Katholieke Universiteit Leuven
Faculty of Medicine
Laboratory of Experimental Immunology

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P.W. Hellings

Thesis submitted in partial fulfillment of the requirements
for the degree of 'Doctor in Medical Sciences'
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Promoter: Prof. Dr. J.L. Ceuppens
Co-promoter: Prof. Dr. M. Jorissen

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LIST OF ABBREVIATIONS

Ag	antigen
AHR	airway hyperresponsiveness
APC	antigen-presenting cell
BAL	broncho-alveolar lavage
BSA	bovine serum albumin
CD	cluster of differentiation
CD40L	CD40 ligand
cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
d	day
DC	dendritic cell
ELISA	enzyme-linked immunoabsorbent assay
Eo	eosinophil
EU	experimental units
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
KO	knock out
LPR	late phase response
Lympho	lymphocyte
Mch	metacholine
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
Mono	monocyte
MRI	magnetic resonance imaging
Neutro	neutrophil
NF-κB	nuclear factor-kappa B
NL	nasal lavage
OVA	ovalbumin
PBLN	peribronchial lymph nodes
Penh	enhanced Pause
Sal	saline
SN	supernatant
TGF-β	transforming growth factor-β
Th	T helper
Thp	T helper precursor
Tr1	T regulatory 1
WT	wild-type

CHAPTER 1. INTRODUCTION

1.1 Allergic asthma

Asthma is a complex and heterogeneous airway disease that affects millions of people worldwide. Its major characteristics include intermittent and reversible airflow obstruction, bronchial hyperresponsiveness and airway inflammation (1). Among asthmatic patients, most have mild to moderate disease that can be controlled with available medication. It is however estimated that up to 10% of patients with asthma have severe disease that is recalcitrant to the classic treatment (2). In the latter group, asthma severely impairs quality of life and has a profound socio-economic impact (3).

The majority of patients with asthma exhibit immediate hypersensitivity responses to defined environmental allergens (4). In

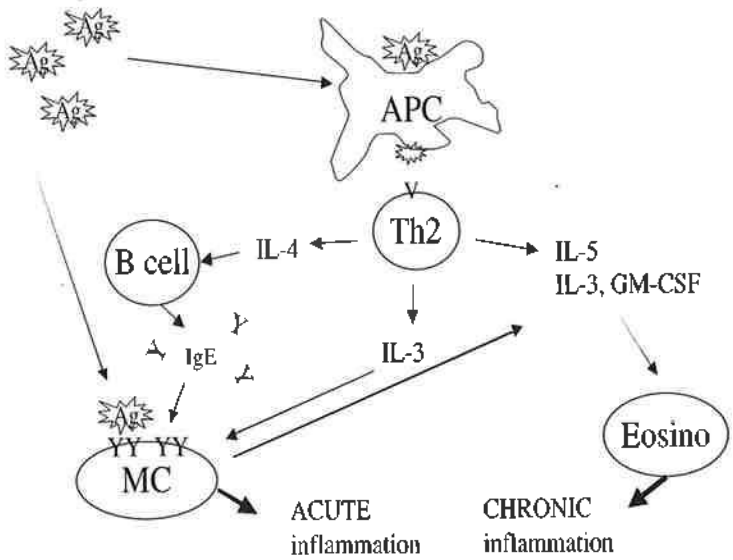

Fig. 1.1 Schematic overview of basic mechanisms involved in the allergic immune response. After presentation of allergen (Ag) by antigen-presenting cells (APC), T helper 2 (Th2) cells release interleukins (IL) and granulocyte macrophage colony-stimulating factor (GM-CSF) that stimulate IgE production by B cells, growth of mast cells (MC) and generation and recruitment of eosinophils. Upon ligation of IgE on MC with Ag, MC release inflammatory mediators that mediate the acute allergic response. Eosinophils are recruited to the site of allergen encounter and release mediators involved in chronic inflammation.

these atopic subjects, inhalation of allergens leads to activation of mast cells through binding of allergens to receptor-bound immunoglobulin E (IgE) on the surface of mast cells (Fig. 1.1). This process activates mast cells to release inflammatory mediators and hence evokes a biphasic response, comprising acute and late phase reactions separated in time by several hours. The clinical feature of these reactions is airflow obstruction, which is believed to be the direct result of bronchial inflammation. Therefore, allergy is acknowledged as a major risk factor for asthma (4). However, only 25 to 30 % of atopic subjects progress to clinical asthma. Therefore, other factors must be involved in the development of asthma in allergic individuals. Organ-specific factors such as thresholds for activation of epithelial or mesenchymal cells may be involved, as suggested by the development of allergic asthma after lung transplantation in non-asthmatic patients (5). On the other hand, external triggers such as viral infections, cigarette smoke or air pollution have been suggested to trigger the progression of the atopic state towards clinical airway inflammation (6).

It is also important to acknowledge that besides allergy, alternative inflammatory pathways may be responsible for asthma development (6). The best-characterized examples are intrinsic asthma and occupational asthma. No role for IgE has been found in these diseases, despite the presence of cellular infiltration and associated cytokine production in airway wall biopsies, which closely resemble the Th2-biased pattern that is typical of atopic asthma (7). The reported incidence of asthma is increasing dramatically during the last decades until approximately 8% of adult population and 20% of children in Europe, North America and Australia (8). This increase may partly result from changes in diagnostic labeling, but there are strong indications of a real increase. At present one can only speculate on factors responsible for

this phenomenon. In spite of the involvement of genetic factors in the development of allergic diseases (6), they cannot explain the rapidly rising prevalence. The hypothesis that currently finds most favour is the influence of hygiene in Western lifestyle (9) that deprives the developing immune response of important signals for developing protective, i.e. anti-allergic, immune responses. This hypothesis results from the insight in factors that determine the differentiation of T helper (Th) cells towards a particular subset of Th cells, called Th2 cells. In the production of IgE and manifestation of allergic asthma (Fig. 1.1), activated Th2 cells play an important role via the secretion of so-called Th2 cytokines (4). Therefore, Th2 cells are considered to be main orchestrators of the allergic inflammatory response (1).

1.2 Subsets of T helper cells

Our perception of the mechanism underlying allergic airway inflammation has changed dramatically over the past few years. Following rapidly on the heels of pioneering work on Th cell heterogeneity in the mouse (10), T cell cloning studies revealed major variations in cytokine production by allergen-specific Th memory cells within the human population. T lymphocytes of atopic patients express a cytokine pattern similar to murine Th2 cells (1). In contrast, T cells involved in so-called Th1-cytokine mediated diseases (10) such as rheumatoid arthritis, type 1 diabetes mellitus, Crohn s disease, multiple sclerosis and sarcoidosis, show a different cytokine production profile. The concept of Th cell subsets has become a paradigm in cellular immunology and provides an elegant framework for understanding the mechanism underlying T cell-mediated inflammatory diseases.

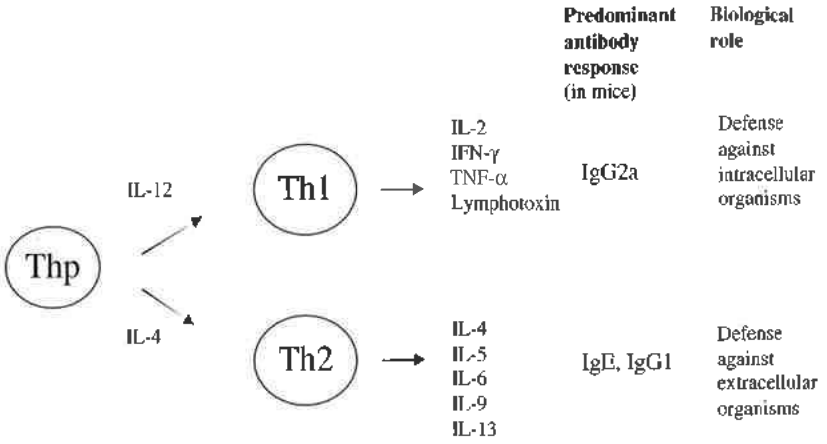

Fig. 1.2 T helper (Th) cell subsets with their cytokine profile and major immunologic functions. At the time of activation by antigenic peptide, Th precursor (Thp) cells differentiate into Th1 or Th2 cells. Interleukin (IL)-12 and IL-4 direct the differentiation process of Thp cells towards the Th1 or Th2 lineage. Th1 and Th2 cells have different cytokine profiles and are involved in diffferent immunologic defense systems.

Th1 cells (Fig. 1.2) secrete interferon-gamma (IFN-γ), IL-2, tumor necrosis factor-alpha (TNF-α), lymphotoxin, and other cytokines that together mobilize cellular and humoral defense mechanisms against intracellular pathogens and antagonize IgE responses (11). They have the capacity to induce inflammatory reactions, to give help to B cells for the production of complement-fixing and opsonizing antibodies, as well as to activate macrophages by enhancing their microbicidal effector functions. As a consequence, Th1-mediated responses are generally characterized by inflammatory reactions that often lead to tissue damage and destruction, such as in rheumatoid arthritis. In contrast, Th2 cells produce a functionally distinct repertoire of cytokines (Fig. 1.2, (11)), including IL-4, IL-5, IL-6, IL-9 and IL-13 and provide help to the B cell for production of non-complement-fixing IgG4 and IgE. Due to the production of IL-4 and IL-13, which are switch factors for the production

of IgE by B cells, and of IL-5, a potent growth and differentiation factor for eosinophils, Th2 cells play an important role in the elimination of large extracellular pathogens such as parasites. From an evolutionary perspective, the primary function of the allergic response may be an anti-parasitic protective mechanism (12). Hence, the development of allergic diseases may be the undesirable reaction towards otherwise inoffensive environmental substances. Most of the characteristic features of human allergic asthma are summarized in Fig. 1.3 and result from the combined effects of Th2 cytokines (1). Therefore, differentiation of Th precursor (Thp) cells towards the Th2 lineage represents a crucial step in the initiation and manifestation of allergic diseases.

Different factors are involved in the differentiation of Thp cells (11). The nature and dose of the antigen, type of dendritic cell and route of antigen administration determine the outcome of Th differentiation. However, the cytokine milieu present during the initiation of a $CD4^+$ T cell response has been put forward as one of the major variables influencing this process (11). IL-12 and IFN-γ play an important role in the differentiation of Th cells towards the Th1 profile, whereas IL-4 promotes Th2 differentiation (Fig. 1.2). Furthermore, there is increasing evidence that costimulatory interactions between T cells and APCs represent an alternative mechanism for skewing the differentiation of Th cells (11).

Several transmembrane receptors have been shown to be differentially expressed on Th subsets. The CC chemokine receptors (CCR) CCR3 and CCR4 are expressed on Th2 cells (13), whereas CCR1 and CCR5 are expressed on the Th1 effector population (13, 14), providing an attractive mechanism by which Th subsets are preferentially recruited to distinct inflammatory sites. In addition, the IL-18 receptor is expressed on Th1 cells, whereas T1/ST2 has recently been demonstrated

to be overexpressed on Th2 cells. Finally, Th2 cells preferentially up-regulate transcription factors GATA-3 (15) and c-maf (16).

It should be kept in mind that classification of allergic asthma as a Th2-mediated disease is far too simplistic. For example, T cells in stable chronic asthmatic patients are also of the Th0 phenotype, producing a mixture of Th1 and Th2 cytokines (17), and of the Th1 phenotype. Indeed, IFN-γ levels are elevated in the serum of patients with severe asthma and in BAL fluid after allergen challenge (1). In addition to Th1 and Th2 cells, Groux et al. (18) described a subset of Th cells that differ from the classic Th1 and Th2 T cell clones. These cells, termed T regulatory 1 (Tr1) cells, differentiate in the presence of IL-10 and inhibit the proliferative response of bystander cells. A similar population of $CD4^+$ T lymphocytes with strong suppressive activity, called Th3 cells, has been described by Chen et al. (19). Much still has to be learned about these elusive subpopulations of $CD4^+$ T lymphocytes and their possible role in allergic diseases.

1.3 T helper lymphocytes orchestrate allergic airway inflammation in asthma

At the cellular epicentre of the clinical triad of allergen-specific IgE production, bronchial eosinophilic inflammation and non-specific AHR, are activated cluster of differentiation (CD)-4^+ Th lymphocytes (1). During both antigen priming as well as development of allergic airway inflammation, Th cells orchestrate the allergic inflammatory response through the production of an array of cytokines.

The first process, called sensitization, involves the development of allergen-specific immunologic memory against inhaled allergens. In a subset of individuals, this results in the development of Th0/Th2-polarized immunologic memory. To initiate synthesis of IgE, inhaled

allergens must encounter dendritic cells that line mucosal surfaces. After migration of antigen-loaded dendritic cells to draining lymph nodes (20), they present processed antigen to B cells and T cells. Antigen-presentation activates $CD4^+$ cells to provide two accessory signals necessary for the Ig isotype switch of the B cell towards IgE (21). Activated $CD4^+$ cells secrete IL-4 and IL-13, which bind their receptors on B cells and activate signal transduction and activation of transcription (STAT) factor 6. The second signal is provided by CD40L on activated $CD4^+$ cells that ligates CD40 on B cells. Additional interactions between other pairs of ligands and receptors, e.g. CD28-B7 and $\alpha_L\beta_2$ integrin-ICAM-1, may complement or up-regulate T cell-dependent activation of B cells.

After the immune system is sensitized, repeated mucosal contact with the allergen initiates, in susceptible subjects, airway inflammation characterized by the presence of activated T lymphocytes, eosinophils, neutrophils, mast cells, and macrophages (1). In this inflammatory milieu, T cell recruitment is thought to represent a key factor in the development and phenotype of allergic asthma in view of the cytokines they release. The principal cytokines implicated in T cell-mediated allergic airway pathology are the so-called Th2 cytokines IL-4, IL-5, IL-13, IL-6 and IL-9 (Fig. 1.3). Their major role in allergic disease will briefly be reviewed.

IL-4 plays a crucial pro-inflammatory role in allergic inflammation during antigen priming as well as during inflammation (1). IL-4 is a cricital factor for skewing Th cell commitment to the Th2 phenotype in parallel with down-regulation of Th1 development. Furthermore, IL-4 plays an essential role in the isotype switch of B cells towards IgE. In ongoing inflammation, IL-4 stimulates eosinophil trafficking by activating adhesion systems at the vascular endothelium.

In conjunction with IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF), IL-5 regulates proliferation and differentiation of eosinophils, promotes chemotaxis of eosinophils and

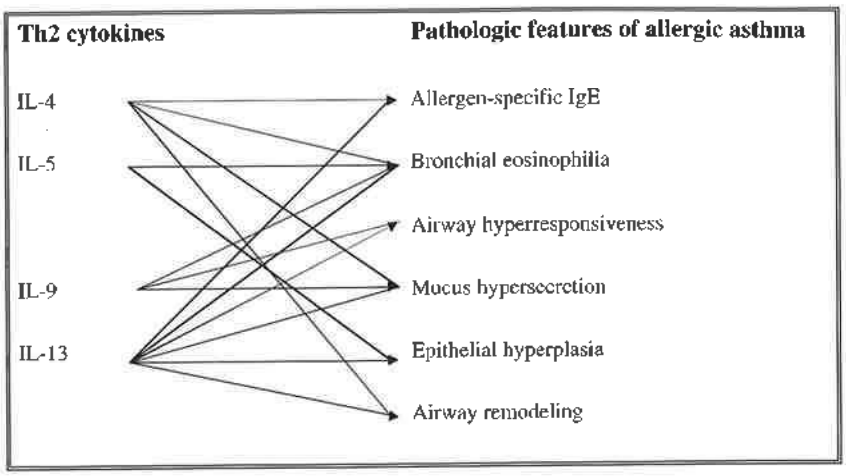

Fig. 1.3 Overview of the involvement of Th2 cytokines (interleukins (IL)) in the pathology of allergic asthma.

primes them for responses to exogenous stimuli. While the biological activities of IL-3 and GM-CSF extend to other cell types, IL-5 primarily influences eosinophil biology. Therefore, IL-5 secretion by activated $CD4^+$ cells represents a key factor in the induction of bronchial eosinophilia and may be involved in the induction of AHR. Indeed, eosinophils release mediators that damage airway epithelium and hence expose sensory nerve endings to stimulation by inflammatory mediators and exogenous triggers. However, conflicting data exist on the role of eosinophils in the development of AHR. Indeed, AHR can occur in the absence of eosinophils (22, 23) and vice versa (24, 25). In a recent clinical trial, longterm reduction of blood and sputum eosinophilia by anti-IL-5 mAb failed to alter AHR (26). Hence, the role of IL-5 and eosinophils in the development of AHR remains incompletely

understood. In this regard, it is interesting to mention that two independent groups reported that IL-13 is involved in the induction of AHR in mice (27, 28).

IL-13 has a number of actions similar to IL-4 as a result of sharing the IL-4 receptor alpha chain (29). Both cytokines are involved in the regulation of the isotype class switching in B cells to IgE synthesis, induction of expression of vascular cell adhesion molecule 1, induction of chemokine production for eosinophil recruitment and mucus hypersecretion. Of note, IL-13 is unable to drive the differentiation of naive Th cells towards a Th2 phenotype. This is likely because of the fact that functional IL-13 receptors have not been detected in activated T cells (29).

The finding of potentiation of IL-4-mediated IgE production by IL-6 (30), also suggests a role for IL-6 in atopy and Th2 cytokine-mediated responses. Transgenic mice that overexpress IL-9 in the lung illustrate that IL-9 may be involved in the development of bronchial eosinophilia and AHR (31).

Although increased T cell numbers have been demonstrated in bronchi of asthmatic individuals after allergen provocation, direct evidence for their role in allergic airway inflammation has first been provided by mice models. In several reports, pharmacologic or genetic depletion of T cells (22, 32) or interference with T cell costimulatory pathways (33) abrogated experimental allergic airway inflammation. Subsequently, Kon et al. reported that also in asthmatic patients targeting the $CD4^+$ population may represent an alternative approach for treating patients with severe asthma (34).

It should however be noted that inflammation in human chronic asthma is far more complex than a simple Th cell-mediated phenomenon. All cells in the airways, including $CD8^+$ cells, natural killer cells,

epithelial cells, endothelial cells, fibroblasts and smooth muscle cells become activated after allergen inhalation (35) and may contribute significantly to cytokine and chemokine production in this chronic inflammatory disease. In view of the fact that Th cells are a principal source of Th2 cytokines in asthmatic bronchi (7) and seem to be essential for the development of allergic airway inflammation (22, 32), allergic asthma can be considered a Th2 cell-mediated disorder with IgE as an important trigger.

1.4 Mouse models of asthma

In recent years, animal models have been proven to be useful in improving our insight into the pathophysiology of different diseases. For immunologic research, mice are advantageous in view of the fact that many tools are available, such as inbred murine strains, transgenic mice, antibodies and recombinant proteins. Moreover, several features characteristic of human allergic asthma can be induced in mice: allergen-specific IgE, non-specific AHR, bronchial eosinophilic inflammation, mucus hypersecretion and epithelial hyperplasia. Furthermore, the biphasic protocol for induction of allergic airway inflammation in mice, i.e. systemic sensitization and airway challenge of sensitized mice, offers the opportunity to study mechanisms during both stages of disease development. Therefore, experiments conducted in mice allow the dissection of complex immunologic responses *in vivo* and help us to elaborate new therapeutic strategies for human asthma.

However, several caveats should be kept in mind when interpreting results obtained in mice. Asthma appears to be a uniquely human disease and mouse models of asthma are only mimicking some aspects of this chronic and complex inflammatory disorder. In addition, mice and humans differ in significant details of immunological and

inflammatory responses that may influence the outcome of airway inflammation. For example, the dichotomy between Th1 and Th2 cells seems to be more absolute in polarized immune responses in mice as compared with humans (36). Human Th1 clones are able to produce some IL-4 and Th2 clones can secrete IL-2 and IFN-γ, depending on their mode of activation. Whereas murine IL-10 production is restricted to Th2 cells, IL-10 is also produced by human Th1 clones. In addition, different mediators may be involved in inflammatory responses of asthmatic patients and mice with experimentally induced airway inflammation. For example, chemo-attraction of neutrophils is largely dependent on IL-8 in humans, whereas this is mediated via granulocyte chemotactic protein-2 (GCP-2) (37) and/or macrophage-inflammatory protein-2 (MIP-2) (38) in mice. Furthermore, on should always interpret data generated in animal models in the context of the model used. Indeed, murine models often differ in a number of factors, including murine strain, choice of antigen, experimental protocol and means of evaluating bronchial responses. Taken together, the extent to which findings in experimental asthma in mice actually reflect the situation in human asthma needs to be investigated in clinical trials.

Recently, the relevance of mouse models of allergic asthma has been questioned by the apparent discrepancy in data obtained in mouse models and in asthmatic patients using anti-IL-5 monoclonal antibodies (mAb). Anti-IL-5 mAb abrogated bronchial eosinophilia concomitant with reduction of AHR (39, 40) and late phase asthmatic response (LPR) (41) in mice, whereas this therapy failed to affect functional bronchial responses in asthmatic patients (26). One should however note here that several reports in mice also demonstrated that AHR can persist in spite of reduced bronchial eosinophilia after anti-IL-5 mAb treatment (23, 42, 43). In addition, other groups have also shown a causal dissociation

between bronchial eosinophilia and AHR in mice (44, 45). One should also realize that in the clinical trial with anti-IL-5 mAb (26), anti-IL-5 mAb had been given for a short period to a relatively small group of mild asthmatic patients. Therefore, we think that data generated in this study cannot be extrapolated to the whole population of patients with allergic asthma. Finally, one should remember that results obtained in a mouse model of asthma, in which homogenous and genetically identical mice are used, may yield information that is relevant to allergic airway disease in a subgroup of asthmatic patients, without being representative of human asthma in general.

Reservations notwithstanding, murine models of asthma have contributed significantly to our understanding of the pathogenesis of asthma, and represent a useful instrument to unravel immunologic mechanisms underlying the pathology of allergic asthma. The murine model we used here mimics both the chronic and airborne character of human allergen exposure and may therefore provide information that is more relevant to human asthma than the more acute models. Apart from induction of cardinal features of human allergic asthma, i.e. allergen-specific IgE, bronchial eosinophilia and non-specific bronchial hyperresponsiveness, our mice also develop ultrastructural changes of the bronchial wall, including epithelial hyperplasia, thickening of the basement membrane and goblet cell hyperplasia, in response to chronic inhalatory allergen challenge.

1.5 The link between upper and lower airways

Epidemiologic studies have consistently shown that allergic asthma and rhinitis often coexist in the same patients (46). In addition, both airway diseases share the same trend of increasing incidence, predisposing factors, pathophysiological mechanisms following allergen

encounter and beneficial effect of treatment with topical corticosteroids (47). Therefore, allergic rhinitis and asthma seem to be manifestations of the same disease in two parts of the respiratory tract, with asthma at the more severe end of the spectrum. The mechanisms connecting upper and lower airway dysfunction have not been well established. Factors such as increased mouth breathing due to nasal blockage with increased exposure of lower airways to allergens, spread of inflammatory mediators to the lungs through postnasal drip, and bronchoconstriction induced by stimulation of sensory nerves and nasobronchial reflexes, may all play a role in the lower airway dysfunction of patients with rhinitis (47).

In order to unravel the mechanisms linking inflammation in nose and lung, studies in mice with experimentally induced allergic airway inflammation are useful. However, recent murine models of allergic rhinitis (48-51) and all studies on experimental allergic asthma in mice have focussed on one part of the airway in isolation without studying rhinitis in the context of asthma.

1.6 IL-17 and neutrophil biology

IL-17 is a 20-30-kD protein that is secreted primarily by memory $CD4^+$ T cells and that shares homology with a protein encoded by the open reading frame 13 of *Herpesvirus saimiri* (52). Neither IL-17 nor its receptor have sequence similarity with any known cytokine or cytokine receptor (53). Due to the ubiquitous distribution of its receptor, IL-17 exerts pleotropic biological activities. Ligation of its receptor initiates the transcription of nuclear factor-kappa B (NF-κB) (54) and c-Jun NH_2-terminal kinase via tumor necrosis factor receptor-associated factor 6 (55). Recent reports illustrate that IL 17 plays a pivotal pro-inflammatory role in several inflammatory responses such as experimental autoimmune neuritis (56), organ allograft rejection (57), tumorigenicity of cervical

tumors (58) and bacterial pneumonia (38). In patients, expression of IL-17 is found in inflamed sites of rheumatoid arthritis (59), multiple sclerosis (60), psoriasis (61), allergic contact dermatitis (62) and allergic asthma (63).

Increasing evidence suggests that IL-17, acting either directly or indirectly, significantly stimulates neutrophil maturation, migration and function (Fig. 1.4). Overexpression of IL-17 results in massive peripheral neutrophilia associated with increased levels of granulocyte colony-stimulating factor (G-CSF) and enhanced granulopoiesis (64). Furthermore, impaired IL-17 receptor signaling lead to delayed bronchial recruitment of neutrophils and impaired clearance of *Klebsiella pneumoniae* infection (38).

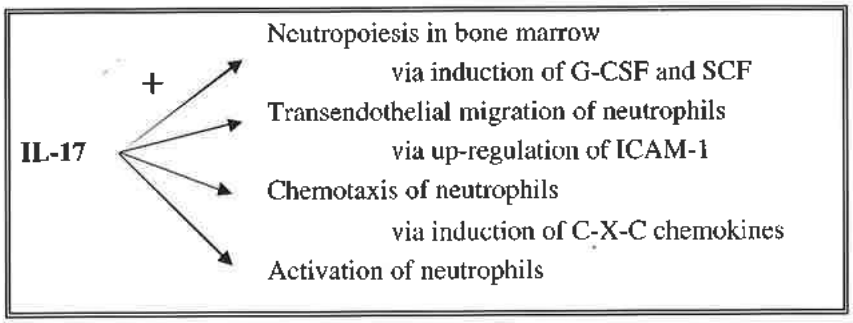

Fig. 1.4 Overview of the functional role of IL-17 in neutrophil biology.

In several cell systems, IL-17 induces the release of C-X-C chemokines (65) and up-regulates IFN-γ-mediated ICAM-1 expression (62). These pro-inflammatory effects of IL-17 are further potentiated by the induction of TNF-α and IL-1 (66), which are involved in up-regulating cellular adhesion molecules and inflammatory cell recruitment. In addition, IL-17 has been reported to activate neutrophils in association with their recruitment to the airways (67). In allergic asthma, inhalation of allergens leads to an inflammatory cascade in which

bronchial eosinophilic influx represents the hallmark of this disease (1). Besides eosinophils, recent evidence is emerging for neutrophil participation in allergic inflammation (68). Indeed, bronchial neutrophilia has been reported in severe asthma attacks (69-72) and after bronchial allergen challenge in asthmatic patients (73). *In vitro* studies showed that neutrophilic proteases are important mucin secretagogues (74), mediate activation of epithelial cells and heighten vascular permeability (75). Bronchial neutrophils may also be involved in airway remodeling in asthma as they represent a potentially important source of TGF-β (76).

Experimental data in a rat model of allergic asthma suggest that T lymphocytes are essential for the development of bronchial neutrophilia (77). However, factors responsible for this T lymphocyte-mediated bronchial neutrophilic influx remain to be identified. In this regard, we hypothesized that IL-17, a T cell-derived cytokine whose biologic functions are beginning to be elucidated, would represent the link between activated $CD4^+$ cells and bronchial neutrophilic influx in allergic asthma.

1.7 T cell activation and costimulatory pathways

Upon encounter with specific antigen, naive Th cells become activated. This event is regulated not only by the engagement of the T cell receptor (TCR) with peptide presented in the context of major histocompatibility complex (MHC) II molecules, but also by a second signal (Fig. 1.5) (11). Interaction between costimulatory molecules on the surface of APCs and Th cells serves as a second obligatory signal for T cell activation. Indeed, absence of signaling through costimulatory molecules leads to aborted activation and depletion of antigen-activated Th cells, or a prolonged state of anergy or clonal unresponsiveness (11). We gained interest in the field of costimulation because of the capacity of

costimulatory interactions to modulate both the activation and differentiation of Th cells (11). Therefore, costimulatory interactions may potentially play an important role in the allergic inflammatory response *in vivo*.

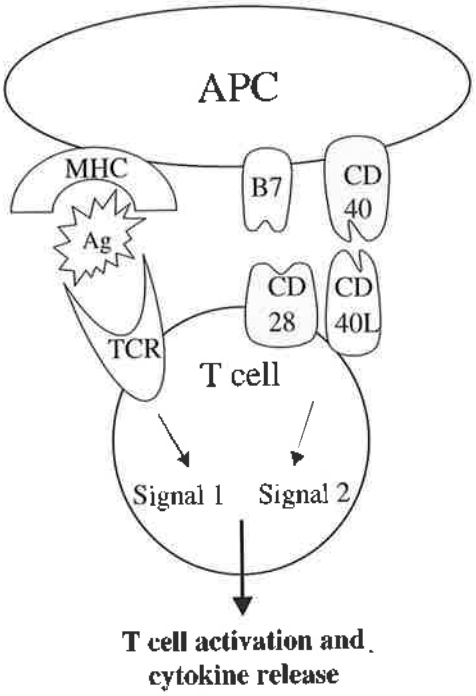

Fig. 1.5 Schematic representation of T cell activation.
Besides ligation of the T cell receptor (TCR) by antigenic peptide (signal 1), T cells become fully activated when surface molecules on T cells can interact with their counter-ligands on antigen-presenting cells (APC, signal 2).

Several ligand-receptor pairs, such as CD40-CD40L, B7-CD28/cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), B7-related protein-1 (B7-RP-1)-inducible costimulator (ICOS), CD58-CD2, LFA-1 (CD18)-ICAM-1 (CD54), OX40L-OX40, 4-1BBL-4-1BB and others, are expressed on APC and T cells respectively and determine the fate of both cells after antigenic stimulation. Interactions between B7-

CD28/CTLA-4 and CD40-CD40L molecules have been well characterized and will be discussed in the next paragraph.

1.8 The role of B7-CD28/CTLA-4 interaction in the immune response

CD28 is a molecule that is constitutively expressed on the surface of T lymphocytes and that binds molecules of the B7 family on APCs, i.e. B7-1 (CD80) and B7-2 (CD86, Fig. 1.6). Engagement of CD28

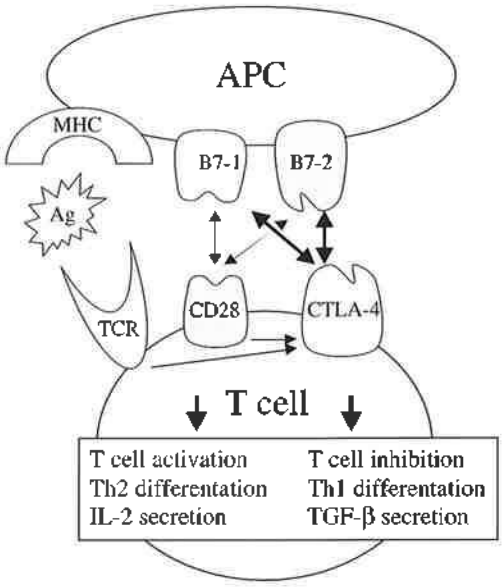

Fig. 1.6 Schematic overview of B7-CD28/CTLA-4 interaction.
Ligation of the constitutively expressed CD28 on T cells by B7 molecules generates a positive signal for T cell activation, whereas ligation of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) on activated T cells provides a negative signal opposing T cell activation.

provides a critical signal for IL-2 production and clonal expansion of T cells. Furthermore, B7-CD28 interaction has been implicated in the differentiation of naive cells towards the Th2 lineage (78).

A homologue of CD28, CTLA-4 or CD152 (78), is primarily expressed as an intracellular molecule that cycles to the cell surface where it can interact with B7 counterligands (B7-1 or CD80 and B7-2 or CD86, Fig. 1.6). CTLA-4 expression is up-regulated by T cell activation which normally requires signals through both the TCR and CD28 costimulatory pathway (78). Once expressed, CTLA-4 interacts with B7 molecules with much higher affinity than CD28 (79). In contrast to CD28, ligation of CTLA-4 generates an inhibitory signal for T cell activation. Taken together, ligation of the CD28 molecule activates T cells, leading to up-regulation of CTLA-4 on their surface. Signaling through CTLA-4 will then dampen T cell activation and hence prevent T cell hyperactivity.

Several mechanisms of action have been postulated for CTLA-4-mediated inhibition of T cell responses (79). Cross-linking of CTLA-4 leads to impaired IL-2 production and cell cycle progression of activated T cells, through shutting off signaling events downstream T cell receptor and/or CD28, i.e. activation of microtubule-associated protein kinases (MAPK) ERK and JNK. In addition, CTLA-4 antagonizes CD28 function by competing for CD80/CD86 molecules, inhibits CD28-mediated TCR reorganization, and/or exerts down-regulatory function through secretion of the anti-inflammatory TGF-β. Each of these non-mutually exclusive mechanisms may be involved in the negative regulation of T cell activity by CTLA-4. The balance between opposing signals elicited by CD28 and CTLA-4 is central to the regulation of T cell responsiveness and homeostasis (79).

The pivotal role of CTLA-4 as a negative regulator of T cell activation is highlighted *in vivo* by the fate of CTLA-4-deficient mice who develop lethal lymphoproliferation (80). In addition, blockade of CTLA-4 with mAb prevents the development of tolerance to soluble

antigen, augments anti-tumor responses, exacerbates auto-immune disease and can even induce auto-immunity in normal mice (79). Recently, involvement of CTLA-4 in Th cell differentiation was suggested by the Th2 phenotype of $CD4^+$ T cells that lack functional CTLA-4 (81). Alternatively, CTLA-4 ligation polarizes naive $CD4^+$ T cells towards Th1 predominance (82).

In mice, functional interaction between B7 and CD28 molecules is essential for the manifestation of allergic airway inflammation. Indeed, CD28 deficient mice show impaired development of experimental allergic asthma (83) and treatment of mice with soluble CTLA-4-Ig fusion protein or anti-B7 mAbs (84) protects them from disease initiation (33). In contrast, the *in vivo* relevance of CTLA-4 expressed on $CD4^+$ T cells in asthmatic bronchi (85) still remains to be elucidated. Based on *in vitro* studies, one could speculate that the CTLA-4 molecule represents a key factor involved in dampening Th2 differentiation and Th2 cytokine-mediated inflammation. Consequently, neutralization of CTLA-4 by blocking mAbs would aggravate Th2 cytokine related diseases. However, neutralization of CTLA-4 has been shown to inhibit Th2-mediated graft-versus-host disease (86) and enhance Th1-mediated diseases such as auto-immune diabetes (87) and experimental auto-immune encephalomyelitis (88).

1.9 Role of CD40-CD40L in the immune response

CD40 is a 45-50 kDa integral membrane glycoprotein that belongs to the tumor necrosis factor (TNF) receptor family. Initially considered as a B-cell-specific receptor, CD40 is now recognized as being widely distributed (89). Its ligand, CD40L or CD154, is a 35 kDa type II transmembrane protein of the TNF family that is expressed mainly on activated $CD4^+$ cells, but also on activated $CD8^+$ cells, mast cells,

basophils, NK cells, B cells, eosinophils, dendritic cells and platelets (89). Apart from its 33kDa transmembrane form, the CD40L molecule is associated with 2 shorter versions of the protein, which are soluble CD40L (sCD40L) fragments (90). sCD40L is released after activation of $CD4^+$ T cells (90) and can replace membrane-bound CD40L on $CD4^+$ T cells for activation of B cells in vitro (91). Little is known so far about the effects of sCD40L *in vivo*.

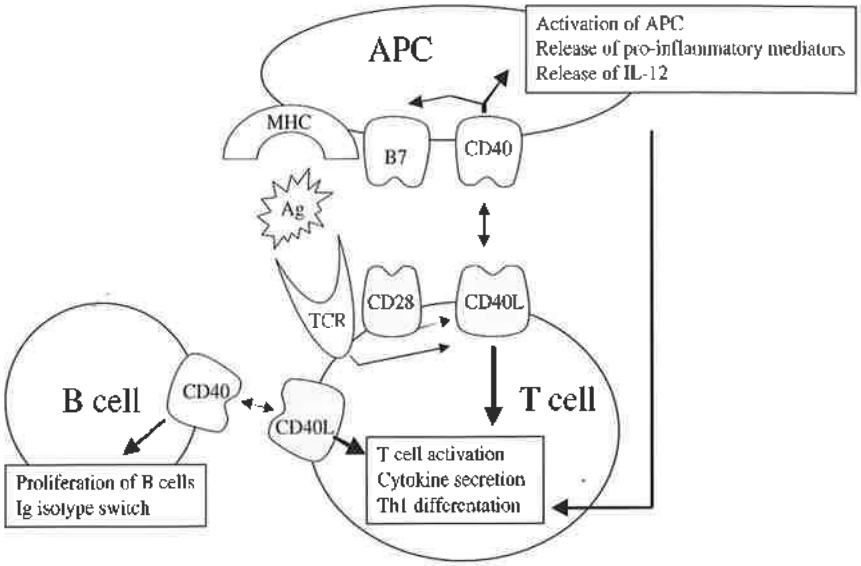

Fig. 1.7 Schematic overview of CD40-CD40L interaction. Ligation of CD40 activates monocytes and B cells, leading to the release of pro-inflammatory cytokines and Ig isotype class switch respectively. Binding of CD40L to CD40 generates a positive signal for T cell acitvation

CD40 ligation activates several second messenger systems (92), resulting in the activation of various transcription factors including NF-κB. Besides isotype switch, activation of CD40 has major effects on B cell biology: proliferation of immature and mature B-cell subsets, differentiation and Ig production, rescue from apoptosis, phenotypic differentiation into germinal center cells and skewing of the maturation

of germinal center cells into memory rather than plasma cells (92). On $CD34^+$ haematopoietic progenitors, ligation of CD40 induces cellular proliferation as well as differentiation into DC (93). CD40 signaling in monocytes and DC (Fig. 1.7) results in rescue from apoptosis and secretion of proinflammatory mediators such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α and MIP-1. Of note, the capacity of CD40 signaling to induce IL-12 production, which biases the Th differentiation towards a Th1 profile, is relevant for Th-mediated diseases (94). Furthermore, ligation of CD40 up-regulates the expression of costimulatory and MHC class II molecules, thereby enhancing Ag-presenting capability (95). Finally, CD40 mediates up-regulation of intercellular adhesion molecules (ICAM), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells (89), and hence contributes to leucocyte recruitment. In T cells (Fig. 1.7), CD40L signaling triggers the production of both Th1 and Th2 cytokines (96). Taken together, these data indicate that interaction between CD40-CD40L generates a bidirectional signal that activates both cell types involved in the interaction. This phenomenon necessitates the existence of strict control mechanisms on the expression of CD40L. *In vitro* stimulation of T cell clones has revealed transient expression of surface CD40L with a peak at 3 to 6 h after stimulation (92). CD28 engagement has been shown to synergize with anti-CD3 to induce and maintain CD40L expression on activated T cells. Some cytokines, e.g. IL-2 and IL-12, act synergistically with anti-CD3 to enhance CD40L expression (92). In addition, ligand-mediated activation of the glucocorticoid receptor has been reported to upregulate the expression of CD40L on lymphocytes (97), whereas cyclosporin A and prostaglandin E2 do the opposite. The expression of CD40L, as well as CD40L mRNA production, is downregulated as a result of CD40L

ligation by membrane bound or soluble CD40 during T cell-B cell recognition (92).

The demonstration of a critical role of CD40-CD40L interactions originated from the finding of a mutation in the CD40L gene in hyper-IgM syndrome (98). This syndrome is characterized by severe impairment of T cell-dependent antibody responses and leads to enhanced susceptibility to opportunistic infections. Consistent with findings in the hyper-IgM syndrome, transgenic mice lacking either CD40 or CD40L failed to mount antigen-specific Ig production in response to immunization with thymus-dependent antigen (99).

The functional relevance of CD40-CD40L interaction has been linked primarily to Th1 cytokine-mediated experimental diseases (92). For example, mAb against CD40L prevented the induction of rheumatoid arthritis, diabetes, lupus nephritis, auto-immune encephalitis (EAE) and oophoritis. Impaired Th1 responses have been reported in mice lacking CD40L (100), whereas CD40 or CD40L deficiency failed to affect Th2-mediated inflammation (25, 101, 102). Apart from antigen priming, CD40-CD40L interaction is also functionally involved in ongoing inflammation. Administration of anti-CD40L mAb down-regulates secondary immune responses in experimental colitis (103), experimental allergic encephalomyelitis (104), lung fibrosis (105) and lupus nephritis (106) and prevents disease recurrence in EAE (107) and diabetes (108). The mechanism behind the beneficial effects of blockade of CD40-CD40L interaction is believed to be down-regulation of IL-12 production (103). However, Zhou et al. (109) reported that CD40L is not essential for induction of protective Th 1 immune responses to *Histoplasma capsulatum*.

Alternatively, stimulation of CD40 signaling induces IL-12 production (110) and hence skews the immune response towards Th1

differentiation concomitant with downregulation of the Th2 cytokine response (94). Indeed, agonistic anti-CD40 Ab induce protective Th1 responses to parasitic infections (111, 112), downregulate pathogenic Th2 inflammation in response to a Th2-eliciting stimulus (112) and prevent Th2-dependent neonatal transplantation tolerance (110). In addition, stimulation of CD40 signaling boosts immune responses (92), provides protective responses against B cell lymphomas (113) and promotes antigen-specific immunotherapy for cancer (114). Consistently, vaccination with CD40L/trimer induces protective immunity to *Leishmania major* infection and provides resistance to metastatic tumor growth (115). Unexpectedly, Mauri et al. (116) reported down-regulation of experimental collagen-induced arthritis by anti-CD40 mAb.

The relevance of CD40-CD40L interaction in Th2-mediated allergic airway inflammation remains speculative. Based on *in vitro* studies, one can speculate that blockade of CD40L during antigen priming would enhance Th2-mediated pathology (117). In addition, anti-CD40L mAb profoundly skews the ratio of IL-4 versus IFN-γ-producing T cells towards the Th2 phenotype in graft-versus-host disease (118). This phenomenon however does not apply to Th2-mediated allergic responses in CD40L (25) or CD40-deficient mice (101). Consistently, blockade of CD40L did not affect Th2 cytokine production in bronchial explants of atopic asthmatic patients (85). Also in experimental atopic dermatitis, CD40 deficiency did not alter cutaneous inflammation nor IL-4 expression (102). However, Lei et al. (119) found decreased bronchial inflammation in CD40L-deficient compared to WT mice in association with reduced expression of VCAM-1. Mehlhop et al. (25) demonstrated that the occurrence of AHR in response to sensitization to *Aspergillus fumatus* was dependent on functional CD40L. In atopic asthma, the recently described correlation between CD40 expression on airway

macrophages and production of IL-12 (120) suggests that CD40-CD40L interaction may be of importance in atopic asthma. In this regard, it is interesting to acknowledge that this CD40 molecule may represent an important target for modulation of the allergic inflammatory response. Indeed, stimulation of CD40 signaling with agonistic anti-CD40 mAb had Th1-deviating capacity, and results in down-regulation of eosinophilic inflammation after injection of *Schistosoma mansoni* eggs (112).

CHAPTER 2. AIMS AND RATIONALE

The principal aim of the present work was to study modulation of the immune response in allergic asthma *in vivo*. To this purpose, we developed a mouse model of allergic asthma that allows the evaluation of both functional as well as inflammatory parameters of allergic airway inflammation. The allergen that we used to sensitize the mice in our study is ovalbumine (OVA), a food allergen (121) that is routinely used in mouse models of allergic asthma. After systemic sensitization of mice with adjuvant-free OVA, mice repeatedly inhaled aerosols containing OVA. This experimental protocol mimics both the chronic as well as the airborne character of allergen exposure of asthmatic patients. We extended this classic biphasic experimental protocol by performing an acute allergen exposure of mice with existing airway inflammation as a result of sensitization and chronic allergen inhalation. This provocation allowed the study of allergen-induced bronchial and systemic inflammatory responses in mice with inflamed airways.

After establishment of the murine model of allergic asthma, we intended to study the upper respiratory tract in this model **(chapter 4.1)**. The aim of this part of the work was to evaluate the development of upper airway inflammation and to correlate it with lower airway inflammation. Therefore, mice were sacrificed at different time-points during the challenge phase and at different time-points after the acute provocation. For the study of upper airway inflammation in mice, 2 new experimental techniques were developed, i.e. nasal lavage and magnetic resonance imaging (MRI) of the murine nose.

We found that bronchial responses after provocation with OVA were characterized by a remarkable increase in bronchial tone and prominent bronchial neutrophilic influx. As the function of IL-17 has been linked to neutrophil biology (64), we investigated whether IL-17

would be involved in the bronchial neutrophilic influx after allergen inhalation **(chapter 4.2)**. Secondly, we studied whether neutralization of IL-17 by anti-IL-17 mAb would affect the functional or cellular characteristics of the inflammatory response after acute provocation. We then investigated the effects of chronic neutralization of IL-17 on bronchial and systemic inflammatory parameters.

Interaction between costimulatory molecules on APC and Th cells has been shown to be critically involved in the generation of cell-mediated as well as humoral immunity (78, 122). However, the precise role of CTLA-4 and CD40-CD40L interaction in allergic asthma is still poorly understood. Therefore, we used the murine model to investigate the role of these molecules in experimental asthma. We evaluated the effects of injection of blocking mAb against CTLA-4 during sensitization on allergen-specific Ig production as well as on the subsequent manifestation of allergic airway disease **(chapter 4.3)**. In view of the potential involvement of genetic factors in CTLA-4-mediated modulation of disease manifestation, the effects of anti-CTLA-4 mAb treatment were evaluated in high and low IgE-responding murine strains, i.e. BALB/c and C57BL/6 mice (123). In addition, anti-CTLA-4 mAb were also injected during the challenge phase of sensitized BALB/c mice and the function of lymphocytes was evaluated *in vitro*. Both cytokine production by lymphocytes from peribronchial lymph nodes (PBLN) and lymphoproliferation after allergen-specific stimulation were evaluated in *in vitro* assays at the time of sacrifice.

In order to unravel the functional relevance of CD40-CD40L interaction in allergic airway inflammation **(chapter 4.4)**, we injected neutralizing mAb against CD40L (MR1) or agonistic anti-CD40 (FGK45) mAb during either systemic sensitization or repeated challenges of sensitized mice with OVA. As the effects of anti-CD40 mAb (FGK45)

are believed to be mediated via induction of IL-12 (112), the effects of anti-CD40 mAb administration to IL-12-deficient mice were studied.

CHAPTER 3. MATERIALS AND METHODS

3.1 Experimental protocol

For induction of allergic airway inflammation, we used a biphasic protocol that was adapted from an existing protocol (124). Mice were sensitized by intraperitoneal (i.p.) injections of adjuvant-free ovalbumin (OVA, Sigma, St Louis, MO) on alternate days (d) from d 1 until d 13 (Fig. 3.1). In a second phase, sensitized mice were exposed to nebulized OVA (10 mg/ml) or saline (Sal) daily from d 33 until d 40. The degree of sensitization and induction of airway inflammation were evaluated by different parameters on d 28 and d 41 respectively (Fig. 3.1). Mice were sacrificed on d 41, i.e. 24 h after the 8th allergen challenge.

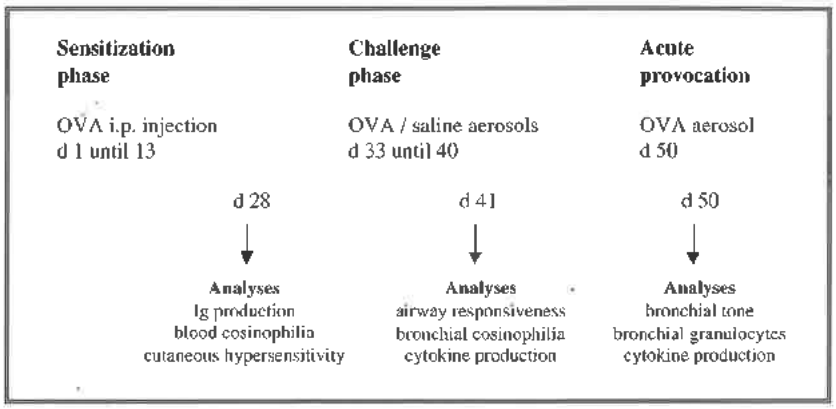

Fig. 3.1 Experimental protocol for induction of allergic airway disease and time-points of analyses.

In addition to the chronic exposure to nebulized OVA, we extended the experimental protocol with an acute allergen provocation (Fig. 3.1). This third phase consisted of exposure of sensitized mice with previously existing airway inflammation to nebulized OVA on d 50. This provocation was performed with low (10 mg/ml for 5 min, chapter 4.1) and high doses (200 mg/ml for 10 min, chapter 4.2) of nebulized OVA.

Mice were sacrificed before and at different time-points after provocation (15 min, 1, 3, 6, 12, 24, 48 and 72 h).

The OVA-mediated nature of observed effects after high dose OVA provocation of OVA-sensitized mice was verified by exposure of OVA-sensitized mice to nebulized saline or bovine serum albumin (BSA, Sigma) at 200 mg/ml for 10 min, and by exposure of naive BALB/c mice to nebulized OVA at 200 mg/ml for 10 min. To exclude a major contribution of endotoxins contaminating the OVA solution, OVA-sensitized mice were exposed for 10 min to nebulized saline containing endotoxin at 5 μg/ml, i.e. the endotoxin content of OVA solution of 200 mg/ml.

3.2 Cutaneous hypersensitivity reaction

On d 28, mice were injected in the right aural concha with OVA (1 μg in 10 μl of saline) and with an equal volume of saline in the contralateral concha. After 1 h, the thickness of both conchae was measured using a Mitutoyo Multimeter (Veenendaal, The Netherlands). The increase in thickness of the right aural concha was expressed as a percentage of the thickness of the contralateral concha. Pilot studies showed that intradermally injected BSA (1 μg in 10 μl of saline) failed to elicit any swelling of the aural concha.

3.3 Eosinophilia in peripheral blood and bone marrow

Mice were anaesthetized with i.p. injection of urethane (2.1 g/kg) at 24 h after the 8th allergen inhalation (d 41). A retro-orbital bleed was performed and blood smears were made. After dissection of one femur, bone marrow (BM) was removed by flushing the femoral shaft with RPMI 1640 (BioWhittaker, Walkersville, MD) and smears were made. Smears were stained with the classic May-Grünwald-Giemsa (MGG)

stain and differential cell counts performed on 300 cells within the white cell population.

3.4 ELISA for measuring OVA-specific IgE, IgG1 and IgG2a levels

After anaesthesia with urethane (i.p., 2.1 g/kg), a retro-orbital bleed was performed on d 0, 28 and at the time of sacrifice. For ELISA, 96-well plates were first coated overnight with rat anti-mouse IgE (10 g in 100 l of phosphate-buffered saline (PBS), PharMingen, San Diego, CA), rat anti-mouse IgG1 (20 g in 100 l PBS, PharMingen) or rat anti-mouse IgG2a (20 g in 100 l PBS, PharMingen). Remaining binding sites were blocked and plates were incubated with 100 l of diluted serum (1:5 for IgE, 1:1000 for IgG1, and 1:10 for IgG2a). After washing, following substances were sequentially added, incubated and washed: OVA (1 g/100 l, Sigma), peroxidase-labelled rabbit anti-OVA IgG (240 ng/100 µl, Rockland, Gilbertsville, PA) and buffer containing tetramethylbenzidin dihydrochloridhydrate (1 µl/ 100 µl, ACROS, New Jersey, NJ) and H_2O_2 (1 µl/ 100 µl). The peroxidase reaction was interrupted by the addition of H_2SO_4 and the optical density measured at 450 nm. Ig levels of a reference pool of serum of highly OVA-sensitized BALB/c mice were arbitrarily assigned a value of 100 experimental units (EU) per ml.

3.5 Measurement of bronchial tone

On d 41, bronchial responsiveness to inhaled metacholine (Mch) was measured using whole body plethysmography (Buxco¡, EMKA Technologies, Paris, France) as described (125). The parameter that is used for bronchial tone is the enhanced pause (Penh). Penh = (Te/Tr – 1) x (Pef/Pif) (Te, expiration time; Tr, relaxation time; Pef, peak expiratory flow; Pif, peak inspiratory flow). This technique has been validated and

compared to more standardized techniques for measuring bronchial tone by Hamelmann et al. (126). Penh values were calculated for each mouse under resting conditions and during 3 min after nebulizing saline and incremental doses of Mch (2,5, 5, 10, 20 and 50 mg/ml) for 1 min. The mean Penh value of each mouse was calculated during one min of maximal response after each aerosol.

On d 50, long-term measurements of bronchial tone were performed using whole body plethysmography. Unrestrained mice that had been sensitized to OVA and repeatedly challenged with OVA, were acutely exposed to nebulized OVA (200 mg/ml), bovine serum albumin (BSA, Sigma, 200 mg/ml) or saline. Penh values were recorded under resting conditions and during 3 h after an acute allergen provocation. The mean Penh value was calculated for each 5 min during the 1st h and for each 10 min during the following 8 h.

3.6 Nasal and broncho-alveolar lavages

Mice were anaesthetized by i.p. injection of urethane (2.1 g/kg) and their 4 limbs and cheeks were tightly attached to the operation table in supine position with hyperextended neck. A polyethylene catheter (∅ 0.85 mm) connected to a syringe was gently inserted in each nostril. One ml of PBS supplemented with 5 % of BSA (Sigma) at 37¡C, was slowly injected into one nasal cavity while this fluid was simultaneously aspirated via the catheter in the contralateral nostril. In this way, both nasal cavities were flushed and on average, 0.7 ml of lavage fluid was obtained. The nasal lavage (NL) fluid was centrifuged (4000 *xg*, 5 min) and the supernatant stored at -20¡C until analysis. The pellet was suspended in 100 µl of PBS for cell counting. Then the lungs were lavaged 5 times with 1-ml aliquots of pyrogen-free PBS at 37¡C through a polyethylene tracheal catheter (∅ 0.85 mm). The first broncho-alveolar

lavage (BAL) was performed with 1ml PBS supplemented with 5 % BSA, centrifuged (4000 xg, 5 min) and the supernatant stored at —20¡C until analysis. The cellular pellet was added to the subsequent 4 lavages, each one performed with 1 ml of PBS. The BAL fluid pool was centrifuged (4000xg, 5 min) and the pellet washed and suspended in 100 l of PBS. Ten l of cell suspension from NLF and BALF were added to 90 l of T rk s solution (Merck Diagnostica, Darmstadt, Germany) and the total number of cells counted in a B rker-T rk chamber. For differential cell counts, cytospin preparations were stained according to the May-Gr nwald-Giemsa method and 300 cells differentiated into eosinophils, neutrophils, monocytes and lymphocytes.

3.7 ELISA for measuring IL-4, IL-5, IFN-γ, IL-10 and TGF-β levels

Levels of IL-5, IFN-γ and IL-4 were measured in 1:2 diluted BAL fluid and undiluted NL fluid, serum and culture supernatants by sandwich ELISA using paired matched Abs from PharMingen (IL-5 and IFN-γ) and Biosource (IL-4 and IL-10, Fleurus, Belgium) according to the manufacturer s instructions. TGF-β was detected in BAL fluid (1:2 diluted) and culture supernatants (1:20 diluted) using an ELISA kit from Biosource. Lower limits of detection were 4.5, 1.2, 1.2, 39.0 and 4.0 pg/ml for IL-5, IFN-γ, IL-4, IL-10 and TGF-β respectively.

3.8 Histologic analysis

After performing BAL, the right lungs were dissected and fixed overnight in buffered formalin (5%). After dehydration and embedding in paraffin, 5- m sections were stained with the classic haematoxylin and eosin (H&E). On H&E-stained sections, eosinophils could be easily recognized by their polyglobular nucleus and bright cytoplasmic granules. For assessment of neutrophilic influx, a classic

myeloperoxidase (MPO) stain was performed as described (127). The maximal thickness of the inflammatory infiltrates around 2 bronchioli and 2 arterioli in the lung were measured using an eyepiece graticule at a magnification of 25. The average of these 4 values was calculated for each mouse and expressed in 10^{-2}/mm.

3.9 Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) assay

MPO activity in BAL fluid was measured using a method described by Bradley et al. (128). In brief, 100 µl of undiluted BAL fluid were incubated for 15 min with 2.9 ml of PBS containing 0.0005 % of H_2O_2 and 0.167 mg/ml of o-dianisidine dihydrochloride at pH 6.0. The reaction was stopped by the addition of 100 µl of NaN_3 1% and the optical density was measured at 460 nm. EPO activity in BAL fluid was assessed by incubation of undiluted BAL fluid (100 µl) with Tris-HCl buffer (50 µl) for 10 min. Then, 100 µl of substrate solution (2 mg/ml o-phenylenediamine and 1,3 µl/ml of H_2O_2 in Tris-HCl buffer) were added. The reaction was interrupted by addition of H_2SO_4 and the optical density was measured at 492 nm.

3.10 Real time quantitative reverse transciptase PCR (RT-PCR) for cytokine mRNA expression

Part of the left lung was dissected at 3 h after acute allergen provocation, immediately frozen in liquid nitrogen and stored at –80¡C. Total RNA was extracted using TRIzol (Life Technologies, Gaithersburg, MD). A constant amount of 1 µg of target RNA was reverse transcribed using 100 U Superscript II RT (Life Technologies) at 42¡ C for 80 min in the presence of 5 µM Oligo$(dT)_{16}$. Real time quantitative RT-PCR was performed for IL-17, IL-4, IL-5, IL-10, IL-13, IFN-γ, IL-12 p40 and IL-1β mRNA in the ABI prism 7700 Sequence detector (Applied

Biosystems, Foster City, CA) as described (129). The primer and probe for mouse IL-17 were designed based on the published sequence (130), and are as follows: IL-17 forward primer, 5 GCTCCAGAAGGCCCTCAGA 3 ; IL-17 reverse primer, 5 AGCTTTCCCTCCGCATTGA 3 ; IL-17 Taqman probe 5 FAM-CTCTCCACCGCAATGAAGACCCTGA-TAMRA 3 . The amplified PCR-fragment is 142 bp in length, located from bp 229 to 370. The primer and probe sequences used for IL-4, IL-5, IL-10, IL-13, IFN-γ, IL-12, IL-1β and TGF-β were as previously published (129). Each PCR amplification was performed in triplicate wells, using the following conditions: 10 min at 94¡C, followed by a total of 40 or 45 two-temperature cycles (15 sec at 94¡C and 1 min at 60¡C).

3.11 Magnetic resonance imaging (MRI) of murine nasal mucosa

After anaesthesia as above, coronal MRI was performed on mice on d 41, in an experimental setting that was specifically designed for making high resolution ($3x10^{-2}$mm) scans of murine tissues. We used a 30-cm bore horizontal superconducting 4.7-T magnet (Bruker BIOPSEC) equipped with a microscopy gradient insert with 6-cm bore. T1 weighted coronal images were acquired using a spin-echo sequence (TR / TE = 1000 / 15 ms), with a field of view of 10mm, matrix of 256 x 256, slice thickness of 1mm and 16 acquisitions. The mice were positioned in such a way that the nose fitted under a horizontal transmit/receive surface coil with a diameter of 17 mm.

3.12 Lymphoproliferation assay and evaluation of *in vitro* cytokine production

Peribronchial lymph nodes (PBLN) and spleens were dissected on d 41 and homogenized using a cell strainer (Falcon®, Becton Dickinson,

Franklin Lakes, NJ) and suspended in RPMI 1640 (Biowhittaker) supplemented with fetal calf serum (5%). The homogenates were washed twice with PBS / FCS and centrifuged at 1500 rounds per min for 10 min. Spleen homogenates were incubated for 1 min with 5 ml of lysis buffer containing NH_4Cl, $KHCO_3$ and Na_2EDTA, and then washed with PBS/FCS. Cells were counted using a Coulter Counter (Analis, Gent, Belgium).

For evaluation of lymphoproliferation, 2 x 10^5 PBLN cells were cultured alone or cocultured with 2 x 10^5 splenocytes as a source of APC, in the absence or presence 10 µg/ml of OVA or BSA (as negative control) in 200 µl of RPMI with 10 % FCS supplemented with penicillin, streptomycin, L-glutamine and 0.1% of 2-mercapto-ethanol in a sterile 96-well plate at 37¡C for 72 h. Wells were pulsed with 1 µCi (^{3}H)-thymidine (ICN, Costa Mesa, CA) and incubated for 8 h. Cells were harvested and thymidine incorporation was counted in a beta scintillation counter (Packard, Meriden, CT). Proliferation of cells was expressed as the mean cpm of quadruplicate cultures. For assessment of cytokine production *in vitro*, splenocytes and PBLN cells (1 x 10^6/ml each) were incubated in 24-well plates in the presence of 10 µg of OVA or BSA (as negative control) in 1 ml of culture medium (as described above) for 5 d at 37¡C. The supernatants (SN) were collected and stored at —20¡C until cytokine measurement.

3.13 Statistical analysis of results

Data are expressed as means – SEM. Statistical analyses were carried out using the *Mann-Whitney* test. A difference was considered to be significant when $p < 0.05$. A *Spearman* correlation test was performed for correlations between analyses of nasal and bronchial lavage fluids and histology.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Eosinophilic rhinitis accompanies the development of lower airway inflammation and bronchial hyperresponsiveness in sensitized mice

4.1.1 Introduction

At present, it is not clear to what extent allergic rhinitis coexists in allergic asthma. There are also no animal models studying rhinitis in the context of asthma. Here, we study the induction of upper airway inflammation in a murine model of allergic asthma. In contrast to local allergen application as done in existing murine models of allergic rhinitis (48-51), mice in our study inhaled nebulized allergen solution both during the induction of airway allergy (d 33 until 40) as well as during the acute allergen provocation (d 50). In order to investigate the pattern of induction of airway inflammation in both upper and lower airways, we studied inflammatory parameters in upper and lower airways of sensitized BALB/c mice that were sacrificed at different time-points during the development of eosinophilic airway inflammation and at several time-points before and after acute allergen provocation.

4.1.2 Results

4.1.2.1 Induction of OVA-specific IgE and airway hyperresponsiveness (AHR)

Sensitization of mice by i.p. injection of OVA on alternate d from d 1 until 13 induced production of OVA-specific IgE antibodies. On d 28, serum levels of OA-specific IgE were higher than before sensitization (91.5 ± 13.4 vs 0.5 ± 0.3 EU/ml, $p < 0.05$). Repeated exposure of sensitized mice to nebulized OVA (OVA mice) for 8 consecutive days caused a further but non-significant ($p > 0.05$) increase in OVA-specific

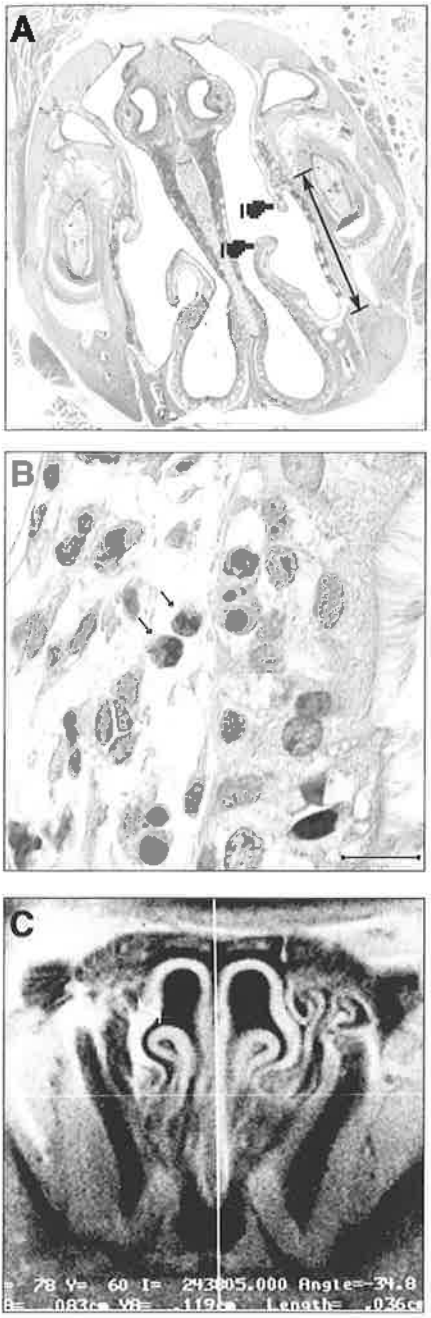

Fig. 4.1.1 Microscopic and radiologic images of the murine nose. (**A**) Light microscopic image (10-fold magnification) of a coronal section through the sinonasal skeleton showing the murine nasal anatomy. (**B**) A representative image (magnification of 1500, Axioplan microscope, Zeiss) of the nasal epithelium and subepithelial layer of mice that have been sensitized by intraperitoneal injections of ovalbumine (OVA) from day (d) 1 until 13 and then repeatedly challenged with nebulized OVA from d 33 until d 40. For quantification of eosinophilic inflammation on d 41, eosinophils (indicated with black arrows on **B**) were counted in a defined region of the nasal mucosa between the lower edge of the middle nasal turbinate (upper index on **A**) and the upper edge of the lower turbinate (lower index on **B**). (**C**) Coronal MRI of the murine skull on which measurements of thickness of nasal mucosa were performed on d 41 at the lateral superior nasal wall (white line) using an interactive computerized program.

IgE levels (109.0 ± 8.7 EU/ml) and induced AHR. Penh values in OVA mice (n = 6) rose to higher levels than in Saline mice (n = 6) after inhalation of nebulized Mch at 10 mg/ml (2.4 ± 0.8 versus 1.2 ± 0.4, $p < 0.05$), 20 mg/ml (3.4 ± 0.9 vs 2.1 ± 0.7, $p < 0.05$) and 50 mg/ml (9.2 ± 3.1 vs 3.7 ± 1.2, $p < 0.05$). Penh values were not different between both groups at rest (0.39 ± 0.1 and 0.37 ± 0.2 respectively) nor after inhalation of nebulized physiologic saline (0.40 ± 0.4 and 0.36 ± 0.3 respectively).

4.1.2.2 Repeated exposure to aerosolized allergen induces nasal inflammation in parallel with lower airway inflammation

Airway inflammation in nose and lung was studied histologically after 1, 4 and 8 daily airway challenges with OVA or saline. The murine nose is anatomically similar to the human, including the nasal septum, turbinates and paranasal sinuses (Fig. 4.1.1 A). The nasal inflammatory infiltrate in OVA mice was present mainly in the subepithelial layer along the lower part of the nasal septum and lateral nasal wall, and consisted primarily of eosinophils and mononuclear cells (Fig. 4.1.1 B). Eosinophil infiltration was quantified by counting eosinophils in the mucosa between the lower edge of the middle turbinate and the superior edge of the lower turbinate (Fig. 4.1.1 A). As shown in Fig. 4.1.2 A, eosinophil counts in the defined nasal region ranged from 6.0 ± 1.9 to 10.1 ± 2.2 and 19.4 ± 2.2 after 1, 4 and 8 OVA challenges (i.e. on d 34, 37 and 41 respectively, n = 6 for each group). On d 41, nasal eosinophil counts were higher in OVA than in Sal mice (19.4 ± 2.2 vs 6.3 ± 2.0, $p < 0.05$, Fig. 4.1.2 A). In the lung, the mean thickness of perivascular and peribronchiolar inflammatory infiltrates increased from 22.5 ± 0.5 $x10^{-2}$ to 90.0 ± 7.5 $x10^{-2}$ and 130.0 ± 7.5 $x10^{-2}$ mm after 1, 4 and 8 challenges with OVA respectively (Fig. 4.1.2 B). On d 41, OVA mice showed more bronchial

inflammation than Sal mice (130.0 ± 7.5 $x10^{-2}$ vs 20.0 ± 5.0 $x10^{-2}$mm, $p <$ 0.05).

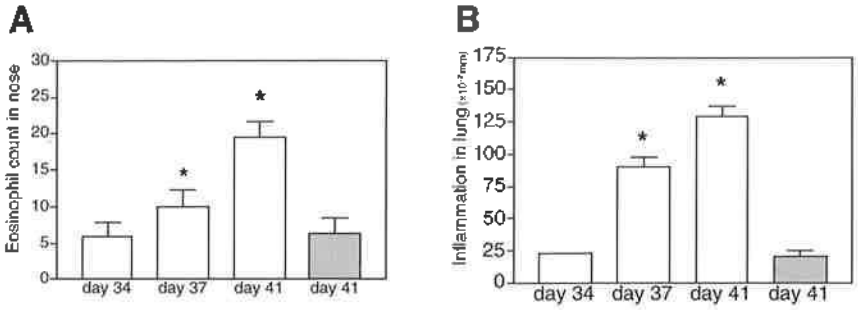

Fig. 4.1.2 Histologic analysis of upper and lower airway inflammation of sensitized mice during the induction of airway inflammation. Mice were sacrificed after 1 (day 34), 4 (day 37) or 8 (day 41) challenges with nebulized ovalbumine (OVA, open bars) or after 8 exposures to nebulized saline (d 41, filled bars). (**A**) Eosinophils were counted in the nasal mucosa in the defined region and (**B**) eosinophilic inflammation was scored in the lower airways by measuring the thickness of peribronchiolar and perivascular inflammatory infiltrates. Each group consisted of 6 mice. One representative experiment out of 2 is presented. *, $p <$ 0.05 compared to saline-challenged mice (filled bars).

To study nasal mucosal changes *in vivo*, a MRI study of the nose and paranasal sinuses was performed on d 41, which is 24 h after the 8th aerosol with saline or OVA (n = 5 per group). The mucosal thickness at the level of the superior lateral nasal wall (Fig. 4.1.1 C) was measured with the aid of a computerized processing program on magnified MRIs. This region was chosen for measurement because of the presence of eosinophilic inflammation on histologic examination and the clear visualization of this mucosa on MRI. Measurements of mucosal thickness were performed on each nasal side and on 2 scans of each mouse. The mean mucosal thickness was 27.4 ± 1.6 $x10^{-2}$ and 19.1 ± 1.2 $x10^{-2}$mm in OVA and Sal mice respectively, which represented a significant ($p <$ 0.05) increase of 42% in OVA mice. Pilot studies demonstrated that the mucosal swelling we found on MRI scans in OVA mice was an OVA-

mediated phenomenon in OVA-sensitized mice. Indeed, 8 daily inhalations of nebulized OVA (10 mg/ml) by sham-sensitized mice (mean: 20.1 x 10^{-2}/ml, n = 2) or 8 daily inhalations of nebulized BSA solution (10 mg/ml) by OVA-sensitized mice (mean: 19.2 x 10^{-2}/ml, n = 2) failed to induce any nasal mucosal swelling on MRI scans compared to Sal mice (19.1 ± 1.2 x10^{-2}mm, n = 5).

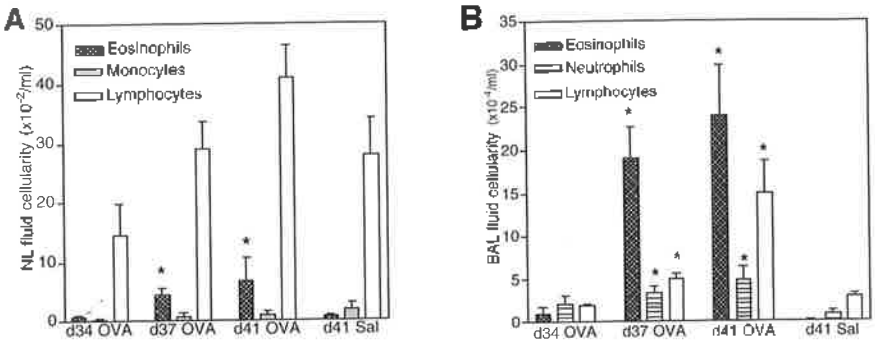

Fig. 4.1.3 Cellular analyses of lavage fluids of upper and lower airways in the course of repeated challenges (day (d) 33 till 40) of sensitized mice with either ovalbumine (OVA) or saline (Sal). Differential cell counts were performed on cytospin preparations of nasal lavage (NL, **A**) and broncho-alveolar lavage (BAL, **B**) fluids after 1 (d 34), 4 (d 37) and 8 (d 41) exposures of sensitized mice to nebulized OVA or Sal. Each group consisted of 6-8 mice. One representative experiment of 2 is shown. *, $p < 0.05$ compared to similar cell type in lavage fluid of Sal mice on d 41.

In NL fluid of OVA mice, eosinophil counts progressively rose in the course of repeated inhalations of OVA (Fig. 4.1.3 A), whereas saline had no effects on eosinophil counts. On d 41, eosinophil counts in NL fluid of OVA mice were significantly ($p < 0.05$) higher than in Sal mice (Fig. 4.1.3 A). Lymphocyte counts were also increased in OVA mice on d 34, 37 and 41, but the difference between OVA and Sal mice on d 41 was not significant ($p > 0.05$, Fig. 4.1.3 A). Monocyte counts were very low and did not differ among the 4 groups of mice analyzed: 0.3 ± 0.2 x10^2,

$0.8 \pm 0.5 \times 10^2$, $1.0 \pm 0.7 \times 10^2$ and $2.0 \pm 1.0 \times 10^2$/ml for OVA mice on d 34, 37 and 41 and Sal mice on d 41 respectively. Neutrophils constituted the most frequent cell type in NL fluid (mean: 75 %) and their number did not differ among the groups analyzed: $6.7 \pm 0.6 \times 10^3$, $6.0 \pm 1.1 \times 10^3$ and $4.8 \pm 0.9 \times 10^3$/ml in OVA mice on d 34, 37 and 41 respectively and $6.9 \pm 0.6 \times 10^3$/ml in Sal mice on d 41. Total cell counts in NL fluid were similar in all groups: $7.5 \pm 0.4 \times 10^3$, $9.4 \pm 0.9 \times 10^3$ and $9.7 \pm 0.8 \times 10^3$/ml in OVA mice on d 34, 37 and 41, and $9.9 \pm 0.7 \times 10^3$/ml in Sal mice on d 41 respectively.

In BAL fluid, the number of eosinophils increased progressively in the course of repeated exposures to OVA (Fig. 4.1.3 B). On d 41, eosinophil counts in BAL fluid of OVA mice were higher than in Sal mice ($24.0 \pm 5.8 \times 10^4$/ml vs $0.1 \pm 0.1 \times 10^4$/ml, $p < 0.05$, Fig. 4.1.3 B). Also neutrophil counts were higher in BAL fluid of OVA than Sal mice on d 41 ($4.8 \pm 1.5 \times 10^4$ vs $0.8 \pm 0.5 \times 10^4$/ml, $p < 0.05$). Lymphocyte numbers increased from $2.1 \pm 0.2 \times 10^4$ over $5.0 \pm 0.5 \times 10^4$ to $15.0 \pm 3.8 \times 10^4$/ml in BAL fluid of OVA mice on d 34, 37 and 41 respectively. On d 41, lymphocytes in BAL fluid of OVA mice ($15.0 \pm 3.8 \times 10^4$/ml) significantly outnumbered lymphocytes in BAL fluid of Sal mice ($3.0 \pm 0.2 \times 10^4$/ml, $p < 0.05$, Fig. 4.1.3 B). Monocytes (not illustrated) were the most frequent cell type in BAL fluid of Sal mice ($3.7 \pm 1.3 \times 10^5$/ml) and their number did not change upon challenge with OVA (data not shown). Neither did the total number of cells in BAL fluid differ among the groups of mice analyzed: $4.5 \pm 1.6 \times 10^5$, $6.6 \pm 1.9 \times 10^5$ and $7.2 \pm 2.3 \times 10^5$/ml after 1, 4, and 8 OVA challenges respectively and $4.0 \pm 1.2 \times 10^5$/ml after 8 saline exposures.

4.1.2.3 Effects of acute allergen exposure

To mimic the situation in which a patient with chronic airway allergy is acutely exposed to allergen, OVA sensitized (d 1 until 13) and chronically challenged (d 33 until 40) mice, inhaled nebulized OVA (10 mg/ml) for 5 min on d 50. This allergen-free interval of 10 d was arbitrarily chosen because inflammatory cells in BAL fluid and bronchial tissue had largely resided at this time-point, and therefore allows studying new cellular influx.

Acute allergen exposure resulted in a systemic increase in IL-5 and eosinophils as evidenced by blood analysis. The level of IL-5 was 9.2 ± 2.1 pg/ml of serum before provocation and increased significantly ($p < 0.05$) at 6 h (81.1 ± 0.4 pg/ml) and 12 h (35.1 ± 8.5 pg/ml) after provocation. No increase was recorded at 3 h (7.3 ± 1.1 pg/ml) or at any other time-point after provocation (15 min, 1, 6, 12, 24, 48 and 72 h). The percentage of eosinophils in blood was 4.3 ± 0.4 before provocation and transiently increased until 13.0 ± 2.5 ($p < 0.05$) at 6 h after allergen provocation.

The respiratory tract was analyzed at several time-points after provocation, i.e. 15 min, 1, 3, 6, 12, 24, 48 and 72 h. We only present findings of mice sacrificed at 6 h after provocation (Post mice) because nasal inflammation was maximal at that time-point and no increase in lower airway inflammation was measured at any other time-point after provocation. Data of Post mice were compared to mice sacrificed on d 50 before provocation (Pre mice). In the defined region of the nose, an eosinophil count of 7.2 ± 1.4 was found at 6 h after provocation (Post, Fig. 4.1.4 A), which represented a significant increase compared to mice analyzed before provocation (Pre, 1.1 ± 0.3, $p < 0.05$). The mean thickness of peribronchial and perivascular inflammation was not altered by this single allergen provocation (Fig. 4.1.4 B).

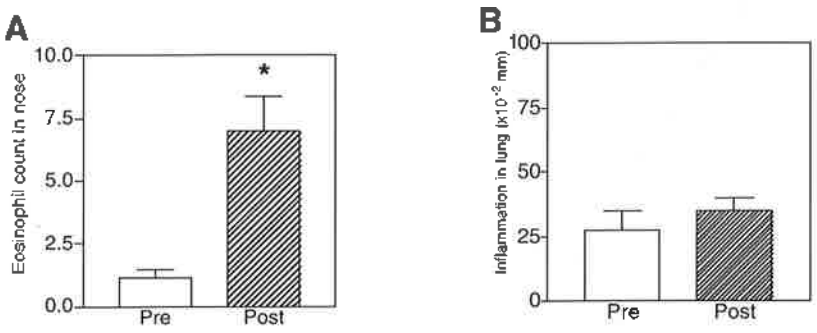

Fig. 4.1.4 Histologic analysis of upper and lower airway inflammation after allergen provocation. Mice that had been sensitized and challenged with ovalbumine (OVA) were exposed to nebulized OVA (10 mg/ml for 5 min) on day 50. The inflammation in upper and lower airway tissue before (Pre) and at 6 h after provocation (Post) was quantified by counting the number of eosinophils in the defined mucosal region of the nose (**A**) and by measuring the thickness of the peribronchial and perivascular eosinophilic inflammatory infiltrate in the lungs (**B**). Each group consisted of 6 mice. One representative experiment out of 2 is presented. *, $p < 0.05$ compared to Pre mice.

In NL fluid, an eosinophil count of 3.0 ± 0.8 x10^2/ml before provocation increased to a maximum of 10.8 ± 3.4 x10^2/ml ($p < 0.05$) at 6 h after provocation (Fig. 4.1.5 A). Lymphocyte numbers were maximal at 12 h (46.4 ± 6.1 x10^2/ml) after provocation, which represented a significant increase compared to counts before provocation (22.6 ± 5.2 x10^2/ml, $p < 0.05$, Fig. 4.1.5 A). Monocyte numbers in NL fluid were not influenced by OVA provocation. Neutrophil counts in NL fluid (not illustrated) were similar in the different groups: 56.7 ± 7.1 x10^3, 62.0 ± 8.6 x10^3, 47.3 ± 6.6 x10^3, 34.8 ± 10.5 x10^3 and 14.8 ± 3.8 x10^3/ml for mice analyzed before and at 3, 6, 12 and 24 h after provocation respectively. Total cell counts in NL fluid were 59.4 ± 6.7 x10^3/ml before provocation and their counts did not change upon provocation. The level of IL-5 in NL fluid of mice analyzed at 6 h after allergen provocation was

18.8 ± 2.1 pg/ml whereas no IL-5 (lower limit of detection: 4.5 pg/ml) could be detected in NL fluid of mice at any other time-point after provocation. Results of mice sacrificed at 15 min, 1, 48 and 72 h after provocation are omitted because of the lack of any increase in cell counts in lavage fluids compared to Pre mice.

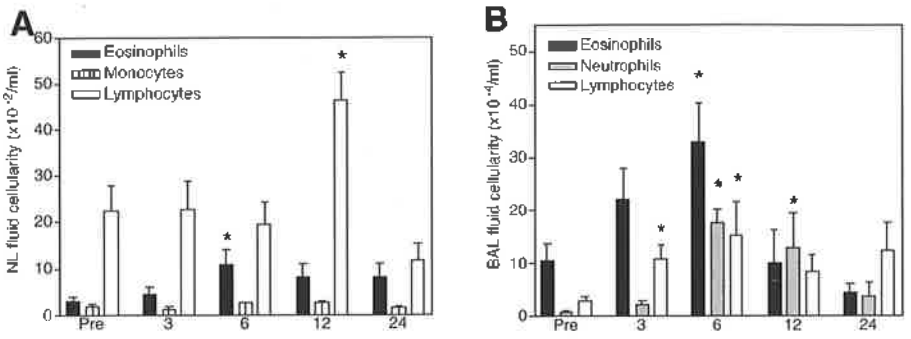

Fig. 4.1.5 Cellular analysis of lavage fluids of upper and lower airways before and after acute allergen provocation with ovalbumine (OVA, 10 mg/ml for 5 min) on day (d) 50. Differential cell counts in nasal lavage (NL, **A**) and broncho-alveolar lavage (BAL, **B**) fluids were compared between mice sacrificed before (Pre) and at 3, 6, 12 and 24 h after provocation. Each group consisted of 6 mice. One representative experiment out of 2 is shown. *, $p < 0.05$ compared to similar cell type in lavage fluid of Pre mice.

In BAL fluid, eosinophils reached their maximal number (33.1 ± 7.3 x10^4/ml) at 6 h after provocation, up from 10.7 ± 3.2 x10^4/ml before provocation ($p < 0.05$, Fig. 4.1.5 B). Neutrophil counts in BAL fluid were increased at 6 h (17.6 ± 2.6 x10^4/ml) and 12 h (13.1 ± 6.5 x10^4/ml) after provocation as compared to mice analyzed before provocation (0.8 ± 0.3 x10^4/ml, $p < 0.05$). Lymphocyte counts were 3.0 ± 0.9 x10^4/ml before provocation and increased ($p < 0.05$) to 10.8 ± 2.7 x10^4 and 15.3 ± 6.3 x10^4/ml at 3 and 6 h after OVA inhalation respectively. Monocytes were the most numerous cell type in BAL fluid (not illustrated) and their counts did not differ among groups: 8.7 ± 2.3 x10^5, 13.0 ± 2.5 x10^5, 12.1 ± 2.3 x10^5, 23.1 ± 6.5 x10^5 and 10.0 ± 17.1 x10^5/ml in mice analyzed before

and at 3, 6, 12 and 24 h after provocation respectively. Total cell counts in BAL fluid were 10.2 ± 2.4 x10^5/ml before provocation and did not change upon provocation. IL-5 levels in BAL fluid increased ($p < 0.05$) after OVA provocation at 3 h (20.9 ± 3.4 pg/ml) and 12 h (29.3 ± 4.4 pg/ml) in comparison to the level before provocation (11.7 ± 3.2 pg/ml).

4.1.2.4 Comparison of parameters of eosinophilic inflammation

Positive correlations were found between eosinophil counts in BAL fluid and inflammatory scores of lung tissue in individual mice exposed to nebulized OVA, i.e. OVA mice on d 34, 37, 41, and 50 (n = 22, r = 0.61, $p < 0.05$). Eosinophil counts in NL fluid and in nasal tissue were positively correlated in these mice (n = 19, r = 0.68, $p < 0.05$). A highly positive correlation (n = 18, r = 0.81, $p < 0.05$) was also observed between eosinophil counts in NL and BAL fluids of OVA-exposed mice.

4.1.3 Conclusion

In this study on nasal allergy, we have used a model in which mice were actively sensitized with allergen in the absence of adjuvant, and then repeatedly (8 times) exposed to nebulized allergen. These mice develop an airway disease with features reminiscent of human allergic asthma (1): allergen-specific IgE production, blood eosinophilia, AHR and peribronchial eosinophilic inflammation. In these mice, allergic rhinitis could be demonstrated by analysis of different parameters of the inflammatory spectrum: IL-5 and eosinophils in NL fluid, histologic evidence of eosinophilic inflammation and increased mucosal thickness on MRI. Moreover, subsequent acute allergen exposure of these mice after an interval of 10 d led to a systemic reaction as evidenced by increased IL-5 levels in serum and eosinophils in blood, with concomitant

re-induction of eosinophilic inflammation in nasal tissue and eosinophilic influx in both upper and lower airway lumen.

Two new techniques were developed to demonstrate nasal inflammation in mice. First, nasal lavage was developed to investigate cellular and cytokine contents in nasal secretions. Analyses of NL fluids showed infiltration of eosinophils in mice that inhaled OVA, and the percentage of eosinophils (8.6 %) in NL fluid of mice was comparable to that in patients with allergic rhinitis (131, 132). Moreover, the eosinophil count in NL fluid proved to be a valuable parameter for evaluation of nasal inflammation as it correlated well with histologic eosinophil count in nasal mucosa. Depending on the aim of studies, one could therefore consider omitting histologic analyses in future studies because histologic examination is more time-consuming and laborious than nasal lavage. On the other hand, the murine nose is small and thus the area of inflammation limited, which results in low numbers of cells in NL fluid. Despite their dilution, cytokines could still be detected in NL fluid at 6 h after acute provocation. Apart from IL-5, IL-4 levels (data not shown) were maximal at 6 h after provocation. The technique of nasal lavage in mice therefore emerges as a valuable method for evaluating the content of nasal secretions and it represents a new parameter for research on allergic rhinitis. In addition to nasal lavages, MRI of the murine skull represents a new, non-invasive but technically demanding tool to investigate the nasal mucosa *in vivo*. Increased thickness of the nasal mucosa was observed at the superior lateral nasal wall of OVA-sensitized and challenged mice. This phenomenon was demonstrated here to be allergen-specific and was consistent with the inflammation found on histology. Apart from mucosal swelling, no other abnormalities or signs of sinusitis, commonly observed in asthmatics (133), were found in OVA-challenged mice. More

prolonged allergen exposure or contact with pathogenic micro-organisms may be required for the development of sinusitis.

Interestingly, allergic inflammation was simultaneously and progressively induced in the upper and lower airways in the chronic model with daily allergen exposure, and parameters of inflammation were positively correlated in the upper and lower respiratory tract. In contrast, acute allergen provocation induced inflammation at the site of major allergen deposition, i.e. the nasal mucosa. Pilot studies showed that 83 % of inhaled particles are retained within the nose. Therefore, we speculate that allergen deposition in the nose is primarily involved in eliciting the observed systemic response, and may hence contribute to the development of bronchial inflammation. In this regard, Gaspar Elsas et al. (134) reported that intranasal application of OVA in sensitized mice caused a rapid systemic response characterized by increased circulating levels of IL-5 and bone-marrow eosinophilia. Furthermore, Braunstahl et al. (135) nicely demonstrated in humans that segmental bronchial provocation resulted in enhanced blood eosinophilia and nasal eosinophilic inflammation Taken together, these data point to a remarkable systemic response following mucosal allergen encounter. In spite of the systemic increase in IL-5 levels in our study, no renewed peribronchial tissue inflammation was found after a single provocation. This finding suggests that induction of lower airway inflammation requires a chronic exposure or a higher dose of allergen, while upper airways are more sensitive to a single exposure.

Taken together, our murine model of allergic asthma and rhinitis offers a particularly valuable investigative tool to study pathophysiologic mechanisms underlying global airway allergy and to test potential new therapies for allergic airway diseases. Our results show for the first time simultaneous induction of eosinophilic inflammation in nose and lung and

support the viewpoint that allergic rhinitis and asthma are part of global airway allergy. The coexistence of rhinitis and asthma in patients with respiratory allergy may have important implications for managing airway allergy.

4.2 IL-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma

4.2.1 Introduction

We here investigated whether IL-17 is involved in the bronchial recruitment of neutrophils and increase in bronchial tone after acute allergen provocation of mice with eosinophilic airway inflammation. Neutralizing mAb against IL-17 (clone 50104.11, R&D, Minneapolis, MN) or control rat IgG (Rockland, Gilbertsville, PA) were injected i.p. at a dosage of 50 µg 30 min before allergen provocation (n = 12 per group). As pilot studies showed that both neutrophilic influx and increase in bronchial tone were maximal at 3 h after provocation on d 50, this time-point was chosen for analysis. To compare effects of blockade of the pro-inflammatory cytokine IL-17 with dexamethasone, dexamethasone was injected (i.p., 3 mg/kg) 30 min before allergen inhalation on d 50 in a separate group of mice (n = 6). In addition to single neutralization of IL-17, we investigated the effects of repeated administrations of anti-IL-17 mAb during the development of airway inflammation. Anti-IL-17 mAb (50 µg) or control rat IgG (50 µg) were injected i.p. on alternate d from d 32 until 40, i.e. during the phase of chronic OVA inhalation (d 33 until 40, n = 6 per group). Mice were sacrificed on d 41, i.e. 24 h after the 8th inhalation of OVA.

4.2.2 Results

4.2.2.1 Allergen provocation in mice with eosinophilic airway inflammation induces bronchial neutrophilic influx, increase in bronchial tone and IL-17 expression

Provocation of sensitized and OVA-challenged mice on d 50 with a high dose of nebulized OVA (200 mg/ml for 10 min) induced an early

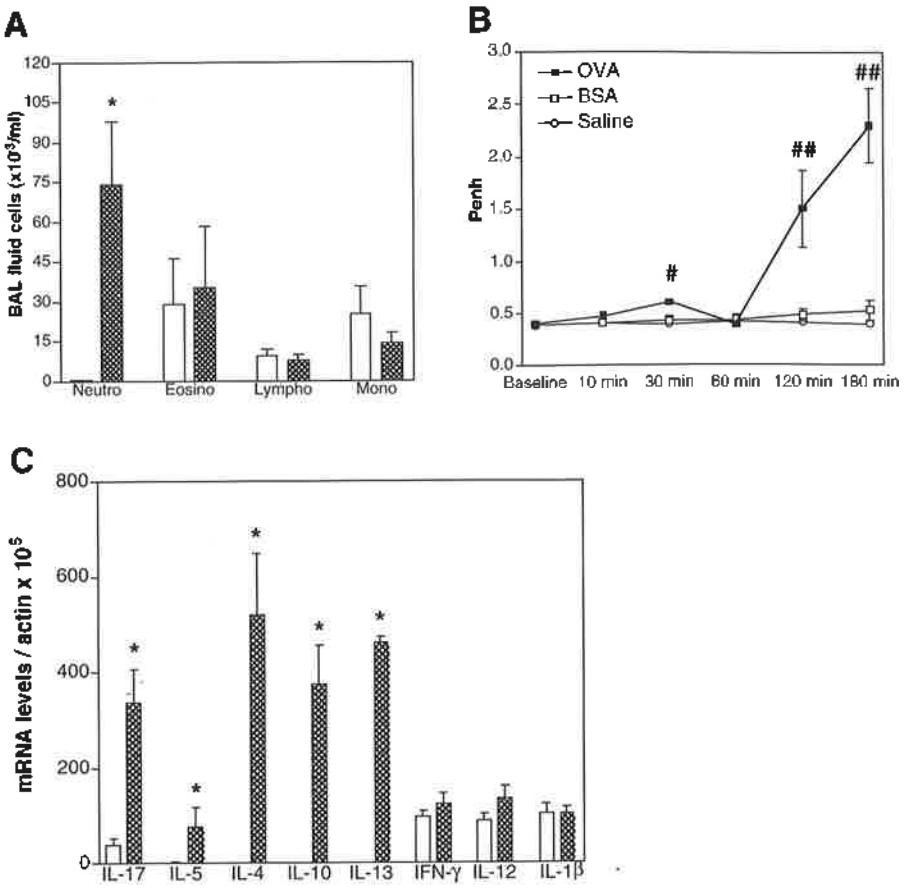

Fig. 4.2.1 Bronchial responses in mice after acute allergen provocation on day (d) 50. Mice were first sensitized (d 1 until 13) and chronically exposed to nebulized ovalbumine (OVA, d 33 until 40). On d 50, an acute provocation was performed by inhalation of nebulized OVA (200 mg/ml for 10 min). (**A**) Mice were sacrificed before (open bars) and at 3 h after acute allergen (filled bars) provocation. Differential cell counts were performed on cytospin preparations of broncho-alveolar lavage (BAL) fluid. (**B**) Long-term measurements of enhanced Pauses (Penh) were performed on d 50 after inhalation of OVA in unrestrained mice using whole body plethysmography (Buxco®). Similarly pretreated mice that inhaled nebulized saline of bovine serum albumin (BSA, 200 mg/ml) for 10 min on d 50 served as negative controls. (**C**) mRNA levels of interleukin (IL)-17 and different cytokines were measured using real-time RT-PCR on lung homogenates before (open bars) and at 3 h after provocation with OVA (filled bars). Data represent means +/- SEM. Each group consists of 10 to 12 mice. *, $p < 0.05$ vs mice sacrificed before provocation; #, $p < 0.05$ and ##, $p < 0.005$ vs saline or BSA.

prominent neutrophilic influx (Fig. 4.2.1 A) and remarkable increase in bronchial tone that peaks at 3 h (Fig. 4.2.1 B). Therefore, this time-point was chosen for further analyses in this study.

At 3 h after provocation, neutrophils constituted the majority of cells (51.2 %, Fig. 4.2.1 A) in BAL fluid. As neutrophils were hardly present in bronchi before provocation (0.4 ± 0.1 x10^3/ml of BAL fluid), their prominent influx in the broncho-alveolar lumen at 3 h after provocation (74.3 ± 23.6 x10^3/ml of BAL fluid, $p < 0.05$, Fig. 4.2.1 A) was striking. In contrast to the prominent neutrophilic influx, numbers of eosinophils and lymphocytes in BAL fluid were not altered by allergen provocation at 3 h after provocation (Fig. 4.2.1 A). It was only at 12 and 24 h after OVA provocation that a significant increase in bronchial eosinophil and lymphocyte counts was observed (data not shown). The OVA-mediated nature of bronchial neutrophilic influx and increase in bronchial tone was illustrated by extended pilot studies. Exposure of sensitized and OVA-challenged mice to nebulized saline (Fig. 4.2.1 B), BSA at 200 mg/ml for 10 min (Fig. 4.2.1 B), or endotoxins (Sigma) at 5 ug/ml, i.e. the concentration present in the OVA solution of 200 mg/ml, induced no or negligible neutrophilic influx (data not shown) without increase in bronchial tone. Furthermore, these bronchial responses were specific for OVA-sensitized mice as single exposure of naive mice to nebulized OVA at 200 mg/ml for 10 min failed to elicit any bronchial response of notice (data not shown).

Fig. 4.2.1 C shows that IL-17 mRNA levels in lung tissue were significantly increased by allergen provocation on d 50 ($p < 0.05$). Furthermore, mRNA of IL-5, IL-4, IL-10 and IL-13 was also elevated in inflamed airways after allergen provocation (Fig. 4.2.1 C). Interestingly, allergen inhalation did not affect mRNA levels of IFN-γ, IL-12 p40 and IL-1β (Fig. 4.2.1 C).

4.2.2.2 Effects of anti-IL-17 mAb administration prior to allergen provocation on bronchial responses

When anti-IL-17 mAb were injected i.p. 30 min prior to OVA provocation, this therapy affected the bronchial cellular infiltrate without change in total cell count. Neutrophilic influx into the bronchial lumen was significantly reduced by anti-IL-17 mAb compared to the control ($p < 0.05$, Fig. 4.2.2 A). In line with this observation, we found lower MPO activity in BAL fluid after injection of anti-IL-17 mAb (55.8 ± 5.5 vs 97.8 ± 21.4 OD, Fig. 4.2.2 B) and smaller MPO-positive infiltrates in bronchial tissue (25.3 ± 5.2 $x10^{-2}$ vs 75.3 ± 7.9 $x10^{-2}$mm, $p < 0.05$, Fig. 4.2.3, A and B). The circulating pool of neutrophils was also reduced after administration of anti-IL-17 mAb (44.4 ± 1.2 % vs 50.2 ± 1.3 %, $p < 0.05$). Interestingly, anti-IL-17 mAb inhibited bronchial neutrophilic influx in an equipotent way as did dexamethasone therapy (Fig. 4.2.2 A). Of note, the anti-inflammatory effect of anti-IL-17 mAb was limited to neutrophilic influx whereas dexamethasone also had anti-inflammatory potential on eosinophils (Fig. 4.2.2 A).

Surprisingly, OVA-induced eosinophilic airway influx was enhanced by anti-IL-17 mAb injection ($p < 0.05$, Fig. 4.2.2 A). This observation was consistent with elevated EPO activity in BAL fluid ($p < 0.05$, Fig. 4.2.2 B) and thicker peribronchial eosinophilic infiltrates in anti-IL-17-treated mice compared to controls (69.3 ± 9.3 $x10^{-2}$ vs 28.3 ± 7.3 $x10^{-2}$mm, $p < 0.05$, Fig. 4.2.3, C and D). Administration of anti-IL-17 mAb failed to influence blood eosinophilia (data not shown) nor did it modulate the increase in bronchial tone following provocation with OVA (Fig. 4.2.2 C). In contrast, dexamethasone completely abrogated the late phase increase in bronchial tone (Fig. 4.2.2. C)

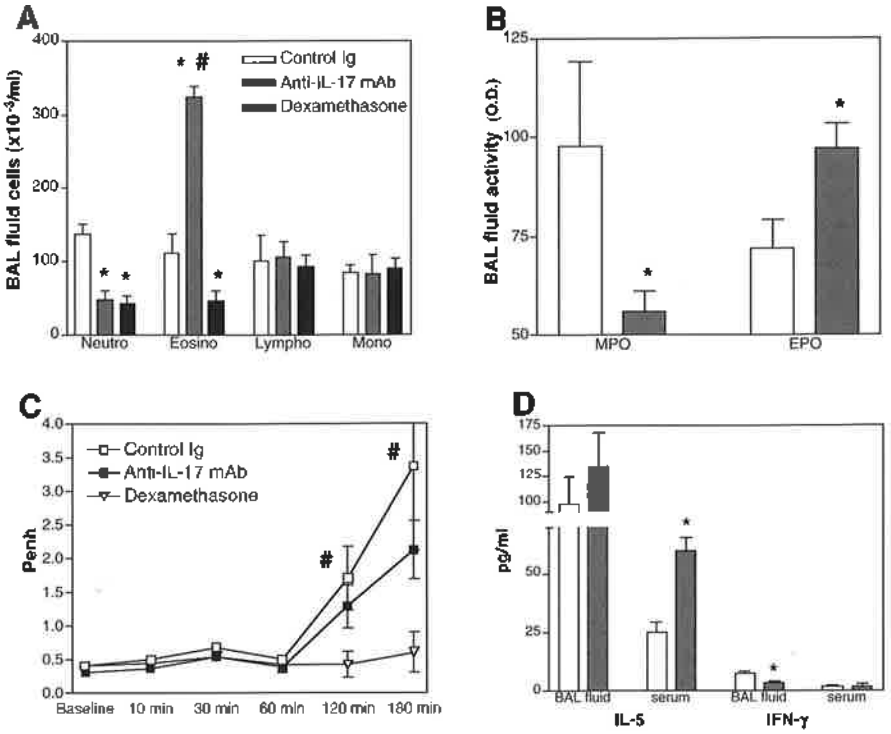

Fig. 4.2.2 Effects of anti-interleukin (IL)-17 mAb on bronchial responses following provocation with nebulized ovalbumine (OVA). Mice that had been sensitized to OVA (day (d) 1 until 13) and challenged with nebulized OVA (d 33 until 40) were injected with control Ig (open bars), anti-IL-17 mAb (grey bars) or dexamethasone (black bars) 30 min prior to inhalation of nebulized OVA (200 mg/ml for 10 min) on d 50. Mice were sacrificed 3 h after this provocation. (**A**) Differential cell counts were performed on cytospin preparations of broncho-alveolar lavage (BAL) fluid and (**B**) the activity of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) measured in BAL fluid. (**C**) Long-term measurements of enhanced Pauses (Penh) were performed after acute OVA provocation using whole body plethysmography (Buxco®). (**D**) Levels of IL-5 and IFN-γ were measured in BAL fluid and serum in control Ig or anti-IL-17 mAb-treated mice. Data represent means +/- SEM. Each group consisted of 6 to 12 mice. *, $p < 0.05$ vs control mice; #, $p < 0.05$ vs dexamethasone.

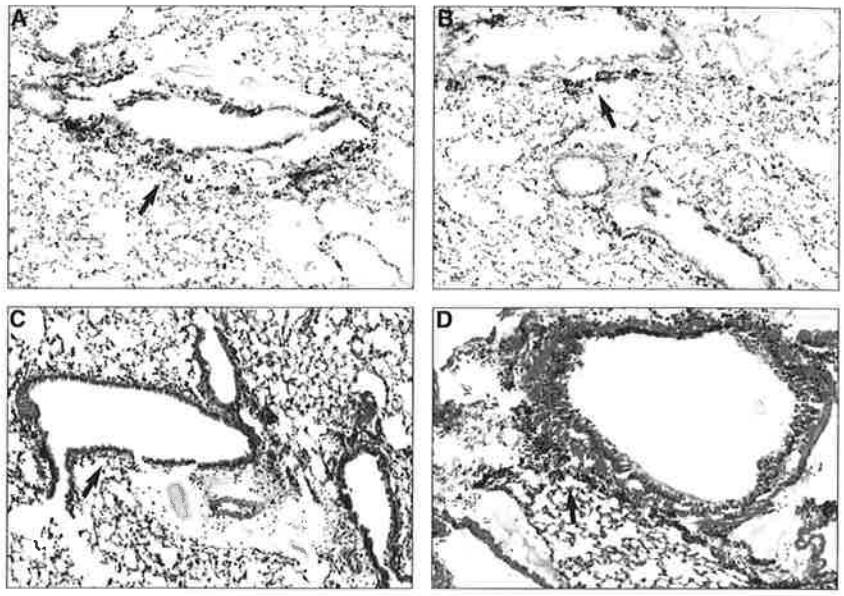

Fig. 4.2.3 Effects of anti-IL-17 mAb on bronchial inflammation. Mice that had been sensitized with OVA (d 1 until 13) and repeatedly inhaled nebulized OVA (d 33 until 40), were exposed to nebulized OVA at d 50. Control Ig (**A** and **C**) or anti-IL-17 mAb (**B** and **D**) were injected prior to acute provocation on d 50. Neutrophils can be visualized on myeloperoxidase-stained lung sections of peribronchial tissue (**A** and **B**) and are indicated with black arrows. On haematoxylin and eosin-stained lung sections (**C** and **D**), eosinophilic peribronchial influx can be assessed. Representative histologic sections are presented.

4.2.2.3 Effect of anti-IL-17 mAb on IL-5 production in vivo

In order to explain the remarkable effects of neutralizing IL-17 on bronchial eosinophilia, IL-5 levels were measured. Levels of circulating IL-5 were elevated in anti-IL-17-treated mice compared to controls (59.7 ± 5.8 vs 25.0 ± 4.1 pg/ml respectively, $p < 0.05$, Fig. 4.2.2 D). Also in BAL fluid, higher levels of IL-5 were found in mice injected with anti-IL-17 mAb, without reaching levels of significance ($p > 0.05$, Fig. 4.2.2 D). As far as IL-4 production is concerned, anti-IL-17 mAb enhanced IL-4 levels in serum (19.8 ± 4.1 vs 12.3 ± 2.1 pg/ml) and BAL fluid (42.2 ± 5.0 vs 32.4 ± 2.4 pg/ml) in a non-significant way ($p > 0.05$). In contrast to Th2 cytokines, IFN-γ levels in BAL fluid were reduced by anti-IL-17

mAb (2.9 ± 1.1 vs 7.0 – 1.3 pg/ml, $p < 0.05$, Fig. 4.2.2 D) and systemic levels of IFN-γ were under the detection limit (1.2 pg/ml) in both groups of mice.

4.2.2.4 Effects of repeated anti-IL-17 mAb administration on the development of allergic airway inflammation

As observed in mice that were given anti-IL-17 mAb prior to provocation, repeated administration of anti-IL-17 mAb during the allergen inhalation phase significantly reduced the bronchial neutrophilic influx (Fig. 4.2.4 A). Here, counts of other inflammatory cells in BAL fluid were not altered by anti-IL-17 mAb. Furthermore, this therapy led to enhanced bronchial IL-5 and IL-4 production ($p > 0.05$, Fig. 4.2.4 B) whereas IFN-γ production remained equally low in both groups (Fig. 4.2.4 B). Serum levels of IL-5, IL-4 and IFN-γ were below detection limit (data not shown) and AHR to inhaled Mch was not altered by chronic administration of anti-IL-17 mAb (Fig. 4.2.4 C).

When granulocytes were counted in different biologic compartments, we found that the reduction of bronchial neutrophilia corresponded with a significant decrease in percentages of neutrophils in bone marrow and peripheral blood (Fig. 4.2.5 A). In contrast, cosinopoiesis was significantly enhanced in mice given anti-IL-17 mAb (Fig. 4.2.5 B), resulting in elevated eosinophilia in peripheral blood (Fig. 4.2.5 B). In BAL fluid, eosinophil percentages were equally high in both groups (Fig. 4.2.5 B).

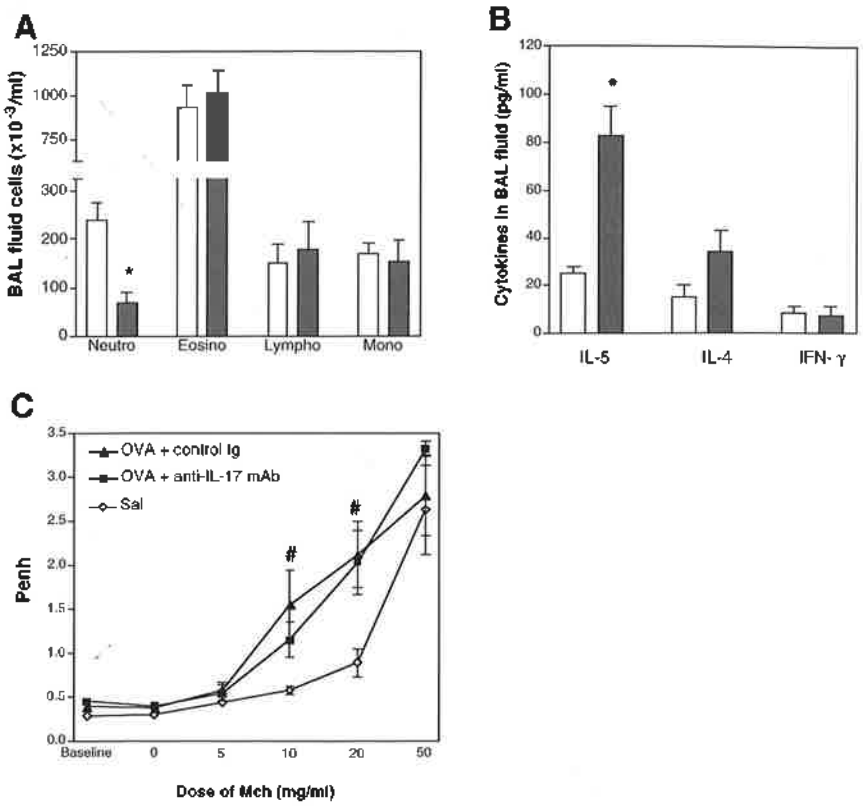

Fig. 4.2.4 Effect of repeated anti-interleukin (IL)-17 mAb administration on the development of allergic airway inflammation. Mice that had been sensitized (day (d) 1 until 13) were injected with control Ig (open bars) or anti-IL-17 mAb (grey bars) on d 32 and 36,. i.e. during the phase of repeated exposures to nebulized ovalbumine (OVA) or saline (d 33 until 40). Mice were sacrificed on d 41. (**A**) Differential cell counts were performed on cytospin preparations of broncho-alveolar lavage (BAL) fluid. (**B**) Cytokine levels in BAL fluid were measured using sandwich ELISA. (**C**) Airway responsiveness to inhaled Mch was evaluated by measuring enhanced Pauses (Penh) using whole body plethymography (Buxco©). Data represent means +/- SEM of 6 - 8 mice per group. *, $p < 0.05$ vs control Ig-treated mice; #, $p < 0.05$ vs saline-challenged mice.

4.2.3 Conclusion

We report for the first time up-regulation of IL-17 mRNA in inflamed airways after allergen inhalation. Besides IL-17, accumulation of mRNAs for typical Th2 cytokines, i.e. IL-4, IL-5 and IL-13, were

increased after allergen inhalation, illustrating the Th2 cytokine milieu of bronchial neutrophilic influx. IL-17 production does not seem to segregate into a distinct subset of $CD4^+$ cells. IL-17-producing T cell clones belong to the Th1 phenotype in rheumatoid arthritis (136) and to both Th1 and Th2 phenotypes after priming in the presence of *Borrelia burgdorferi* (137) and in allergic contact dermatitis (138). Here, the simultaneous induction of mRNA for IL-17 and Th2, and not Th1 cytokines, suggests that activated Th2 lymphocytes are involved in IL-17 secretion.

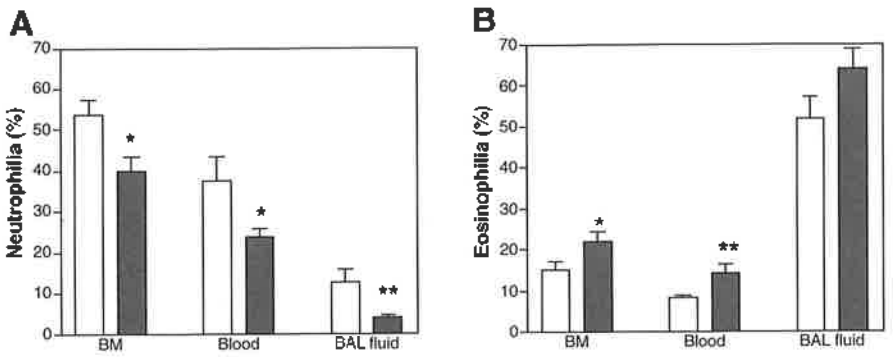

Fig. 4.2.5 Effects of repeated anti-interleukin (IL)-17 mAb administration on granulocyte biology in different compartments. Mice that had been sensitized to OVA (day (d) 1 until 13) were treated with control Ig (open bars) or anti-IL-17 mAb (grey bars) before and during the phase of repeated inhalations of nebulized OVA (d 33 until 40). Mice were sacrificed on d 41 and percentages of neutrophils (**A**) and eosinophils (**B**) determined in bone marrow (BM), blood and broncho-alveolar lavage (BAL) fluid. Each group consists of 8 mice and data represent means +/- SEM. *, $p < 0.05$ and **, $p < 0.005$ vs control.

In view of the link between IL-17 and neutrophil biology (64), we intended to inhibit bronchial neutrophilic influx by neutralizing IL-17 and to study the effects of this therapy on bronchial responses. Together with the finding of bronchial recruitment of neutrophils by intratracheal instillation of IL-17 in rats (65), our data on significant reduction of bronchial neutrophilic influx by anti-IL-17 mAb illustrate that IL-17 is

essential for mediating bronchial influx of neutrophils. Therefore, our observations are in line with a recent report on the requirement for IL-17 receptor signaling for neutrophil migration to the lung (38). Several mechanisms may underlie the inhibition of neutrophilic recruitment by anti-IL-17 mAb. First of all, we demonstrated that prolonged absence of functional IL-17 resulted in defective neutropoiesis in bone marrow leading to lower levels of circulating neutrophils. This phenomenon may be related to reduced production of G-CSF and stem cell factor in anti-IL-17-treated mice (64, 139) and highlights the key role of IL-17 in regulating neutropoiesis in the bone marrow. In addition, this therapy may have impaired attraction of neutrophils to murine bronchi due to lower production of chemokine KC (the murine homologue of human growth-related oncogene alpha, (140)) and/or macrophage inflammatory protein-2 (65). Also in human tissue, IL-17 is involved in the migration of neutrophils through production of IL-8 (61, 139) and growth-related oncogene-α (140). Here, levels of granulocyte chemotactic protein-2, a C-X-C chemokine that is the most potent murine neutrophil-chemo-attractive protein and is nearly as effective as human IL-8 in attracting neutrophils (37), remained unchanged in BAL fluid after anti-IL-17 therapy (unpublished observation). Therefore, decreased production of other factors such as pro-inflammatory cytokines IL-1β and TNF-α (141), or impaired upregulation of ICAM-1 (61, 66) may explain decreased bronchial influx of neutrophils in anti-IL-17-treated mice.

Interestingly, reduced bronchial neutrophilia was dissociated from beneficial effects on AHR. One reason may be that the reduction of bronchial neutrophilia was not absolute, allowing no firm conclusion concerning the lack of involvement of neutrophils in airway contractile properties. In addition, both bronchial eosinophilia and IL-5 production, which both have been linked to AHR (39-41), were enhanced after anti-

IL-17 mAb administration. However, our observations of neutrophil-independent AHR are consistent with data obtained in a rat model of allergic asthma (77) and in a mouse model of acute respiratory distress syndrome (142).

Apart from orchestrating bronchial granulocyte influx, our data illustrate that IL-17 has immunomodulatory capacity in allergic asthma. Anti-IL-17 therapy significantly enhanced systemic and bronchial IL-5 and to a lesser extent IL-4 levels. As circulating IL-5 is one of the major stimulators of eosinopoiesis (134) and eosinophil migration towards airways (143), enhanced IL-5 production may here explain the aggravation of bronchial eosinophilia. *In vitro* studies demonstrated that IL-17 has no direct influence on IL-5 production by allergen-stimulated lymphocytes (unpublished observation). We therefore speculate on indirect mechanisms involved in the increase in IL-5 levels and bronchial and systemic eosinophilia. In this regard, IL-17 has been reported to inhibit TNF-α and IFN-γ-mediated production of RANTES (62). However, it remains to be elucidated to what extent absence of IL-17 may favor RANTES production.

Whether targeting IL-17 may have therapeutic potential in human airway disease remains speculative. Neutralizing anti-IL-17 mAb decreases the bronchial influx of neutrophils in an equipotent way as did dexamethasone. In view of the non-specific mode of action of dexamethasone, targeting IL-17 might represent a new, more specific, therapeutic approach for airway diseases with major neutrophilic inflammation such as acute severe asthma attacks and chronic obstructive pulmonary diseases (144). It remains to be determined to what extent acute and/or chronic reduction in bronchial neutrophilia may lead to decreased mucus secretion, bronchial remodeling and/or symptom relief. Therefore, better insight into the pathophysiologic role of bronchial

neutrophils is required before one can delineate the potentially beneficial effect of anti-IL-17 mAb in human airway disease.

4.3 Blockade of CTLA-4 enhances allergic sensitization and development of eosinophilic airway inflammation in genetically predisposed mice

4.3.1 Introduction

The involvement of CTLA-4 in allergic asthma remains speculative. Since CTLA-4 has been regarded as an important negative signaling molecule opposing T cell activation (79), we speculated that blockade of CTLA-4 signaling would enhance T cell activation and hence aggravate allergic airway disease. Therefore, a murine model of allergic asthma was utilized to investigate the role of this molecule in the pathogenesis of asthma. In order to be able to evaluate a potential aggravation of airway disease by anti-CTLA-4 mAb, we shortened the experimental protocol by reducing the number of OVA injections during sensitization to 5 (one injection on alternate d from d 1 until 9) and by limiting the number of exposures to nebulized OVA to 5 (once daily from d 33 until 38). In addition to induction of experimental asthma, 250 μg of neutralizing mAb against CTLA-4 (UC10-4F10-11 (145)) or control hamster Ig (Rockland, Gilbertsville, PA) were administered on d 0, 4, 8 and 12, allowing the study of effects of this treatment on sensitization as well as on subsequent manifestation of allergic airway disease. To evaluate whether genetic factors are involved in the observed effects of anti-CTLA-4 mAb injection, high and low IgE-responding murine strains, i.e. BALB/c and C57BL/6 mice (123), were used in this study. Finally, neutralizing anti-CTLA-4 mAb or control hamster Ig have also been injected i.p. at 250 μg in sensitized BALB/c mice on d 32 and 36, i.e. during the phase of repeated inhalations of nebulized OVA. All mice in this study were sacrificed on d 39, i.e. 24 h after the 5th OVA challenge.

4.3.2 Results

4.3.2.1 Blockade of CTLA-4 during antigen priming potentiates anti-OVA IgE production in BALB/c but not in C57BL/6 mice

At the onset of experiments (d 0), no OVA-specific Igs were

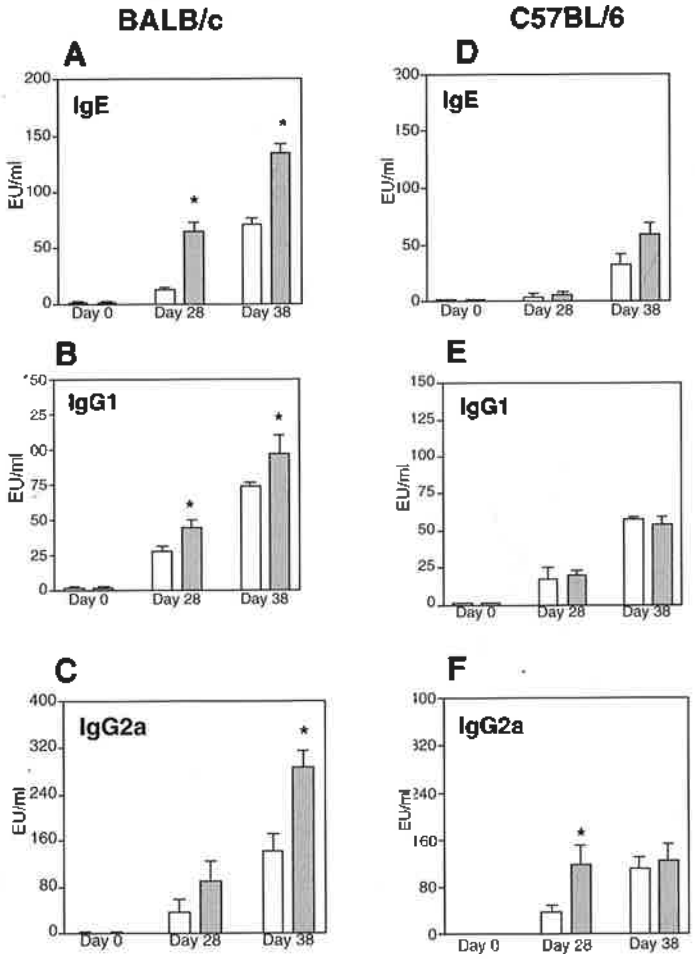

Fig. 4.3.1 Serum levels of ovalbumin (OVA)-specific Igs in BALB/c and C57BL/6 mice treated with anti-CTLA-4 mAb during sensitization. BALB/c (**A-C**) and C57BL/6 (**D-F**) mice were sensitized by intraperitoneal injections of OVA on alternate days (d) from d 1 until 9 and inhaled nebulized OVA daily from d 33 until 37. In addition, mice were injected with control hamster Ig (open bars) or anti-CTLA-4 mAb (filled bars) every 4 d from d 0 until 12. Blood was taken before (d 0) and after sensitization (d 28), and after the OVA challenge phase (d 38). Serum levels of OVA-specific Igs were measured by sandwich ELISA. Each group consisted of 12 mice. Data are expressed as means +/- SEM. *, $p < 0.05$ vs value o fcontrol Ig-treated mice on the same d.

present (Fig. 4.3.1) and blood eosinophilia was low (Fig. 4.3.2). Sensitization of BALB/c mice with OVA induced production of OVA-specific IgE, IgG1 and IgG2a by d 28 (Fig. 4.3.1, A-C). Cutaneous hypersensitivity reactions to OVA, which correlate with the production of allergen-specific IgE (146), were consistently observed after intradermal injection of OVA (data not shown).

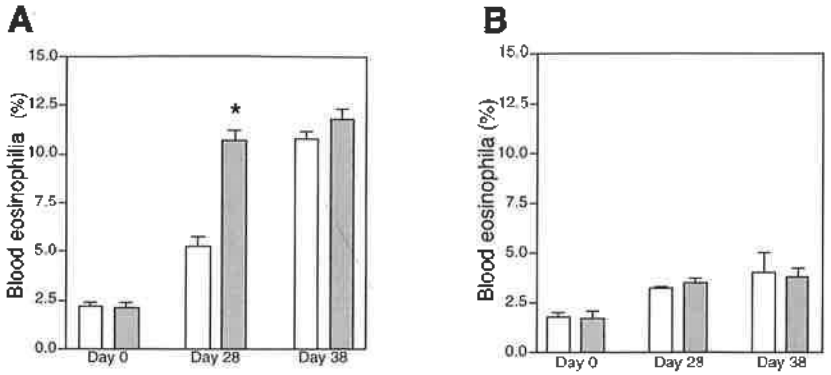

Fig. 4.3.2 Blood eosinophilia in BALB/c (**A**) and C57BL/6 (**B**) mice before (day (d) 0) and after sensitization (d 28), and after the challenge phase with nebulized ovalbumine (OVA, d 38). Mice were injected with control Ig (open bars) or anti-CTLA-4 mAb (filled bars) during the sensitization phase. Eosinophil percentages in the total white blood cell population were determined on smears of peripheral blood. Data represent means +/- SEM. Each group consisted of 6 animals. One representative experiment out of 2 is shown. *, $p < 0.05$ vs control Ig-treated mice on the same day.

Interestingly, BALB/c mice that were injected with anti-CTLA-4 mAb had elevated production of OVA-specific IgE and IgG1 by d 28 (Fig. 4.3.1, A and B), whereas production of IgG2a was not significantly affected by this treatment (Fig. 4.3.1 C). In addition to enhanced IgE production, anti-CTLA-4 mAb increased blood eosinophilia as compared to control Ig on d 28 (10.7 ± 0.5 vs 5.25 ± 0.4 %, $p < 0.05$, Fig. 4.3.2 A). Both groups of BALB/c mice showed similar cutaneous hypersensitivity reactions to OVA (data not shown). When sensitized BALB/c mice

repeatedly inhaled nebulized OVA solutions from d 33 until 37, this allergen challenge boosted OVA-specific Ig production, as reflected by higher levels of OVA-specific IgE, IgG1 and IgG2a on d 38 than d 28 (Fig. 4.3.1, A-C). Of note, anti-CTLA-4 mAb injection resulted in a stronger boosting effect on Ig production of the 3 Ig isotypes than control Ig (Fig. 4.3.1, A—C).

Results in C57BL/6 mice were strikingly different. Sensitization of C57BL/6 mice with adjuvant-free OVA failed to induce allergen-specific IgE production on d 28 (Fig. 4.3.1 D) and did not enhance blood eosinophilia (Fig. 4.3.2 B). The lack of allergen-specific IgE production in C57BL/6 mice after sensitization was consistent with the absence of cutaneous hypersensitivity reaction after intradermal injection of OVA (data not shown). Here, anti-CTLA-4 treatment augmented allergen-induced IgG2a production by d 28 ($p < 0.05$, Fig. 4.3.1 F), without affecting OVA-specific IgE and IgG1 production (Fig. 4.3.1, D and E) nor blood eosinophilia (Fig. 4.3.2 B). Repeated allergen inhalations then boosted OVA-specific Ig production (Fig. 4.3.1, D-F) and induced a similar cutaneous hypersensitivity reaction by d 38 in both control and anti-CTLA-4-treated C57BL/6 mice (data not shown). The low IgE-producing capacity of C57BL/6 in comparison to BALB/c mice has previously been reported (146) and can apparently not be reversed by blockade of CTLA-4.

Taken together, our findings reveal that blockade of CTLA-4 during sensitization potentiates IgE and IgG1 production, but only in the genetically predisposed strain, i.e. BALB/c mice, and also leads to enhanced systemic eosinophilia in this strain.

4.3.2.2 Blockade of CTLA-4 during sensitization enhances eosinophilic airway inflammation upon repeated allergen challenge of BALB/c mice

We subsequently studied the effects of repeated OVA inhalations on airway inflammatory disease in mice that had been treated with either anti-CTLA-4 mAb or control Ig during sensitization. In BALB/c mice, we found AHR in sensitized mice that had been exposed to nebulized OVA compared to saline (Fig. 4.3.3 A). Furthermore, OVA-challenged mice that had been injected with anti-CTLA-4 mAb showed enhanced AHR compared to the control Ig-treated group (Fig. 4.3.3 A). Indeed, higher Penh values ($p < 0.05$, Fig. 4.3.3 A) were found in the former group after inhalation of nebulized Mch at dosages of 2.5 mg/ml (0.65 ± 0.07 vs 0.35 ± 0.03) and 5 mg/ml (1.28 ± 0.10 vs 0.85 ± 0.17).

In addition to elevated AHR, aggravated eosinophilic inflammation was found in anti-CTLA-4-treated mice than in the controls, in BAL fluid (Fig. 4.3.3 B) as well as in peribronchial tissue (285.2 ± 34.3 $x10^{-2}$ vs 110.2 ± 23.4 $x10^{-2}$mm, $p < 0.05$, Fig. 4.3.4 B). More severe bronchial eosinophilia was confirmed by elevated activity of EPO in BAL fluid of anti-CTLA-4 compared to control mice (86.6 ± 10.3 vs 57.8 ± 5.4 pg/ml, $p < 0.05$). Also higher total cell counts were found in BAL fluid of anti-CTLA-4 mice (25.6 ± 5.5 $x10^5$ vs 11.8 ± 2.8 $x10^5$/ml respectively, $p < 0.05$). Thus, blockade of CTLA-4 during antigen priming of BALB/c mice enhances not only IgE production but also subsequent AHR and development of eosinophilic airway inflammation following inhalatory OVA challenges. Increased eosinophilia in BAL fluid of anti-CTLA-4-treated BALB/c mice corrresponded with elevated levels of IL-5 in both BAL fluid and serum ($p < 0.05$, Table 4.3.1). As a result of CTLA-4 blockade, the ratio of IL-4 / IFN-γ in BAL fluid was significantly enhanced compared to control Ig-treated mice (data not shown). This

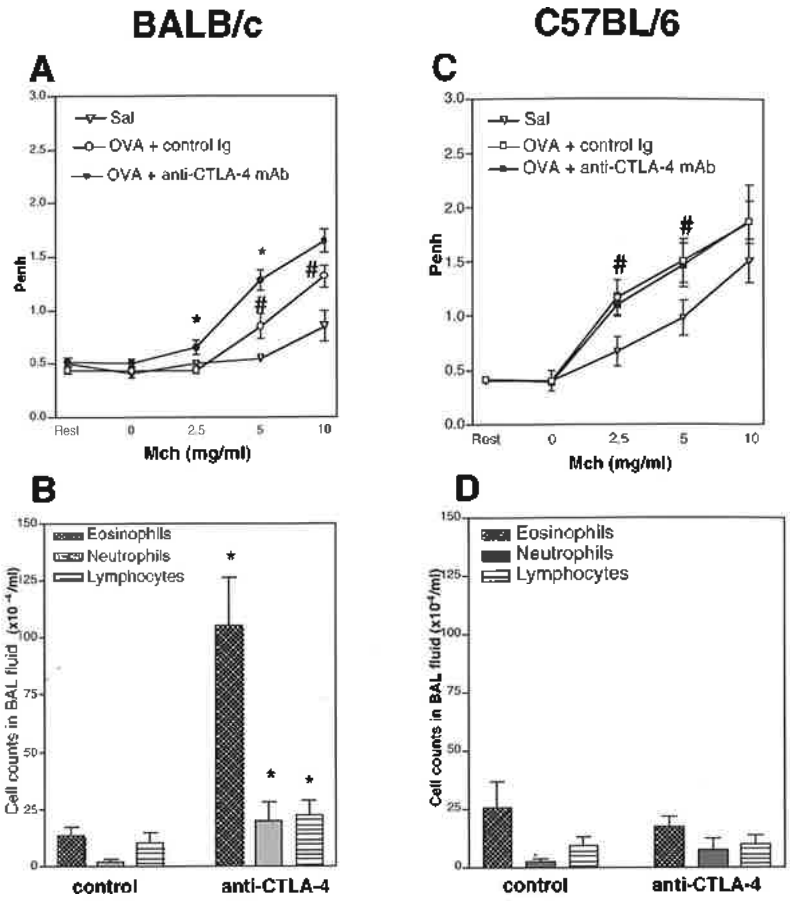

Fig. 4.3.3 Effects of anti-CTLA-4 mAb administration during sensitization on the subsequent development of airway hyperresponsiveness (AHR) and bronchial inflammation in BALB/c (**A** and **B**) and C57BL/6 (**C** and **D**) mice. Mice were sensitized with ovalbumine (OVA) from d 1 until 9, and repeatedly inhaled saline (Sal) or OVA from d 33 until 37. In addition, OVA-challenged mice had been injected with anti-CTLA-4 mAb or control Ig during sensitization. Airway responsiveness to incremental doses of nebulized Mch was evaluated by measuring enhanced Pauses (Penh) using whole body plethymography (**A** and **C**). Differential cell counts were performed on cytospin preparations of broncho-alveolar lavage (BAL) fluid of OVA-challenged mice (**B** and **D**). Each group consisted of 12 mice. Data are expressed as means +/- SEM. *, $p < 0.05$ vs control Ig-treated, OVA-challenged mice; #, $p < 0.05$ vs saline-challenged mice.

enhanced Th2 cytokine profile in anti-CTLA-4-treated mice mainly resulted from elevated bronchial and systemic IL-4 production (Table 4.3.1) and was in keeping with previous reports on CTLA-4-mediated dampening of Th2 differentation and cytokine production (147).

In C57BL/6 mice, OVA challenges to sensitized animals induced AHR (Fig. 4.3.3 C) and bronchial eosinophilic inflammation (Fig. 4.3.3 D). Unlike BALB/c mice, administration of anti-CTLA-4 mAb to C57BL/6 mice did not modulate the severity of bronchial inflammation in response to inhalatory OVA challenges (Fig. 4.3.3, C and D). The lack of effect of blockade of CTLA-4 on AHR and eosinophilic inflammation in C57BL/6 mice was in line with the finding of similar IL-4, IL-5 and IFN-γ levels and IL-4 / IFN-γ ratios in BAL fluid of anti-CTLA-4 and sham-treated C57BL/6 mice.

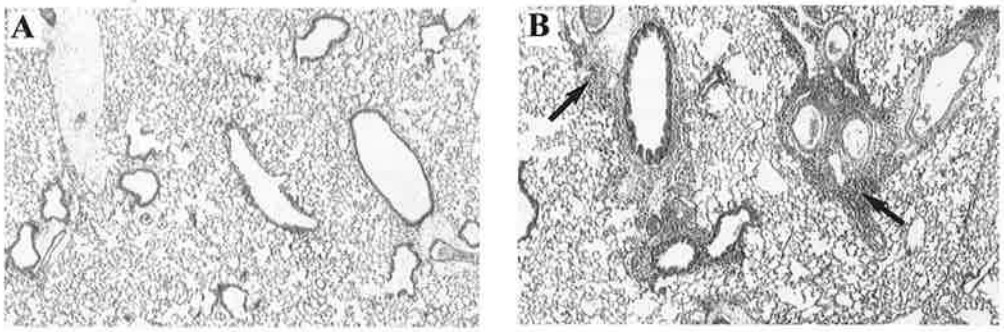

Fig. 4.3.4 Effects of anti-CTLA-4 mAb on bronchial inflammation in BALB/c mice. BALB/c mice that had been injected with control Ig (**A**) or anti-CTLA-4 mAb (**B**) during sensitization (day (d) 1 until 9), repeatedly inhaled nebulized ovalbumin (OVA, d 33 until 37). Mice were sacrificed on d 41. Sections of inflamed airways were stained with the classic haematoxylin and eosin stain and the thickness of peri-bronchial and perivascular inflammatory infiltrates (indicated with black arrows on **B**) was measured.

It is relevant to mention that the difference in response of BALB/c and C57BL/6 mice to anti-CTLA-4 mAb administration is probably not related to differences in levels and kinetics of CTLA-4-expression. Both

strains of mice had similar percentages and kinetics of CTLA-4-expressing cells in resting as well as on activated $CD4^+$ cells from PBLNs (data not shown).

Table 4.3.1. Cytokine profile in broncho-alveolar lavage (BAL) fluid and serum of BALB/c mice treated with control Ig or anti-CTLA-4 mAb during sensitization

Treatment[a]	Control Ig	Anti-CTLA-4 mAb
IL-5[b]		
BAL fluid	10.8 ± 2.2	89.1 ± 21.5*
serum	9.7 ± 3.9	103.2 ± 44.7*
IL-4		
BAL fluid	20.8 ± 4.6	56.3 ± 16.6*
serum	< 2.0	7.1 ± 3.1
IFN-γ		
BAL fluid	10.1 ± 3.8	8.6 ± 11.6
serum	2.1 ± 0.7	1.7 ± 0.5

[a]BALB/c mice were sensitized by repeated intraperitoneal injections of ovalbumine (OVA) from d 1 until 9 and repeatedly challenged with nebulized OVA from d 33 until 37. In addition, control Ig or anti-CTLA-4 mAb had been injected during sensitization. Mice were sacrificed on d 38.

[b]Cytokine levels were measured in BAL fluid and serum using sandwich ELISA and expressed in pg/ml.

[c]Results are shown as means ± SEM of 12 mice per group. Statistical analyses were performed with the *Mann-Whitney* test with *, $p < 0.05$ vs control Ig-treated mice.

4.3.2.3 In vitro analysis of peribronchial lymph nodes of mice injected with anti-CTLA-4 mAb during sensitization

To further document the effects of blockade of CTLA-4 during sensitization on the allergic inflammatory response, we isolated PBLN lymphocytes of mice with eosinophilic airway inflammation and restimulated them *in vitro* with OVA. Lymphoproliferation of PBLN cells obtained from anti-CTLA-4-treated BALB/c mice was highly elevated in comparison to the controls, in conditions with and without OVA ($p <$

0.05, Fig. 4.3.5 A). PBLN lymphocytes of anti-CTLA-4-treated mice also produced more IL-5, IL-4 and IL-10 in response to OVA stimulation (Fig. 4.3.5 B). Stimulation of PBLN of OVA-sensitized mice with BSA *in vitro* did not induce lymphoproliferation nor cytokine production (data not shown).

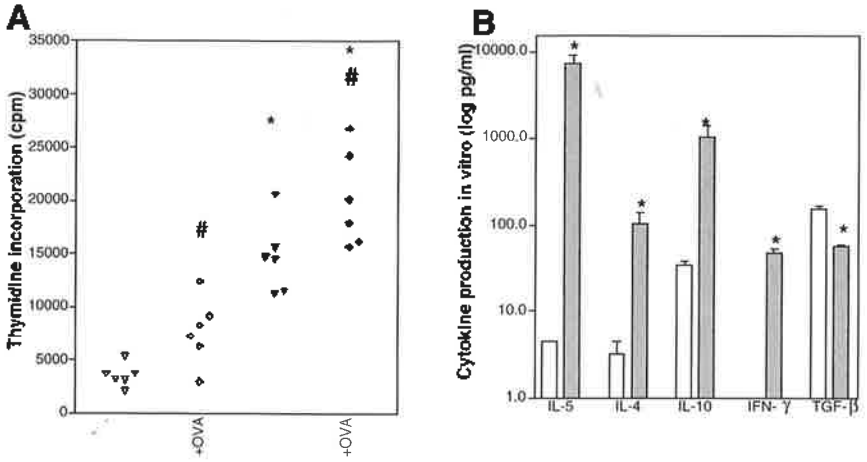

Fig. 4.3.5 Effect of anti-CTLA-4 treatment during sensitization of BALB/c mice on lymphocyte function *in vitro* . BALB/c mice were injected with control Ig or anti-CTLA-4 mAb during systemic sensitization with ovalbumine (OVA, d 1-9). All mice were then repeatedly exposed to nebulized OVA (d 33-37) and sacrificed on d 38 for disssection of peribronchial lymph nodes (PBLN). (**A**) PBLN cells of control Ig (open symbols) or anti-CTLA-4-treated mice (filled symbols) were cocultured with an equal number of splenocytes as antigen-presenting cells in the absence (triangles) or presence (squares) of OVA. After 72 h lymphoproliferation was evaluated by thymidine incorporation. Each value represents the mean of quadruplicate cultures. (**B**) PBLN cells of control Ig (open bars) or anti-CTLA-4-treated mice (filled bars) were incubated with splenocytes and OVA for 5 d. Cytokine production was evaluated in the culture supernatants using sandwich ELISA. Data represent the mean +/- SEM of 6 to 8 values. *, $p < 0.05$ vs control Ig-treated mice; #, $p < 0.05$ vs conditions containing PBLN cells of similarly treated mice without OVA.

Our observation of enhanced production of IL-10 in anti-CTLA-4 mice with severe airway inflammation apparently contrasts to the anti-inflammatory capacity of IL-10 in eosinophilic airway inflammation (148). However, the role of IL-10 in dampening eosinophilic airway inflammation remains controversial (44). It is also noteworthy to mention

that levels of mRNA for IL-10 in lung homogenates were not affected by anti-CTLA-4 injections (data not shown). Compared to the large increase in Th2 cytokine production, the enhancement of IFN-γ production by PBLN cells from anti-CTLA-4-treated BALB/c mice was rather weak (Fig. 4.3.5 B). Of note, enhanced IFN-γ secretion by PBLN cells can explain the elevated IgG2a production (149) in anti-CTLA-4-treated BALB/c mice. Interestingly, we found less TGF-β production after OVA stimulation of PBLN cells of anti-CTLA-4-treated BALB/c mice (Fig. 4.3.1 B). These *in vitro* data of enhanced Th2 cytokine production together with reduced TGF-β production following anti-CTLA-4 treatment could explain the aggravation of Th2-mediated pathology we observed *in vivo*.

In C57BL/6 mice, activated lymphocytes of PBLN also produced more IL-5 (1,522.2 ± 565.0 vs 5.2 ± 0.5 pg/ml, $p < 0.05$) and proliferated more (27,323 ± 8,452 vs 8,525 ± 2,156 cpm in OVA-stimulated conditions, $p < 0.05$) in the anti-CTLA-4-treated compared to the control group. The discrepancy between enhanced IL-5 production *in vitro* and the lack of effect of anti-CTLA-4 on airway inflammation *in vivo* may relate to the fact that C57BL/6 mice did not have enhanced levels of circulating IL-5 in C57BL/6 mice (data not shown). In addition, the absolute IL-5 production by PBLN cells of C57BL/6 mice *in vitro* was still relatively low compared to similarly treated BALB/c mice. Finally, activated PBLN cells of C57BL/6 mice produced more IL-4 and IFN-γ after anti-CTLA-4 mAb injection without reaching levels of significance (data not shown), whereas TGF-β and IL-10 production were similar in both groups (data not shown).

4.3.2.4 Anti-CTLA-4 mAb injection decreases bronchial TGF-β production in BALB/c mice

Several mechanisms could explain how CTLA-4 triggering controls airway inflammation in BALB/c mice. As mentioned earlier, TGF-β production by PBLN cells of anti-CTLA-4-treated BALB/c mice was significantly reduced (Fig. 4.3.5 B), suggestive of an association between blockade of CTLA-4, low TGF-β production and severe airway inflammation. Indeed, cross-linking of CTLA-4 in the presence of TCR ligation has been shown to lead to TGF-β secretion by purified naive $CD4^+$ T cells (150). *In vivo* evidence for a functional link between

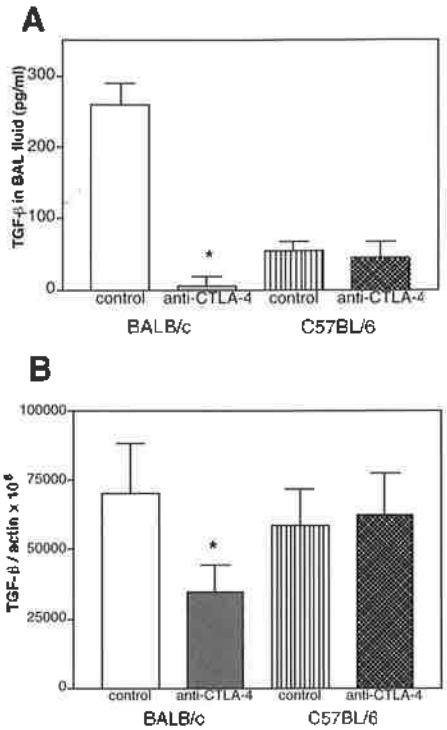

Fig. 4.3.6 Bronchial production of transforming growth factor (TGF)-β in BALB/c and C57BL/6 mice with allergic airway inflammation. On day 38, levels of TGF-β were measured in broncho-alveolar lavage (BAL) fluid of BALB/c and C57BL/6 mice that had been treated with control Ig or anti-CTLA-4 mAb during sensitization (**A**). At the same occasion, mRNA levels of TGF-β were measured on lung homogenates using real-time RT-PCR (**B**). Each group consisted of 6 mice. Data are expressed as meas +/- SEM. *, $p < 0.05$ vs control-Ig treated BALB/c mice.

CTLA-4 signaling and TGF-β secretion was provided by Gomes et al. (151) in visceral leishmaniasis in BALB/c mice.

We further sought support for a functional link between CTLA-4 signaling and TGF-β secretion by quantifying TGF-β protein in BAL fluid and TGF-β mRNA in lung tissue. Aggravated airway inflammation in anti-CTLA-4-treated BALB/c mice was found in association with lower TGF-β levels in BAL fluid (Fig. 4.3.6 A) and less mRNA for TGF-β in homogenized lung tissue (Fig. 4.3.6 B). Therefore, our results suggest that CTLA-4 signaling during antigen priming exerts a disease-limiting role in allergic airway inflammation through prolonged secretion of TGF-β. Indeed, TGF-β favors differentiation of na ve murine $CD4^+$ T cells toward the Th1 subset (82, 152), inhibits eosinophil survival and function (153), down-regulates tracheal eosinophilia (154) and reverses allergen-induced airway hyperresponsiveness and inflammation (155).

In contrast to BALB/c mice, we found no influence of CLTA-4 neutralization on TGF-β production in C57BL/6 mice. Bronchial production of TGF-β was similar in sham and anti-CTLA-4-treated C57BL/6 mice (Fig. 4.3.6, A and B). These data are in line with observations by Sullivan et al. (156), who reported that CTLA-4 ligation does not regulate TGF-β production in T cells of C57BL/6 mice.

4.3.2.5 Effects of anti-CTLA-4 mAb injection during allergen challenges of sensitized BALB/c mice

Finally, we investigated whether anti-CTLA-4 mAb injections during inhalatory OVA challenges of previously sensitized BALB/c mice would modulate the development of airway inflammation. In this experimental setting, anti-CTLA-4 treatment failed to influence the allergic phenotype in BALB/c mice. No differences were observed in functional or cellular characteristics of the allergic inflammatory response

in the airways between control and anti-CTLA-4-treated mice (data not shown). This observation suggests that memory T lymphocytes are refractory to CTLA-4-mediated inhibition of allergic responses and is in line with earlier reports on the confinement of CTLA-4 s role to disease onset in experimental diabetes (87) and allergic encephalomyelitis (157).

4.3.3 Conclusion

This study shows that *in vivo* blockade of CTLA-4 with mAb enhances allergic sensitization in mice with a genetic predisposition to produce IgE and to develop Th2-mediated pathology, i.e. BALB/c mice. Absence of functional CTLA-4 signaling during sensitization in these mice leads to elevated IgE production and potently shifts the allergen-induced cytokine profile towards Th2 preponderance. This phenomenon results in more severe eosinophilic airway inflammation and AHR following chronic airborne allergen challenges. Therefore, our data provide *in vivo* support for the notion that CTLA-4 is important in setting, at the time of initiation, the tune of the immune response (158). Although the mechanism responsible for the potent effect of anti-CTLA-4 mAb on favoring Th2 differentiation is still unclear, we would argue that TGF-β is involved. Indeed, severe airway inflammation in anti-CTLA-4-treated BALB/c mice correlated with a marked reduction of TGF-β production, both *in vivo* and *in vitro*. Reduced TGF-β production may explain the observed aggravation of airway inflammation (159).

Interestingly, the effect of anti-CTLA-4 mAb on TGF-β secretion was observed long after cessation of anti-CTLA-4 treatment and anti-CTLA-4 treatment during the effector phase of the disease had no influence on airway inflammation. Taken together, these data suggest that anti-CTLA-4 treatment leads to impaired function and/or expansion of an immunoregulatory T cell population at the time of sensitization. In this

regard, T helper 3 (Th3) cells have been reported to prevent and/or cure auto-immune diseases through production of TGF-β (160). On the other hand, ligation of the constitutively expressed CTLA-4 on $CD4^+CD25^+$ cells, also called regulatory T cells (Treg), has been found to prevent the development of pathogenic responses to both self (161) and intestinal antigens (162), through production of TGF-β (163). So far, the relevance of these regulatory T cell populations in allergic inflammation and in the effect of anti-CTLA-4 treatment as observed in our model, still needs to be elucidated.

In contrast to BALB/c mice, IgE production, airway inflammation and TGF-β secretion were not affected by administration of anti-CTLA-4 mAb in C57BL/6 mice. The presence or absence of CTLA-4-mediated TGF-β secretion may thus underlie the difference in inflammatory responses in BALB/c and C57BL/6 mice after blockade of CTLA-4. Therefore, CTLA-4-mediated TGF-β production may represent one link between genetic predisposition and asthma development. Research on genetic and environmental factors that influence CTLA-4 signaling at the time of antigen priming is warranted in order to improve our insight into mechanisms involved in allergic sensitization and development of asthma.

4.4 Down-regulation of allergic airway inflammation in mice by agonistic anti-CD40 mAb

4.4.1 Introduction

Interaction between CD154 (CD40 ligand, CD40L) and its receptor CD40 has been shown to be critically involved in the generation of cell-mediated as well as humoral immunity (92). CD40-CD40L interaction is critical for the induction of IgE but it is not clear whether this interaction is also important for Th2-dependent airway inflammation. We studied the involvement of CD40-CD40L interaction during antigen priming and during the effector phase of airway inflammation. Agonistic anti-CD40 mAb (FGK45, 250 µg, (164)), neutralizing anti-CD40L (MR1, 250 µg, (165)) or control Ig (250 µg) were injected in BALB/c mice either on d 0, 4, 8 and 12, i.e. during either systemic sensitization with OVA (d 1 until 13), or on d 32 and 36, i.e. during repeated inhalatory challenges with OVA (d 33 until 40). All mice were sacrificed on d 41, i.e. 24 h after the 8th allergen inhalation. We further investigated whether the effects of agonistic anti-CD40 mAb would be mediated via induction of IL-12 as was the case in a mouse model of *Schistosoma mansoni* infection (112). To this purpose, bronchial inflammatory responses were compared between IL-12-deficient BALB/c mice and wild-type littermates that were injected with agonistic anti-CD40 mAb or control rat Ig during sensitization.

4.4.2 Results

4.4.2.1 Inhibition of the development of eosinophilic airway inflammation by agonistic anti-CD40 mAb (FGK45) administered during sensitization

In all experiments, BALB/c mice were sensitized by i.p. injection of OVA on alternate days (d 1 until 13) and then daily challenged with

nebulized OVA for 8 d (d 33 until 40). When FGK45 was injected during sensitization with OVA, this therapy markedly attenuated the severity of allergic inflammation in response to repeated inhalatory OVA challenges (d 33 until 40). FGK45 injections reduced total cell counts (66.7 ± 9.3 vs 108.2 ± 12.1 x10^5/ml), eosinophil and lymphocyte counts in BAL fluid (Fig. 4.4.1 A). On the other hand, monocyte counts in BAL fluid of FGK45-treated mice were twice as high as in controls (23.6 ± 5.6 vs 12.9 ± 15.4 x10^5/ml, Fig. 4.4.1 A). Similar cellular shifts were observed in peripheral blood, where lower percentages of eosinophils (4.3 ± 0.7 vs 12.2 ± 1.1 % of white cell count, $p < 0.05$) were found in parallel with more monocytes (23.4 ± 2.1% vs 7.3 ± 2.1% of white cell population, $p < 0.05$).

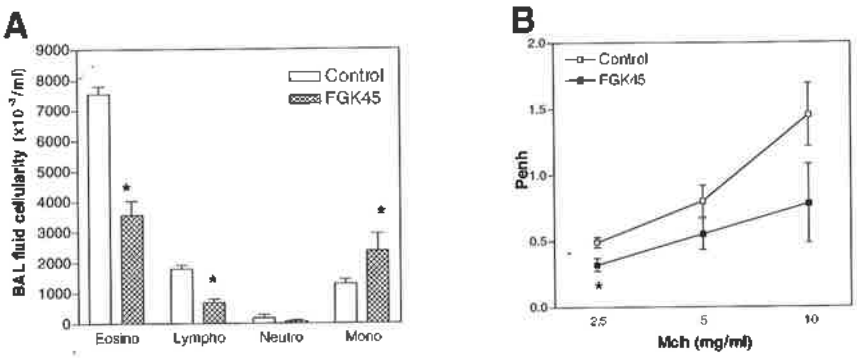

Fig. 4.4.1 Effects of agonistic anti-CD40 mAb (FGK45) administered during sensitization on the subsequent development of allergic airway inflammation. During sensitization to OVA (day (d) 1 until 13), mice were injected with 250 µg of FGK45 or control Ig on d 0, 4, 8 and 12. Thereafter, eosinophilic airway inflammation was induced by repeated inhalatory OVA challenge (d 33 until 40). On d 41, differential cell counts were performed on cytospins of broncho-alveolar lavage (BAL) fluid (**A**) and airway responsiveness to incremental doses of Mch was measured using whole body plethysmography (Buxco°, **B**). One representative experiment out of 3 is shown. Each group consists of 6 mice. Data represent means +/- SEM. *, $p < 0.05$ vs control mice.

On tissue level, peribronchial eosinophilic infiltrates were significantly smaller in FGK45 than control mice (35.3 ± 23.3 $x10^{-2}$ vs 170.3 ± 13.2 $x10^{-2}$ mm respectively, $p < 0.05$). In addition, functional analysis revealed that FGK45 administered during sensitization reduced bronchial responsiveness to inhaled Mch, as demonstrated by a shift of the dose-response curve of Penh values to the right (Fig. 4.4.1 B).

FGK45 treatment during sensitization significantly reduced OVA-specific IgG1 production (65.7 ± 11.7 vs 94.1 ± 1.3 EU/ml on d 28, $p < 0.05$) whereas the opposite trend was found for OVA-specific IgG2a (362.3 ± 90.2 vs 257.3 ± 63.2 EU/ml on d 28, $p > 0.05$). Both OVA-specific IgE production and cutaneous hypersensitivity reaction were reduced by FGK45 treatment, without reaching levels of significance (data not shown). Repeated inhalatory OVA challenges boosted Ig production (125), ultimately leading to similar levels of OVA-specific IgG1, IgE and IgG2a in both treatment groups on d 41. Data on FGK45-mediated effects on cytokine production will be dealt with in the next paragraph.

4.4.2.2 IL-12-independent inhibition of eosinophilic airway inflammation by anti-CD40 mAb administered during sensitization

In accordance with the reported induction of IL-12 by FGK45 (112), mice that were treated with FGK45 during sensitization had elevated levels of circulating IL-12 (3,395 ± 269 vs 725 ± 425 ng/ml in controls, $p < 0.05$) and had more mRNA for IL-12 in inflamed lung tissue (185.2 ± 3.8 vs 127.2 ± 4.6, $p < 0.05$) on d 41. Therefore, we investigated whether incapability to produce IL-12 would abrogate the down-regulatory effects of FGK45 on allergic airway inflammation.

FGK45 or control Ig were injected during sensitization in IL-12-deficient (knock-out or KO) BALB/c mice and WT controls. As expected

(94), IL-12 KO mice responded to sensitization and inhalatory OVA challenges (d 33 until 40) with more severe influx of eosinophils and lymphocytes in bronchi than WT littermates (Fig. 4.4.2 A). In the former group, eosinophils constituted up to 90.1 ± 2.3 % of cells in BAL fluid compared to 72.1 ± 2.1 % ($p < 0.05$) in WT controls. In BAL fluid, IL-5 levels were higher in IL-12 KO mice (Fig. 4.4.2 B) as was the expression of IL-4 mRNA in inflamed lung tissue (Fig. 4.4.2 C). Consistent with an enhanced Th2 cytokine profile, OVA-stimulated PBLN cells of IL-12-deficient mice produced more IL-4 and IL-5 and less IFN-γ than their WT controls (Fig. 4.4.2 D).

Unexpectedly, FGK45 treatment during sensitization also down-regulated eosinophilic inflammation in IL-12 KO mice, suggestive of an IL-12-independent mechanism of action of FGK45. Both IL-12 KO and WT mice responded to FGK45 therapy with a potent reduction of both bronchial eosinophilia (Fig. 4.4.2 A) and IL-5 levels (Fig. 4.2.2 B). As was observed in WT mice (Fig. 4.4.3, A and B), FGK45 therapy reduced the size of peribronchial inflammatory infiltrates in IL-12 KO mice (45.3 ± 23.2 $x10^{-2}$/mm vs 210.2 ± 32.3 $x10^{-2}$/mm, $p < 0.05$, Fig. 4.4.3, C and D). In the latter group, FGK45 reduced bronchial mRNA expression of IL-4, IL-5 and IL-13 (Fig. 4.4.2 C), and lowered production of IL-4 and IL-5 by PBLN in vitro (Fig. 4.2.2 D). FGK45 did not alter IFN-γ production on both mRNA and protein level (Fig. 4.4.2, C and D).

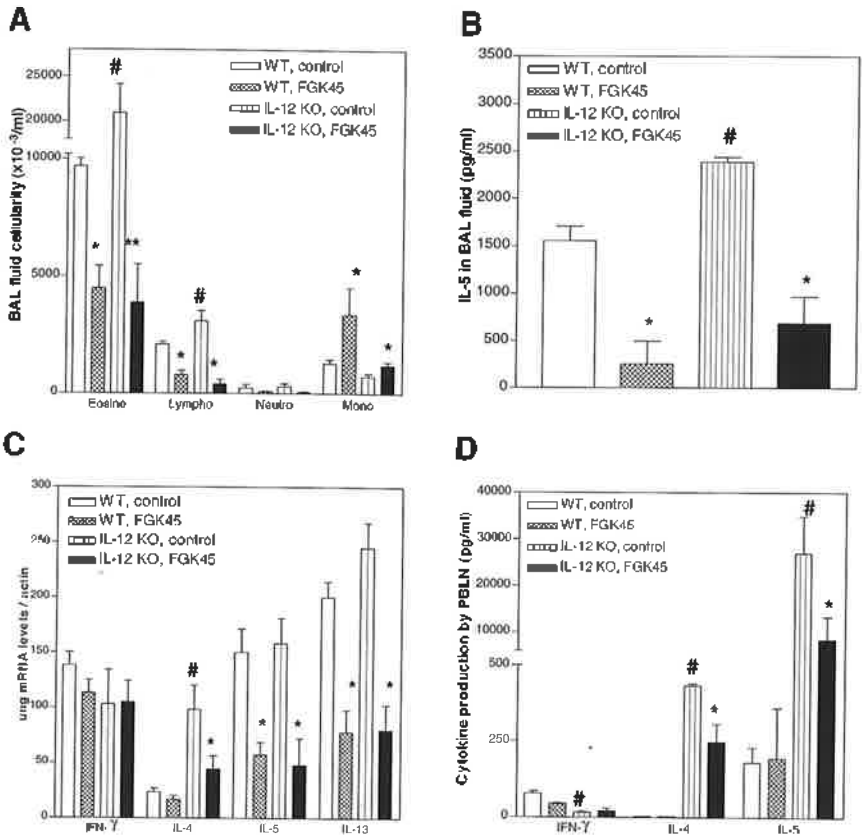

Fig. 4.4.2 Effects of administration of anti-CD40 mAb (FGK45) during sensitization on allergic airway inflammation in interleukin (IL)-12 knock-out (KO) and wild-type (WT) BALB/c mice. Both IL-12 KO and WT mice were injected on day (d) 0, 4, 8 and 12 with 250 μg of FGK45 or control Ig during sensitization (d 1 until 13) and then daily challenged with nebulized OVA (d 33 until 40). On d 41, mice were sacrificed and differential cell counts in broncho-alveolar lavage (BAL) fluid performed on cytospin preparations (**A**). IL-5 levels in BAL fluid were measured with sandwich ELISA (**B**). mRNA levels of IFN-γ, IL-4, IL-5 and IL-13 were measured on lung homogenates with real-time RT-PCR. The level of mRNA for different cytokines was expressed relative to mRNA for actin, and multiplied by 10^5 for IL-4 and IL-13, and by 10^6 for IFN-γ and IL-5 (**C**). Production of IFN-γ, IL-4 and IL-5 by OVA-stimulated peribronchial lymph node (PBLN) cells was measured in culture supernatants using sandwich ELISA (**D**). Each group consisted of 4 to 8 mice. #, $p < 0.05$ vs WT control Ig-treated mice; *, $p < 0.05$ and **, $p < 0.005$ vs control Ig-treated mice of similar background.

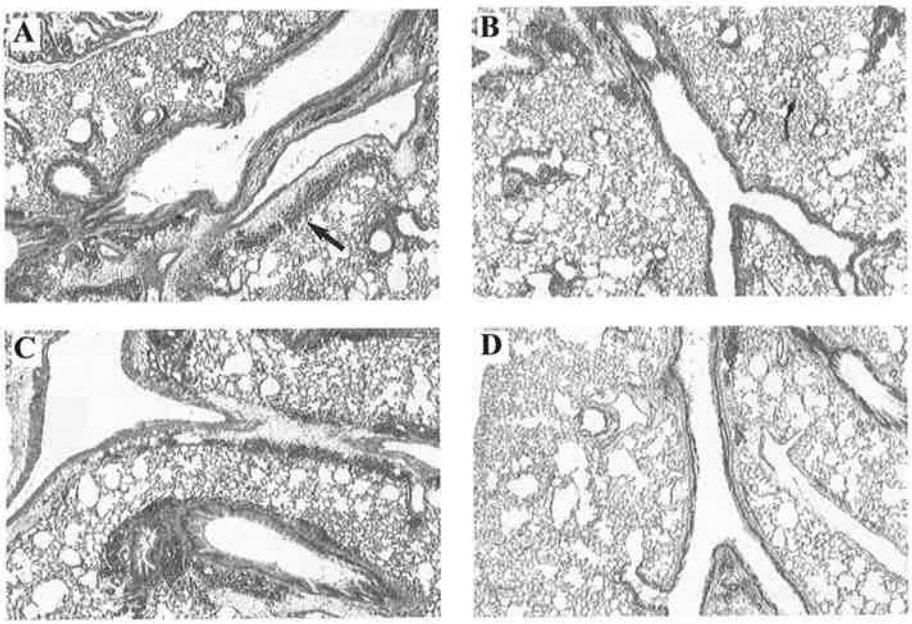

Fig. 4.4.3 Effects of agonistic anti-CD40 mAb (FGK45) on bronchial inflammation in IL-12 wild-type (**A** and **B**) and IL-12-deficient (**C** and **D**) BALB/c mice. Mice that had been injected (d 0, 4, 8 and 12) with control Ig (**A** and **C**) or anti-CD40 mAb (FGK45, **B** and **D**) during sensitization (d 1 until 13), repeatedly inhaled nebulized OVA (d 33 until 40). On d 41, histologic sections of lung tissue were stained with the classic haematoxylin and eosin stain and the degree of peribronchiolar and perivascular inflammation (black arrow, **A**) measured. Representative sections are presented.

4.4.2.3 Inhibition of the development of eosinophilic airway inflammation by agonistic anti-CD40 mAb administered during airway challenges of sensitized mice

In order to evaluate whether FGK45 treatment has down-regulatory capacity on allergic inflammation at the time of development of airway inflammation, sensitized mice were injected with FGK45 mAb or control Ig twice (d 32 and 36) during the phase of daily challenges with nebulized OVA (d 33 until 40). FGK45 potently down-regulated allergic airway disease both on the cellular as well as on the functional level. Bronchial

eosinophilia was significantly lower in FGK45-treated mice (Fig. 4.4.4 A), as were percentages of circulating eosinophils (4.5 ± 1.2 % vs 13.2 ± 0.9 %, $p < 0.05$).

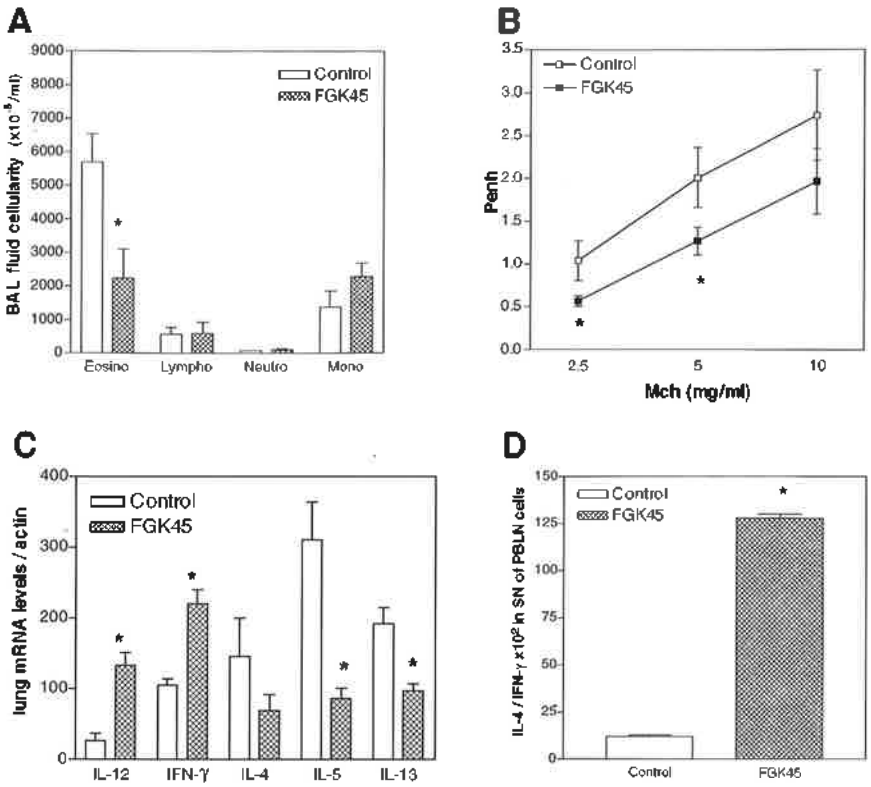

Fig. 4.4.4 Effects of agonistic anti-CD40 mAb (FGK45) administered during inhalatory OVA challenge of sensitized mice on the development of allergic airway inflammation. Mice that had been sensitized to ovalbumine (OVA, from day (d) 1 until 13) repeatedly inhaled nebulized OVA (d 33 until 40). In addition, FGK45 or control Ig were injected at 250 µg on d 32 and 36. On d 41, differential cell counts were performed on cytospin preparations of broncho-alveolar lavage (BAL) fluid (**A**). Airway responsiveness to inhalation of incremental doses of Mch was measured using whole body plethysmography (Buxco°, **B**). mRNA levels of cytokines were measured on d 41 on lung homogenates using real-time RT-PCR. The level of mRNA for different cytokines was expressed relative to mRNA for actin, and multiplied by 10^5 (**C**). IL-4 and IFN-γ levels were measured by sandwich ELISA in culture supernatants of OVA-stimulated peribronchial lymph node (PBLN) cells. The mean ratio of IL-4 / IFN-γ is presented (**D**). Each group consisted of 6 mice and data represent means +/- SEM . One representative experiment out of 2 is presented. *, $p < 0.05$ vs control Ig-treated mice.

Consistently, smaller peribronchial infiltrates were observed in FGK45-treated mice (55.3 ± 23.2 $x10^{-2}$ vs 145.2 ± 32.6 $x10^{-2}$/mm, $p < 0.05$). FGK45 also reduced AHR, as demonstrated by significantly lower Penh values after inhalation of nebulized Mch at 2.5 and 5 mg/ml (Fig. 4.4.4 B). In keeping with down-regulation of allergic inflammation, FGK45 lowered IL-5 levels in the broncho-alveolar lumen (388.2 ± 89.3 vs 721.3 ± 132.3 pg/ml of BAL fluid) and in serum (undetectable levels in FGK45 mice vs 16.3 ± 1.2 pg/ml in the control). Levels of IL-4 were under the detection limit in serum and BAL fluid (data not shown). With real time RT-PCR, we demonstrated FGK45-mediated downregulation of IL-5 and IL-13 mRNA levels in lung homogenates ($p < 0.05$, Fig. 4.4.4 C). Also IL-4 mRNA levels were lower in FGK45 mice without reaching levels of significance ($p > 0.05$, Fig. 4.4.4 C). In contrast to Th2 cytokines, mRNA levels of IL-12 p40 and IFN-γ were significantly up-regulated by administration of FGK45 (Fig. 4.4.4 C), as were circulating levels of IL-12 p40 (data not shown). IFN-γ could not be detected in serum.

In keeping with the predominant Th1 profile in bronchi of FGK45-treated mice, lymphocytes obtained from PBLN produced more IFN-γ relative to IL-4 after stimulation with OVA. A 10-fold increase in the IFN-γ / IL-4 production ratio was observed in culture SN of OVA-stimulated PBLN cells (1.28 ± 0.02 vs 0.12 ± 0.01, $p < 0.05$, Fig. 4.4.4 D).

Furthermore, the boosting effect of inhalatory challenges on OVA-specific IgE production (125) was reduced in FGK45-treated mice compared to the control (127.2 ± 13.1 vs 234.8 ± 34.5 EU/ml for IgE, $p < 0.05$). On d 41, levels of OVA-specific IgG1 and IgG2a were not significantly affected by FGK45 treatment during repeated inhalatory challenges (data not shown).

Of note, 2 injections of FGK45 (d 32 and 36) induced a marked splenomegaly, reflected by a 3-fold increase in splenic weight and 8-fold

increase in total splenic cell counts (data not shown). This phenomenon proved to be transient and independent of the induction of allergic airway inflammation. Indeed, this splenomegaly was not observed in mice treated with FGK45 (d 0 until 12) during sensitization and sacrificed on d 41. In addition, na ve BALB/c mice developed similar splenomegaly as the above-mentioned group with allergic airway inflammation when sacrificed 5 d after similar the 2nd injection with FGK45 (data not shown).

4.4.2.4 Anti-CD40L mAb inhibit OVA-specific Ig production during sensitization but fail to modulate allergic inflammation

After demonstration of the anti-inflammatory effects of agonistic mAb against CD40 on the phenotype of allergic inflammation, we investigated whether interaction between CD40-CD40L is essential for the induction of airway inflammation. For this purpose, anti-CD40L mAb (MR1) were injected during either sensitization or repeated inhalatory allergen challenges of sensitized mice.

When MR1 was injected during sensitization, this therapy significantly inhibited OVA-specific IgE production as demonstrated on d 28 (18.3 ± 8.3 vs 51.2 ± 12.2 EU/ml, $p < 0.05$, Fig. 4.4.5 A). This reduction of circulating OVA-specific IgE was accompanied by impaired cutaneous hypersensitivity reactions after intradermal injection of OVA on d 28 (Fig. 4.4.5 B), which is an IgE-dependent phenomenon (146). Apart from IgE, also OVA-specific IgG1 (data not shown) and IgG2a (Fig. 4.4.5 A) production were significantly inhibited, as expected from previous demonstrations that CD40-CD40L interaction is crucial for Ig isotype switch (89). When these mice repeatedly inhaled OVA, this allergen challenge boosted Ig production in both control and MR1-treated mice in a similar way (Fig. 4.4.5 A) and induced cutaneous hypersensitivity reactions in MR1-treated mice (Fig. 4.4.5 B). Of note,

MR1 administered during sensitization did not affect the increase in peripheral blood eosinophilia induced by sensitization, nor did it modulate the subsequent development of allergic airway disease in terms of AHR, bronchial eosinophilia nor Th2 cytokine production (data not shown).

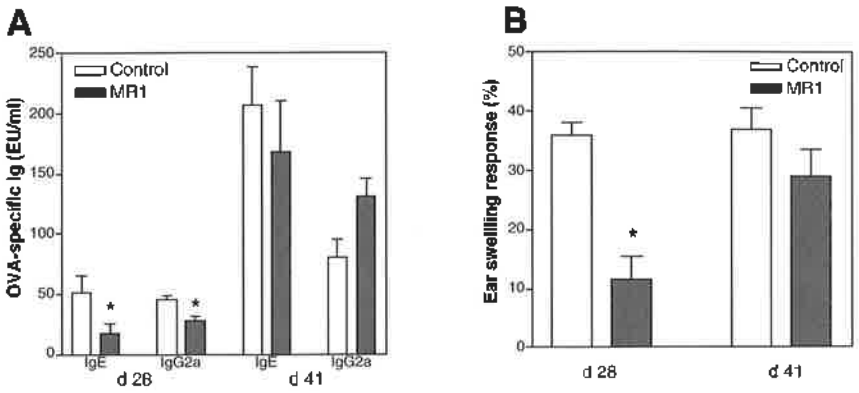

Fig. 4.4.5 Effects of MR1 administered during sensitization on ovalbumine (OVA)-specific Ig production and cutaneous hypersensitivity reaction to OVA. Mice were sensitized to OVA by intraperitoneal injections on alternate d from day (d) 1 until 13. In addition, mice were injected with 250µg of MR1 or control Ig on d 0, 4, 8 and 12. In a second phase, mice were daily exposed to nebulized OVA from d 33 until 40. OVA-specific Igs were determined in serum using sandwich ELISA and expressed in EU/ml (**A**). On the same occasion, cutaneous hypersensitivity to OVA was evaluated by intradermal injection of OVA in the right aural concha and saline in the contralateral side (**B**). After 1 h, the increase in thickness of the right concha was expressed in percentage of thickness of the left concha. Data are expressed as means +/- SEM. Each group consists of 12 mice. *, $p < 0.05$ vs control Ig.

Blockade of CD40-CD40L interaction during the inhalatory challenge phase of sensitized mice also failed to affect the severity of allergic airway inflammation as evaluated by the same parameters as above (data not shown).

4.4.3 Conclusion

Stimulation of CD40 during antigen priming resulted in a reduction of bronchial eosinophilia and airway hyperresponsiveness

following subsequent inhalatory allergen challenges. Both phenomena are accompanied by and can potentially be explained by decreased production of IL-4, IL-5 and IL-13. In view of enhanced bronchial and systemic production of IL-12 after administration of anti-CD40 mAb and the reported IL-12-dependency of effects of anti-CD40 mAb in another model of Th2 cytokine-mediated pathology (112), we hypothesized that the beneficial effects of anti-CD40 mAb would be mediated via induction of IL-12. Indeed, IL-12 has the capacity to inhibit Th2 differentiation at the time of antigen priming (94) and to reduce bronchial eosinophilia in allergic inflammation (166). To our surprise, FGK45 down-regulated the allergic inflammatory response in IL-12-deficient mice, indicative of alternative IL-12-independent pathways. We therefore speculate on CD40-mediated induction of other mediators with inhibitory potential on Th2 differentiation. In this regard, Martin et al. reported that IFN-γ is necessary for anti-CD40-induced Th2 cytokine inhibition in *Schistosoma mansoni* infection (112). In our model of allergic airway inflammation, IFN-γ is unlikely involved in downregulating Th2 cytokine production as both bronchial and systemic production of IFN-γ remained low after FGK45-therapy. Here, production of TGF-β, another cytokine with anti-inflammatory capacity in allergic asthma (155), was not altered by anti-CD40 treatment (unpublished observation). CD40 signaling has been reported to mediate production of IL-10 *in vitro* (95) as well as *in vivo* (116). Mauri et al. (116) found up-regulation of IL-10 production by activated antigen-specific T lymphocytes from mice treated with FGK45. Here, we found high levels of circulating IL-10 after FGK45 therapy in both IL-12-deficient and wild-type mice (unpublished observation). IL-10 has powerful anti-inflammatory activities (167) and could explain the down-regulatory capacity of FGK45 on allergic airway inflammation (148) and rheumatoid arthritis (116). However, contradictory results have

been reported regarding the potential immunosuppressive capacity of IL-10 in allergic airway inflammation (44, 148). Finally, CD40-mediated induction of IL-18 may also represent a mechanism by which allergic airway inflammation is down-regulated. Indeed, IL-18 has been reported to reduce eosinophilic airway inflammation and replace an established Th2-biased immune response with a Th1-biased response (168).

In parallel with down-regulating the eosinophilic and lymphocytic influx, anti-CD40 mAb administered during sensitization enhanced bronchial monocyte counts. We speculate that this phenomenon represents a late effect of IL-12-induced stimulation of extramedullary haematopoiesis (169) with expansion of the circulating pool of monocytes as was suggested by the finding of higher percentages of monocytes in peripheral blood.

When agonstic mAb against CD40 was injected during the phase of repeated allergen challenge of sensitized mice, this therapy similarly attenuated bronchial eosinophilia and AHR in association with a marked shift of cytokine production away from Th2, and towards Th1 cytokine predominance. This FGK45-mediated cytokine switch from Th2 towards Th1 profile was documented both *in vivo* as well as *in vitro*. It is noteworthy to mention that, in our hands, beneficial effects on allergic inflammation occur concomitantly with induction of Th1 cytokines, but not IL-10 (data not shown). The association between amelioration of allergic airway inflammation and induction of Th1 cytokines is supported by clinical studies showing increased IFN-γ levels after succesful immunotherapy of allergic patients (170) and reduction of bronchial eosinophilia by exogenous IL-12 (171). Our finding of reduced bronchial hyperresponsiveness may be related to decreased bronchial IL-13 production (28).

The marked splenomegaly we observed after FGK45 injection may result from CD40-mediated stimulation of haematopoiesis by IL-12 (169) and/or GM-CSF (172), and raises questions about the clinical applicability of anti-CD40 mAb. However, systemic administration of anti-CD40 mAb did not cause any sign of wasting disease and the observed splenomegaly was transient.

Taken together, anti-CD40 mAb administered during either sensitization or during the effector phase suppressed bronchial eosinophilia and non-specific hyperresponsiveness in an equipotent way. Hence, our data underline the critical role of ligation of CD40 in the negative control of allergic inflammation during both initiation of immune responses as well as during the effector phase of inflammation.

The reduction of OVA-specific Ig production by anti-CD40L mAb during sensitization indicates that blockade of CD40-CD40L interaction during antigen priming impairs Ig isotype switch, consistent with data in CD40 or CD40L-deficient mice (25, 101). Reduction of OVA-specific IgE levels was reflected by lower cutaneous hypersensitivity reaction to OVA and was a transient phenomenon, confirming the reversible character of the inhibitory effect of anti-CD40L mAb on Ig isotype switch (173). This finding also suggests that CD40L is probably not involved in the process of T and B cell priming. Furthermore, the development of bronchial inflammation in the latter mice is consistent with CD40/CD40L-independent Th2 differentiation during antigen priming (174). In addition, we showed that CD40-CD40L interaction during the effector phase of allergic disease is not involved in the boosting effect on IgE production nor in the induction of airway inflammation. The lack of effect of anti-CD40L mAb on OVA-specific IgE levels suggests that newly recruited, na ve B cells contribute little to IgE production after OVA challenge and/or that ligation of CD40 on

memory B cells is not essential for boosting Ig production. In keeping with our data, Han et al. reported failure of MR1 to modify Ig production during secondary immune responses (175). Our finding that CD40-CD40L interaction is not required for development of bronchial inflammation or responsiveness apparently contrasts to data obtained in CD40 (25) or CD40L-deficient mice (119). In contrast to the use of trangenic mice, CD40-CD40L interaction was interrupted here by administration of mAb during one phase of disease induction, i.e. sensitization or inhalatory challenge.

In conclusion, we have demonstrated that CD40-CD40L interaction is not essential for the development of allergic airway inflammation. However, stimulation of CD40 signaling during either sensitization or repeated allergen inhalations, redirects the allergic immune response away from the Th2 cytokine phenotype normally induced in allergic airway inflammation.

CHAPTER 5. DISCUSSION AND CONCLUDING REMARKS

In this work, we demonstrate that experimental allergic asthma in a mouse model is part of an inflammatory disorder of the whole respiratory tract, in which costimulatory interactions between APC and Th cells during antigen priming and/or restimulation determine the severity of airway disease. In this final chapter, we will highlight some of the novel findings that have been described in previous chapters of this thesis.

Our data on simultaneous induction of upper and lower airway inflammation in sensitized mice (**chapter 4.1**) support the hypothesis of the existence of global airway allergy. Since the majority of inhaled allergens are deposited onto the nasal mucosa, we hypothesize that allergen deposition in the nose triggers the production and systemic release of IL-5 in sensitized individuals, thereby facilitating inflammation of the lower airways in response to a minute amount of allergen reaching the lower airways (Fig. 5.1). In this regard, Braunstahl et al. recently reported that nasal allergen provocation of patients with allergic rhinitis upregulates adhesion molecules in both nose and lung (176). As a consequence of the concept of the nose being primarily involved in eliciting bronchial inflammation in asthma, we should consider modifying our diagnostic and therapeutic attitude towards patients with allergic asthma. For instance, studies on the effectiveness of nasal versus bronchial therapy, or nasal versus systemic immunotherapy, seem warranted in allergic patients in order to evaluate the potential advantage of treating the nose in patients with allergic asthma. It is noteworthy to mention that nasal therapy has already been shown to prevent the seasonal increase in bronchial hyperresponsiveness in patients with allergic rhinitis and asthma (177).

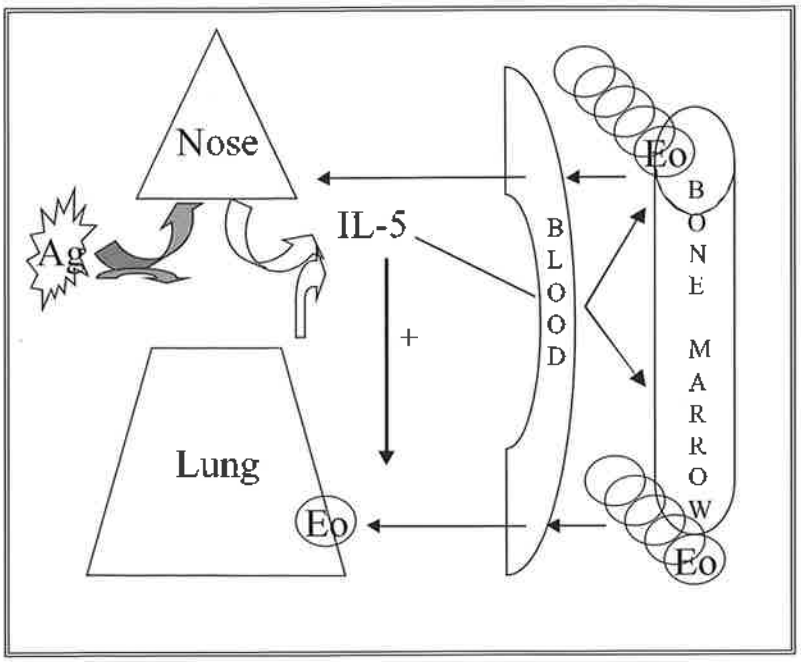

Fig. 5.1 Scheme of the systemic character of the allergic response following allergen inhalation. Deposition of allergens onto airway mucosal surfaces leads to the systemic release of IL-5, that stimulates eosinopoiesis in bone marrow. Newly generated eosinophils (Eo) are released from the bone marrow and enter inflamed airways.

One should however bear in mind that humans are not obligate nose-breathers like mice and therefore, these data should be interpreted with caution. In addition, one could also speculate that the presence of rhinitis in patients with asthma impairs nasal deposition of inhaled allergens. However, there seems to be no alteration of median total nasal airflow during the pollen season in pollen-allergic rhinitis patients (178). Therefore, the presence of allergic rhinitis does not necessarily prevent nasal deposition of allergens. It is also noteworthy to mention that, besides nasal deposition of allergens, nebulized allergens reach the immune system in the gut. Indeed, nasally deposited allergens are transported towards the oropharynx by the muco-ciliary transport system

(180), and particles that are deposited onto the skin, are licked and swallowed. In this way, the immune system in the gut, which is a site for induction of tolerance (179), may modify immune responses to inhaled allergens. The phenomenon of oral tolerance has been reported to play an important role during antigen priming (179), but is unlikely involved in the early local and systemic immune responses following allergen provocation of sensitized animals.

The next question will be to delineate the cellular source of systemic IL-5 after mucosal allergen inhalation (**chapter 4.1 and 4.2**). Our *in vitro* data (**chapter 4.3 and 4.4**) illustrate that lymphocytes from peribronchial lymph nodes of mice with eosinophilic airway inflammation represent a significant source of cytokines. To what extent intra-epithelial lymphocytes or other inflammatory cells in bronchi or circulation contribute to the release of IL-5 after allergen inhalation, remains to be determined. Of note, the concept of systemic release of cytokines after allergen contact (**chapter 4.1 till 4.4**) fundamentally changes our insight into the function of cytokines. Besides paracrine effects, cytokines may be involved in a much broader spectrum of effects, including stimulation of haematopoiesis in the bone marrow. For example, IL-5 stimulates eosinopoiesis in the bone marrow (3) and is highly effective at releasing eosinophils and their precursors from the bone marrow (4). In addition, circulating IL-5 is one of the key factors involved in the recruitment of eosinophils towards airways (5). As bronchial eosinophilic inflammation represents one of the hallmarks of allergic asthma, elucidation of the factors responsible for the generation, recruitment and survival of eosinophils in bronchi may improve our understanding of allergic airway inflammation. In addition, one also needs to explore the mechanisms that control the recruitment and differentiation of mucosal eosinophil progenitors at the site of

inflammation (181). It will be of utmost importance to delineate the extent to which the allergen-driven increase in tissue eosinophilia is caused by systemic recruitment of eosinophils and/or local differentiation of progenitors in the mucosa. This will enhance our insight into systemic and local inflammatory factors responsible for the development and perpetuation of airway inflammation.

Our data on IL-17-mediated influx of granulocytes in bronchi (**chapter 4.2**) after inhalation of allergen, necessitates further research on the relevance of both IL-17 and granulocytes in allergic asthma. The fact that IL-17, which is secreted primarily by activated memory T cells, is essential for the bronchial recruitment of neutrophils, has recently been confirmed by a study in mice (38). Indeed, defective IL-17 receptor signaling results in a delayed influx of neutrophils into the lungs. Therefore, targeting IL-17 may represent a new therapeutic option in neutrophil-mediated diseases. Of note, neutralization of IL-17 in our asthma model unexpectedly enhanced allergen-induced IL-5 production, eosinopoiesis in bone marrow and bronchial eosinophilia. The mechanism behind this anti-IL-17 mAb-mediated enhanced IL-5 production still remains obscure. *In vitro* studies failed to show any significant effect of IL-17 on IL-5 production by lymphocytes (data not shown). Therefore, other indirect mechanisms are probably involved in the observed elevation of IL-5 production. In order to exclude the eosinophil from being the major source of this IL-5, experiments are currrently being performed in which granulocyte-depleted mice are injected with anti-IL-17 mAb prior to provocation. Furthermore, the results of these experiments will reveal the extent to which both neutrophils and eosinophils are involved in the generation of the late phase response following allergen provocation of mice with pre-existing eosinophilic airway inflammation. Apart from studying the effects of

acute depletion of granulocytes on the bronchial allergic response, it will be of interest to investigate what beneficial or deleterious effects the chronic inhibition of bronchial influx of granulocytes will have on the outcome of allergic airway disease. Indeed, the role of both eosinophils and neutrophils in allergic asthma is incompletely understood. In spite of their abundant presence and potential role in the development of airway hyperresponsiveness and late phase asthmatic response (41), prolonged reduction of bronchial eosinophilia failed to have beneficial effects in allergic asthma (26). As far as neutrophils are concerned, their potential contribution to disease in asthma has been neglected until recent years, and their role in allergic asthma *in vivo* remains to be elucidated. For this purpose, it will be of interest to study the effects of acute and/or chronic reduction of bronchial eosinophilia or neutrophilia on bronchial mucus secretion, bronchial remodeling and/or AHR in mouse models. Only then will it be possible to delineate the potentially beneficial effects of targeting IL-17 in allergic asthma.

We further illustrate that the bone marrow participates in the pathogenesis of allergic airway disease (**chapter 4.2**). Modulation of bone marrow granulopoiesis correlated with a shift of granuylocyte populations in the bronchial inflammatory infiltrate. In another series of experiments (182), we found that aggravation of bronchial eoisnophilia corresponded with an increase in eosinophil colony forming units in the bone marrow. Therefore, it seems important to gain insight into bone marrow responses after allergen inhalation and to identify factors involved in this process. This may further enhance our insight into the development of global airway inflammation after local allergen deposition.

Furthermore, we demonstrate that modulation of the immune response during antigen priming has a high impact on the severity of

airway inflammation following allergen inhalation. Blockade of CTL-4 at the time of sensitization resulted in the development of severe airway inflammation following allergen inhalation (**chapter 4.3**). In contrast, agonistic anti-CD40 mAb markedly attenuated bronchial inflammation and hyperresponsiveness, irrespective of administration during sensitization or inhalatory challenge (**chapter 4.4**). We therefore propose a hypothetical scheme depicting the role of CD40-CD40L and B7-CD28/CTLA-4 interactions at the time of sensitization on the outcome of allergic airway disease (Fig. 5.2). Our observations demonstrate that modulation of the function of costimulatory molecules at the time of antigen priming determined the cytokine production profile at the time of new contact with the same allergen. Therefore, we speculate on genetic imprinting of cytokine production in differentiated memory Th2 cells, or modulation of effector functions of a regulatory T cell (Treg) population (Fi. 5.2).

As events during antigen priming significantly affect the subsequent manifestation of allergic airway disease, research on environmental and genetic factors that influence the developing immune system in general and costimulatory interactions between APCs and Th cells in particular, seems necessary. In this regard, Coscoy et al. (183) provide evidence for a virus-mediated alteration of expression of costimulatory molecules and outcome of immune responses. Further research on the interplay between the environment and the immune system will undoubtedly yield information that is necessary in order to understand the increasing prevalence of allergic asthma.

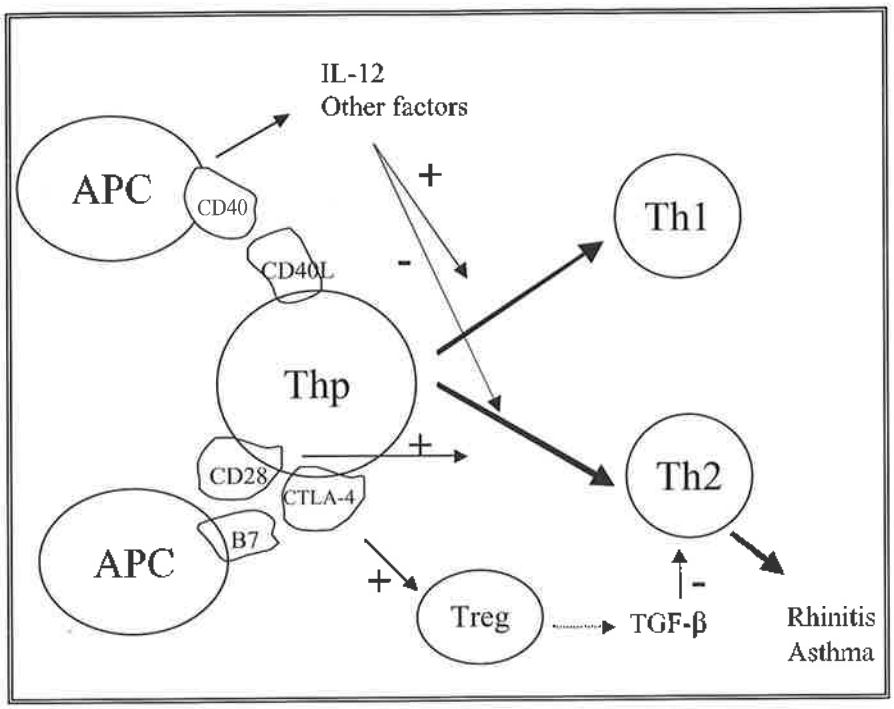

Fig. 5.2 Schematic overview of the functional relevance of CD40-CD40L and B7-CD28/CTLA-4 interactions between **Th** precursor (Thp) cells and antigen-presenting cells (APC) during sensitization. Ligation of CD40 results in the release of mediators that favor Th1 and suppress Th2 differentiation. Binding of CD28 is necessary for differentiation of Thp cells towards the Th2 lineage. CTLA-4 regulates, at the time of antigen priming, the degree of Th2 differentiation and disease manifestation.

In view of our data on aggravation of experimental asthma by blockade of CTLA-4 (**chapter 4.3**) and attenuation of experimental asthma by stimulation of CD40 signaling (**chapter 4.4**), we propose stimulation of CTLA-4 or CD40 molecules on the surface of T cells and monocytes respectively, as novel pathways for asthma therapy. In addition, it will be of interest to determine which factors are involved in

the anti-CD40-mediated reduction of allergic airway inflammation we observed in our mouse model. Futhermore, investigating the role of other costimulatory interactions between Th cells and APCs, such as the recently discovered receptor-ligand pairs ICOS-B7RP1 (184), TRANCE-RANK (185), OX-40-OX40L (186) and 4-1BB-4-1BBL (187), will undoubtedly enhance our insight into the complex immunologic mechanisms underlying allergic airway disease.

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SUMMARY

Allergic diseases, such as asthma, rhinitis, conjunctivitis and eczema, are reaching epidemic proportions in the Western world. The reason for the increasing prevalence of allergic diseases remains elusive and present therapies for these diseases are symptomatic without curation. Therefore, insight in the pathophysiology of the allergic inflammatory response will aid us in optimizing currently available therapies and may ultimately lead to reverse the trend of rising prevalence.

Our understanding of immunologic mechanisms underlying allergic diseases has changed dramatically since the discovery that, in response to stimulation with allergenic peptide, T helper (Th) lymphocytes of allergic patients produce a restricted array of cytokines (IL-4, IL-5, IL-9 and IL-13). These so-called Th2 cells play a central role in the induction of IgE production and manifestation of airway allergy via the production of the Th2 cytokines. Another Th subtype, Th1, is involved in different pathologic conditions and tends to antagonize the allergic response. Therefore, insight into the process of differentiation of naive Th cells towards Th1 or Th2-cytokine producing cells, represents a key factor in the development of allergic diseases. Presentation of peptides of allergens by antigen-presenting cells (APC) leads to the activation and differentiation of naive Th cells. Recent evidence suggests that interactions between surface molecules on APCs and Th cells, like CD40-CD40 ligand (CD40L) and B7-CD28/cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) interactions, are involved in the differentiation of Th cells. In order to unravel the functional relevance of these so-called costimulatory molecules in allergic asthma *in vivo*, mouse models of allergic asthma are useful tools for research.

We developed a mouse model of allergic asthma in BALB/c mice, a murine strain with the well-known characteristic of IgE production after sensitization. The asthma model that is used in this work, is particular in that it resembles both the chronic and airborne exposure of humans to allergens and allows the study of allergic rhinitis in the context of allergic asthma. In view of the simultaneous induction of allergic inflammation in both upper and lower airways of mice, our data confirm the hypothesis of the existence of global airway allergy. In addition, mice show a late phase bronchial response after inhalation of nebulized allergen, characterized by bronchial granulocyte influx and increase in bronchial tone. As many of the features of this chronic asthma model are reminiscent of human allergic asthma, data obtained in this model yield information that has relevance for human airway allergy.

We demonstrate here that blockade of CTLA-4 with anti-CTLA-4 monoclonal antibodies (mAb) at the time of systemic sensitization with allergen results in the development of more severe airway inflammation following inhalatory allergen challenges. In addition, different responses of BALB/c and C57BL/6 mice to blockade of CTLA-4 highlight the importance of the genetic background in the effects of anti-CTLA-4 mAb on the generation of allergic airway disease. Therefore, research focussed on factors involved in modulating the function of CTLA-4 at the time of first contact with allergens in susceptible subjects, may provide important information regarding the development of allergic diseases. In contrast to CTLA-4, neutralization of CD40L during sensitization or challenge has no effect on the development of allergic airway inflammation. However, stimulation of the CD40 molecule on monocytes, macrophages and/or APC during sensitization markedly impaired the subsequent development of allergic airway inflammation following repeated allergen inhalations. Both bronchial hyperresponsiveness and eosinophilia were significantly

reduced. Taken together, these data illustrate the potentially important impact of costimulatory interactions between APCs and Th cells at the time of sensitization on the subsequent development of allergic airway disease.

With regard to the development of new therapeutic strategies for asthma, we provide experimental evidence for a potential role of agonistic anti-CD40 mAb in the treatment of allergic airway disease. Indeed, anti-CD40 mAb severely reduced the phenotype of allergic airway inflammation when administered to previously sensitized mice during the phase of allergen inhalations. Both bronchial hyperresponsiveness as well as bronchial eosinophilia were markedly reduced. In contrast to our expectations, the beneficial effect of anti-CD40 mAb therapy was independent of the induction of IL-12, a typical Th1 inducing cytokine with inhibitory potential on allergic inflammation. Future research will elucidate which factors are responsible for the anti-CD40 mAb-mediated beneficial effects on experimental asthma and to what extent this approach may have therapeutic value in human allergic asthma. Therefore, these findings open up new venues for therapy of allergic asthma and allergic diseases in general.

SAMENVATTING

Allergische aandoeningen zoals astma, rhinitis, conjunctivitis en atopisch eczeem komen alsmaar vaker voor in Westerse, geïndustrialiseerde landen. De oorzaak van deze stijgende incidentie blijft ongekend. Daarnaast zijn de huidige behandelingen voor allergische aandoeningen gericht op het verlichten van de symptomen, zonder mogelijkheid tot genezen van deze aandoeningen. Gezien de grote morbiditeit van allergische aandoeningen is het van kapitaal belang onderzoek te verrichten naar immunologische mechanismen die aan de basis liggen van het ontstaan en het chronische karakter van deze ziekten.

Het inzicht in de pathofysiologie van allergische aandoeningen is grondig gewijzigd sinds de ontdekking dat T helper (Th) cellen van allergische patiënten voornamelijk interleukine (IL)-4, IL-5, IL-9 en IL-13 aanmaken. Secretie van deze zogeheten T helper (Th) 2 cytokinen door Th2 cellen, leidt tot de aanmaak van immuunglobuline E (IgE) en tot het ontstaan van luchtwegpathologie bij allergisch astma. Allergie van de lagere luchtwegen wordt hoofdzakelijk gekenmerkt door de episoden van luchtwegvernauwing, luchtweghyperreactiviteit en bronchiale eosinofiele inflammatie. In tegenstelling tot Th2 cellen, produceren Th1 cellen cytokinen die de allergische immuunrespons onderdrukken. Om het ontstaan van allergische aandoeningen te begrijpen, is het daarom nuttig onderzoek te verrichten naar factoren die de differentiatie van naïeve Th cellen naar Th1 of Th2 cellen beïnvloeden. Recent *in vitro* onderzoek toont aan dat interacties tussen antigen-presenterende cellen (APC) en Th cellen de aktivatie en differentiatie van naïeve Th cellen bepalen. Hierbij spelen interacties tussen moleculen aan het oppervlak van APC en Th cellen, o.a. CD40-CD40 ligand (CD40L) en B7-C28/cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), een belangrijke rol. Om de functie van deze moleculen *in vivo* te bestuderen,

maken we gebruik van een diermodel van allergisch astma. Een muismodel van allergisch astma is zeer nuttig om inzicht te verwerven in immunologische mechanismen van humaan astma. Zoals allergische patiënten maken muizen van de BALB/c stam na sensibilisatie allergeen-specifiek IgE aan en stijgt de eosinofilie in het bloed. Wanneer gesensibiliseerde muizen vervolgens herhaaldelijk een verstoven oplossing met allergenen inhaleren, treden bronchiale overgevoeligheid en eosinofiele luchtwegontsteking op. Naast inductie van deze bronchiale ontsteking, leidt allergeeninhalatie bij gesensibiliseerde muizen tot het ontstaan van een nasaal eosinofiel ontstekingsinfiltraat. Deze vondst bevestigt de hypothese over het bestaan van globale luchtwegallergie en opent nieuwe mogelijkheden voor onderzoek en therapie bij patiënten met allergisch asthma. Daarnaast leidt allergeeninhalatie bij muizen met een bestaande eosinofiele luchtwegontsteking tot een laattijdige toename van bronchiale tonus, die in de tijd correleert met een bronchiale influx van neutrofielen. Dit bronchiaal antwoord na allergeeninhalatie vertoont sterke gelijkenissen met ernstige astma-aanvallen die optreden bij een subpopulatie van asthmatische patiënten, enkele uren na allergeeninhalatie. Omwille van al deze gelijkenissen met humaan astma is het chronische muismodel van allergische luchtwegontsteking goed bruikbaar om modulatie van immunologische responsen na allergeencontact te bestuderen.

In het muismodel tonen we aan dat blokkeren van de functie van het CTLA-4 molecule aan het oppervlak van geactiveerde T lymphocyten door middel van neutraliserende anti-CTLA-4 monoclonale antistoffen (mAs) tijdens sensibilisatie, de sensibilisatie versterkt, en leidt tot een uitgesproken toename van eosinofiele luchtweginflammatie na herhaalde allergeeninhalatie. Hierbij spelen genetische factoren een belangrijke rol, aangezien er duidelijke verschillen worden waargenomen in de effecten

van neutraliserende anti-CTLA-4 mAs tussen BALB/c en C57BL/6 muizen. In tegenstelling tot CTLA-4, beïnvloedt blokkeren van het CD40L molecule op geactiveerde T lymfocyten tijdens sensibilisatie, noch tijdens herhaalde allergeeninhalatie, het ontstaan van eosinofiele luchtweginflammatie en luchtweghyperreactiviteit. Wanneer echter het CD40 molecule aan het oppervlak van de APC gestimuleerd wordt tijdens de sensibilisatie, onderdrukt dit sterk de ontwikkeling van experimenteel astma. Inderdaad, agonistische anti-CD40 mAs verhinderen het ontstaan van zowel eosinofiele ontsteking van de luchtwegen als van bronchiale hyperreactiviteit. Deze experimenten tonen aan dat interacties tussen APC en T lymfocyten tijdens het eerste contact met allergenen, tot op een zeker niveau bepalend kunnen zijn voor het immunologisch antwoord bij herhaald allergeencontact. Het lijkt daarom zinvol om in de toekomst onderzoek te verrichten naar factoren die het immunologische antwoord in het algemeen, en costimulatorische interacties in het bijzonder, beïnvloeden tijdens de sensibilisatie. Inzicht in deze mechanismen zal ongetwijfeld leiden tot een beter begrip van de toename in prevalentie van allergische aandoeningen en is noodzakelijk om deze opwaartse trend om te buigen.

Met het oog op de ontwikkeling van nieuwe behandelingen voor patiënten met allergisch astma, is het interessant dat anti-CD40 mAs de luchtweginflammatie onderdrukken, ook wanneer zij toegediend worden aan gesensibiliseerde muizen tijdens hun blootstelling aan allergenen. In tegenstelling tot onze verwachting, zijn de anti-CD40-gemediëerde effecten onafhankelijk van de inductie van IL-12, het prototype Th1 cytokine dat productie van Th2 cytokines onderdrukt. Verder onderzoek zal uitwijzen welke factoren verantwoordelijk zijn voor het therapeutische effect van anti-CD40 mAs in het muismodel, en of stimulatie van het CD40 signaal ook bij patiënten met allergisch astma de

luchtwegpathologie kan onderdrukken. Dit laatste luik van het onderzoek opent nieuwe therapeutische mogelijkheden voor allergisch astma en andere chronische allergische aandoeningen.

CURRICULUM VITAE

The author of this manuscript was born in Aalst on 27th April 1971. From 1983, he attended secondary school at the 'Sint-Jozefscollege' in Aalst. The city of Aalst awarded him with the gold medal for piano in 1988. In 1989, he entered medical school at the Katholieke Universiteit Leuven where he graduated magna cum laude in 1996. Under the supervision of Professor Dr. L. Feenstra, he started his specialty training in otorhinolaryngology in 1996 at the University Hospitals of the Katholieke Universiteit Leuven. In 1997, he was the recipient of a grant from the F.W.O. (Fonds voor Wetenschappelijk Onderzoek) Vlaanderen. From then, he was a research fellow in the Laboratory of Experimental Immunology under the guidance of Professor Dr. J. Ceuppens. For his experimental work, he received the Glaxo Wellcome Award for Experimental Rhinology in 1998 and the Award of the Belgian Society for Allergy and Clinical Immunology in 1999. In 1998, he obtained the certificate of the Educational Commission for Foreign Medical Graduates (ECFMG) of the United States Medical Licence Examination (USMLE) Committee. From October 2001, he will continue his clinical training in the Department of Otorhinolaryngology, Head and Neck Surgery at the University Hospitals of Leuven under the supervision of Professor Dr. M. Jorissen.

He is married to Ingeborg Stalmans and is the father of Peter-William and Nicolas.

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