosphorylation and activation[13], we show that interaction *per se* does not lead to autophosphorylation of P38 MAPK in human neutrophils and eosinophils.

The endogenous TAB1 is known to be constitutively associated with transforming growth factor-beta (TGF-beta)-activated kinase 1 (TAK1) in unstimulated cells without affecting TAK1 kinase activity[34]. TAK1 can be involved as upstream activator of both the MAPK and nuclear factor-κB (NF-κB) pathways and has mitogen-activated protein kinase kinase kinase (MAPKKK) properties[34-37]. Upon e.g. IL-1 receptor activation, the TAB1-TAK1 complex is recruited to intracellular TNF receptor-associated factor 6 (TRAF6) and TAK1 is supposed to be activated via TAB1-mediated TAK1 autophosphorylation[34-37]. It is tempting to speculate that TAB1 has a similar role in P38 MAPK activation in eosinophils and neutrophils and is a *conditio sine qua non* for autophosphorylation. Recruitment of the P38 MAPK-TAB1 complex to cytokine receptors might therefore be a crucial step in facilitation of P38 MAPK autophosphorylation. This alternative pathway is not identified in neutrophils because MKKs can phosphorylate P38 MAPK and in this way bypass autophosphorylation and mask the importance of autophosphorylation in these cells.

In this study we have shown that identical cytokine receptors in different granulocytes can induce two distinct pathways towards the phosphorylation and activation of P38 MAPK. In neutrophils a MKK-driven activation of P38 MAPK dominates whereas in eosinophils a MKK-independent autophosphorylation regulates P38 MAPK activation. TAB1 was constitutively associated with P38 MAPK in both neutrophils and eosinophils and is likely an essential but not a sufficient condition for the autophosphorylation of P38 MAPK. Our data clearly show that the cellular context of cytokine receptors determines which signalling pathways control the P38 MAPK pathway in primary cells.

References

1. Kumar S, Boehm J, Lee JC: p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat.Rev.Drug Discov. 2: 717-726, 2003
2. Barnes PJ: Novel signal transduction modulators for the treatment of airway diseases. Pharmacol.Ther. 109: 238-245, 2006
3. Underwood DC, Osborn RR, Bochnowicz S, Webb EF, Rieman DJ, Lee JC, Romanic AM, Adams JL, Hay DW, Griswold DE: SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am.J.Physiol Lung Cell Mol.Physiol 279: L895-L902, 2000
4. Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW, Griswold DE: SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. J.Pharmacol.Exp.Ther. 293: 281-288, 2000
5. Wong WS: Inhibitors of the tyrosine kinase signaling cascade for asthma. Curr.Opin.Pharmacol. 5: 264-271, 2005
6. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL: Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201, 2000
7. Kankaanranta H, De Souza PM, Barnes PJ, Salmon M, Giembycz MA, Lindsay MA: SB 203580, an inhibitor of p38 mitogen-activated protein kinase, enhances constitutive apoptosis of cytokine-deprived human eosinophils. J.Pharmacol.Exp.Ther. 290: 621-628, 1999
8. Oudijk EJ, Lammers JW, Koenderman L: Systemic inflammation in chronic obstructive pulmonary disease. Eur.Respir.J.Suppl 46: 5s-13s, 2003
9. Douwes J, Gibson P, Pekkanen J, Pearce N: Non-eosinophilic asthma: importance and possible mechanisms. Thorax 57: 643-648, 2002
10. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ: Neutrophilic inflammation in severe persistent asthma. Am.J.Respir.Crit Care Med. 160: 1532-1539, 1999
11. Gibson PG, Simpson JL, Saltos N: Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119: 1329-1336, 2001
12. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L: Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005
13. Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J: MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295: 1291-1294, 2002
14. Salvador JM, Mittelstadt PR, Guszczynski T, Copeland TD, Yamaguchi H, Appella E, Fornace AJ, Jr., Ashwell JD: Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. Nat.Immunol. 6: 390-395, 2005
15. Kumar S, Jiang MS, Adams JL, Lee JC: Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem.Biophys.Res.Commun. 263: 825-831, 1999
16. Gum RJ, McLaughlin MM, Kumar S, Wang Z, Bower MJ, Lee JC, Adams JL, Livi GP, Goldsmith EJ, Young PR: Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. J.Biol.Chem. 273: 15605-15610, 1998
17. Frantz B, Klatt T, Pang M, Parsons J, Rolando A, Williams H, Tocci MJ, O'Keefe SJ, O'Neill EA: The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. Biochemistry 37: 13846-13853, 1998
18. Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR: Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. Biochem.Biophys.Res.Commun. 235: 533-538, 1997
19. Hale KK, Trollinger D, Rihanek M, Manthey CL: Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. J.Immunol. 162: 4246-4252, 1999

20. Kim L, Del Rio L, Butcher BA, Mogensen TH, Paludan SR, Flavell RA, Denkers EY: p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. J.Immunol. 174: 4178-4184, 2005
21. Tanno M, Bassi R, Gorog DA, Saurin AT, Jiang J, Heads RJ, Martin JL, Davis RJ, Flavell RA, Marber MS: Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. Circ.Res. 93: 254-261, 2003
22. Suzuki K, Hino M, Hato F, Tatsumi N, Kitagawa S: Cytokine-specific activation of distinct mitogen-activated protein kinase subtype cascades in human neutrophils stimulated by granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha. Blood 93: 341-349, 1999
23. Bracke M, Coffer PJ, Lammers JW, Koenderman L: Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. J.Immunol. 161: 6768-6774, 1998
24. Thomas PS: Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol.Cell Biol. 79: 132-140, 2001
25. Ritz SA, Stampfli MR, Davies DE, Holgate ST, Jordana M: On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends Immunol. 23: 396-402, 2002
26. Oudijk EJ, Nijhuis EH, Zwank MD, Van de Graaf EA, Mager HJ, Coffer PJ, Lammers JW, Koenderman L: Systemic inflammation in COPD visualised by gene profiling in peripheral blood neutrophils. Thorax 60: 538-544, 2005
27. Kotlyarov A, Yannoni Y, Fritz S, Laass K, Telliez JB, Pitman D, Lin LL, Gaestel M: Distinct cellular functions of MK2. Mol.Cell Biol. 22: 4827-4835, 2002
28. Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, Blaser K, Walker C: An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J.Immunol.Methods 145: 105-110, 1991
29. Matsuyama W, Faure M, Yoshimura T: Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-beta-activated protein kinase 1 binding protein 1 beta/p38 alpha mitogen-activated protein kinase signaling cascade. J.Immunol. 171: 3520-3532, 2003
30. Dong C, Davis RJ, Flavell RA: MAP kinases in the immune response. Annu.Rev.Immunol. 20: 55-72, 2002
31. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, Karin M: Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268: 286-290, 1995
32. Ganiatsas S, Kwee L, Fujiwara Y, Perkins A, Ikeda T, Labow MA, Zon LI: SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proc.Natl.Acad.Sci.U.S.A 95: 6881-6886, 1998
33. Avdi NJ, Nick JA, Whitlock BB, Billstrom MA, Henson PM, Johnson GL, Worthen GS: Tumor necrosis factor-alpha activation of the c-Jun N-terminal kinase pathway in human neutrophils. Integrin involvement in a pathway leading from cytoplasmic tyrosine kinases apoptosis. J.Biol.Chem. 276: 2189-2199, 2001
34. Kishimoto K, Matsumoto K, Ninomiya-Tsuji J: TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. J.Biol.Chem. 275: 7359-7364, 2000
35. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K: The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 398: 252-256, 1999
36. Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K: TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol.Cell 5: 649-658, 2000
37. Sakurai H, Miyoshi H, Mizukami J, Sugita T: Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. FEBS Lett. 474: 141-145, 2000

Chapter 7

General Discussion

Phenotyping of asthma based on peripheral blood leukocyte priming can be used to link better understanding of the pathogenesis of the disease with development of improved pharmacotherapy.

Definition of asthma - Several definitions have been proposed to describe asthma[1,2]. The most accepted definition was put forward by the NHLBI/WHO Global Initiative for Asthma (GINA)[3]:

'*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment*[4].'

This definition clearly describes asthma as a chronic disease, in which several inflammatory cells might influence the pathology of the airways[1]. The mechanisms underlying this induction of the chronic persistent phase in allergic asthma are poorly defined, but this *switch to chronicity* is not a unique characteristic for allergic asthma as it is also found in other chronic inflammatory diseases such as rheumatoid arthritis and chronic inflammatory bowel disease (see e.g. review of Buckley CD *et al.*[5]).
The GINA definition of (allergic) asthma is descriptive and, therefore, imperfect. It does e.g. not include difficult-to-treat patients or asthma severity. However, it is well known that the presentation of asthma severity varies from patient to patient ranging from mild intermittent to severe persistent based on patient's lung function indications, symptoms and medication requirements[2,4]. The characterisation based on these clinical symptoms is not clear-cut leading to potential misclassification of patients[2]. Therefore, it is currently uncertain whether all these subtypes of asthma represent variable expressions of one single disease (i.e. asthma) or that patients represent distinct diseases with similar symptomatology[6].

Guidelines - The practical implication of guidelines that can be used to establish the clinical status of an asthmatic and its pharmacotherapy is challenging.

Implementation is hindered by the variable nature of the disease, minimization of symptoms by patients and a poor compliance to pharmacotherapy (Chapter 1; Section 5)[7]. This had led to the need of identification of objective inflammatory biomarkers to characterise asthma phenotypes and manage pharmacotherapy according to established phenotypes. Recently, much emphasis is directed to the search of disease predicting genes or genetic marker, such as gene ADAM33 on chromosome 20[8] (see also Chapter 1; Section 2). However, at this moment it is not possible to characterize asthma patients based on such gene(tic) candidates, due to the inherent complexity and heterogeneity of the disease[9-11].

Markers - Several (bio)markers that might be useful in the characterisation of asthma patients have been identified during the last decades. These markers are mainly derived from analyses of bronchial biopsies, bronchoalveolar lavage (BAL), sputum induction, breath constituents, urine and/or peripheral blood and include e.g. 1) changes in levels of inflammatory cells, such as eosinophils or neutrophils, in the different compartments, 2) changes in cell-derived protein levels in the BAL, (induced) sputum, urine or blood or 3) changes in exhaled constituents, such as exhaled nitric oxide (NO) (these markers are extensively reviewed in ref.[12-19]). Several of these markers are discussed below and compared with the methods we used in this thesis.

One of the methods used in management of asthma is the measurement of airway hyperresponsiveness (AHR)[20]. However, AHR measurements via determination of PC_{20}-values were not sufficient in our hands to phenotype asthmatics, because we could not detect clear differences in AHR, measured via PC_{20} to either methacholine or histamine, between well-controlled and difficult-to-treat asthmatics (Chapter 3). Another method used to direct pharmacotherapy is on guidance of sputum eosinophils[21-23]. However, this method is not ideal for the establishment of asthma phenotypes that include non-eosinophilic inflammation[24]. Furthermore, this technique can not routinely be used by general practitioners. Measuring exhaled NO is a less invasive method in the management of asthma treatment compared with AHR-measurements and sputum inductions[7,15,22]. On the other hand, exhaled NO has a small potential in the prediction of asthma control and exacerbations and is influenced by changes in lung function[22,23].

Peripheral blood markers - Investigation of (potential) biomarkers in peripheral blood is, next to exhaled NO, a less-invasive method that might be used

in the management of asthma[12]. Peripheral blood markers in asthma include e.g. the levels of inflammatory cells, their activation status and their derived inflammatory mediators[12,16]. Especially eosinophil cationic protein (ECP) levels in peripheral blood have been associated with allergic inflammation and might serve as a biomarker that predicts an increase in asthma severity and disease activation[25,26]. However, ECP is an eosinophil derived protein and might therefore only predict the inflammatory status in a subset of asthmatic phenotypes in which eosinophils are involved[27]. In addition, the method of measuring ECP is variable and very sensitive to environmental factors[28].

Priming of leukocytes as biomarker - The studies in this thesis were started with the realization that we have to develop (novel) biomarkers to better characterize asthma phenotypes that can be used to design tailor-made pharmacotherapy in different patient groups. We approached this research focus by investigation of the (pre-)activation status of peripheral blood leukocytes as a read-out for inflammation in asthmatics with special emphasis on the innate immune cells: eosinophils, neutrophils and monocytes (Chapter 1; Section 4). The activation responses in peripheral blood of asthmatics *in vivo* are subtle and do not lead to clear activation of cells in terms of cytotoxicity. This priming process converts resting leukocytes, which are refractory to activation, into cells that are prone to activation by many (patho)physiological stimuli[29,30]. Priming or pre-activation is a key event in the regulation of leukocyte activation *in vivo*. Under normal conditions, leukocytes exhibit a non-primed phenotype in the circulation. This is an essential condition as it provides protection against non-specific activation of the highly cytotoxic responses of granulocytes and monocytes. However, under various pathological conditions, such as found in chronic inflammatory diseases (e.g. asthma), granulocytes and monocytes are present in the circulation with a primed phenotype (Chapter 2 and 3)[31]. The primed phenotype *in vivo* is very similar to the phenotype observed when normal leukocytes are treated *in vitro* with inflammatory cytokines or chemokines[29,32]. Despite these well-defined observations *in vitro*, little is known about the contribution of priming mechanisms that lead to distinct phenotypes in asthmatics and the effects of allergen exposures on these phenotypes.

We hypothesized that the priming responses on distinct populations of asthmatics reflect different phenotypes of asthma and are, therefore, less-invasive biomarkers to characterize the disease status of these patients. In this thesis *in vivo* priming

was measured by determination of: 1) cellular expression profiles of priming associated epitopes on leukocytes, such as epitopes recognized by monoclonal phage antibodies (MoPhab)s A17 and A27[33] or monoclonal antibodies CD11b (integrin-subunit: αm) and CD62L (L-selectin) or 2) via gene expression analysis. These methods of determination of leukocyte priming seem to have a better potential in the characterisation of the inflammation in asthmatics compared with the direct measurement of single cytokines or chemokines that are associated with priming in peripheral blood, because:

1) Many factors can influence the actual presence of bioactive cytokines and/or chemokines in the systemic compartment such as proper passage of these mediators to the circulation, local degradation of these mediators, their short half-lives, their binding to (decoy) receptors, their systemic clearance and availability of proper bioassays[12,34,35];
2) Leukocyte priming is most probably the result from an interplay between *unique combinations* of inflammatory mediators which results in a synergistic action on target cells. The resulting primed phenotype of leukocytes is, therefore, a better representation of the signals that act on these cells compared with the mere presence of individual mediators;
3) Leukocytes might have picked up signals near or at inflammatory sites that are not present in peripheral blood.

The latter issue (3) might include the recirculation of leukocytes from inflammatory sites back into the circulation. Priming of inflammatory cells, such as eosinophils, in peripheral blood of asthmatics *in vivo* is particularly associated with increased adhesion, transendothelial migration and chemotaxis[36-38]. Therefore, enhanced priming of these cells is thought to result in increased and preferential homing of these cells to the (inflamed) tissues. Data about recirculation of inflammatory cells is scarce but two studies showed convincing evidence that murine eosinophils can leave the lung tissue and home to draining lymph nodes through the lymphatic system[39,40]. A mechanism of recirculation was shown by Buckley *et al*. as they provided evidence that neutrophils can reversely transmigrate over a layer of endothelial cells[41]. This latter study clearly showed that the expression profile of membrane expressed proteins differs between non- and retro-migrated neutrophils.

Well-controlled asthma - Using priming of leukocytes *in vivo* as readout for inflammatory processes in the pulmonary tissue, we identified and confirmed (Chapter 2 and 3)[31] that (mild) asthmatics with well-controlled disease patterns were characterized by a phenotype with clear systemic inflammatory responses that involved primed eosinophils, sublte primed neutrophils and unprimed monocytes in peripheral blood (Fig. 1). The eosinophil and subtle neutrophil priming were further enhanced after allergen challenge in well-controlled asthmatics who experienced a late asthmatic reaction (LAR), which was identified using respectively MoPhab A17 and gene expression analysis. In addition, also the stimulated neutrophil priming, measured via MoPhab A17, was enhanced in these cells of patients with a LAR. This was in contrast to patients with only an early asthmatic reaction (EAR), who did not have enhanced eosinophil or neutrophil priming with or without stimulation after allergen challenge (Chapter 2). This indicates that patients with and without a LAR can be phenotyped on basis of enhanced priming of both eosinophils and neutrophils after allergen challenge.

Poorly-controlled asthma - Poorly-controlled asthmatics, which were relatively difficult-to-treat, were characterised by primed neutrophils and monocytes (Chapter 3). Remarkably, priming of neutrophils was subtle in well-controlled asthmatics, which could only be identified using gene expression analysis (Chapter 2), whereas priming of neutrophils was more pronounced and associated with increased levels of priming associated epitopes in poorly-controlled/difficult-to-treat patients (Chapter 3; Fig. 1). This indicates that several priming stadia can be present in peripheral blood granulocytes of patients with different phenotypes of asthma. It is still unclear whether poorly-controlled asthma is a distinct asthma phenotype or whether it develops from a ‘normal’ controlled asthma by yet undefined

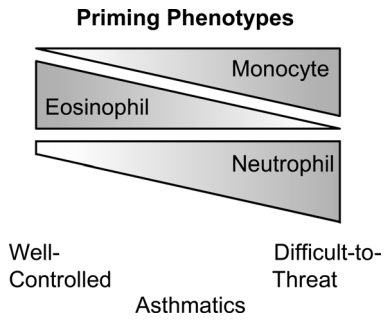

Figure 1. Model for the primed status of leukocytes in asthma phenotypes.

mechanisms. However, potential factors that might have contributed to the development of poorly-controlled asthma include:

1) Poor compliance/suboptimal treatment[42];
2) Adaptation mechanisms caused by long-term treatment with high concentrations of corticosteroids;
3) Unique interactions between certain genotypes of asthma and risk factors in the environment.

Further evidence related to the existence of specific characteristics of circulating leukocytes in distinct asthma phenotypes was shown by the study of Mann and Chung[43]. They showed that severe steroid dependent asthma patients have elevated levels of αm (CD11b) expressed on their peripheral blood neutrophils. Their findings are in agreement with the association of neutrophil inflammation with chronic inflammation in asthmatics[44-47].

Priming responses - The priming responses can be divided into two groups: 1) acute priming, visualized by enhanced expression of priming epitopes recognized by MoPhab A17 (or A27) and 2) chronic or long-term priming, characterized by enhanced expression of integrins (e.g. αm) on leukocytes. The distinction between these two priming-stadia was derived from correlations of celepitope expressions with AHR and leukocyte responses after allergen changes. Acute priming marker A17 did not correlate with AHR but was very sensitive in showing acute priming signals induced by e.g. allergen challenges, while the chronic priming marker αm (CD11b) correlated with AHR and was relatively insensitive for acute signals induced by allergen challenges (Chapter 2).
Eosinophils were characterized by enhanced MoPhab A17 (and A27) priming without enhanced expression of αm (CD11b) in well-controlled asthmatics (Chapter 2 and 3), which suggests that ongoing acute priming signals, induced by a continuous changing environment, are present in mild asthmatics before chronic inflammatory processes occur. As acute priming is not correlated to (changes in) AHR, priming measurements of peripheral blood leukocytes might be more sensitive in prediction of inflammation in asthmatics compared with AHR measurements.

We conclude from these patient data that the determination of priming of leukocytes is a biomarker, which has a high potential in the characterisation of asthma phenotypes and might help the development of useful and more tailor-

made pharmacotherapies for the different asthma phenotypes. Further characterisation of priming associated epitopes of asthmatics will likely reveal even more (different) phenotypes of these patients (e.g. severe vs. mild and moderate) and will help in better stratification of patients' therapies.

Steroid effects on primed granulocytes

To further characterise the cellular mechanisms and effects that are responsive to the inflammation induced priming of granulocytes we evaluated the granulocyte responses to anti-inflammatory steroids. Several studies suggested that steroids can interact with innate immune cells and modulate their functionality[48,49].

Eosinophil apoptosis - A recurrent issue in the literature concerning eosinophil and neutrophil responsiveness to steroids is the potential of steroids to inhibit eosinophil survival[50-55], while they can enhance neutrophil survival[50,51]. We also investigated the effects of steroid treatment on eosinophil apoptosis, but could not find any effect of steroids during overnight (16h) incubation of these cells with steroid dexamethasone (Dex) upon eosinophil survival in the presence or absence of survival cytokines such as IL-5 (Chapter 4). Although the onset time of induction of eosinophil apoptosis by steroids varies between studies[50-54], also others clearly showed that eosinophils can survive incubation with steroids up to 16h whether or not co-incubated with survival cytokines[50,52]. Data derived from studies in which steroid-induced eosinophil apoptosis was investigated in the co-presence of particular survival factors, such as IL-5, suggest that the concentration of such a factor determines the actual outcome of steroid-induced apoptosis[51,53-56]. For example, Bloom *et al.*[56] showed that steroids in combination with suboptimal concentrations of IL-5 reduced cellular survival, but enhanced eosinophil survival in the presence of optimal concentrations of IL-5. In addition, it is reasonable to assume that the variation in effects of steroids on eosinophil apoptosis are dependent on the presence of certain environmental conditions that are poorly defined. For example, subtle differences in donor phenotypes (e.g. donors with atopic diseases or slight eosinophilia vs. normal donors) or cell culture conditions (e.g. distinct compositions of sera and different durations of cellular incubation with steroids) might have contributed to these distinct findings upon eosinophil survival. Interestingly, it seems unlikely that steroids induce significant eosinophil apoptosis

in the airways of asthmatics, because apoptotic eosinophils are suggested to be absent or rarely present in respiratory tract tissue[57]. This issue concerning the absence of apoptotic eosinophils in tissues is still poorly understood, but might in part be explained by:

1) Clearance of eosinophils through mucociliary transport[57];
2) Technical issues concerning determination of apoptosis in tissue dwelling eosinophils, although tissue eosinophil apoptosis could clearly be visualized in tissue obtained from nasal polyps[58];
3) Recirculation of cells (already mentioned above).

Overall, more research is needed to clarify the absence of apoptotic eosinophils in airway tissues and the contribution of steroids to putative induction of eosinophil apoptosis in the lungs of asthma patients.

Neutrophil apoptosis - In contrast to eosinophils, but in agreement with the literature[50,51,59-61], we found that neutrophil survival is enhanced by steroids (Table 1). There were no significant differences between similar concentrations of three investigated steroids (i.e. Dex, beclomethasone dipropionate and methylprednisolone) in induction of neutrophil survival, indicating that these three steroids have on average similar potencies in the induction of neutrophil survival. In contrast, Croxtall *et al.* showed different potencies of these three steroids in inducing genomic or non-genomic effects[62]. They showed that Dex induced both genomic and non-genomic effects, beclomethasone dipropionate induced only genomic effects, while methylprednisolone induced only non-genomic effects in A549 cells treated with different concentrations of steroids (10^{-10}-10^{-5}M) in the co-presence of IL-1. It is currently not known if these three steroids exert their actions via their supposed differential mechanisms in granulocytes. In addition, the time-frame of treatment with steroids might have influenced the onset of genomic and non-genomic actions. Croxtall *et al.* treated the A549 for 3h, while we treated neutrophils for 16h (Table 1).

The steroid enhanced neutrophil survival was dependent on the glucocorticoid receptor (GCR) for the three investigated steroids, because the GCR antagonist RU38486 inhibited steroid-induced neutrophil survival significantly (data not shown). These neutrophil survival responses might be a contributing factor to the neutrophil inflammation that is present in difficult-to-treat asthmatics who received high concentrations of steroids (Chapter 3). Neutrophils are also characterized as cells that do not well respond to steroids. The reason for this low responsiveness is

Table 1. Percentage apoptotic neutrophils after incubation with several concentrations of steroids.[#]

	-	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M	10^{-9}M
Dexamethasone	66.9±1.6	44.7±2.1*	45.2±1.1**	43.8±1.7**	55.6±3.8*	70.9±5.0
Beclomethasone Dipropionate	66.9±1.6	45.2±2.4*	48.1±1.6*	47.2±0.1*	59.9±1.0*	71.2±3.5
Methylprednisolone	66.9±1.6	42.5±1.1*	45.8±2.0**	49.7±0.9**	60.0±2.1	69.9±1.2

[#] Purified neutrophils (<3% eosinophils) were incubated in IMDM (Life Technolgies; Breda, The Netherlands) supplemented with 10% FCS in the presence or absence of several concentrations (10^{-5}M-10^{-9}M) of steroids for 16h at 37°C. Hereafter, the cells were labelled with Annexin-V-FITC (Kordia Life Sciences, Leiden, The Netherlands). The percentage of apoptotic cells was determined as the percentage of annexin-V positive cells using flowcytometry (FACScalibur; Becton & Dickinson, San Jose, CA). Results are shown as mean percentage of apoptotic neutrophils (±SEM) of three independent experiments. Statistical analysis was performed using paired t-tests (SPSS vs. 11.0, Chicago, Ill; *P<0.05, **P<0.005 compared with sample without steroid).

unknown, but might be related to the relative high expression of a decoy GCR in human neutrophils, the GCRβ, which is likely involved in escaping steroid-induced cell death in these cells[63] .

Steroid effects on eosinophil recruitment - As steroids *per se* do not seem to influence eosinophil survival in the presence of priming cytokines, other mechanisms must be operational in mediating the decrease in eosinophil presence in the lung tissue of asthmatics after steroid treatment[64,65]. Our focus was subsequently directed to processes involved in eosinophil recruitment to inflammatory sites, which include eosinophil adhesion and migration mediated by (adhesion) molecules such as β2-integrins[66-69]. Interestingly, steroids inhibited both responses of eosinophils (Chapter 4), provided that the experiments were performed in the presence of priming cytokines. These data are consistent with a model that eosinophils *in vivo* are only inhibited by steroids after priming (or activation) by inflammatory mediators. This mechanism would prevent inappropriate effects of steroids upon eosinophil functions unless they have been pre-activated *in vivo*. These data are a further indication that priming of inflammatory eosinophils is a crucial intermediate state of eosinophil functionality. Priming makes the cells prone to pick up activating or inhibitory signals[70].
The mechanism(s) regulating these steroid-mediated inhibitions of eosinophil adhesion and migration under primed conditions needs to be elucidated, but includes the involvement of the glucocorticoid receptor (Chapter 4). Liu *et al.*[71] showed a central role for annexin-1 expression and cytosolic phospholipase A2

(cPLA2) translocation in steroid-induced inhibition of eosinophil β2-mediated adhesion. In their model steroids block eosinophil adhesion by upregulation of annexin-1 on the plasma membrane, which subsequently leads to blocking of cPLA2 translocation to the nuclear membrane. In contrast to our study, these effects of steroids already occurred in the absence of cytokines. However, they used eosinophils derived from mild atopic patients, which might already have been primed *in vivo* and might therefore, in agreement with our model, be susceptible for steroids. Eosinophils used in our study were from donors with no atopic diseases.

Rapid effects of steroids - Next to the long-term effects of steroids in asthma, several recent studies have shown rapid effects of steroids in this disease. For example, steroid budenoside could inhibit asthma symptoms within 10 min in guinea pigs[72]. We obtained data showing similar rapid effects in human asthmatics treated with fluticasone[73]. Furthermore, acute vasoconstriction in the bronchial vasculature by corticosteroids is thought to be an important mechanism in the resolution of acute asthma complaints by corticosteroid treatment[74,75]. These acute and rapid effects of steroids can occur within 30 min. These responses are likely mediated via non-genomic mechanisms as the first genomic effects of steroids are identified after prolonged incubation (>30 min)[76,77].

However, the exact mechanisms of these rapid effects are poorly defined. Several mechanisms have been proposed by which corticosteroids can induce these rapid effects[76,77]:

1) By specific interaction with the cytosolic corticosteroid receptor resulting in fast but poorly defined mechanisms;
2) By specific interaction with membrane bound corticosteroid receptors, which are not cloned and not (sufficiently) functionally characterized;
3) As a result of non-receptor mediated physicochemical interactions with cellular membranes.

We investigated potential non-genomic effects of steroids on several functional responses of eosinophils. Incubation of eosinophils with Dex for 15 min resulted in a clear functional upregulation of the immunoglobulin (Ig)A receptor (FcαR), without influencing the IgG receptor. In contrast, short term incubation with Dex (<30 min) did not influence eosinophil migration, adhesion or respiratory burst in the presence or absence of IL-5. These data imply that long-term and short-term interactions of eosinophils with steroids lead to different mechanisms: e.g. 1) steroids inhibit long-term priming of eosinophil migration and adhesion whereas

they do not affect short-term induced adhesion; 2) the functionality of the IgA receptor seems hardly influenced after prolonged incubation with steroids,[78] but is enhanced after a short incubation time (15 min) with steroids.
It seems likely that the Dex-induced selective priming of the FcαR will have an important impact on the mechanisms that are operational in the control of eosinophils in allergic diseases such as asthma. Both eosinophils and IgA are abundantly present at mucosal surfaces and IgA is an important secretagogue of these immune cells. In addition, allergen specific IgA is found at both mucosal and systemic sites in asthmatics[79,80]. Eosinophils from asthma patients show increased FcαR activity compared with healthy donors[81]. Therefore, it is tempting to speculate that steroids will potentiate the IgA-induced eosinophil responses in asthmatics.

Inside-out signalling by steroids - Most research regarding eosinophil activation through receptors was focused on the receptor functionality that was based on 'outside-in' regulation of functional responses: i.e. the ability of these receptors to transmit signals ('signal transduction') into the cells after binding of a ligand to the receptor. In immune cells the functionality of several important receptors is controlled by modulating intracellular signals that are induced from heterologous receptors. This so-called 'inside-out' regulation in innate immune cells is particularly evident for the functions of adhesion and immunoglobulin receptors[82-86]. After the identification of the steroid-induced priming of the FcαR we focused on the underlying 'inside-out' signals that mediated this priming response. These investigations showed that steroid-induced FcαR activation was regulated via the GCR, because the response could be inhibited by the steroid receptor antagonist RU38486. Further characterisation of the 'inside-out' signalling that mediated the priming by Dex showed a central role for P38 MAPK in the signal transduction towards the functionality of FcαR. This is in agreement with previous work form our lab in which the 'inside-out' regulation of the FcαR was dependent on activation of intracellular P38 MAPK after cytokine priming[87].

Regulation of P38 MAPK among granulocytes

Because a central role of P38 MAPK was found in the priming of the functionality of the FcαR in eosinophils, we subsequently investigated the regulation of this kinase in these cells. Studies using small inhibitors of P38 MAPK have shown that P38

MAPK might play an important role in the development and persistence of inflammatory diseases, such as asthma[88-91]. In addition, P38 MAPK is often involved in priming of inflammatory cells *in vitro* and *in vivo*[87,92,93]. Therefore, it is reasonable to assume that the P38 MAPK pathway must be regulated properly to control inflammatory responses *in vivo*. We further investigated the regulation of steroid-induced priming of P38 MAPK in eosinophils to understand the underlying mechanism of P38 MAPK activation in these cells.

The most common known upstream kinases involved in P38 MAPK activation and regulation are the MAPK kinase kinases (MKK)3 and MKK6[94]. These MKKs phosphorylate P38 MAPK on a conserved Thr180-X-Tyr182 motif. This phosphorylation of P38 MAPK is associated with increased activation of this kinase towards several downstream kinases such as MAPK-activated protein kinase 2 (MK2). Investigation of the phosphorylation of this target can be used as read-out for the functionality of P38 MAPK *in situ*. The phosphorylation of P38 MAPK mediated via MKKs is insensitive to the P38 MAPK inhibitor SB203580, due to the fact that SB203580 occupies the ATP-binding pocket in this kinase without affecting its phosphorylation sites[95-98].

Recently, two other mechanisms of P38 MAPK activation have been described in which the phosphorylation of P38 MAPK is sensitive to SB203580, indicating that P38 MAPK autophosphorylation occurs. The first mechanism consists of an association of the P38-isoform P38α MAPK with the adaptor protein TAK1-binding protein 1 (TAB1)[94]. Initial studies showed that TAB1 associates with P38 MAPK after cellular activation and that this association is sufficient to induce P38α MAPK autophosphorylation[99-101]. The other mechanism of P38 MAPK autophosphorylation was identified in T-cells and was dependent on the phosphorylation of a previously unrecognized site of P38 MAPK (Tyr323)[102,103].

Although the regulation of P38 MAPK is extensively studied in many cell types, the regulation of P38 MAPK in eosinophils is less clear. The only known upstream activator of P38 MAPK in eosinophils is phophatidylinoisitol 3'-kinase (PI3K), which was identified in IL-4 and IL-5 induced IgA-binding in eosinophils[87]. In Fig. 2 we show that the phosphorylation and activation (determined by investigation of the upper band in the blots that were probed with anti-P-MK2; see Fig. 2) of P38 MAPK by Dex or IL-5 are sensitive to SB203580, indicating that these priming agents regulate P38 MAPK via autophosphorylation in eosinophils.

Further characterisation on the regulation towards P38 MAPK in eosinophils was done in comparison with the regulation of P38 MAPK in human neutrophils. In

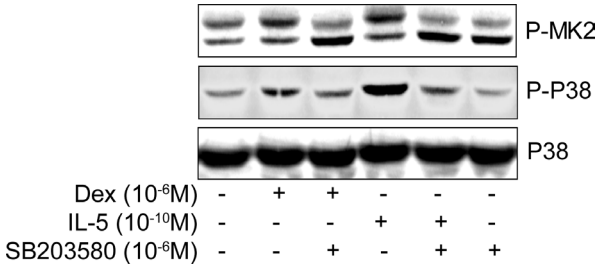

Figure 2. P38 MAPK phosphorylation and activation are mediated via autophosphorylation after priming with Dex or IL-5. Eosinophils ($0.5x10^6$ in HEPES buffered RPMI-1640 medium supplemented with L-glutamine (Life Technologies, Breda, The Netherlands) and 10% FCS) were preincubated with or without P38 MAPK catalytic site inhibitor SB203580 (10^{-6}M) for 15 min and stimulated for 15 min with or without Dex (10^{-6}M) or Il-5 (10^{-10}M). Subsequently, cells were lysed and protein samples were analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P (Millipore; Bedford, MA). The blots were incubated with antiphospho-P38 MAPK (P-P38; 1/1000; Cell Signaling, Beverly, MA) and, antiphospho-MK2 (P-MK2; 1/1000; Cell Signaling, Beverly, MA) or anti-P38 MAPK (P38; 1/1000; Santa Cruz, CA). After incubation with the first antibody, the blots were washed and were incubated with the second antibody (HRP-coupled swine anti-rabbit; 1/3000; Dako, Denmark). Detection of all western blots was performed by enhanced chemiluminescence plus (Amersham; UK) and using a Typhoon 9410 (Amersham; UK). The experiment shown is representative for at least three experiments.

preliminary data we could not identify proper P38 MAPK activation in human neutrophils after 15 min incubation with steroids, suggesting that steroids do not act on neutrophil P38 MAPK in a similar way as steroids do in eosinophils. We, therefore, focused on the comparison with P38 MAPK phosphorylation and activation between these cells using the asthma-related cytokines GM-CSF and TNFα. Receptors of these cytokines are expressed on both eosinophils and neutrophils. We used GM-CSF instead of IL-5 because the GM-CSF receptor is the only member of the common β-chain containing receptors[104,105] that is present on mature neutrophils (Chapter 1; Section 6.1.2.).

In our comparative study (Chapter 6), we showed that activation of MKK3/MKK6 most probably controls P38 MAPK phosphorylation in neutrophils stimulated by TNFα or GM-CSF, because the kinetics of phosphorylation of MKK3/MKK6 and P38 MAPK were similar in stimulated neutrophils[106]. In addition, we showed that this P38 MAPK phosphorylation was insensitive to SB203580 where its activation *in situ,* visualized by MK2 phosphorylation, was sensitive to the inhibitor (Chapter 6). In contrast to neutrophils but in agreement with the data in Fig. 2 we also found that TNFα and GM-CSF induced autophosphorylation of P38 MAPK in eosinophils. Remarkably, the cytokine-induced autophosphorylation of eosinophil P38 MAPK was irrespective of the phosphorylation of MKK3/MKK6, which were constitutively phosphorylated in eosinophils. This suggests that either P38 MAPK can not interact with MKK3/MKK6 (intracellular localisation; see below) or that a crucial

intermediate protein is missing that regulates MKK3/MKK6-mediated activation of P38 MAPK. The latter seems unlikely, because MKK3 and MKK6 can directly activate P38 MAPK[107].

Concerning P38 MAPK isoforms, only P38α MAPK can be involved in the MKK3/MKK6 driven process in neutrophils, because[95,108,109]:

1) only isoforms P38α MAPK and P38δ MAPK are identified in neutrophils;
2) only isoforms P38α MAPK and P38β MAPK can phosphorylate MK2;
3) SB203580 inhibits only isoforms P38α MAPK and P38β MAPK.

For eosinophils, P38 MAPK phosphorylation and activation were sensitive to SB203580 after stimulation with cytokines GM-CSF and TNFα, which indicates that only P38α and/or P38β isoforms of P38 MAPK could be involved.

Further characterisation on the regulation towards autophosphorylation of P38α and/or P38β in eosinophils was performed by investigation of the autophosphorylation processes that occur via either an association of P38 MAPK with TAB1 or Tyr323 phosphorylation in P38 MAPK. Unfortunately, due to the lack of a proper working antibody (similar as used for the identification of P-Tyr323[102]) against the phosphorylated form of Tyr323 we could not study phosphorylation of Tyr323 on P38 MAPK in eosinophils.

Remarkably, we found using co-immunoprecipitations that TAB1 was constitutively associated with P38 MAPK in eosinophils and not only with the phosphorylated form of P38 MAPK after cellular activation with TNFα, GM-CSF (Chapter 6), IL-5 or Dex (data not shown). The regulation of the phosphorylated form of P38 MAPK in association with TAB1 was similar to the regulation of P38 MAPK that was determined in total cell lysates of eosinophils. These data are in contrast to recent literature in which TAB1 was only associated with phosphorylated P38 MAPK after cellular activation[94,99,100]. Currently, the reasons for this discrepancy are unknown but several issues should be taken into account: 1) TAB1 can be a downstream target of P38 MAPK. Cheung *et al.*[110] described TAB1 as a substrate of P38 MAPK and could not find indications for *in vitro* P38 MAPK autophosphorylation or activation via TAB1. They showed that TAB1 is phosphorylated on three sites by P38α *in vitro* and *in vivo* in response to cellular stresses, proinflammatory cytokines and LPS. TAB1 is in this model not responsible for autophosphorylation, but its association is a result of distinct P38 MAPK phosphorylation and activation. 2) TAB1 association with P38 MAPK must be investigated using antibodies against the non-phosphorylated form of P38 MAPK in association studies (in particular

when co-immunoprecipitations or pull-down assays are used). In the study of Kim *et al.*[100] an antibody against the phosphorylated form of P38 MAPK was used in their pull-down assays with TAB1 in mouse macrophages. They can, therefore, not exclude that unphosphorylated P38 was constitutively associated with TAB1 in these inflammatory cells.

It is tempting to hypothesize that TAB1 is responsible for activation of P38 MAPK in eosinophils due to facilitation of the recruitment of the TAB1-P38 MAPK complex to receptor elements, which can transduce signals from activated receptors into the cell. Supportive for this hypothesis is the role of TAB1 in activation of transforming growth factor-beta (TGFβ)-activated kinase 1 (TAK1). Endogenous TAB1 is known to be constitutively associated with TAK1 in unstimulated cells without TAK1 kinase activity[111]. TAK1 is involved as upstream activator of the MAPK and nuclear factor-κB (NF-κB) pathways and has mitogen-activated protein kinase kinase kinase (MAPKKK) properties[111-114]. Upon e.g. IL-1 receptor activation, the TAB1-TAK1 complex is recruited to TNF receptor-associated factor 6 (TRAF6) and TAK1 is subsequently activated via TAB1-mediated TAK1 autophosphorylation[111-114].

In addition, TAB1 interactions with P38 MAPK might lead to a redirection of the localisation of P38 MAPK and lead to (negative) modulation of MKK-dependent P38 MAPK activation[115]. MKK- vs. TAB1-mediated P38 MAPK activation might via these regulatory effects result in different activation of downstream targets of P38 MAPK. Activation of different substrates of P38 MAPK was also suggested for P38 MAPK autophosphorylation when mediated via Tyr323 phosphorylation compared with MKK-dependent P38 MAPK activation in T-cells[103]. Therefore, TAB1 appears to have unique signalling functions:

1) involvement in regulation of MKK-independent activation of P38 MAPK activity and
2) scaffold protein that modulates intracellular localisation and signal complex interactions of the activated P38 MAPK[115].

A (re)direction of the TAB1-P38 MAPK complex is most likely involved in eosinophil P38 MAPK activation, because this P38 MAPK activation is most probably not regulated via MKK3/MKK6. In comparison with eosinophils, neutrophils could have both a MKK-dependent and TAB1-dependent pathway towards P38 MAPK (Chapter 6). Further research is needed 1) to identify if these two pathways lead to different activation of downstream targets of P38 MAPK in neutrophils and 2) to identify the specific targets of P38 MAPK mediated via TAB1 binding to this kinase in both neutrophils and eosinophils.

P38 MAPK inhibition as therapeutic intervention

Current treatment options for asthma are mainly directed to bronchodilation through β2-agonists and inhibition of inflammation by glucocorticoids. Poor compliance to steroid therapy and the potential of induction of adverse effects by steroids have encouraged the search for new therapeutic options in the management of asthma (reviewed in ref.[116]). In addition, approximately 5-10% of asthma patients are still poorly-controlled by current therapies.

Successful development of new therapeutic options is mainly achieved by improving corticosteroids. On the other hand, the development of new compounds, which include single mediator antagonists and biologicals that antagonize cytokine mediated signals, is still very disappointing. The reasons for this lack of success are uncertain, but it is likely that distinct (poorly) recognized phenotypes of asthma are differentially sensitive for these new compounds. In addition, the pathogenesis of asthma is likely caused by a complex network of inflammatory signals without involvement of a single dominant signal. Therefore, these new single mediator antagonists are considered to be too specific to be effective in complex inflammatory diseases such as asthma[116]. This is underlined by the work in this thesis, in which the data suggest that priming mechanisms reflect ongoing inflammation, which is most probably the result of an interplay between many inflammatory mediators and not just of one mediator. Inhibition of a common (most probably intracellular) point of convergence for multiple signalling processes from these mediators might therefore be an important strategy in the design of new anti-inflammatory drugs. One of these targets is P38 MAPK, which regulates the expression of several inflammatory mediators that are relevant to asthma and can also be activated by several of these pro-inflammatory mediators (e.g. cytokines)[116-118]. Although the overall effect of P38 MAPK inhibition might be beneficial in inflammatory diseases such as asthma, careful evaluation of the effects of P38 MAPK inhibition is needed, because several P38 MAPK-dependent responses can also be beneficial to the resolution or modulation of these diseases[118]. But, as already mentioned above, the use of small inhibitors of P38 MAPK has already identified a central role of this protein in several inflammatory processes that are also seen in asthma[88-91,117].

Regarding safety concerns about the use of P38 MAPK inhibitors, Branger *et al*. showed that the P38 MAPK inhibitor they used in their study (BIRB 796 BS) could

safely be used in humans (*in vivo*) to decrease inflammatory responses[119].
Interestingly, blocking of P38 MAPK seems to have a preferential inhibitory effect on synthesis of Th2-cytokines compared with Th1-cytokines, which is a further indication that blocking of P38 MAPK can be an important new therapeutic option in the treatment of atopic diseases[116,120]. Furthermore, inhibition of P38 MAPK decreases the survival capacity of eosinophils[121] and neutrophils[122] via activation of apoptotic pathways. Potentially, blocking P38 MAPK activity might also help to decrease the onset of steroid-resistance, because activated P38 MAPK was shown to have the potential of inhibiting the glucocorticoid receptor[123,124]. Inhibition of P38 MAPK might also have an additional advantage in the resolution of the inflammatory responses in asthmatics due to downregulation of eosinophil FcαR-priming mediated either via cytokines or steroids. Therefore, inhibitors that reduce a common point of convergence of several inflammatory pathways, such as P38 MAPK, can be very efficacious in treatment of asthma.

Conclusions

1) *Different phenotypes of allergic asthma are associated with distinct priming profiles of inflammatory cells in peripheral blood (Chapters 2 and 3; Fig. 1).*
2) *Glucocorticoids inhibit primed eosinophil migratory and adhesive responses after prolonged incubation (16 hrs) without influencing these responses under non-primed conditions (Chapter 4).*
3) *Glucocorticosteroids can rapidly prime innate immune cells via inside-out regulation of functional receptors, such as FcαR (Chapter 5).*
4) *P38 MAPK activation is distinctly regulated between neutrophils and eosinophils (Chapter 6).*

Future Perspectives

Studies performed in this thesis have provided evidence for distinct systemic responses of leukocytes in different phenotypes of asthma and gave more insight in the regulation of leukocyte priming and the effects of steroids on leukocyte functions. Despite these thorough investigations, the data presented in this thesis have raised several interesting questions for additional studies:

- Are priming profiles of peripheral blood leukocytes distinctly regulated in increased severity of asthma (from mild to severe)?
- Is long-term treatment with high doses of steroids causing the redirection of the treatment responsive Th2/eosinophil-type of allergic asthma towards a more treatment resistant neutrophil/monocyte-type of asthma?
- Is short-term corticosteroid-induced upregulation of the mucosal immune response (IgA binding responses of eosinophils) beneficial or detrimental to the inflammatory processes that occur in asthma?
- What are the upstream activators of P38 MAPK from either cytokine receptors or the glucocorticoid receptor in eosinophils?
- What is the functional role of the TAB1 association with P38 MAPK in granulocytes?
- Is the differential activation of P38 MAPK among granulocytes causing different downstream effects in eosinophils and neutrophils?
- Can therapeutic treatment with P38 MAPK inhibitors reduce chronic inflammation in asthmatics?

References

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM: Asthma. From bronchoconstriction to airways inflammation and remodeling. Am.J.Respir.Crit Care Med. 161: 1720-1745, 2000
2. Fabbri LM, Stoloff S: Is mild asthma really 'mild'? Int.J.Clin.Pract. 59: 692-703, 2005
3. Skrepnek GH, Skrepnek SV: Epidemiology, clinical and economic burden, and natural history of chronic obstructive pulmonary disease and asthma. Am.J.Manag.Care 10: S129-S138, 2004
4. National Heart, Lung, and Blood Institute. Global strategy for asthma management and prevention NHLBI/WHO Workshop Report. 1995. National Institutes of Health. Updated April, 2002. National Institutes of Health Bethesda, MD. Publication No. 95-3659.
5. Buckley CD, Filer A, Haworth O, Parsonage G, Salmon M: Defining a role for fibroblasts in the persistence of chronic inflammatory joint disease. Ann.Rheum.Dis. 63 Suppl 2: ii92-ii95, 2004
6. Bel EH: Clinical phenotypes of asthma. Curr.Opin.Pulm.Med. 10: 44-50, 2004
7. Deykin A: Targeting biologic markers in asthma-is exhaled nitric oxide the bull's-eye? N.Engl.J.Med. 352: 2233-2235, 2005
8. Howard TD, Postma DS, Jongepier H, Moore WC, Koppelman GH, Zheng SL, Xu J, Bleecker ER, Meyers DA: Association of a disintegrin and metalloprotease 33 (ADAM33) gene with asthma in ethnically diverse populations. J.Allergy Clin.Immunol. 112: 717-722, 2003
9. Carroll W: Asthma genetics: pitfalls and triumphs. Paediatr.Respir.Rev. 6: 68-74, 2005
10. Illig T, Wjst M: Genetics of asthma and related phenotypes. Paediatr.Respir.Rev. 3: 47-51, 2002
11. Ober C: Perspectives on the past decade of asthma genetics. J.Allergy Clin.Immunol. 116: 274-278, 2005
12. Bousquet J, Corrigan CJ, Venge P: Peripheral blood markers: evaluation of inflammation in asthma. Eur.Respir.J.Suppl 26: 42S-48S, 1998
13. Fabbri LM, Durham S, Holgate ST, O'Byrne PM, Postma DS: Assessment of airway inflammation: an

overview. Eur.Respir.J.Suppl 26: 6S-8S, 1998
14. Kips JC, Fahy JV, Hargreave FE, Ind PW, In 't Veen JC: Methods for sputum induction and analysis of induced sputum: a method for assessing airway inflammation in asthma. Eur.Respir.J.Suppl 26: 9S-12S, 1998
15. Gustafsson LE: Exhaled nitric oxide as a marker in asthma. Eur.Respir.J.Suppl 26: 49S-52S, 1998
16. Virchow JC, Jr., Kroegel C, Walker C, Matthys H: Cellular and immunological markers of allergic and intrinsic bronchial asthma. Lung 172: 313-334, 1994
17. Koller DY: Sampling methods: urine/blood analysis. Am.J.Respir.Crit Care Med. 162: S31-S33, 2000
18. Kharitonov SA, Barnes PJ: Exhaled markers of pulmonary disease. Am.J.Respir.Crit Care Med. 163: 1693-1722, 2001
19. Scheinmann P, Pedersen S, Warner JO, De Blic J: Methods for assessment of airways inflammation: paediatrics. Eur.Respir.J.Suppl 26: 53S-58S, 1998
20. Sont JK, Willems LN, Bel EH, Van Krieken JH, Vandenbroucke JP, Sterk PJ: Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am.J.Respir.Crit Care Med. 159: 1043-1051, 1999
21. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID: Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet 360: 1715-1721, 2002
22. Brightling CE, Green RH, Pavord ID: Biomarkers predicting response to corticosteroid therapy in asthma. Treat.Respir.Med. 4: 309-316, 2005
23. Jatakanon A, Lim S, Barnes PJ: Changes in sputum eosinophils predict loss of asthma control. Am.J.Respir.Crit Care Med. 161: 64-72, 2000
24. Simpson JL, Scott R, Boyle MJ, Gibson PG: Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology. 11: 54-61, 2006
25. Sorkness C, McGill K, Busse WW: Evaluation of serum eosinophil cationic protein as a predictive marker for asthma exacerbation in patients with persistent disease. Clin.Exp.Allergy 32: 1355-1359, 2002
26. Venge P, Bystrom J, Carlson M, Hakansson L, Karawacjzyk M, Peterson C, Seveus L, Trulson A: Eosinophil cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. Clin.Exp.Allergy 29: 1172-1186, 1999
27. Venge P: Monitoring the allergic inflammation. Allergy 59: 26-32, 2004
28. Pronk-Admiraal CJ, Bartels PC: Effect of clotting temperature and eosinophil concentration on the eosinophil cationic protein concentration in serum. Scand.J.Clin.Lab Invest 54: 185-188, 1994
29. Coffer PJ, Koenderman L: Granulocyte signal transduction and priming: cause without effect? Immunol.Lett. 57: 27-31, 1997
30. Hallett MB, Lloyds D: Neutrophil priming: the cellular signals that say 'amber' but not 'green'. Immunol.Today 16: 264-268, 1995
31. Luijk B, Lindemans CA, Kanters D, Van der Heijde R, Bertics P, Lammers JW, Bates ME, Koenderman L: Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J.Allergy Clin.Immunol. 115: 997-1003, 2005
32. Koenderman L, Van der Bruggen T, Schweizer RC, Warringa RA, Coffer P, Caldenhoven E, Lammers JW, Raaijmakers JA: Eosinophil priming by cytokines: from cellular signal to in vivo modulation. Eur.Respir.J.Suppl 22: 119s-125s, 1996
33. Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, De Kruif J, Logtenberg T: Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. J.Leukoc.Biol. 68: 58-64, 2000
34. Diez-Ruiz A, Tilz GP, Zangerle R, Baier-Bitterlich G, Wachter H, Fuchs D: Soluble receptors for tumour necrosis factor in clinical laboratory diagnosis. Eur.J.Haematol. 54: 1-8, 1995
35. Bemelmans MH, van Tits LJ, Buurman WA: Tumor necrosis factor: function, release and clearance. Crit Rev.Immunol. 16: 1-11, 1996
36. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L: In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 79: 1836-1841, 1992

37. Sehmi R, Cromwell O, Wardlaw AJ, Moqbel R, Kay AB: Interleukin-8 is a chemo-attractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. Clin.Exp.Allergy 23: 1027-1036, 1993
38. Moser R, Fehr J, Olgiati L, Bruijnzeel PL: Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. Blood 79: 2937-2945, 1992
39. Shi HZ, Humbles A, Gerard C, Jin Z, Weller PF: Lymph node trafficking and antigen presentation by endobronchial eosinophils. J.Clin.Invest 105: 945-953, 2000
40. Van Rijt LS, Vos N, Hijdra D, De Vries VC, Hoogsteden HC, Lambrecht BN: Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells. J.Immunol. 171: 3372-3378, 2003
41. Buckley CD, Ross EA, McGettrick HM, Osborne CE, Haworth O, Schmutz C, Stone PC, Salmon M, Matharu NM, Vohra RK, Nash GB, Rainger GE: Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. J.Leukoc.Biol. 79: 303-311, 2006
42. Strek ME: Difficult asthma. Proc.Am.Thorac.Soc. 3: 116-123, 2006
43. Mann BS, Chung KF: Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. Respir.Res. 7: 59, 2006
44. Wenzel SE, Balzar S, Cundall M, Chu HW: Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J.Allergy Clin.Immunol. 111: 1345-1352, 2003
45. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ: Neutrophilic inflammation in severe persistent asthma. Am.J.Respir.Crit Care Med. 160: 1532-1539, 1999
46. 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. 22: 470-477, 2003
47. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ: Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am.J.Respir.Crit Care Med. 156: 737-743, 1997
48. Belvisi MG: Regulation of inflammatory cell function by corticosteroids. Proc.Am.Thorac.Soc. 1: 207-214, 2004
49. Caramori G, Adcock I: Anti-inflammatory mechanisms of glucocorticoids targeting granulocytes. Curr.Drug Targets.Inflamm.Allergy 4: 455-463, 2005
50. Meagher LC, Cousin JM, Seckl JR, Haslett C: Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J.Immunol. 156: 4422-4428, 1996
51. Zhang X, Moilanen E, Adcock IM, Lindsay MA, Kankaanranta H: Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. Life Sci. 71: 1523-1534, 2002
52. Lamas AM, Leon OG, Schleimer RP: Glucocorticoids inhibit eosinophil responses to granulocyte-macrophage colony-stimulating factor. J.Immunol. 147: 254-259, 1991
53. Wallen N, Kita H, Weiler D, Gleich GJ: Glucocorticoids inhibit cytokine-mediated eosinophil survival. J.Immunol. 147: 3490-3495, 1991
54. Zhang X, Moilanen E, Kankaanranta H: Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide, and beclomethasone. Eur.J.Pharmacol. 406: 325-332, 2000
55. Adachi T, Motojima S, Hirata A, Fukuda T, Kihara N, Kosaku A, Ohtake H, Makino S: Eosinophil apoptosis caused by theophylline, glucocorticoids, and macrolides after stimulation with IL-5. J.Allergy Clin.Immunol. 98: S207-S215, 1996
56. Bloom JW, Chacko J, Lohman IC, Halonen M, Martinez FD, Miesfeld RL: Differential control of eosinophil survival by glucocorticoids. Apoptosis. 9: 97-104, 2004
57. Uller L, Persson CG, Kallstrom L, Erjefalt JS: Lung tissue eosinophils may be cleared through luminal entry rather than apoptosis: effects of steroid treatment. Am.J.Respir.Crit Care Med. 164: 1948-1956, 2001
58. Davidsson A, Anderson T, Hellquist HB: Apoptosis and phagocytosis of tissue-dwelling eosinophils in sinonasal polyps. Laryngoscope 110: 111-116, 2000

59. Liles WC, Dale DC, Klebanoff SJ: Glucocorticoids inhibit apoptosis of human neutrophils. Blood 86: 3181-3188, 1995
60. Cox G: Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. J.Immunol. 154: 4719-4725, 1995
61. Zhang X, Moilanen E, Kankaanranta H: Beclomethasone, budesonide and fluticasone propionate inhibit human neutrophil apoptosis. Eur.J.Pharmacol. 431: 365-371, 2001
62. Croxtall JD, Van Hal PT, Choudhury Q, Gilroy DW, Flower RJ: Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br.J.Pharmacol. 135: 511-519, 2002
63. Strickland I, Kisich K, Hauk PJ, Vottero A, Chrousos GP, Klemm DJ, Leung DY: High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. J.Exp.Med. 193: 585-593, 2001
64. Giembycz MA, Lindsay MA: Pharmacology of the eosinophil. Pharmacol.Rev. 51: 213-340, 1999
65. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST: Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. Am.Rev.Respir.Dis. 145: 669-674, 1992
66. Resnick MB, Weller PF: Mechanisms of eosinophil recruitment. Am.J.Respir.Cell Mol.Biol. 8: 349-355, 1993
67. Weber C, Kitayama J, Springer TA: Differential regulation of beta 1 and beta 2 integrin avidity by chemoattractants in eosinophils. Proc.Natl.Acad.Sci.U.S.A 93: 10939-10944, 1996
68. Yamamoto H, Sedgwick JB, Busse WW: Differential regulation of eosinophil adhesion and transmigration by pulmonary microvascular endothelial cells. J.Immunol. 161: 971-977, 1998
69. Yamamoto H, Nagata M: Regulatory mechanisms of eosinophil adhesion to and transmigration across endothelial cells by alpha4 and beta2 integrins. Int.Arch.Allergy Immunol. 120 Suppl 1: 24-26, 1999
70. Grutters JC, Brinkman L, Aslander MM, Van den Bosch JM, Koenderman L, Lammers JW: Asthma therapy modulates priming-associated blood eosinophil responsiveness in allergic asthmatics. Eur.Respir.J. 14: 915-922, 1999
71. Liu J, Zhu X, Myo S, Lambertino AT, Xu C, Boetticher E, Munoz NM, Sano M, Cordoba M, Learoyd J, Meliton A, Johnson M, Leff AR: Glucocorticoid-induced surface expression of annexin 1 blocks beta2-integrin adhesion of human eosinophils to intercellular adhesion molecule 1 surrogate protein. J.Allergy Clin.Immunol. 115: 493-500, 2005
72. Zhou J, Kang ZM, Xie QM, Liu C, Lou SJ, Chen YZ, Jiang CL: Rapid nongenomic effects of glucocorticoids on allergic asthma reaction in the guinea pig. J.Endocrinol. 177: R1-R4, 2003
73. Luijk B, Kempsford RD, Wright AM, Zanen P, Lammers JW: Duration of effect of single-dose inhaled fluticasone propionate on AMP-induced bronchoconstriction. Eur.Respir.J. 23: 559-564, 2004
74. Kumar SD, Brieva JL, Danta I, Wanner A: Transient effect of inhaled fluticasone on airway mucosal blood flow in subjects with and without asthma. Am.J.Respir.Crit Care Med. 161: 918-921, 2000
75. Mendes ES, Pereira A, Danta I, Duncan RC, Wanner A: Comparative bronchial vasoconstrictive efficacy of inhaled glucocorticosteroids. Eur.Respir.J. 21: 989-993, 2003
76. Goulding NJ: The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus. Curr.Opin.Pharmacol. 4: 629-636, 2004
77. Buttgereit F, Scheffold A: Rapid glucocorticoid effects on immune cells. Steroids 67: 529-534, 2002
78. Kita H, Abu-Ghazaleh R, Sanderson CJ, Gleich GJ: Effect of steroids on immunoglobulin-induced eosinophil degranulation. J.Allergy Clin.Immunol. 87: 70-77, 1991
79. Pilette C, Durham SR, Vaerman JP, Sibille Y: Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A? Proc.Am.Thorac.Soc. 1: 125-135, 2004
80. Delacroix DL, Dive C, Rambaud JC, Vaerman JP: IgA subclasses in various secretions and in serum. Immunology 47: 383-385, 1982
81. Bracke M, Van de Graaf E, Lammers JW, Coffer PJ, Koenderman L: In vivo priming of FcalphaR functioning on eosinophils of allergic asthmatics. J.Leukoc.Biol. 68: 655-661, 2000
82. Kinashi T: Intracellular signalling controlling integrin activation in lymphocytes. Nat.Rev.Immunol. 5: 546-559, 2005

83. Bracke M, Lammers JW, Coffer PJ, Koenderman L: Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. Blood 97: 3478-3483, 2001
84. Nagarajan S, Fifadara NH, Selvaraj P: Signal-specific activation and regulation of human neutrophil Fc gamma receptors. J.Immunol. 174: 5423-5432, 2005
85. Ginsberg MH, Partridge A, Shattil SJ: Integrin regulation. Curr.Opin.Cell Biol. 17: 509-516, 2005
86. Ortiz-Stern A, Rosales C: Cross-talk between Fc receptors and integrins. Immunol.Lett. 90: 137-143, 2003
87. Bracke M, Coffer PJ, Lammers JW, Koenderman L: Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. J.Immunol. 161: 6768-6774, 1998
88. Wong WS: Inhibitors of the tyrosine kinase signaling cascade for asthma. Curr.Opin.Pharmacol. 5: 264-271, 2005
89. Barnes PJ: Novel signal transduction modulators for the treatment of airway diseases. Pharmacol.Ther. 109: 238-245, 2006
90. Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW, Griswold DE: SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. J.Pharmacol.Exp.Ther. 293: 281-288, 2000
91. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL: Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201, 2000
92. Brown GE, Stewart MQ, Bissonnette SA, Elia AE, Wilker E, Yaffe MB: Distinct ligand-dependent roles for p38 MAPK in priming and activation of the neutrophil NADPH oxidase. J.Biol.Chem. 279: 27059-27068, 2004
93. Wyman TH, Dinarello CA, Banerjee A, Gamboni-Robertson F, Hiester AA, England KM, Kelher M, Silliman CC: Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. J.Leukoc.Biol. 72: 401-409, 2002
94. Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J: MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295: 1291-1294, 2002
95. Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL: Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47: 185-201, 2000
96. Kumar S, Jiang MS, Adams JL, Lee JC: Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem.Biophys.Res.Commun. 263: 825-831, 1999
97. Gum RJ, McLaughlin MM, Kumar S, Wang Z, Bower MJ, Lee JC, Adams JL, Livi GP, Goldsmith EJ, Young PR: Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. J.Biol.Chem. 273: 15605-15610, 1998
98. Frantz B, Klatt T, Pang M, Parsons J, Rolando A, Williams H, Tocci MJ, O'Keefe SJ, O'Neill EA: The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. Biochemistry 37: 13846-13853, 1998
99. Tanno M, Bassi R, Gorog DA, Saurin AT, Jiang J, Heads RJ, Martin JL, Davis RJ, Flavell RA, Marber MS: Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. Circ.Res. 93: 254-261, 2003
100. Kim L, Del Rio L, Butcher BA, Mogensen TH, Paludan SR, Flavell RA, Denkers EY: p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. J.Immunol. 174: 4178-4184, 2005
101. Zhou H, Zheng M, Chen J, Xie C, Kolatkar AR, Zarubin T, Ye Z, Akella R, Lin S, Goldsmith EJ, Han J: Determinants That Control the Specific Interactions between TAB1 and p38{alpha}. Mol.Cell Biol. 26: 3824-3834, 2006
102. Salvador JM, Mittelstadt PR, Guszczynski T, Copeland TD, Yamaguchi H, Appella E, Fornace AJ, Jr., Ashwell JD: Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases.

Nat.Immunol. 6: 390-395, 2005
103. Rudd CE: MAPK p38: alternative and nonstressful in T cells. Nat.Immunol. 6: 368-370, 2005
104. Geijsen N, Koenderman L, Coffer PJ: Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. Cytokine Growth Factor Rev. 12: 19-25, 2001
105. Dewachi O, Joubert P, Hamid Q, Lavoie JP: Expression of interleukin (IL)-5 and IL-9 receptors on neutrophils of horses with heaves. Vet.Immunol.Immunopathol. 109: 31-36, 2006
106. Suzuki K, Hino M, Hato F, Tatsumi N, Kitagawa S: Cytokine-specific activation of distinct mitogen-activated protein kinase subtype cascades in human neutrophils stimulated by granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha. Blood 93: 341-349, 1999
107. Keesler GA, Bray J, Hunt J, Johnson DA, Gleason T, Yao Z, Wang SW, Parker C, Yamane H, Cole C, Lichenstein HS: Purification and activation of recombinant p38 isoforms alpha, beta, gamma, and delta. Protein Expr.Purif. 14: 221-228, 1998
108. Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR: Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. Biochem.Biophys.Res.Commun. 235: 533-538, 1997
109. Hale KK, Trollinger D, Rihanek M, Manthey CL: Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. J.Immunol. 162: 4246-4252, 1999
110. Cheung PC, Campbell DG, Nebreda AR, Cohen P: Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha. EMBO J. 22: 5793-5805, 2003
111. Kishimoto K, Matsumoto K, Ninomiya-Tsuji J: TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. J.Biol.Chem. 275: 7359-7364, 2000
112. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K: The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 398: 252-256, 1999
113. Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K: TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol.Cell 5: 649-658, 2000
114. Sakurai H, Miyoshi H, Mizukami J, Sugita T: Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. FEBS Lett. 474: 141-145, 2000
115. Lu G, Kang YJ, Han J, Herschman HR, Stefani E, Wang Y: TAB-1 modulates intracellular localization of p38 MAP kinase and downstream signaling. J.Biol.Chem. 281: 6087-6095, 2006
116. Barnes PJ: New drugs for asthma. Nat.Rev.Drug Discov. 3: 831-844, 2004
117. Kumar S, Boehm J, Lee JC: p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat.Rev.Drug Discov. 2: 717-726, 2003
118. Newton R, Holden N: Inhibitors of p38 mitogen-activated protein kinase: potential as anti-inflammatory agents in asthma? BioDrugs. 17: 113-129, 2003
119. Branger J, Van den Blink B, Weijer S, Madwed J, Bos CL, Gupta A, Yong CL, Polmar SH, Olszyna DP, Hack CE, Van Deventer SJ, Peppelenbosch MP, Van der Poll T: Anti-inflammatory effects of a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. J.Immunol. 168: 4070-4077, 2002
120. Schafer PH, Wadsworth SA, Wang L, Siekierka JJ: p38 alpha mitogen-activated protein kinase is activated by CD28-mediated signaling and is required for IL-4 production by human CD4+CD45RO+ T cells and Th2 effector cells. J.Immunol. 162: 7110-7119, 1999
121. Kankaanranta H, De Souza PM, Barnes PJ, Salmon M, Giembycz MA, Lindsay MA: SB 203580, an inhibitor of p38 mitogen-activated protein kinase, enhances constitutive apoptosis of cytokine-deprived human eosinophils. J.Pharmacol.Exp.Ther. 290: 621-628, 1999
122. Alvarado-Kristensson M, Porn-Ares MI, Grethe S, Smith D, Zheng L, Andersson T: p38 Mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities have opposite effects on human neutrophil apoptosis. FASEB J. 16: 129-131, 2002
123. Szatmary Z, Garabedian MJ, Vilcek J: Inhibition of glucocorticoid receptor-mediated transcriptional

activation by p38 mitogen-activated protein (MAP) kinase. J.Biol.Chem. 279: 43708-43715, 2004

124. Irusen E, Matthews JG, Takahashi A, Barnes PJ, Chung KF, Adcock IM: p38 Mitogen-activated protein kinase-induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid-insensitive asthma. J.Allergy Clin.Immunol. 109: 649-657, 2002

Chapter 8

Samenvatting in het Nederlands

Astma

Astma is een chronische ziekte van de luchtwegen waar ongeveer 300 miljoen mensen wereldwijd last van hebben. De verspreiding van deze ziekte varieert van 2 tot 18% tussen landen. Er is een stijging in het aantal mensen dat astmaklachten krijgt. Hoewel er geen duidelijke verklaring voor deze stijging is, lijkt het erop dat de stijging mede wordt veroorzaakt door een toename in verstedelijking en doordat steeds meer gemeenschappen moderne levensstijlen aannemen.

Astma wordt gekenmerkt door vernauwing van de luchtwegen door prikkelende stoffen (allergenen). Het merendeel van de astmapatiënten is allergisch voor huisstofmijt, honden, katten, stuifmeel, gras- en/of boompollen. Daarnaast kan een astma-aanval optreden na een zware inspanning of na inhalering van koude lucht, maar ook door het gebruik van bepaalde medicijnen, zoals aspirine. Kenmerkend voor de vernauwing van de luchtwegen bij astma is dat dit samengaat met een piepende ademhaling, kortademigheid, benauwdheid, hoesten en het opgeven van slijm.

De ernst van de ziekte varieert per patiënt en verschilt van licht tot levensbedreigend. Sommige patiënten hebben dan ook continu klachten, terwijl anderen geen of weinig luchtwegklachten ondervinden tussen de aanvallen. Doordat de aanvallen onprettig en beangstigend zijn heeft astma gevolgen voor de kwaliteit van leven en kan het de dagelijkse activiteiten beïnvloeden. Meestal ontstaan de klachten bij astmapatiënten al op jonge leeftijd, maar ook is het mogelijk dat astmasymptomen pas op latere leeftijd optreden. Hoewel veel mensen deze ziekte hebben, is astma nog niet te genezen. Door het gebruik van geneesmiddelen kunnen de klachten verlicht en onder controle gehouden worden. De medicatie die een astmapatiënt gebruikt hangt samen met de ernst van de ziekte. In veel gevallen worden luchtwegverwijders gebruikt tijdens astma-aanvallen. Binnen deze klasse van medicijnen vallen de β2-agonisten. Omdat allergisch astma een ziekte is die samen gaat met een chronische ontsteking van de luchtwegen moeten veel patiënten ook ontstekingsremmers innemen. Deze medicatie dient, indien voorgeschreven, ook ingenomen te worden als de klachten niet aanwezig zijn, om zo de onderliggende ontsteking te remmen en de kans op aanvallen te verminderen. Binnen deze klasse van medicijnen worden vaak

ontstekingsremmende corticosteroïden voorgeschreven. Zowel de β2-agonisten als de corticosteroïden worden veelal ingenomen via inhalatoren.

Duidelijke oorzaken voor het krijgen van astma zijn nog niet aan te wijzen, maar diverse factoren zijn reeds beschreven die de kans op het krijgen van astma vergroten. Een van deze factoren is erfelijkheid. Diverse studies hebben aangetoond dat bepaalde genetische factoren de kans op astma vergroten. Indien ouders deze genetische factoren bezitten, is de kans groter dat kinderen ook astma krijgen. Echter het lijkt er sterk op dat er niet één, maar meerdere genetische factoren nodig zijn voor het verkrijgen van een verhoogde kans op astma. Naast erfelijkheid blijkt ook de samenstelling van omgevingsfactoren de kans op astma te beïnvloeden. Zo zijn o.a. theorieën ontwikkeld die stellen dat een toename in hygiëne en/of een tekort aan blootstelling aan ziekteverwekkers ervoor zorgt dat kinderen een verhoogde kans op astma hebben. Daarnaast lijkt blootstelling aan (tabaks)rook en het gebruik van bepaalde diëten de kans op astma te vergroten. Hoewel voor geen van de risicofactoren een 100% causale relatie bestaat met de ontwikkeling van astma, is het zeer waarschijnlijk dat een combinatie van genetische en omgevingsfactoren ervoor zorgt dat astma ontstaat.

Ontsteking bij astma

De reactie met allergenen zorgt er in de luchtwegen van astmapatiënten voor dat, naast het samentrekken van de spieren van de luchtwegen (vernauwing) en het opzwellen van de slijmvliezen (verhoogde slijmproductie), een complex ontstekingsproces plaatsvindt. Dit proces wordt in gang gezet doordat ontstekingsbevorderende stoffen vrijkomen in de longen na allergeen inhalatie. Deze stoffen zorgen er o.a. voor dat inflammatoire cellen uit het bloed naar de longen gaan en er een scala aan inflammatoire interacties plaatsvindt. De ontstane ontsteking kan chronisch worden. Dit chronische proces kan ertoe leiden dat er veranderingen optreden in de structuur van het longweefsel, wat resulteert in een verlaging van de longfunctie bij een astmapatiënt.
Hoewel de ontsteking bij astmapatiënten zich voornamelijk lijkt te concentreren in het longweefsel, is het aannemelijk dat de ontsteking ook plaatsvindt buiten de longen in het bloed (het systemisch compartiment). Zo zijn bijvoorbeeld inflammatoire stoffen in het bloed gemeten die verhoogd aanwezig zijn bij

astmapatiënten en niet bij gezonde controles. Daarnaast zijn in het bloed de ontstekingscellen bij astmapatiënten verhoogd gevoelig voor het uittreden naar de longen en reageren deze cellen overmatig op een activerende stimulus t.o.v. cellen van gezonde personen. Deze verhoogde gevoeligheid wordt pre-activatie of 'priming' genoemd. Priming van ontstekingscellen lijkt een essentiële tussenstap te zijn voor het lichaam om ervoor te zorgen dat deze cellen niet direct door ontstekingsstoffen geactiveerd worden, maar dat ze verhoogd gevoelig zijn om op de plek van bestemming (waar de activerende ontstekingsstoffen aanwezig zijn) actief te zijn. Het bepalen van de priming van deze cellen in het bloed is een goede uitleesmogelijkheid voor de mate van activatie van de ontsteking bij astmapatiënten.

De effecten van allergenen kunnen bij astmapatiënten bestudeerd worden aan de hand van allergeen provocatie testen. Hierbij inhaleren astmapatiënten allergenen en kan het verloop van de reactie op deze stoffen bepaald worden door diverse uitleesmogelijkheden, zoals via het meten van diverse longfunctieparameters, maar ook via het bepalen van priming van ontstekingscellen in het bloed. Na het inhaleren van een allergeen ontstaat bij de astmapatiënten een vroege reactie (binnen een uur). Bij een deel van de patiënten treedt ook een late reactie op (tussen 3 en 24 uur, optimaal 6-8 uur na provocatie). De processen die bij de late reactie een rol spelen lijken veel op die van de chronische ontsteking bij astma. Zo gaan o.a. inflammatoire cellen vanuit het bloed naar de longen.

Het uittreden van cellen vanuit het bloed naar de longen geschiedt d.m.v. verschillende fasen: Fase 1. Cellen in het bloed worden afgeremd in de bloedstroom doordat specifieke adhesiemoleculen (selectins) op de cellen binden aan suikerstructuren op cellen die wand van het bloedvat bekleden (endotheelcellen). Door deze interacties gaan cellen rollen over de wand van het bloedvat. Fase 2. Indien er inflammatoire stoffen aanwezig zijn waarop cellen specifiek reageren dan binden deze stoffen aan hun specifieke receptor op de cellen. De cellen worden hierdoor geactiveerd om naar de plek van de inflammatie te gaan. Daarom worden de signaalmoleculen die dit veroorzaken ook wel chemoattractanten genoemd. Fase 3. De activatie van de cellen resulteert in versterkte hechting van de cellen doordat de cellen van binnenuit ('inside-out') een andere klasse van adhesiemoleculen, de integrines, activeren. De cellen stoppen hierdoor met rollen en zijn sterk gehecht aan het endotheel. Fase 4. De geactiveerde cellen bewegen zich over het endotheel en vervolgens (op de juiste plek) tussen de endotheelcellen door naar het onderliggende weefsel waarbij de

(concentratie van) chemoattractanten hun richting bepaalt. Dit bewegen van de cellen naar de plaats van de ontsteking wordt migratie genoemd. Dit hele proces van uittreden van cellen is schematisch weergegeven in Fig. 1 van **Hoofdstuk 1**.

Priming van inflammatoire cellen bij diverse fenotypes van astma

Bepaalde witte bloedcellen, zoals de eosinofiele en neutrofiele granulocyten, en monocyten, spelen een belangrijke rol in het ontstekingsproces van astmapatiënten. Het zijn voornamelijk de eosinofielen die verantwoordelijk zijn voor het inflammatoire proces in astmapatiënten met een milde tot middelmatige ernst van de ziekte. Recent onderzoek heeft aangetoond dat neutrofielen voornamelijk betrokken zijn bij de meer ernstige vormen van astma, alhoewel er ook neutrofiel-inflammatie aanwezig kan zijn bij de mildere vormen van astma. De kennis over de rol van de monocyten m.b.t. astma is beperkt.

In diverse studies is reeds verhoogde priming van eosinofielen aangetoond bij astma. De kennis over neutrofiel en monocyt priming in bloed van astmapatiënten is beperkt. In **Hoofdstuk 2** hebben we de priming van granulocyten (eosinofielen en neutrofielen) onderzocht bij astmapatiënten en gezonde controles door te kijken naar binding van antistoffen op deze cellen. De antistoffen die hierbij werden gebruikt waren gericht tegen eiwitten op het celoppervlak, die betrokken zijn bij hechting (adhesie) en uittreding van cellen. Deze antistoffen waren anti-CD11b en anti-CD62L en herkennen respectievelijk de adhesiemoleculen integrine-subunit αm en L-selectin. Ook werd de priming onderzocht via de nieuw ontwikkelde antistof A17, die specifieke priming herkent van inflammatoire cellen in het perifere bloed. Deze priming werd gemeten in het bloed zonder de cellen te isoleren uit het bloed. Isolatie van cellen kan ervoor zorgen dat cellen verhoogde priming krijgen. Door gebruik te maken van A17 hebben we aangetoond dat t.o.v. gezonde controles eosinofielen een verhoogde priming hebben bij milde astmapatiënten die geen medicijnen gebruikten (zij waren tenminste 4 weken voor de studie gestopt met de inname van medicatie). Deze priming was verder verhoogd 6 uur na allergeen-inhalatie. In tegenstelling tot eosinofielen hadden neutrofielen geen verhoogde A17 priming in astmapatiënten t.o.v. neutrofielen van controles en was de A17 priming niet beïnvloed door allergeen inhalatie. Om nu uit te sluiten dat ofwel neutrofielen geen rol spelen of dat ze een priming hebben die zo subtiel is

dat A17 het niet kan meten, hebben we de cellen geactiveerd om het verschil in gestimuleerde priming te onderzoeken. Nu bleek echter dat neutrofielen een verhoogde gestimuleerde A17 priming hadden bij astmapatiënten t.o.v. de neutrofielen van controles. Neutrofielen van astmapatiënten waren dus wel degelijk geprimed. We hebben dit verder onderzocht door te kijken naar verschillen in genexpressies tussen de neutrofielen van astmapatiënten en controles, waarbij de cellen niet gestimuleerd waren. Hierbij werd gevonden dat de neutrofielen bij astmapatiënten een ander genexpressiepatroon hadden t.o.v. de genexpressies van deze cellen bij controles. Dit genexpressieprofiel van de neutrofielen van astmapatiënten leek sterk op het genexpressieprofiel zoals gevonden werd bij stimulatie van neutrofielen van gezonde mensen met het met astma-geassocieerde cytokine GM-CSF.

Daarnaast was bij milde astmapatiënten een verschil in A17 priming van neutrofielen en eosinofielen gevonden tussen patiënten die een vroege reactie hadden en patiënten die een late reactie hadden op allergeen inhalatie. Alleen eosinofielen van patiënten met een late reactie hadden verhoogde A17 priming t.o.v. de eosinofielen van patiënten met alleen een vroege reactie. Na stimulatie van de cellen bleek echter dat alleen neutrofielen van de patiënten met een late reactie verhoogde A17 priming hadden t.o.v. deze cellen van patiënten met alleen een vroege reactie. Ook was het genexpressieprofiel van ongestimuleerde neutrofielen (zie hierboven) versterkt na allergeen provocatie bij astmapatiënten met een late reactie. Door de overeenkomsten tussen het meten van neutrofiel priming via A17-expressie na stimulatie en via het bepalen van genexpressie zonder stimulatie, kan geconcludeerd worden dat het meten van genexpressie van neutrofielen een goede uitleesmogelijkheid van de priming van deze cellen is zonder dat de cellen vooraf gestimuleerd hoeven te worden.

Hoewel priming goed te meten was met A17, werd er geen verschil gevonden tussen astmapatiënten en controles wat betreft de ongestimuleerde of gestimuleerde celexpressies van CD11b en CD62L op zowel eosinofielen als neutrofielen. Wel vonden we sterke positieve correlaties tussen CD11b en CD62L niveaus op beide celtypen in het bloed van astmapatiënten. De aanwezigheid van deze positieve correlaties zou kunnen betekenen dat cellen met hoge CD11b en hoge CD62L niveaus extra gevoelig zijn voor rollen en hechten aan het endotheel, waarna de cellen kunnen uittreden uit de bloedbaan. Ook werd er een duidelijke correlatie gevonden tussen CD11b niveaus op beide celtypen en luchtweggevoeligheid, wat een mogelijke marker is voor het bepalen van de

chroniciteit van de ontsteking bij astma. Er was geen verband tussen luchtweggevoeligheid en A17 priming. Het niveau van CD11b (en mogelijk ook L-selectin) op deze cellen lijkt daarom een uitleesmogelijkheid te zijn van de chroniciteit van de ontsteking bij astmapatiënten, terwijl A17 en de genexpressie voornamelijk de (acute) priming verschillen weergeven.

In **Hoofdstuk 3** is onderzocht of het meten van priming bij witte bloedcellen gebruikt kan worden als marker bij het bepalen van astma-fenotypes. Hiervoor werden 2 groepen astmapatiënten geïncludeerd, die klinisch nauwelijks van elkaar verschilden, maar die verschillende medicatie nodig hadden voor de stabilisatie van hun ziekte. De eerste groep patiënten was een vergelijkbare groep zoals die beschreven is in **Hoofdstuk 2** (geen medicatie 4 weken voor aanvang van de studie). De tweede groep astmapatiënten bestond uit een groep patiënten die hoge hoeveelheden steroïden nodig hadden voor stabilisatie van hun ziekte. Deze groep wordt ook wel aangeduid als moeilijk-te-behandelen en omvat 5-10% van alle astmapatiënten. Bij deze patiënten werd de priming gemeten bij eosinofielen, neutrofielen en monocyten door wederom te kijken naar de celexpressies van A17 en CD11b. Daarnaast is bij deze cellen gekeken naar de celexpressies van een andere nieuw ontwikkelde antistof, genaamd A27. Er werden nauwelijks verschillen gevonden tussen de celexpressies van A17 en A27 op de cellen van controles, milde patiënten of moeilijk-te-behandelen patiënten. Gebaseerd op de celexpressies van A17 en A27 vertoonden de milde patiënten zonder medicatie wederom een eosinofiel-priming en geen neutrofiel- of monocyt-priming. De cellen van milde patiënten vertoonden geen van alle verhoogde celexpressies van CD11b. Echter moeilijk-te-behandelen astmapatiënten vertoonden verhoogde expressies van A17 en A27, maar ook van CD11b op hun neutrofielen en monocyten. Dit is opmerkelijk, omdat CD11b bij de milde patiënten geen verschillen vertoonde. Er werd geen eosinofiel-priming gevonden bij moeilijk-te-behandelen astmapatiënten. Na activatie van de cellen waren er nauwelijks verschillen in priming tussen de 2 fenotypes van astma. We hadden geen ‘tussengroep’ van astmapatiënten geïncludeerd die middelmatige hoeveelheden steroïden gebruikten, omdat uit preliminaire data was gebleken dat deze hoeveelheden steroïden nauwelijks effect hebben op de priming. Vanwege medisch ethische restricties was het niet mogelijk om de patiënten met hoge hoeveelheden steroïden van hun medicatie af te halen, om zo te kijken wat het effect hiervan is op de priming. Daarom weten we niet of de verhoogde neutrofiel-

en monocyt-priming is veroorzaakt door de ontsteking of door de hoge hoeveelheid steroïden in moeilijk-te-behandelen astmapatiënten. Aan de hand van de priming data beschreven in **Hoofdstukken 2 en 3** is geconcludeerd dat het bepalen van de priming van leukocyten gebruikt kan worden bij het fenotyperen van astmapatiënten. Als uitsluitend gekeken wordt naar de A17 en A27 celepitoop expressies dan hebben milde astmapatiënten een eosinofiel-priming, terwijl moeilijk-te-behandelen patiënten een neutrofiel- en monocyt-priming hebben.

Om de effecten van steroïden te onderzoeken op processen die betrokken zijn bij het uittreden van leukocyten vanuit het bloed naar de longen zijn er experimenten gedaan waarbij onderzocht is wat de effecten waren op de migratie en adhesie van eosinofielen na een lange termijn incubatie (16 uur) met steroïden. Dit onderzoek is beschreven in **Hoofdstuk 4**. Migratie van cellen werd gemeten door cellen door kleine poriën in filters te laten ‘lopen’ waarna gekeken werd hoever de cellen in het filter gekomen waren. Adhesie van cellen werd gemeten door te kijken naar de binding van ICAM-1 gecoate beads (kleine bolletjes) aan de integrine αm/β2 van eosinofielen. ICAM-1 is een natuurlijk voorkomend ligand van deze integrine. De experimenten waarin de lange termijn incubatie-effecten van steroïden zijn bestudeerd werden uitgevoerd met steroïd dexamethasone (Dex) in aan- en afwezigheid van IL-5. IL-5 is een belangrijke inflammatoire stof bij astma en is betrokken bij de zowel de priming van eosinofielen als de rijping van deze cellen vanuit het beenmerg. IL-5 induceerde migratie en adhesie van eosinofielen. Dex was in staat om de IL-5 geprimede migratie en adhesie te remmen alleen als cellen waren geïncubeerd voor 16 uur in aanwezigheid van IL-5. Dex incubatie zonder IL-5 had geen invloed op de migratie en adhesie van eosinofielen als ze na 16 uur werden blootsgesteld aan IL-5. De celexpressie niveaus van de integrine αm/β2 en de IL-5-receptor werden gemeten om uit te zoeken of de Dex-geïnduceerde remming van de lange termijn IL-5 priming veroorzaakt werd doordat integrines en/of IL-5-receptoren verdwenen van het celoppervlak van eosinofielen tijdens de incubatie. Dit bleek niet het geval, omdat Dex geen invloed had op het expressieniveau van de integrine of IL-5-receptor op het celoppervlak. Daarnaast bleek dat de door Dex geremde integrine nog goed te activeren was door een integrine activerend cation. Dit betekent dat Dex de integrine αm/β2 remt via een ‘inside-out’ mechanisme, maar alleen als eosinofielen zich bevinden in een priming stimulerende omgeving. Ook geven de data in **Hoofdstuk 4** heel duidelijk aan dat priming een intermediaire status is van eosinofielen: aan de ene kant kan het de

responsen van de cellen verhogen na activatie, terwijl het aan de andere kant geremd kan worden door anti-inflammatoire behandelingen d.m.v. bijv. steroïden.

Naast het lange termijn remmend effect van steroïden is in de recente literatuur aangetoond dat steroïden ook snelwerkende effecten kunnen hebben. Lange termijn effecten van steroïden gebeuren over het algemeen doordat steroïden aangrijpen op de genexpressies van inflammatoire mediatoren, zodat deze verminderd worden. De snelste genomische processen zijn waargenomen na 30 minuten incubatie met steroïden. Snellere (<30 min) effecten van steroïden gebeuren daarom hoogstwaarschijnlijk via niet-genomische mechanismen. Snelle effecten van steroïden zijn in **Hoofdstuk 5** onderzocht op diverse functies van eosinofielen. Hieruit bleek dat een kwartier incubatie met steroïd Dex geen effect had op eosinofiel migratie, adhesie of activatie van de productie van zuurstofradicalen. Echter bleek deze korte incubatietijd met Dex de functionaliteit van de antilichaam/immunoglobulin (Ig)A receptor te primen. Dit proces is opmerkelijk, omdat IgA een antilichaam is dat voornamelijk voorkomt in mucosale oppervlakken, zoals die ook aanwezig zijn in het longweefsel. Tevens zijn er specifieke IgA moleculen tegen allergenen beschreven die een rol kunnen spelen bij astma. IgA binding aan cellen kan ervoor zorgen dat de cellen geactiveerd worden en daardoor hun toxische effecten kunnen vertonen. Hoewel dit snelle effect van steroïd Dex op de priming van de IgA-receptor was gevonden is het op dit moment onbekend of deze priming door steroïden een ziekteverslechterend effect heeft bij astmapatiënten.
De data in **Hoofdstukken 4 en 5** laten tegengestelde effecten van Dex zien op eosinofiele responsen. Zoals we in **Hoofdstuk 4** hebben aangetoond werkt een steroïd remmend op de lange termijn eosinofiel priming, terwijl in **Hoofdstuk 5** is aangetoond dat een korte incubatie met een steroïd een primend effect kan hebben op de functionalitiet van eosinofielen. Hoewel in het verleden is aangetoond dat lange termijn incubatie met steroïden nauwelijks effecten heeft op de IgA-receptor, waren de lange termijn effecten van steroïden duidelijk aanwezig op de priming van integrines. Aan de andere kant zijn integrines niet gevoelig voor korte termijn effecten van steroïden, terwijl de IgA-receptor dit wel was.

Veel immunologische reacties in de mens worden veroorzaakt door de activatie van de productie van specifieke IgG's. Echter Dex had geen invloed op de IgG-receptor, wat de specificiteit van het snelle effect van steroïd Dex op de IgA-

receptor benadrukt. De regulatie van de IgA-receptor is in het verleden bestudeerd door Madelon Bracke (co-promotor). Zij heeft hierbij laten zien dat cytokines IL-4 en IL-5 de IgA receptor kunnen primen door 'inside-out' regulatie van signaalmoleculen PI3K en P38 MAPK. Bij bestudering van het snelle effect van steroïd Dex op de IgA-receptor is gebleken dat naast PI3K vooral P38 MAPK het 'inside-out' signaal verzorgde naar de IgA-receptor. Dit is onderzocht door gebruik te maken van een remmer van P38 MAPK (SB203580). Tevens is gebleken dat het signaal van Dex naar de IgA-receptor ook afhankelijk was van de glucocorticosteroïd receptor, omdat een remmer van deze receptor dit signaal kon blokkeren.

P38 MAPK wordt geactiveerd door de fosforylering van twee aminozuren in de structuur van P38 MAPK. Deze activatie kan geschieden door MKK3/MKK6. MKK3/MKK6 zijn twee signaalmoleculen die in de activatie/priming cascade tussen de geactiveerde receptor en P38 MAPK staan. Doordat P38 MAPK wordt gefosforileerd ondergaat het een verandering in structuur waardoor P38 MAPK ATP kan binden. P38 MAPK heeft ATP nodig voor het uitoefenen van de activiteit. De reeds genoemde remmer van P38 MAPK, SB203580, kan binden in de ATP site. Hierdoor is P38 MAPK niet actief, maar kan nog steeds gefosforileerd zijn, omdat SB203580 geen invloed heeft op de fosforilatieplekken van P38 MAPK. Naast MKK3/MKK6 is er een mechanisme van P38 MAPK activatie beschreven waarbij SB203580 er ook voor zorgt dat P38 MAPK niet meer gefosforileerd wordt. Deze vorm van activatie van P38 MAPK wordt ook wel het autofosforilatie-proces genoemd. Bij autofosforilatie heeft P38 MAPK zijn eigen ATP bindingsplek nodig om zichzelf te kunnen fosforileren en activeren. Bij autofosforilatie zorgt SB203580 er dus voor dat P38 MAPK niet meer actief kan zijn om zichzelf te fosforileren. Dit autofosforilatie-proces is ook gezien in de studie van **Hoofdstuk 5** bij de bestudering van het snelle effect van steroïd Dex op P38 MAPK in eosinofielen. Om beter uit te zoeken hoe dit autofosforilatie-proces verliep bij eosinofielen, is de activatie van P38 MAPK vergeleken tussen eosinofielen en neutrofielen. Dit onderzoek is beschreven in **Hoofdstuk 6**. Voor neutrofielen bestonden bij aanvang van de studie reeds aanwijzingen dat daar vooral MKK3/MKK6 de P38 MAPK fosforilatie reguleren. Voor eosinofielen was niets bekend over de betrokkenheid van MKK3/MKK6 bij de activatie van P38 MAPK. De regulatie van P38 MAPK werd bestudeerd door het toedienen van cytokine GM-CSF of TNFα aan zowel eosinofielen als neutrofielen. Naast GM-CSF is ook TNFα geassocieerd met het

inflammatoire proces in astma. Korte termijn activatie dmv. steroïden konden we niet gebruiken in deze vergelijkende studie, omdat toediening van steroïden geen activatie van P38 MAPK gaf in neutrofielen. In de vergelijkende studie werd gevonden dat neutrofielen P38 MAPK fosforileren en activeren door MKK3/MKK6, omdat na stimulatie van neutrofielen de kinetiek van MKK3/MKK6-fosforilatie gelijk was aan die van P38 MAPK en omdat SB203580 wel de activiteit van P38 MAPK kon remmen, maar niet de MKK3/MKK6-gereguleerde fosforilatie. Echter bij eosinofielen was het proces van P38 MAPK autofosforilatie verantwoordelijk voor de fosforilatie en activiteit van P38 MAPK, omdat SB203580 beide processen kon remmen in eosinofielen en omdat er geen verband was tussen de kinetiek van MKK3/MKK6-fosforilatie en P38 MAPK-fosforilatie in deze cellen. Dit was in overeenstemming met de Dex-geïnduceerde P38 MAPK autofosforilatie bij eosinofielen.
Voor het proces van autofosforilatie is bekend dat dit gemedieerd kan worden door binding van TAB1 aan P38 MAPK. Echter in **Hoofdstuk 6** is gevonden dat TAB1 aan P38 MAPK bond bij zowel eosinofielen als neutrofielen. Mogelijk is het zo dat TAB1-gereguleerde P38 MAPK fosforilatie in neutrofielen overschaduwd wordt door de MKK3/MKK6-gereguleerde fosforilatie. Verder onderzoek is nodig om de rol van TAB1 in deze cellen vast te leggen. Tevens blijft de brandende vraag waarom deze cellen verschillende mechanismen bezitten van P38 MAPK-fosforilatie.

Naast de effecten op signaalmoleculen, zoals P38 MAPK, kunnen steroïden ook effecten hebben op de celdood van eosinofielen en neutrofielen. In **Hoofdstuk 4** was echter geen enkel effect van steroïden gevonden op de overleving van eosinofielen. Dit is in tegenspraak met sommige artikelen die over dit onderwerp zijn verschenen. Ondanks het feit dat anderen wel versnelde celdood bij eosinofielen hebben laten zien is door ons aangetoond dat deze cellen wel degelijk bij hoge hoeveelheden steroïden kunnen overleven gedurende lange termijn (16 uur) incubatie. Verder zijn er vanuit de literatuur aanwijzingen dat steroïden ook geen verhoogde eosinofiel-dood geven in het longweefsel. In overeenkomst met de literatuur is door ons gevonden dat neutrofielen langer blijven leven door lange termijn incubatie met steroïden (**Hoofdstuk 7**). Dit proces wordt gezien als een mogelijke drijvende factor achter de neutrofiel-inflammatie van ernstige astmapatiënten. Deze ernstige patiënten gebruiken veelal (hoge doseringen) steroïden.

Conclusies

In dit proefschrift is aangetoond dat:

1) Verschillende fenotypen van allergisch astma geassocieerd zijn met specifieke priming profielen van inflammatoire cellen in het perifere bloed van astmapatiënten (**Hoofdstukken 2 en 3**);
2) Glucocorticosteroïden de migratie en adhesie van eosinofielen alleen kunnen remmen als deze cellen zich (gedurende lange tijd; 16 uur) in een priming-stimulerende omgeving bevinden (**Hoofdstuk 4**);
3) In eosinofielen glucocorticosteroïden een snel (< 30 min) primend effect hebben op de ‘inside-out’ regulatie van functionele receptoren, zoals de IgA-receptor (**Hoofdstuk 5**);
4) P38 MAPK verschillend gereguleerd wordt tussen eosinofielen en neutrofielen (**Hoofdstuk 6**).

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Curriculum Vitae

Willem ten Hove werd op 13 september 1979 geboren te IJsselmuiden. Hij behaalde in 1997 het gymnasium diploma aan het Carolus Clusius College (thans Agnieten College) te Zwolle. In hetzelfde jaar is hij begonnen met de studie Biologie aan de Universiteit Utrecht. Binnen deze studie zijn door hem de volgende afstudeeronderwerpen voltooid:

Afstudeeronderwerp 1:	Fundamentals of Business and Economics Begeleiders: Prof.dr. A.G.M. Tielens en Prof.dr. A. Buijs Binnen dit afstudeeronderwerp is stage gelopen bij de afdeling Business Development van OctoPlus B.V. (thans OctoPlus N.V.) te Leiden. Stage-begeleider: Dr. H. Luessen Stage-onderwerp: Potential of OctoPlus' Proprietary Polymers in the investigated market(s).
Afstudeeronderwerp 2:	Moleculaire Microbiologie Afdeling Moleculaire Microbiologie, Universiteit Utrecht Begeleiders: Dr. J.P. van Ulsen en Prof.dr. J.P.M. Tommassen Onderwerp: Characterisation at molecular level of gene NMB1985 of *Neisseria meningitidis.*

In maart 2002 studeerde hij af en in mei van datzelfde jaar is hij begonnen aan het in dit proefschrift beschreven onderzoek bij de divisie Farmacoepidemiologie en Farmacotherapie van de afdeling Farmaceutische Wetenschappen te Utrecht onder begeleiding van Prof.dr. H.G.M. Leufkens en Dr. M. Bracke. Dit onderzoek werd uitgevoerd in samenwerking met de afdeling Longziekten van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof.dr. L. Koenderman. In het vierde jaar van de promotie is de begeleiding door Prof.dr. H.G.M. Leufkens overgenomen door Prof.dr. J.A.M. Raaijmakers. Per 1 augustus 2006 is Willem ten Hove in dienst getreden bij OctoPlus N.V. te Leiden.