Interleukin 16 in a mouse model of allergic asthma

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Interleukine 16 in een muizenmodel voor allergische astma
(met een samenvatting in het Nederlands)

Proefschrift

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Joris Johannes de Bie

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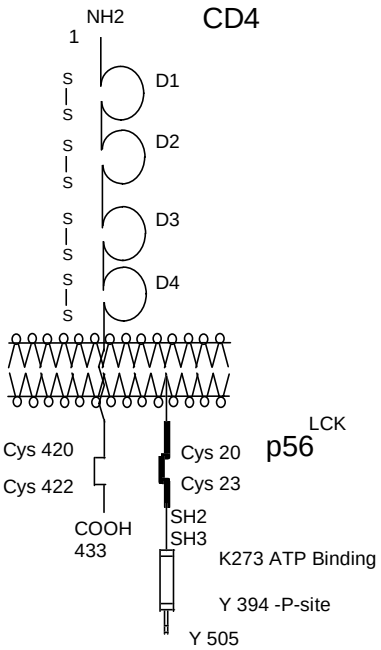
General Introduction

Introduction

Allergic asthma affects approximately 5% of the adult population and 10-15% of children in developed countries (1). This disease can be characterized by increased airway responsiveness to a variety of specific and nonspecific stimuli, accompanied by a chronic inflammatory response in the airways and excessive mucus production (2-4). The inflammatory response consists mainly of an influx and subsequent activation of eosinophils and T lymphocytes (reviewed in 5). Thereby, many different mediators are released, leading eventually to symptoms such as airway obstruction and airway remodeling (reviewed in 5). Furthermore, in serum of asthmatic patients increased levels of antigen-specific immunoglobulin (Ig)E can be measured (4), which can bind to Fcε-R1 receptors on mast cells. Upon antigenic-provocation IgE can be cross-linked leading to mast cell degranulation, thereby causing an acute bronchoconstrictive response (6).
Recent epidemiological studies have demonstrated that there is an increase in prevalence of asthma in western society and especially hospitalization due to asthma has increased dramatically (7). Factors that could contribute to the rise in asthma prevalence include environmental factors, e.g. increased presence of house-dust mites in newly build, well heated and isolated homes, airway pollution and decreased exposure to bacterial infections in early childhood. Furthermore, lack of physical activity is thought to contribute to this phenomenon (reviewed in 1).
Over the past decades some of the immunological disorders that contribute to this disease have been revealed. One of the most important findings was that a subset of $CD4^+$ T lymphocytes, T helper (Th)-2 cells are the main regulatory cells of the inflammatory response. Th2 cells were first described by Mosmann *et al.* (8) in the mouse and by Wierenga *et al.* in humans (9). This subset of lymphocytes is characterized by the production of certain cytokines, including interleukin (IL)-4, and IL5, whereas Th1 cells mainly produce IL2 and IFNγ (reviewed in 10).
IL4 is important for B-cell IgE production whereas IL5 plays an important role in recruitment and priming of eosinophils (11, 12). Both IL4 and IL5 play an important role in development of airway hyperresponsiveness and inflammation in several different animal models of allergic asthma. IL4 receptor blockade has been shown to inhibit development of airway hyperresponsiveness in mice (13, 14), whereas IL5 has been demonstrated to be crucial for induction of inflammation and airway hyperresponsiveness in guinea pigs and mice (12, 15, 16). However, controversy still exists about the exact role of these cytokines since IL5 has also been shown not to be necessary for development of airway hyperresponsiveness (13). Furthermore, IL4-independent airway hyperresponsiveness has been observed (17). Recently, several new cytokines have been discovered in murine models and human asthmatics that could be related to the pathogenesis of this disease, e.g. IL9, IL10 and IL13 (reviewed in 18,19-24).
Another cytokine that could be related to the pathology of asthma is IL16. It has been observed that a large upregulation in expression and presence of IL16 in BAL fluid and bronchial mucosa occurs after challenge with antigen in asthmatics but not in healthy volunteers or atopic subjects (25, 26). Furthermore, IL16 uses CD4 as its receptor (27, 28) and induces chemotaxis of $CD4^+$ T cells *in vitro* (reviewed in 29) and might therefore be of importance in the pathogenesis of $CD4^+$ T-cell mediated diseases such as allergic asthma.
Current research is focusing on possible ways to decrease the number of antigen-specific T lymphocytes or to redirect the Th2-type cytokine production towards a Th1 profile. Other possibilities include modulation of antigen-presenting cells, inhibition of various co-stimulatory pathways which are necessary for optimal T-cell stimulation, or use of allergen-immunotherapy. Furthermore, it could be beneficial to induce unresponsiveness or anergy in $CD4^+$ T lymphocyte subsets that are thought to be the cause of the pathology associated with allergic asthma. A generally accepted definition of anergy is "A cellular state in which a lymphocyte is alive but fails to display certain functional responses when optimally stimulated through both its antigen-specific receptor and any other receptors that are normally required for full activation" (30). One of the ways to induce anergy in $CD4^+$ T cells is the use of ligands for CD4 molecules, which are present on these T cells.

Figure 1



Schematic representation of the CD4 molecule and protein tyrosine kinase p56^{LCK} molecule. Shown are the four Ig-like domains (D1-D4) with their disulfide bonds (S-S) between cysteine residues. Tyrosine residues Y394 (autophosphorylation site) and Y505 (negative p56^{LCK} regulation site) on p56^{LCK} are shown as well as the cysteine residues (two on each molecule) that mediate CD4-p56^{LCK} interaction. (adapted from Gaubin *et al.*, 31).

Structure and function of CD4

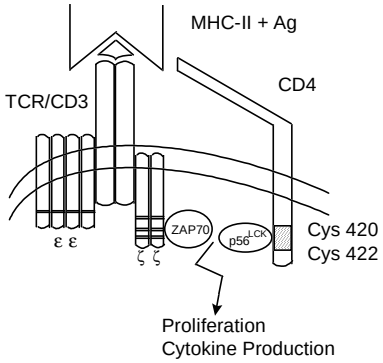
CD4 is a 55 kDa cell surface glycoprotein that consists of 4 extracellular immunoglobulin (Ig)-like domains (D1-D4), a transmembrane region and a cytoplasmic tail (32-36, Figure 1). The CD4 molecule exists as a monomer molecule on the cell membrane (reviewed in 37). The src-related protein tyrosine kinase (PTK) p56lck can be non-covalently associated with the cytoplasmic tail (38, 39, Figure 1). Initially, the CD4 molecule was considered to be a marker for Th subsets of lymphocytes (40). These lymphocytes respond to antigenic stimulation by proliferating and production of different cytokines which stimulate antibody responses or lead to macrophage activation depending on the subclass of Th cells. Later, it was discovered that CD4 molecules on T cells can associate with class II major histocompatibility complex (MHC-II) molecules on antigen-presenting cells (APC).

A possible function of this CD4-MHC-II molecule interaction could be to increase the avidity of association between the APC and the T cell. This hypothesis is called 'the accessory molecule' hypothesis (37, 41). The TCR-MHC interaction itself has a typical half-life that would be too short for effective T cell activation (42), which could be increased by CD4-MHC interactions.

Another possible function for CD4 could be amplification of the TCR/CD3 complex-induced signaling. During the antigen presentation process by MHC-II molecules, a single antigen-containing MHC-II molecule interacts with both the TCR and CD4 (at the $\alpha 2$-$\beta 2$ site, reviewed in 43), leading to co-clustering of TCR and CD4 (44, 45). CD4 enhances the T cell activation in this hypothesis by serving as a co-receptor for the TCR/CD3 complex (46, 47) since co-localization of the CD4 and TCR to the same ligand (MHC-II) would generate a more potent activating signal then signaling via the TCR alone (37, 41). The association between CD4 and the CD3/TCR complex does not occur in the resting state, but only after MHC-TCR interaction, leading to CD3/TCR complex activation (48). This 'signal-amplification by CD4' hypothesis is sustained by more recent data in which it has been demonstrated that CD4-TCR transduction cascade does not differ from the transduction cascade induced by TCR-TCR cross-linking, which implicates that there is a pure synergistically amplifying function for CD4 on TCR-mediated signaling (49).

CD4 could directly enhance CD3/TCR signaling via recruitment of protein tyrosine kinases (PTK) including p56lck (50, 51). Upon activation, these PTKs become autophosphorylated and subsequently several other substrates are phosphorylated, including the cytoplasmic tails of CD3 ε- and ζ-chains (39, 52). These chains then serve as docking sites for ZAP70, a 70 kDa tyrosine phosphoprotein (53), which subsequently becomes phosphorylated and thus activated (54, Figure 2). Tyrosine phosphorylation of so called immunoreceptor tyrosine activation motifs (ITAMs) at the CD3 ζ-chains is

Figure 2



Schematic representation of CD4 interaction with MHC and CD3-TCR complex. Antigen-induced T cell activation requires CD4-binding to MHC-II as well as TCR/CD3-recognition of the antigen in the context of MHC-class II. (adapted from Gaubin *et al*., 31).

crucial for this ZAP-70/CD3 interaction and it has been suggested that different of these ITAMs play a role in peripheral T cell activation, either in a quantitative or in a qualitative manner (55-57). Tyrosine-phosphorylation therefore is an early (58, 59) and obligatory (60, 61) event in signaling cascade of the TCR.

Upon phosphorylation and subsequent activation of ZAP70 several different signaling pathways become activated, most notably the activation of phospholipase Cγ1 (PLCγ1, reviewed in 62). Phosphorylation and activation of PLCγ1 leads to enhanced hydrolysis of phosphatidylinositol 4,4-biphosphate (PIP2), giving rise to the second messengers diacylglycerol (DAG) and inostol 1,4,5-triphosphate (IP3). These second messengers than activate protein kinase C (PKC) and trigger the release of Ca^{2+} from intracellular sources (reviewed in 62). This rise in intracellular Ca^{2+} concentrations can be further enhanced by Ca^{2+} influx via cell surface voltage-independent Ca^{2+} channels which become activated upon TCR activation (63). Since CD3/TCR activation finally results in increases in intracellular calcium, calcium ionophores (e.g. ionomycin) or PKC activators (e.g. phorbol esters) can mimic TCR activation.

TCR/CD3-coupled PTKs are also involved in the activation of pathways regulated by p21ras (a GTP-binding protein) and PI-3 kinase. A series of serine/threonine kinase cascades is controlled by p21ras. The direct downstream targets of PI-3 kinase include phospho-inositides which can be phosphorylated on the D-3 position of the inositol ring. One of these, phosphatidylinositol tri-phosphate (PIP-3) has been demonstrated to bind SH2 domains of many other signaling molecules (64).

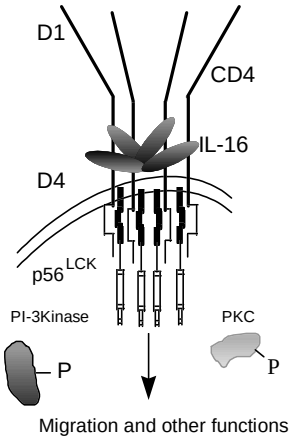
Upon activation of these three different pathways, TCR signaling finally results in proliferation and expression of different cytokine-encoding genes, e.g. IL2, via regulation of different transcription factors (e.g. NF-AT, AP-1 and NF-κB, reviewed in 65). While CD4 could thus contribute to T cell activation, immunosuppressive effects exerted by CD4 have also been observed. When CD4 is bound independently of the TCR/CD3 complex, CD3/TCR activation results in suppressed T cell proliferation and cytokine production (66, 67). Recently, IL16, a natural occurring soluble ligand for CD4 (29, 68) has been described to have immunosuppressive properties when administered before TCR activation (69, 70). This is in agreement with the effects of other ligands for CD4 which include monoclonal antibodies to CD4 and HIV-gp 120. Furthermore, the CD4 molecule has been implicated to play a role in T cell differentiation.

The role of CD4 in T cell differentiation

CD4 signaling is not only important for TCR-dependent T cell activation but also regulates differentiation of lymphocytes into either Th1 or Th2 lymphocytes (71). In a system using peptides with different affinities for the TCR the cytokine profile of restimulated T cells is dependent on the strength of the initial stimulation (72). Peptides that are based on the normal ligand for the TCR turn naive TCR-transgenic cells into Th1-type cytokine producing cells upon restimulation, whereas initial stimulation with an altered peptide ligand (with low affinity for the TCR) differentiates naive T cells to a Th2-type profile upon restimulation with the high affinity ligand (72). The role of CD4 in this process was determined using TCR-transgenic mice that were crossed with either CD4 mutant mice or mice that expressed a truncated form of CD4. Absence of CD4 did not influence development of Th1 cytokine profiles, however, Th2 development was impaired (71). These results indicate that CD4

signaling is necessary for the generation of Th2-type-inducing signals. In these experiments exogenously added IL4 could restore impaired Th2 development caused by CD4 absence. However, CD4 deficient cells cultured in presence of IL4 still produce less IL4 than wild type T cells that were differentiated towards a Th2-type phenotype (19). Therefore absence of CD4 could influence Th2 development in two different ways: either CD4 expression influences TCR-induced signaling or CD4 can modulate IL4-receptor induced signaling (71). In contrast with these observations, others observed that stimulation of naïve T cells with agonist peptides via MHC-II molecules that are unable to interact with CD4, a TCR signaling pattern is induced that is comparable to low affinity peptide signaling. This suggests that absence of CD4 signaling induces Th2-development (73). The discrepancy between these studies could be accounted for by different affinities of the agonist peptides for the TCR.

Figure 3



IL16/CD4 interaction and subsequent signal transduction in T cells. After CD4 ligation by IL16, p56^{LCK} becomes associated with CD4. For IL16-induced migratory signals PKC or PI-3 kinase activation is required (adapted from Center *et al.*, 29).

IL16 as a CD4-ligand

Thus far IL16 is the only known naturally occurring soluble ligand for CD4 (29). Competition studies with F(ab)-fragments of antibodies to CD4 (OKT4) indicated that IL16 is probably binding CD4 in the proximity of the epitope recognized by this antibody (D4-region, Figure 3, 29).

IL16 was first discovered as a lymphocyte chemoattractant factor in 1982 (74, 75). Recently murine, simian and feline IL16 have been cloned and remarkable similarities with human IL16 in both amino acid sequence and biological activity was demonstrated (76-78). IL16 was reported to be produced by $CD8^+$ T cells upon stimulation with 5-HT or histamine (Table I). Later it was observed that $CD4^+$ T cells could also release IL16 after antigen-specific as well as mitogenic stimulation (Table I, 74, 75, 79, 80). However, in contrast to $CD8^+$ T cells, $CD4^+$ T cells do not contain preformed IL16. Furthermore, eosinophils, mast cells and epithelial cells are sources of IL16 (Table I, 26, 81, 82). IL16 mRNA expression as well as immunoreactivity was significantly higher in epithelial cells obtained from biopsies from asthmatic subjects than in biopsies taken from either healthy controls or non-atopics (26). Cultured human eosinophils were demonstrated to constitutively express IL16 mRNA and to contain preformed IL16 which is released into supernatant of unstimulated cultures (81). Mast cells also contain preformed IL16 which is released into supernatant after stimulation with PMA or C5a (82). Furthermore, *de novo* synthesis was increased largely after stimulation of mast cells as was detected by an increase in IL16 mRNA (82). In contrast to other known interleukins, constitutive IL16 mRNA expression is almost exclusively limited to lymphatic tissues, underlining the potential of IL16 as an immunomodulatory molecule (83).

IL16 is encoded by a gene located on human chromosome 15 (15q23.6, 84) and is unrelated to previously described cytokines or chemokines (27, 76). The molecular mass of IL16 (17 kDa) and the occurrence of an open reading frame (ORF) of 390 base pairs suggests that IL16 is produced as a mature 130 amino acid protein with neither a signal peptide or further processing of the protein (27). However, recent observations suggest that the 130 amino acid protein is part of a much longer ORF, thereby indicating that the 17 kDA protein is derived from corresponding precursor molecules (83, 85). This precursor is most likely synthesized as a 67 kDa protein consisting of 631 amino acids. Only cleaved IL16 is biologically active and this cleavage product consists of 121 C-terminal residues of

pro-IL16. Cleavage of pro-IL16 into biologically active IL16 is mediated only by caspase-3 (86). The naturally secreted C-terminal portion of IL16 is proposed to be a cationic homotetramer, suggesting that CD4 cross-linking is necessary for IL16 to be biologically active (29, Figure 3). However, the necessity for tertramerization of IL16 to be biologically active is challenged by a more recent publication in which monomeric forms of IL16 were also able to induce chemotaxis of $CD4^+$ T cells (87).

Besides chemotaxis induction of $CD4^+$ cells, including T cells, eosinophils and monocytes, IL16 has been reported to induce upregulation of IL2 receptor expression, upregulation of HLA-DR and enhanced survival of $CD4^+$ T cells in a synergistic manner with IL2 (Table I, 29, 88-90). Furthermore, IL16 induces a shift from G_0 to G_{1a} in human $CD4^+$ T cells (reviewed in 29). In contrast to these stimulatory effects, IL16 has been found to inhibit proliferation of $CD4^+$ lymphocytes following activation with antigen or antibodies to CD3 (69, 70, 91). Furthermore, IL16 has been reported to inhibit mixed lymphocyte reactions of human peripheral blood mononuclear cells (69). Inhibition of these T cell responses is not likely to be caused by induction of apoptosis since binding of IL16 to CD4 does not induce apoptosis, but is of a protective nature (92). Furthermore, IL16 does not decrease CD4 expression, even after long term incubation (70) as has been reported using other CD4 ligands (93).

The generation of second messengers by IL16 requires the presence of an intact cytoplasmatic tail of CD4 since truncated CD4 molecules were not able to induce any of the described changes in $CD4^+$ T cell functions (100). Binding, and subsequent cross-linking of CD4 molecules by IL16 rapidly induces association of CD4 with p56lck, which is necessary for induction of chemotaxis (101). In agreement herewith, hybridoma cells that express CD4 molecules with point mutations which uncouple the CD4-p56lck association, lack both chemotaxis and p56lck activation induced by IL16. However, it seems to be that only the physical interaction between CD4 and p56lck is sufficient to induce these responses since inhibition of p56lck activation by herbamycin A does not inhibit IL16 induced chemotaxis (101). Furthermore, T cell hybridomas, expressing a chimeric receptor combining the extracellular domain of human CD4 with murine p56lck, which lacks the kinase domain, displayed normal IL16 induced chemotaxis. These findings are consistent with other reports in which a kinase independent role for p56lck has been identified in T cell activation (102, 103). While the kinase activity is not necessary for the induction of chemotaxis, p56lck binding to other signaling molecules (including PLCγ1, Raf-1 and PI-3 kinase) may explain the requirement for p56lck-CD4 association in the motile responses (101).

Besides p56lck, PI-3 kinase is involved in the induction of chemotaxis by IL16 since wortmannin, a selective PI-3 kinase inhibitor has been demonstrated to inhibit this response (29). Furthermore, activation of PKC is associated with CD4-IL16 interaction (100), since CD4 ligation by IL16 increases $[Ca^{2+}]_i$ and the amount of IP-3. This suggestion was corroborated by the finding that IL16 induces a rapid translocation of PKC activity from the cytosol to the membrane in three different $CD4^+$ cell types and that inhibitors of both the regulatory and the catalytic domain of PKC inhibited IL16 induced chemotaxis (104). Finally, activation of PKC alone is sufficient to induce chemotactic responses.

Besides activation of these second messengers, it has also been demonstrated that IL16 activates the stress-activated protein kinase (SAPK) pathway in $CD4^+$ macrophages (105). After stimulation of macrophages with IL16, SEK-1 becomes activated, resulting in activation of the SAPKs p46 and p54. Furthermore, c-jun and p38 MAPK (mitogen-activated protein kinase) become phosphorylated. However, in contrast to expectations, activation of these SAPKs and MAPK did not lead to detectable apoptosis (105). The pathophysiological significance of IL16 is supported by observations that presence of biologically active IL16 is largely upregulated in asthmatics after antigen-specific provocation as well as in several different animal models of diseases including allergic asthma, rheumatoid arthritis and colitis (26, 106-108).

HIV-1 gp120 as a CD4-ligand

The CD4 molecule can also serve as the primary receptor for human immunodeficiency virus type-1 (HIV-1, 32, 109, 110), the cause of the acquired immunodeficiency syndrome (AIDS). High affinity

binding of the viral envelope protein gp120 to CD4 mediates attachment and entry of viral particles (111, 112). Extensive random and site-directed mutagenesis studies revealed that the primary binding site of gp120 is located within the first amino-terminal domain of CD4 (D1).
In agreement with IL16, native and recombinant gp120 have been shown to induce PKC-dependent migration of $CD4^+$ lymphocytes (104, 113). Upregulation of IL2 receptor expression as well as enhanced turnover of phosphatidylinositol into IP-3 and increases in intracellular Ca^{2+} are also observed after gp120-CD4 interaction (100).
CD4-gp120 interaction has been demonstrated to induce activation of p56lck (70, 114), as was measured by enhanced autophosphorylation and the phosphorylation of the exogenous substrate enolase (115). P56lck activation by gp120 required association with CD4 since p56lck activity was not detectable in cells expressing truncated or mutated forms of CD4 (100). However, others failed to observe detectable changes in p56lck activity or phosphorylation of tyrosine residues after incubation of $CD4^+$ T cells with gp120 (116, 117).
Soluble gp120 is shed from HIV infected $CD4^+$ T cells *in vitro* (118). Furthermore, soluble gp120 can be detected in high concentrations in serum derived from patients suffering from AIDS (119). Finally, circulating $CD4^+$ T cells from patients with advanced disease appear to be coated with gp120/anti-gp120 immune complexes (120). Together, these studies suggest that soluble gp120, released into the extracellular compartments *in vivo* could bind to CD4 of uninfected cells and thus interfere with normal $CD4^+$ T cell functions as will be described in the next paragraphs. Besides interference with the normal function of the CD4 molecule, long term independent CD4 ligation by gp120 (or other CD4 binding molecules such as antibodies) can induce downregulation of CD4 expression on the cell surface (93, 121). Moreover, CD4 triggering by these ligands might prime T cells for apoptosis in response to antigen (122, 123) and gp120 could thus interfere with normal $CD4^+$ T cell functioning at multiple levels.

Immunosuppression by different CD4 ligands or anti-CD4 antibodies

I Immunosuppression by IL16
$CD4^+$ T cells, incubated with IL16 before stimulation with anti-CD3 display a decreased proliferation and IL2R expression. Furthermore, exogenous administration of IL2 can not restore this decreased proliferative response (70). The observation that IL16 induces a similar inhibition of proliferation after antigen-specific stimulation but not after PHA stimulation suggests that the observed effects are specific for TCR signaling, since PHA probably induces alternative T cell-activating pathways through CD2 (124). These results also indicate that the inhibitory effects of IL16 are not of a general toxic nature.
Besides inhibition of proliferation, pretreatment with IL16 also altered the Ca^{2+} response by delaying the initial rise following anti-CD3 mAb stimulation and reducing the maximal $[Ca^{2+}]_i$ response by approximately 50% (70). This observation suggests that the mechanisms by which IL16 affects anti-CD3-induced activation are at the level of second messenger generation.

II Immunosuppression by gp120
As already stated, gp120 is a ligand for CD4. Similar to effects observed with IL16, gp120 inhibits the proliferative responses of human $CD4^+$ T cell clones. Furthermore, gp120 is unable to inhibit PHA-induced proliferative responses (125). In contrast with IL16-induced effects, native gp120 or anti-CD4 mAb (Leu3a)-induced inhibition of proliferation is restored by exogenous IL2 (126, 127), whereas IL16 induced decreases in proliferation could not be reversed by IL2 addition (70).
It has also been reported that a secondary antibody to gp120 to cross-link CD4 molecules is needed to inhibit proliferation of $CD4^+$ T cells (45, 127). This could indicate that the human immune system actually contributes to the inhibitory role of gp120 on $CD4^+$ T cell functioning by production of anti-gp120 antibodies (45).

III Immunosuppression by antibodies to CD4

When CD3 and CD4 are independently and simultaneously cross-linked, calcium mobilization is inhibited when compared to that induced by cross-linking CD3 alone (128). Similarly, when CD4 is cross-linked by antibodies (GK1.5 or OKT4D) prior to TCR or CD3 cross-linking, the Ca^{2+} influx is inhibited, suggesting that negative signals generated by CD4 cross-linking are dominant over those generated by TCR/CD3 ligation (45, 129). Finally, when CD3 and CD4 are cross-linked together e.g. via antigen-presentation by MHC-II, Ca^{2+} responses are much higher than upon stimulation via solely CD3, suggesting that CD4 signaling plays an active role in signal transduction of CD3/TCR/MHC/CD4 complexes (114, 129-131).

In agreement with impaired CD3/TCR signaling after blocking CD4, Woods *et al.* demonstrated that T cells, stimulated with anti-CD3 antibodies do not proliferate when pre-treated with either the complete antibody or with F(ab')2 fragments of humanized anti-CD4 antibodies (132). They furthermore found that production of several cytokines, including IL4 and IL10, as well as expression of several activation markers, including CD25 and CD69, were diminished by this treatment (132).

Use of antibodies to CD4 *in vivo*

Several studies in animal models using anti-CD4 antibodies have demonstrated that targeting to the CD4 molecule is an effective way to suppress graft rejection as well as to prevent autoimmune diseases. However, in all these studies depleting antibodies were used and therefore the way they act is self-explanatory; they eliminate $CD4^+$ T lymphocyte subsets. Even though these antibodies are efficacious in these conditions, they do not provide a satisfactory approach since by depletion of $CD4^+$ T cells, patients are prone to infections. Furthermore, disease is likely to reoccur whenever the $CD4^+$ population is restored after ending the treatment. A more satisfactory therapeutic approach would be

Table I: Summary of IL16-producing cells and IL16-induced effects

IL16-Production	**Stimulation**	**References**
$CD8^+$ T cells	Histamine, 5-HT	(79, 80)
$CD4^+$ T cells	antigen, mitogen, anti-CD3	(74, reviewed in 29)
Mast cells	PMA, C5a	(82)
Eosinophils	Unstimulated	(81)
Epithelial cells	Histamine	(26, 94)

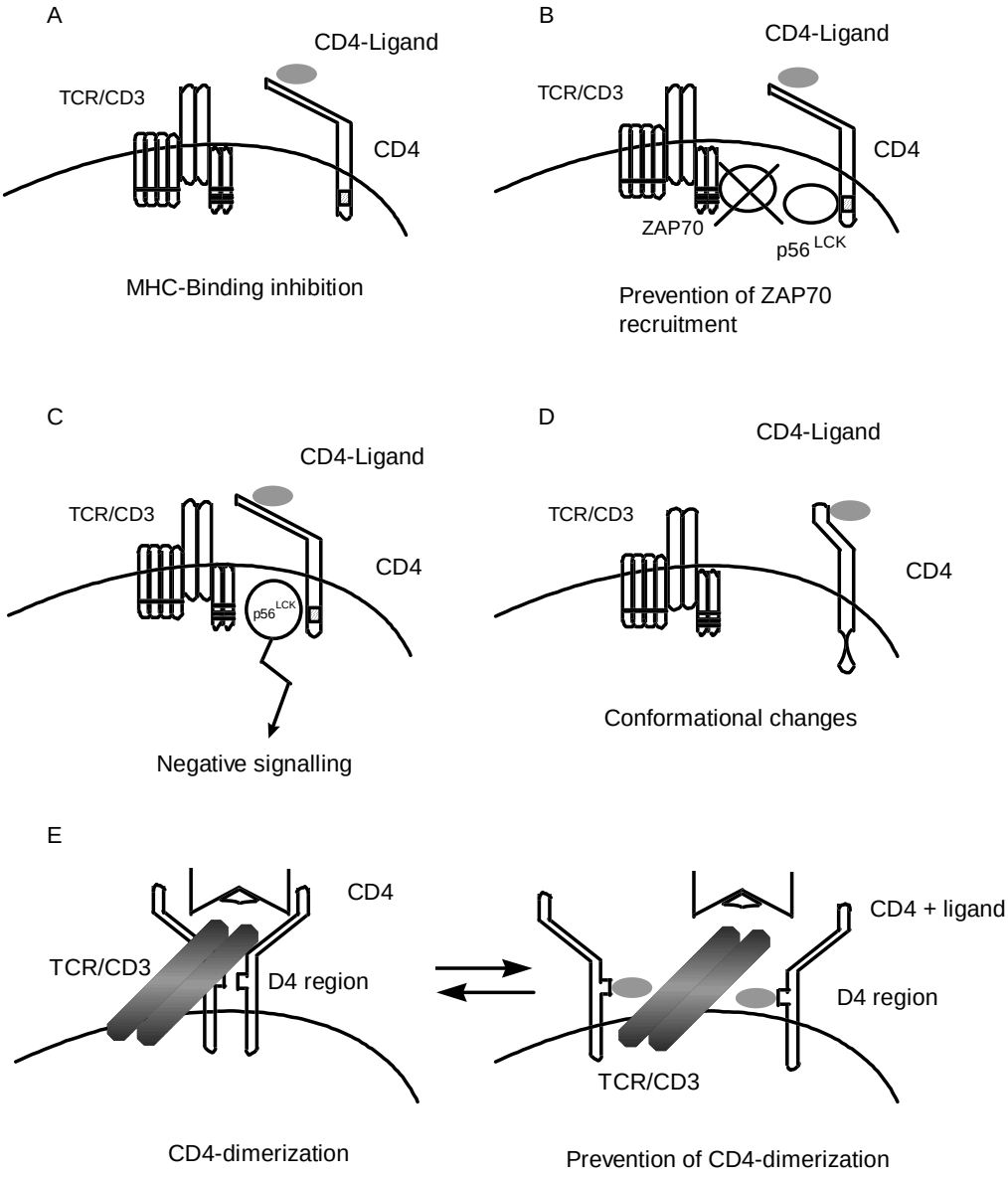
Effects on $CD4^+$ T lymphocytes	**References**
Chemotaxis of $CD4^+$ cells *in vitro,* including eosinophils, T cells and monocytes	(29, 90, 95)
HLA-DR induction	(96)
IL2Rα expression	(27, 96)
G_0-G_1 cell cycle change	(29)
Inhibition of antigen-induced or αCD3-induced T cell activation	(70)
Inhibition of mixed lymphocyte reaction	(69)
Prevention of apoptosis and CD95 (Fas-Receptor) expression	(70, 92)
Synergistic activation of $CD4^+$ T cells with IL2	(88)
Inhibition of IL2 production	(97)
Inhibition of HIV transcription/replication	(92, 98, 99)

modification of the profile of the immune response of pathogenic T cells, thus creating a condition of active control or suppression.
Over the past decade use of non-depleting antibodies in several animal models provided new insights in the use of antibodies to CD4 as a therapy in several different disorders (133-135). Cristopher *et al.* First demonstrated that the use of IgG2a class anti-CD4 antibodies (KT6) did inhibit *in vitro* mixed lymphocyte responses without *in vivo* depletion of $CD4^+$ T lymphocytes (136). *In vivo* use of these antibodies in mice resulted in long-term survival of cardiac grafts that were mismatched for both major and multiple minor histocompatibility antigens (136). Furthermore, use of KT6 inhibits development of collagen-induced arthritis (CIA) as well as adoptive transfer of CIA into severe combined immunodeficicient (SCID) mice (137). Besides inhibition of disease phenotype, antigen-induced cytokine production of lymphocytes derived from KT6-treated mice upon restimulation *in vitro* appeared to be shifted from Th1 type cytokines towards a Th2-type profile (137). However, it could also be postulated that in this study anti-CD4 antibodies did not alter Th1 responses into Th2-type profiles but merely inhibited Th1 responses, thereby allowing the emergence of Th2-type T cells that are specific for the same antigen (137). Whether non-depleting antibodies can really alter T cell responses *in vivo* remains to be established but *in vitro* data suggest that indeed CD4 signaling can determine the T cell cytokine profile (71). Based on such data it could at least be speculated that the use of non-depleting antibodies to CD4 is very beneficial to patients suffering from diseases that are controlled by $CD4^+$ lymphocytes.

Possible CD4-mediated mechanisms of immunosuppression

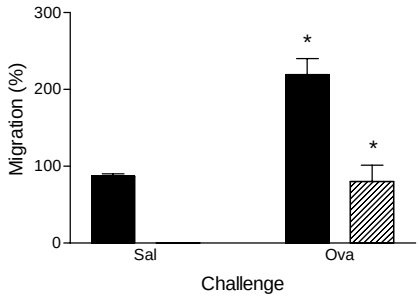
The effects of several different ligands for CD4 molecules described so far confirm that CD4 can function as a signal-transducing receptor which can decrease $CD4^+$ T cell functions when activated independently of the CD3/TCR complex and can augment T cell responses when activated together with this complex. Therefore, timing of the CD4-ligation might dictate whether stimulatory or inhibitory effects on TCR/CD3-mediated signaling are exerted. Based on the presented data, several different mechanisms can be postulated to explain the qualitative immunosuppression by CD4 ligands (Figure 4).
Firstly, CD4 occupation by gp120, anti-CD4 mAbs or IL16 could result in a physical blockade of association of CD4 molecules on T cells and MHC-II molecules on APCs, thereby preventing the formation of a stable TCR/CD4/MHC complex that has a half-life that is long enough for adequate signaling. Such a possibility is plausible since the affinity of MHC-II and CD4 binding is much lower than e.g. gp120/CD4 interaction (32, 111). Evidence has been provided that gp120 impairs binding of CD4 with MHC-II (138). Furthermore, mutational studies by Fleury *et al.* demonstrated that the D4 region of the CD4 molecule is important for CD4-MHC class II binding (reviewed in 43) and IL16 binds CD4 in the D4 region (reviewed in 29). However, this does not explain the ability of CD4 ligands to inhibit T cell responses which are elicited in the absence of MHC-bearing APCs by e.g. antibodies to CD3 (Figure 4A).
Secondly, CD4 ligation could result in a diversion of the CD4-p56lck complex, away from the CD3/TCR complex and their associated second messengers, thereby preventing the formation of an active CD3/TCR signaling complex (48). In respect to several different studies in which a positive participation of p56lck is necessary for optimal TCR signaling (50, 51, 139, 140), it appears very likely that CD4 ligation prior to CD3/TCR activation leads to impaired signaling via sequestration of p56lck. Uncoupling p56lck from the TCR-signaling complex finally leads to prevention of recruitment and activation of ZAP70 (141). ZAP70 is essential for TCR signaling since $CD4^+$ T cells are unable to respond to TCR-mediated stimuli when certain mutations in ZAP70 are present. Such mutations have recently been demonstrated to be the cause of severe combined immunodeficiency syndromes in humans (142, Figure 4B).

Figure 4



Schematic representation of possible working unresponsiveness after CD4-Ligand interaction. See text for explanation.

Figure 5



Migration of lymphocytes induced by BAL fluid samples (black bars) derived from saline (Sal) or ovalbumin (Ova)-challenged animals. The IL16-dependent part (hatched bars) of the induced migration is also depicted. Migration is presented as a percentage of migration induced by medium alone (100%). Results are expressed as arithmetic average ± SEM (n is at least 5 per group). (P<0.05) significantly different from migration induced by BAL fluid derived from saline-challenged animals.*

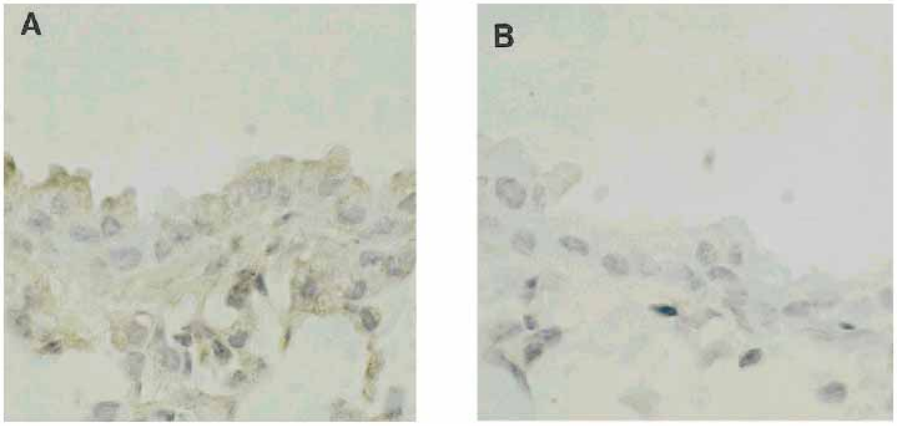
A third explanation could be the transduction of a negative signal initiated by the engagement of CD4, which could interfere with CD3/TCR signaling, as was originally observed with anti-CD4 antibodies (66). A role for p56lck has been suggested in this process. If p56lck is activated in the absence of CD3/TCR activation this kinase might have access to ligands that are normally not available when CD4-associated p56lck is part of the CD3/TCR signaling complex. In this hypothesis, CD4-induced signaling, when turned on in the absence of CD3/TCR activation, might induce a qualitatively different signal which could interfere with subsequent CD3/TCR-induced signals. Alternatively, too early p56lck activation could result in inappropriately timed tyrosine phosphorylation of cytoplasmic proteins and thus inhibit signal transmission or exhaust T cell activation capabilities (Figure 4C).
Binding of CD4-ligands to CD4 could also induce conformational changes of this CD4 molecule (143), thereby preventing e.g. coupling to MHC-II molecule or intracellular signaling (Figure 4D).
Finally, recent evidence revealed that CD4-dimerization (Figure 4E) is necessary for optimal CD4 functioning (43). Furthermore, it has been established that the D4 region is necessary for CD4 oligomerization, and that synthetic peptide analogs derived from the D4 region inhibited CD4-dependent T-cell activation (144). Since it is known that IL16 binds CD4 in the D4 region (29), it could be speculated that IL16/CD4 interaction inhibits CD4 dimerization (Figure 4E).
So far, use of non-depleting antibodies to CD4 have proved to be extremely effective in down-modulating CD4-mediated diseases in animal models and humans (reviewed in 133, 145-147). Since IL16 expression is largely upregulated in $CD4^+$ T cell-mediated diseases, including allergic asthma (26) and since IL16 uses the CD4 molecule as its receptor it could very well be speculated that IL16 serves as some sort of homeostatic protein *in vivo*.

Aim of this thesis

The main objective of our studies was to unravel the role of IL16 in the pathogenesis of allergic asthma. For this purpose we used a murine model for allergic asthma, recently developed by Hessel *et al.* (148). After antigen challenge of ovalbumin-immunized male BALB/c mice, increased airway responsiveness to either methacholine or 5-hydroxytryptamine (5-HT) can be measured, when compared with saline-challenged animals (149). Furthermore, increased numbers of eosinophils can de detected in bronchoalveolar lavage fluid as well as a large increase in the amount of ovalbumin-specific IgE in serum (148, 150). Finally, thoracic lymph node cells (TLN), isolated from antigen-challenged animals, produce Th2-type cytokines such as IL4 and IL5 upon antigenic restimulation *in vitro* (151), whereas TLN cells isolated from saline-challenged animals hardly produce any cytokines upon restimulation with antigen *in vitro*.
In human asthmatics IL16 presence in BAL fluid and expression in bronchial mucosa is increased after antigen-specific stimulation (26). A similar upregulation is observed in the murine model we used in this thesis. Increased levels of biological active IL16 can be detected in bronchoalveolar lavage fluid obtained from antigen-sensitized and challenged mice (Figure 5). Using immunohistochemistry, it was demonstrated that mainly epithelial cells display immunostaining for IL16 after ovalbumin-challenge

(Figure 6). Finally, lung-draining lymph node cells also contain significantly more IL16 protein after antigen-challenge when compared to saline-challenged animals (146 vs 73 pg ml^{-1}). To unravel the functions of IL16 in our murine model we used different approaches.
First, we further characterized the used model using Whole body plethysmograph. In chapter 2 we discuss the use of this recently developed technique to determine airway hyperresponsiveness and the occurrence of early and late asthmatic responses after antigen challenge. Furthermore, development of airway hyperresponsiveness in time was compared with infiltration of eosinophils into the lungs.
Secondly, we determined the role of histamine and 5-hydroxytryptamine (5-HT) on IL16 production in our model since these autacoids are known to be released by murine mast cells upon antigen-specific stimulation (152, 153) and since both histamine and 5-HT can induce production and release of IL16 by lymphocytes and epithelial cells (reviewed in 29, 90). Therefore, we used specific 5-HT or histamine receptor antagonists and determined the effect on antigen-induced IL16 production and subsequent biological effects. IL16 amounts and activity in BAL fluid were determined using a chemotaxis assay. Furthermore, airway hyper-responsiveness and presence of eosinophils in BAL fluid were measured as well as antigen-specific IgE antibody levels in serum (chapter 3).
Thirdly, to explore the function of endogenously produced IL16, animals were treated intranasally with antibodies to IL16. Furthermore, animals were treated intranasally with peptides of which the amino acid sequence was based on different parts of the predicted sequence of rhIL16. First, the effects of these peptides were determined on *in vitro* IL16-induced chemotaxis (chapter 4). Thereafter, both antibodies to IL16 and the different peptides were intranasally applied to ovalbumin-sensitized and saline or ovalbumin-challenged animals. In these experiments development of antigen-induced airway hyperresponsiveness and infiltration of eosinophils into BAL fluid were determined.
Fourthly, we determined the potentially beneficial effects of systemical or local treatment with rmIL16 or rhIL16 *in vivo* (chapter 5) since rhIL16 was shown to inhibit antigen-specific or polyclonal activation of human lymphocytes *in vitro* (69, 70). In the experiments in which effects of systemic administration of IL16 were examined, airway hyperresponsiveness, eosinophilia and serum antigen-specific IgE levels were determined. Furthermore, Th2-type cytokine production by TLN cells derived from *in vivo*

Figure 6



Localization of IL16 immunoreactivity in airway sections of mice sensitized with OVA and exposed to repeated OVA inhalations demonstrating staining in the epithelium (A). Much less staining was observed in sections from ovalbumin-sensitized saline-challenged animals (B, see also 154*).*

IL16-treated animals upon antigen-specific restimulation *in vitro* was measured (chapter 5).
Effects of CD4 cross-linking by either rmIL16 or antibodies to CD4 on antigen-induced proliferation and cytokine production of murine lymphocytes were also examined (chapter 5 and 6).
Finally, *in vitro* effects of IL16 or antibodies to CD4 on differentiation of Th0 into either Th1 or Th2-type T cells were determined using lymphocytes derived from DO11.10 transgenic mice. In this system, presence of IL4 or antibodies to IL4 determines whether naive Th cells differentiate towards a Th2 or a Th1-like cytokine producing pattern (71). During the initial stimulation period, antibodies to CD4 or IL16 were added to the different cultures and effects of either antibodies to CD4 or IL16 were determined on cytokine production after the initial stimulation with antigen or upon antigen-specific restimulation (chapter 6).
In the last chapter, the results are discussed and compared to already published data on functions and effects of IL16 (chapter 7).

Absence of Late Airway Response Despite Increased Airway Responsiveness and Eosinophilia in a Murine Model of Asthma

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Summary

In asthmatics an immediate asthmatic response occurs after antigen provocation. Furthermore, asthmatic patients display airway hyperresponsiveness, accompanied by airway eosinophilia. In some patients late asthmatic responses can be detected. Many controversies still exist about the relations between different airway responses and inflammatory cell infiltration, and we therefore used a murine model to investigate correlations between these phenomena. In this study we show presence of antigen-induced early bronchoconstrictive responses, accompanied by increased serum MMCP-1 levels. However, we were unable to demonstrate late bronchoconstrictive responses either at the time when eosinophils start to infiltrate the lungs or when both airway hyperresponsiveness and eosinophilia are established. With sequential exposures to antigen a correlation exists between development of airway hyperresponsiveness and eosinophilia. In contrast, resolution of this hyperreactivity appears to be dissociated from eosinophilia after stopping the antigen-challenges. Based on these data we conclude that mast cell degranulation is the probable cause of early bronchoconstrictive responses. Furthermore, late bronchoconstrictive responses are not related to the infiltration of eosinophils or development of airway hyperresponsiveness in this murine model. Finally, we conclude that airway hyperresponsiveness and eosinophilia are only associated with each other during the induction phase and not after the final antigen-challenge.

Introduction

Asthma is characterized by a reversible airway obstruction upon encounter with the antigen and an increased airway reactivity to various bronchoconstrictive stimuli (6, 155, 156). Furthermore, a cellular inflammatory response, consisting mainly of eosinophils, can be demonstrated in asthmatic patients after antigen-provocation, which is thought to be caused by the production of Th2-type cytokines including IL4 and IL5 (4). In some patients these phenomena are accompanied by a late asthmatic response (LAR, 157).
The immediate airway response following provocation with antigen is probably caused by the release of bronchoconstrictive mediators including histamine by activated mast cells (158). Besides release of these mediators, mast cells may also contribute to the infiltration of eosinophils and the induction of airway hyperresponsiveness, e.g. via production and release of cytokines such as IL4 (159).
The development of LAR in human asthmatics occurs as a spectrum ranging from no LAR to severe late asthmatic bronchoconstricitive responses following antigen challenge, which seems to be at least partially dependent on the dose of antigen (reviewed in 157). Furthermore, it is thought that there is a direct correlation between infiltration of eosinophils and the occurrence of LAR in asthmatic patients (157, 160). However, others observed that eosinophilia or the presence of mediators released by eosinophils could not predict the emergence of LAR (161). Eosinophilic infiltration and subsequent activation of these inflammatory cells has also been associated with the development of airway hyperresponsiveness, possibly via destruction of epithelial layers in the lungs (162). Finally, it has been postulated that occurrence and severity of LAR contributes to the development of airway hyperresponsiveness (157).
In the past decade, several different murine models have been developed that display some of the features of asthmatic patients and the use of these models has gained insight in the pathogenesis underlying these phenomena (149, 163, 164). In the present study we used a well established murine model in which airway hyperresponsiveness and eosinophilia occur after antigen-challenge. Furthermore, presence of these phenomena is accompanied by an enhanced production of Th2-type cytokines by ovalbumin-specific T cells derived from lung draining lymph nodes and an increased level of antigen-specific IgE in serum (149, 165).
To address questions raised by the controversies in literature about the relations between changes in airway functions and inflammatory cell infiltration, we used whole body plethysmography to measure airway bronchoconstrictions. Whole body plethysmography has recently been adapted for measuring airway responses in mice (166) and using this technique it is possible to determine the kinetics of development of antigen-induced airway hyperresponsiveness in the same animals. This technique can also be used to measure direct effects of antigen provocation and to monitor airway functions for long periods of time and thus to determine whether late bronchoconstrictive responses occur in this murine model. We therefore used this technique to determine if a relationships exist between the occurrence of early and late bronchoconstrictive responses, development of airway hyperresponsiveness and the cellular inflammatory response in antigen-sensitized and challenged mice.

Materials and Methods

Sensitization and challenge

Specified pathogen free male BALB/c mice (age 6-8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (Utrecht, The Netherlands). All experiments were approved by the animal care committee of the Utrecht University. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen free saline on alternate days. Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in saline) or to 8 saline aerosols for 5 min, on consecutive days (1 aerosol day^{-1}). The aerosols were generated with a jet nebulizer (Pari IS-2, Pari-Werk GmbH, Starnberg, Germany) connected to a Plexiglas exposure chamber with a volume of 5 l in which a

maximum of 6 animals was placed. In some experiments the animals were not challenged by aerosol but by intranasal application of 50 µl of either vehicle (saline) or ovalbumin (10 mg ml^{-1}) to increase antigen-deposition. Before the intranasal application the mice were exposed to a short-lasting inhalation anesthetic (Halothane, ALBIC BV, Maassluis, the Netherlands).

Whole body plethysmography

Airway responsiveness was determined using whole body plethysmography (Buxco Corp., Sharon, CT, USA). Pressure differences between the chamber containing the animal and a reference chamber were calibrated using a steady flow. Differences in pressure, measured with a transducer (M45, Validyne Engineering Corp. Northridge, CA, USA), are caused mainly by changes in volume during a respiratory cycle (166).
As a measure of bronchoconstriction PENH (enhanced pause) is used, a dimensionless, empirically established value which is defined as: $\left[\frac{PEP}{PIP}\right]\times\left[\frac{T_e - T_r}{T_r}\right]$ (see 166). During bronchoconstriction due to either methacholine or antigen, several characteristic changes occur in the wave form of the flow signal (166). The most striking differences in signal form occur during the expiration phase of a breath cycle, including changes in time of expiration (T_e) and relaxation time (T_r), finally resulting in $\left[\frac{T_e - T_r}{T_r}\right]$ (166). An increase in bronchoconstriction is accompanied by changes in both peak expiratory and inspiratory pressure (PEP and PIP, respectively), which is reflected in the formula for PENH by $\left[\frac{PEP}{PIP}\right]$.

Before measuring airway responses to bronchoconstrictive stimuli with whole body plethysmography, basal PENH values were measured for 3 minutes (10 valid breaths were averaged per PENH value). Aerosol was generated using a jet-nebulizer (Pari IS-2). Ovalbumin-sensitized and saline- or ovalbumin-challenged animals were subjected to aerosols containing increasing concentrations of methacholine (ranging from 1.5 to 50 mg ml^{-1}) for 3 min and subsequently data were collected on breathing patterns during periods which lasted 3 min (protocol adapted from Hamelmann *et al.*, 166).

Development and resolution of airway hyperresponsiveness and kinetics of eosinophilia

The kinetics underlying development and resolution of airway hyperresponsiveness were monitored during and after the challenge period. Using the same animals, changes in airway responsiveness to nebulized methacholine were measured at 24 h after the second and fourth antigen or saline-challenge and at 1, 4 and 6 days after the last challenge. In a separate series of experiments the kinetics of eosinophil infiltration into the bronchoalveolar lavage fluid were determined. Lungs of ovalbumin-sensitized and saline- or ovalbumin-challenged animals were lavaged as previously described (149). Lavages were performed at the same days at which airway responses were measured.

Detection of antigen-induced early airway responses

Antigen-induced immediate airway responses in ovalbumin-sensitized mice were determined. Basal PENH value was measured and thereafter the mice were exposed to a saline or ovalbumin aerosol which lasted for 5 min, followed by a measuring period (20 min). To increase antigen deposition into the lungs, the mice were intranasally treated with 50 µl of either vehicle (saline) or ovalbumin (10 mg ml^{-1}) and subsequently PENH values were recorded for 20 minutes. The PENH values during this measuring period were averaged per 2 minutes. From the same animals serum was collected 30 min after intranasal challenge and mucosal mast cell protease-1 (MMCP-1) contents were measured using an MMCP-1 enzyme linked immunosorbent assay (Moredun Scientific Ltd. Midlothian, UK), according to the manufacturer's instructions.

Detection of antigen-induced late bronchoconstrictive responses

In order to measure late bronchoconstrictive responses, basal PENH value was recorded in ovalbumin-sensitized mice for 10 minutes. Thereafter, the mice were challenged with either nebulized saline or ovalbumin (2 mg ml^{-1}) for 5 minutes or by intranasal application of 50 µl of either saline or ovalbumin (10 mg ml^{-1}) and subsequently PENH values were collected overnight.
Animals were also i.p. injected with ovalbumin (4 mg kg^{-1}) as a booster at 5 days before overnight monitoring of PENH. This booster of ovalbumin was used to increase antigen-induced responses (167). Furthermore, these animals were treated with either vehicle or metyrapone (30 mg kg^{-1}, Sigma Chemical Company, St. Louis, MO, USA) at 3 days before the measurement, since inhibition of endogenous corticosterone production has been shown to increase the incidence of late bronchoconstrictive responses in dogs (168) and to increase antigen-specific immune responses in rats (169). Subsequently the animals were exposed to either saline or ovalbumin (2 mg ml^{-1}) aerosol on two separate days. Immediately after the second aerosol, airway functioning was monitored overnight for changes in PENH values.
Animals were also monitored for occurrence of late bronchoconstrictive responses after eight consecutive aerosol challenges with either saline or ovalbumin because then eosinophilia and airway hyperresponsiveness are maximal in our model as was demonstrated in the kinetics study. Presence or absence late bronchoconstrictive responses was determined by calculating the area under the individual tracings using the following formula: $f(x) = \sum_{n-1}^{i=1} 1/2\left[y_{i+1} + y_i\right] \times \left[x_{i+1} - x_i\right]$.

Statistical analysis

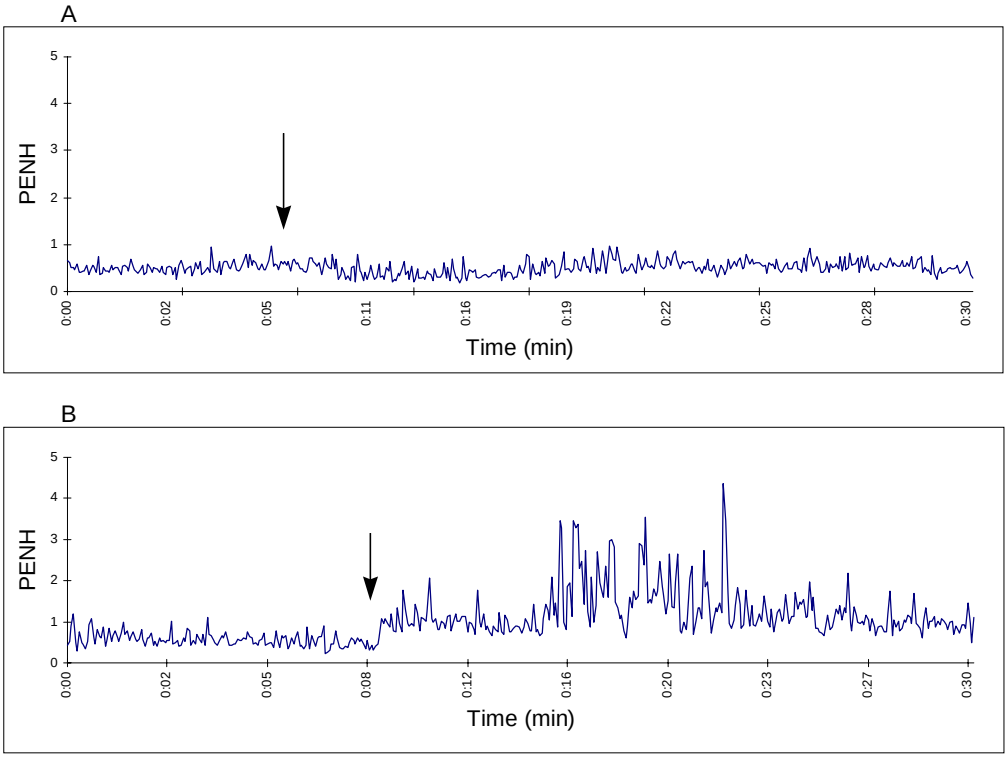
Comparisons between different groups of animals were made using an analysis of variance (ANOVA) followed by a Student's *t*-test (unpaired, two-tailed). Total BAL cell numbers and the number of various cell types were tested with an analysis of variance (ANOVA). For cell types with low numbers in control animals (i.e. neutrophils and eosinophils) a Poisson distribution was assumed. Data are expressed as arithmetic average ± SEM and a difference was considered to be significant when $P<0.05$). Statistical analyses were carried out using SPSS/PC^{+}, version 4.0.1 or Microsoft Excel version 7.0.

Results

Detection of antigen-induced early responses

Intranasal application of ovalbumin in sensitized mice induced an increase in PENH values, when compared to PENH-readings obtained after saline-instillation (Figure 1). After aerosol provocation with ovalbumin an increase in PENH values could also be observed when compared to saline challenge, however this increase did not reach statistical significance (data not shown). Maximal increases in PENH values (approximately 3-fold) were reached at 8 min after the intranasal instillation of ovalbumin and were resolved at 16 min after antigen challenge (Figure 2A). From the same animals serum was collected 30 min after intranasal challenge and MMCP-1 levels were measured. In ovalbumin-sensitized saline-exposed animals MMCP-1 levels in serum amounted to 5.6±0.7 ng ml^{-1} whereas a 5-fold increase ($P<0.05$) in MMCP-1 levels in ovalbumin-sensitized and exposed animals was detected (25.3±3.4 ng ml^{-1}, Figure 2B).

Figure 1

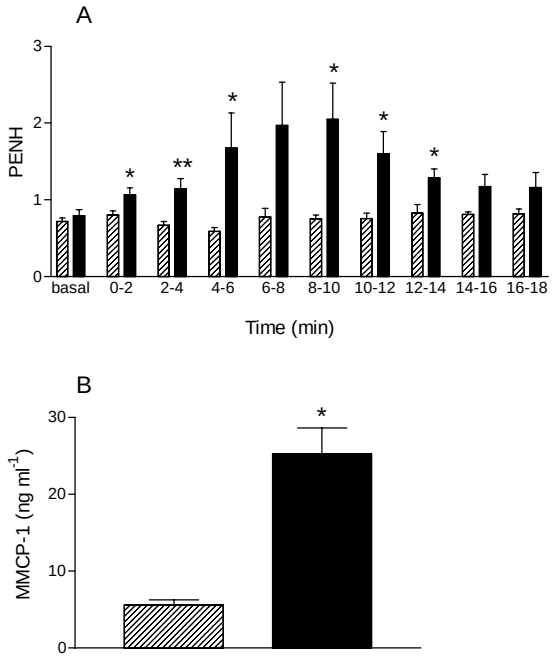


Representative examples of changes in PENH values measured after intranasal application (50 µl) of saline (panel A) or ovalbumin (10 mg ml^{-1}, panel B) in ovalbumin-sensitized mice. Basal recordings were made for at least 3 min and starting immediately after the challenge, PENH values were recorded for an additional 20 minutes. Arrows indicate the moment of challenge.

Kinetics of airway hyperresponsiveness and eosinophilia

One of the advantages of measuring airway functions with whole body plethysmography in conscious mice is the possibility of monitoring development of airway hyperresponsiveness during and after the challenge period. We therefore measured airway responses to increasing concentrations of methacholine in ovalbumin-sensitized and either saline or ovalbumin-challenged mice. At all different time points complete concentration response curves were made of which a representative example, measured at 1 day after the last challenge is shown in Figure 3A. Development of airway hyperresponsiveness was monitored at days 3 and 5 of the challenge period, as well as at 1, 3 and 6 days after the last challenge. Already at 24h after the second challenge (day 3), ovalbumin-challenged animals displayed a significant (P<0.05) increase in responsiveness at the highest concentration of methacholine (50 mg ml^{-1}), when compared to saline-challenged animals (4.7±0.3 vs 3.1±0.4, respectively, Figure 3B). This hyperresponsiveness to methacholine was further increased at 24h after the fourth antigen-challenge (day 5; 6.0±0.8 vs 3.2±0.3, P<0.05, Figure 3B) and was maximal at 24h after the last antigen-challenge (day 9; 7.0±0.6 vs 4.6±0.4, P<0.05, Figure 3A and B). Furthermore, it was observed that airway hyperresponsiveness to methacholine in ovalbumin-challenged animals was

Figure 2



*Increase in PENH values (A) and MMCP-1 serum levels (B) after intranasal instillation of saline (hatched bars) or ovalbumin (black bars) in ovalbumin-sensitized mice. Baseline PENH values were obtained by measuring for at least 3 minutes. The effect of either ovalbumin or vehicle was determined by averaging for 2 minutes during a 20 minute period following intranasal application. MMCP-1 levels were measured in serum collected at 3h after intranasal instillation of either ovalbumin (black bars) or saline (hatched bars). Results are expressed as arithmetic average ± SEM of 7-9 animals per group. * (P<0.05); ** (P<0.01) significantly different from ovalbumin-sensitized saline-exposed animals.*

still present at day 4 days after the last exposure to methacholine, whereas this phenomenon was resolved after 6 days (Figure 3B). No significant differences were observed in responsiveness to methacholine in the saline-challenged animals at all different time-points.

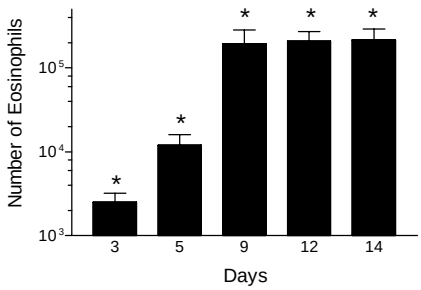
In a parallel series of experiments eosinophil infiltration into BAL fluid was determined. Lungs from ovalbumin-sensitized and saline challenged animals were lavaged at 24h after the last challenge and hardly any eosinophils were present in lungs from these animals ($0.2\pm0.2 \times 10^3$). At day 3 during the challenge period eosinophils started to be present in BAL fluid derived from ovalbumin-challenged animals (Figure 4). The number of eosinophils was further increased at day 5 (Figure 4) and was maximal at 24h after the last (eight) antigen challenge (day 9, Figure 4). At both 4 and 6 days after the last antigen-challenge eosinophils remained present in BAL fluid derived from ovalbumin-sensitized animals (Figure 4). No differences were observed between the numbers of either neutrophils or mononuclear cells present in lungs of either saline or ovalbumin-challenged animals.

Detection of antigen-induced late bronchoconstrictive responses

Several different protocols were used to determine whether late bronchoconstrictive responses could be measured after antigen challenge. No differences in airway functioning monitored for 15h after a single ovalbumin aerosol challenge or after intranasal instillation with ovalbumin were observed (n=6 per treatment group, data not shown) when compared to control animals.

In a different protocol to determine late bronchoconstrictive responses, animals were i.p. treated with a booster before the ovalbumin challenge to enhance immune responses (167). Furthermore, these animals were treated with either vehicle or metyrapone (30 mg kg^{-1}) at 3 days before the measurement to inhibit production of endogenous corticosterone (149). As was demonstrated in the kinetic study, eosinophils have already infiltrated the lungs at 24h after the second challenge and therefore it is very likely that during the measurement period eosinophils are either infiltrating into or already present in the lungs. However, no differences between saline and ovalbumin-challenged vehicle or metyrapone-treated animals could be observed in PENH values, when monitored overnight (n=6 per treatment group, data not shown).

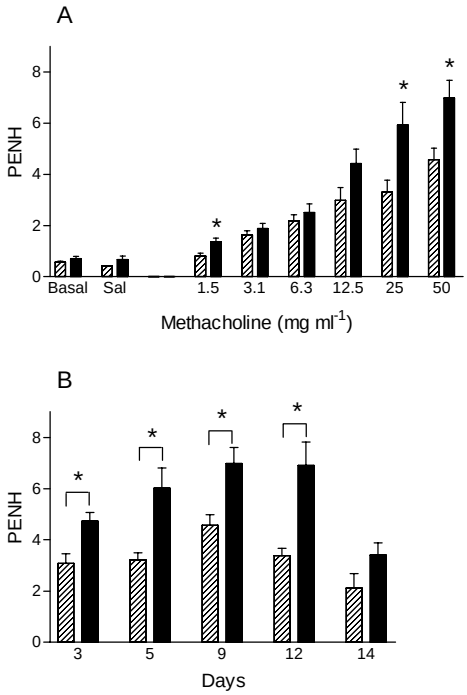
Figure 4



*Kinetics of infiltration of eosinophils into bronchoalveolar lavage (BAL) fluid at different time-points. Lungs from ovalbumin-sensitized and challenged animals were lavaged at 24h after the second (day 3), fourth (day 5) and last (day 9) antigen challenge. Furthermore, lungs were lavaged at 4 (day 12) and 6 days (day 14) after the final challenge with antigen. Lungs from ovalbumin-sensitized and saline-challenged animals were lavaged at 24h after the last challenge and hardly any eosinophils were present in the BAL fluid of these animals (0.2±0.2 x 10^3 eosinophils, data not shown). Results are expressed as arithmetic average ± SEM of 5-6 animals per group. * (P<0.05) significantly different from saline-challenged animals.*

Finally, animals were monitored for presence of late bronchoconstrictive responses after eight consecutive aerosol challenges because then eosinophils and airway hyperresponsiveness are definitely present in our model (Figures 3 and 4). However, no differences in PENH values between saline (n=7) or ovalbumin-challenged (n=8) animals were observed (Figure 5A and 5B).

Figure 3



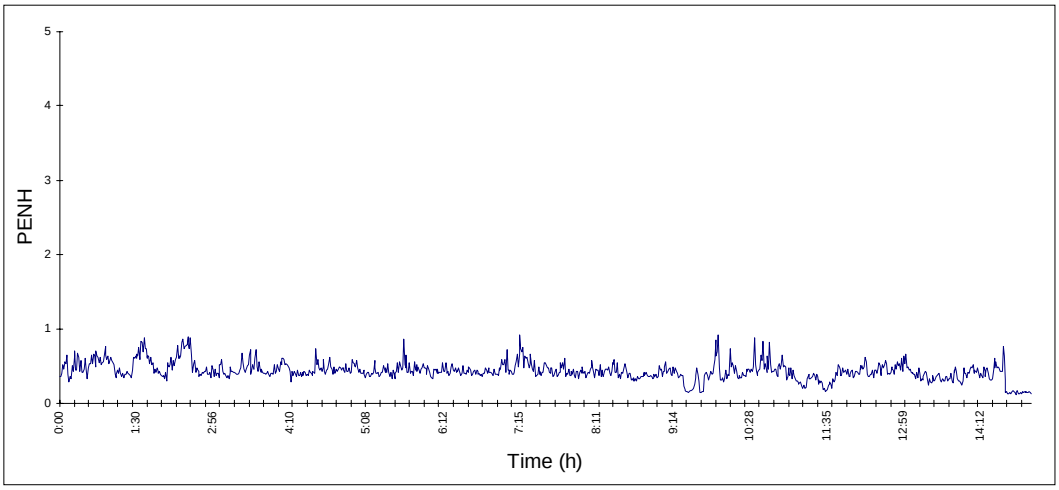
*Kinetics of the development of airway hyperresponsiveness to nebulized methacholine at different time-points. Dose response curves to methacholine were measured in the same animals throughout the experiment. PENH values were recorded for 3 minutes after each exposure to methacholine. Shown are the complete dose response curves measured at 24h after the last challenge (A) and PENH values measured at different days after the highest concentration (50 mg ml^{-1}) of methacholine (B). Dose response curves were made at 24h after the second (day 3), fourth (day 5) and last (day 9) antigen (black bars) or saline-challenge (hatched bars). Furthermore, airway responsiveness to methacholine was determined at 4 (day 12) and 6 days (day 14) after the final antigen or saline-challenge. Results are expressed as arithmetic average ± SEM of 6 animals. * (P<0.05) significantly different from PENH values measured in saline-challenged animals.*

Discussion

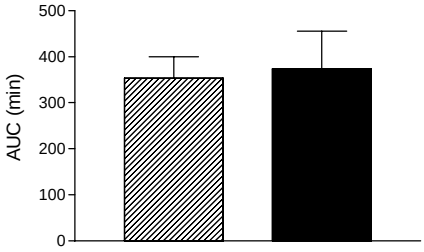
In this study we demonstrate the presence of antigen-induced early but not late bronchoconstrictive responses, even at a time when both airway hyperresponsiveness and eosinophilia are well established. Furthermore, a correlation exists between the increase in airway hyperresponsiveness to methacholine and the number of eosinophils in bronchoalveolar lavage fluid upon sequential antigen exposures. In contrast, resolution of airway hyperresponsiveness

Figure 5

A



B



Representative example of changes in PENH in an ovalbumin-sensitized and repeatedly (8x) ovalbumin-challenged animal (A). Measurements started at 1h after the last challenge and lasted for 15h during which the animal had access to food and water. From the measurements obtained after the last (eight) challenge with either saline (hatched bars, n=7) or ovalbumin (black bars, n=8) areas under the curve (AUC) were calculated (B). Results are expressed as arithmetic average ± SEM.

appears to be dissociated from airway eosinophilia. We observed in the present study that intranasal application of ovalbumin in sensitized mice induced a marked increase in PENH value, whereas challenge with aerosolized antigen did not cause a significant increase in PENH value. The difference in results between the methods of delivering antigen to the lungs are very likely due to limited antigen deposition in the lungs after aerosol challenge. In contrast with these observations we previously showed that aerosolized ovalbumin did cause an immediate bronchoconstrictive response as was determined by forced oscillatory techniques (170), which therefore appears to be a more sensitive technique than whole body plethysmography. In the present study we observed that the increase in PENH after antigen instillation is accompanied by an increase in MMCP-1 levels, which is a result of mast cell degranulation (171).

In agreement herewith we previously showed that exposure to ovalbumin causes massive mast cell degranulation as was detected by electron-microscopy analysis of airway tissue (170). In humans it is well known that upon mast cell degranulation histamine is released which is thought to be responsible for causing the immediate bronchoconstriction upon antigen provocation (158). However, it is very unlikely that histamine release is the cause of the immediate increase in PENH values in mouse models, even though murine mast cells do release histamine upon activation (152). It has been demonstrated that histamine can only induce smooth muscle contraction

in murine tracheas at very high doses *in vitro* (172) and is unable to induce any detectable bronchoconstriction in murine airways *in vivo* (J.J. de Bie, unpublished observations). Besides histamine, 5-hydroxytryptamine (5-HT) which is a well known constrictive agent for murine airway smooth muscle cells (149), can be released by activated murine mast cells (153). In BALB/c mice 5-HT induces a dose-dependent increase in airway pressure overflow (149) and PENH values (J.J. de Bie, unpublished observations). Based upon these data it can be speculated that mast cell degranulation and subsequent release of 5-HT, is related to the observed antigen-induced early bronchoconstrictive response in mice.

An advantage of whole body plethysmography when compared to invasive methods of measuring airway responses, is the possibility to monitor development of airway hyperresponsiveness in time after repeated exposures to antigen in the same animals (166). Our data indicate that airway hyperresponsiveness is already present after 2 antigen-challenges. The difference between saline- and ovalbumin-challenged animals was maximal at 24h after the last (eight) challenge in our model and from previous experiments it is known that airway hyperresponsiveness does not further increase when challenges are continued (J.J. de Bie, unpublished observations). Furthermore, we demonstrate that ovalbumin-induced airway hyperresponsiveness remains present for at least 4 days after the last antigen-exposure and is resolved at 6 days after the final ovalbumin-inhalation. In human asthmatics the increase in non-specific airway hyperresponsiveness observed after a single antigen-challenge can also persist for several days, lasting up to weeks in some individuals (reviewed in 157).

It can be concluded that early bronchoconstrictive responses can be detected using whole body plethysmography and correlate with mast cell degranulation after antigenic provocation. However, this early bronchoconstrictive responses does not predict occurrence of late bronchoconstrictive responses. Furthermore, no causal relations between the infiltration of eosinophils or development of airway hyperresponsiveness and the emergence of late bronchoconstrictive responses could be the detected in this model. Finally, we conclude, based on our study on kinetics of airway hyperresponsiveness and eosinophilic infiltration, that these two parameters are only associated with each other during the induction phase but not during the resolution of these symptoms after stopping the antigen challenges.

Modulation of Airway Hyperresponsiveness and Eosinophilia by Selective Histamine and 5-HT Receptor Antagonists in a Mouse Model of Allergic Asthma

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Summary

Since both histamine and 5-hydroxytryptamine (5-HT) can be released by murine mast cells, we investigated the possible role of these autacoids on airway hyperresponsiveness, eosinophil infiltration and serum-IgE levels in a murine model of allergic asthma. Ovalbumin-sensitized mice were exposed to either ovalbumin or saline aerosols on 8 consecutive days. Starting one day before the challenge, animals were injected i.p. twice a day with a 5-HT type 1 (5-HT_1) or type 2 (5-HT_2) receptor antagonist (methiotepine and ketanserin, respectively) or a histamine-type 1 (H_1) or type 2 (H_2) receptor antagonist (mepyramine and cimetidine, respectively). Furthermore, animals were injected with a combination of cimetidine and ketanserin or with an α-adrenoceptor antagonist (phentolamine). In vehicle-treated ovalbumin-challenged animals airway responsiveness to intravenous injections of methacholine *in vivo* was significantly increased when compared to vehicle-treated saline-challenged animals. Furthermore, ovalbumin challenge of vehicle-treated animals induced a significant increase in both eosinophil numbers in bronchoalveolar lavage (BAL) fluid and ovalbumin-specific IgE levels in serum compared to saline-challenged mice. Virtually no eosinophils could be detected in saline-challenged animals after all different treatments. Treatment with either ketanserin or cimetidine resulted in a partial but significant decrease of the ovalbumin-induced airway hyperresponsiveness when compared to ovalbumin-challenged controls and reduced eosinophil infiltration after ovalbumin. Increasing the dose of cimetidine did not further reduce ovalbumin-induced eosinophilia. Treatment with a combination of ketanserin and cimetidine almost completely abolished antigen-induced airway hyperresponsiveness, whereas eosinophilia was not further decreased when compared to either antagonist alone. No effects of these antagonists were observed on IL16 levels in BAL fluid or on serum antigen-specific IgE levels. Treatment with either the H_1-receptor, the 5-HT_1-receptor or the α-adrenoceptor antagonist, did not decrease the observed ovalbumin-induced airway responsiveness or eosinophilia in vehicle-treated animals. However, higher doses of either methiotepine or mepyramine did decrease ovalbumin-induced eosinophil whereas no effects of these antagonists were observed on ovalbumin-specific IgE levels in serum. From these data it can be concluded that both histamine and 5-HT play a role in antigen-induced airway hyperresponsiveness and eosinophilia.

Introduction

Upon antigen challenge IgE-mediated mast cell degranulation occurs, which leads to an immediate bronchoconstrictive reaction in patients with allergic asthma (6). Besides an immediate bronchoconstriction, allergic asthma in humans is characterized by antigen-specific IgE in serum, airway hyperresponsiveness and inflammation of lung tissue (5, 183). Furthermore, Th2 cells are thought to play an important regulatory role in this disease (reviewed in 10, 184). We have developed a mouse model to investigate several asthma related phenomena. Increased antigen-specific IgE levels in serum are present and non-specific airway hyperresponsiveness and eosinophil infiltration in bronchoalveolar lavage (BAL) fluid have been demonstrated (149, 170). It has also been shown that mast cell degranulation upon antigen challenge occurs accompanied by an immediate bronchoconstriction and an increase in mucosal exudation (170). Besides a role in early asthmatic reactions, mast cells have also been implicated in the development of airway hyperresponsiveness. Using mast cell deficient mice, Nagai *et al.* demonstrated that mast cells play an important role in the onset of airway hyperresponsiveness (15). Furthermore, mast cell activation enhances antigen-induced airway hyperresponsiveness to methacholine in mice (173). Besides a role in airway hyperresponsiveness, mast cells are also believed to play a central role in allergic inflammation via release of cytokines, including IL4 and IL5 (185). In contrast, using mast cell deficient mice, mast cells were shown not to be important for the induction of chronic airway inflammation (15, 186).
Murine mast cells are known to release both histamine and serotonin (5-hydroxytryptamine, 5-HT) upon stimulation *in vivo* (152, 153). Both histamine and 5-HT have been shown to induce IL16 production by $CD8^+$ T cells (79, 80). IL16, previously known as lymphocyte chemoattractant factor (LCF, 90), is a potent chemoattractant for human eosinophils (95) and $CD4^+$ T cells (90), which are thought to play a central role in the pathogenesis of asthma (10). Interestingly, we recently have shown that, upon antigen-challenge, presence of IL16 in BAL fluid derived from ovalbumin-sensitized and challenged animals can be detected (154), which is in agreement with the presence of IL16 in human asthmatics (26). In addition, treatment with neutralizing antibodies to IL16 has an inhibitory effect on airway hyperresponsiveness in our animal model (154). Other pro-inflammatory actions by histamine and 5-HT include an increase in vascular permeability, mucus production and various T-cell related effects (158, 187-190).
Since both histamine and 5-HT can be released by murine mast cells upon antigen challenge (152, 153), we wanted to investigate the role of histamine and 5-HT on asthma-related features. We therefore measured the presence of IL16 in BAL fluid, *in vivo* airway responsiveness to methacholine, inflammatory cell infiltration into the airways and serum levels of antigen-specific IgE after treatment with selective 5-HT or histamine receptor antagonists in a murine model of allergic asthma.

Materials and Methods

Sensitization and challenge

Specified pathogen free male BALB/c mice (age 6-8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (GDL), Utrecht, The Netherlands. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Active sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen free saline on alternate days. This sensitization procedure has been shown to induce high titres of total immunoglobulin E antibodies in the serum, of which 80% was ovalbumin-specific (152). Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in saline) or to 8 saline aerosols for 5 min, on consecutive days (1 aerosol per day). The aerosols were generated with a jet nebulizer (Pari IS-2, Pari-Werk GmbH, Starnberg, Germany) connected to a plexiglas exposure chamber with a volume of 5 l in which a maximum of 6 animals was placed. One day before the challenge started and during the entire challenge period, the mice were injected intraperitoneally twice a day with selective 5-HT-type 1 ($5\text{-}HT_1$) or type 2 ($5\text{-}HT_2$) receptor antagonists (methiotepine, 1.25 mg kg^{-1}or 2.0 mg kg^{-1}; ketanserin, 12 mg kg^{-1}) or selective histamine-type 1 (H_1) or type 2 (H_2) receptor antagonists (mepyramine, 12 mg kg^{-1} or 20 mg kg^{-1};

cimetidine, 10 mg kg^{-1} or 20 mg kg^{-1}). Doses used were adapted from other studies in which mice were treated with these receptor antagonists (191-193) or according to the manufacturer's advice. Furthermore, animals were injected with a combination of cimetidine (10 mg kg^{-1}) and ketanserin (12 mg kg^{-1}) or with an α-adrenoceptor antagonist (phentolamine, 5 mg kg^{-1}) since ketanserin also has effects on these receptors (194). Control mice were injected with 0.25 ml sterile saline.

Airway responsiveness in vivo

Airway responsiveness was measured *in vivo* 24h after the last aerosol exposure using a modified plethysmograph as described by Corry *et al.* (13). In short; mice were anesthetized by intraperitoneal injection of urethane (2 g kg^{-1}), and placed on a heated blanket (30°C). Then, the trachea was cannulated and a small polyethylene catheter was placed in the jugular vein for intravenous administrations. Spontaneous breathing of the animals was suppressed by intravenous injection of tubocurarine chloride (3.3 mg kg^{-1}). When the breathing stopped, the tracheal cannula was attached to a ventilator (C.F. Palmer, London, UK). The inflation volume of the ventilator was 0.8 ml of which the mouse inhales approximately 0.15 ml per breath with a rate of 200 breaths per min. Under these conditions, mice maintain physiologic arterial blood gas parameters (data not shown). Changes in resistance were measured by use of a plethysmograph, coupled to a pressure transducer (M45, Validyne Engineering Corp. Northridge, CA, USA). By use of a pulmonary mechanics analyzer (Model 6, Buxco Corp., Sharon, CT, USA), lung resistance (R_L) was measured by quantitating $\Delta P_t \bullet \Delta V^{-1}$ (ΔP_t = change in tracheal pressure, ΔV = change in flow) at points of equal volume (70% tidal volume). Changes in tracheal pressure were measured using a pressure transducer connected to the tracheal ventilation cannula, changes in flow were measured by use of a pressure transducer connected to the plethysmograph (pressure changes were calibrated to changes in volume over the physiologic range studied). At time intervals of at least 4 min and after the response had returned to baseline level, doses of methacholine ranging from 40 μg kg^{-1} to 640 μg kg^{-1} were administered via the jugular catheter. Concentrations of methacholine were prepared in saline and kept on ice for the duration of the experiment. For each dose of methacholine the increase in airway resistance was measured at its peak and expressed in cm H_2O ml^{-1} s^{-1}. At least 6 mice were evaluated per experimental group.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in the same animals that were used for airway hyperresponsiveness measurements. In pilot experiments it was found that combining these techniques had no effect on the total number of cells derived from the lavage nor on the appearance of the different cell types. Mice were lavaged 5 times through the tracheal cannula with 1 ml aliquots of pyrogen free saline at 37°C. The first aliquot was supplemented with the protease inhibitor aprotinine (2 mg ml^{-1}) and after centrifugation the supernatant of this first ml was stored at -80°C for cytokine measurements. The BAL cells were washed with cold phosphate-buffered saline (PBS, 400x*g*, 4°C, 5 min) and the pellet was resuspended in 200 μl cold PBS. The total number of BAL cells was counted by use of a Bürker-Türk chamber. For differential BAL cell counts cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Düdingen, Switzerland). After coding, all cytospin preparations were evaluated by one observer using oil immersion microscopy. Cells were identified and differentiated into mononuclear cells, neutrophils and eosinophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated.

Determination of ovalbumin-specific immunoglobulin E serum levels

Ovalbumin-specific IgE was measured in serum derived from blood isolated from mice after airway responsiveness measurements were completed. Ninety-six well microplates (Nunc A/S, Roskilde, Denmark) were coated with 2 μg ml^{-1} chimeric fusion protein of the human high affinity immunoglobulin E receptor and human immunoglobulin G (FcεR1-IgG) diluted in PBS. After 12-24h incubation at 4°C the

plates were washed 5 times with PBS supplemented with 0.05% Tween-20 (PBT). Thereafter, the plates were blocked with ELISA buffer (2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.5% BSA , 0.05% Tween-20, pH 7.2) and left to incubate at room temperature for 1 h on an orbital shaker. Serum samples derived from ovalbumin-challenged animals were diluted 1:10, samples from saline-challenged animals 1:5 in ELISA buffer. Then, diluted serum samples were added to the plates and left to incubate on an orbital shaker at room temperature for 2h. Ovalbumin-IgE reference standard dilution series were treated the same as the serum samples. The standard was obtained by intraperitoneal immunization of mice with ovalbumin, and arbitrarily assigned a value of 1,000 units ml^{-1} ovalbumin-specific immunoglobulin E (195). After washing, 10 $\mu g\ ml^{-1}$ of ovalbumin in ELISA buffer was added to each well and after incubation at room temperature for 1h washing procedures were repeated. Horse-radish peroxidase-conjugated goat anti-ovalbumin antibody was diluted in ELISA buffer and added to each well. Incubation was continued for 1 h followed by washing procedures. The last step was a 15-30 min incubation at room temperature with 10 mM OPD substrate solution after which the reaction was stopped by adding H_2SO_4 (4M). OD was read at λ 492 nm using a Titertek Multiskan (Flow Labs., Irvine, UK).

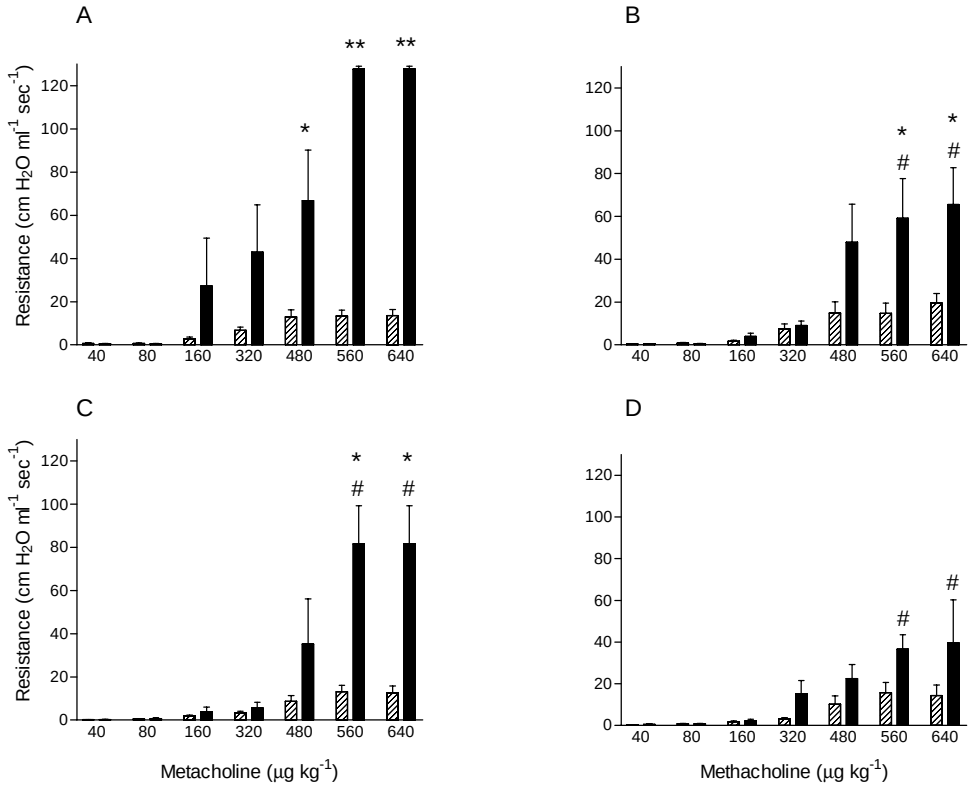
IL16 migration assay

Since IL16 levels in BAL are to low for detection in a specific ELISA (detection limit 40 pg ml^{-1}) a modified Boyden chemotaxis chamber was used for IL16 measurements (75). In short, human lymphocytes were isolated from heparinized venous blood samples of healthy normal volunteers by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ, USA). The cell layer containing peripheral blood mononuclear cells was recovered and washed 3 times in Medium 199 supplemented with 25 mM Hepes buffer, 100 units ml^{-1} penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin. The cells were incubated on a nylon wool column at 37°C and 5% CO_2 for 45 min. The cell population eluted from the column contained >97% T-lymphocytes as determined by fluorescent staining with anti-CD3 monoclonal antibody (Becton-Dickinson, Mountain View, CA, USA). 30 µl of BAL fluid derived from ovalbumin or saline-challenged mice was placed in the lower well and the lymphocytes ($1x10^7$ cells in 50 ml Medium 199 enriched with 0.4% bovine serum albumin) were loaded into the upper well of the Boyden chamber. For blocking experiments, rabbit-anti-human IL16 polyclonal antibody was added to the lower well as well. In previous experiments it was shown that 5 $\mu g\ ml^{-1}$ anti-IL16 antibody neutralizes 0.1 nM of recombinant human IL16 protein. Antibodies to IL16 were only added if the migration was enhanced compared to medium induced migration. The upper and lower well were separated by a nitrocellulose filter with a pore size of 8 µm. The chamber was incubated for 3h and afterwards the filter was fixed and stained with hematoxylin. Migration was quantified by counting the number of cells that migrated beyond a depth of 50 µm utilizing an Optomax automated image analyzer (Burlington, MA, USA). All migration data are expressed as percent values of cell migration in Medium 199 enriched with 0.4% bovine serum albumin, which was normalized to 100%. All BAL samples were tested in triplicate.

Drugs and chemicals

Ovalbumin (chicken egg albumin crude grade V), o-phenylenediamine, 3-amino-1,2,4-triazole and mepyramine were purchased from Sigma Chemical Company (St. Louis, MO, USA), recombinant human FcεR1-IgG, horse-radish peroxidase-conjugated goat anti-ovalbumin antibody and ovalbumin-IgE reference standard were generously provided by Dr. P.M. Jardieu, Genentech Inc. (South San Francisco, CA, USA). Urethane and methacholine (acetyl-ß-methylcholine) were purchased from Janssen Chimica (Beerse, Belgium), tubocurarine chloride from Nogepha (The Netherlands), Tween-20 from Merck (Darmstadt, Germany) and phentolamine from Ciba Geigy (Basel, Switzerland). Cimetidine was purchased from SmithKline Beecham (Irvine, U.K.) and ketanserin and methiotepine from ICN (Costa Mesa, CA, USA).

Figure 1

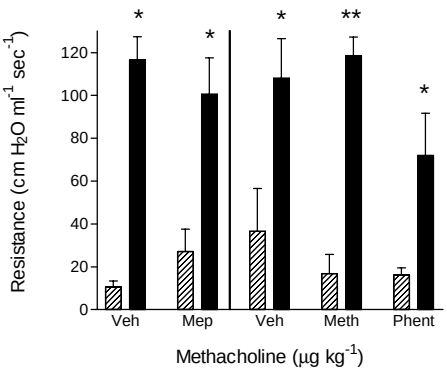


*Airway responsiveness to intravenous administration of methacholine in ovalbumin-sensitized mice at 24 h after the last challenge with saline (hatched bars) or ovalbumin (black bars) and treated with vehicle (A), cimetidine (10 mg kg^{-1}, B), ketanserin (12 mg kg^{-1}, C) or a combination of cimetidine (10 mg kg^{-1}) and ketanserin (12 mg kg^{-1}, D). Results are expressed as arithmetic average ± SEM (n=6 per group). * (P<0.05); ** (P<0.01) significantly different from saline-challenged animals. # (P<0.05) significantly different from vehicle-treated ovalbumin-challenged animals.*

Statistical analysis

Whole concentration-response curves, total BAL cell number and the numbers of the various BAL cell types were tested with an analysis of variance (ANOVA). For cell types with a very low number in control animals (i.e. neutrophils and eosinophils) a Poisson distribution was assumed. Comparisons between 2 groups were made using a Student's *t*-test (unpaired, two-tailed). Data are expressed as arithmetic average ± SEM and a difference was considered to be significant when $P<0.05$. Statistical analyses were carried out using SPSS/PC^{+}, version 4.0.1 (SPSS Inc., Chicago, IL, USA) or GLIM, version 4.0 (NaG Inc., Oxford, UK).

Figure 2



*Increase in airway resistance at 640 µg kg^{-1} of methacholine in ovalbumin-sensitized and saline (hatched bars) or ovalbumin-challenged (black bars) animals at 24h after the last challenge. Animals were intraperitoneally injected with either vehicle (Veh), methiotepine (1.25 mg kg^{-1}, Meth) or phentolamine (5 mg kg^{-1}, Phent) or in a different experiment with vehicle (Veh) or mepyramine (12 mg kg^{-1}, Mep) during the challenge period. Results are expressed as arithmetic average ± SEM (n=6 per group). * (P<0.05); ** (P<0.01) significantly different from saline-challenged animals.*

Results

Airway resistance in vivo

Intravenous methacholine (480 µg kg^{-1}) produced a 5-fold increase in airway responsiveness after ovalbumin challenge in vehicle-treated animals (P<0.05), whereas a 9-fold increase was observed both at 560 µg kg^{-1} and at 640 µg kg^{-1} methacholine, compared to vehicle-treated saline-challenged mice (P<0.01, Figure 1A). Cimetidine-treated (10 mg kg^{-1}) ovalbumin-challenged animals showed a partial but significant decrease in airway hyperresponsiveness compared to vehicle-treated ovalbumin-challenged mice (Figure 1A and B). At the highest dose of methacholine (640 µg kg^{-1}) the observed decrease was 49% (P<0.05). However, airway hyperresponsiveness was still present (P<0.05) compared to cimetidine-treated saline-challenged animals (Figure 1B). The dose of cimetidine used in this study inhibited histamine-induced decrease in blood pressure (data not shown). Ketanserin-treated animals (12 mg kg^{-1}) also showed a partial but significant decrease in airway responsiveness compared to vehicle-treated ovalbumin-challenged mice (Figure 1A and C). At the highest dose of methacholine (640 µg kg^{-1}) the decrease was 36% (P<0.05). This decrease however, was still significantly enhanced compared to ketanserin-treated saline-challenged animals (P<0.05, Figure 1C). Higher doses of ketanserin could not be used to further inhibit development of airway hyperresponsiveness since it was observed that ketanserin has sedative actions in mice. No airway hyperresponsiveness could be observed in animals treated with a combination of ketanserin (12 mg kg^{-1}) and cimetidine (10 mg kg^{-1}) compared to their saline-challenged controls (Figure 1D). This combination caused a decrease in airway responsiveness of 69% which was significantly different from vehicle-treated ovalbumin-challenged animals (P<0.05, Figure 1A and D).

Complete dose-response curves were also made in animals treated with either a selective H_1-receptor antagonist (mepyramine, 12 mg kg^{-1}), a 5-HT_1-receptor antagonist (methiotepine, 1.25 mg kg^{-1}) or an α-adrenoceptor antagonist phentolamine (5 mg kg^{-1}, results not shown). At the highest dose of methacholine (640 µg kg^{-1}) a significant airway hyperresponsiveness to methacholine was observed in vehicle-treated mice after ovalbumin challenge (P<0.05) compared to saline-challenged animals (Figure 2). Treatment with either the H_1-receptor, the 5-HT_1-receptor or the α-adrenoceptor antagonist did not decrease the observed ovalbumin-induced airway hyperresponsiveness (Figure 2).

Eosinophil infiltration

Cells in bronchoalveolar lavage fluid of animals collected at 24 h after the last challenge were differentiated by light microscopy. No differences in infiltration of mononuclear cells or neutrophils between all different antagonist treatments could be detected after either ovalbumin or saline challenge (Tables I and II). Virtually no eosinophils could be detected in BAL fluid derived from saline-challenged animals treated with vehicle, ketanserin, cimetidine or the combination of antagonists (Tables I and II).

Table I: Number of leukocytes in bronchoalveolar lavage fluid

Treatment	Challenge	Mononuclear cells x 10^5	Eosinophils x 10^4	Neutrophils x 10^4
Vehicle	Sal	3.0±0.4	0.0±0.0	0.1±0.1
	Ova	3.3±0.7	15±5.9 (*)	0.0±0.0
Cimetidine	Sal	2.9±0.4	0.0±0.0	0.1±0.1
	Ova	2.9±0.5	6.2±3.9 (*)	0.0±0.0
Ketanserin	Sal	2.6±0.3	0.0±0.0	0.0±0.0
	Ova	2.6±0.4	6.6±2.7 (*)	0.1±0.0
Cimetidine + Ketanserin	Sal	2.5±0.2	0.1±0.1	0.1±0.0
	Ova	2.4±0.5	8.0±5.0 (*)	0.1±0.1

*Total number of various cell types in BAL fluid recovered at 24h after the last ovalbumin (Ova) or saline (Sal) challenge in ovalbumin-sensitized mice treated with vehicle, cimetidine (10 mg kg^{-1}), ketanserin (12 mg kg^{-1}) or a combination of ketanserin (12 mg kg^{-1}) and cimetidine (10 mg kg^{-1}). Results are expressed as arithmetic average ± SEM (n=6 per group). * (P<0.05) significantly different from saline-challenged animals.*

After ovalbumin challenge of vehicle-treated animals a significant migration of eosinophils into the BAL fluid was observed (15.0±5.9 x 10^4 cells, P<0.05). Treatment with either cimetidine (10 mg kg^{-1}) or ketanserin (12 mg kg^{-1}) diminished the infiltration of eosinophils with 60% (6.2±3.9 x 10^4 cells) and 58% (6.6±2.7 x 10^4 cells) respectively, compared to the number of eosinophils present in BAL fluid of vehicle-treated ovalbumin-challenged animals. The combination of cimetidine and ketanserin inhibited the eosinophil infiltration with 49% (8.0±5.0 x 10^4 cells, Table I). The higher dose of cimetidine (25 mg kg^{-1} did not further diminish eosinophil infiltration (data not shown). No differences were observed in eosinophil infiltration between vehicle-treated ovalbumin-challenged animals and methiotepine-, phentolamine-, or mepyramine-treated ovalbumin-challenged animals (Table II). However, higher doses of either methiotepine (2.0 mg kg^{-1}) or mepyramine (20 mg kg^{-1}) did decrease eosinophil infiltration with 67% (1.7±1.3 x 10^5 cells, P=0.09) and 73% (1.4±1.0 x 10^5 cells, P<0.05) when compared to eosinophil numbers in BAL fluid derived form vehicle-treated ovalbumin-challenged animals (5.3±1.4 x 10^5 cells).

Ovalbumin-specific IgE production

Serum levels of ovalbumin-specific IgE were determined in animals treated with vehicle, cimetidine (10 mg kg^{-1}), ketanserin (12 mg kg^{-1}) or with a combination of both ketanserin (12 mg kg^{-1}) and cimetidine (10 mg kg^{-1}). A significant increase in the level of ovalbumin-specific IgE was found after ovalbumin challenge of vehicle-treated animals (P<0.05, Table III). No significant differences were observed between the ovalbumin-induced increase in serum ovalbumin-IgE levels in the groups of mice treated with different antagonists. Furthermore, no significant differences were observed between the ovalbumin-induced increase in ovalbumin-specific IgE levels in serum obtained from animals treated with the higher doses of either mepyramine or methiotepine (data not shown).

Table II: Number of leukocytes in bronchoalveolar lavage fluid

Treatment	Challenge	Mononuclear cells x 10^4	Eosinophils x 10^3	Neutrophils x 10^3
Vehicle	Sal	2.4±0.3	0.0±0.0	0.3±0.3
	Ova	3.1±0.4	6.9±1.5 (*)	0.0±0.0
Methiotepine	Sal	2.7±0.2	0.0±0.0	0.0±0.0
	Ova	2.5±0.1	6.0±2.0 (*)	0.2±0.2
Phentolamine	Sal	2.5±0.4	0.0±0.0	0.2±0.2
	Ova	2.4±0.2	6.7±2.4 (*)	0.3±0.2
Vehicle	Sal	2.3±0.1	0.0±0.0	0.2±0.1
	Ova	2.1±0.3	6.6±2.7 (*)	0.0±0.0
Mepyramine	Sal	2.0±0.4	0.0±0.0	0.0±0.0
	Ova	2.1±0.3	6.9±2.2 (*)	0.0±0.0

*Total number of various cell types in BAL fluid recovered at 24h after the last ovalbumin (Ova) or saline (Sal) challenge in ovalbumin-sensitized mice treated with vehicle, methiotepine (1.25 mg kg^{-1}) or phentolamine (5 mg kg^{-1}). In a different experiment, animals were treated with vehicle or mepyramine (12 mg kg^{-1}) Results are expressed as arithmetic average ± SEM (n=6 per group). * (P<0.05) significantly different from saline-challenged animals.*

Table III: Serum ovalbumin-specific IgE levels

Treatment	Saline	Ovalbumin
Vehicle	157±69	619±171 (*)
Cimetidine	235±84	960±182 (*)
Ketanserin	377±118	801±223
Combination	402±122	1141±220 (*)

*Ovalbumin-specific Immunoglobulin E (IgE) levels (arbitrary units ml^{-1}) measured by ELISA in serum after saline or ovalbumin challenge in ovalbumin-sensitized mice after treatment with vehicle, cimetidine (10 mg kg^{-1}), ketanserin (12 mg kg^{-1}) or a combination of cimetidine (10 mg kg^{-1}) and ketanserin (12 mg kg^{-1}). Results are expressed as arithmetic average ± SEM (n is at least 10 per group). * (P<0.05) significantly different from immunoglobulin E level in serum from saline-challenged animals.*

IL16 levels in BAL

Migration of human lymphocytes induced by BAL fluid collected from vehicle-treated saline-challenged animals was significantly less (87±3%) than migration induced by medium (100%, P<0.05), indicating that inhibitory factors are present in these BAL fluid samples. BAL fluid samples from ovalbumin-challenged vehicle-treated animals induced a significant increase (219±21%, P<0.05) of lymphocyte migration compared to both medium controls and BAL fluid from saline-challenged vehicle-treated animals. Of this migration 80±21% appeared to be due to IL16 as determined by neutralizing polyclonal antibodies. BAL fluid derived from ovalbumin-challenged animals treated with either cimetidine, ketanserin or the combination of these two antagonists did not induce a significantly

Table IV: Migration of lymphocytes induced by BAL fluid samples

Treatment	Total migration	IL16 dependent part of migration
Vehicle/Sal	87±3%	0±0%
Vehicle/Ova	219±21% (*)	80±21%
Cimetidine/Sal	153±41%	36±30%
Cimetidine/Ova	176±42%	79±%35
Ketanserin/Sal	108±16%	13±12%
Ketanserin/Ova	198±51%	53±27%
Combination/Sal	112±15%	7±6%
Combination/Ova	214±46%	99±27%

Migration of lymphocytes induced by BAL fluid samples derived from saline (Sal) or ovalbumin (Ova)-challenged animals treated with either vehicle, cimetidine (10 mg kg^{-1}), ketanserin (12 mg kg^{-1}) or a combination of cimetidine (10 mg kg^{-1}) and ketanserin (12 mg kg^{-1}). Furthermore, the IL16 dependent part of the induced migration is depicted. Results are expressed as arithmetic average ± SEM (n is at least 5 per group). (P<0.05) significantly different from migration induced by BAL fluid derived from saline-challenged vehicle-treated animals.*

different lymphocyte migration when compared to BAL fluid derived from vehicle-treated ovalbumin-challenged animals (Table IV). Furthermore, no significant differences were observed in the IL16 dependent part of this migration. Finally, no differences were observed between both the lymphocyte migration and the IL16 attributable part of this migration induced by BAL fluid samples obtained from saline-challenged animals of all different treatments (Table IV).

Discussion

Both the selective 5-HT_2 receptor antagonist ketanserin and the H_2 receptor antagonist cimetidine were capable of partially decreasing airway hyperresponsiveness and eosinophil infiltration, whereas a combination of ketanserin and cimetidine completely inhibited airway hyperresponsiveness without further decreasing eosinophil numbers in BAL fluid. No effects of either antagonist alone or the combination of antagonists on ovalbumin-specific IgE serum levels or on IL16 levels in BAL fluid could be detected.

Previously we have developed a murine model of allergic asthma that shows several characteristics which are also observed in human allergic asthma. Non-specific airway hyperresponsiveness, eosinophil infiltration and an increase in antigen-specific serum IgE levels are commonly observed in human allergic asthma and can also be detected in this mouse model (148, 149, 170). Furthermore, upon antigen challenge, an early bronchoconstrictive reaction together with an increase in mucosal exudation can be measured, which is believed to be caused by mast cell degranulation after IgE cross-linking (170). In humans, mast cell degranulation has also been observed after antigen challenge (6). This mast cell degranulation is believed to play a pivotal role in the early asthmatic reaction and is implicated in the onset of the inflammation via e.g. TNFα, IL4 and IL5 release (185, 196). Murine mast cells have been shown to be capable of releasing both histamine and 5-HT (152, 153) but little is known about the role of these autacoids in phenomena such as inflammation, IgE production and airway hyperresponsiveness. Therefore, we treated mice with several different selective histamine or 5-HT receptor antagonists to determine the role of histamine or 5-HT on these parameters.

A possible explanation for the results after treatment with H_2 or 5-HT_2 receptor antagonists or the combination of these receptor antagonists could be impaired production of IL16. Upon stimulation of H_2 and 5-HT_2 receptors, IL16 is released by $CD8^+$ T cells (79, 80). IL16 has been reported to be a very potent chemoattractant for human eosinophils (95) and $CD4^+$ T cells (90), which are believed to contribute to airway hyperresponsiveness and inflammation (reviewed in 10, 184). In agreement herewith, the development of ovalbumin-induced airway hyperresponsiveness was partially impaired by treatment with monoclonal antibodies to IL16 (154), however, eosinophilia was not affected by treatment with IL16.

Thus, a likely explanation for the observed effects on airway hyperresponsiveness after treatment with cimetidine and ketanserin could be that the release of IL16 is blocked by the antagonists used in this study. However, in the present study IL16 levels in BAL fluid after ovalbumin challenge were not decreased by treatment with either antagonist alone or the combination. Thus, it is not very likely that the observed effects on airway hyperresponsiveness were caused by a decrease in IL16 release. Besides $CD8^+$ T cells, epithelial cells are a well known source of IL16 in asthmatics (26). Furthermore, IL16 can also be produced by mitogen and antigen-stimulated mononuclear cells (29), and it is therefore possible that IL16 levels in BAL were not decreased by H_2 and 5-HT_2 antagonists.

As was previously stated, mast cells can secrete both histamine and 5-HT (152, 153) and mast cells degranulate upon antigen challenge in our model (170). Furthermore, it is well known that mast cells are important in the onset of airway hyperresponsiveness (15, 173). Together with the observed effects of both cimetidine and ketanserin on airway hyperresponsiveness, it is very tempting to speculate that mast cells are involved in development of airway hyperresponsiveness via release of both histamine and 5-HT upon antigen challenge. Our observation that treatment with ketanserin can decrease airway hyperresponsiveness to methacholine is in agreement with observations made by Cazzola *et al.* (194) who demonstrated that 5-HT_2 receptor blocking can cause a small but significant modification of airway hyperresponsiveness in human asthmatic patients. Besides being a 5-HT_2 receptor antagonist, ketanserin also has antagonistic activity on α-adrenoceptors (197). However, the observed inhibition of airway hyperresponsiveness in our experiments after treatment with ketanserin was 5-HT_2 receptor specific since no effects were observed when either a 5-HT_1 receptor antagonist or an α-adrenoceptor antagonist were used. Treatment with cimetidine resulted in a partial inhibition of both airway hyperresponsiveness and eosinophil infiltration. However, cimetidine is also capable of binding to H_1 receptors although the affinity for H_1 receptors is less than the affinity for H_2 receptors (197). Furthermore, H_2 receptor antagonists prevent release of histamine and therefore it is difficult to analyze *in vivo* results of treatment with such antagonists (198). The observation that H_1 as well as 5-HT_1 receptor antagonists could reduce eosinophil infiltration at higher doses could be due to nonselective effects associated with these high doses, e.g. on other histamine or 5-HT receptors. However, these data do not exclude the involvement of H_1 and 5-HT_1 receptors in eosinophil infiltration upon antigen challenge.

An explanation for the observed effects on eosinophil infiltration and airway hyperresponsiveness could be that both histamine and 5-HT have several pro-inflammatory effects. Therefore inhibition of the action of these autacoids could lead to a decrease in inflammation and airway hyperresponsiveness. Histamine e.g., has been reported to augment activation of cytotoxic T lymphocytes *in vivo* and production of IL2 *in vitro* (199). Furthermore, histamine has been shown to inhibit IFNγ production in a H_2 receptor restricted manner (200). IFNγ is well known for its capabilities to down-regulate Th_2-mediated responses such as eosinophil infiltration and airway hyperresponsiveness (reviewed in 184). In contrast, histamine also has immunosuppressive capacities such as inhibition of antigen- and mitogen-induced T cell responses and production of cytokines including IL2, IL1 and TNFα (196). Besides lymphocyte-mediated effects histamine appears to be chemotactic for eosinophils when studied under *in vivo* and *in vitro* circumstances, which is thought to be mediated via both H_1 and H_2 receptors (198). This would implicate that the effects observed by either the H_1 or H_2 receptor antagonist are due to nonselective inhibition of histamine receptors, which is in agreement with e.g. the observation that mepyramine only has an effect on eosinophil infiltration when administered in high doses.

5-HT is involved in T cell activation (189), local recruitment and activation of $CD4^+$ T helper cells (187, 188) and B cell proliferation (190). Therefore, it could be speculated that cimetidine and ketanserin exert their activity via inhibition of lymphocyte functions. Little is known about the role of either histamine or 5-HT in antibody production by B cells. However, since antigen-specific IgE levels in serum were not affected by the different treatments it is not very likely that B cell functioning is impaired by one of the antagonists alone or by the combination of cimetidine and ketanserin.

Another characteristic that could have contributed to the effects caused by cimetidine and ketanserin as well as the higher doses of mepyramine and methiotepine, is that both histamine and 5-HT are able to increase vascular permeability (158, 201). 5-HT has been clearly demonstrated to increase vascular permeability via 5-HT_2 receptor stimulation (201) whereas histamine has been reported mainly to induce

vascular leakage upon H_1 receptor stimulation. This seems to be in contrast with our observations that an H_2 receptor antagonist caused a decrease in airway hyperresponsiveness, whereas an H_1 receptor antagonist (12 mg kg^{-1}) did not cause any effect. However, H_1 receptor antagonists alone are less able to prevent the increase in vasopermeability than combinations of H_1 and H_2 receptor antagonists in preventing the increase in vasopermeability (158). Combined with the observations that cimetidine can bind to both H_2 and to H_1 receptors (197) and that H_2 receptor antagonists prevent histamine release (198), it could therefore be speculated that cimetidine as well as ketanserin prevented the antigen-induced increase in vasopermeability after ovalbumin challenge (170). An increase in vascular permeability could not only lead to oedema of the airway wall which contributes to increased airway hyperresponsiveness (197) but also to leakage of plasma proteins into the airways, which leads to an increase of the viscosity of mucus (reviewed in 5). This increase in viscosity retards the mucociliary clearance of mucus which constitutes an integral part of the inflammatory response (5). Moreover, histamine is believed to play a direct stimulatory role on the mucus production in the lower airways via H_2 receptor stimulation (158). It has previously been established in the used model that an ovalbumin-induced increase in mucus exudation as well as goblet cell hyperplasia occurs (150). The observed mucus production could very well predispose ovalbumin-challenged animals to air trapping, especially after administration of a well known secretagogue for mucus such as methacholine. Thus, it could be possible that the effects of either the histamine or 5-HT receptor antagonist on airway hyperresponsiveness are caused by a decrease in antigen-induced mucus production or exudation.

The combination of both cimetidine and ketanserin did completely abolish airway hyperresponsiveness without further decreasing eosinophil infiltration. The observation that eosinophilia and airway hyperresponsiveness are not always closely related is in agreement with observations made both in humans and animal models (148, 181). The observed effects of the combination of ketanserin and cimetidine, could be due to a complete inhibition of the antigen-induced increase in vascular permeability, mucus production and possibly T lymphocyte functioning by both histamine and 5-HT. However, since eosinophil infiltration was not further decreased by the combination of antagonists it is not very likely that the main effect of these antagonists is impairment of immune cell functions. If these antagonists would have impaired T lymphocyte functions such as cytokine production, eosinophil infiltration would have been completely blocked since it is generally accepted that Th_2 type cytokines are crucial for development of eosinophilia (reviewed in 184).

In conclusion, our data suggest that 5-HT and histamine are mediators of antigen-induced airway hyperresponsiveness and, to a lesser degree, of inflammation in the mouse. Furthermore, it can be speculated that these actions probably take place via 5-HT_2 and H_2 receptors. Since a combination of both antagonists completely inhibited airway hyperresponsiveness it can also be concluded that these antagonists act in an additive way. However, the exact mechanism by which these antagonists exert their action on asthma related features and via which receptors remains to be elucidated.

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Effect of IL16-blocking Peptide on Parameters of Allergic Asthma in a Murine Model

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Summary

In this study we examined whether peptides based on the hydrophilic CD4-binding part of the amino acid sequence of hIL16 can block IL16-induced chemotaxis of murine lymphocytes *in vitro.* Next, we compared the effects of intra-airway administration of these peptides with antibodies to IL16 on antigen-induced features in a murine model of allergic asthma. Peptide 3 inhibited IL16-induced chemotaxis of murine splenocytes *in vitro*. Furthermore, intra-airway administration of peptide 3 largely inhibited the development of antigen-induced airway hyperresponsiveness while airway eosinophilia was not affected. Similar effects were observed after intranasal application of antibodies to IL16. These results indicate that treatment with peptide 3 causes the same effects as antibodies to IL16, possibly via inhibition of interaction between IL16 and its receptor CD4. Therefore, peptide 3 could be useful as a lead compound in attempting to limit airway hyperresponsiveness via binding to CD4.

Introduction

Human allergic asthma is characterized by airway hyperresponsiveness and infiltration of lymphocytes and eosinophils in the lungs (10). There is an increasing amount of evidence that $CD4^+$ T cells play a crucial role in orchestrating these different phenomena by production of Th2-type cytokines, including IL4 and IL5 (10, 159).
Besides IL4 and IL5 many other cytokines have been associated with the pathology of asthma, one of them being IL16 (29). Interleukin-16, has been demonstrated to use the CD4 molecule as its receptor. Upon binding and cross-linking of CD4 molecules, activation of several second messengers, including p56lck and PKC, occurs (101, 202, 203). Furthermore, IL16 can evoke different functional responses in $CD4^+$ cells. One of the most extensively described actions of CD4 cross-linking by IL16 is the induction of chemotaxis *in vitro* (74). It has been demonstrated that IL16 can induce such responses in various $CD4^+$ cells, including eosinophils, monocytes and T helper cells (29, 90). Other biological effects of IL16 include induction of IL2R expression and upregulation of HLA-DR expression in human lymphocytes (29, 90).
Both $CD4^+$ and $CD8^+$ T cells constitutively express messenger RNA for IL16 and the biologically active protein is secreted from $CD8^+$ T cells after stimulation with either histamine or 5-hydroxytryptamine (5-HT, 79, 80). Furthermore, $CD4^+$ T cells release IL16 after mitogen, antigen or anti-CD3 stimulation (29). Bioactive IL16 can also be produced by eosinophils, mast cells and epithelial cells (81, 82, 94). These data suggest that IL16 may be involved in the pathophysiology of asthma. Indeed, in patients with allergic asthma as well as in a murine model of allergic asthma IL16 expression in epithelial cells and presence of bioactive IL16 in bronchoalveolar lavage fluid after antigen-challenge has been observed (26, 154). Interestingly, inhibition of endogenous IL16 by intraperitoneal administration of antibodies to IL16 has been demonstrated to partially decrease airway hyperresponsiveness but did not affect the number of eosinophils in bronchoalveolar lavage fluid in a murine model of allergic asthma (154).
In a previous study, Keane *et al.* demonstrated that peptide 3 which is based on the predicted amino acid sequence of one of the hydrophilic C-terminal regions of IL16 is capable of partially inhibiting rhIL16-induced chemotaxis of PBMCs (76). They also demonstrated that this peptide displaced OKT4 binding to CD4 molecules. These data suggest that the C-terminal hydrophilic domain of IL16 is involved in binding to CD4 and is critical for induction of chemotaxis in $CD4^+$ cells (76). Additionally, these data suggest that this peptide might inhibit IL16-induced activity by binding to CD4 molecules, thereby preventing cross-linking of CD4 molecules by IL16. Finally, sequence homology of murine and human IL16 is over 80% and cross-specificity is nearly 100% since identical effects were observed on

Figure 1

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1     MDYDFDTTAE DPWVRISDCI KNLFSPIMSE NHGHMPLQPN ASLNEEEGTQ GHPDGTPPKL
61    DTANGTPKVY KSADSSTVKK GPPVAPKPAW FRQSLKGLRN RASEPRGLDP PALSTQPAPA
121   SREHLGSHIR ASSSSSSIRQ RISSFETFGS SQLPDKGAQR LSLQPSSGEA AKPLGKHEEG
181   RFSGLLGRGA APTLVPQQPE QVLSSGSPAA SEARDPGVSE SPPPGRQPNQ KTFPPGPDPL
241   LRLLSTQAEE SQGPVLKMPS QRARSFPLTR SQSCETKLLD EKTSKLYSIS SQVSSAVMKS
301   LLCLPSSISC AQTPCIPKAG ASPTSSSNED SAANGSAETS ALDTGFSLNL SELREYTEGL
361   TEAKEDDDGD HSSLQSGQSV ISLLSSEELK KLIEEVKVLD EATLKQLDGI HVTILHKEEG
421   AGLGFSLAGG ADLENKVITV HRVFPNGLAS QEGTIQKGNE VLSINGKSLK GTTHHDALAI
481   LRQAREPRQA VIVTRKLTPE AMPDLNSSTD SAASASAASD VSVESTAEAT VCTVTLEKMS
                            peptide 1
541   AGLGFSLEGG KGSLHGDKPL TINRIFKGAA SEQSETVQPG DEILQLGGTA MQGLTRFEAW
                                        peptide 2
601   NIIKALPDGP VTIVIRRKSL QSKETTAAGDS
                          peptide 3
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Alignment of the predicted human pro-IL16 amino acid sequence of the longest open reading frame (Adapted from Keane et al., (76). *Genbank database accession number M90391. Underlined sequences represent the predicted amino acid sequences on which the different oligopeptides were based.*

migration of human or murine $CD4^+$ T lymphocytes by either rhIL16 or rmIL16 and this chemoattracting activity could be blocked by anti-human IL16 mAb (clone 14.1, 76).
In the present study we examined the effect of different peptides based on the predicted amino acid sequence of IL16 on rmIL16-induced chemotaxis of lymphocytes *in vitro*. Next, we examined the effects of intranasal administration of these peptides on airway hyperresponsiveness and eosinophilia in a murine model of asthma. In addition we compared the effects of local administration of these peptides with administration of antibodies to IL16 on these parameters.

Materials and Methods

Peptides and antibodies

Synthetic oligopeptides corresponding to three hydrophilic domains identified within the hIL16 sequence were used in this study. The single letter amino acid codes of the different peptides are: MPDLNSSTDSA (based on amino acids 502-512, Figure 1), designated peptide 1, AASEQSETVQPGDEIL (based on amino acids 569-584, Figure 1), designated peptide 2 and RRKSLQSKETTAAGDS, peptide 3 (based on amino acids 616-631, Figure 1). Peptides were produced as previously published (76). Monoclonal antibodies to hIL16 (clone 14.1) were verified by western blotting with rhIL16 and shown to be neutralizing by inhibition of chemoattractant activity, IL2 receptor expression and human immunodeficiency virus (HIV)-1 repression in IL16-treated cells (68, 69). Furthermore, it is known that amino acid sequences of rhIL16 and rmIL16 are over 80% homologous and share several biological functions, including induction of chemotaxis with no apparent differences in cross-specificity (76).

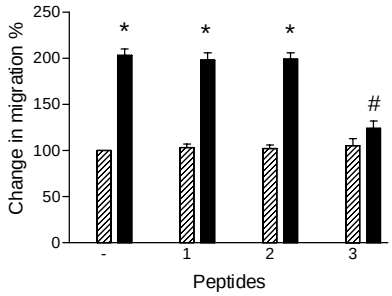
Cell isolation and chemotaxis assay

Murine splenocytes were isolated from euthanized BALB/c mice and maintained in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS and 100 units ml^{-1} of both penicillin and streptomycin (complete medium). Erythrocytes were lysed by suspension in one part complete medium to three parts Gey's solution. This mixture was incubated on ice for 2 min. The reaction was stopped by the addition of 10 parts complete medium and the cells were then washed twice in complete medium before use in experiments.
Chemotaxis was performed using a modified Boyden chamber assay as described previously (74, 75). Cells were suspended at $5x10^6$ ml^{-1} in complete medium and incubated for 15 min with or without the different peptides at a concentration of 5 μg ml^{-1}. A nitro-cellulose membrane with a pore size of 12 μm separated cells in the upper wells from control buffer or experimental supernatants in the lower wells. Supernatants in the lower wells consisted of rmIL16 (100 nM) or medium alone. Chambers were incubated at 37°C for 4h, then the membranes were removed, stained with hematoxylin and dehydrated by sequential washes in ethanol, propanol, and finally xylene. Cell migration was quantitated by light microscopy, counting the number of cells migrating below a depth of 50 μm. All samples were tested in duplicate, and four high power fields were examined in each duplicate. Chemotaxis induced by medium alone was normalized to 100%. In previous experiments it was shown that 5 μg ml^{-1} anti-IL16 antibody (clone 14.1) neutralizes chemotaxis induced by 0.1 nM of recombinant human IL16 protein.

Sensitization and challenge

Specified pathogen free male BALB/c mice (age 6-8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (The Netherlands). All experiments were approved by the animal care committee of the Utrecht University. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Active sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen free saline on alternate days. Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in saline) or saline aerosols for 5 min, on consecutive days (1 aerosol per day). The aerosols were generated with a jet nebulizer (Pari IS-2, Pari-

Figure 2



*The effects of different peptides on IL16-induced (100 nM) chemotaxis (black bars) was tested. Chemotaxis of mouse splenocytes was compared to complete medium (hatched bars,-) or to the effect of the different peptides without IL16 (hatched bars, 1, 2 or 3). Results are expressed as arithmetic average ± SEM. * (P<0.05) significantly different from control chemotaxis induced by medium or by the different peptides alone. # (P<0.05) significantly different from IL16-induced chemotaxis.*

Werk GmbH, Starnberg, Germany) connected to a plexiglas exposure chamber with a volume of 5 l in which a maximum of 6 animals was placed. Approximately 30 min before each antigen exposure, the mice were treated intranasally with peptides based on the predicted sequence of IL16 (76) after inhalation of a short lasting anesthetic (Halothane, ALBIC BV, Maassluis, The Netherlands). Peptides 1 and 3 were tested at a dose of 100 μg day^{-1}. In a different series of experiments peptides 2 and 3 were tested in higher doses (240 μg day^{-1}). The effects of local administration of anti-hIL16 or control antibodies were also determined. In this experiment animals were intranasally treated on the first and fifth day of the challenge period with either mIgG2a or anti-IL16 monoclonal antibodies (500 μg per mouse) at 30 min before the antigen or saline challenge.

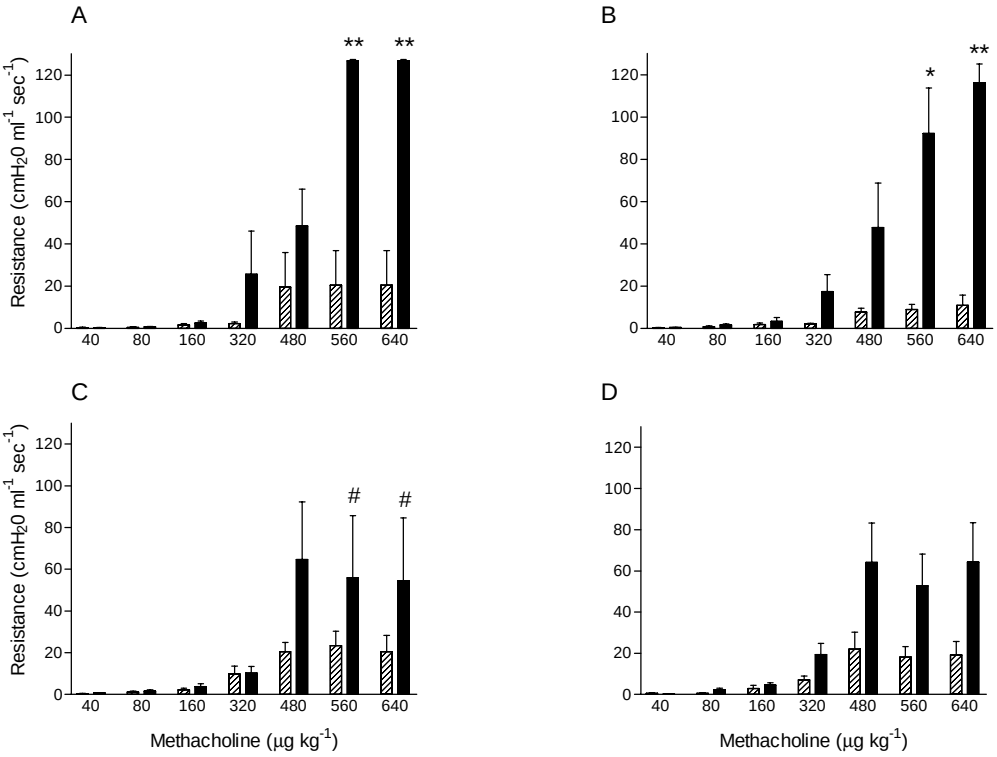
Airway responsiveness in vivo

Airway responsiveness was measured *in vivo* 24 h after the last aerosol exposure using a modified plethysmograph as described previously (177). In short: mice were anaesthetized by intraperitoneal injection of urethane (2 g kg^{-1}), and placed on a heated blanket (30°C). The trachea and jugular vein were cannulated. Spontaneous breathing of the animals was suppressed by i.v. injection of tubocurarine chloride (3.3 mg kg^{-1}). The tracheal cannula was attached to a ventilator (model 687, Harvard Apparatus, Southnatick, MA, USA). The mice maintain physiologic arterial blood gas parameters (data not shown). Changes in flow and pressure were measured using a plethysmograph coupled to pressure transducers (M45, Validyne Engineering Corp. Northridge, CA, USA). Lung resistance (R_L) was measured by quantitating $\frac{\Delta V}{\Delta P_t}$ (ΔP_t = change in tracheal pressure, ΔV = change in flow) at points of equal volume (70% tidal volume) using specific software (Model 6, Buxco Corp., Sharon, CT, USA). After the response had returned to baseline level (after at least 4 min), doses of methacholine ranging from 40 to 640 μg kg^{-1} were administered via the jugular catheter. For each dose of methacholine the increase in airway resistance was measured at its peak and expressed in cm H_2O ml^{-1} sec^{-1}. At least 6 mice were evaluated per experimental group.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in the same animals that were used for airway hyperresponsiveness measurements. Mice were lavaged 5 times through the tracheal cannula with 1 ml aliquots of pyrogen free saline at 37°C. The BAL cells were washed with cold phosphate-buffered saline (PBS, 400x*g*, 4°C, 5 min) and the pellet was resuspended in 200 μl cold PBS. The total number of BAL cells was counted by use of a Bürker-Türk chamber. For differential BAL cell counts cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Düdingen, Switzerland). After coding, all cytospin preparations were evaluated by one observer using oil immersion microscopy. Cells were identified and differentiated into mononuclear cells, neutrophils and eosinophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated.

Figure 3

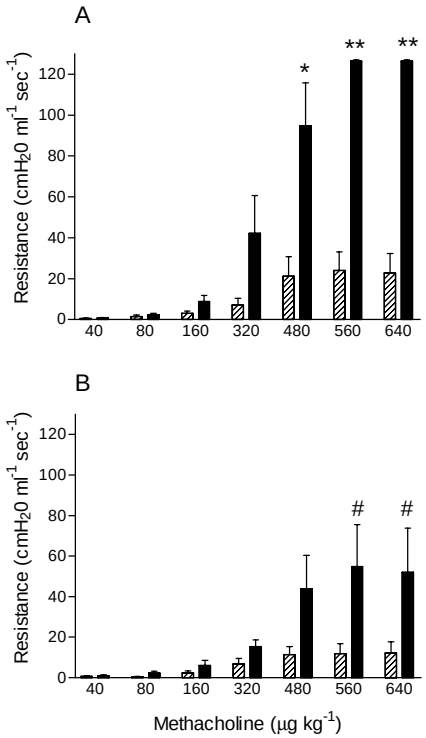


*Airway resistance after intravenous administration of methacholine in ovalbumin-sensitized mice at 24 h after the last challenge with saline (hatched bars) or ovalbumin (black bars) and intranasally treated daily during the challenge period with control peptides (100 µg day^{-1}, A or 240 µg day^{-1}, C). Furthermore, animals were treated with peptide 3 (100 µg day^{-1}, B or 240 µg day^{-1}, D). Results are expressed as arithmetic average ± SEM of 5-6 animals per group. * (P<0.05); ** (P<0.01) significantly different from saline-challenged animals. # (P<0.05) significantly different from control peptide-treated ovalbumin-challenged animals.*

Drugs and chemicals

Ovalbumin (chicken egg albumin crude grade V) and o-phenylenediamine were purchased from Sigma Chemical Company (St. Louis, MO, USA). Urethane and methacholine (acetyl-ß-methylcholine) were purchased from Janssen Chimica (Beerse, Belgium), tubocurarine chloride from Nogepha (The Netherlands) and Tween-20 from Merck (Darmstadt, Germany).

Figure 4



*Airway resistance after intravenous administration of methacholine in ovalbumin-sensitized mice at 24 h after the last challenge with saline (hatched bars) or ovalbumin (black bars) and i.n. treated on the first and fifth day of the challenge period with control antibody (mIgG2a, A) or antibodies to IL16 (500 µg per animal, B). Results are expressed as arithmetic average ± SEM of 5-6 animals per group. * (P<0.05); ** (P<0.01) significantly different from saline-challenged animals. # (P<0.05) significantly different from mIgG2a-treated ovalbumin-challenged animals.*

Statistical analysis

Total BAL cell number and the numbers of the various BAL cell types were tested with an analysis of variance (ANOVA). For cell types with low numbers, e.g. eosinophils or neutrophils, a Poisson distribution was assumed. Data on airway responsiveness were tested for significance using ANOVA followed by post-hoc comparison using Bonferroni. Data are expressed as arithmetic average ± SEM and a difference was considered to be significant when $P<0.05$. Statistical analyses were carried out using SPSS/PC$^+$ (SPSS inc. Chicago, IL).

Results

Chemotaxis assay

The effects of peptides 1, 2 and 3 were measured on rmIL16-induced mouse lymphocyte motility. Lymphocyte motility induced by medium alone was normalized to 100%. The IL16-induced chemoattractive activity amounted to 203±7%, which was significantly ($P<0.05$) inhibited by peptide 3 to 124±8% (Figure 2). Incubation of the target cells with either peptide 1 or 2 did not result in any detectable decrease in IL16-induced chemotaxis (Figure 2). Furthermore, peptides 1, 2 and 3 did not induce any chemotaxis by themselves when compared to chemotaxis induced by medium alone (Figure 2).

Airway resistance in vivo

Peptides 1 and 2 did not inhibit chemotaxis *in vitro* (Figure 2) and were therefore used as control peptides in the *in vivo* studies. Peptide 1 was used as control peptide at a dosage of 100 µg day^{-1}, whereas peptide 2 was used as control at a higher dose (240 µg day^{-1}). In animals treated daily intranasally with control peptides airway hyperresponsiveness after ovalbumin challenge was observed when compared with saline-challenged animals. In animals treated with control peptide (100 µg day^{-1}) airway resistance in ovalbumin-challenged animals was significantly ($P<0.01$) enhanced to 127±1 cm H_2O ml^{-1} sec^{-1}, as compared with saline-challenged animals (20±16 cm H_2O ml^{-1} sec^{-1}, Figure 3A). In animals treated with control peptide at a higher dose (240 µg day^{-1}) airway resistance in response to 640 µg kg^{-1} methacholine amounted to 116±9 cm H_2O ml^{-1} sec^{-1} after ovalbumin challenge, whereas in saline-challenged animals airway resistance amounted to 11±5 cm H_2O ml^{-1} sec^{-1} ($P<0.05$, Figure 3C). Treatment with peptide 3 (100 µg day^{-1}) however, significantly ($P<0.05$) reduced the ovalbumin-induced airway hyperresponsiveness with 57% to 54±30 cm H_2O ml^{-1} sec^{-1} at the highest dose of methacholine, when compared with ovalbumin-challenged control peptide-treated mice (Figure 3B). A higher dose of peptide 3

Table I: Number of inflammatory cells in lavage fluid

Treatment/ Challenge	Dosage (μg day^{-1})	Eosinophils (%)
Control/Sal	100	2±1
Control/Ova	100	42±11 (*)
Peptide 3/Sal	100	1±1
Peptide 3/Ova	100	54±10 (*)
Control/Sal	240	1±0
Control/Ova	240	14±2 (*)
Peptide 3/Sal	240	1±0
Peptide 3/Ova	240	23±5 (*)
mIgG2a/Sal	500	0±0
mIgG2a/Ova	500	14±9 (*)
anti-IL16/Sal	500	2±1
anti-IL16/Ova	500	21±8 (*)

*Percentage of eosinophils present in bronchoalveolar lavage fluid of ovalbumin-sensitized and saline (Sal) or ovalbumin (Ova)-challenged mice. Data are expressed as mean ± SEM. During the challenge period, the mice were intranasally treated on a daily basis with control peptide or peptide 3 (100 μg day^{-1}, 30 min before each aerosol challenge). In a different experiment mice were treated daily during the challenge period with either control peptide or peptide 3 (240 μg day^{-1}, 30 min before each aerosol challenge) or with either mIgG2a or antibodies to IL16 (500 μg mouse^{-1} on the first and fifth day of the challenge period). * (P<0.05) significantly different from saline-challenged animals.*

(240 μg day^{-1}) did not further reduce airway resistance to methacholine (64±19 cm H_2O ml^{-1} sec^{-1} at the highest dose of methacholine, Figure 3D). No significantly different effects were observed between all differently-treated saline-challenged animals.
In animals treated intranasally with mIgG2a and challenged with saline airway responses to the highest dose of methacholine (640 μg kg^{-1}) amounted to 23±10 cm H_2O ml^{-1} sec^{-1}, whereas in ovalbumin-challenged animals airway responses were significantly (*P*<0.01) enhanced to 126±1 cm H_2O ml^{-1} sec^{-1} (Figure 4A). Treatment of ovalbumin-challenged mice with antibodies to IL16 (500 μg at day 1 and 5) significantly decreased airway resistance with 59%, when compared to mIgG2a-treated ovalbumin-challenged animals. However, this airway resistance was still significantly (*P*<0.05) enhanced when compared to saline-challenged anti-IL16-treated animals (12±5 cm H_2O ml^{-1} sec^{-1}, Figure 4B).

Cellular infiltration into bronchoalveolar lavage fluid

No or hardly any eosinophils could be detected in BAL fluid derived from saline-challenged animals treated with the different peptides, mIgG2a or anti-IL16 (Table I). In all differently-treated animals ovalbumin challenge caused an infiltration of eosinophils into the BAL fluid (Table I). However, no significant effects of the different treatments of ovalbumin-challenged animals on this eosinophilic infiltration were detected. Furthermore, no significant differences in infiltration of mononuclear cells or neutrophils were observed between different treatment groups (data not shown). Finally, no differences were observed in total number of cells between the different treatment groups (data not shown).

Discussion

The study described in this report illustrates that peptide 3, which is based on the predicted amino acid sequence of hIL16 is capable of inhibiting rmIL16-induced chemotaxis of murine splenocytes. Peptide 3 also largely inhibits antigen-induced airway hyperresponsiveness in a murine model of allergic asthma, whereas eosinophilic infiltration is not significantly altered. Furthermore, we demonstrate that local administration of antibodies to IL16 also decreased airway hyperresponsiveness without affecting eosinophil infiltration, which is in agreement with previous experiments in which antibodies to IL16 were intraperitoneally administered (154). Peptides 1 and 2 were based on different C-terminal located regions of the predicted amino acid sequence of hIL16. These peptides have been demonstrated not to interfere with IL16-induced chemotaxis *in vitro* in this study or with OKT4 binding to CD4 in another study (76). Therefore we have used these two peptides as control peptides.

Recently, it has been observed that in antigen-exposed asthmatics as well as in our murine model for allergic asthma, IL16 expression in epithelial cells and presence of bioactive IL16 in BAL fluid is upregulated (26, 154). IL16 is a natural soluble ligand for CD4, which activates several CD4-associated second messengers and induces chemotaxis of $CD4^+$ cells, including T-lymphocytes, monocytes and eosinophils *in vitro* (29, 90). So far, no evidence exists on the induction of chemotaxis of $CD4^+$ cells *in vivo*. The amino acid sequence of peptide 3 is based on a hydrophilic C-terminal region of hIL16. This region of hIL16 is very likely to be exposed on the surface of the molecule (76). In this study we demonstrated that peptide 3 was capable of inhibiting rmIL16-induced chemotaxis of mouse splenocytes *in vitro*. In agreement herewith, it was shown in previous experiments that peptide 3 is capable of decreasing rhIL16 induced chemotaxis of human $CD4^+$ cells (76). Furthermore, peptide 3 can partially prevent the binding of monoclonal antibodies to CD4 (76). These data, together with the observation that IL16 uses CD4 as receptor, suggest that the C-terminal hydrophilic region of IL16 is involved in binding of CD4. Furthermore, these data suggest that peptide 3 interacts with CD4, thereby inhibiting cross-linking by IL16 and subsequent signaling.

Recently, it has been observed that intraperitoneal administration of antibodies to IL16 can partially inhibit airway hyperresponsiveness, whereas eosinophilia was not affected (154). In the present study it was demonstrated that inhibition of IL16 interaction with CD4 by peptide 3 has similar effects as inhibition of IL16-induced effects by intraperitoneal administration of anti-IL16. Furthermore, as described in this study, local administration of anti-IL16 also largely inhibits airway hyperresponsiveness without affecting eosinophil infiltration.

It has been demonstrated that IFNγ plays an important role in the induction of airway hyperresponsiveness in our murine model since administration of antibodies to IFNγ can inhibit development of airway hyperresponsiveness without affecting eosinophil numbers in BAL fluid (148). From that study it can thus be concluded that airway hyperresponsiveness and eosinophilia are not causally related in this model as has also been observed before in another murine model of allergic asthma (13). Production of IFNγ by human peripheral blood mononuclear cells can be detected after incubation *in vitro* with a combination of IL16 and IL2 (88). Therefore, if peptide 3 inhibits cross-linking of CD4 molecules by IL16 and subsequent local IFNγ production, it could be speculated that airway hyperresponsiveness is decreased, whereas eosinophilia is not affected. The same could be true for the effects of antibodies to IL16. In contrast, others have demonstrated that IFNγ is a potent down regulator of development of airway hyperresponsiveness in different animal models (204, 205). Therefore, at present no definite conclusions can be drawn on how IL16 plays a role in development of airway hyperresponsiveness in this murine model. Furthermore, if peptide 3 decreases airway hyperresponsiveness via prevention of IL16-CD4 interaction, it can also not be excluded that peptide 3 has direct effects on other $CD4^+$ cells, e.g. monocytes or dendritic cells.

In this study airway eosinophilia was not inhibited by intra-airway treatment with either anti-IL16 or peptide 3. Endogenous IL16 could be important for the induction of airway hyperresponsiveness via recruitment of $CD4^+$ cells, including eosinophils. However, intraperitoneal or local administration of antibodies to IL16 did not affect eosinophilia in this and other studies (154). From previous studies it is known that murine eosinophils do not express CD4 on their membrane as determined by FACS analysis (E.M. Hessel, personal communication). Furthermore, it has been described that IL16 does not prime or activate eosinophils *in vitro* (95). Finally, *in vivo* chemotaxis of $CD4^+$ cells, including eosinophils, by IL16 could not be detected (J.J. de Bie, unpublished observations) and therefore inhibition of chemotaxis of eosinophils or lymphocytes by peptide 3 or the antibodies to IL16 is not very likely.

Previously, it was demonstrated that inhibition of endogenously produced IL16 by intraperitoneal treatment with anti-IL16 inhibits production of antigen-specific IgE (154). In agreement herewith, local administration of anti-IL16 also inhibited the upregulation of antigen-specific IgE levels in serum after ovalbumin-challenge (data not shown). However, no effects of peptide 3 on IgE production were observed (data not shown). It could thus be speculated that peptide 3 has a lower stability or half-life than antibodies to IL16. However, further research is necessary to elucidate the exact mechanism by which antibodies to IL16 do inhibit IgE production whereas peptide 3 does not.

In conclusion, our data suggest that peptide 3, which is based on the predicted amino-acid sequence of IL16, can significantly inhibit airway hyperresponsiveness, whereas eosinophilia is not affected. Our results also show that treatment with peptide 3 has the same effects as antibodies to IL16, possibly via inhibition of interaction between IL16 and its receptor CD4. Therefore, peptide 3 could be useful as a lead compound in attempting to limit airway hyperresponsiveness via binding to CD4.

IL16 inhibits Antigen-induced Airway Hyperresponsiveness, Eosinophilia and Th2-type Cytokine Production in Mice

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Summary

Allergic asthma is characterized by chronic eosinophilic airway inflammation and by airway hyperresponsiveness to bronchoconstrictive stimuli. $CD4^+$ type-2 T cells are thought to regulate these allergic manifestations via production of cytokines such as IL4, IL5 and IL13. IL16 has been described as a natural soluble ligand for the CD4 molecule with immunosuppressive effects on $CD4^+$ T lymphocytes *in vitro*. However, little is known about the effect of IL16 on $CD4^+$ T lymphocyte mediated immune responses *in vivo*. In the present study, we examined the potential therapeutic effect of treatment with IL16 on antigen-induced allergic inflammation and airway hyperresponsiveness. Treatment with IL16 resulted in a complete inhibition of antigen-induced airway hyperresponsiveness as well as a strong decrease (>90%) in the number of eosinophils in bronchoalveolar lavage. Next, we determined whether these effects were mediated by modulation of $CD4^+$ T lymphocytes. First, it appeared that thoracic lymph node (TLN) cells from *in vivo* IL16-treated ovalbumin-challenged animals produced significantly less IL4 (77% reduction) and IL5 (85% reduction) when compared to vehicle-treated mice. Second, preincubation of TLN cells isolated from ovalbumin-challenged animals with IL16 *in vitro* induced a reduction in Th2 type cytokine production and proliferation. It can be concluded that IL16 is able to inhibit antigen-induced allergic airway responses, which is probably mediated by inhibition of Th2-type cytokine production. Thus, it can be speculated that IL16 may be of therapeutic importance in the treatment of allergic asthma.

Introduction

IL16 is released by $CD4^+$ T cells following mitogen, antigen or anti-CD3 stimulation (29). However, T-lymphocytes are not the only cellular source of IL16 since it has been shown that both eosinophils and epithelial cells can release bioactive IL16 (26, 81, 94). Messenger RNA for IL16 is constitutively expressed in $CD4^+$ and $CD8^+$ T cells and the biologically active protein is secreted from $CD8^+$ T cells upon stimulation with either histamine or 5-hydroxytryptamine (5-HT, 79, 80).
In previous studies we demonstrated that IL16 immunoreactivity is present in epithelial cells of ovalbumin-sensitized mice but not in those from naïve animals (154). Upon antigen-challenge, an increase in epithelial IL16 expression was observed, as well as IL16 expression in inflammatory cell infiltrates (154). Moreover, presence of IL16 bioactivity in bronchoalveolar lavage (BAL) fluid was detected. Interestingly, in patients with allergic asthma, IL16 expression in epithelial cells and presence of bioactive IL16 in BAL fluid after antigen challenge has also been observed (25, 26).
It has been clearly demonstrated that IL16 uses the CD4 molecule as its receptor and exerts diverse functional activities via CD4 signaling in lymphocytes, monocytes, and eosinophils (29, 90). In human lymphocytes CD4 ligation by IL16 leads to activation of p56lck and a rise in intracellular IP_3 and subsequent mobilization of Ca^{2+} (29, 90). The functional consequences of IL16 in resting $CD4^+$ T cells are the upregulation of IL2R and HLA-DR expression as well as a shift from G_0 to G_{1a} (29). In addition, IL16 synergistically stimulates proliferation of $CD4^+$ T cells when combined with IL2 (76). Finally, it has been demonstrated that IL16 is a chemoattractant for $CD4^+$ cells *in vitro* (29). In contrast to these stimulatory effects, IL16 has been found to inhibit proliferation of $CD4^+$ lymphocytes following activation with antigen or anti-CD3 (70). Furthermore, IL16 has been reported to inhibit mixed lymphocyte reactions of human peripheral blood mononuclear cells (69). Thus, IL16 appears to have both stimulatory and suppressive effects on T-lymphocytes *in vitro*.
Since IL16 has immunosuppressive actions *in vitro*, we were interested in the potential beneficial effects of IL16 on the development of airway symptoms in a murine model of allergic asthma. Here, we demonstrate that treatment with IL16 during the antigen challenge period of previously sensitized mice, inhibits allergic airway inflammation and hyperresponsiveness. Furthermore, we show that these effects are accompanied by a decreased production of Th2-type cytokines by TLN cells derived from IL16-treated animals. Moreover, we found that IL16 can inhibit cytokine production by lymphocytes upon antigen-specific stimulation *in vitro*.

Materials and Methods

Sensitization and challenge

Specified pathogen free male BALB/c mice (age 6-8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (Utrecht, The Netherlands). All experiments were approved by the animal care committee of the Utrecht University. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen free saline on alternate days. Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in saline) or to 8 saline aerosols for 5 min, on consecutive days (1 aerosol per day). The aerosols were generated with a jet nebulizer (Pari IS-2, Pari-Werk GmbH, Starnberg, Germany) connected to a plexiglas exposure chamber (5 l). Before each aerosolized antigen challenge the mice were injected intraperitoneally with 1 μg rmIL16 or rhIL16 (in 0.25 ml saline) or with vehicle. From literature data and our observations *in vitro* as well as *in vivo* it is known that both structure and biological effects of IL16 of either murine or human origin are well conserved (76).

Airway responsiveness in vivo

Airway responsiveness was determined in animals treated with either vehicle or rhIL16 by measuring airway resistance to intravenously administered methacholine as previously published (177). In short, at 24h after the last aerosol challenge animals were anaesthetized by intraperitoneal injection of urethane (2 g kg^{-1}) and trachea and jugular vene were intubated with small catheters. Spontaneous breathing of the animals was suppressed by intravenous injection of tubocurarine chloride (3.3 mg kg^{-1}). Subsequently, animals were attached to a ventilator and changes in resistance were measured by use of a plethysmograph, coupled to a pressure transducer (M45, Validyne Engineering Corp. Northridge, CA, USA). By use of a pulmonary mechanics analyzer (Model 6, Buxco Corp., Sharon, CT, USA), lung resistance (R_L) was measured by quantitating $\Delta P_t \bullet \Delta V^{-1}$ (ΔP_t = change in tracheal pressure, ΔV = change in flow) at points of equal volume (70% tidal volume). Changes in tracheal pressure were measured using a pressure transducer connected to the tracheal ventilation cannula, changes in flow were measured by use of a pressure transducer connected to the plethysmograph.
In experiments in which effects of rmIL16 on airway responsiveness were measured barometric plethysmography was used (166, Buxco Corp.). Before measuring pressure differences between the chamber containing the animal and a reference chamber, pressure differences, measured with a pressure transducer (M45), were calibrated using a steady flow. Differences in pressure between box and reference chamber are caused by changes in volume during a respiratory cycle. Baseline PENH values were established by averaging for 3 minutes (10 valid breaths were averaged per PENH value). Mice were then subjected to a saline aerosol, followed by increasing concentrations of nebulized methacholine (1.5-50 mg ml^{-1}) for 3 minutes, followed by a measuring period of 3 minutes after each aerosol (protocol adapted from Hamelmann *et al.*, 1997, 166). Aerosol was generated using a jet-nebulizer (Pari IS-2).

Bronchoalveolar lavage

Bronchoalveolar lavages were performed in the same animals that were used for airway responsiveness measurements. Mice were lavaged 5 times through the tracheal cannula with 1 ml aliquots of pyrogen free saline at 37°C. The BAL cells were washed with cold PBS (400x*g*, 4°C, 5 min) and resuspended in 200 µl. The total number of BAL cells was counted by use of a Bürker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential BAL cell counts cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Düdingen, Switzerland). After coding, all cytospin preparations were evaluated by one observer using oil immersion microscopy. Cells were identified and differentiated into mononuclear cells, neutrophils and eosinophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated.

Ovalbumin-specific IgE ELISA

Ninety-six well microplates (Nunc A/S, Roskilde, Denmark) were coated with 2 µg ml^{-1} chimeric fusion protein of the human high affinity IgE receptor and IgG (FcεR1-IgG) diluted in PBS. After 24h incubation at 4°C the plates were washed 5 times with PBS supplemented with 0.05% Tween-20 (PBT). Thereafter, the plates were blocked with ELISA buffer (2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.5% BSA, 0.05% Tween-20, pH 7.2) for 1h. Diluted serum samples were added to the plates and left to incubate at room temperature for 2h. Serial dilutions were made of the standard, which was obtained by intraperitoneal immunization of mice with ovalbumin and arbitrarily assigned a value of 1,000 units ml^{-1} ovalbumin-specific IgE. After washing, 10 µg ml^{-1} of ovalbumin in ELISA buffer was added to each well and after incubation washing procedures were repeated. Horse-radish peroxidase-conjugated goat anti-ovalbumin antibody was diluted in ELISA buffer and added to each well. Incubation was continued for 1h followed by washing procedures. 10 mM OPD substrate solution was added and the reaction was ended at the appropriate time (after approximately 10 min) by adding H_2SO_4 (4M). OD was read at λ 492 nm using a Titertek Multiskan (Flow Labs, Irvine, UK).

Cytokine production by thoracic lymph node (TLN) cells in vitro

TLN cells were isolated as described previously (206) from animals treated *in vivo* with either vehicle or IL16 and challenged with saline or ovalbumin. A single cell suspension was prepared and resuspended in RPMI (supplemented with 10% FCS, 5 x 10^{-5} M 2-mercaptoethanol, 50 mg l^{-1} gentamycine and 1% glutamax). After isolation these TLN cells were cultured in the presence or absence of ovalbumin (40 μg ml^{-1}) at 1 x 10^6 cells per ml (in 100 μl). After 5 days, supernatants were harvested and stored at -20°C for cytokine analysis. IL4, IL5 and IFNγ levels in supernatants of the TLN cell cultures were determined with ELISA according to the manufacturer's instructions (Pharmingen, San Diego, CA, USA).

Preincubation of TLN cells with IL16 in vitro

To determine the effect of IL16 on antigen-specific stimulation of lymphocytes *in vitro*, TLN cells were isolated from ovalbumin-sensitized and ovalbumin-challenged mice. Cells (1 x 10^6 per ml in a total volume of 100 μl) were preincubated for 1h with medium or IL16 (0.01-1 μg ml^{-1}). Thereafter, cells were cultured in the presence or absence of ovalbumin (40 μg ml^{-1}) and after 5 days supernatant was harvested for cytokine analysis. IL16 remained present during this culture period. Separate cultures were used to determine proliferation. [^{3}H]thymidine (0.3 μCi per well, Amersham, Freiburg, Germany) was added to each well 48h after stimulation and left to be incorporated. After 18h, cells were harvested and [^{3}H]thymidine uptake was determined by liquid scintillation counting.

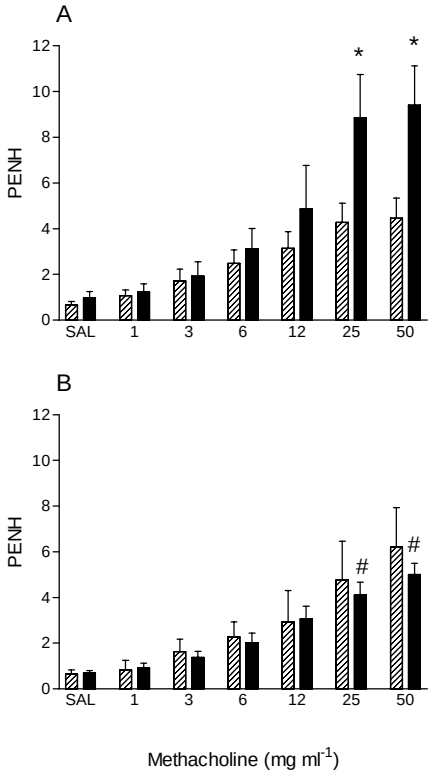
Chemicals

Ovalbumin (chicken egg albumin crude grade V), o-phenylenediamine and 3-amino-1,2,4-triazole were purchased from Sigma Chemical Company (St. Louis, MO, USA), recombinant human FcεR1-IgG, horse-radish peroxidase-conjugated goat anti-ovalbumin antibody and ovalbumin-IgE reference standard were generously provided by Dr. P.M. Jardieu (Genentech Inc., South San Francisco, CA, USA). Urethane and methacholine (acetyl-ß-methylcholine) were purchased from Janssen Chimica (Beerse, Belgium), tubocurarine chloride from Nogepha (The Netherlands), Tween-20 from Merck (Darmstadt, Germany). rhIL16 and rmIL16 used in the cultures were obtained from Preprotech (The Netherlands) and Pharmingen (San Diego, CA, USA), respectively, rhIL16 and rmIL16 used *in vivo* were obtained from Dr. W.W. Cruikshank (Pulmonary Center, Boston, MA, USA) and produced as previously published (76).

Data analysis

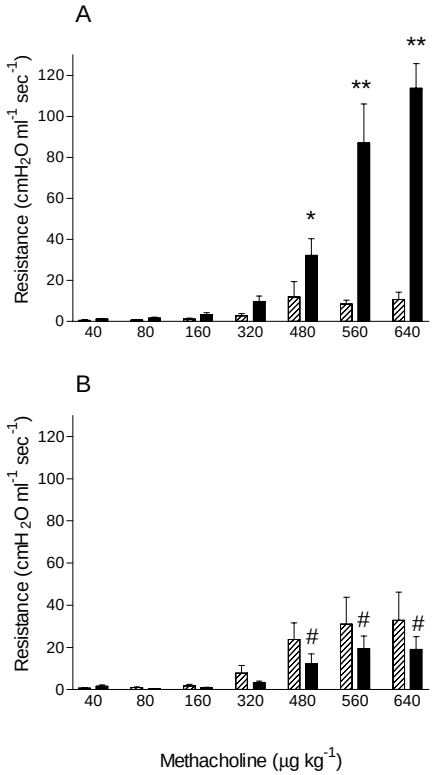
Comparisons between different treatment groups were made using an ANOVA followed by post-hoc comparisons using Bonferroni. Data are expressed as arithmetic average ± SEM and a difference was considered to be significant when $P<0.05$. Data on proliferation or cytokine levels were analyzed using a paired student's *t*-test. Total BAL cell numbers and the number of various cell types were tested with an analysis of variance (ANOVA). For cell types with low numbers in control animals (i.e. neutrophils and eosinophils) a Poisson distribution was assumed. Statistical analyses were carried out using SPSS/PC$^+$, version 4.0.1 (SPSS Inc., Chicago, IL).

Figure 1



*Increase in PENH values after exposure to nebulized saline or increasing concentrations of methacholine (ranging from 1.5 to 50 mg ml^{-1}) in ovalbumin-sensitized mice at 24 h after the last challenge with saline (hathed bars) or ovalbumin (black bars) and treated daily i.p. during the challenge period with vehicle (A) or rmIL16 (B). Results are expressed as arithmetic average ± SEM of 5-6 animals per group. * (P<0.05) significantly different from saline-challenged animals. # (P<0.05) significantly different from vehicle-treated ovalbumin-challenged animals.*

Figure 2



*Increase in airway resistance after exposure to increasing intravenously administered dosages of methacholine (ranging from 40 to 640 µg kg^{-1}) in ovalbumin-sensitized mice at 24 h after the last challenge with saline (hatched bars) or ovalbumin (black bars) and i.p. treated daily during the challenge period with vehicle (A) or rhIL16 (B). Results are expressed as arithmetic average ± SEM of 6-7 animals per group. * (P<0.05); **(P<0.01) significantly different from saline-challenged animals. # (P<0.05) significantly different from vehicle-treated ovalbumin-challenged animals.*

Results

Effect of treatment with IL16 on airway hyperresponsiveness in vivo

In vehicle-treated ovalbumin-challenged mice the increase in airway responsiveness after exposure to aerosolized methacholine was significantly potentiated when compared to saline-challenged animals (Figure 1A). At the highest concentration of methacholine (50 mg ml^{-1}) PENH value measured in saline-treated animals was 4.5±0.9 whereas in ovalbumin-challenged animals PENH value amounted to 9.4±1.7 (P<0.05). Administration of rmIL16 during the aerosol challenge-period completely prevented (P<0.05) ovalbumin-induced airway hyperresponsiveness (Figure 1B). At the highest concentration of methacholine the PENH value in rmIL16-treated ovalbumin-challenged animals was 5.0±0.5, whereas PENH value in saline-challenged mice was 6.2±1.7.
In experiments in which we determined the effect of rhIL16 we measured airway responsiveness to intravenously administered methacholine (ranging from 40 to 640 μg kg^{-1}). In vehicle-treated ovalbumin-challenged mice the increase in airway resistance after intravenous administration of methacholine was significantly (P<0.01) potentiated when compared to saline-challenged animals (Figure 2A). Treatment with rhIL16 significantly (P<0.05) inhibited development of ovalbumin-induced airway hyperresponsiveness to methacholine (Figure 2A and B).

Effect of treatment with IL16 on number of eosinophils in BAL

Ovalbumin challenge of vehicle-treated animals resulted in a significant (P<0.05) increase in the number of eosinophils in BAL fluid compared to vehicle-treated saline-challenged mice (162±85 x 10^3 vs 0.4±0.3 x 10^3 cells, respectively, Figure 3A). Treatment with rmIL16 during the aerosol challenge period resulted in a significant (P<0.05) decrease of ovalbumin-induced eosinophil infiltration into BAL fluid compared to vehicle-treated ovalbumin-challenged animals (13.9±7.5 x 10^3 cells, Figure 3A). No significant differences in infiltration of mononuclear cells or neutrophils were observed between different groups of animals. Furthermore, no differences were observed in total numbers of cells that were present in BAL fluid (Table I). A significant (P<0.05) inhibition of antigen-induced eosinophil infiltration was also observed when rhIL16 (1 μg kg^{-1}) was administered during the challenge period (Figure 3B).

Table I: Absolute numbers of different cells in BAL fluid

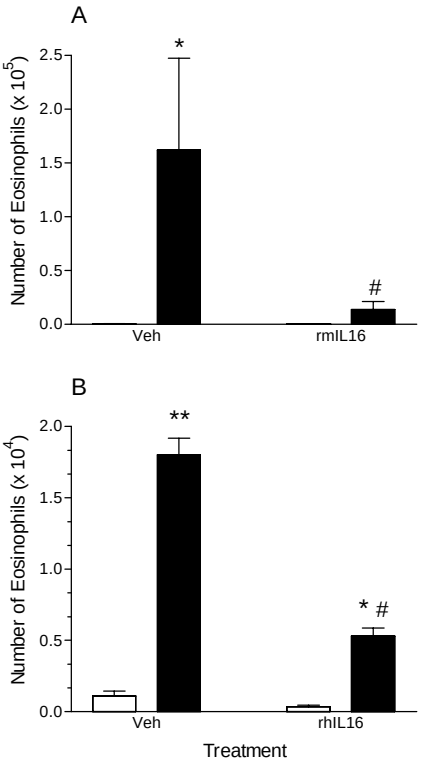
Treatment/ challenge	Mononuclear cells x 10^5	Neutrophils x 10^2	Total number of cells x 10^5
Veh/Sal	3.6±0.6	6.3±3.3	3.6±0.6
Veh/Ova	4.9±1.6	37.7±29.1	6.6±2.2
IL16/Sal	3.5±0.5	2.8±1.8	3.5±0.5
IL16/Ova	4.0±0.5	7.5±3.2	4.1±0.5

Animals were treated i.p. with either vehicle (Veh) or rmIL16 (1 μg day^{-1}), before each ovalbumin (Ova) or saline (Sal) aerosol. Data are expressed as arithmetic average ± SEM of at least 6 animals per group.

Effect of treatment with IL16 on ovalbumin-specific IgE levels in serum

Ovalbumin challenge of vehicle-treated animals resulted in an almost 5-fold increase (P<0.05) in serum levels of ovalbumin-specific IgE when compared to saline-challenged mice (745±209 vs 174±55 units ml^{-1} respectively, n=5). Ovalbumin challenge of rhIL16-treated animals also resulted in a significant (P<0.05) increase of serum IgE levels when compared to saline-challenged animals (768±50 vs 271±84 units ml^{-1}, respectively, n=5). No significant differences were observed between serum IgE levels of vehicle or rhIL16-treated animals challenged with either saline or

Figure 3



*Number of eosinophils in BAL fluid derived from ovalbumin (black bars) or saline-challenged (open bars) animals after i.p. treatment with vehicle (VEH) or rmIL16 (A) and after i.p. treatment with either vehicle (VEH) or rhIL16 (B). Results are expressed as arithmetic average ± SEM of at least 6 animals per group. * (P<0.05); ** (P<0.01) significantly different from saline-challenged animals). # (P<0.05) significantly different from vehicle-treated ovalbumin-challenged animals.*

ovalbumin.

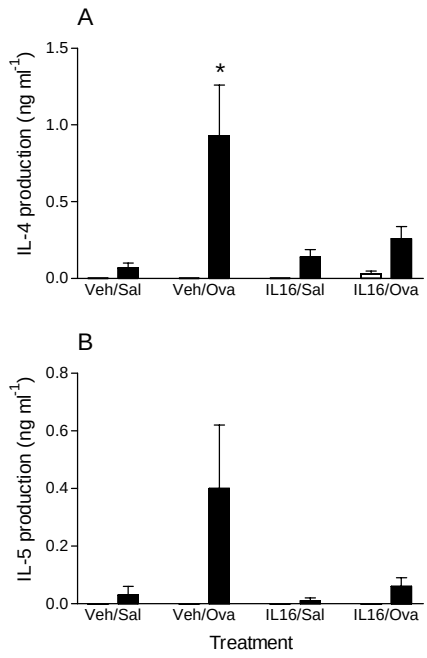
Effect of treatment with IL16 in vivo *on antigen-specific cytokine production* in vitro

No significant differences were observed in the total number of cells derived from lymph nodes from either vehicle or IL16-treated saline or ovalbumin-challenged animals (data not shown). TLN cells, derived from different treatment groups, were cultured in the presence or absence of ovalbumin. After five days of culturing, supernatants were harvested and the presence of IFNγ, IL4 and IL5 was determined by ELISA. No significant differences in IFNγ levels were detected in supernatant of TLN cells derived from vehicle-treated, saline or ovalbumin-challenged animals, cultured *in vitro* in the presence or absence of ovalbumin (data not shown). Similarly, *in vivo* administration of rmIL16 during the aerosol challenge period did not significantly change IFNγ production (detection limit 0.3 ng ml^{-1}) in TLN cultures (data not shown). TLN cells derived from vehicle-treated ovalbumin-challenged animals produced more IL4 (P<0.05) and IL5 after culturing in the presence of antigen (0.93±0.33 ng ml^{-1} and 0.40±0.22 ng ml^{-1}, respectively) when compared to cells derived from saline-challenged animals (0.07±0.03 ng ml^{-1} and 0.03±0.03 ng ml^{-1}, respectively, Figure 4A and B). *In vivo* treatment with rmIL16 during the aerosol challenge period with ovalbumin largely inhibited the production of IL4 (by 72%) and IL5 (by 85%, to 0.26±0.08 ng ml^{-1} and 0.06±0.03 ng ml^{-1}, respectively) after stimulation with ovalbumin *in vitro* (Figure 4A and B). No differences were observed in IL4 and IL5 production by TLN cells between saline-challenged animals, treated with rmIL16 (0.14±0.05 ng ml^{-1} and 0.01±0.01 ng ml^{-1}, respectively) or with vehicle (0.07±0.03 ng ml^{-1} and 0.03±0.03 ng ml^{-1}, respectively, Figure 4A and B). When cultured in the absence of ovalbumin hardly any IL4 or IL5 could be detected in supernatant of TLN cultures (Figure 4A and B).

Effect of preincubation with IL16 on TLN cells in vitro

Antigen-specific restimulation *in vitro* of TLN cells derived from ovalbumin-sensitized and challenged animals resulted in an increase in IL4 production when compared to cells cultured with medium alone (Figure 5A). Preincubation with rmIL16 dose-dependently decreased this IL4 production up to 56% at 1 μg ml^{-1}. IL5 production was also enhanced (P<0.05) by ovalbumin stimulation *in vitro* when compared to cells cultured with medium alone (Figure 5B). Preincubation of TLN cells *in vitro* with rmIL16 dose-dependently decreased (P<0.05) ovalbumin-induced IL5 production up to 77% at 1 μg ml^{-1} (Figure 5B). TLN cells stimulated with ovalbumin *in vitro* displayed a significant (P<0.05) increase in proliferation when compared to cells cultured without ovalbumin (Figure 5C). Preincubation of

Figure 4



Cytokine production by thoracic lymph node cells isolated from ovalbumin-sensitized animals challenged with saline (Sal) or ovalbumin (Ova). Animals were treated i.p. during the challenge period with either vehicle (Veh) or rmIL16 in vivo. TLN cells were restimulated in vitro *in the absence (open bars) or presence of ovalbumin (black bars). Hardly any cytokine production was detectable in supernatant of TLN cells cultured in the absence of ovalbumin. Depicted are IL4 (A) and IL5 production (B). Results are expressed as arithmetic average ± SEM of 5-6 animals per group. * (P<0.05) significantly different from vehicle-treated saline-challenged animals.*

TLN cells with rmIL16 (1 μg ml^{-1}) significantly (P<0.05) decreased *in vitro* proliferation with 55% after stimulation with ovalbumin (Figure 5C). Culturing TLN cells with IL16 in the absence of ovalbumin did not induce IL4 or IL5 production or proliferation (data not shown). Similar inhibitory effects on antigen-induced cytokine production or proliferation of TLN cells were observed after incubation with rhIL16 instead of rmIL16 (data not shown).

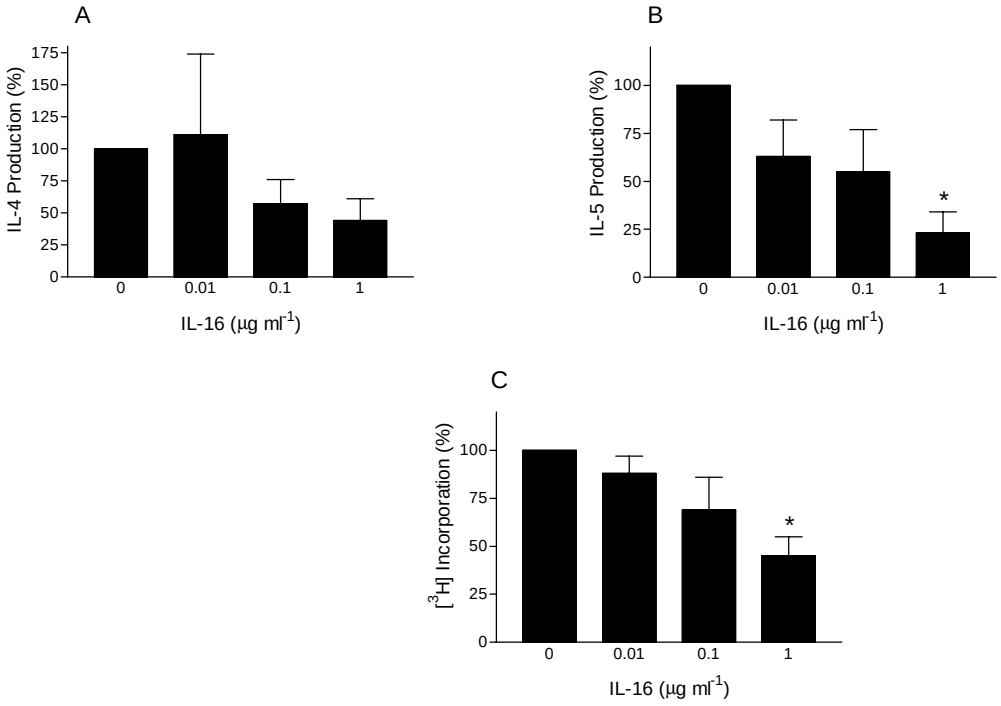
Discussion

In this study we are the first to demonstrate that IL16 administration during the antigen-challenge period has potent immunosuppressive effects in a murine model of allergic asthma. Intraperitoneal as well as intranasal (rhIL16, 1 μg day^{-1}, 63% reduction, data not shown) IL16 administration decreased antigen-induced eosinophil infiltration but did not affect upregulation of antigen-specific IgE levels in serum. Furthermore, treatment with IL16 inhibited development of airway hyperresponsiveness. The beneficial effects of IL16 appears to be caused by inhibition of proliferation and cytokine production of antigen-specific type-2 lymphocytes.

Several studies have demonstrated via depletion of $CD4^+$ T cells in animal models that these lymphocytes are crucial for the development of asthma-like features, including airway hyperresponsiveness and eosinophilic inflammation (207-209)**.** In addition, using adoptive transfer experiments it has been clearly demonstrated that reconstitution with solely $CD4^+$ T cells is sufficient to restore antigen-induced airway hyperresponsiveness, eosinophilia and goblet cell hyperplasia (210-212). It is now widely accepted that type-2 $CD4^+$ T cells are also important for inflammatory cell infiltration and airway hyperresponsiveness in patients with allergic asthma. Since IL16 uses CD4 as its receptor and exerts its action via cross-linking of several CD4 molecules (29), the $CD4^+$ T lymphocyte is a likely target for the beneficial effects of IL16.

Type-2 $CD4^+$ T cells are characterized by production of cytokines such as IL4, IL13 and IL5 upon antigen-specific stimulation (8, 20). In murine models it has been shown that IL4 is essential for antigen-induced airway hyperresponsiveness, eosinophilia and increases in goblet cell numbers since receptor blocking antibodies can decrease these phenomena (14). Furthermore, it has been demonstrated in IL4 deficient mice that antigen-specific IgE, eosinophils and airway hyperresponsiveness were absent (213). IL4 has also been shown to play a critical role in recruitment of Th2 cells to the lung (212). Depletion of IL5 by monoclonal antibodies completely abolished airway hyperresponsiveness and eosinophilia in an animal model of allergic asthma (12). Similar observations were made when IL5 deficient mice were used (16). *Vice versa*, intranasal administration of IL5 to mice leads to eosinophil infiltration followed by the development of airway hyperresponsiveness and an increase in mucosal

Figure 5.



*Thoracic lymph node (TLN) cells from ovalbumin-sensitized and challenged animals were cultured in the presence of ovalbumin. Results are presented as a percentage of the response of untreated TLN (0) cells in the presence of ovalbumin (100%). TLN cells cultured in the presence of ovalbumin were preincubated with increasing doses of rmIL16 (ranging from 0-1 µg ml^{-1}). Depicted are IL4 (A), IL5 production (B) and proliferation of TLN cells (C). In supernatant harvested from cells cultured in the absence of ovalbumin, no IL4 or IL5 production by TLN cells could be detected (data not shown). Proliferation in the absence of ovalbumin amounted to 17±1 %, when compared to cells cultured in the presence of ovalbumin (100%). Results are expressed as arithmetic average ± SEM of four separate experiments. * (P<0.05) significantly different from the response of untreated TLN cells cultured in the presence of ovalbumin.*

exudation (214). In contrast, others did not find a role for IL5 in development of antigen-induced airway hyperresponsiveness (13, 148). Recent data suggest that the Th2-type cytokine IL13 could also play an important role in the pathology of asthma (20).

At present, nothing is known about the effect of IL16 on type-2 cytokine production by T lymphocytes. In this study, we demonstrate that treatment with IL16 *in vivo* inhibits antigen-stimulated production of IL4 and IL5 by lymphocytes isolated from lung draining lymph nodes. Moreover, preincubation of TLN cells with IL16 *in vitro* also inhibits antigen-specific Th2-type cytokine production as well as proliferation. No significant differences in IFNγ levels upon antigen restimulation could be detected in TLN cultures either from *in vivo* IL16-treated animals or from TLN cultures which were *in vitro* preincubated with IL16 (data not shown). This strongly suggests that IL16 does not induce a shift from a Th2-type cytokine profile towards production of Th1-type cytokines by $CD4^+$ T cells. However, based on these data we can not rule out an effect by IL16 on IFNγ production. Furthermore, our data strongly support the concept that the $CD4^+$ T cells is a target cell for the beneficial effects of IL16 on manifestations of allergic asthma in the

model. However, we can not exclude that IL16 exerts its *in vivo* immunosuppressive actions on other CD4-expressing cells such as monocytes (96).
In our study, antigen-specific IgE production was not inhibited by administration of IL16, whereas IL4 production by TLN cells was decreased. This seems to be in contradiction with the essential role of IL4 in IgE-synthesis by B-lymphocytes (11). However, it has been shown that after a second encounter with antigen, upregulation of immunoglobulin E production by B-cells is less dependent on cognate B-T cell contact and on $CD4^+$ T-cell derived cytokines such as IL4 (215). Therefore IL4 could be involved mainly in the induction of isotype class switching to IgE production by B-cells during sensitization. Moreover, it has been demonstrated that IL4-independent class switch to IgE in the mouse can occur (216).
In agreement with our data on the suppressive effects of IL16 on T-cell activation *in vitro*, IL16 has been shown to suppress the mixed lymphocyte reaction (69) as well as antigen or anti-CD3-induced proliferation of human $CD4^+$ T cells *in vitro* when added prior to stimulation (70). These down-regulatory actions of IL16 are consistent with the inhibitory actions of other CD4 binding molecules including multivalent HIV-1 gp120 or CD4-antibodies (41, 202). Both monoclonal antibodies to CD4 and gp120 have been shown to induce unresponsiveness after antigen-specific or polyclonal stimulation of $CD4^+$ T cells *in vitro* (45, 217). Furthermore, non-depleting antibodies to CD4 can cause T cell unresponsiveness *in vivo*, thereby preventing graft rejection (134, 136). It has also been demonstrated that rheumatoid arthritis can be inhibited when non-depleting anti-CD4 antibodies are used in a murine model (137). With the present study, we are the first to show that a natural soluble ligand for CD4 also has immunosuppressive actions *in vivo* as well as *in vitro*. Whether the mechanism of action is similar to that of antibodies to CD4 remains to be elucidated.
One of the mechanisms that could account for the observed antigen-specific unresponsiveness after treatment with IL16 could be that TCR signaling is impaired by CD4 ligation. From literature data it is known that cross-linking of CD4, e.g. by gp120, can inhibit subsequent T cell activation via the TCR/CD3 complex (127). Binding of CD4 molecules by IL16 leads to the activation of p56lck (101). p56lck plays a role in TCR signaling via recruitment and subsequent phosphorylation of ZAP70, after TCR ζ-chain phosphorylation (141). Therefore, it has been speculated that when CD4 is cross-linked independently from TCR activation, subsequent antigen-specific TCR stimulation is inhibited since no p56lck is present to provide help for TCR/ZAP70 phosphorylation (141, 218). Besides ZAP70, p56lck activation also leads to p120 phosphorylation, which is implicated in T cell activation (131). Thus, after CD4 ligation, prior to TCR/antigen interaction, the signaling might be impaired (219), which could explain the immunosuppressive effects of IL16 on antigen-induced symptoms *in vivo* as well as *in vitro*.
Another explanation for the observed decrease in proliferation and cytokine production upon antigen-specific stimulation could be the induction of apoptosis. In several studies it has been observed that CD4 engagement by either antibodies to CD4 or gp120 induces Fas-dependent cell death (122, 220). Similarly, induction of Fas-dependent apoptosis could explain our observations that IL16 has immunosuppressive effects. However, others have reported that preincubation of $CD4^+$ T cells with IL16 *in vitro* does not lead to increased CD95 expression or subsequent apoptotic cell death (70) and thus it is very unlikely that the IL16-mediated effects are caused by this mechanism. Furthermore, recent data clearly demonstrated that IL16 actually protects cells against activation-induced cell death (92).
Previously, we have demonstrated that biologically active IL16 is present in BAL fluid of sensitized mice early after antigen-challenge (154). It could be speculated that this endogenously produced IL16 also has immunosuppressive effects in our animal model. However, administration of anti-IL16 during the challenge period partially decreased bronchial hyperresponsiveness whereas eosinophilic infiltration was not affected (154). These data argue against an immunosuppressive role for endogenously produced IL16 *in vivo*. However, this discrepancy with our present findings can be explained by the observation that endogenously secreted bioactive IL16 can only be detected after antigen exposure whereas in this study IL16 was injected prior to the first ovalbumin inhalation. Furthermore, the amount and localization of exogenously administered or endogenously produced IL16 are probably different.
As already mentioned, IL16 is a potent inducer of $CD4^+$ cell chemotaxis *in vitro*, including T cells, eosinophils and monocytes (74, 90). Therefore, it can be argued that the inhibitory effects of IL16 on airway hyperresponsiveness and eosinophilia are caused by an infiltration of $CD4^+$ cells into the

intraperitoneal cavity instead of airway tissues. However, when IL16 was administered intranasally instead of intraperitoneally during the aerosol challenge period, similar effects were observed on eosinophil infiltration (data not shown). Moreover, intraperitoneal administration of IL16 did not induce any detectable accumulation of eosinophils into the peritoneal cavity, nor did intratracheal administration of IL16 induce any increase in number of mononuclear cells or eosinophils (data not shown). Therefore, it is not very likely that IL16 is a potent inducer of $CD4^+$ cell chemotaxis *in vivo*. This observation is further sustained by the fact that administration of antibodies to IL16 do not have any effect on the number of eosinophils or mononuclear cells in BAL fluid after antigen-challenge in this murine model (154).

In conclusion, we are the first to demonstrate that IL16, a natural CD4 ligand, has immunosuppressive effects *in vivo* on both antigen-induced allergic inflammatory responses as well as airway hyperresponsiveness. Although the precise mechanism remains to be elucidated, we demonstrate that *in vivo* immunosuppressive effects of IL16 are associated with inhibition of Th2-type cytokine production. It is therefore tempting to speculate about the potential therapeutic effects of IL16 in allergic asthma and other $CD4^+$ T cell-mediated diseases.

Effects of IL16 on stimulation and differentiation of lymphocytes *in vitro*

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Summary

IL16 is the only known soluble ligand for CD4 that is endogenously produced. In a previous study we observed that treatment with IL16 completely inhibited development of antigen-induced airway hyperresponsiveness to methacholine whereas infiltration of eosinophils was largely decreased in a murine model of allergic asthma. Furthermore, *in vivo* treatment with IL16 reduced IL4 and IL5 production by TLN cells upon antigen-specific restimulation *in vitro*.
In the present study we examined the effects of CD4 cross-linking by antibodies to CD4 or IL16 on T cell responses *in vitro*. First, we determined the effects of either IL16 or antibodies to CD4 on proliferation of polyclonally or antigen-specifically stimulated T cells. Secondly, we examined the effect on cytokine production by lymphocytes isolated from antigen-sensitized and challenged animals. Thirdly, since the CD4 molecule has been shown to play a role in T cell differentiation, we determined effects of antibodies to CD4 or IL16 in priming cultures of naive T cell receptor transgenic (DO11.10) $CD4^+$ T cells towards effector cells that produce a Th0, Th1 or a Th2-type cytokine profile upon restimulation with antigen.
In this study we clearly demonstrate that CD4 cross-linking by either IL16 or antibodies to CD4 inhibits anti-CD3-induced proliferation of lymphocytes. Furthermore, antigen-induced proliferation and production of Th2-type cytokines, such as IL4, IL5 and IL10 are also inhibited by CD4 cross-linking. Finally, we demonstrate that the presence of IL16 or antibodies to CD4 in priming cultures of DO11.10 $CD4^+$ T cells display a decreased capacity to produce IL4 and IL5 upon restimulation, whereas production of IL10 and IFNγ are not altered. These *in vitro* results provide us with novel insights in the mechanisms by which IL16 inhibits antigen-induced allergic airway responses *in vivo*.

Introduction

IL16 is a cytokine that has been discovered in the early 80's (29, 90) and has been shown to use the CD4 molecule as its receptor. Most *in vitro* and *in vivo* data obtained so far propose that IL16 serves as a pro-inflammatory cytokine (29, 90, 154). IL16 has been found to be a very potent inducer of chemotaxis *in vitro* of $CD4^+$ cells, including T cells and eosinophils (90, 95). Furthermore, IL16 may play a role in $CD4^+$ T cell mediated diseases such as asthma. It has been demonstrated that IL16 expression is increased in asthmatics upon antigen challenge and that this increased expression correlates with disease status (26). However, little is known about the effects of IL16 *in vivo*. Recently, we have shown that exogenously administered IL16 can inhibit antigen-induced airway hyperresponsiveness and eosinophilia in a murine model of allergic asthma (59). Furthermore, we found that this *in vivo* inhibitory action of IL16 was associated with decreased production of Th2-type cytokines by lymphocytes upon restimulation with antigen *in vitro* (221). In agreement herewith, Klimiuk *et al.* demonstrated that exogenous IL16 can serve as an anti-inflammatory cytokine in an animal model for human synovitis (222).
It has been extensively demonstrated that IL16-induced effects require cross-linking of CD4 molecules (29). It is well documented that other ligands for CD4 such as antibodies or gp120 can induce T cell unresponsiveness (45, 127, 136, 223). Therefore, it could be speculated that IL16-induced CD4 signaling also induces an unresponsive state in T cells. In order to test this hypothesis, we isolated splenocytes and determined the effects of IL16 or antibodies to CD4 on polyclonal stimulation of lymphocytes. Furthermore, we isolated TLN cells from animals sensitized to and challenged with ovalbumin and measured the effect of IL16 or antibodies to CD4 on antigen-specific proliferation as well as on antigen-induced cytokine production *in vitro*.
The observed anti-inflammatory action of IL16 administration *in vivo* could also be explained by effects of IL16 on T cell differentiation. It has been clearly demonstrated that besides an accessory function in TCR-induced signaling, the CD4 molecule also plays a role in differentiation of naive T cells into effector cells (71). It was shown by Leitenberg *et al.* that the presence of a functional CD4 molecule is necessary for induction of Th2 cells whereas CD4-signaling is not a prerequisite for Th1 T cell differentiation (71). To explore possible effects of IL16 or anti-CD4 antibodies on differentiation of naive $CD4^+$ T cells, we used an artificial *in vitro* T cell skewing system (224). In this experimental set-up, differentiation of naive T cells towards the development of effector cells that display a Th0, Th2 or Th1-type cytokine profile upon restimulation is directed by culturing in presence of medium only, IL4 or antibodies to IL4, respectively.

Materials and Methods

Animals

Specified pathogen free male BALB/c mice (age 6-8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (Utrecht, The Netherlands). All experiments were approved by the animal care committee of the Utrecht University. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade V) in 0.5 ml pyrogen free saline on alternate days. Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in saline) or to 8 saline aerosols for 5 min, on consecutive days (1 aerosol per day). The aerosols were generated with a jet nebulizer (Pari IS-2, Pari-Werk GmbH, Starnberg, Germany) connected to a plexiglas exposure chamber (5 l). For T cell differentiation experiments, spleens from DO11.10 T cell receptor transgenic mice were used. DO11.10 mice were generously donated by L. Adorini (Department of Inflammation/Autoimmune Diseases, Hoffmann-La Roche Inc., Nutley, NJ, USA) and maintained at the Central Animal Laboratory (Utrecht, The Netherlands).

Proliferation of lymphocytes with anti-CD3

Anti-CD3-coated plates were prepared by incubating 96-wells plates with anti-CD3 antibodies (10 μg well^{-1}) for 24h at 4°C. Plates were washed twice with phosphate-buffered saline (PBS) before use. Single cell suspensions were made from spleens derived from naive BALB/c mice. Lymphocytes were then isolated using a Lympholyte M gradient according to the manufacturer's instructions (800x*g*, 20 min, 21°C, Cedarlane, NJ, USA). Thereafter CD4$^+$ T-lymphocytes were negatively selected using a commercially available CD4$^+$ T cell isolation kit (R&D systems, Minneapolis, MN, USA). CD4-enriched cells were cultured in medium (RPMI, supplemented with 10% FCS, 5 x 10^{-5} M β-mercaptoethanol, 50 mg l^{-1} gentamycine and 1% glutamax) at a concentration of 1 x 10^6 cells ml^{-1}. Thereafter cells were incubated for 1h at 37°C and 5% CO_2 with medium, rhIL16, rmIL16 (0.06-0.6 μg ml^{-1}) or antibodies to CD4 (GK1.5, 0.01-1 μg ml^{-1}). Subsequently, cells (1 x 10^5 cells well^{-1}) were added to the anti-CD3-coated wells without washing. After 48h at 37°C and 5% CO_2, [^{3}H] thymidine (0.3 μCi per well) was added to each well and left to be incorporated for 18h. Thereafter, cells were harvested and [^{3}H] thymidine uptake was determined by liquid scintillation counting (1450 microbeta plus, Wallac Oy, Turku, Finland). All experiments were performed at least in triplicate.

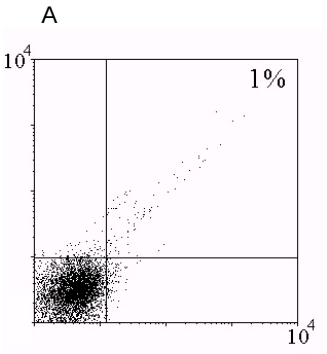
Antigen-specific restimulation of thoracic lymph node cells in vitro

Thoracic lymph node (TLN) cells were isolated from ovalbumin-sensitized and ovalbumin-challenged BALB/c mice as previously described (151). TLN cells (1 x 10^6 per ml, 100 μl well^{-1}) were incubated for 1h at 37°C and 5% CO_2 with either medium alone (RPMI), rhIL16, rmIL16 (0.01-1 μg ml^{-1}) or anti-CD4 antibodies (GK1.5, 0.01-1 μg ml^{-1}). Thereafter, TLN cells were cultured in the presence or absence of ovalbumin (40 μg ml^{-1}) and after 5 days supernatant fractions were harvested for cytokine analysis. IL16 or antibodies to CD4 remained present during this culture period. Separate cultures were used to determine proliferation. [^{3}H]thymidine (0.3 μCi per well) was added to each well 48h after stimulation and left to be incorporated. After 18h, cells were harvested and [^{3}H]thymidine uptake was determined by liquid scintillation counting. All experiments were performed at least in triplicate.

In vitro *T-cell differentiation*

DO11.10 CD4$^+$ T cells were isolated using immunomagnetic negative selection (71, 225). In short, single cell suspensions were made from spleens derived from DO11.10 mice. Lymphocytes were isolated using a lympholyte M gradient (800x*g*, 20 min, 21°C, Cedarlane, NJ, USA). Lymphocytes were left to incubate (1 x 10^7 cells ml^{-1}) with antibodies to MHC-II (MKD6), CD8 (YTS69), Thy 1 (YTS154), B220 (RA3-6B2) and CD16/32 (2.4G2) in previously established optimal dilutions determined by flow cytometry analysis. After 1h incubation at 4°C on a rotating platform, cells were washed twice in cold medium (RPMI) and resuspended with a mixture of magnetic beads coupled to antibodies to mIgG2a, rIgG2b and mIgM (Perseptive Biosystems, MA, USA) and left to incubate for 30 min at 4°C on a rotating platform. Negative immunomagnetic selection was then performed using a magnetic plate (10 min at 4°C, Perseptive Biosystems, MA, USA) and non-bound cells were washed twice with medium. The resultant CD4 population, as assessed by standard fluorescent staining was >90% pure (data not shown), the percentage of CD3-CD4 double positive cells was 80-90% pure (Figure 1). Fluorescent staining was performed as follows; cells were washed, resuspended in PBS with 1% azide supplemented with hamster and rat serum (10%) and left to incubate for 30 min at 4°C. After washing, cells were stained (20 min, 4°C) with phycoerythrin (PE)-conjugated anti-CD3 antibodies (145-2C11) and Cy-Cychrome-conjugated anti-CD4 antibodies (RM4-5) or control antibodies. After washing, surface expression was determined (FACS, Becton Dickinson and Co., Mountain View, CA, USA).
In order to detect possible effects of IL16 on the differentiation of naive T cells into either Th1 or Th2-type lymphocytes, a slightly modified system first described by Hsieh *et al.* was used (224). Splenocytes from BALB/c mice were used (1 x 10^6 ml^{-1}) as antigen presenting cells (APC) and these cells were cultured

Figure 1



CD3 and CD4 expression of purified T cells from spleens of DO11.10 mice. Expression was determined using phycoerythrin-conjugated anti-CD3 (FL-2, x-axis) and Cy-cychrome-conjugated anti-CD4 (FL3, y-axis). Presented are background staining for FL-2 and FL-3 (A) and staining with antibodies to CD3 and CD4 (B). Numbers indicate the percentage of double positive cells.

with $CD4^+$ DO11.10 T cells (5×10^5 ml^{-1}) in the presence of $ovalbumin_{323-339}$ ($OVA_{323-339}$) peptide (0.3 μM) in a total volume of 1,5 ml $well^{-1}$. Furthermore, cultures were supplemented with either IL4 (200 units ml^{-1}), antibodies to IL4 (10 μg ml^{-1}) or medium only (control). After 4 days of culturing, supernatant fractions were harvested, non viable cells were removed using Lympholyte M gradient and cells were resuspended in medium at 5×10^5 cells ml^{-1}. Subsequently, cultures were restimulated using APC's (1×10^6 ml^{-1}) and $OVA_{323-339}$ (0.3 μg ml^{-1}) without adding IL4 or antibodies to IL4. After two additional days of culturing, supernatants were again harvested for cytokine analysis. During the first antigen stimulation either rmIL16 (1 μg ml^{-1}) or antibodies to CD4 (GK1.5, 0.1 μg ml^{-1}) were added to the different cultures to determine the effects of CD4 cross-linking on differentiation of DO11.10 T cells into different Th subsets. Experiments were performed at least in triplicate.

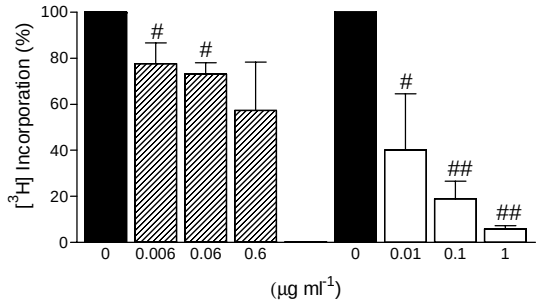
Chemicals

Ovalbumin (chicken egg albumin crude grade V) was purchased from Sigma Chemical Company (St. Louis, MO, USA), $OVA_{323-339}$ was obtained from the Dept. of Immunology, Faculty of Veterinary Sciences, Utrecht University, Tween-20 from Merck (Darmstadt, Germany). Cy-Cychrome-conjugated anti-CD4 (RM4-5) and PE-conjugated anti-CD3 (145-2C11) as well as rmIL16 were obtained from Pharmingen (San Diego, CA, U.S.A). rhIL16 was purchased from Preprotech. All antibodies used for cell isolations were purified, using standard protein A or G column affinity, from hybridomas maintained at Veterinary Faculty, Dept. of immunology, Utrecht University, The Netherlands. Antibodies used in this study were MKD6 (anti-$I\text{-}A^d$, mIgG2a), YTS169 (anti-CD8, rIgG2b), YTS154 (anti-Thy 1, rIgG2b), RA3-6B2 (anti-B220, rIgG2b), 2.4G2 (anti-muFc_γRII; CD16/32, rIgG2b), GK1.5 (anti-CD4, rIgG2b) and 11B11 (anti-IL4, rIgG1). IL4 and IL2 were generously donated by dr. W. P. Paul, NIAID, NIH, Bethesda, USA.

Data analysis

Comparisons were made using a Student's *t*-test (paired, two-tailed). Data are expressed as arithmetic average ± SEM and a difference was considered to be significant when $P<0.05$. Statistical analyses were carried out using Microsoft Excel 7.0.

Figure 2



Effect of CD4 cross-linking on proliferation of T cells isolated from naive BALB/c mice induced by immobilized anti-CD3 (black bars). Depicted are the effects of different concentrations of rmIL16 (hatched bars, average of 4 separate experiments) or antibodies to CD4 (open bars, average of 3 separate experiments) on immobilized anti-CD3-induced proliferation. Results are expressed as arithmetic average ± SEM. # (P<0.05); ## (P<0.01) significantly different from proliferation induced by anti-CD3 antibodies (100%). Proliferation of unstimulated cells was <1%.

Results

Effect of CD4 cross-linking on anti-CD3-induced proliferation

Immobilized anti-CD3 significantly enhanced proliferation of $CD4^+$ lymphocytes when compared to cells cultured in medium only (41,600 ± 14,300 vs 191 ± 48 counts min^{-1}, *P*<0.05). Both rmIL16 and anti-CD4 antibodies concentration-dependently inhibited immobilized anti-CD3-induced proliferation with maximal effects at the highest concentration tested (0.6 and 1 μg ml^{-1}, respectively, Figure 2). Anti-CD3-induced proliferation was inhibited by 44% to 18,068 ± 7,476 counts min^{-1} and by 95% (*P*<0.01) to 2,493 ± 1,310 counts min^{-1}, respectively, when compared with proliferation induced by anti-CD3 alone. rhIL16 also significantly decreased anti-CD3-induced proliferation at the highest concentration (0.6 μg ml^{-1}) tested (>30%, *P*<0.05, data not shown).

Effect of CD4 cross-linking on antigen-specific T cell stimulation

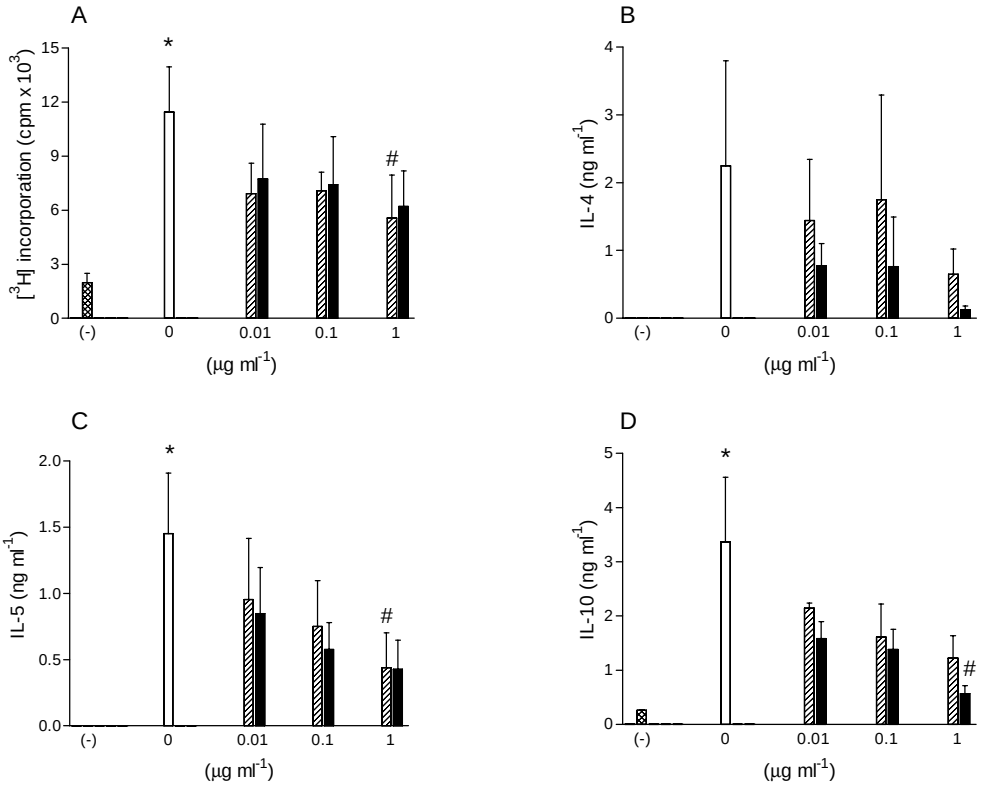
Stimulation of TLN cells isolated from OVA-sensitized and challenged animals with ovalbumin *in vitro*, significantly enhanced proliferation of these cells when compared with unstimulated cells (*P*<0.05, Figure 3A). Incubation with rmIL16 (1 μg ml^{-1}) inhibited ovalbumin-induced proliferation with more then 50%, when compared with proliferation induced by ovalbumin alone (*P*<0.05). Anti-CD4 antibodies (1 μg ml^{-1}) also inhibited ovalbumin-induced proliferation (45% inhibition), when compared with control (Figure 3A).
Cytokine levels in supernatants of these cultures were also determined. Both rmIL16 and antibodies to CD4 inhibited production of IL4, IL5 and IL10 in a concentration-dependent manner after antigen-specific stimulation of TLN cells (Figure 3B, C and D), with maximal effects at the highest

Table I: The effect of rhIL16 on antigen-induced cytokine production and proliferation.

Stimulation	Proliferation (cpm)	IL4 (ng ml^{-1})	IL5 (ng ml^{-1})	IL10 (ng ml^{-1})
-	1,981±524	0±0	0±0	0.3±0.0
Ova	11,447±2,509 (*)	2.25±1.55	1.45±0.46 (*)	3.4±1.2 (*)
Ova + rhIL16	5,954±1,624 (#)	0.53±0.24	0.51±0.29 (#)	1.1±0.2

*TLN cells were isolated from OVA-sensitized and challenged animals and cultured in absence (-) or presence of ovalbumin (Ova). Shown are the effects at the highest concentration of rhIL16 (1μg ml^{-1}) on antigen-induced production of IL4 and IL5 as well as on proliferation. Results are presented as arithmetic average ± SEM of 4 separate experiments. * (P<0.05) significantly different from proliferation or cytokine production induced by medium alone. # (P<0.05) significantly different from proliferation or cytokine production induced by ovalbumin.*

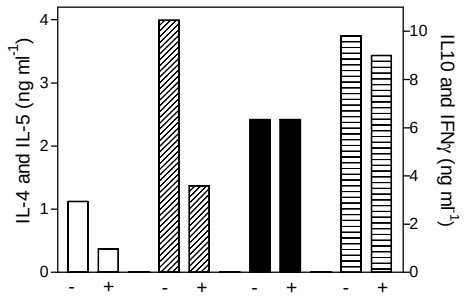
Figure 3



*TLN cells were isolated from OVA-sensitized and challenged animals and cultured in absence (crosshatched bars) or presence of ovalbumin (open bars). Shown are the effects of different concentrations of either antibodies to CD4 (black bars) or rmIL16 (hatched bars) on antigen-induced proliferation (A), IL4 (B), IL5 (C) and IL10 production (D). Results are expressed as arithmetic average ± SEM of 4 separate experiments. * (P<0.05) statistically significant difference in proliferation or cytokine production by cells stimulated with antigen without CD4 ligands, when compared with cells cultured in medium alone. # (P<0.05) significantly different from proliferation or cytokine production induced by ovalbumin.*

concentration of either antibodies to CD4 or rmIL16 (1 μg ml^{-1}). Incubation with antibodies to CD4 or rmIL16 (both at 1 μg ml^{-1}) decreased IL4 production by 95% and 71%, respectively, after stimulation with ovalbumin. Production of IL5 was decreased by 70% (antibodies to CD4) and 70% (rmIL16, $P<0.05$) and IL10 by 83% (antibodies to CD4, $P<0.05$) and 64% (rmIL16), when compared with cytokine production after culturing in presence of only ovalbumin (Figure 3). No significant differences were observed in IFNγ levels in supernatants (data not shown). Similar effects were observed on antigen-induced cytokine production and proliferation when lymphocytes were preincubated with rhIL16 (Table I).

Figure 4



$CD4^+$ T cells, isolated from DO11.10 mice were stimulated with $OVA_{323-339}$ presented by splenocytes derived from BALB/c mice and cultured in the presence (+) or absence (-) of IL16. After 5 days non-viable cells were removed and cells were restimulated with splenocytes and $OVA_{323-339}$ and subsequently cultured for 2 days. Presented are levels of IL4 (open bars), IL5 (hatched bars), IL10 (black bars) and IFNγ (horizontally striped bars) after the second culturing period. Presented is a representative example of four independent experiments.

Effect of CD4 cross-linking on T-cell differentiation

$CD4^+$ T cells were isolated from DO11.10 mice and cultured in presence of APCs and $OVA_{323-339}$. In order to induce differentiation of these DO11.10 T cells towards either a Th0, Th2 or a Th1-type cytokine producing effector cells, priming cultures were maintained for four days in medium only, IL4 or antibodies to IL4, respectively. During this period, IL16 or antibodies to CD4 were added. Thereafter, non-viable cells were removed and remaining cells were restimulated with antigen and freshly isolated APCs and cultured for an additional 2 day period, without the skewing agents or CD4 ligands. Cytokine levels in supernatant fractions were determined both at the end of the first stimulation period and after the second stimulation period with $OVA_{323-339}$.

I Th0-type cytokine production

At the end of the first culturing period, cells maintained in medium and stimulated with $OVA_{323-339}$ produced some IL5, hardly any IL10 and a high amount of IFNγ (Table II). After the second stimulation with $OVA_{323-339}$, DO11.10 $CD4^+$ T cells cultured in medium alone produced Th2-type cytokines IL4, IL5 and IL10 as well as the Th1-type cytokine IFNγ (Table II), indicating a Th0 type cytokine profile.

Cytokine levels in supernatant fractions isolated after the first stimulation period from cells cultured in medium with rmIL16 did not differ from levels measured in cultures without rmIL16 (Table II). However, upon restimulation with antigen, these cells produced significantly less IL4 (P<0.05) and IL5 (P<0.05), whereas IL10 and IFNγ levels in these supernatant fractions were not significantly altered (Figure 4, Table II).

Cytokine levels in supernatant fractions isolated after the first stimulation period from cells cultured in medium with antibodies to CD4 did also not differ from levels measured in cultures maintained in medium only (data not shown). Upon restimulation, a decreased production of both IL4 (P<0.05, from 0.2±0.1 to 0±0 ng ml^{-1}), and IL5 (from 3.1±1.1 to 1.9±0.5 ng ml^{-1}) was also observed, when compared with cells cultured in medium alone. Furthermore, no significant differences were found in IL10 and IFNγ production by these cultures (8.5±2.0 vs 7.0±1.6 ng ml^{-1} and 10.4±0.5 vs 11.0±0.7 ng ml^{-1}, respectively).

II Th2-type cytokine production

At the end of the first culturing period in the presence of IL4 and $OVA_{323-339}$, IL5 and IL10 production were enhanced, although not statistically significant, whereas IFNγ levels were significantly (P<0.05) decreased, when compared with cytokine levels in supernatants of cells cultured with antigen alone (Table II). Upon restimulation of these cells, IL4 (P<0.05), IL5 and IL10 (P<0.01) production was largely upregulated, whereas IFNγ production was almost completely inhibited (P<0.01), indicating a Th2-type cytokine profile (8, Table II).

The presence of rmIL16 in the priming cultures enhanced production of IL5 (P<0.05) and of IL10 (P=0.06) when compared with cells cultured without rmIL16 (Table II). Furthermore, IFNγ levels were not significantly decreased anymore, as was observed in supernatant fractions derived from cells cultured

Table II: Cytokine production during different stages of T cell differentiation

Without IL16 present during the first antigen-specific stimulation.

Cytokine	IL4		IL5		IL10		IFNγ	
Stimulation Period/ Skewing conditions	I	II	I	II	I	II	I	II
-	0.01±0.0	0.9±0.2	1.4±0.5	8.4±3.7	0.0±0.0	7.0±0.4	10.0±0.1	9.2±0.7
IL4	nd	1.4±0.0 (*)	2.5±0.3	14.0±3.0	1.3±0.7	10.7±0.4 (**)	8.6±0.2 (*)	2.5±0.9 (**)
anti-IL4	nd	0.1±0.1 (*)	1.1±0.8	1.0±0.5 (*)	0.1±0.1	1.9±0.5 (**)	9.8±0.1	10.0±0.1

With IL16 present during the first antigen-specific stimulation.

Cytokine	IL4		IL5		IL10		IFNγ	
Stimulation Period/ Skewing conditions	I	II	I	II	I	II	I	II
-	0.01±0.0	0.6±0.2 (#)	1.5±0.5	5.5±3.2 (#)	0.6±0.3	8.3±1.2	9.8±0.3	8.5±1.1
IL4	nd	1.4±0.1	4.5±0.6 (#)	12.6±2.7	$3.4\pm1.5^{(P=0.06)}$	10.9±0.2	9.1±0.2	2.0±0.3
anti-IL4	nd	0.0±0.0	1.7±1.0	1.6±1.1	0.3±0.2	3.8±1.0	9.8±0.1	9.7±0.1

*Cytokine levels (ng ml^{-1}) in supernatant fractions from DO11.10 $CD4^+$ T cells, stimulated with $OVA_{323-339}$ presented by BALB/c splenocytes after the first culturing period (I) with or without IL16 (1.0 μg ml^{-1}) and after the second stimulation period (II). Cells were cultured during the first stimulation period in medium alone, in the presence of IL4 or in the presence of antibodies to IL4 (anti-IL4). During the second stimulation period with $OVA_{323-339}$, cultures were maintained in medium only. Results are expressed as arithmetic average ± SEM of 4 separate experiments. IL4 levels after the first stimulation period were not determined (nd). * ($P<0.05$); ** ($P<0.01$) significantly different from the cytokine level measured in supernatant fractions from cells cultured in medium. # ($P<0.05$) significantly different from the cytokine level measured in supernatant fractions from cells cultured without IL16 present during the first antigen stimulation.*

without rmIL16 (Table II). No differences were observed in IL4, IL5, IL10 and IFNγ levels in supernatants harvested after the second stimulation, when compared with cells cultured without rmIL16 (Table II).
Adding antibodies to CD4 during the first stimulation period of these cultures also enhanced production of both IL10 and IL5 (2.8±2.0 vs 4.9±1.6 ng ml^{-1} and 1.7±1.3 vs 3.4±2.5 ng ml^{-1}, respectively), although this did not reach statistical significance. Again, no differences in cytokine production were observed in supernatants upon restimulation (data not shown).

III Th1-type cytokine production
No significant differences were observed between cytokine levels in supernatant fractions obtained from cells cultured in presence of antibodies to IL4 when compared to cells maintained in medium alone at the end of the first stimulation (Table II). When antibodies to IL4 were present during the initial phase, only IFNγ was produced upon restimulation (Table II), which is indicative of a Th1-type cytokine profile (8).
No significant differences in cytokine levels were observed between cultures supplemented with rmIL16 after the first stimulation with antigen, when compared with cells cultured in presence of only antibodies to IL4 (Table II). Furthermore, no differences in cytokine production due to rmIL16 were observed upon restimulation (Table II).
Presence of antibodies to CD4 also did not alter cytokine levels measured in supernatant fractions harvested either after the first stimulation or upon restimulation, when compared to cells that were initially cultured in presence of only antibodies to IL4 (data not shown).

Discussion

In this study we demonstrate that IL16 and antibodies to CD4 concentration-dependently inhibit proliferation of lymphocytes that are either polyclonally or antigen-specifically stimulated. Moreover, Th2-type cytokine production by these antigen-stimulated cells is also significantly decreased. In addition, upon restimulation of priming cultures that were maintained in presence of IL16 or antibodies to CD4, TCR transgenic T cells display a marked reduction in IL4 and IL5 production, but not IL10 and IFNγ, when compared with effector cells that were generated in absence of IL16.
Previously, we found that treatment with IL16 inhibits airway hyperresponsiveness and decreases infiltration of eosinophils after treatment with either rhIL16 or rmIL16 in a murine model of allergic asthma (226). It has been clearly demonstrated that development of airway hyperresponsiveness and eosinophilia in different animal models of allergic asthma, as well as the Th2-type cytokine production upon *in vitro* restimulation are caused by $CD4^+$ T cells (151, 227, 228). IL16 is a cytokine that uses the CD4 molecule as its receptor and cross-linking CD4 by IL16 has been shown to be a prerequisite for IL16 to exert its biological activities (29, 90). Thus the $CD4^+$ T cell is likely to be affected by treatment with IL16 *in vivo*. In agreement herewith, we demonstrated previously that treatment with IL16 *in vivo* largely decreased IL4 and IL5 production by TLN cells upon antigen-specific restimulation *in vitro* (221). This $CD4^+$ T cell inhibitory activity by IL16 treatment is supported by a recent publication in which it was demonstrated that *in vivo* administration of rhIL16 is capable of inhibiting $CD4^+$ T cell mediated responses in a murine model for human rheumatoid synovitis (222). However, the mechanisms via which IL16 exerts these anti-inflammatory actions are not known yet.
In the present study we examined the effects of IL16 on T cell responses *in vitro*. Here, we demonstrate that CD4 cross-linking with IL16 or with antibodies to CD4 decreased anti-CD3-induced proliferation of T cells. These observations are in agreement with the inhibitory effects of rhIL16 on proliferation of freshly isolated PBMCs after stimulation with immobilized anti-CD3 (69). More importantly, inhibition of antigen-induced T cell proliferation by rhIL16 has also been demonstrated (69, 70). Furthermore, we found that both antibodies to CD4 and IL16 inhibit antigen-induced proliferation as well as production of the Th2-type cytokines produced by $CD4^+$ lymphocytes. It is well known that Th2 type cytokines such as IL4 and IL5 are necessary for induction of allergic manifestations in murine models of allergic asthma (11, 13, 163, 229, 230). Therefore, decreased antigen-induced proliferation and cytokine production by antigen-specific T cells could very well be the cause of the inhibition of airway hyperreactivity and eosinophilia after *in vivo* treatment with IL16 (chapter 5). In agreement with these findings, binding of CD4 molecules by e.g.

antibodies to CD4 or gp120, prior to T cell receptor-mediated activation of T cells also causes the T cell to become unresponsive (45, 127, 136, 223).
The observed anti-inflammatory action of IL16 administration *in vivo* could also be explained by effects of IL16 on T cell differentiation. It has been clearly demonstrated that besides an accessory function in TCR-induced signaling, the CD4 molecule also plays a role in differentiation of T cells into Th2-type effector cells (71). In the present study, we demonstrate that DO11.10 $CD4^+$ effector T cells, raised in presence of IL16 without skewing agents during priming cultures, display reduced production of IL4 and IL5 upon antigen-specific restimulation, whereas IL10 and IFNγ levels are not affected. Similar observations were made after CD4 cross-linking with antibodies to CD4. Differentiation of naive $CD4^+$ T cells towards effector cells is likely to occur during *in vivo* antigen challenge. In previous studies we showed that TLN cells, isolated from ovalbumin-sensitized saline-challenged mice do not produce any Th2-type cytokines upon antigen-specific restimulation *in vitro*, whereas TLN cells isolated from antigen challenged animals do (151, 206). It can thus be speculated that in this murine model naive T cells are recruited to lymph nodes upon antigen challenge and subsequently differentiate towards effector cells that produce cytokines such as IL4 and IL5. Decreased IL4 and IL5 production, combined with normal levels of IL10 and IFNγ could offer an explanation for the results obtained after *in vivo* treatment with IL16. As already mentioned, both IL4 and IL5 are necessary for development of allergy associated parameters. Both IL10 and IFNγ have been demonstrated to be potent inhibitors of Th2-type T cell-mediated responses (231, 232). IL10 has been clearly shown to inhibit antigen-dependent T cell activation, probably via effects on APCs (233, 234).
Furthermore, IL10 administration inhibits antigen-induced responses in different murine models of allergic asthma (231, 232). IFNγ also is a potent suppressor of development of antigen-induced airway hyperresponsiveness and eosinophilia in murine models (165, 235). However, after *in vivo* treatment with IL16 of antigen-sensitized and challenged mice, we were unable to detect enhanced antigen-induced IFNγ production by TLN cells (unpublished observations). Therefore, IFNγ is not very likely to be the cause of the inhibitory effects of treatment with IL16 in our murine model of allergic asthma.
The observation that we did not detect any effect of IL16 in priming cultures on restimulation of T cells that were skewed towards a Th2 profile seems to be in contrast with a recent report stating that CD4-signaling is crucial for Th2 development (71). However, in our experimental set-up presence or absence of IL4 during the initial stimulation determines T cell differentiation (224), whereas in the study by Leitenberg *et al*. the affinity of the ligand that is used during the initial stimulation for the TCR determines the cytokine profile that is produced upon antigen-specific restimulation (71). In agreement with our observations, it was shown in the latter system that IL4 is capable of bypassing the necessity for CD4-signaling in development of Th2-type cytokine responses (71). Furthermore, in the ovalbumin model in BALB/c mice approximately half of the T and B cell responses is directed against other epitopes of ovalbumin (with other affinities for the TCR) than against the immunodominant epitope $OVA_{323-339}$. Therefore our *in vitro* skewing conditions could be different from circumstances that determine T cell differentiation *in vivo* (E. M. Janssen *et al.*, Am. J. Respir. cell Mol. Biol., *in press*).
The observation that IL16-treated DO11.10 cells under non-skewing circumstances do not display decreased cytokine production at the end of the first antigen stimulation, seems to be in contrast with our observation that proliferation and cytokine production by antigen-specific T cells, isolated from antigen-sensitized and challenged animals, are decreased by CD4 ligation *in vitro*. However, it is well documented that memory cells in particular are susceptible to CD4-induced unresponsiveness, whereas naive T cells are not (236). An explanation for this difference in sensitivity could be the level of CD4 expression since this level increases if T cells are exposed to antigen (i.e. become effector/memory cells). Freshly isolated DO11.10 $CD4^+$ T cells indeed demonstrate a naive phenotype, as was indicated by high expression of $CD45^{RB}$ and CD62L/L-selectin (data not shown), whereas TLN cells, isolated from antigen-sensitized and repeatedly challenged animals are likely to have a memory/effector phenotype and are therefore more susceptible to CD4-mediated unresponsiveness. Interestingly, recent evidence suggests that the lymphocytes that are responsible for the passive transfer of allergic inflammation indeed have a $CD4^{high}$, $CD62L^{low}CD25^+$ phenotype (211).

Cells that were skewed towards a Th2-type cytokine profile and cultured in presence of IL16 have an increased production of IL5 and IL10 during the first stimulatory period and a similar tendency was observed with antibodies to CD4. The fact that Th2 differentiation is enhanced by CD4 cross-linking is in agreement with observations by others (237). However, no differences were observed after the second stimulation with antigen of cells that were initially cultured in presence of IL4. Thus, this effect appears to be short-lasting and depending on the presence of CD4 ligands. As already mentioned, the observed increase in IL10 levels could be an explanation for the IL16-induced effects *in vivo*. In contrast, enhanced IL5 production, as was observed *in vitro* after culturing with IL16 under Th2-skewing circumstances, would actually increase the antigen-induced responses *in vivo*. It has been clearly demonstrated that IL5 is important for antigen-induced airway hyperresponsiveness and eosinophilia in different animal models of allergic asthma (12, 214, 229). However, this enhanced Th2-type cytokine production by CD4 ligands was only observed under very strict circumstances that promote differentiation towards a Th2-type T cell (224). Since *in vivo* treatment with IL16 inhibits development of antigen-induced airway hyperresponsiveness and eosinophilia, it is not very likely that IL16 induces production of IL5 *in vivo* upon antigen-specific stimulation. Moreover, this would be completely opposite to the observation that TLN cells isolated from IL16-treated animals display decreased production of IL5 upon antigen restimulation *in vitro*. It is not known at present which *in vitro* conditions accurately reflect the microenvironment of T cell differentiation *in vivo.* However, based on our *in vivo* results it seems to be that the conditions in which no skewing agents are present resemble the *in vivo* conditions in our model most.

In conclusion, the *in vitro* data presented in this study provide novel insights in how IL16 can inhibit antigen-induced responses *in vivo*. However, further research is necessary to explore which of these possibilities is the cause of the inhibition by IL16 of antigen-induced airway hyperresponsiveness and eosinophilia.

Summary and General Discussion

Introduction on allergic asthma

Allergic asthma is a disease which affects an increasing number of people. Patients with this illness suffer from variable airflow obstruction and increased airway responsiveness to a variety of stimuli. Furthermore, an inflammatory response in the lungs occurs, accompanied by enhanced mucus production (10). In serum of these patients enhanced levels of IgE antibodies can be detected (reviewed in 238, 239).
Currently, several different drugs are available for people who suffer from asthma. These medicines include corticosteroids, β_2-agonists, leukotriene antagonists and phosphodiesterase inhibitors (240-244). Corticosteroids are the most effective and most used drug at this moment in the treatment of asthma to reduce the inflammatory component and hyperresponsiveness (245). However, corticosteroids are not very selective and affect a whole range of inflammatory and non-inflammatory cells (246-248). More importantly, all drugs that are available for people who suffer from allergic asthma do not cure these patients but only reduce the clinical symptoms of this disorder. Therefore, current research is focused on development of strategies that treat asthmatics at a more causal level of their illness.
It is now widely accepted that T-helper type 2 (Th2) lymphocytes play a causal role in allergic asthma (10, 249, 250). Th2 cells produce a limited set of cytokines including interleukin-3 (IL3), IL4, IL5, IL9, IL10 and IL13. Both in human asthmatic patients and murine models of allergic asthma Th2 subsets have been detected (8, 9) and it is extensively documented that the cytokines produced by these cells are crucial for development of allergic symptoms. New strategies could therefore be aimed at specifically targeting this T cell subset or the cytokines produced by these T cells (18, 251, 252).

Murine models of allergic asthma

Over the past decades several different animal models have been developed which display phenomena characteristic of allergic asthma (13, 20, 149, 164, 166, 186, 253). These models have been demonstrated to be very useful for the development of new therapeutic strategies for the cure of allergic asthma and to gain insight in the immunological disorders underlying the cause of this disease. In the murine model we have used in our studies, several allergy associated parameters can be measured, including increased airway hyperresponsiveness to non-specific bronchoconstrictive agents as well as an inflammatory response in the lungs (148-151). Furthermore, elevated levels of antigen-specific IgE upon challenge and production of Th2-type cytokines by $CD4^+$ T cells isolated from lung-draining lymph node cells can be detected (149, 151, 165, 254). As is the case in human allergic disease (reviewed in 255), several of these parameters can be influenced by treatment with corticosteroids (149).

Detection of Early and Late Bronchoconstrictive Responses and kinetics of airway hyperresponsiveness and eosinophilia

One of the characteristics of allergic asthma in humans is the occurrence upon contact with allergens of the so-called acute bronchoconstrictive response (256). In the animal model we use, we can also measure increased airway constriction upon provocation with antigen, using either forced oscillatory techniques (257) or whole body plethysmography (chapter 2). This acute airway response is believed to be caused by mediators released by mast cells. These inflammatory cells degranulate upon cross-linking of antigen-specific IgE molecules bound to high affinity Fcε receptors present on the cell membrane. It is possible to determine mast cell degranulation via measurement of MMCP-1 levels, an enzyme normally present in mucosal mast cells (171). Increased serum MMCP-1 levels could be detected after antigen challenge in murine the model we use (chapter 2) indicating that mast cells degranulate. This is in agreement with previous experiments in which a clear mast cell degranulation was observed upon a single antigen challenge, using electron microscopy (170). Besides the early bronchoconstrictive responses, late asthmatic responses (LAR) have been reported in humans, which

are associated with the infiltration of inflammatory cells into the lungs. The development of LAR in human asthmatics occurs as a spectrum ranging from no LAR to severe late asthmatic bronchoconstricitive responses following antigen challenge, which seems to be at least partially dependent on the dose of antigen (reviewed in 157). Furthermore, it is thought that there is a direct correlation between infiltration of eosinophils and the occurrence of LAR in asthmatic patients (157, 160). However, others observed that eosinophilia or the presence of mediators released by eosinophils could not predict the emergence of LAR (161). This LAR usually occurs at several hours after contact with the antigen. Decreased serum corticosteroid levels during night time (258-260) could be the explanation for the observation that this response typically occurs during the sleeping period. We used several different approaches to induce such late bronchoconstrictive responses in the murine model. Our attempts included inhibition of endogenous corticosteroid production using metyrapone (149), which has been demonstrated to increase occurrence of late responses in dogs (168) and to increase antigen-induced responses in rats (169). Furthermore, metyrapone has also been found to increase both the number of eosinophils and the degree of airway hyperresponsiveness after ovalbumin-challenge in the used murine model (149). Since correlations between infiltration of eosinophils and the occurrence of LAR in asthmatic patients and between the dose of antigen and LAR have been reported to exist (157), we measured bronchoconstrictive responses after increasing antigen deposition via local administration of ovalbumin and at times when eosinophils are already present in the lungs. Despite these attempts, we were unable to demonstrate such late bronchoconstrictive responses in the animal model, even at times when eosinophils are already abundantly present (chapter 2). This might be due to the fact that eosinophils are not or only mildly activated in this model of allergic asthma. Indeed, it has been demonstrated that eosinophils in murine models of allergic asthma were not activated since no major basic protein release (178) or eosinophil peroxidase in cell free bronchoalveolar lavage (BAL) fluid could be detected (E.M. Hessel, unpublished observations). However, others did report eosinophil activation and subsequent morphologic changes of the airways upon antigen-challenge (179). Another reason for not detecting late bronchoconstrictive responses whereas both airway hyperresponsiveness and eosinophilia are present could be that these phenomena are not causally related in mice. This could accurately reflect the situation in human asthmatics, because numerous patients do exhibit airway hyperresponsiveness or eosinophilia upon antigen-provocation without detectable LAR (157, 161).

Besides detection of early and late asthmatic responses, we measured development of airway hyperresponsiveness and eosinophilia in time. It appeared that eosinophilia and airway hyperresponsiveness are only correlated during the period in which animals are daily challenged with antigen. After ending antigen provocations, airway hyperresponsiveness declines within 5 days whereas eosinophils are still present in the lungs at that time (chapter 2). From previous experiments it is well known that eosinophilia is not always correlated to airway hyperresponsiveness in this murine model (148). In particular, treatment with antibodies to IL5 in this and an other model revealed that airway hyperresponsiveness can still develop even though no detectable eosinophilia is present (13, 148). This dissociation between presence of eosinophils and airway hyperresponsiveness has also been reported for human allergic patients (reviewed in 180, 181, 261-263). It has e.g. been described that patients have eosinophils in the bronchoalveoar lavage fluid without displaying airway hyperresponsiveness (181). Furthermore, patients have been described that suffer from airway hyperresponsiveness, whereas no eosinophils could be detected in the lungs (262). However, others did observe positive correlations between airway hyperresponsiveness and presence of eosinophils (reviewed in 5, 264).

IL16 in the murine model; effects of histamine and 5-HT receptor antagonists

Using this murine model of allergic asthma, we explored the function of IL16, a cytokine that has been reported to be associated with $CD4^+$ T cell mediated diseases. In asthmatics, it was observed that IL16 levels in BAL fluid increased upon antigen challenge, as well as after histamine provocation (26, 29). Furthermore, in atopic dermatitis, a positive association between the number of $CD4^+$ T cells and IL16 levels was detected (265). A similar correlation was observed in synovium from patients suffering from rheumatoid arthritis (107). Finally, a correlation between disease activity index and levels of circulating IL16 has been detected in systemic lupus erythematosus (SLE, 266). These data suggest that IL16 is involved in several different $CD4^+$ T cell-mediated diseases.
The first discovered source of IL16 was the T cell (74, 75). $CD8^+$ T cells secrete IL16 following stimulation with either histamine or serotonin (5-hydroxytryptamine, 5-HT, 79, 80) whereas $CD4^+$ T cells release IL16 following mitogen, antigen or anti-CD3 stimulation (29). In contrast to $CD8^+$ T cells, $CD4^+$ T cells do not contain preformed IL16 (29). In agreement herewith, it has recently been shown that resting $CD8^+$ T cells contain active capsase-3 that is capable of cleaving pro-IL16, whereas $CD4^+$ T cells require activation for the appearance of active capsase-3 (267). Caspase-3 is necessary for the processing of pro-IL16 into biologically active IL16 (86). Besides T-lymphocytes, it has been shown that both eosinophils and epithelial cells can release bioactive IL16 (26, 81, 94), whereas in rheumatoid arthritis (RA) $CD68^-$ synovial fibroblasts have been shown to be the source of IL16 (107). One of the major sources of IL16 in the airways could very well be epithelial cells, which have been demonstrated to contain IL16 protein upon antigen-challenge in both humans (26, 29, 90) and the murine model of allergic asthma we use (154). Furthermore, biologically active IL16 can be measured in BAL fluid within hours after antigen-challenge (154). From literature it is known that preformed IL16 is released from $CD8^+$ T cells after stimulation via histamine type 2 (H_2) or 5-HT type 2 ($5\text{-}HT_2$) receptors and by epithelial cells upon stimulation with histamine (29, 79, 80). Since murine mast cells are known to release both histamine and 5-HT upon stimulation *in vivo* (152, 153) and since murine mast cells degranulate upon antigen challenge (chapter 2), it can be speculated that both 5-HT and histamine are set free, which induces release of IL16. Therefore we tried to inhibit this release using different histamine or 5-HT receptor-specific antagonists (chapter 3). Both eosinophil infiltration and airway hyperresponsiveness were partially decreased after blocking either the H_2 (using cimetidine) or $5\text{-}HT_2$ receptors (using ketanserin), whereas after blocking H_1 and $5\text{-}HT_1$ receptors (using mepyramine and methiotepine, respectively) these effects were not observed (chapter 3). Treatment with a combination of cimetidine and ketanserin completely inhibited development of airway hyperresponsiveness, whereas eosinophilia was not further decreased.
As was previously stated, mast cells can secrete both histamine and 5-HT (152, 153) and mast cells degranulate upon antigen challenge in our model. Furthermore, it is well known that mast cells are important in the onset of airway hyperresponsiveness (15, 173) and that mast cells play a pivotal role onset of the inflammation via e.g. TNFα, IL4 and IL5 release (185, 196). Together with the observed effects of both cimetidine and ketanserin on airway hyperresponsiveness, it is very tempting to speculate that mast cells are involved in development of airway hyperresponsiveness and eosinophilia via release of both histamine and 5-HT upon antigen challenge.
We did not detect any differences in presence of biologically active IL16 levels in bronchoalveolar lavage fluids after treatment with either the receptor antagonists alone or after treatment with the combination of antagonists (chapter 3). It is therefore very likely that autacoids, such as histamine or 5-HT are not the only inducers of IL16 release *in vivo*. Other sources of IL16 could e.g. be the $CD4^+$ T lymphocyte, which has been demonstrated to release IL16 after challenge with antigen (29, 90). Moreover, eosinophils constitutively release IL16 (81) and IL16 presence has been shown in inflammatory cell infiltrates, which contain eosinophils, in lung tissue upon antigen-challenge (154). It could thus be speculated that even though specific histamine and 5-HT receptor subtypes that were reported to mediate IL16 release *in vitro*, were blocked, other cells, such as T cells and eosinophils are major sources of IL16 in the mouse model upon antigen provocation. Finally, mast cells

themselves have been shown to release IL16 upon stimulation with C5a or PMA and could thus be a source of IL16 upon activation (82).

IL16 in the murine model; effect of blocking peptides or antibodies

Another approach to determine the role of endogenous IL16 in development of allergy associated parameters in the murine model, was to use either antibodies to IL16 or different peptides of which the amino acid sequences were based on different parts of the predicted sequence of human IL16 (76). Peptides 1 and 2 were based on different C-terminal located regions of the predicted amino acid sequence of rhIL16. These peptides have been demonstrated not to interfere with rmIL16-induced chemotaxis of murine lymphocytes *in vitro* in our study (chapter 4) or with OKT4 binding to CD4 or rhIL16-induced chemotaxis in another study (76). Peptide 3, which was based on the C-terminal hydrophilic region of human IL16 was shown to decrease rhIL16 induced chemotaxis of human $CD4^+$ T cells (76). Furthermore, peptide 3 can partially prevent the binding of monoclonal antibodies to CD4 (76). These data, together with the observation that IL16 uses CD4 as receptor, suggest that the C-terminal hydrophilic region of IL16 is involved in binding of CD4. Furthermore, based on these data it could be suggested that peptide 3 interacts with CD4, thereby inhibiting cross-linking by IL16 and subsequent signaling. Cross-linking of CD4 molecules has been demonstrated to be a prerequisite for IL16 to be biologically active (29, 90). We therefore intranasally treated mice either with these peptides or with antibodies to IL16 during the antigen-challenge period to inhibit effects caused by endogenously produced IL16. Using the different peptides, we demonstrated that the *in vitro* IL16-blocking peptide was also active *in vivo* (chapter 4). Intranasal administration during the antigen challenge period of either peptide 3 or antibodies to IL16 partially decreased airway hyperresponsiveness whereas inflammation was not affected (chapter 4). These results indicate that treatment with peptide 3 causes the same effects as antibodies to IL16, possibly via inhibition of interaction between IL16 and its receptor CD4. Therefore, peptide 3 could be useful as a lead compound in attempting to limit airway hyperresponsiveness via binding to CD4.
These results also indicate that endogenous IL16 at least has an effect on airway hyperresponsiveness *in vivo*, since we observed a decrease in development of airway hyperresponsiveness by treatment with either antibodies to IL16 or peptide 3. However, this was not accompanied by a decreased inflammatory response. From this observation it could be concluded that endogenously produced IL16 is not a crucial factor for chemotaxis of eosinophils in the murine model. These data appear to be in contrast with *in vitro* studies in which pro-inflammatory properties of IL16 have been detected. IL16 in resting $CD4^+$ T cells upregulates expression of IL-2R and HLA-DR and induces a shift in cell cycle from G_0 to G_{1a} (29). In addition, IL16 synergistically stimulates proliferation of $CD4^+$ T cells when combined with IL2 (76) and induces chemotaxis of $CD4^+$ cells, including T cells, monocytes and eosinophils (29, 90). Furthermore, IL16 can function as a competence growth factor for $CD4^+$ T cells (reviewed in 268). Finally, it has been observed that upon antigen challenge IL16 is the most important chemoattracting factor present in BAL fluid in human asthmatics after antigen-challenge (25) and that antibodies to IL16 can protect against induction of colitis in mice (108). It could be speculated that a great redundancy is present in the inflammatory process that regulates the infiltration of inflammatory cells and that therefore antibodies to IL16 do not reduce antigen-induced eosinophilia in this model. Furthermore, based on the observation that decreased airway hyperresponsiveness does not correlate with decreased presence of eosinophils in BAL fluid it can be concluded that these two phenomena are not necessarily causally related in this model.

Effect of treatment with IL16 *in vivo* and *in vitro*

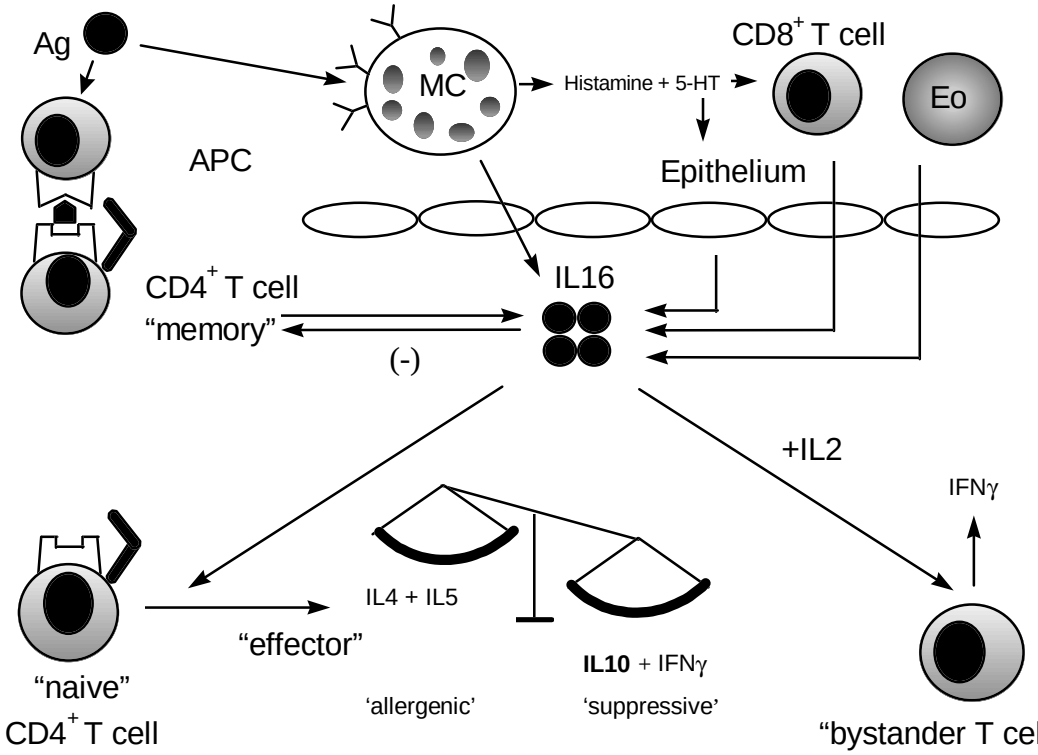
Besides blocking endogenously produced IL16, we also administered IL16 in this murine model during antigen challenge. As demonstrated in chapter 5, intraperitoneal administration of IL16 before each antigen challenge inhibits development of airway hyperresponsiveness and infiltration of eosinophils. Intranasal application of IL16 also inhibits infiltration of eosinophils upon antigen challenge (data not

shown). IL16 uses the CD4 molecule as its receptor and the antigen-specific $CD4^+$ Th2-type T cell has abundantly been proposed to be the regulatory cell in asthma-related pathology. In agreement herewith, antigen-specific restimulation of thoracic lymph node cells appears to be affected by *in vivo* treatment with IL16 since these cells display a decreased Th2-type cytokine production upon *in vitro* restimulation with antigen. Thus IL16, probably via inhibition of $CD4^+$ T cell functioning, has potential beneficial properties in the treatment of features reminiscent of allergic asthma. Interestingly, no effects were observed on production of antigen-specific IgE by *in vivo* treatment with IL16, indicating that memory B cell responses were not affected.
When we further examined this T cell unresponsiveness-inducing property of CD4 cross-linking, we observed that lymph node cells which were preincubated *in vitro* with either IL16 or antibodies to CD4, were unable to respond to antigen (chapters 5 and 6) or to polyclonal stimulation of lymphocytes (chapter 6). This IL16-induced T cell unresponsiveness is in agreement with observations by others who demonstrated that IL16 inhibits T cell activation (69, 70). Analogous to this observation, other CD4 ligands, such as antibodies to CD4 or gp120, can induce anergy (see chapter 1). It has been clearly demonstrated in several animal models that use of non-depleting antibodies to CD4 *in vivo* can be of potential therapeutic importance for different $CD4^+$ T cell-mediated disorders (133, 136, 137, 269). It has been shown e.g. that CD4 cross-linking by antibodies to CD4 results in long-term survival of cardiac grafts in mice (136) and inhibits development of collagen-induced arthritis (CIA) as well as adoptive transfer of CIA into SCID mice (137).
Since the CD4 molecule plays a role in T cell differentiation (71), besides acting as an accessory molecule in TCR-mediated T cell activation (37, 41), we also determined effects of CD4 cross-linking on T cell differentiation. It was shown by Leitenberg *et al*. that the CD4 molecule is required for induction of Th2 type T cell differentiation, whereas it is not for development of Th1 type T cells (71). To explore possible effects of IL16 or anti-CD4 antibodies on differentiation of naive $CD4^+$ T cells into effector cells with a Th0, Th1 or a Th2-type cytokine producing phenotype we determined effects of these CD4 agonists in an artificial *in vitro* T cell skewing system. In this experimental set-up, medium only, IL4 or antibodies to IL4 are used to direct differentiation of naive TCR transgenic (DO11.10) T cells towards the production upon restimulation of Th0, Th2 or Th1-type cytokines, respectively (224).
When naive DO11.10 T cells were cultured with IL16 or antibodies to CD4, and stimulated with antigen without skewing agents, no apparent effects were noted on cytokine production. However, when these cells were restimulated with antigen, the capacity to produce IL4 and IL5 was markedly reduced, whereas IL10 and IFNγ levels were not affected in these cultures (chapter 6). This observation could offer an explanation for the results obtained after *in vivo* treatment with IL16. Both IL4 and IL5 are crucial for development of allergy associated parameters, whereas both IL10 and IFNγ have been demonstrated to be potent inhibitors of Th2-type T cell-mediated responses (reviewed in 251). IL10 has been clearly demonstrated to inhibit antigen-dependent T cell activation, probably via effects on antigen presenting cells (APC, 233, 234). Furthermore, IL10 administration has been shown to inhibit antigen-induced responses in different murine models of allergic asthma (231, 232). IFNγ has also been shown to be a potent inhibitor of development of antigen-induced airway hyperresponsiveness and eosinophilia (165). Furthermore, IFNγ receptor knock-out mice have an impaired ability to resolve antigen-induced inflammation of the airways and sustained Th2-type cytokine production by lymphocytes (235). IFNγ production has also been demonstrated to be increased in a synergistical manner when T cells were incubated with a combination of IL16 and IL2 (88). In agreement herewith, we observed that IL16 combined with IL2 enhances IFNγ production by murine $CD4^+$ lymphocytes (unpublished observations). This synergistical enhancing effect by the combination of IL2 and IL16 on IFNγ could be caused by an IL16-induced upregulation of IL2 receptor expression (29, 90).

Hypothesis on effects of exogenously administered IL16

These observations lead us to the following hypothesis on the effects induced by *in vivo* treatment with IL16. When IL16 is administered before proper T cell receptor stimulation via MHC-II and antigen interaction, IL16-induced CD4 cross-linking could induce T cell unresponsiveness in memory cells

Figure 1



Possible mechanisms of action of IL16 in a murine model of allergic asthma (see text for explanation). Abbreviations used: AHR; Airway Hyperresponsiveness, IL; Interleukin, Ag; Antigen, APC, Antigen-Presenting Cell, MC; Mast Cell, TCR, T Cell Receptor; Eo, Eosinophil; CD, Cluster of Differentiation, (-) inhibitory effect.

(Figure 1). On the other hand, naive T cells that are stimulated with antigen in presence of IL16 may have a decreased capacity to produce the Th2-type cytokines IL4 and IL5 upon restimulation, whereas production of IL10 and IFNγ is not affected (Figure 1). It can be speculated that in the murine model we use, naive T cells are continuously recruited to lymph nodes during repetitive antigen challenge. Differentiation of naive $CD4^+$ T cells towards effector cells is likely to occur during *in vivo* antigen challenge. In previous studies we showed that thoracic lymph node (TLN) cells, isolated from ovalbumin-sensitized saline-challenged mice do not produce any Th2-type cytokines upon antigen-specific restimulation *in vitro*, whereas TLN cells isolated from antigen challenged animals do (151, 206). It could thus be suggested that the balance between production of allergy-associated cytokines (such as IL4 and IL5) and anti-inflammatory cytokines (such as IFNγ and IL10) by effector cells, is disrupted by exposure to IL16 under priming conditions (Figure 1). Moreover, non-specific "bystander" T cells could be synergistically activated by IL16 and endogenously produced IL2, leading to production of IFNγ. In BAL fluid derived from asthmatic patients with bronchial asthma increased IL2 concentrations were detected when compared with normal subjects (270). This IL2 could be produced by eosinophils, epithelial cells or T lymphocytes (271-273).

In its turn, IFNγ has been demonstrated to be a potent inhibitor of Th2-type T cell responses (165). However, no IFNγ could be detected when TLN cells, derived from *in vivo* IL16-treated animals, were stimulated with antigen. Production of IFNγ is also associated with increases in IgG2a antibody production (274, 275) and we were unable to detect any increase in IgG2a levels after treatment with IL16 (unpublished observations). Therefore, IFNγ production, induced by *in vivo* treatment with IL16, is probably not the cause of the observed effects. Treatment with IL16 of IL10 knock-out (276) mice or co-administration of IL16 and antibodies to IL10 or its receptor in the model could resolve the issue whether IL10 is responsible for the IL16-induced effects.
One of the issues that remains unanswered by our studies is the fact that *in vivo* small amounts of IL16 (1 μg animal^{-1} day^{-1}) are capable of inducing anti-inflammatory effects whereas *in vitro* concentrations up to 1 μg ml^{-1} are necessary to change CD4$^+$ T cell functions, such as antigen-induced proliferation and cytokine production. An explanation for this discrepancy could be that IL16 has synergistical effects *in vivo* with other mediators which are not present in our *in vitro* systems. Furthermore, it could very well be suggested that other CD4$^+$ cells, such as monocytes or different subsets of dendritic cells are affected by *in vivo* IL16 treatment, instead of CD4$^+$ T cells. IL16 has been demonstrated to induce CD4-dependent signaling in macrophages (105) and these cells have been demonstrated to play an important regulatory role in development of features reminiscent of allergic asthma (reviewed in 277). Alveolar macrophages have e.g. been demonstrated to produce both IL10 and IL12 (278) and these cytokines have been extensively documented to inhibit allergic manifestations in murine models (151, 231, 232). However, contradictory evidence on the function of macrophages exists since alveolar macrophages increase Th2-type cytokine production by lymphocytes upon stimulation with antigen (279). Besides macrophages, a subset of dendritic cells has the CD4 molecule on its membrane (280). Dendritic cells are of crucial importance for the development of chronic eosinophilic airway inflammation in response to antigen (281). Therefore, future experiments focused at other CD4$^+$ cells than T cells might further unravel the mechanisms via which treatment with IL16 reduces antigen-induced responses *in vivo*.

Hypothesis on endogenously produced IL16

The observation that intratracheal or intraperitoneal treatment with IL16 does not lead to an inflammatory response by itself (chapter 5), indicates that IL16 might not be a potent *in vivo* chemotactic factor for CD4$^+$ cells. This theory is further supported by the observation that IL16 transgene mice do not display any signs of inflammation due to local overexpression in the lungs of IL16 (G. Chubb, personal communications). Furthermore, antibodies to IL16 do not inhibit development of antigen-induced inflammation (chapter 4). Finally, other CD4 binding molecules, such as antibodies to CD4 or gp120 also induce *in vitro* chemotaxis of CD4$^+$ cells, e.g. of eosinophils, (95), without having pro-inflammatory properties *in vivo*. As already mentioned this is not in agreement with *in vitro* evidence which suggests that IL16 can attract CD4$^+$ T cells (29).
We (chapters 5 and 6) and others (222) have clearly demonstrated that exogenously administered IL16 has anti-inflammatory activity. In contrast, endogenously produced IL16 has also been found to have pro-inflammatory effects (reviewed in 90) and (29, 25). Furthermore, we demonstrated that inhibition of endogenously produced IL16, using either an antibody to IL16 or peptide 3 which inhibits interaction between IL16 and its receptor, partially decreased induction of airway hyperresponsiveness, whereas eosinophilia remained present (chapter 4). This is in agreement with previous experiments in which systemic treatment with antibodies to IL16 also partially inhibited development of airway hyperresponsiveness, without decreasing eosinophilia (154). It has also been shown that IL16 can protect lymphocytes from apoptosis (92) and is a competence growth factor for CD4$^+$ lymphocytes (268), which implicates that IL16 plays a role in accumulation of CD4$^+$ antigen-specific T cells at the site of allergen deposition.
Thus IL16 could have pro-inflammatory properties on the one hand, whereas IL16 has also been demonstrated to have immunosuppressive properties. Both the timing and local concentrations of IL16 in the microenvironment of e.g. CD4$^+$ T cells may be important factors between either playing a role in development of airway hyperresponsiveness or induction of T cell unresponsiveness. Furthermore, as

already mentioned, we only focused on effects of CD4 cross-linking by IL16 on T cells in our studies, whereas other $CD4^+$ cells, such as monocytes or dendritic cells, can not be excluded to play a role in causing the inhibitory effects observed after treatment with IL16 *in vivo*.
Another possible explanation for the results obtained with peptide 3 (chapter 4) could be that by binding CD4 molecules, monovalent peptide 3-CD4 interaction actually induces similar responses as treatment with IL16. This would contradict the observation that CD4 cross-linking is necessary for IL16 to be biologically active (29). However, recent literature suggests that CD4 cross-linking is not a prerequisite for IL16-induced biological activity (87). According to literature, it is not unlikely that monovalent CD4 binding instead of CD4 cross-linking is capable of inhibiting $CD4^+$ T cells functions. Using gp120 as a CD4 ligand it has been extensively demonstrated that monovalent CD4 binding can inhibit stimulation of T cells by either antigen of polyclonal stimulation (45, 126, 282-284). It has also been shown that monovalent interaction with CD4 does not necessarily lead to p56lck activation and that p56lck-independent inhibition of T cell responses can occur (117, 285-287), suggesting a regulatory role for the extracellular domains of CD4. In contrast, others did observe activation of p56lck upon CD4-gp120 interaction (70, 114), as was e.g. measured by enhanced autophosphorylation and the phosphorylation of the exogenous substrate enolase (115). Furthermore, it has been reported that a secondary antibody to gp120 to cross-link CD4 molecules was needed to inhibit proliferation of $CD4^+$ T cells (45, 127). Therefore, it remains disputable whether CD4 cross-linking is an absolute requirement for induction of CD4-mediated T cell unresponsiveness. If peptide 3 indeed has similar biological effects as IL16, it could be suggested that preincubation of $CD4^+$ lymphocytes with peptide 3 would logically inhibit IL16-induced migration. In contrast with the hypothesis that CD4 cross-linking is not necessary for IL16 induced effects is the observation that peptide 3 by itself does not induce any detectable chemotaxis *in vitro* as does IL16 (76).
Based on the observation that peptide 3 is capable to bind to CD4 (76), it could be postulated that peptide 3 (or monomeric IL16) inhibits T cell activation via inhibition of dimerization of CD4 or inhibition of MHC-II/CD4 interaction (see chapter 1; Figure 4A and E). The amino acid sequence of peptide 3 is based on the part of IL16 that binds CD4 and peptide 3 has been shown to partially inhibit binding of antibodies to CD4. Furthermore, it is known that IL16 binds CD4 in the D4 region and this particular region has been shown to be of importance for CD4 dimerization and CD4/MHC-II interaction and thus to be important for optimal T cell activation (43).
The observation that development of airway hyperresponsiveness was partly impaired after administration of antibodies to IL16 (chapter 4) could also be explained by enhancing half-life of IL16 instead of capturing endogenously produced IL16. Similar life-time enhancing effects have been described with other antibodies. It has been clearly shown that antibodies can actually function as carrier proteins, thereby prolonging the circulatory half-life of molecules such as insulin (reviewed in 288). Furthermore, it has been demonstrated that antibodies to IL4, IL3 and IL7 are capable of enhancing biological activity of the cytokines they are directed against (289, 290). In agreement herewith, it has been found that either autoantibodies or monoclonal antibodies against IL6 stabilize the cytokine and thus constitute a reservoir of masked, but potentially active cytokine (291-293). Alternatively, the immunecomplex of IL16 with antibodies could induce an immune response by itself (294), thereby causing the observed inhibitory effects on airway hyperresponsiveness.
This could implicate that endogenously produced IL16 has similar inhibitory effects on antigen-induced immune responses as were observed after treatment with IL16. If so, e.g. local concentrations of endogenously produced IL16 might just not be high enough to inhibit development of antigen-induced characteristics of allergic asthma. This hypothesis would provide an alternative explanation for the correlation between disease severity and levels of circulating IL16 (266). Furthermore, if endogenously produced IL16 indeed serves as an anti-inflammatory molecule, the mechanism that causes induction of airway hyperresponsiveness must be more susceptible to IL16-induced inhibitory effects than eosinophilia. If endogenously produced IL16 serves as an immunoinhibitory molecule, it could be suggested that timing of IL16 treatment is not of crucial importance. This would be in agreement with the study by Klimiuk *et al.*, who clearly demonstrated that treatment with IL16 is capable of inhibiting an already established inflammatory response (222). Future experiments, using e.g. IL16 knock-out

mice, F(abs) of anti-IL16 antibodies, or cocktails of different antibodies to IL16 will have to provide the answers to these questions. Furthermore, to resolve the issue on possible importance of timing of treatment, effects of IL16 therapy will have to be determined in already established inflammatory responses.

IL16 as a treatment for $CD4^+$ T cell-mediated diseases

Based on our results it is very tempting to speculate on the use of IL16 as a cure for allergic diseases, or more broadly, as a therapeutic for any $CD4^+$ T cell mediated disease. Indeed, it has recently been demonstrated that exogenously administered IL16 (1 μg $animal^{-1}$ day^{-1}) is capable of inhibiting $CD4^+$ T cell mediated responses in a murine model for human rheumatoid synovitis (222). In this particular study it was demonstrated that IL16, derived from $CD8^+$ T cells, inhibits inflammatory responses in transplanted human inflamed synovium into non-obese diabetic (NOD)- severe combined immunodeficient (SCID) mice. Furthermore, it was found that administration of rhIL16 inhibited tissue cytokine production in human synovium-SCID mouse chimeras, suggesting a down-regulatory function of IL16 in these immune responses (222). In our studies we observed an inhibitory effect by treatment with IL16 on antigen-induced airway hyperresponsiveness and eosinophilia in a murine model of allergic asthma. This decrease in allergic manifestations was accompanied by decreased production of IL4 and IL5 by TLN cells upon restimulation *in vitro*, suggesting that $CD4^+$ T cells are involved in the mechanism via which treatment with IL16 causes these effects. Interestingly, no effects were observed on production of antigen-specific IgE, indicating that memory B cell responses are not affected. The observation that production of IgE is not affected whereas development of both airway hyperresponsiveness and eosinophilia are inhibited is similar to effects caused by treatment with corticosteroids (149). It has been clearly demonstrated that the $CD4^+$ T cell is one of the main targets of therapy with corticosteroids in both human asthmatics and murine models (255, 295-298). Therefore, it could be suggested that patients who respond badly to treatment with steroids could benefit from additional treatment with IL16.
Based on *in vitro* data it could be suggested that IL16-induced T cell unresponsiveness only occurs upon subsequent stimulation of the T cell via the TCR. It has e.g. been shown that PHA-induced T cell responses are not inhibited by treatment with IL16, whereas polyclonal or antigen-specific stimulation is decreased (this thesis, 69, 70). This would implicate that simultaneous immunotherapy, combined with IL16 treatment could be beneficial for patients who suffer from allergic asthma.
Both on our *in vitro* observations and literature data, it can be theorized that only memory cells are susceptible for IL16-induced CD4 cross-linking. We clearly demonstrate that no direct effects can be observed on naive T cells when stimulated with antigen. However, upon restimulation, these cells display decreased cytokine production which appears to be limited to IL4 and IL5. Therefore, caution is necessary since, even though CD4 cross-linking before stimulation of $CD4^+$ T cells with antigen could lead to clonal anergy, other CD4 molecules on either naive or memory T lymphocytes would also be affected. Thereby, unwanted side effects could occur since as long as IL16 binds to CD4, clonal anergy could be induced against e.g. any opportunistic infection which needs $CD4^+$ T cell help for clearance and is resident during or after the treatment. Furthermore, research will have to provide answers on duration of IL16-induced unresponsiveness and long term effects of IL16 as a therapy.

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List of Abbreviations

5-HT	5-hydroxytryptamine
Ag	antigen
AIDS	Acquired immunodeficiency virus
ANOVA	analysis of variance
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CD	cluster of differentiation
CIA	collagen-induced arthritis
ConA	concanavalin A
CPM	counts per minute
DAG	diaglycerol
ELISA	enzyme-linked immunosorbent assay
Eo	eosinophil
EU	experimental units
FACS	fluorescent adhesion cell sorter
FCS	fetal calf serum
FITC	fluorescent isothiocyanate
gp	glycoprotein
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
IP3	inositol tri-phosphate
ITAM	immunoreceptor tyrosine activation motifs
LAR	late asthmatic response
LCF	lymphocyte chemoattractant factor
Lck	lymphocyte-specific tyrosine kinase
LT	leukotriene
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MC	mast cell
MHC	major histocompatibility complex
MMCP-1	mucosal mast cell protease-1
mRNA	messenger ribonucleic acid
n	number
nd	not determined
NOD	non-obese diabetic
OD	optical density
ORF	open reading frame
Ova	ovalbumin
$OVA_{323-339}$	ovalbumin-peptide$_{323-339}$
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

List of Abbreviations (continued)

PCR	polymerase chain reaction
PE	phycoerythrin
PENH	enhanced pauze
PGE_2	prostaglandin E_2
PHA	phytohaemagglutin
PIP2	phosphatidylinositol bi-phosphate
PIP3	phosphatidylinositol tri-phosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
PTK	protein tyrosine kinase
rh	recombinant human
rm	recombinant murine
Sal	saline
SAPK	stress-activated protein kinase
SCID	severe combined immunodeficient
SEM	standard error of the mean
TCR	T cell receptor
Tc	cytotoxic T cell
Th	T helper cell
TLN	thoracic lymph node
Veh	vehicle
WBP	whole body plethysmography
ZAP	ζ-chain associated protein

Nederlandse samenvatting

Introductie

In de geïndustrialiseerde landen lijdt naar schatting 10% van de bevolking aan allergische astma en dit aantal neemt nog steeds toe. Het merendeel van de patiënten met allergische astma vertoont overgevoeligheidsreacties voor gras- of boompollen of voor de uitwerpselen van de huisstofmijt (dit zijn dan de antigenen). Patiënten die lijden aan allergische astma kampen met een verhoogde gevoeligheid van de luchtwegen voor niet-specifieke mediatoren zoals histamine en serotonine maar ook mist (de zogenaamde luchtweg hyperreactiviteit). Tevens wordt in het merendeel van deze patiënten een ontstekingsreactie in de longen waargenomen die voornamelijk bestaat uit eosinofiele granulocyten. Gedacht wordt dat er een causaal verband is tussen het voorkomen van deze eosinofiele granulocyten en de aanwezigheid van de luchtweg hyperreactiviteit. Deze ontstekingsreactie gaat ook vaak gepaard met een verhoogde productie van slijm (mucus).
In het serum van deze mensen kunnen antistoffen, zogenaamde immuunglobulines, van het type E worden gemeten. Dit IgE, wat kan binden via specifieke receptoren op mest cellen, kan een interactie aangaan met het antigeen en dien tengevolge activatie veroorzaken van deze mest cellen. Dit leidt tot de afgifte van mediatoren (waaronder histamine en serotonine), waardoor de luchtwegen vernauwen. Dit is de zogenaamd acute astmatische reactie, die zich gewoonlijk voltrekt binnen enkele minuten en tot enkele uren na contact met het antigeen manifest kan zijn. Naast de acute reactie wordt er bij een select deel van de patiënten ook een laat astmatische reactie onderscheiden die gewoonlijk na enkele uren optreedt en meestal binnen 24 uur verdwijnt. Deze respons wordt geassocieerd met de infiltratie van verschillende ontstekingscellen.
Een van de cellen waaruit het immuunsysteem is opgebouwd is de CD4 positieve ($CD4^+$) T helper 2 (Th2) cel en deze cel wordt geacht een belangrijke rol te spelen in de pathologie die wordt waargenomen bij allergische aandoeningen. Deze $CD4^+$ Th2 cellen produceren verschillende eiwitten waarmee deze cellen “communiceren” met andere cellen van het immuunsysteem. Deze boodschapper moleculen worden cytokines genoemd. De $CD4^+$ Th2 cel wordt gekenmerkt door de productie van een aantal van deze cytokines, waaronder interleukine (IL)-4, 5, 9, 10 en 13. Dit profiel van interleukines wordt gemeten bij patiënten met allergische astma na contact met het allergeen en het is uitgebreid beschreven dat deze interleukines een belangrijke rol spelen bij het ontstaan van op allergie lijkende reacties in verschillende diermodellen voor deze aandoening.
Een van deze diermodellen hebben wij gebruikt in de experimenten die beschreven zijn in dit proefschrift. In dit model worden muizen gesensibiliseerd voor een lichaams vreemd eiwit (ovalbumine, OVA) en vervolgens blootgesteld aan òf een nevel waarin datzelfde eiwit is opgelost òf aan een nevel die slechts bestaat uit het oplosmiddel (saline, SAL). Door het gebruik van een nevel wordt het antigeen in de longen gebracht. Het blijkt dat deze muizen een hyperreactiviteit van de luchtwegen vertonen, dat ontstekingscellen (voornamelijk eosinofiele granulocyten) infiltreren in het longweefsel en dat er een evidente toename detecteerbaar is in OVA-specifieke antilichaam concentraties in het serum van deze dieren, na herhaaldelijke blootstelling van OVA-gesensibiliseerde muizen aan OVA-nevel.

Detectie van de acute en laat astmatische reactie

Recentelijk is er een nieuwe techniek ontwikkeld waarmee op een niet invasieve manier luchtweg reactiviteit van muizen bepaald kan worden. Deze techniek heeft als belangrijkste voordeel dat dieren herhaaldelijk onderworpen kunnen worden aan metingen en dat dan dus de ontwikkeling van luchtwegovergevoeligheid in de tijd gemeten kan worden. Daarnaast is het mogelijk met deze techniek om de luchtweg responsen gedurende langere tijdsperioden te volgen om te bepalen of de zogenaamde acute en laat astmatische respons optreden in dit diermodel. Van de acute respons wordt verondersteld dat deze wordt veroorzaakt door het vrijkomen uit mest cellen van mediatoren die glad spierweefsel in de luchtwegen aanzetten tot contractie. De laat astmatische reactie wordt

geassocieerd met de infiltratie van ontstekingscellen (eosinofiele granulocyten) als gevolg van antigeen inhalatie.
Zoals ook bij patiënten met allergische astma gebeurt na inhalatie van het allergeen, zijn acute luchtwegobstructies meetbaar in dit model (hoofdstuk 2). Tevens werd op het moment dat deze obstructie optreed een verhoging van MMCP-1 gemeten in het serum. Dit is een sterke indicator dat mest cellen degranuleren als gevolg van antigeen-inhalatie. De laat astmatische reactie, zoals die wel wordt waargenomen in een deel van de patiënten met allergische astma, hebben wij niet kunnen detecteren in dit diermodel. Zelfs wanneer de endogene productie van corticosteroïden werd voorkomen door toediening van metyrapone, gecombineerd met verhoogde depositie van antigeen in de longen en versterking van de immuunrespons door gebruik te maken van zogenaamde “booster” (versterker) antigeen-injecties, werden deze responsen niet waargenomen (hoofdstuk 2). Dit zou verklaard kunnen worden door de hypothese dat in het door ons gebruikte model eosinofiele granulocyten niet of niet-detecteerbaar geactiveerd worden door antigeen, hoewel dit wel is aangetoond in andere, soortgelijke, modellen.

Interleukine 16 en het muizenmodel voor allergische astma

Het hoofddoel van dit proefschrift was het bepalen van de rol van IL16 in de pathologie van allergische astma. IL16 wordt aangetroffen in zowel patiënten met allergische astma als ook in het diermodel na contact met het antigeen. Van IL16 is aangetoond dat het geproduceerd kan worden door epitheel cellen, mest cellen, eosinofiele granulocyten, $CD8^+$ en $CD4^+$ T cellen. Een van de eerst ontdekte eigenschappen van IL16 was dat IL16 een aantrekkende (chemotactische) werking heeft op $CD4^+$ cellen. Deze eigenschap, samen met de observatie dat verhoogde hoeveelheden IL16 aanwezig zijn na antigeen contact leidde tot de hypothese dat IL16 bijdraagt aan de formatie van met allergie geassocieerde klachten.
Een van de belangrijkste bronnen van IL16 zou de epitheelcel kunnen zijn omdat hiervan, onder andere middels histologie, is aangetoond dat deze cellen veel IL16 bevatten. Tevens is bekend dat epitheel cellen IL16 produceren na stimulatie met histamine terwijl $CD8^+$ T cellen IL16 afgeven na stimulatie met histamine of serotonine. Aangezien mest cellen in muizen zowel histamine als serotonine vrijzetten na stimulatie middels IgE-antigeen interactie, werd geprobeerd de IL16 afgifte te remmen door toediening van selectieve histamine en serotine receptor antagonisten (hoofdstuk 3). Gebruik van de histamine-type 2 receptor antagonist, zowel als gebruik van de serotonine 2 receptor antagonist, resulteerde in een gedeeltelijke remming van de luchtweg hyperreactiviteit als ook een gedeeltelijke verlaging van de hoeveelheid eosinofiele granulocyten in de longen. Gebruik van deze twee receptor antagonisten samen resulteerde in een volledige remming van de luchtwegovergevoeligheid zonder de aantallen eosinofiele granulocyten verder te verlagen. Metingen van de hoeveelheid IL16 in de longen bracht aan het licht dat deze onveranderd waren. Het is daardoor niet erg waarschijnlijk dat histamine en serotonine de enige inductoren zijn voor de productie en afgifte van IL16.
Een andere methodologische aanpak om de rol van endogeen (door het lichaam zelf aangemaakt) IL16 te bepalen bestond uit het gebruik van zogenaamde monoclonale antilichamen gericht tegen IL16. Daarnaast werd ook gebruik gemaakt van peptides (eiwit fragmenten) waarvan de sequentie (volgorde van de aminozuren, “bouwstenen”) gebaseerd was op IL16 (hoofdstuk 4). Van een van deze peptides was reeds aangetoond dat deze de werking van IL16 *in vitro* (in glas) kan remmen, wat hoogst waarschijnlijk een gevolg is van het feit dat dit peptide bindt aan de receptor voor IL16 (het CD4 molecuul) zonder deze cellen dan te activeren.
Gebruik van zowel de antilichamen, als het IL16 inhiberende peptide, gedurende de blootstellingsperiode middels inhalatie van het antigeen, resulteerde in een partiële remming van de luchtweg hyperreactiviteit, zonder dat een effect werd waargenomen op de mate van ontsteking. Deze experimenten tonen aan dat endogeen geproduceerd IL16 in ieder geval een rol speelt in de inductie van luchtweg hyperreactiviteit als gevolg van antigeen inhalatie. Tevens tonen deze experimenten aan dat IL16 geen rol speelt in de chemotaxie van eosinofiele granulocyten.

Een geheel andere methodologie werd ook aangewend om de effecten van IL16 in het muizenmodel te bestuderen. In verschillende experimenten werd IL16 toegediend gedurende de antigeen-blootstellings periode (hoofdstuk 5). Toediening van IL16 voor iedere antigeen expositie resulteerde in een sterke inhibitie van antigeen-geïnduceerde fenomenen. Een volledige reductie werd waargenomen van zowel de luchtweg hyperreactiviteit als ook nagenoeg complete inhibitie van de ontstekingsrespons, bestaande uit de infiltratie van eosinofiele granulocyten. De productie van antigeen-specifieke antilichamen (in het bijzonder IgE) was niet aangetast door behandeling met IL16, waaruit geconcludeerd kan worden dat de zogenaamde geheugen-B cel respons niet beïnvloed wordt door IL16.
Een ander interessant fenomeen na behandeling van muizen met IL16 bestond uit de observatie dat cellen uit lymfeknopen, geïsoleerd uit IL16 behandelde en aan antigeen blootgestelde dieren, minder capabel waren om IL4 en IL5 te produceren na stimulatie met antigeen *in vitro* als deze productie werd vergeleken met cellen die geïsoleerd waren uit controle dieren. Van zowel IL4 als IL5 is aangetoond dat dit belangrijke interleukines zijn voor de inductie van allergische symptomen in zowel patiënten als diermodellen. Tevens werd geobserveerd dat deze cellen minder goed in staat zijn tot proliferatie (vermenigvuldiging) wanneer zij in aanraking zijn geweest met IL16 *in vivo* (in het intacte dier). Naast deze effecten van IL16 *in vivo*, hebben wij ook gekeken naar mogelijke effecten van IL16 behandeling op antigeen-specifieke T cellen *in vitro*. Ook na voorbehandeling van $CD4^+$ T cellen *in vitro* werd een inhiberend effect waargenomen op zowel antigeen-geïnduceerde cytokine productie als op proliferatie (hoofdstuk 6).
Aangezien het CD4 molecuul de receptor is voor IL16 en aangezien de belangrijkste bron van IL4 en IL5 deze $CD4^+$ Th2 cel is, is het erg aanlokkelijk om te speculeren op een mogelijk effect van IL16 op deze T cel populatie. Geheel in lijn met deze gedachtengang zijn de observaties dat andere CD4-bindende eiwitten, zoals antilichamen tegen CD4 en gp120 (HIV enveloppe eiwit), ook in staat zijn $CD4^+$ T cel responsen te remmen. Het is bijvoorbeeld goed gedocumenteerd dat antilichamen tegen CD4 kunnen beschermen tegen afstotingsreacties na orgaantransplantatie en tegen de inductie van collageen-geïnduceerde gewrichtsontsteking.
Aangezien het CD4 molecuul niet alleen een rol speelt bij de activatie van T cellen, maar ook bij de differentiatie van naïeve T cellen in T cellen met een Th2 cytokine profiel, waarvan bekend is dat deze een cruciale rol vervullen in de pathologie van allergische astma, hebben wij ook gekeken naar de rol van IL16 in dit soort responsen (hoofdstuk 6). Hiervoor hebben wij een artificieel systeem gebruikt waarbij de aan of afwezigheid van IL4 (de sturende factoren) tijdens de eerste stimulatie met antigeen *in vitro* bepaald of T cellen na restimulatie differentiëren richting een Th1 of Th2 cytokine producerende cel. Wanneer naïeve T cellen werden blootgesteld aan IL16, zonder de sturende factoren, was er geen effect waarneembaar op de cytokine productie na de eerste stimulatie met antigeen. Echter, na restimulatie met antigeen produceerden deze cellen minder IL4 en IL5, wanneer een vergelijk werd gemaakt met cellen die initieel niet in contact waren geweest met IL16. De productie van IFNγ en IL10 was echter niet verandert in de IL16 behandelde cellen. Geen effecten veroorzaakt door aanwezigheid van IL16 werden waargenomen op de cytokine productie na restimulatie met het antigeen, als de differentiatie van T cellen werd gestuurd richting een Th1 of Th2 cytokine profiel.
Het effect van IL16 op de differentiatie van naïeve T cellen als gevolg van herhaaldelijke blootstelling aan antigeen zonder dat daarbij sturende factoren aanwezig zijn, zou een mogelijke verklaring kunnen zijn voor de effecten van IL16 behandeling *in vivo*. Zoals reeds vermeld, is het wel bekend dat zowel IL4 en IL5 belangrijk zijn voor de inductie van met allergie geassocieerde fenomenen, terwijl van zowel IL10 als IFNγ bekend is dat deze interleukines potente inhiberende eigenschappen bezitten.
Het zou heel wel mogelijk kunnen zijn dat IL16 deze effecten veroorzaakt door binding aan het CD4 molecuul. Het CD4 molecuul speelt een belangrijke rol bij de interactie tussen T cel en de cellen die het antigeen aan de T cel laten zien (antigeen-presenterende cel, APC). Dit proces van antigeen presentatie aan de T cel is noodzakelijk in het immuunsysteem aangezien op deze manier gewaarborgd wordt dat de T cel op een gecontroleerde manier geactiveerd wordt (m.a.w. alleen wanneer het antigeen aanwezig is). Het CD4 molecuul zorgt ervoor dat de interactie tussen APC en T

cel gestabiliseerd wordt en dat de T cel de juiste signalen krijgt. Wanneer het CD4 molecuul onafhankelijk van de APC-T cel interactie wordt gebonden dan krijgt de T cel de verkeerde signalen waardoor de T cel anerg kan worden (dit wil zeggen; niet meer reageert op prikkels waarop de cel normaal gesproken wel zou reageren). Dit zou veroorzaakt kunnen worden door de signalen die de T cel krijgt als het gevolg van zg. cross-linking (het verbinden van meerdere CD4 moleculen tegelijkertijd). In theorie kan IL16 vier CD4 moleculen tegelijkertijd cross-linken terwijl een intact antilichaam tegen CD4 er twee tegelijkertijd zou kunnen verbinden. In tegenspraak hiermee is de observatie dat gp120 ook dit soort responsen kan opwekken in CD4 cellen, terwijl gp120 slechts een monovalente (één op één) interactie kan aangaan met het CD4 molecuul. In het geval waarbij IL16 toegediend werd voor iedere antigeen inhalatie, zou het dus kunnen zijn dat de CD4 moleculen op $CD4^+$ Th2 cellen reeds gebonden zijn door IL16, waardoor de interactie APC-T cel niet correct kan plaats vinden óf dat er negatieve signalen worden doorgegeven aan de T cel.
Een van de observaties die hiermee niet verklaard kunnen worden is het feit dat voor de realisatie van de *in vivo* effecten door IL16 slechts weinig van dit IL16 nodig is, terwijl *in vitro* effecten van IL16 pas bij veel hogere concentraties worden waargenomen. Dit zou veroorzaakt kunnen worden doordat IL16 *in vivo* synergistische (elkaar versterkende) effecten heeft met andere mediatoren die niet aanwezig zijn in de *in vitro* kweken. Een andere mogelijkheid zou kunnen zijn dat IL16 interacties aangaat met andere cellen dan de T cel, bijvoorbeeld met de macrofaag, die ook CD4 moleculen tot expressie brengt op de celmembraan. Macrofagen zijn in grote hoeveelheden aanwezig zijn in de longen en zouden dus mogelijk beïnvloed kunnen worden door therapie met IL16.
De observatie dat antilichamen tegen IL16 een gedeeltelijke remming geven van de luchtweg hyperreactiviteit is niet in overeenstemming met de observatie dat toediening van IL16 zelf een volledige remming van de luchtwegovergevoeligheid geeft. Een verklaring hiervoor kan zijn dat de tijd van toediening van essentieel belang is voor de uitkomst van de behandeling. Op het moment dat de antilichamen werden toegediend is er nog geen detecteerbare hoeveelheid IL16 aanwezig in de luchtwegen van deze muizen. Pas na een aantal dagen antigeen blootstelling kan IL16 gemeten worden in de longen van gesensibiliseerde muizen. Bij toediening van IL16 is er sprake van een situatie waarbij IL16 aanwezig is in het lichaam op een tijdstip waarop dit normaal gesproken niet het geval is, waardoor deze discrepantie in resultaten verklaard zou kunnen worden. Een andere mogelijke verklaring voor dit fenomeen zou kunnen zijn dat in plaats van het wegvangen van IL16 door de antilichamen tegen IL16, er een verhoging van de halfwaarde tijd plaatsvindt, waardoor het door het lichaam gevormde IL16 langer aanwezig blijft in plaats van te verdwijnen. Dit is ook waargenomen bij het gebruik van andere antilichamen tegen verschillende andere interleukines.
Het feit dat het IL16 blokkerende peptide een gedeeltelijke remming laat zien van de luchtwegovergevoeligheid zou verklaard kunnen worden door te veronderstellen dat het peptide dezelfde activiteit heeft na binding met het CD4 molecuul als het intacte IL16. Het is inderdaad gepubliceerd dat voor T cel inhiberende effectiviteit van CD4-bindende substanties het binden van slechts een CD4 molecuul tegelijkertijd, in plaats van het koppelen van verschillende CD4 moleculen, voldoende is.

IL16 als mogelijke therapie voor allergische astma

Omwille van de bovenstaande resultaten is het erg verleidelijk om te speculeren op het gebruik van IL16 als mogelijke therapie bij de behandeling van allergische astma. Inderdaad is recentelijk aangetoond dat toediening van IL16 in een diermodel, in dezelfde dagelijkse dosis als die door ons is gebruikt, het ontstaan van ontsteking (synovitis) kan tegengaan. Bovendien werd in die studies aangetoond dat dit effect gepaard ging met een verlaging van de aanmaak van boodschapper RNA voor diverse interleukines. Deze resultaten zijn in overeenstemming met de door ons waargenomen effecten van therapie met IL16 in het muizenmodel voor allergische astma. Op grond van de literatuur en het voorgestelde werkingsmechanisme kan geconcludeerd worden dat IL16 therapie niet alleen de antigeen-specifieke T cellen beïnvloed maar alle $CD4^+$ T cellen. Als deze andere T cellen het antigeen tegenkomen waar zij specifiek op reageren (bijvoorbeeld een opportunistische infectie) dan zijn deze T

cellen dus ook niet meer in staat zijn hierop een adequate respons te genereren, wat onvermijdelijk zal lijden tot voor de patiënt kwalijke gevolgen. Verder is therapie met IL16 alleen zinvol indien de geinduceerde veranderingen in $CD4^+$ T cellen van lange duur zijn. Verder onderzoek is dus noodzakelijk om IL16 als therapeuticum verder te ontwikkelen.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 1 december 1970 te Veghel. In augustus 1989 behaalde hij het Gymnasium-β diploma aan “Gymnasium Bernrode” te Heeswijk-Dinther. In hetzelfde jaar werd door hem een aanvang gemaakt met de studie medische Biologie aan de Faculteit Geneeskunde van de Universiteit van Utrecht. In augustus 1990 behaalde hij het propedeutisch examen, gevolgd door het doctoraal examen in augustus 1995. Tijdens deze studie heeft hij een drietal wetenschappelijke stages volbracht. Onder begeleiding van Prof. Dr. A.J.P. Schrijvers (UU, Fac. Geneeskunde, vakgroep Algemene gezondheidszorg & Epidemiologie) werd een studie volbracht naar de gevolgen van een beleidsverandering in het Westeinde Ziekenhuis te ‘s Gravenhage, als onderdeel van het bijvak getiteld; “Beleid en Management van de Gezondheidszorg”.
Onder begeleiding van Dr. E.M. Hessel en Dr. A.J.M. van Oosterhout (UU, Fac. Farmacie, vakgroep Farmacologie en Pathofysiologie) werd gedurende negen maanden onderzoek gedaan met als titel “modulatie van bronchiale hyperreactiviteit en ontsteking in een muizenmodel voor allergische astma”. Hierna werd een extra-curriculaire stage volbracht onder supervisie van Dr. P. Okunieff (NHI, NCI, Radiation Oncology Branch, USA) naar de voorspelbaarheid van genezing op grond van zuurstof verdeling in verschillende humane plaveiselcel carcinomen als het gevolg van het aanbieden van radiotherapie. Na het behalen van het doctoraal examen begon hij aansluitend met een door Glaxo-Wellcome gesubsidieerd promotie onderzoek naar de rol van interleukine 16 in een muizenmodel voor allergische astma. Dit onderzoek werd uitgevoerd aan de Fac. Farmacie, vakgroep Farmacologie en Pathofysiologie in samenwerking met mw. Ing. E.H. Jonker onder supervisie van Dr. A.J.M. van Oosterhout en Dr. P.A.J. Henricks. Als promotor fungeerde Prof. Dr. F.P. Nijkamp. Het resultaat van dit onderzoek ligt thans voor U. Inmiddels is de schrijver dezes verbonden aan het Department of Clinical Trial Operations van N.V. Organon.

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