Dendritic Cells in Asthma:

a function beyond sensitization

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Dendritische cellen in astma:
meer dan sensitisatie alleen

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Chapter 1

Introduction and aim of the thesis

Asthma

Asthma is a chronic inflammatory disease characterized by the clinicopathologic symptoms of intermittent and reversible airway obstruction, enhanced mucus production, chronic bronchial inflammation with eosinophils, and bronchial smooth muscle cell hypertrophy. The severity of asthma can vary from intermittent, mild persistent, moderate persistent to severe persistent with increasing frequencies of symptoms and exacerbations. As a result of the chronic inflammation, airway tissue is continuously being injured and healed resulting in structural changes (remodeling) of the airway tissue which may account for the observed irreversible decline in airway function as seen in asthmatic patients over several years[1]. Affected individuals may suffer considerable morbidity, and asthma causes death in some patients.

Genetic predisposition

The etiology of asthma is complex and multifactorial, but it is believed that there is a genetic factor in asthma. Genetic studies of asthma have begun to identify distinct regions within the human genome, which are closely associated with atopy, airway hyperresponsiveness (AHR) and asthma. However, no simple pattern of Mendelian inheritance has been established. Genome wide polymorphism studies identified several proteins on different chromosomes significantly associated with asthma, atopy, and bronchial hyperreactivity. Recently, a polymorphism in the ADAM33 gene on chromosome 20 was detected. This gene has been identified as an asthma-susceptibility gene[2,3]. The ADAM33 gene encodes for ADAM proteins, which are membrane-anchored metalloproteases with diverse functions, which include the shedding of cell surface proteins such as cytokines and cytokine receptors and are expressed in lung fibroblasts and airway smooth muscle but not in airway epithelium. Another protein associated with asthma was detected on chromosome 2 next to the IL-1 cluster, DPP10. DPP10 encodes a homologue of dipeptidyl peptidases (DPPs) that cleave terminal dipeptides from cytokines and chemokines[4]. Genetic factors also determine whether or not environmental factors can offer protection to the development of asthma. The receptor for hepatitis A virus (HAV) on T cells is believed to be involved in the development of tolerance, which is protective against the induction of allergic diseases. HAV can bind to TIM1, the cell-surface receptor used by HAV to infect human cells, expressed by T cells, which directly alters Th2 differentiation and limits the development of Th2 cells. Subjects with a particular

polymorphism in this TIM1 gene were protected after HAV infection against the development of atopy[5].

Several regions on the genome contain candidate genes implicated in the asthma pathogenesis[6]. Polymorphisms in these genes could contribute to the variable susceptibility to asthma between subjects. Chromosome 5 contains the IL-4 gene cluster that also includes IL-3, IL-5, IL-9, IL-13 and GM-CSF, all of which are involved in orchestrating the IgE mast cell and eosinophil response characteristic of allergic responses. In addition, this gene region contains the β_2-adrenoceptor and the glucocorticoid receptor. Chromosome 11 contains the β subunit of the high-affinity IgE receptor. Chromosome 12 contains IFNγ (cytokine counterbalancing Th2 reactions), NF-κB (transcription factor) and a gene controlling for the synthesis of NO. Chromosome 6 contains the genetic information for tumor necrosis factor α, a proinflammatory cytokine[7]. However direct relationships between polymorphisms of these genes and the development of asthma have not been elucidated yet.

Allergens and Environmental Factors

However not only genetic factors account for the induction of asthma. The rate of increase of reported asthma cases in the last three decades is such that it can only be accounted for by environmental changes and not by genetic factors alone (see Fig 1).

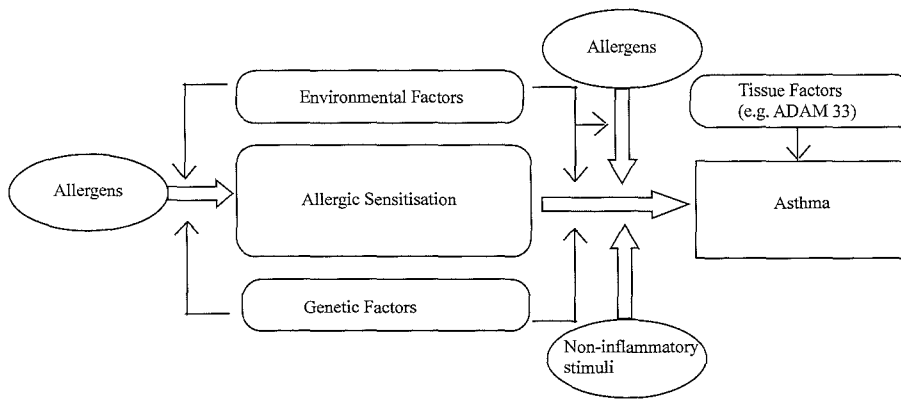

Figure 1. Factors influencing sensitization to allergen and subsequent development of asthma.

In the Netherlands the prevalence of asthma has increased from 5 per 1000 persons in 1984 to 26-31 per 1000 persons in 1999[8]. One of the most important predisposing factors for asthma

is allergy as indicated by the strong association between serum IgE and the risk for asthma[9]. It is currently believed that a dysregulated cellular (primed T cells) and humoral (Immunoglobulin (Ig) E antibodies) immune response to allergen is the basis for atopy[10-12]. In 70% of asthma patients a common aeroallergen, by itself harmless, like house dust mite, birch pollens, cat dander, and moulds, can provoke a positive skin prick test and in most cases bronchoconstriction after inhalation. The presence of activated eosinophils and Th2 cells in asthmatic airway biopsies[13,14] further emphasizes the importance of allergy in asthma pathogenesis. In most non-atopics, aeroallergens evoke a (mild) Th1 reaction which does not lead to clinical symptoms, while in contrast atopic subjects react via the Th2 pathway[15]. Th1 cells are characterised by the production of IFN-γ, TNF-α and IL-2, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13[16]. These distinct cytokine patterns direct different subsets of inflammatory cells. Th1 cells activate macrophages to fight intracellular microbes whereas Th2 cells recruit B cells, mast cells and eosinophils. The Th2 pathway is generally induced to eliminate extracellular pathogens like helminthes.

The observation that the incidence of atopy and asthma has increased tremendously the last two decades in developed countries, while in less-developed countries there is a lower incidence, and the observation that the highest prevalence of asthma is reported among children without older siblings and who have consequently lower infection rates at a young age, has led to the so-called hygiene hypothesis[17,18]. The improved hygiene would have, besides its beneficial effects, a paradoxical allergy-promoting effect[19]. According to the hygiene theory, the increase in asthma incidence can be attributed to a combination of changes in environmental factors, such as reduced risk of childhood infections[20], increased environmental pollution[21], increased allergen exposure[22] and altered diet[23], but also changes in other factors including increased report of asthma, improved diagnostics and psycho-social influences. Exposure to microbial pathogens at an early age can protect against the development of atopy and asthma [24-26] by skewing the immune system from a Th2 ('allergic') default pathway to a response that is more in balance with Th1 cells (See Fig 2). Most intracellular pathogens evoke a Th1 response, which can teach the immune system to react via Th1 pathway. The classical immunological explanation for the hygiene hypothesis has been that in the absence of the Th1 provoking infections, the balance shifts towards Th2 responses,

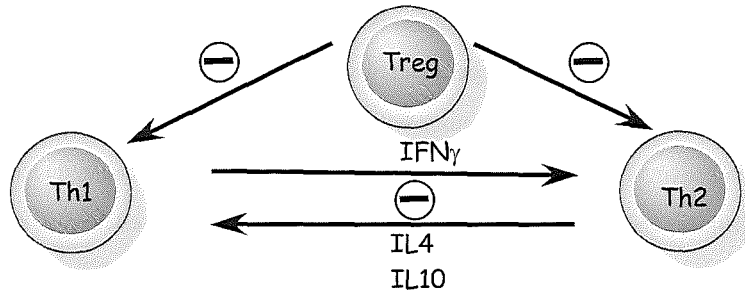

Figure 2. Balance between Th1, Th2 and regulatory T cells

thus provoking allergic reactions to harmless antigens such as allergens. Another explanation offered for the protective effect of infections would be the development of regulatory factors, which are secreted during an infection to down regulate the degree of inflammation[27]. Most bacteria, Gram-negative and Gram-positive, as well as most helminthes upregulate IL-10 secretion by several cell types, consequently suppressing an underlying predisposition to allergy. Although it has been described that IL-10 can have pro-Th2 effects *in vitro*, the principal biological role of IL-10 *in vivo* seems to be a homeostatic one. This is illustrated by experiments in mice with a genetic deletion of IL-10, which are highly susceptible to acute inflammatory processes[28]. *In vivo* a particular role of IL-10-producing regulatory T cells in controlling allergy has been suggested[29]. Conditions of improved hygiene diminish the number of infections but would also inhibit the development of IL-10-producing regulatory T cells. This explanation would also explain the rising trends in Th1 associated auto-immune disease with conditions of improved hygiene, as regulatory T cells producing IL-10 also suppress Th1 responses[27].

However, 10-30%[30] of the asthma patients are not demonstrably atopic, and here the term non-atopic (intrinsic) asthma is used. Intrinsic asthma patients show negative skin tests and there is no history of allergy. Furthermore, serum IgE levels are within normal ranges[31], however there is some evidence for local IgE switching[32]. In contrast, eosinophils that are classically associated with 'allergic' inflammation, are also abundant in the bronchial submucosa of intrinsic asthmatics[13,31,33] and patterns of airways responses are very similar to that observed in atopic asthma.

Immunological basis for asthma

In most asthma patients, there are one or more aeroallergens that can provoke an early response, almost immediately after allergen exposure, inducing direct bronchial constriction. In approximately 50% of patients exposed to allergens, a late phase reaction occurs about 4-12 hours after allergen exposure which is characterized by infiltration of eosinophils in the bronchial mucosa[34, 35]. Although the inflammatory basis of asthma is well established[36], the mechanisms involved in Th2 sensitization are not totally elucidated. Allergens have passed several levels of the pulmonary defense mechanisms before they can induce asthmatic symptoms. First, allergens have to deal with the non-specific innate defense responses before they can evoke a specific, adaptive primary immune response, which will lead to sensitization to the allergen. After established sensitization, repeated encounter with the allergen will evoke a more vigorous allergen-specific secondary immune response, responsible for the symptoms seen in asthma. In the next paragraphs, the subsequent stages that are passed by an allergen will be discussed.

Non-specific immune response

After inhalation into the airways, allergens first encounter the anatomical barriers, the cough reflex, the mucociliary apparatus and the airway epithelium. The epithelium forms the lining of the luminal surface of the airways and is a barrier between the lumen and the pulmonary parenchyma. The epithelium is impermeable through formations of tight junctions between epithelial cells. The epithelium is lined with cilliary cells which enables the airways to dispose of inhaled particles. After a particle has been covered with mucus, it can be transported by the upward movement of the cilia back to the mouth, where it can be swallowed. It has been shown that some allergens (e.g. Der p 1) have the potential to break down tight junctions, in this way gaining access to the processes of dendritic cells that are located in the basal cellular channels surrounding basal epithelial cells[37].

Sensitisation

Inhaled antigens that reach the epithelium are taken up by antigen presenting cells[38]. Allergens are processed by antigen presenting cells into small antigenic peptides that can be presented to antigen-specific T cells in the context of MHCII molecules. Animal models for asthma have shown that dendritic cells are the most potent allergen presenting cells in the

lung[39]. If the antigen is a microbial protein or is administered with an adjuvant (e.g. diesel particles[40]), it elicits a local innate immune response, during which inflammatory cytokines are produced. These cytokines act on DCs[41], which will migrate into the lymphatic vessels to the T cell zones in the draining lymph nodes of the lung. Naïve $CD4^+$ helper T cells recirculate through the secondary lymphoid organs in the body where they will encounter these antigen presenting cells. After antigen recognition, T cells secrete IL-2 and upregulate the IL-2 receptor. IL-2 is the principal autocrine growth factor of naïve T cells and secretion of IL-2 will lead to proliferation (clonal expansion) to generate the large number of antigen-specific cells which are required to control the response or eliminate the antigen. The mechanism by which allergens induce differentiation of naïve T cells towards a Th2 phenotype is still unclear. However, several factors like cytokine environment, strength of antigen-MHCII/TCR stimulation and presence of costimulatory molecules are involved in the differentiation to Th2 cells. When allergen presentation results in the differentiation of naïve T cells into Th2 cells, these activated Th2 cells will migrate to the B cell zone in the draining lymph node where they will induce B cell Ig heavy chain class switching of IgM to the IgE isotype. Binding of IL-4 and IL-13 on the IL-4 receptor α chain on B cells activates the JAK/STAT6 pathway inducing germ line gene transcription resulting in IgE switch[42]. The presence of IgE in the serum of patients is the hallmark of Th2-dependent sensitization that is so typical for patients with allergy. This is classically measured using the radio allergosorbent test (RAST) or by specific skin testing using allergen extracts (see below).

The early phase in the effector response

Secreted IgE will bind to mast cells present in the periphery, expressing high affinity receptors for IgE (FcεRI). An encounter with allergen can lead to cross linking of two IgE molecules inducing cellular activation and the release of inflammatory products within minutes. Histamine is one of the mediators released by mast cells. Histamine is preformed and stored in granules and can therefore be released within minutes. Histamine can bind to H1 receptors which are associated with bronchoconstriction of the airway smooth muscle and increased vascular permeability, whereas binding to H2 receptor activation leads to increased airway mucus secretion[43]. The bronchoconstriction during the early phase reaction is therefore attributed to mast cells[44]. Recently, it has been shown that the localization of mast cells in the

smooth muscle layer surrounding the airways is an important predictor of bronchial hyperreactivity, discriminating asthma from eosinophilic bronchitis[45]. A generally used test to determine sensitization to several antigens is based on this mast cell induced reaction. A range of possible allergens is injected at different spots in the skin. When mast cells are present in the skin with bound allergen specific IgE, the antigen will crosslink the IgE molecules inducing degranulation of the mast cells and the secreted mediators will lead to a red, swollen skin where the antigen was injected. In this way, sensitization to allergens can be determined by a skin-prick test.

The late phase in the effector response

The immune response to inhaled allergen seen in asthma consists not only of an immediate early phase reaction but in approximately 50% of the patients also a late phase reaction occurs, 4-12 hours after antigen exposure. The late phase airway response is characterized by a recurrence in clinical symptoms, mediator release and leukocyte recruitment. During the late phase response eosinophils, basophils and lymphocytes are recruited. The late phase reaction is orchestrated by recruited Th2 cells at the site of the allergen-induced immune response (See Fig 3).

Activated Th2 cells will leave the lymph node, recirculate and will be recruited from the bloodstream at the site of inflammation by an increased vascular permeability and the upregulation of adhesion molecules on the endothelium[41]. Effector Th2 cells will secrete several cytokines. IL-4 and IL-13 are both involved in the development of goblet cell hyperplasia and enhanced mucus production *in vivo* by binding the IL-4 Rα. IL-4 is also a growth factor for mast cells and differentiated Th2 cells. One of the other major functions of IL-4 secreted by effector Th2 cells during allergic airway inflammation might be the upregulation of the vascular cell adhesion molecule-1 (VCAM-1) expression at endothelial cells for recruiting very late antigen-4 positive eosinophils[46]. IL-4 is however not sufficient to cause all aspects of the asthmatic phenotype.

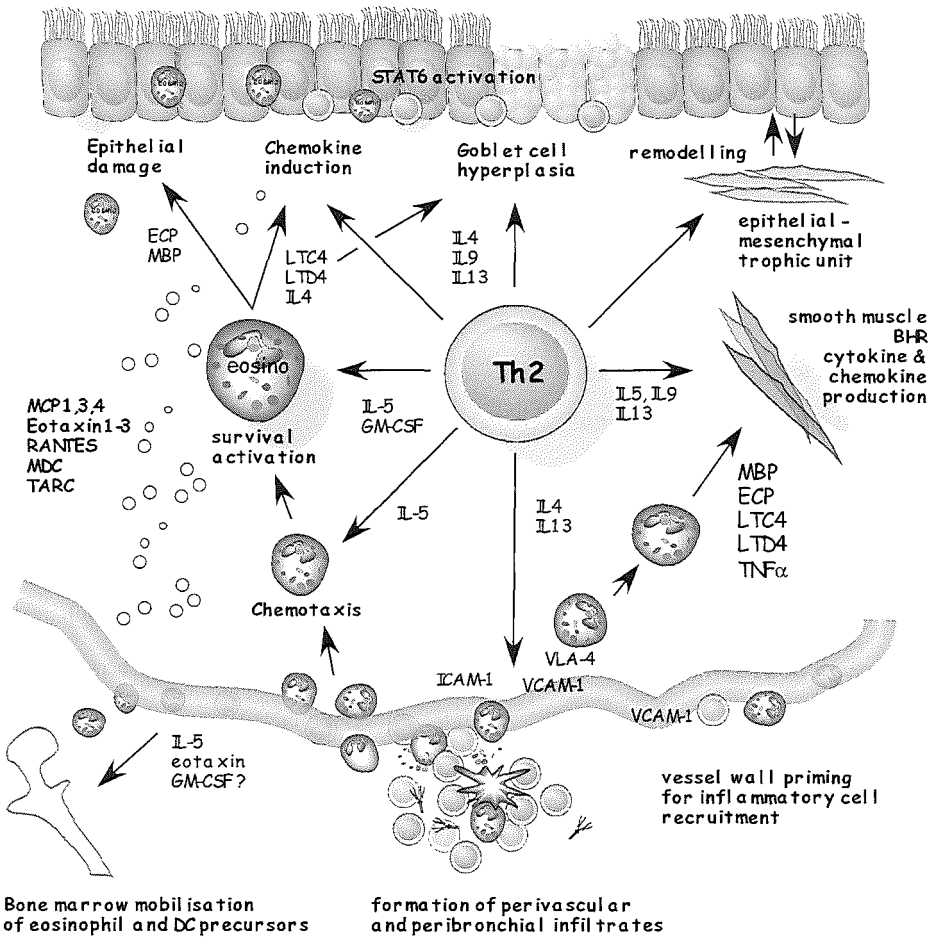

Figure 3. Effector functions of Th2 cells

This is illustrated in mice, which transgenically expressed IL-4 in the airways. Some mild degree of peribronchial eosinophilic inflammation was induced but no AHR[47].

Infiltration with eosinophils in the lung mucosa, characteristic for the late phase response, is mediated by IL-5 in concert with CCR3 active chemokines like Eotaxin, which is a selective chemoattractant for eosinophils which express the eotaxin receptor CCR3[48]. IL-5, released by effector Th2 cells, is an important mediator for the proliferation, activation, survival and recruitment of eosinophils. IL-5 is not only increased at the inflammatory site but as well at the serum level, exerting a systemic effect by increasing the production of eosinophil precursors in bone marrow[49]. Eosinophils contain granules with cationic proteins, like

eosinophil peroxidase (EPO), Major Basic Protein (MBP), Eosinophil cationic protein (ECP) and eosinophils-derived neurotoxin EDN, which are toxic and damaging to the lung epithelium upon degranulation. Eosinophilia in humans has been correlated to the severity of asthma symptoms[14, 50]. The role of eosinophils in murine models for asthma is still a matter of debate, mostly focused on the observed lack of degranulation by murine eosinophils[51].

The role of mast cells is not restricted to the release of histamine during the early phase reaction only, mast cells also secrete neutral proteases bound to proteoglycans. Neutral proteases are the most abundant proteins in mast cell granules and are represented by tryptase, chymase, and carboxypeptidase A. The proposed functions for these proteases include tissue remodeling by activation of matrix metalloproteases, activation of protease-activated receptors, increased mucus secretion and leukocyte recruitment[52, 53]. Also leukotrienes (LTB4, LTC4, LTE4) and prostaglandins (PGD2) are released, involved in bronchoconstriction and recruitment of T cells, basophils, eosinophils[54], and DCs[55] important during the late phase. The role of the many secreted cytokines by mast cells *in vivo* remains to be elucidated.

Infiltration with basophils is observed in lungs of patients with fatal asthma. Basophils are recruited to the site of antigen during the late phase response. Basophils in asthma release similar inflammatory products as mast cells, within minutes after cross linking IgE bound to FcεRI on the cell[56].

It has been shown that epithelial cells also play a very important role by recruitment of inflammatory cells by the expression of adhesion molecules[57] and production of chemokines (e.g. MCP-1[58], TARC[59]). In addition, epithelium of asthma patients produces more GM-CSF[60], a known maturation factor for DCs. An enhanced survival or number of dendritic cells in the epithelium, can contribute to the maintenance of the inflammatory response.

Memory T cell response

Effector cells rapidly decline after antigen clearance[61]. However, besides differentiation to effector T cells also some cells differentiate into memory T cells. These cells do not secrete cytokines and are in a resting state. In contrast to effector T cells, memory T cells survive for long periods. Memory T cells are thought to consist out of two subsets, one subset (central memory T cells) which circulates in the body through the secondary lymphoid organs, while

another subset ('effector' memory T cells) resides in the peripheral tissue where sensitisation took place. Both subsets are on stand-by for a re-encounter with the antigen[62, 63].

On subsequent encounters with the same antigen, memory T cells can induce an enhanced and accelerated immune response. It is hypothesized that effector memory T cells in the periphery can differentiate into effector T cells, secreting cytokines to activate and recruit innate effector cells, like mast cells and eosinophils. Central memory T cells recirculating through lymph nodes are activated by antigen presenting cells carrying the immunogenic peptide from the periphery[64]. In the draining lymph nodes, these memory T cells will proliferate and differentiate into new waves of effector T cells, which are recruited to sites of allergen exposure, enhancing the immune response. Restimulated memory T cells can provoke a quantitatively and qualitatively different immune response with a second encounter with allergen.

Protective mechanisms against asthma

The earlier mentioned possible mechanisms that would protect against the development of Th2 responses to harmless antigens are relatively limited (See Fig 4).

First, counterbalancing the Th2 reaction with Th1 cells has some difficulties. Th1 cells are able to inhibit the development of Th2 cells but not the function of Th2 effector cells. Th1 cells provide pro-inflammatory signals, most likely by secreting IFNγ, rather than counterbalancing Th2[65]. Therefore Th1 cells are unlikely to provide protection to the development of asthma once Th2 cells have been induced. Second, the role of regulatory T cells in suppressing or regulating the allergic reaction has been the focus of multiple studies[27]. Several regulatory cell types have been described. Tr1, $CD4^+CD25^+$ naturally occurring Treg, Th3 and NKT cells have been reviewed all for their role in the development of allergic disease and asthma (reviewed in [66]). Tr1 cells have been shown to inhibit Th2 responses as well as Th1 responses, and have been shown to regulate the development of Th2 cells and AHR. Naturally occurring $CD4^+CD25^+$ cells have been shown to inhibit the development of airway eosinophilia[67] but do not appear to inhibit the development of AHR[68]. Th3 cells are

possibly more involved in regulating tolerance in gastrointestinal (GI) disease than in the

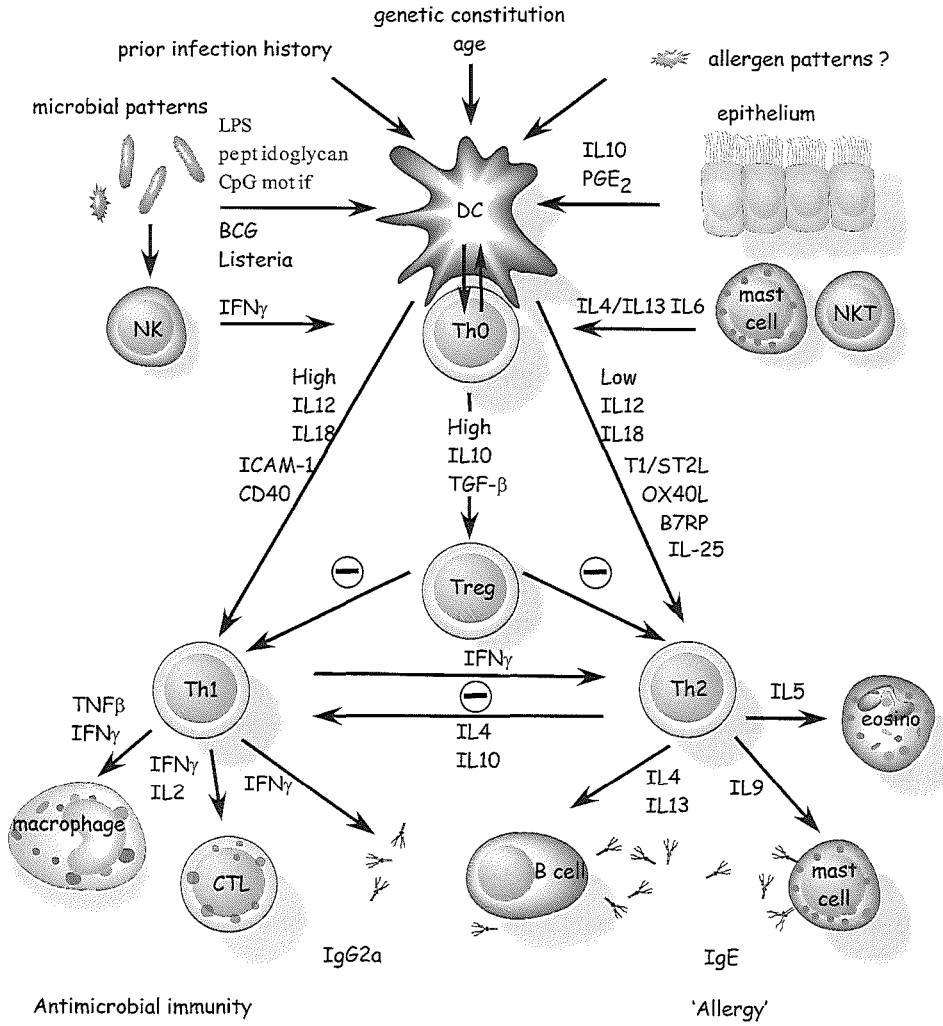

Figure 4. Counterbalancing the Th2 response

respiratory tract as they are induced by GI-DCs producing TGF-β and IL-10, and not by respiratory DCs producing IL-10 exclusively[69]. TGF-β might be required for the induction of Th3 cells. Another regulatory cell type, however not suppressive, are NKT cells, which can produce large amounts of IL-4 and IL-13, and appear to exacerbate the development of asthma in mice, and seem to be essential for the induction of AHR[70].

Dendritic cells in asthma

Dendritic cells in the sensitization to allergen

The inflammatory cascade aimed at the clearance of the allergen, is initiated by the antigen presentation to antigen specific T cells. In the lung, dendritic cells are believed to be the most important antigen presenting cells[39]. The role of dendritic cells in allergic Th2 sensitization, believed to be one of the main risk factors for developing asthma, has received a lot of attention in the last two decades[71]. The position of the DC in lung tissue, above and under the basal membrane forming a network, enables this cell to sample the epithelium for inhaled antigens[72-74]. Dendritic cells are able to pass the epithelium with their dendrites without interrupting the barrier exchange by producing tight junction proteins as shown in the gut[75]. Dendritic cells in the mucosa of the airways are in an immature state, associated with a high capability of allergen uptake and processing[76]. Dendritic cells can take up antigen in three ways, a receptor-mediated manner, macropinocytosis or phagocytosis[77]. In addition, there can also be a passive transfer of macrophage-derived peptides to DCs[78] or Ag-MHCII complexes from one DC[79] to another. When antigen is degraded in short immunogenic peptides and is loaded on newly synthesized or recycled MHCII molecules, antigen can be presented to naïve specific T cells. Dendritic cells have to migrate to the T cell rich area of draining lymph nodes to interact with recirculating naïve T cells[38, 80]. With antigen uptake, dendritic cells undergo a developmental program called maturation. During this process dendritic cells loose their capacity to take up antigen and acquire a phenotype of professional antigen presenting cells and exchange their CC chemokine receptors CCR1, -5 and –6 for CXCR4 and CCR7 allowing them to migrate through the afferent lymphatics expressing the CCR7 ligand CCL-19 (secondary lymphoid chemokine) and are directed to the T cell zone of the lung draining lymph nodes where the second CCR7 ligand CCL-21 (MIP-3β) is expressed[81]. In the draining lymph nodes, the predominant function of dendritic cells is antigen presentation. To attract naïve T cells, dendritic cells secrete MIP-3β and DCCK-1[82,83]. Ligation of an MHCII-antigen complex with an antigen specific TCR (signal 1) will not lead to complete activation, a so-called signal 2 is required which is provided by costimulatory molecules. In support of their new function, dendritic cells upregulate costimulatory molecules after antigen uptake.

Dendritic cells express several different costimulatory molecules; B7.1(CD80), B7.2(CD86), ICOS-L, CD40, 4-IBBL, OX-40L, PD-L1, PD-L2[84-86](See Fig 5).

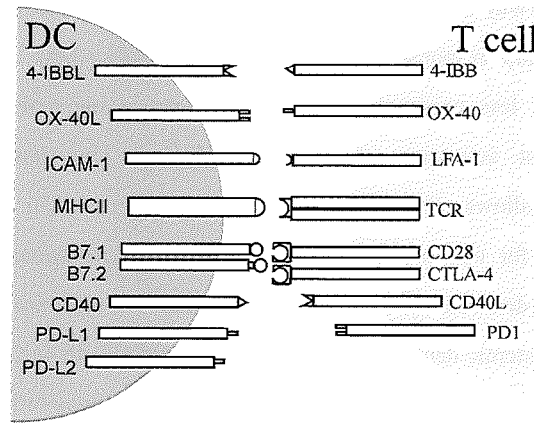

Figure 5. Interaction of DC and T cell by TCR and antigen bound MHCII complex in combination with costimulatory molecules

Dependent on several factors including the cytokine environment of the T cell/DC encounter, naïve T cells will differentiate in a Th1 or a Th2 cell or will become anergic in case of a too weak ligation. There is evidence that dendritic cells can influence polarization of T cells by a specific costimulatory molecule expression and cytokine secretion pattern, depending on their lineage, their maturation status and the consequent costimulatory molecule pattern and the environment they are in[87, 88]. Also the nature of an antigen can instruct the dendritic cell to influence the polarization of T cells by ligation of specific receptors during uptake[55, 87]. The way in which allergens are handled by DCs is fundamentally different between atopics and non-atopics. Dendritic cells derived from peripheral blood monocytes of house dust mite-allergic patients preferentially upregulated CD86 and IL-10 after incubation with Der p 1 in contrast to DCs of healthy donors which upregulated CD80. Upregulation of CD86 and IL-10 secretion is associated with the polarization to Th2 cells. In support, after incubation with Der p 1, dendritic cells of house dust mite-allergic donors preferentially induced a Th2 response in naïve T cells[55].

Dendritic cells in the maintenance of asthma

The late phase response in asthma is characterized by an influx of activated Th2 cells and eosinophils into the airway mucosa. The mechanisms underlying transient T cell activation during this inflammatory response is unclear. In-vitro studies have shown that effector memory T cells need less costimulation by antigen presenting cells in contrast to the costimulation-dependent activation of naive T cells[89,90]. This lower dependency on costimulatory ligation allows other antigen presenting cells present in the lung to possibly play a role as APCs for memory T cells. However, there are some intriguing findings supportive of a role for dendritic cells during secondary immune responses.

A ganciclovir-based systemic depletion of all myeloid dendritic cells in OVA-sensitized mice immediately before OVA challenge abolished the eosinophilic airway inflammation as seen in control animals with normal numbers of DCs[73], indicating that other antigen presenting cells are not capable for replacing dendritic cells in activation of memory T cells. This indicates that DCs are involved in the activation of previously primed T cells. These experiments did not address whether local lung DCs were also important for maintaining airway inflammation when effector T cells are present in the lungs.

It is not clear yet at which location DCs activate T cells, mainly at the draining lymph nodes of the lung or also in the periphery. The short time frame in which a Th2-driven eosinophilic airway infiltration occurs in the airway mucosa following allergen challenge, supports the concept of local activation of memory T cells by DCs. Activation of memory T cells into effector T cells at distal locations such as the draining lymph nodes of the lung, which in their turn have to migrate back to the site of antigen deposition and subsequent recruitment of eosinophils by secretion of cytokines, would take considerably more time. In a rat asthma model, 2 hours after OVA exposure more CD86 expressing DCs were present in the tracheal mucosa compared with the number of DCs seen in naïve animals. In this model, DCs were co-localizing with activated T cells. In-vitro co-culture of tracheal DCs and memory T cells or naïve T cells revealed that only memory T cells were able to upregulate CD86 on DCs[91]. These results therefore suggest a role for DCs in local antigen presentation to memory T cells. In support of this rat study, the number of airway DCs in bronchial biopsies of patients with

stable allergic asthma, is elevated compared with healthy controls and local allergen challenge leads to rapid accumulation of $CD1a^+$ $HLA\text{-}DR^+$ DCs in the airway lamina propria[72,92].

In humans, two subtypes of DCs have been characterized. It has been hypothesized that myeloid DCs would favor polarization of T cells towards Th1 while plasmacytoid DCs would favor development into Th2[93]. Therefore it is noteworthy that in patients with asthma more plasmacytoid DCs are present in peripheral blood suggesting an involvement of the pDC:mDC ratio for producing the Th2 dominant immune phenotype in asthma[91]. Studies in which pDCs were studied locally in the lungs[72] however do not support a predominant role for pDCs in the maintenance of airway inflammation in contrast to allergic rhinitis[94].

Other antigen presenting cells in asthma?

Other antigen presenting cells in the lung like B cells, macrophages and epithelial cells are believed to be less important in antigen presentation in the airway in steady state[95, 96]. However, this can not be extrapolated to the inflamed situation, when more and normally absent potential APCs are recruited. During a strong Th2 reaction in the lung as seen in asthma, eosinophils accumulate in airway mucosa. Eosinophils are mainly seen as effector cells releasing numerous pro-inflammatory mediators and cytokines as well as cationic proteins, which are toxic to the lung epithelium and account for many of the histopathologic abnormalities of asthma[97]. However, studies showing expression of MHCII and the costimulatory molecules CD80 and CD86 by eosinophils[95,98-103], suggested a possible function as antigen presenting cell. Murine eosinophils derived from BALF of sensitized and challenged mice were reported to stimulate sensitized Th2 cells *in vivo* although stimulation was read out ex-vivo[101]. Eosinophils derived from the peritoneal cavity of IL-5 transgenic mice were capable of sensitizing mice after repeated intraperitoneal injections, although the mechanism of T cell priming was not investigated in a direct manner[102]. This possible antigen presenting function of eosinophils would shed a whole new light over the understanding of the maintenance of the secondary immune response to inhaled allergen in asthma.

Aim and design of this study

The aim of this thesis is to characterize the involvement of dendritic cells in the induction and maintenance of the secondary immune response leading to an eosinophilic airway

inflammation as seen in asthma. Special attention was attributed to the mechanisms by which these cells accumulate in the airways of challenged mice, to their interaction with primed $CD4^+$ T cells as well as to their functional contribution to primed T cell activation. These questions were addressed in a well-established murine model of eosinophilic airway inflammation[104].

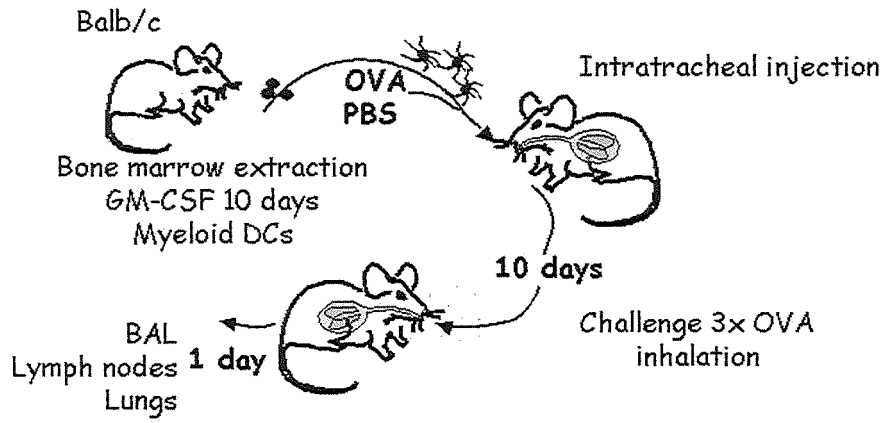

Figure 6. Schematic presentation of the used murine model for asthma.

Balb/c mice were sensitized to OVA by an intratracheal injection of OVA-pulsed bone marrow-derived DCs. Ten days post-sensitization, mice were challenged with an aerosol of the same antigen resulting in an eosinophilic airway inflammation as shown by histological analysis of lungs revealing peribronchial and perivascular inflammatory infiltrates and goblet cell hyperplasia, increased numbers of eosinophils in bronchoalveolar lavage fluid and Th2 cytokine production by draining lymph nodes of the lung (See Fig 6).

In **Chapter 2**, we give an overview of the literature on the involvement of dendritic cells in the sensitization and the secondary immune response to inhaled allergen. Dendritic cells are initiators of the inflammatory cascade resulting in an eosinophilic airway inflammation, therefore we also discussed the role of other players of the game; $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, NK cells and mast cells in the induction and maintenance of an eosinophilic airway inflammation.

In **Chapter 3**, we described a new method to determine the cellular composition of bronchoalveolar lavage fluid. The number of eosinophils is often used as a marker for the

degree of eosinophilic airway inflammation. In general, the cellular composition is determined based on morphological characteristics on histological stained cytospins. A method that is laborious and can have large inter-observer variations. We developed a new flowcytometric method in which T cells, B cells, neutrophils, eosinophils, macrophages and DCs can be identified reliably in one single staining. This identification is based on cell size, cell scatter patterns and the expression of specific cell markers. Using this method, we were also able to quantitate the number of DCs in BALF during the secondary immune response.

In **Chapter 4,** it was described that the number of dendritic cells in the airways increased during the secondary immune response in an allergen dose-dependent manner. The experiments described in this chapter were designed to elucidate the mechanisms involved in the observed increase of DCs. There are several not mutually exclusive possibilities. First, the increase could be caused by enhanced recruitment of DCs from the bloodstream into the site of airway inflammation. To support an enhanced demand for DCs in the inflamed airways, the bone marrow might enhance its output of DCs or DC progenitors, similar to what is seen for the release of eosinophil progenitors from bone marrow. This bone marrow response is indicative of a systemic effect of local airway inflammation. Secondly, enhanced local differentiation of freshly recruited monocytes into DCs could enhance the number of DCs. Finally, also emigration patterns of DCs were investigated because a small decrease in the migration of DCs to the draining lymph nodes could lead to a rapid accumulation of DCs. These experiments revealed an increased migration of DCs during the secondary immune response. This increased migration of DCs, raises the possibility that more antigen reaches the draining lymph nodes of the lungs, the site where naïve T cells and central memory Th2 cells can be stimulated.

In **Chapter 5,** we determined the functional role of the accumulation of dendritic cells seen during the secondary immune response to inhaled antigen. We used CD11c-Diphtheria toxin receptor transgenic mice, in which a short ablation of airway DCs can be induced by an intratracheal injection of Diphtheria Toxin. Airway DCs were depleted in OVA-sensitized mice before OVA challenge to determine the requirement of DCs as antigen presenting cells to activate memory T cells. The requirement of DCs to maintain effector T cell function was studied by depleting DCs during an ongoing inflammation. These experiments showed the

involvement of DCs in the induction as well as in the maintenance phase of the secondary immune response. In the absence of DCs, previously primed Th2 cells had a greatly diminished production of effector cytokines. Moreover, we determined that DCs have the intrinsic capacity to induce a secondary immune response in sensitized mice, leading to eosinophilic airway inflammation and bronchial hyperreactivity.

In **Chapter 6,** we determined which factors expressed by DCs would be important during the secondary immune response. We focused on the role of CD80/CD86 costimulation by DCs for activating memory T cells *in-vivo*. This was studied by an intratracheal injection of $CD80/86^{-/-}$ DCs in OVA-sensitized mice. Lungs were analyzed for the induction of airway eosinophilia, goblet cell hyperplasia and Th2 cytokine production.

In **Chapter 7,** we investigated the recent proposition that besides dendritic cells also eosinophils have an antigen presenting capacity. We determined the antigen presenting capacity of BALF eosinophils by using OVA TCR Tg T cells as an extremely sensitive *in-vivo* and *in-vitro* read-out for antigen presentation. Eosinophils were isolated from bronchoalveolar lavage fluid of OVA-sensitized and challenged mice by a new flowcytometric sorting method (described in **Chapter 3**) avoiding contamination with other antigen presenting cells present in the lung.

Based on the observations described in Chapters 3-7, we propose that dendritic cells have an important role in the induction and maintenance of the secondary immune response leading to eosinophilic airway inflammation in sensitized mice. This activation seems to be independent of CD80/86 costimulation but is possibly compensated by other costimulatory molecules provided by dendritic cells.

In **Chapter 8,** we discussed the results obtained in the previous chapters in the context of relevant literature, with emphasis on the role of dendritic cells in activating several subsets of T cells (Naïve T cells, Central Memory T cells and Effector Memory T cells) locally as well as at a more distant site at the T cell zone of the draining lymph nodes of the lung.

RESEARCH QUESTIONS

To determine the role of the dendritic cell in the secondary immune response to inhaled allergen the following research questions were addressed in this thesis using a murine model for asthma:

- Does the number of dendritic cells in the airways increase during a secondary immune response? (Chapter 4)
- Is an intratracheal injection of antigen pulsed dendritic cells sufficient to induce a secondary immune response in already sensitized mice? (Chapter 5 en 6)
- Does depletion of dendritic cells in sensitized mice before and during challenge inhibit the development of an eosinophilic airway inflammation? (Chapter 5)
- Does the capacity of dendritic cells to provide CD80/86 costimulation contribute to the function of dendritic cells in the secondary immune response? (Chapter 6)
- Do eosinophils play a role as antigen presenting cells during the secondary immune response? (Chapter 7)

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Chapter 2

Role of dendritic cells and Th2 lymphocytes in asthma: lessons from eosinophilic airway inflammation in the mouse

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Role of Dendritic Cells and Th2 Lymphocytes in Asthma: Lessons From Eosinophilic Airway Inflammation in the Mouse

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KEY WORDS dendritic cells; asthma; Th2 lymphocyte; antigen presentation; mouse model; pathophysiology; eosinophilic airway inflammation

ABSTRACT Asthma is a chronic disorder of the airways characterized by variable airway narrowing, mucus hypersecretion, and infiltration of the airway wall with eosinophils. It is now believed that asthma is controlled by Th2 lymphocytes producing cytokines such as IL-4, IL-5, IL-9, and IL-13. Animal models of eosinophilic airway inflammation and airway hyperreactivity have been developed to study the contribution of cells or mediators in the pathogenesis of asthma. In this review, we discuss the role of antigen presenting cells, $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, NK cells, and mast cells in the induction and maintenance of eosinophilic airway inflammation, mucus hypersecretion, and airway hyperreactivity. *Microsc. Res. Tech. 53:256–272, 2001.*

HUMAN ASTHMA AS A Th2-DRIVEN DISORDER

Asthma is a chronic disorder of the airways in which symptoms such as shortness of breath, cough, and dyspnea are primarily related to airway obstruction. Increased airway responsiveness to provocative stimuli, termed airway hyperresponsiveness (AHR), and mucus hypersecretion by goblet cells are two of the principal causes of airway obstruction observed in asthma patients. Although asthma is a very complex disorder, our understanding of the pathogenesis of this disease has evolved substantially over the past decade. Following bronchial allergen exposure in atopic individuals, crosslinking of IgE on mast cells leads to immediate degranulation of mast cells and to synthesis of prostaglandins, leukotriens, and cytokines. Mast cells release a variety of pre-formed mediators known to directly constrict bronchial smooth muscle, irritate local nerve endings, dilate blood vessels, and increase leakage of plasma into the airways, leading to the occurrence of airway narrowing within 15 minutes after challenge. This form of IgE-mediated allergy is classically called type I immediate hypersensitivity according to the classification of Gell and Coombs. In about 50% of challenged individuals, this early response is followed by a second, late bronchoconstrictive response that occurs 4–8 hours after challenge and is characterized by tissue infiltration with mononuclear cells, T cells, and eosinophils. It is now believed that this late phase response (LPR) of cellular inflammation is a form of type IV hypersensitivity that is mediated by T lymphocytes. Cytokines produced by T helper (Th) cells promote the activation of inflammatory cells and their recruitment to late phase reaction inflammatory sites. In contrast to delayed type hypersensitivity (DTH), in which Th1 cytokines are clearly involved (see other articles in this issue), the LPR is controlled by Th2 cells. T-helper lymphocyte responses can indeed be operationally divided on the basis of the cytokines produced after encounter of antigen-specific T cells with antigen. Th1 lymphocytes predominantly secrete IL-2, IFN-γ, and TNF-β to activate macrophages and to induce a strong cellular immune response typical of the DTH reaction. Conversely, Th-2 lymphocytes secrete IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and GM-CSF to induce a humoral immune response and possibly to induce antiparasitic defence mechanisms (Abbas et al., 1996). Th-2 cytokines have also been implicated in the pathophysiology of the LPR. IL-4 leads to upregulation of cell adhesion molecules on inflammatory endothelium leading to the recruitment of inflammatory cells and stimulates the production of IgE by B cells (Brusselle et al., 1995). IL-5 is important for the growth, differentiation, and activation of tissue eosinophils (Foster et al., 1996). IL-9 is important for mast cell growth and activation (McLane et al., 1998). IL-13 is important for bronchial hyperreactivity, goblet cell hyperplasia, and IgE synthesis (Grunig et al., 1998; Wills-Karp et al., 1998). Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the growth of eosinophils and the activation of antigen presenting cells (APC). Moreover, cytokines produced by Th2 cells and inflammatory cells can affect the (myo)fibroblasts, epithelia, and smooth muscle cells in the lungs directly or indirectly by stimulating other inflammatory cells to secrete mediators and chemokines.

It is currently held that the changes observed during the LPR after allergen challenge are closely related to the pathophysiology of chronic asthma. Endobronchial biopsy and broncho-alveolar lavage (BAL) studies in patients with chronic and intermittent asthma have indeed revealed eosinophilic mucosal inflammation

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and have also identified activated $CD4^+$ T helper lymphocytes producing a defined set of cytokines. Biopsy studies have demonstrated that the mRNA and/or the protein for IL-3, IL-4, IL-5, GM-CSF, and IL-13 are found in increased amounts in the airways of both atopic and non-atopic asthmatics, the signals co-localizing mainly with T cell markers and/or mast cells, basophils, and eosinophils (Humbert et al., 1999). The induction of the asthmatic phenotype seems to occur in discrete steps (Holt et al., 1999). Sensitization to inhaled antigen occurs in early (sometimes prenatal) life but is not sufficient to cause asthma. Recent studies have indeed identified allergen-reactive Th2 cells in close to 100% of newborns (both non-atopics and atopics) (Prescott et al., 1998; Upham et al., 1995). Later in life, allergen-specific T-lymphocytes continue to produce Th2-type cytokines in atopic individuals as opposed to Th1 cytokines in non-atopics (Holt and Macaubas, 1997; Yabuhara et al., 1997). Although largely derived from in vitro data, these findings suggest that a key etiologic factor in atopic disease may not be the initial acquisition of allergen-specific Th2 immunity per se, but instead may be the efficiency of immune deviation mechanisms, which in non-atopic individuals redirect these responses to a protective Th1 cytokine phenotype. The redirecting of the Th2 response towards a protective Th1 response seems to occur when the immune response is instructed to react to environmental microbial antigens. The hygiene hypothesis has been put forward to explain the East to West gradient of atopic disease, and the influence of social class, rural living and tuberculin reactivity on the occurrence of atopic disease. According to this theory, a reduced propensity towards Th1 responses due to the lack of bacterial stimuli from the environment, e.g., in the form of bacterial LPS from close contact with animals or from naturally occurring infections, is associated with a rising trend in atopy. Although Th2-type sensitization to inhaled allergen (i.e., atopy) is a clearly defined risk factor for the development of asthma, it is clearly not the only pathway towards asthma, as exemplified by the existence of non-atopic (i.e., intrinsic) asthma, that shares many of the immunopathological findings with atopic asthma and occurs later in life (Humbert et al., 1999). Moreover, in countries that have witnessed a rise in the occurrence of atopy, there has not been a similar rise in the occurrence of asthma. It could be that certain (genetically defined?) atopic individuals go on to develop eosinophilic airway inflammation and symptoms of asthma when continuously exposed to high levels of allergens, or when triggered by exogenous factors such as respiratory viral infections (Holt et al., 1999). Moreover, it seems likely that additional factors, such as those derived from bronchial epithelial cells or from tissue resident cells, are necessary before this inflammation becomes a chronic, self-perpetuating process that is characteristic of human asthma. Although asthma is an immunologically mediated disorder, as the disease becomes chronic, it is increasingly regulated by signals within the lung itself that seem to be allergen-independent. An interesting observation in this regard was the transfer of the asthmatic phenotype by transplantation of lungs obtained from asthmatic donors into non-atopic recipients that had never experienced asthma before (Corris and Dark, 1993).

USE OF MURINE MODELS OF EOSINOPHILIC AIRWAY INFLAMMATION

Whereas studies in humans have shown only an association between the asthma phenotype and increased numbers of $CD4^+$ T cells secreting Th2 cytokines, animal models of the disease offer the possibility to show cause and effect (Ray and Cohn, 1999). Experimental models to study the development and function of the immune and inflammatory response have focused mainly on the mouse and have generated a large amount of reagents such as monoclonal antibodies directed against immune cells, cytokines, chemokines, and (anti-) inflammatory mediators. This, together with the availability of inbred wild-type (WT), mutant, gene-knockout (−/−), as well as transgenic (Tg) strains in which the expression of these molecules is genetically altered, has made the mouse the most widely used animal species to study the pathogenesis of asthma (Kips and Pauwels, 1997). Murine models of allergen-induced pulmonary inflammation share many features with human asthma, including the development of antigen-induced pulmonary eosinophilia, AHR, antigen-specific cellular and antibody responses, the elaboration of Th2 cytokines (IL-4 and IL-5), and the expression of chemokines with activity for eosinophils. Of the various antigens, ovalbumin (OVA) is the most widely used antigen to induce eosinophilic airway inflammation, although some investigators have used the more relevant house dust mite or cockroach allergens. The different models reported in the literature vary widely in the strain of mice used, in the method in which sensitization and challenge to OVA is performed and, finally, in the time point after exposure when mice are sacrificed for analysis (see Table 1) (Brewer et al., 1999). In its most simple form, OVA is injected intraperitoneally with or without a Th2 skewing adjuvant, such as alum. This period of "sensitization" to inhaled antigen is followed 10–14 days later by a period of OVA "challenge" to the airways, either in the form of single or repeated inhalation of OVA aerosol or intranasal application of soluble OVA. Before final analysis of airway inflammation by either immunohistology or cellular analysis of BAL fluid, some investigators have been able to demonstrate bronchial hyperreactivity to inhaled or intravenous bronchoconstrictive agents such as metacholine, serotonine, or carbachol, using either invasive or non-invasive (plethysmographic) measurements (Hamelmann, 1997a).

Some debate on the relevance to human asthma continues to surround the use of murine models. A major drawback of the murine model is the observation that plasma extravasation and mucosal edema are not prominent features of challenged airways, in contrast to human asthma. Moreover, murine eosinophils do not seem to degranulate upon encounter with allergen, questioning their role in the induction of bronchial hyperreactivity and induction of tissue damage to the airway mucosa (Stelts et al., 1998). Moreover, most models use fairly acute exposure to inhaled OVA, leading to a form of acute inflammation that may not be relevant to chronic asthma. Despite these drawbacks, murine models of eosinophilic airway inflammation have elucidated parts of the complex inflammatory processes of the asthmatic airways and have provided us

TABLE 1. Variable parameters in mouse models of asthma

Variable	Most common variations	Remarks
Mouse strain	Balb/c C57BL/6 A/J C3H	Genetic influence can be marked, especially when related to degree of Th2 cytokine production, cellular inflammation, and AHR.
Sensitization		
Antigen	Ovalbumin Sheep red blood cells *Dermatophagoides pteronyssinus* *Nippostrongyloides*	Most naturally occurring allergens have enzymatic activity. OVA and sheep red blood cells are inert.
Route	Intraperitoneal Inhalational Epicutaneous Subcutaneous	Depending on the route, different APCs present the Ag to naive T cells. Cytokine milieu differs depending on route.
Adjuvant	Alum Ricin None	Adjuvants stimulate the maturation and migration of DCs. Most adjuvants used skew the response to Th2.
Schedule	Single injection Booster injection	Booster usually 1 week after first injection.
Challenge		
Exposure	15, 30, 60 minutes Single Repeated (3–7 days)	Models on chronic exposure to allergen are lacking.
Route	Aerosol Nasal aspiration	Aerosol particle size can vary. Low volumes required.
Endpoint analysis		
Time point	3, 6, 24, 48 hours after last exp.	Marked effect on cell recovery by BAL.
Eosinophilia	BAL fluid cell count EPO levels BAL Flow cytometry Histology	DiffQuick stain, May-Grunwald-Giemsa. Can be measured by ELISA. Also allows detection of T, B, DC, macro. H&E stain, cyanide resistant peroxidase, MBP moAb.
AHR	Invasive measurement Body plethysmograph In vitro tracheal rings	R_L or APTI index P_{enh} value or forced oscillation technique.
Cytokine levels	BAL fluid Mediastinal lymph nodes Intracellular	Levels in BAL fluid can be low. Three day culture w/o or w OVA. Intracellular staining allows single cell analysis.

with an important tool to help identify the potential contribution of individual inflammatory molecules and cells to specific pathophysiologic processes that are hallmarks of this disorder. Proving that a particular cell or mediator is functionally important for the pathogenesis of a given disease usually follows Koch's postulates. When applied to an animal model of allergen-induced asthma, this would imply (1) that the given cell or mediator is found in increasing amounts in the challenged airways of animals with "asthma," (2) that administration of the cell or mediator induces phenotypic changes observed after allergen challenge, and (3) that antagonizing the cell or mediator partially or completely blocks the allergen-induced changes and features of the disease (Kips and Pauwels, 1997). In the remainder of this review, special attention will be given to the interaction between antigen presenting dendritic cells (DCs) and Th2 cells, which ultimately leads to infiltration of the airways with eosinophils.

ROLE OF ANTIGEN PRESENTING CELLS IN THE SENSITIZATION PROCESS TO INHALED ANTIGEN

The paradigm that asthma is a Th2 mediated disorder has largely ignored the fact that T cells need antigen presenting cells (APC) to become fully activated and respond to allergens. The recognition of allergen by APC is in fact the first step of immune recognition that eventually leads to the formation of Th2 immunity to inhaled allergen and development of asthma. During the development of sensitization, APCs capture antigen and process it into small peptides of defined length for presentation on MHC molecules and presentation to the T cell receptor (TCR) on naive T cells. Moreover, APCs express co-stimulatory molecules such as CD80, CD86, ICAM-1, heat stable antigen, etc., to provide a second signal for optimal induction of T cell activation, division, and differentiation. In the absence of such co-stimulatory signals, the outcome of TCR ligation is anergy rather than immunity. A number of professional APCs such as DCs, B cells, and macrophages are present in the airways and have the cellular specialization to capture and process antigen for presentation to T cells. It is, however, increasingly clear that the network of DCs is particularly important for inducing the primary immune response to inhaled allergen that eventually leads to sensitization. Dendritic cells are bone marrow–derived cells that continuously patrol the mucosae, skin, and internal organs of our body in search of foreign antigens (Banchereau and Steinman, 1998). Upon recognition of foreign antigens in peripheral tissues, these cells migrate to the T cell area of draining lymph nodes and report their antigenic cargo to naive T cells, at the same time providing essential costimulatory molecules for inducing the primary immune response. Studies in rodents have demonstrated that the majority of cells expressing MHC class II in the lungs are airway and interstitial DCs that capture

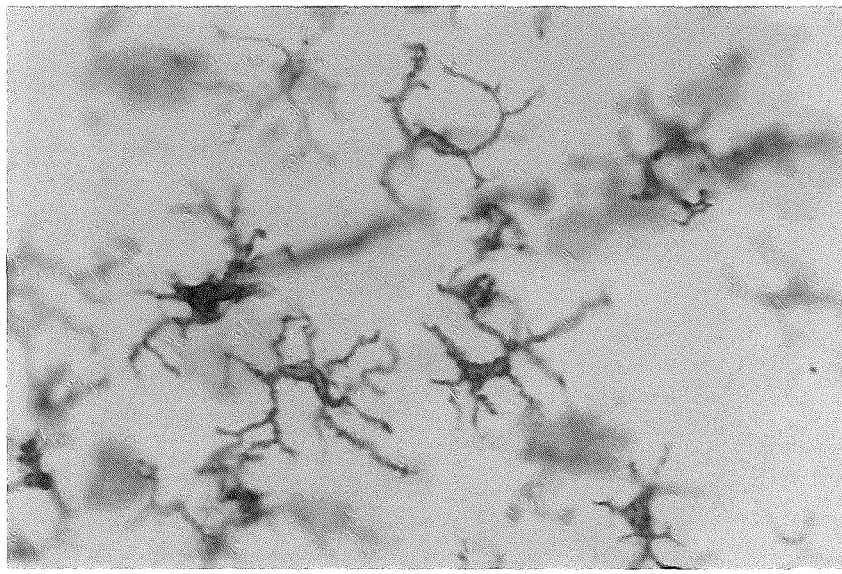

Fig. 1. Mouse airway dendritic cells revealed by MHC class II staining. Mice were fixation-perfused, and the entire trachea was removed, pinned flat on dishes, permeabilized and subsequently stained with an anti-MHC class II antibody. Staining was revealed by peroxidase reaction. The entire trachea ("whole-mount") was mounted under a glass slide, allowing visualization of all layers of the trachea. Numerous MHC class II-positive cells with a dendritic morphology can be seen to form a network. The density of this network approaches 600 cells/mm^2 in the upper 1/3 of the trachea. Original magnification, 1,000×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhaled soluble OVA and present it to primed T cells in vitro (see Fig. 1) (Holt et al., 1988; Schon-Hegrad et al., 1991). We have studied the contribution of DCs to the sensitization process in a murine model of asthma. Other investigators have used the intraperitoneal route of injection of OVA with or without alum adjuvant to sensitize mice systemically to OVA, leading to accumulation of OVA antigen in the spleen and mediastinal lymph nodes, draining the peritoneal cavity as well as the lungs. Realising that this may not be the relevant route for naturally occurring sensitization and that the use of adjuvant might be artificial, we have injected Ag pulsed myeloid DCs into the airways of naive animals and have studied the occurrence of a naive immune response in the draining lymph nodes (Lambrecht et al., 2000a–c). Dendritic cells were cultured from the bone marrow in GM-CSF, had long surface extensions (Fig. 2), and expressed MHC class II and costimulatory molecules CD80, CD86, CD40, ICAM-1, and heat stable antigen. To allow detection of the primary naive T cell response following injection of antigen-pulsed DCs, we intravenously transferred a cohort of naive antigen-specific TCR transgenic T cells, that were labeled with a mitosis-sensitive dye called carboxy fluorescein succinimidyl ester (CFSE) allowing the calculation of T cell division number in vivo (Lambrecht et al., 2000b). As soon as 12 hours after intratracheal injection, DCs migrated into the draining mediastinal lymph nodes (MLN) and started to interact with naive T cells, as evidenced by the upregulation of the early activation marker CD69 on naive antigen-specific T cells. There was no migration beyond this first draining LN station, illustrating the compartmentalization of the primary immune response induced by DCs in the lung. Forty-eight hours after injection, antigen-specific T cells were recruited from the bloodstream into the MLN and some antigen-specific T cells had already undergone 2 cell divisions. It was only between 72 and 92 hours after injection that divided Ag specific T cells emigrated from the draining MLN into the bloodstream and the peripheral tissues and spleen. The phenotype of these cells was remarkable in that they had divided at least 4 times and had universally high levels of the memory marker CD44 while totally lacking expression of the early activation marker CD69, a phenotype most consistent with activated effector T cells. Dendritic cells were remarkably efficient in selecting and activating those T cells that were specific for antigen, as none of the changes observed in TCR Tg T cells was seen in aspecific T cells. Together, these data were a first indication that DCs induce sensitization to inhaled Ag in vivo.

To prove that OVA-pulsed DCs could induce the development of effector T cells that had the capacity to orchestrate airway inflammation, we challenged OVA-DC immunized mice with an aerosol of OVA

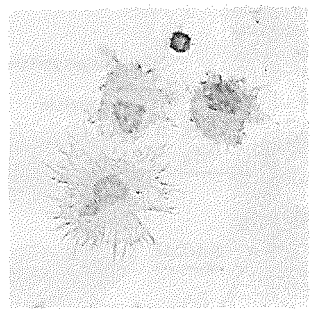

Fig. 2. Bone marrow–derived dendritic cells stained with MHC class II reagent to detect cytoplasmic processes. Bone marrow cells were depleted of $CD4^+$, $CD8^+$, $B220^+$, and $Gr\text{-}1^+$ cells by magnetic depletion, and subsequently cultured in the presence of GM-CSF. After 8 days of culture, cells arise with a clear dendritic morphology. Almost all cells arising in these cultures express MHC class II, as revealed by brown staining of their cytoplasmic processes. Original magnification 700×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

14 days after primary immunization. In contrast to PBS-DC immunized mice, OVA-DC immunized mice developed peribronchial and perivascular infiltrates of eosinophils, T cells, and mononuclear cells (Fig. 3a and b). Goblet cell hyperplasia was also consistently observed in OVA-DC immunized and challenged mice (Fig. 3c). The presence of goblet cell hyperplasia and eosinophils is dependent on IL-4 receptor signalling and IL-5, respectively, and was already a first indication that a CD4 Th2 response was being induced by DCs in the airways. When intracellular cytokine production was studied on individual $CD4^+$ and $CD8^+$ T cells within the BALF compartment, it was clear that $CD4^+$ cells produced IL-4 and IL-5 but also IFN-γ, whereas $CD8^+$ T cells exclusively produced IFNγ (see below). Although antigen presentation by adoptively transferred DCs in the lung seems to induce Th2-dependent sensitization to inhaled antigen, one might argue that this is not a physiological system. Despite this, a number of studies have recently supported this concept by showing that activation of endogenous DCs leads to sensitization when mice are exposed to OVA aerosol. Primary aerosolization with OVA is normally a tolerogenic event that leads to downregulation of the IgE response and eosinophilic airway inflammation. Although suppressor cells that express the γδ T cell receptor have been implicated in this antigen-specific downregulation of the immune response, the precise mechanisms are unknown (McMenamin et al., 1994; Seymour et al., 1998). When the expression of GM-CSF was increased by adenoviral-mediated gene transfer into the airways, the numbers and activation status of airway DCs were greatly enhanced, rendering mice sensitive to primary OVA aerosol and leading to the abolition of inhalational tolerance and to development of Th2-dependent airway eosinophilia (Stampfli et al., 1998). Similarly, upregulation of airway DC function was clearly associated with the augmentation of OVA

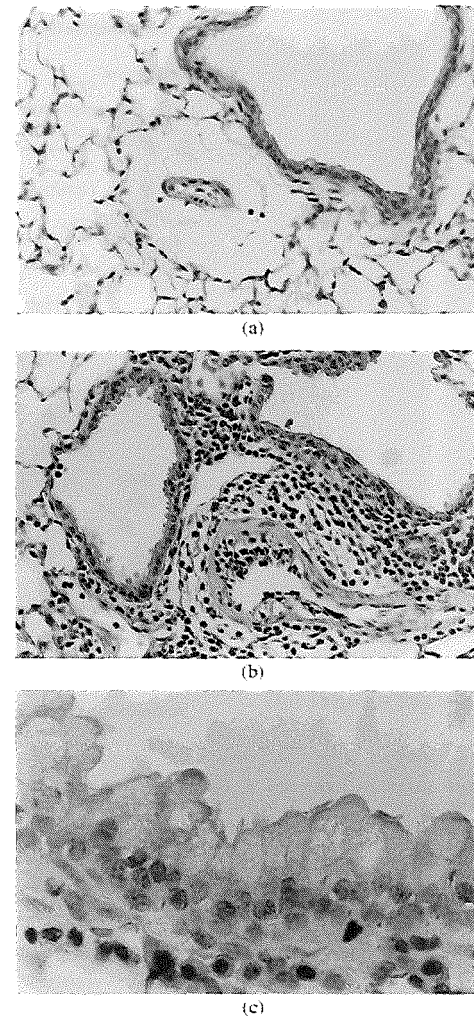

Fig. 3. Airway histology of mice immunized with PBS-pulsed DCs (a) or OVA-pulsed DCs (b and c) and subsequently exposed to OVA aerosol. **a:** There are no peribronchial or perivascular changes in animals exposed to OVA when animals were immunized with control PBS-DC (400×). **b:** Actively immunized mice demonstrate peribronchial and perivascular infiltrates that contain mononuclear cells, eosinophils and T cells. Some inflammatory cells adhere to the vessel wall (400×). **c:** On higher magnification, there is a marked increase in the number of goblet cells and the height of the airway epithelial cells in actively immunized mice (1,000×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

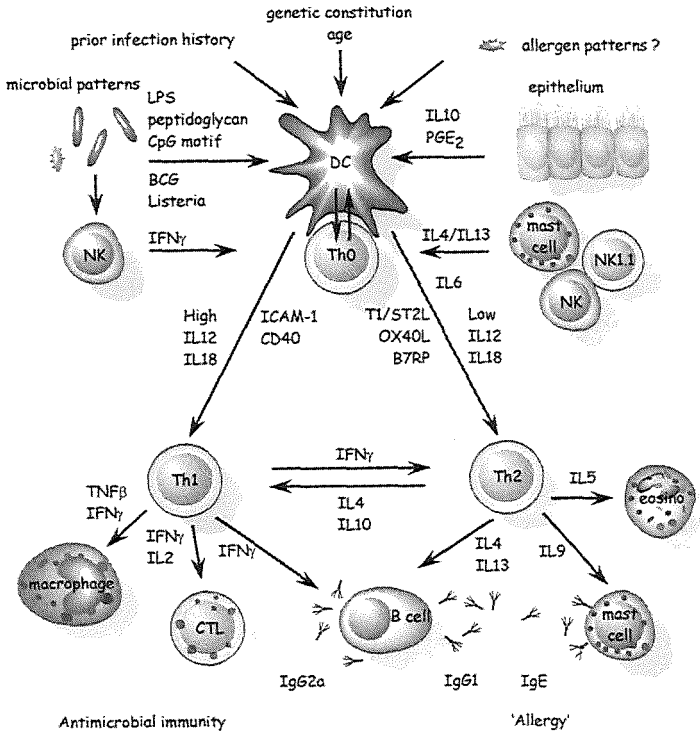

Fig. 4. Differentiation of T-helper responses induced by dendritic cells. See text for explanation.

sensitization by influenza A virus infection in mice (Yamamoto et al., 2000). It will be interesting to study whether other known sensitizers, such as respiratory syncitial virus (RSV), mold-derived β-1,3-D-glucan, cigarette smoke, or diesel particulate matter also affect the DC network of the lung (Schwarze et al., 1997; Seymour et al., 1997). Dendritic cells have been shown to induce both Th1 and Th2 types of responses upon transfer in vivo, depending on a variety of factors, of which the level of IL-12 production seems to be the most important (see Fig. 4). Interleukin 12 is essential for stimulating the innate immune system (e.g., NK cells) for IFN-γ production and for the development of a Th1 response, as evidenced by the lack of DTH responses in IL-12 deficient mice (Magram et al., 1996). Why would DCs be inducing Th2 responses in the airways of atopic patients? One possibility is that APC of atopic patients is genetically deficient in IL-12 production (van der Pouw Kraan et al., 1997). One striking observation in murine asthma models was the identification of a gene locus that determines IL-12 production in susceptible and resistant mouse strains, by determining levels of the complement factor C5, a known stimulus for IL-12 production by APCs (Karp et al., 2000). Similarly, there is a striking polymorphism between levels of soluble LPS receptor CD14 and protection against atopy in humans (Baldini et al., 1999). As for C5a, LPS is a known inducer of IL-12 production in DCs. Another possibility is that the environment of the lung favours the development of Th2 responses, by downregulating the production of IL-12 in DCs (Constant et al., 2000). Of the possible candidate factors involved in this process, IL-10 and PGE_2 are produced by airway epithelial cells and known to downregulate IL-12 production. In the presence of these factors, DCs induce Th2 responses (DeSmedt et al., 1997; Kalinski et al., 1998). We have recently found that DCs obtained from IL-$10^{-/-}$ mice fail to induce Th2 responses in the airways (unpublished observations). Conversely, models of intraperitoneal OVA immunization have shown that exogenous administration of recombinant IL-12 reduces Th2 sensitization and subsequent airway eosinophilia in an IFN-γ dependent manner (Kips et al., 1996). Moreover, factors known to increase the levels of IL-12 within APCs such as unmethylated CpG motifs within bacterial DNA and heat killed *Listeria monocytogenes* micro-organisms are able to inhibit sensitization to inhaled OVA (Hansen et al., 2000; Kline et al.,

1998). Thus, it seems that levels of IL-12 during the interaction of DCs and naive T cells are critical for determining Th polarization.

Despite the fact that the absence of IL-12 might predispose to Th2 development, the prototypic Th2 cytokine IL-4 is essential for Th2 development, as evidenced by strongly reduced Th2 responses and airway eosinophilia in anti-IL4 antibody treated mice, IL-4 deficient mice, IL-4 receptor α chain (IL-4Rα) deficient mice, or mice deficient in the critical downstream signal transducer and activator of transcription (STAT)-6 (Brusselle et al., 1995; Corry et al., 1996; Coyle et al., 1995b; Grunig et al., 1998; Kuperman et al., 1998). Closer analysis of these various knock-out mouse strains has, however, suggested that there may be IL-4R- and STAT6-dependent, but IL-4-independent mechanisms to generate IL-5 producing $CD4^+$ T cells, IL-13 being a likely candidate for Th2 development in the absence of IL-4 (Grunig et al., 1998; Hogan et al., 1997; Tomkinson et al., 1999; Webb et al., 2000; Wills-Karp et al., 1998). It is interesting to observe that the importance of IL-13 in inducing Th2 development might be related to the route of immunization and the genetic background of the mouse strain. In this regard, sensitization of C57B1/6 mice to OVA via the skin seems to depend exclusively on IL-13, whereas sensitization via the airways depends on IL-4 (Herrick et al., 2000).

The important role of IL-4 and/or IL-13 in the development of Th2 sensitization immediately raises the question as to what cell type initially produces these cytokines. Although DCs do not produce IL-4, some recent reports have demonstrated that human immature DCs can produce IL-13 (Johansson et al., 2000). Our recent studies have, however, demonstrated that murine DCs fail to induce Th2-dependent airway eosinophilia when transferred into IL-4-deficient mice C57B1/6 mice (Lambrecht et al., 2000a). Alternatively, the source of initial IL-4 production could be found within cells of the innate immune system. A population of $NK1.1^+$ cells (natural killer [NK] cells or NK T cells), is known to produce large amounts of this cytokine in vivo (Korsgren et al., 1999b). However, sensitized β_2-microglobulin-deficient or CD1d-deficient mice, which lack functional NK1.1 T cells, have normal Th2-dependent eosinophilic responses to inhaled OVA (Daser et al., 1998; Korsgren et al., 1999b; Zhang et al., 1996). Despite this, depleting NK1.1 antibodies given before sensitization abolish the Th2 response to OVA, suggesting that NK cells may be important (Korsgren et al., 1999b). It has been shown that DCs can directly interact with NK cells, but the relevance of this interaction for initial IL-4 production and allergic sensitization remains to be determined (Fernandez et al., 1999). Alternatively, T cells expressing the γδ TCR could be an early source of initial IL-4, as mice lacking these cells have deficient Th2 responses to OVA (Schramm et al., 2000; Zuany-Amorim et al., 1998). The most likely source of initial IL-4, however, is the naive T cell itself. Under conditions where IL-12 production is low, naive T cells polarize towards IL-4 production in the absence of exogenous IL-4 (Croft and Swain, 1995; Ohshima and Delespesse, 1997). Exposure to inhaled allergens occurs at low concentrations, generating a low MHC-peptide density on the DC. A number of experimental systems suggest that low Ag doses and low avidity interactions between T cells and DCs favour the development of a Th2 response (Constant and Bottomly, 1997). Also, chronic exposure to low levels of allergen might lead to low numbers of DCs continuously reaching the draining lymph nodes. Sustained TCR activation at low stimulator DC/responder T cell ratios independently favours the development of a Th2 response (Lanzavecchia and Sallusto, 2000; Tanaka et al., 2000).

It has been suggested that the particular expression of costimulatory molecules on APCs is critical for Th2 differentiation from naive T cells (Schweitzer et al., 1997). Dendritic cells in the lung express high levels of CD80 and CD86, compared with B cells and macrophages (Masten and Lipscomb, 1999). Airway DC-induced Th2 responses in the lung fail to develop in the absence of CD28, the receptor for CD80 and CD86 expressed on T cells (Lambrecht et al., 2000a). Models of intraperitoneal sensitization to OVA have highlighted that administration of CTLA4-Ig or blocking antibodies to either CD80 and/or CD86 can inhibit airway eosinophilia when given during primary immunization (Haczku et al., 1999; Harris et al., 1997; Tsuyuki et al., 1997). Although CD86 preferentially stimulates Th2 and CD80 stimulates Th1 formation in some disease models, the situation is far from clear in the murine asthma model, CD80 and CD86 having redundant roles (Mark et al., 1998; Mathur et al., 1999). We have recently found that a ligand for the newly described Th2-associated type I IL-1 receptor family member T1/ST2 is expressed on DCs and critically contributes to Th2 induction in the lung (Coyle et al., 1999; Lambrecht et al., 2000a). It will also be interesting to study whether the newly described B7 related proteins (B7rp)-1 and -2, and the Th2-associated tumor necrosis factor (TNF) receptor family member OX40L have direct effects on Th polarization induced by DCs in the lung (Akiba et al., 2000; Coyle et al., 2000; Yoshinaga et al., 1999).

Because of their inability to migrate into the draining lymph nodes and by their low level expression of costimulatory molecules, B cells and macrophages do not seem to play an important role in the sensitization process to inhaled antigen. This is supported by the finding of a normal development of Th2 responses and airway eosinophilia in B cell–deficient mice (Korsgren et al., 1997). Macrophages were unable to induce sensitization to inhaled OVA when adoptively transferred into naive animals (Lambrecht et al., 2000c). Moreover, both alveolar and interstitial macrophages seem to actively suppress the activation and proliferation of naive T cells in the lung by their direct inhibitory effects via nitric oxide and TGF-β on T cell proliferation and DC activation (Holt et al., 1988; Lee et al., 1999). This suppressive effect can, however, be overruled by enhanced expression of GM-CSF (Bilyk and Holt, 1993; Stampfli et al., 1998). Alveolar macrophage elimination in vivo is associated with an increase in the primary pulmonary immune response to inhaled antigen in mice (Thepen et al., 1989).

ROLE OF ANTIGEN PRESENTING CELLS DURING CHALLENGE TO INHALED ANTIGEN IN SENSITIZED MICE

In addition to their contribution to the primary immune response leading to sensitisation, DCs are likely

to contribute to the chronic secondary Th2 immune response that occurs in the airways of asthmatics. Bronchial biopsies have revealed increased numbers of $CD1a^+$ MHC class II^+ DCs in the epithelium and in the subepithelial lamina propria of non-smoking atopic asthmatics compared with non-atopic controls (Moller et al., 1996; Tunon de Lara et al., 1996). We observed a 60-fold increase in the number of BALF DCs in OVA-sensitised and -exposed rats with eosinophilic airway inflammation (Lambrecht et al., 1999). The allergen-induced kinetics of increase of DCs closely resembled those of Th2 cells and eosinophils, suggesting that they were attracted by the same mechanisms. Allergic airway inflammation is, indeed, accompanied by the local release of chemokines and mediators such as RANTES, eotaxin, MIP-1α, MCP-1, MCP-3, MCP-4, and platelet activating factor, which have been shown to attract both inflammatory cells and DCs in vitro and in vivo (McWilliam et al., 1996; Sozzani et al., 1995). Another possibility is the presence of a DC differentiation signal acting on monocytes after transendothelial migration into the airways (Randolph et al., 1998; Suda et al., 1998). In a very elegant study, Hammad et al. demonstrated that human PBMCs of house dust mite (HDM) allergic donors that were injected into severe combined immunodeficiency mice (humanised-SCID model) developed into DCs after inhalation exposure to allergen (Hammad et al., 2000). The classical differentiation signals for monocytes to become DCs in vitro are GM-CSF in combination with IL-4 (Sallusto et al., 1995). These cytokines are likely to contribute to the differentiation of lung DCs in vivo, as mice expressing recombinant GM-CSF in the airways have greatly increased numbers of DCs (Stampfli et al., 1998), and as human airway DCs are found in close proximity with IL4- and GM-CSF producing cell types. Finally, allergen challenge in OVA-sensitised rats and mice induces an increase in the earliest precursor for DCs in the bone marrow, leading to an increased yield of DCs after culture in GM-CSF and an increase in circulating DCs (van Rijt et al., unpublished data; Lambrecht et al., 1999). These studies suggest that the recruitment of DCs to the airways is accompanied by increased production of DCs in the bone marrow.

The increased presence of DCs in the airways of atopic asthmatics and allergen-exposed animals suggests that DCs have a critical contribution to the disease pathogenesis. However, one needs to take into account that asthma is controlled by memory/effector $CD4^+$ T cells. These cells have been shown in vitro to be less dependent on costimulation and can in theory respond to any APC such as B cells, macrophages, and even eosinophils and epithelial cells. To address the contribution of DCs to eosinophilic airway inflammation in sensitised animals, we have used transgenic mice in which the suicide gene thymidine kinase is preferentially expressed in cells of the DC-lineage (Lambrecht et al., 1998). Using this transgenic strain, treatment with the antiviral drug ganciclovir selectively depleted DCs, but not macrophages, B cells, or T cells from the airways of OVA-sensitised mice. In sensitised animals treated with ganciclovir, there was a complete disappearance of eosinophilic airway inflammation and goblet cell hyperplasia induced by OVA. These findings strongly suggest that memory/effector $CD4^+$ Th2 cells were not properly activated in the absence of DCs. Indeed, the levels of the Th2 cytokines IL-4 and IL-5 in the airways were suppressed in animals lacking DCs. Moreover, there was no allergen-induced boosting of OVA-specific IgE levels in the absence of DCs.

Studies on depletion of other APCs during the secondary challenge to inhaled OVA have suggested that elimination of macrophages using clodronate-filled liposomes is associated with an enhancement of Th2 responses and airway inflammation (Thepen et al., 1992). Similarly, B cells do not seem to contribute significantly to the secondary immune response and the development of airway eosinophilia, although they might contribute to plasma extravasation during the early phase response to OVA aerosol and to bronchial hyperreactivity (see below) (Hamelmann et al., 1997b; Korsgren et al., 1997, 1999a). Recently, it was also shown that airway eosinophils express MHC class II and the costimulatory molecules CD80 and CD86, effectively contributing to the stimulation of effector Th2 cells in the lungs and lymph nodes of sensitized mice (Korsgren et al., 1997; Mathur et al., 1999; Shi et al., 2000). However, sensitized IL-5-deficient and anti-IL-5-treated mice that lack eosinophils in the airways, demonstrate normal Th2 responses in the draining lymph nodes (Hamelmann et al., 1999). Therefore, eosinophils might have an enhancing role in antigen presentation, but do not seem to be essential for mounting the Th2 effector response.

Why would DCs be so critical APCs for eosinophilic airway inflammation? (1) One possibility is that memory T cells in vivo are more dependent on co-stimulation than appreciated from in vitro data, and, therefore, rely on professional APCs that can provide these stimuli. Indeed, allergen-induced IL-4 and IL-13 production in bronchial explants and in peripheral blood cells from asthmatics is dependent upon delivery of costimulation via the CD80/CD86 pathway (for review see Djukanovic, 2000). Similarly, animal models have shown that eosinophilic airway inflammation in sensitised mice can be suppressed by blocking the CD80/CD86-CD28 pathway during challenge (Haczku et al., 1999; Harris et al., 1997; Lambrecht et al., 2000a; Mathur et al., 1999; Tsuyuki et al., 1997). In this regard, it was recently shown that the CD28-related molecule *i*nducible *co*stimulator (ICOS) and the T1/ST2 molecule, both expressed on Th2 cells, are critical for Th2 effector function in the lung (Coyle et al., 1999, 2000). (2) Alternatively, DCs may be critical for the recognition of allergen via specific IgE bound to the high-affinity or low-affinity (CD23) receptor for IgE. Indeed, $CD1a^+$ airway DCs express the α chain of the high-affinity IgE receptor (Tunon de Lara et al., 1996). When allergen is recognized via the high-affinity IgE receptor on DCs, it is very efficiently targeted to the MHC class II rich endocytic compartment, the site of peptide loading onto MHC class II (Maurer et al., 1998). It has been suggested that the presence of IgE on DCs lowers the threshold for allergen recognition, boosting the secondary immune response by efficiently stimulating memory Th2 cells (Van der Heijden et al., 1993). In this regard, an interesting study by Coyle et al. demonstrated that memory Th2 lymphocytes of sensitised mice failed to produce Th2 cytokines upon rec-

ognition of inhaled of allergen when allergen-specific IgE was captured by a non-anaphylactogenic anti-IgE antibody (Coyle et al., 1996b). The phenotype of CD23-deficient mice suggests that the low-affinity IgE receptor CD23 is not the most critical receptor for IgE targeting and Th2 cytokine enhancement, although other inhibitory effects of CD23 on IgE synthesis and allergic inflammation might obscure the precise role of this receptor in vivo (Haczku et al., 1997, 2000; Stief et al., 1994). (3) Finally, DCs could be critical for maintaining a chronic localised immune response in the airways. Much emphasis has been put forward that chronic asthma is a localised disease, as evidenced by the transfer of the disease by lung transplantation into non-asthmatic patients. It is tempting to speculate that the presence of chronic inflammation and airway structural changes leads to local maturation of DCs and to antigen presentation occurring in the airways. The cytokines TNFα, GM-CSF, and IL-4 are expressed in asthmatic epithelium and have the potential to upregulate the costimulatory capacity of DCs (Banchereau and Steinman, 1998). Mast cells in asthmatic airways express CD40L and produce cytokines that can activate DCs and prolong their survival in tissues (Bjorck et al., 1997; Caux et al., 1994). The process of airway remodeling leads to the deposition of extracellular matrix components (collagens, fibrobectin, heparan sulphate) that have the capacity to enhance DC differentiation and costimulatory function (Kodaira et al., 2000; Mahnke et al., 1996). In addition to their potential to activate T cells in the airways, myeloid DCs produce chemokines that preferentially attract recently activated Th2 cells. In this regard, monocyte-derived DCs have been shown to produce the CC chemokines *m*onocyte *d*erived *c*hemokine (MDC) and *t*hymus and *a*ctivation *r*egulated *c*hemokine (TARC), which attract recently activated Th2 cells expressing CCR4 (Hashimoto et al., 1999; Imai et al., 1999; Lieberam and Forster, 1999). Activation of DCs induces the mRNA for *m*onocyte *c*hemotactic *p*rotein MCP-4 and I-309, the ligands for CCR3 and CCR8 receptors, also expressed on Th2 cells (Hashimoto et al., 1999; Zhou and Tedder, 1995). In this way, DCs ensure that the recently activated effector cells are attracted and retained in the airways and do not migrate back to the draining lymph nodes (Sallusto et al., 2000). Moreover, it has been shown that chronic antigen presentation by DCs leads to neo-formation of organised lymphoid structures within peripheral tissues, such as the pancreas (Ludewig et al., 1998). These organised follicles contain high endothelial venules, specialized for the extravasation of lymphocytes, and are the sites of local immunoglobulin production. It is speculative that repetitive presentation of antigen to Th2 cells by DCs in the airways would also lead to formation of these aggregates and to local production of IgE. At least in rodents, repetitive inhalation of allergen in sensitised animals leads to the formation of organised lymphoid tissues in the airways (Chvatchko et al., 1996). Switching to IgE production in B cells can be enhanced locally by Th2 cells and mast cells producing IL-4, and expressing CD40L (Scharenberg and Kinet, 1994). Additionally, DCs cells might directly interact with B cells in the airways to enhance immunoglobulin production.

ROLE OF CD4 CELLS AND THEIR CYTOKINE PRODUCTS IN INDUCING EOSINOPHILIC AIRWAY INFLAMMATION AND AIRWAYS HYPERREACTIVITY

The essential role of $CD4^+$ Th2 cells in regulating airway eosinophilia is suggested by the finding that eosinophilic airway inflammation and its accompanying bronchial hyperreactivity is associated with increased amounts of CD4 T cells producing IL-4, IL-5, and/or IL-13 being recovered from the airways of asthmatics and challenged mice (Corry et al., 1996; Garlisi et al., 1996; Humbert et al., 1999; Lambrecht et al., 2000a; Winterrowd and Chin, 1999). Moreover, administration of OVA-specific $CD4^+$ Th2 cells from OVA TCR Tg mice or from OVA-sensitized mice to naive mice was able to transfer the development of airway eosinophilia, bronchial hyperreactivity, and goblet cell hyperplasia upon exposure to OVA aerosol (Cohn et al., 1998, 1999b; Hansen et al., 1999; Hogan et al., 1998a). However, some investigators have suggested that additional factors derived from Th1 cells are essential for inducing the changes of asthma (see below). Finally, and most importantly, elimination of $CD4^+$ T cells by injecting depleting anti-CD4 monoclonal antibodies or absence of $CD4^+$ T cells in MHC class $II^{-/-}$ mice abolishes eosinophilic airway inflammation, AHR, and goblet cell hyperplasia induced by OVA aerosol (Brusselle et al., 1994; Gavett et al., 1994; Hogan et al., 1998b; Nakajima et al., 1992). Therefore, the three essential postulates of Koch are fulfilled for $CD4^+$ T cells in most aspects of murine asthma. Although it is clear that $CD4^+$ T cells mediate many of the asthmatic features, the individual roles of Th2 cytokines IL-4, IL-5, IL-6, IL-9, and IL-13 are less clearly defined, and vary depending on the type of experimental protocol, mouse strain, as well as the parameter (eosinophilia vs. AHR vs. goblet cell hyperplasia) that is being studied.

The prototypical Th2 cytokine IL-4 induces polarization of Th0 cells into Th2 cells, enhances mast cell activation, induces IgE class switching in B cells, enhances the expression of vascular cell adhesion molecule (VCAM)-1 on endothelial cells, and induces expression of the mucin 5AC gene in epithelial cells. In most, but not all, animal models of asthma it seems that IL-4 is most important for inducing the Th2 response, having a more redundant function during the challenge period of the airways. Indeed, it was shown that AHR and airway eosinophilia were not abolished when antibodies to IL-4 were given during challenge of the airways (Corry et al., 1996; Coyle et al., 1995b; Hogan et al., 1998b; Webb et al., 2000). Transgenic expression of IL-4 in the airways induces some mild degree of peribronchial eosinophilic inflammation, upregulates expression of the MUC5AC gene, but does not induce AHR, illustrating that IL-4 is not sufficient to cause all aspects of the asthmatic phenotype (Rankin et al., 1996; Temann et al., 1997). One of the major effector functions of IL-4 during allergic inflammation might be the enhanced upregulation of VCAM-1, the counter receptor on endothelial cells for recruiting *v*ery *l*ate *a*ntigen (VLA)-4 positive eosinophils (Cohn et al., 1997).

The importance of IL-5 in regulating lung eosinophilia is underscored in $IL\text{-}5^{-/-}$ and in mice treated

with blocking anti-IL-5 antibodies that show strongly reduced blood and tissue eosinophilia (Foster et al., 1996; Kung et al., 1995b). When IL-$5^{-/-}$ $CD4^+$ Th2 cells were transferred into naive mice, they failed to induce airway eosinophilia yet induced normal mucus hypersecretion (Cohn et al., 1999a). Studies in IL-$5^{-/-}$ mice have shown that IL-5 is required for growth and differentiation of eosinophils in the bone marrow and that it co-operates with eotaxin to generate a chemotactic signal that attracts eosinophils into the lung (Kung et al., 1995b; Mould et al., 2000; Wang et al., 1998). Consistent with this, administration of IL-5 to the lung and transgenic expression of IL-5 in the lung leads to profound airway eosinophilia (Lee et al., 1997; Lefort et al., 1996; Van Oosterhout et al., 1995). Despite the fact that IL-5 is critical for eosinophilia, it has been a very complex issue to study whether IL-5 and its ensuing eosinophilic response are critical for the establishment of AHR in mice. Models of administration or transgenic expression of IL-5 to the lung have either shown no effect or an increase in AHR (Lee et al., 1997; Lefort et al., 1996; Van Oosterhout et al., 1995). Depending on the mouse strain, antigen, and protocol used to study the involvement of IL-5, AHR has been shown to be dependent or independent of IL-5 and eosinophils. Studies in the OVA model in C57BL/6 mice have highlighted that IL-$5^{-/-}$ mice and mice treated with anti-IL-5 moAbs have reduced eosinophilia and abolished AHR, whereas those using certain parasite antigens or house dust mite have shown an independence (Coyle et al., 1998; Foster et al., 1996; Hogan et al., 1997, 1998a; Tournoy et al., 2000b).

Studies in the OVA model using Balb/c mice have suggested two pathways to generate AHR. One is dependent on IL-5 and eosinophils (Cieslewicz et al., 1999; Hamelmann et al., 1999; Karras et al., 2000), the other is independent of IL-4, IL-5, and eosinophils, but clearly dependent on $CD4^+$ T cells and signalling through the IL4-R (Corry et al., 1996; Hogan et al., 1998b; Webb et al., 2000; Wilder et al., 1999). Treatment of IL-$4^{-/-}$ mice with blocking anti-IL-5 completely reduced airway eosinophilia but did not abolish AHR (Hogan et al., 1998b). In contrast, treatment of IL-$13^{-/-}$ mice with blocking anti-IL-5 abolished eosinophilia and AHR, suggesting that airway eosinophilia is linked to the mechanism underlying AHR only in the absence of IL-13 (Webb et al., 2000). The fact that eosinophils seem to be of minor importance for AHR could be related to the fact that in some mouse models eosinophils do not degranulate upon recognition of allergen (Stelts et al., 1998). In this scenario, the airways and smooth muscle are not exposed to major basic protein and eosinophil peroxidase.

Interleukin 13 is secreted by T-cells, NK-cells, and mast cells and has a critical role in Th2 development and IgE responses in mice, by signalling through the IL4Rα and STAT-6 (Corry, 1999; McKenzie et al., 1998). In addition to its effects during the primary immune response, IL-13 is also important in the effector response, an effect that is not shared with IL-4 (Corry et al., 1996; Coyle et al., 1995b). Indeed, studies using the antagonist sIL-13Rα2-IgFc have shown that IL-13 is a major cytokine regulating AHR and mucus cell hypersecretion, independently of tissue eosinophilia (Grunig et al., 1998; Wills-Karp et al., 1998). Transgenic expression and administration of IL-13 in the lung of naive mice leads to tissue infiltration with eosinophils and mononuclear cells, goblet cell hyperplasia, subepithelial fibrosis, and, importantly, AHR to metacholine (Wills-Karp et al., 1998; Zhu et al., 1999). Adoptive transfer experiments have shown that IL-13 could be the factor responsible for mucus hypersecretion and goblet cell hyperplasia induced by adoptive transfer of IL-$4^{-/-}$ $CD4^+$ T cells and seems to be the most essential Th2-derived cytokine for inducing eotaxin expression in the lung (Cohn et al., 1999a; Li et al., 1999).

The Th2 cytokine IL-6 has effects on B cell immunoglobulin production and enhances Th2 formation in the presence of IL-4, but also has some anti-inflammatory actions (Rincon et al., 1997). This cytokine is increased in the draining lymph nodes of OVA-exposed mice and transgenic over-expression in the lung leads to impressive subepithelial fibrosis and lymphocytic infiltration, in the absence of AHR or tissue eosinophilia (DiCosmo et al., 1994). However, sensitized IL-$6^{-/-}$ showed normal eosinophilic responses and AHR in response to OVA compared with their WT littermates, questioning the role of IL-6 in the asthma model (Kopf et al., 1995). Transgenic overexpression of the closely related cytokine IL-11 induced subepithelial fibrosis, lymphocytic infiltration, and AHR, in the absence of airways eosinophilia or goblet cell hyperplasia (Kuhn et al., 2000). Moreover, IL-11 was shown to inhibit OVA-induced eosinophilia, Th2-cytokine expression, and VCAM-1 expression (Wang et al., 2000). Interleukin-9 is another Th2 associated cytokine that stimulates mast cell growth and differentiation, goblet cell hyperplasia, and IgE production by B cells. Transgenic mice systemically overexpressing IL-9 had exaggerated tissue eosinophilia, IgE responses, and AHR in response to *Aspergillus fumigatus* challenge (McLane et al., 1998). Mice overexpressing IL-9 in the lungs had increased baseline levels of eosinophils, mast cell hyperplasia, mucus cell hypersecretion, and AHR (Temann et al., 1998). Mechanisms behind the effects of IL-9 have recently been suggested. A genetic trait that genetically determines baseline AHR in mice maps to the IL-9 locus on chromosome 13, suggesting that IL-9 might affect bronchial smooth muscle directly; IL-9 induces the expression of eosinophil selective chemokines and the MUC5AC gene leading to mucus hypersecretion (Louahed et al., 2000).

Although IL-10 is a prototypical Th2 cytokine in the mouse and enhances the formation of Th2 cells by downregulating IL-12 in DCs and other APCs, its effects during challenge of the airways are mainly anti-inflammatory. Consistent with this, administration of IL-10 during challenge was shown to reduce airways eosinophilia, infiltration with DCs, and Th1 and Th2 cytokine production (Stampfli et al., 1999a; van Scott et al., 2000; Zuany-Amorim et al., 1995). However, studies using IL-$10^{-/-}$ mice are somewhat inconsistent, reflecting the dual role of IL-10 in allergy, enhancing Th2 responses when given during priming, while suppressing them when given during challenge. Indeed, it was shown that allergen challenge of IL-$10^{-/-}$ mice either resulted in enhanced airway eosinophilia or did not affect or decreased airway eosinophilia compared

with IL-10$^{+/+}$ controls, with varying effects on AHR (Makela, 2000; Tournoy, 2000a; Yang, 2000).

IS THERE A ROLE FOR Th1 CYTOKINES AND CD8$^+$ CELLS IN ASTHMA?

Studies in humans have suggested that CD4$^+$ T cells producing IFN-γ are also found in increased amounts in the airways of asthmatics (Krug et al., 1996). Similarly, when studied at the single cell level using intracellular staining, it is clear from a number of murine asthma models that CD4$^+$ T cells producing either IFN-γ exclusively or in combination with IL-4 and/or IL-5 can be recovered from the BAL fluid and lungs of OVA-challenged mice (Lambrecht et al., 2000a; Temann et al., 1998). In addition, CD8 cells producing IFN-γ exclusively are attracted into the airways of challenged mice. This raises the important question as to whether IFN-γ derived from CD4$^+$ and/or CD8$^+$ cells contributes to the asthmatic phenotype. Administration of IFN-γ or Th1 cells during allergen challenge has been shown to reduce airway eosinophilia and AHR by reducing the recruitment of eosinophils and by downregulating eotaxin (Cohn et al., 1999b; Iwamoto et al., 1993; Li et al., 1996). Moreover, IFN-γ receptor deficient mice have a reduced clearance of airway eosinophilia following discontinuation of OVA challenge (Coyle et al., 1996a). Other Th1 promoting cytokines also influence eosinophilia. When IL-12 was given during challenge of the airways, there was a reduction in IL-4 and IL-5, airway eosinophilia, and AHR that was independent of IFN-γ (Gavett et al., 1995; Kips et al., 1996; Stampfli et al., 1999b). By analogy, neutralization of IL-12 during challenge in sensitized mice led to enhanced Th2 cytokine production and AHR (Karp et al., 2000; Keane-Myers et al., 1998). In the absence of IL-12 and/or IFN-γ, unmethylated CpG oligonucleotides are less effective in downregulating airway responses to OVA (Kline et al., 1999).

Despite these pieces of evidence for a protective role of Th1 cytokines in asthma, some investigators have suggested that endogenous IFN-γ is essential for the establishment of AHR following OVA challenge in OVA sensitized mice, independently of eosinophilia (Hessel et al., 1997). Moreover, adoptive transfer of OVA-specific Th1 cells together with Th2 cells enhances damage to the airways (Hansen et al., 1999; Randolph et al., 1999a,b). From these data it appears that IFN-γ and CD4$^+$ Th1 cells might contribute to asthma depending on the model studied.

The precise contribution of the Th1-enhancing cytokine IL-18 remains elusive. It was shown that exogenous IL-18 co-operates with IL-12 to reduce tissue eosinophilia, IgE, and AHR in one model of asthma, whereas IL-18 actually enhanced these parameters in other models (Hofstra et al., 1998; Kumano et al., 1999; Wild et al., 2000). It was shown that IL-18$^{-/-}$ mice had enhanced airway responses to OVA (Kodama et al., 2000). The most likely explanation for these discrepant findings is that IL-18 is less of a Th1 skewing factor than initially thought, and that effects of this cytokine might depend on the timing of administration (Robinson et al., 1997). In this regard, it was interesting to note that IL-18 administration to the lung led to primary sensitization to OVA via the airways (Wild et al., 2000).

Increased amounts of CD8$^+$ lymphocytes producing IFN-γ are recovered from the airways of challenged mice in the OVA model (Lambrecht et al., 2000a; Winterrowd and Chin, 1999). Although it was shown that (virus-specific) CD8$^+$ cells can be induced to produce IL-4 and IL-5 within an IL-4 rich environment, the production of these cytokines in the OVA model is absent (Coyle et al., 1995a; Erard, 1993). It was shown that CD8-deficient and β_2-microglobulin deficient mice that lack CD8$^+$ T cells and NK1.1 T cells develop normal eosinophilic responses to inhaled OVA in some models, whereas others have shown that depletion of CD8$^+$ cells using mo Abs led to the disappearance of IL-5 production, eosinophilia, and AHR (Gonzalo et al., 1996b; Hamelmann et al., 1996; Schwarze et al., 1999; Zhang et al., 1996). Part of the explanation might be that CD8 depletion also depletes other CD8$^+$ cells such as γδ-T cells or CD8$^+$ DCs.

IS THERE A ROLE FOR IgE AND MAST CELLS IN ORCHESTRATING AIRWAY EOSINOPHILIA?

Mast cells participate in the initial early phase response after exposure to allergen, but their importance in orchestrating eosinophilia is uncertain. After IgE-triggered activation, mast cells may promote the LPR and inflammation of the airways with eosinophils by producing inflammatory mediators (e.g., IL-1, TNF-α, and leukotriens) and eosinophil-directed cytokines (IL-4, IL-5, and IL-13) (Henderson et al., 1996). These substances in turn induce chemokines that attract eosinophils. Moreover, it has been suggested that $\alpha_E\beta_7$ positive mast cells communicate with $\alpha_E\beta_7^+$ Th2 cells and DCs to enhance ongoing Th2 responses in the airways. However, studies in mast cell-deficient *W/W^v* mice suggested that mast cells do not appear to be required for eosinophilia and/or AHR in some animal models of allergic disease in which adjuvant is used to prime Th2 responses (Brusselle et al., 1994; Kobayashi et al., 2000; Takeda et al., 1997). In protocols in which weaker Th2 responses are induced by injection of OVA in the absence of adjuvant, mast cells are clearly necessary for chronic eosinophilia and AHR in response to inhaled OVA (Kung et al., 1995a; Williams and Galli, 2000).

Although IgE has an established role in mediating mast cell degranulation and the early phase response after allergen challenge, it is questionable whether IgE is necessary in mediating the enhancing effects of mast cells on airway eosinophilia and AHR, and again depends on the model studied. In allergen-sensitized mice with a targeted deletion for the gene for IgE, or treated with anti-IgE antibodies, the recruitment of eosinophils into the lungs and AHR were not impaired after allergen challenge (Hamelmann et al., 1999; Mehlhop et al., 1997). If IgE has a function in chronic eosinophilic inflammation, it is most likely via formation of IgE immune complexes and its enhancing effects of allergen recognition and presentation by DCs and B cells, either via a FcεRI or CD23 mechanism (see above) (Coyle et al., 1996b; Zuberi et al., 2000).

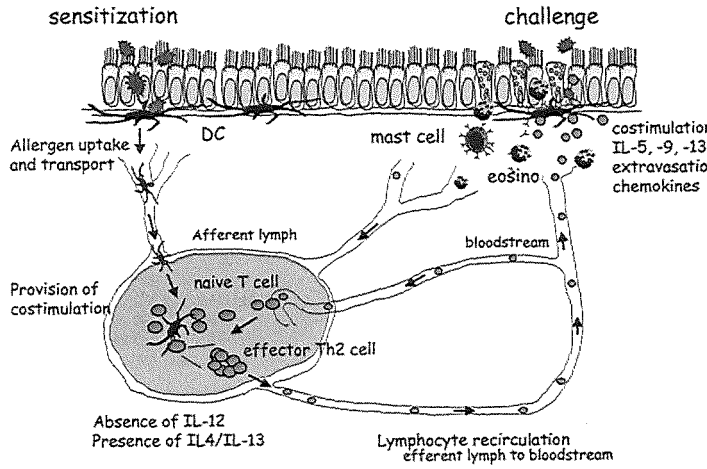

Fig. 5. A model of dendritic cell–T cell interaction inducing and controlling eosinophilic airway inflammation in the mouse. During the sensitization phase of the response, DCs in the periphery of the lung capture allergen. These cells enter the afferent lymphatic vessels and carry the allergen to the draining lymph nodes where it is presented on the surface of MHC molecules, together with costimulatory molecules. Allergen-specific T cells are selected for antigen specificity and induced to proliferate. In the absence of IL-12 and the presence of IL-4 and/or IL-13, DCs induce a primary Th2 response in the draining lymph nodes. Memory/effector $CD4^+$ Th2 cells leave the draining lymph node via the efferent lymphatics and extravasate at areas of inflammation where cell-adhesion molecules and signals for extravasation are encountered during the challenge phase. Once in the tissues, they interact again with IgE-bearing local DCs to increase production of IL-4, IL-5, IL-9, and IL-13. These cytokines are important for inducing tissue eosinophilia, airway hyperreactivity, goblet cell hyperplasia, mast cell activation, and for inducing the production of chemokines that further attract inflammatory cells. During the challenge phase, effector Th2 cells, eosinophils (and possibly DCs) also migrate to the draining lymph nodes.

DOWNSTREAM EVENTS THAT CONTRIBUTE TO THE ASTHMATIC PHENOTYPE IN MICE

In the above paragraphs, we have described the various immune cells and their mediators that are involved in the regulation of airway eosinophilia and AHR. It is increasingly clear that an extremely complex inflammatory cascade is induced by the various actions of DCs, Th2 cells, and mast cells that lead to airway eosinophilia, AHR, and mucus hypersecretion. Extravasation of eosinophils into inflammatory sites is a multi-step process characterized by initial intravascular rolling and firm adhesion to endothelium, followed by sequential eosinophil diapedesis between endothelial cells and chemotaxis into tissues. Rolling of eosinophils on endothelium is mediated via endothelial-expressed P-selectin and, accordingly, P-selectin deficient mice have reduced numbers of eosinophils in challenged airways (Broide et al., 1998). Lymphocyte function antigen (LFA)-1 interacting with endothelial intercellular adhesion molecule (ICAM)-1, or more importantly VLA-4 interacting with VCAM-1 mediates firm adhesion of eosinophils. Accordingly, ICAM-$1^{-/-}$ but not ICAM-$2^{-/-}$ mice have reduced tissue eosinophilia and AHR upon allergen challenge (Broide et al., 1998; Gerwin et al., 1999; Wolyniec et al., 1998). T lymphocytes and IL-4/IL-13 are important for regulating the expression of VCAM-1 on endothelial cells, in conjunction with TNF-α. The expression of VCAM-1 is highly increased on endothelial cells of challenged mice (Nakajima et al., 1994). Mice deficient in VCAM-1 or treated with anti-VCAM-1, anti-VLA-4, or anti-α_4 integrin (CD49d) antibodies had strongly reduced tissue eosinophilia and AHR (Gonzalo et al., 1996b; Henderson et al., 1998; Nakajima et al., 1994). It appears that VLA-4 and LFA-1 are also highly expressed on a population of $CD11c^+$ putative DCs, explaining part of the effectiveness of these antibodies in inhibiting the response to OVA (Henderson et al., 1998).

It cannot be overemphasized that the regulated production of chemokines has an important role in this downstream chain of events. Chemokines and their receptors are classified into CC, CXC, or CX_3C depending upon their molecular structure. Although many immune cells produce chemokines that contribute to the asthmatic phenotype, a dominant source of these molecules are the epithelial cells, endothelial cells, and fibroblasts of the lung (Gonzalo et al., 1996b; Li et al., 1999). Of the various CC chemokines, eotaxin-1 and -2, monocyte chemotactic protein (MCP)-1 and -5, monocyte derived chemokine (MDC), and regulated upon activation normal T cell expressed and secreted (RANTES) seem to be most important for regulating tissue eosinophilia and AHR, sometimes in conjunction with IL-5 (Gonzalo et al., 1996a,b, 1998, 1999; Mould et al., 2000). Signalling through the CCR3 via eotaxin is important for recruiting Th2 cells and eosinophils early in the response, whereas signalling through CCR4 via the MDC or thymus activated and regulated chemo-

kine (TARC) ligand is more important for recruiting Th2 cells and eosinophils during repeated challenge (Lloyd et al., 2000). It will be important to study the asthmatic phenotype of the various chemokine (receptor) knock-out mice.

CONCLUDING REMARKS

Over the past years, there has been an explosion of research on the use of murine models of eosinophilic airway inflammation to study the role of a particular cell or mediator in the pathogenesis of asthma. When Koch's postulates are applied, a model emerges in which DCs are crucial cells in the induction and maintenance of T cell reactivity to inhaled allergen (Fig. 5). Effector $CD4^+$ T cells and mast cells elaborate cytokines that induce the Th2 phenotype (IL-4), tissue eosinophilia (IL-5), mucus hypersecretion (IL-4, IL-13, IL-9), and AHR (IL-5 and IL-13). These studies have identified these cells and mediators as crucial targets for the therapy of asthma.

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Chapter 3

A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma

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Abstract

Mouse models of allergic asthma are increasingly used to study the immunopathology of this complex disorder. The degree and type of airway inflammation is often studied by determination of differential cell counts on cytospins of bronchoalveolar lavage fluid cells stained with May-Grünwald Giemsa, in which the separation of eosinophils from neutrophils and of mononuclear cells from activated T cells can be quite problematic. In this study, we compared differential cell counts based on morphological criteria on May-Grünwald Giemsa stained cytospins with a newly developed flow cytometric method. BAL fluid cells were identified based on forward and side scatter characteristics, auto-fluorescence of macrophages, and simultaneous one step staining with antibodies for T cells (CD3-Cy-Chrome), B cells (B220-Cy-Chrome), eosinophils (CCR3-PE), and dendritic cells (MHCII-FITC, CD11c-APC). The validity of this flow cytometric determination was tested by morphological analysis of flow-sorted cellular subsets. In an animal model of ovalbumin-induced asthma, this new method correlated very well with the differential counts based on cytospins. Flow cytometric determination of the cellular composition of BAL fluid in mouse models of asthma is a rapid and easy method that can replace differential cell counts based on morphology.

Introduction

Mouse models of allergic asthma are increasingly used to study the pathophysiology of this complex disorder[1-3]. In its most simple form, mice are sensitized to ovalbumin using either alum as an adjuvant or by using professional antigen presenting cells such as dendritic cells (DCs) as natural adjuvants[2,4]. Next, mice are challenged 10-14 days later with an aerosol of ovalbumin, and killed 24 h later. At this point, most models use bronchoalveolar lavage to measure the cellular composition of the lungs following allergen challenge. Bronchoalveolar lavage is also used as a clinical diagnostic tool in patients with a variety of lung disorders[5-7] and as a research tool in patients with asthma[8]. In mouse models of asthma, the cellular composition of BAL fluid is determined on cytospins stained with either May-Grünwald Giemsa or hematoxylin-eosin. Using morphological criteria, it is at best possible to discriminate between macrophages/monocytes, lymphocytes, neutrophils and eosinophils. Especially problematic in daily practice, however, is the ability to discriminate between eosinophils and neutrophils and between monocytes and activated T cells. Some authors have therefore used the endogenous cyanide-resistant peroxidase activity of eosinophils or the intracellular presence of major basic protein to enumerate these cells in BAL fluid[9-13]. Also, morphological criteria are insufficient to discriminate between activated T and B cells, and between monocytes and alveolar DCs.

Flow cytometry and cell sorting have been used for several decades to identify and purify cells for different research goals. The development of a wide range of fluorescently labeled monoclonal antibodies has made it possible to analyze several cell types in large numbers of samples in a relatively short time period. Flow cytometry can combine 'morphological' features based on by FSC/SSC channel intensity with expression of cell-specific surface markers using fluorescently labeled Abs. It is therefore surprising that flow cytometry has hardly been used for the purpose of differential cell counting on BAL fluid samples in mice till now. By flow cytometric analysis, a multiplicity of cells can be analyzed compared with the number of cells counted by an investigator (25,000 events vs the traditional 200-400 counted cells in morphological analysis). In addition, staining and analysis of many samples simultaneously is much less time consuming compared with preparing, staining and counting

cytospins. Moreover, analysis by flow cytometry minimizes inter-observer variation by rigorous determination of thresholds to determine the identity of a cell type.

Here we describe a flow cytometric method for differential cell counts of murine bronchoalveolar lavage fluid cells by staining with a combination of commercially available antibodies, MHCII, CCR3, CD3, B220 and CD11c. This combination of antibodies identifies T and B lymphocytes, eosinophils, neutrophils, dendritic cells and monocytes/macrophages in BALF.

Methods

Animals

Female BALB/c (H-2^d) mice (Harlan, Zeist, the Netherlands) were housed under specific pathogen-free conditions at the animal care facility at the Erasmus University Rotterdam. Mice were between 6-8 weeks old. The experimental procedure used in this study was approved by the Erasmus University Committee of Animal Experiments.

Generation of eosinophilic airway inflammation

On day 0 mice were actively immunized with OVA (grade V, Sigma Chemical Co., MO, USA) by intratracheal instillation of $1x10^6$ OVA pulsed bone marrow DCs, and subsequently boosted at day 10 by an i.p injection of 10 µg adsorbed to 1 mg of $Al(OH)_3$ (Sigma), as previously described[14]. Ten days after boosting, mice were challenged 3 times with a daily 30 min OVA or PBS aerosol (OVA grade III, 1% w/vol in Phosphate Buffered Saline (PBS), Sigma). To induce a less severe airway inflammation in some mice the OVA alum boost was omitted.

Flow cytometry

Twenty-four hours after the last aerosol, mice were sacrificed by avertin overdose followed by exsanguination. Bronchoalveolar lavage (BAL) was performed with 3x 1 ml of Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1 mM EDTA (Gibco). Red blood cells (RBCs) were lysed using ammonium chloride lysis buffer[4]. Half of the cells were used for cytospins and the remaining BALF cells were stained for 30 min in a refrigerator in a 96 well flexiplate (ICN, The Netherlands) with 40 µl FACS buffer/well (PBS, 5% BSA, 0.01% sodium azide) containing the final concentrations of the following monoclonal antibodies: MHCII-FITC (2.5

μg/ml; 2G9), allophycocyanin (APC)-labeled CD11c (2 μg/ml, HL3), Cychrome (CyChr)-labeled Abs against CD3 (10 μg/ml, 145-2C11) and B220 (2 μg/ml, RA3-6B2; PharMingen, Becton Dickinson, San Diego, CA, USA) and Phycoerytrin (PE)-labeled CCR3 (0.625 μg/ml, 83101.111, R&D systems, Abingdon, UK). To prevent non-specific binding to Fc receptors 2.4.G2 blocking reagent (6 μg/ml) was added to the monoclonal antibody mix (See Table 1). Cells were sorted on a FACS DIVA and/or subsequently analyzed on a FACS Calibur (BD biosystems) using CellQuest (Becton and Dickinson Immunocytometry Systems, San Jose,

BALF cell differentiation

Flowcytometry	**Cytology**
>100 000 cells/well flexiplate (up to $1*10^6$)	Prewet slides with 1% BSA/PBS 50 000 cells/cytospin Dry overnight
Add 40 μl antibody mix/well: 2.4.G2 (6 μg/ml) MHCII FITC (2.5 μg/ml) CCR3 PE (625 ng/ml) CD3 Cy-Chr (10 μg/ml) B220 Cy-Chr (2 μg/ml) CD11c APC (2 μg/ml) in FACS buffer (PBS, 5% BSA, 0.01%SoAz) Incubate 30' at 4 degrees, dark	3' May-Grunwald 1' 1:1 May-Grunwald:Milli-Q short dip milli-Q 20' 1:4 Giemsa: Milli-Q 2 short dips in Milli-Q dips in 0.01% acetic acid 4 dips in 95% ethanol 4 dips in 100% ethanol 2 dips in xylene
Wash 150 μl FACS buffer Spin down 400xg, 3'] 2x	Dry 1 hour
Resuspend in 150 μl FACS buffer	Mount in entellan
Acquire measurement at FACS Analyse with Flowjo	Dry overnight Count cells by morphological criteria

Table 1: Flowcytometric versus cytological differential cell count protocol.

CA) and FlowJo software (Treestar, Costa Mesa, CA). Lymphocytes were identified as FSC^{lo}/SSC^{lo} and expressing CD3 or B220, B cells were distinguished from T cells by MHCII expression in the $(B220/CD3)^{+}$ gate. Granulocytes were recognized as non-auto-fluorescent highly granular (SSC^{hi}) cells, and within this gate, eosinophils were defined as cells expressing the eotaxin receptor CCR3[15], intermediate levels of CD11c[4], and very low to undetectable expression of MHCII[16], B220 and CD3. Neutrophils had a similar scatter profile as eosinophils but lacked CCR3 expression[15]. Dendritic cells were identified as $(CD3/B220)^{-}$ and expressing high levels of MHCII and CD11c[4, 17]. Alveolar macrophages were identified as large auto-fluorescent cells[18].

Cytology

Cytospins were prepared from cells of the same BALF samples as used for flow cytometry. Slides were treated with 1% Bovine Serum Albumin/PBS solution and 50,000 cells were spun on to the slides. Cytospins were dried overnight and then frozen at –80°C until staining with May-Grünwald Giemsa and were characterized morphologically. In short: cytospins were stained by 3 min in May-Grünwald, 1 min in 1:1 May-Grünwald:milliQ, a short dip in milliQ, 20 min in 1:4 Giemsa:milliQ, 2 short dips in milliQ, dips in 0.01% acetic acid (glacial) until excess stain was removed, 4 dips in 95% ethanol, 4 dips in 100% ethanol and, finally, 2 dips in xylene. Stained cytospins were mounted in Entellan (See Table 1).

For histochemical detection of $MHCII^{+}$ DCs in BALF, cytospin preparations of BALF cells were acetone fixed and blocked with 1% Bovine Serum Albumin/PBS. MHCII expression was detected using a rat anti-mouse MHCII antibody (Ab) (M5.114), followed by goat anti-rat $F(ab')_2$ fragments conjugated to horseradish peroxidase (Serotec, Oxford, U.K.). The signal was developed using diamino benzidine substrate for 10 min in TRIS buffered saline (Sigma). Cytospins were mounted in Entellan mounting medium (Merck, Darmstadt, Germany).

Purification of cell populations from the BALF

Cells were purified from BALF under sterile conditions using a FACS DIVA flow cytometric sorter (Becton Dickinson). BALF cells were stained as described above and different cell types were recognized based on scatter characteristics and expression of specific markers. Purity of the sort was determined at 84-99% by May-Grünwald Giemsa staining on cytospins

of sorted cells. 400 cells were counted per cytospin. The determination of the validity of the sort was extended by admixing sorted eosinophils with BALF cells of naïve mice (which contained 0.73% eosinophils) at increasing concentrations (0, 10, 20, 30, ...100%). These mixed cell populations were measured by flow cytometry and the measured eosinophil percentages were compared with the admixed concentrations. A similar method was used by admixing bone marrow-derived myeloid DCs[4] with BALF cells obtained from naïve mice.

Statistical analysis

Differential cell counts of BALF cells measured by flow cytometry were compared with cell counts obtained by cytology by calculating Spearman's rank correlation coefficient (SPSS 10.0 for Windows). The correlation was considered to be significant if $p<0.05$.

Results

Identification of lymphocytes (T and B cells), eosinophils, neutrophils and mononuclear cells (macrophages and DCs) in BALF by flow cytometry

Mice were sensitized to OVA and subsequently challenged with 3 OVA aerosols from day 10-12. Twenty-four hours after the last aerosol challenge, the lung was lavaged. Bronchoalveolar lavage fluid cells were lysed with ammonium chloride to remove red blood cells. Cells were stained with a combination of antibodies including: MHCII FITC, CCR3 PE, CD3 Cy-Chr, B220 Cy-Chr, CD11c APC. To reduce non-specific binding, cells were incubated with 2.4G2 blocking reagent.

Cells were analyzed using six different parameters (See Fig 1). First, a plot of the Fl3 channel detecting Cy-Chrome (Cy-Chr) and auto-fluorescent signal versus cell size (FSC) was made. B220/CD3-Cy-Chr plotted against FSC was used to distinguish small B220/CD3^{+} lymphocytes from large auto-fluorescent macrophages and small CD3/B220^{-} granulocytes (Fig 1, first column of panels). In the second column of panels, cells were further identified by using specific markers, following gating in the Fl3/FSC plot. Small FSC^{lo} CD3/B220^{-} granulocytes were further divided in a MHCII/CCR3 plot into neutrophils or eosinophils upon expression of the eotaxin receptor CCR3-PE[15]. Within the small (FSC^{lo}CD3/B220^{hi}) lymphocyte gate, MHCII-FITC expression was used to discriminate T cells from MHCII^{+} B cells. FSC^{hi} large cells in BALF include auto-fluorescent macrophages and non-auto-

fluorescent MHCII^{+}/CD11c^{+} dendritic cells (see also Fig 3C, D, E, F). The cell populations in BALF differed not only in size (FSC) but also in granularity (SSC) as shown in the third column of FSC/SSC plots gated on eosinophils, neutrophils, lymphocytes and mononuclear cells. Eosinophils are small cells that demonstrate considerable sideward light scattering properties, indicative of complex granules. Neutrophils, which are of identical size, have considerably less light scatter, illustrating that the granular content is not so complex, but the scatter is still higher than with lymphocytes. Lymphocytes are small cells with very little light scattering ability. Mononuclear cells are large, medium to high light scattering cells, reflecting heterogeneity within this population.

To validate this classification based on specific markers and size/light scatter characteristics, all cell populations were flow cytometrically sorted and stained with May-Grünwald Giemsa

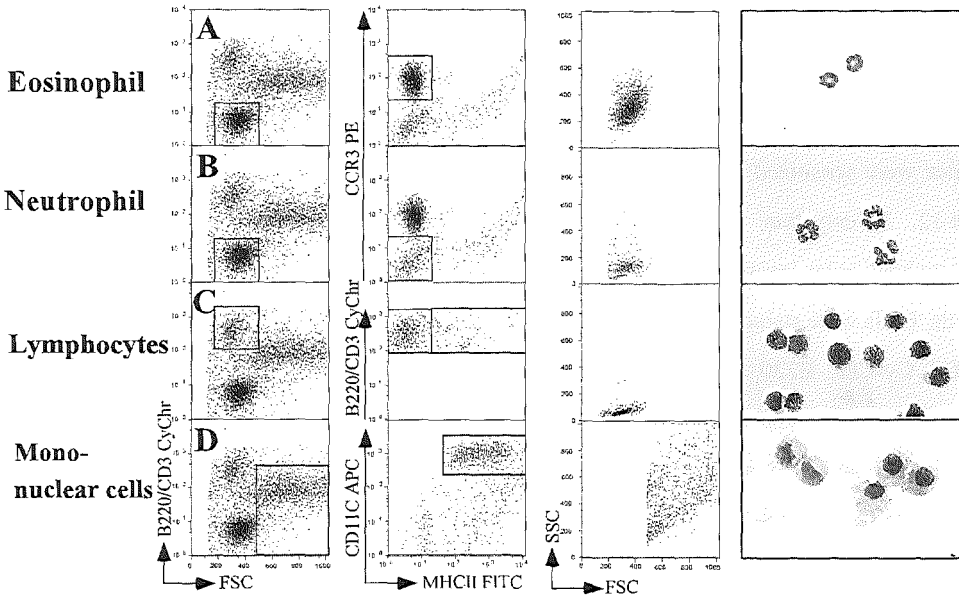

Figure 1. Identification of major BALF cell types using flow cytometry. *BALF cells were obtained from OVA-sensitized and challenged mice. To determine the major BALF cell types: eosinophils (row A), neutrophils (row B), lymphocytes (row C) and mononuclear cells (row D), cells were stained with fluorescently labeled antibodies. First column of panels: CD3/B220 vs Forward Scatter (FSC) plot, a gate was set on (CD3/B220)$^{-}$ granulocytes, (CD3/B220)$^{+}$ lymphocytes or Mononuclear cells (high-auto-fluorescent macrophages and non auto-fluorescent DCs). Second column: Eosinophils were discriminated from neutrophils by the expression of the eotaxin receptor CCR3 within the granulocyte gate. B cells were discriminated from T cells by MHCII expression. Mononuclear cells had a high MHCII^{+}/CD11c^{+} expression. Third column: 'morphological' flow cytometric parameters of the sorted cell types concerning size (FSC) and light scattering indicative of granularity (SSC). Fourth column: May-Grünwald Giemsa stained cytospins of the sorted major BALF cell types, shown at 100x.*

(last column of panels). Eosinophils displayed a characteristic doughnut shaped nucleus and had a pink cytoplasm in May-Grünwald Giemsa staining, indicative of eosinophilic granules. The sort of eosinophils was 93% pure (the remaining cells consisted of 6% mononuclear cells and 1% neutrophils). Neutrophils had a multi-lobed nucleus that contained more lobulations and the cytoplasm was only faintly stained with May-Grünwald Giemsa. The neutrophil sort was 84% pure (4% lympho, 6% mono, 6% eo). Lymphocytes had a rather large appearance and demonstrated a large round and partially lobed nucleus with a very high nuclear to cytoplasmic ratio (99% pure sort with 1% eo). Mononuclear cells were large, sometimes veiled cells with a large cytoplasm (88% pure sort with 9% eo, 2% neutro, 1% lympho).

Correlation of differential cell counts based on cytology and flowcytometry

Cytospins of BALF cells of OVA-sensitized and challenged mice and control mice were stained with May-Grünwald Giemsa and differential cell counts were performed by classification of 400 cells according to the morphological characteristics described above (Fig 2A). BALF cells from the same mice were investigated for cellular composition by flow cytometry (Fig 2B). The two differential cell count methods resulted in a similar cellular composition, and the two methods correlated very well. For each identified cell type, Spearman's rank correlation coefficient rho was calculated. For eosinophils (rho=0.870), neutrophils (rho=0.596), lymphocytes (rho=0.789) and mononuclear cells (rho=0.950) the correlation coefficient was statistically significant (Fig 2C, $p<0.05$). In addition to the validation of the method based on the morphological characteristics of the cell populations, we admixed sorted BALF eosinophils with BALF cells of naïve mice and measured the cell mixtures on the FACS Calibur by the above described method. The premixed percentages of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% eosinophils corresponded very well with the measured percentages (Fig 2D). The same strategy was used by mixing bone marrow DCs with BALF of naïve mice.

Cellular composition of BALF in OVA-sensitized/challenged mice compared to PBS-sensitized/challenged mice

OVA aerosol challenge caused a strong eosinophilic inflammation of the lung of OVA-sensitized mice and this was reflected in the bronchoalveolar lavage fluid. On average $2.05x10^6 \pm 1.95x10^5$ total cells were recovered. Performing the new flow cytometric method

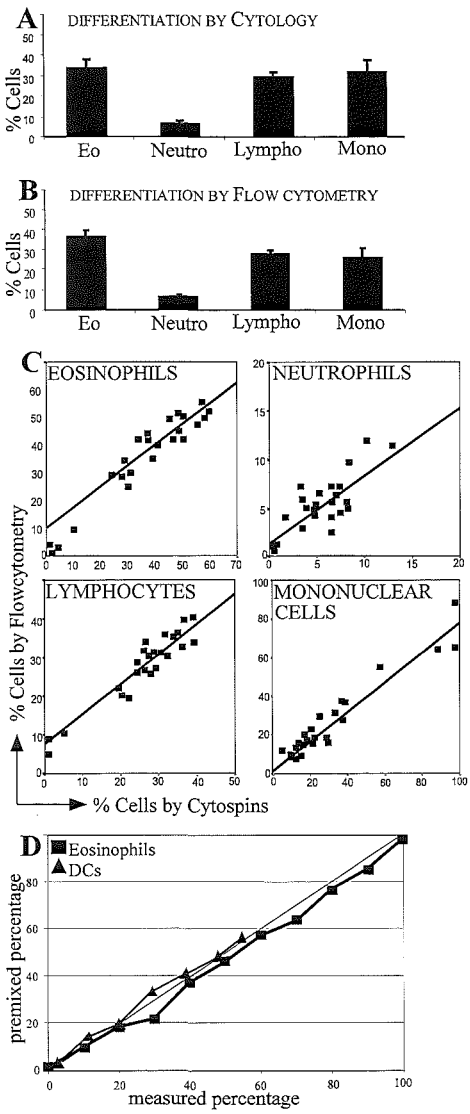

Figure 2. Correlation of differential cell counts of bronchoalveolar lavage fluid cells based on flow cytometry versus cytology. *BALF cells were obtained from OVA-sensitized and challenged mice (n=20) and PBS sensitized/OVA-challenged control mice (n=4). The percentage of eosinophils, neutrophils, lymphocytes and macrophages in the BALF of OVA sensitized and challenged mice were determined by counting 400 cells on May-Grünwald Giemsa stained cytospins using morphological criteria (A) or based on cytometric characteristics (B). Correlation between the percentage of each major cell type determined by FACS analysis and based on histology on BALF cells of OVA sensitized/OVA-challenged mice and control mice (Spearman's correlation coefficient rho was significant for all cell populations ($p<0.05$) (C). In addition to the morphological validation of the method, sorted eosinophils (squares) and cultured dendritic cells (triangles) were admixed each with a pooled BALF sample of 10 naïve mice at increasing percentages and were subsequently measured by flow cytometry (D).*

revealed that eosinophils accounted for 48.7%; macrophages for 13.8%; neutrophils for 9.5% and lymphocytes for 20.3% of the total cell count. In PBS-sensitized/challenged animals, BALF cell composition consisted for the largest part of alveolar macrophages (Fig 3A, B, C) with some contaminating red blood cells and total cell counts were much lower ($9.0x10^4 \pm 1.8x10^4$). In the B220/CD3 against FSC plot (Fig 3A) and FSC/SSC plot (Fig 3B) the shift in cellular composition from mainly macrophages (M) during steady state towards granulocytes (G) and lymphocytes (L) during inflammation is clearly seen. Most of the

granulocytes were $CCR3^+$ eosinophils (see also Fig 1A). Another distinct difference between PBS and OVA-sensitized/challenged mice concerns the phenotype of mononuclear cells. In the steady state condition, mononuclear cells are mainly $CD11c^{int/hi}/MHCII^{int}$ macrophages, while in OVA-sensitized and challenged mice large mononuclear cells demonstrated high CD11c and $MHCII^{int/hi}$ expression, consistent with a DC phenotype In order to demonstrate the presence of DCs in BAL fluid, MHCII staining on BALF cells was performed. This staining did indeed reveal (2.5% of total cells) large MHC class II strongly positive mononuclear cells with a dendritic morphology (Fig 3D). The percentage by

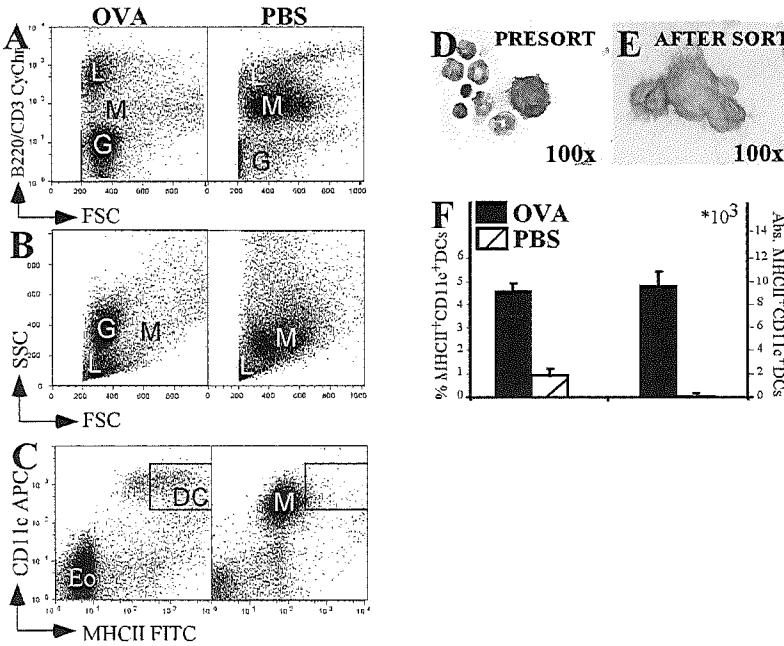

Figure 3. Flow cytometric characteristics and cellular composition of BALF cells in OVA sensitized/challenged mice compared with PBS sensitized/challenged mice. *Cellular composition of BALF cells of OVA-sensitized/challenged mice (left column) compared with PBS-sensitized/challenged mice (right column). (A) Ungated BALF cells in a B220/CD3 vs FSC plot. In PBS-sensitized/challenged mice mainly auto-fluorescent macrophages ('M') were recovered from the bronchoalveolar compartment. In OVA-sensitized/challenged mice granulocytes (mainly eosinophils, see Fig 1) ('G') and lymphocytes ('L') were attracted to the site of inflammation. (B) Distinction of eosinophils, lymphocytes and macrophages based on the morphological flow cytometric parameters FSC and SSC. (C) CD11c vs MHCII plot on ungated cells. In OVA-sensitized/challenged mice, $CD11c^{lo}/MHCII^{lo}$ eosinophils and $CD11c^{hi}/MHCII^{hi}$ DCs are present. In PBS-sensitized/challenged mice, macrophages have a $CD11c^{int}/MHCII^{int}$phenotype and $CD11c^{hi}/MHCII^{hi}$ DCs are absent. (D) MHCII staining on unsorted pooled BALF cells of OVA-sensitized/challenged mice. (E) MHCII staining on sorted $CD11c^{hi}$ $MHCII^{hi}$ cells showing large dendrites, typical of DCs. (F) The number and percentage of $CD11c^{hi}/MHCII^{hi}$ DCs in BALF of OVA-sensitized/challenged mice compared to PBS-sensitized/challenged mice.*

morphological analysis corresponded well with the 2.02% measured using flow cytometric analysis. Cells in the DC gate were sorted and MHCII staining was performed. The distinction between macrophages and DCs was made based on the higher level of MHCII expression and the smaller and/or lobed nucleus compared with macrophages. DC processes were observed after a period of 1 h at 37°C in TCM containing 2% FCS (Fig 3E). At least 84% of the sorted cells had a DC morphology (14.6% macrophages, 0.6% eos, 0.8% lympho). In OVA-sensitized mice the number of these putative DCs was significantly enhanced, relatively as well as absolutely (Fig 3F).

Discussion

Differential cell counting on BAL fluid cells is often used to ascertain the degree and type of inflammation induced by allergen challenge in mouse models of asthma[3]. BAL fluid sampling is thought to reflect the degree of inflammation within and around the bronchial lumen and deeper alveolar compartments. Cell types are classically identified based on morphological criteria in May-Grünwald Giemsa staining using a combination of cell size, nuclear shape and staining of the cytoplasm. In daily practice, the differentiation of eosinophils from neutrophils can be quite problematic due to heterogeneity in cytoplasmic staining and the possibility that eosinophils can loose their cytoplasmic staining due to degranulation[19]. Eosinophils are classically discriminated from neutrophils by the presence of eosinophilic cytoplasm, or by the presence of a ring-shaped nucleus. Another common problem in these differential cell counts of BAL fluid can be the discrimination between large activated lymphocytes and monocytes. Using a combination of fluorescently labeled antibodies, added in a single step staining reaction, we have been able to identify the major cell type populations in BAL fluid by flow cytometry. Using a combination of cell size and granularity, autofluorescence, an antibody to the CCR3 receptor for eotaxin expressed on eosinophils, CD3 expressed on T cells, B220 expressed on B cells, MHCII expressed on B cells and DCs and CD11c expressed on DCs, we were able to identify 6 cell types. Eosinophils were found to be granular, non-autofluorescent, $CCR3^{+}$ and $CD11c^{dim}$ cells lacking expression of MHCII, B220 and CD3. Neutrophils, on the other hand, were also granular but lacked expression of CCR3 or CD11c. Lymphocytes were small non-granular, non-autofluorescent cells that expressed either CD3

(T cells) or B220 and MHCII (B cells). Dendritic cells were larger non-autofluorescent cells expressing high levels of CD11c and MHCII, while lacking expression of CCR3, CD3 or B220. Finally, macrophages were large autofluorescent cells expressing intermediate levels of CD11c. As far as we know, this is the first report of murine BALF cell differentiation using flow cytometry instead of cytological differentiation on cytospins. The validity of our staining reaction was ascertained by flow cytometrically sorting the various populations identified and looking at the morphology of the cells. The validation of the method was furthermore verified by flow cytometry and admixing sorted eosinophils (purity 98%, based on flow cytometric analysis of the sorted sample) or cultured dendritic cells with BALF cells of naïve mice at increasing concentrations. The admixed percentages corresponded very well to the measured percentages. We also compared the flow cytometric method with the histological method to determine cellular composition of BALF using the same samples. The resulting differential cell counts of BALF based on flow cytometric characteristics correlated very well to those based on the classical morphological criteria. In particular, the degree of BAL fluid eosinophilia, a primary endpoint in most studies on murine asthma, correlated extremely well across a broad range making this method very useful indeed.

The use of specific antibodies made it possible to characterize further several distinct cell types not classically mentioned in manual counts of cytospins. Within the B220/CD3-positive lymphocyte gate, formed by gating on small non-granular cells expressing CyChr labeled B220 and CD3, expression of MHCII made it possible to distinct B cells from T cells instead of gathering the two cell types as lymphocytes. In mice, in contrast to humans, activated T cells do not express MHC class II allowing differentiation based on this marker.

Mononuclear cell types are often lumped together in manually counted differential formulae. In the lung, these cells consist mainly of alveolar macrophages with a very high degree of auto-fluorescence. However, immunostaining for MHC class II revealed that numerous cells with long dendrites could be found in the airways of allergen-challenged mice, most likely representing DCs[4,20]. By using the DC-specific marker CD11c in combination with high level expression of MHC class II on flow cytometry, we were able to show that DCs were also contained in the population of cells classically enumerated as mononuclear cells or monocytes/macrophages. Immunohistological staining of MHCII on sorted $MHCII^{hi}CD11c^{hi}$

cells showed cells with, unsurprisingly, a high MHCII expression, a low nucleus/cytoplasm ratio and a smaller and/or lobed nucleus compared with the round and larger nucleus of macrophages, and numerous long dendrites. The percentage DCs in BALF of mice that were OVA-sensitized and challenged, based on cytospins (2.5%) corresponded well to the percentage found by flow cytometry on the same BALF cells (2.02%). In baseline conditions, mononuclear cells consisted mainly of autofluorescent $CD11c^{hi}$ $MHC\text{-}II^{int}$ alveolar macrophages. It is very hard to study the specific expression of fluorescent markers on alveolar macrophages that spontaneously emit light at all the detection channels of the flow cytometer, and therefore it cannot be excluded completely that part of the signal of CD11c and MHC class II is due to auto-fluorescence[18]. However, others have reported weak CD11c expression and MHC class II by 10-30% of alveolar macrophages[21]. As we challenged the lungs with allergen, the cellular composition of BALF shifted from mainly macrophages to recruited eosinophils and non-auto-fluorescent $CD11c^{hi}$ $MHC\text{-}II^{hi}$ cells. It has been shown that macrophages can mature into dendritic cells by incubating macrophages overnight in the presence of GM-CSF[22-26]. We hypothesize therefore that these $CD11c^{hi}/MHCII^{hi}$ cells are mature dendritic cells (Fig 3C) that were either recently recruited from the bloodstream[4] or that developed from $CD11c^{hi}/MHCII^{int}$ alveolar cells under the influence of locally produced cytokines. Additional experiments will have to demonstrate whether alveolar macrophages indeed give rise to DCs under the inflammatory conditions offered by eosinophilic airway inflammation.

Although the method described in this report is relatively simple, certain points should be addressed. First, alveolar macrophages display a high level of auto-fluorescence, which can be measured at all detectors to various extents, and can significantly disturb measurements of cell specific markers[27]. Therefore, care should be taken to exclude these cells from the granulocyte and lymphocyte gate. Secondly, degranulated eosinophils probably have a different light scattering phenotype compared to eosinophils that have not degranulated. After degranulation, eosinophils give a much lower side scatter signal due to the expulsion of complex granules[19]. In our hands, all $CCR3^{+}$ cells had a highly granular phenotype on FSC/SSC plots arguing against significant degranulation. Indeed, in several asthma models the degranulation of eosinophils has been questioned[28,29]. However, it could be that in other

asthma models, the degranulation of eosinophils would give rise to $CCR3^+$ cells of low side scatter profile.

Identification of the major cell type populations by flow cytometry automatically leads to the possibility to isolate these cells by flow-sorting. In this way contamination with other cell types can be prevented specifically. Others have used the characteristic of eosinophils to emit polarized scattered light to sort these cells from lavage fluid[30]. We have studied several BALF cell types identified on flow cytometric characteristics[4,16]. Using the same criteria to isolate BAL fluid cells as described here, we were able to investigate the presumed antigen presenting capacity of BAL fluid eosinophils without contamination of other antigen presenting, like B cells, macrophages and DCs. Using this method we were able to sort large quantities of eosinophils at a very high purity (>98%). Other applications are the sorting of BAL fluid subsets (e.g. DCs or T cells) to perform subsequent RT-PCR of expressed genes (unpublished observations).

In conclusion, we have developed a new, rapid and simple method to determine the cellular composition of BALF cells in a murine model for asthma using flow cytometry. This method correlated very well with differential cell counts based on morphological characteristics and may prove to be a time saving, investigator independent and more detailed method for general use in experimental asthma research.

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Chapter 4

Allergen-induced accumulation of airway dendritic cells is supported by an increase in $CD31^{hi}Ly\text{-}6C^{neg}$ bone marrow precursors in a mouse model of asthma

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Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31^{hi}Ly-6C^{neg} bone marrow precursors in a mouse model of asthma

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Airway dendritic cells (DCs) are held responsible for inducing sensitization to inhaled antigen, leading to eosinophilic airway inflammation, typical of asthma. However, less information is available about the role of these cells in ongoing inflammation. In a mouse model of asthma, sensitization to ovalbumin (OVA) was induced by intratracheal injection of myeloid OVA-pulsed DCs. Upon OVA aerosol challenge and induction of eosinophilic airway inflammation in sensitized mice, there was a time-dependent and almost 100-fold increase in the number of MHCII$^+$ CD11b$^+$ CD11c$^+$ endogenous airway DCs as well as CD11b$^+$ blood DCs. The mechanism of this increase was studied. Adoptive transfer experiments demonstrated that accumulation of airway DCs was not due to reduced migration to the mediastinal lymph nodes. Rather, the massive increase in airway and lymph node DCs was supported by an almost 3-fold expansion of myeloid CD31^{hi}Ly-6C^{neg} hematopoietic precursor cells in the bone marrow (BM). There was no change in any of the other 5 populations revealed by CD31/Ly-6C staining. When these CD31^{hi}Ly-6C^{neg} BM precursors were sorted and grown in granulocyte macrophage–colony-stimulating factor, they differentiated into MHCII$^+$ CD11c$^+$ DCs. The same CD31^{hi}Ly-6C^{neg} precursors also expressed the eotaxin receptor CCR3 and differentiated into eosinophils when grown in interleukin 5. Serum levels of eotaxin were doubled in mice with inflammation. These findings in an animal model of asthma suggest that the BM increases its output of myeloid precursors to meet the enhanced demand for DCs and eosinophils in inflamed airways. (Blood. 2002; 100:3663-3671)

Introduction

The role of the dendritic cell (DC) as a professional antigen-presenting cell (APC) in the primary immune response is now well established.[1] Airway DCs form a network in the epithelium, capture inhaled antigen (Ag), and migrate to the mediastinal lymph nodes (MLN) where Ag is presented to recirculating naive T cells.[2-5] Not surprisingly, these cells have been implicated to cause sensitization to inhaled allergens, typical of Th2-mediated allergic asthma. In a mouse model of asthma, intratracheal immunization with ovalbumin (OVA)–pulsed DCs generates Th2 effector cells that control eosinophilic airway inflammation, goblet cell hyperplasia, and bronchial hyperreactivity upon repeated challenge with OVA aerosol.[6,7] These observations indicate that airway DCs are essential in the early steps of sensitization. However, less information is available on the role of DCs in stimulating memory and/or effector Th2 cells upon repeated encounter with allergens.[8] In patients with stable allergic asthma, the number of airway DCs is elevated compared with healthy controls, and local allergen challenge leads to rapid accumulation of CD1a$^+$ HLA-DR$^+$ DCs in the airway lamina propria, suggesting that DCs also present allergens to T cells in the secondary immune response leading to airway inflammation.[9]

The mechanisms by which DC numbers increase in asthmatic airways include several not mutually exclusive possibilities. First, the increase could be caused by enhanced recruitment of DCs from the bloodstream into the site of airway inflammation. To support an enhanced demand for DCs in the inflamed airways, the bone marrow (BM) might enhance its output of DCs or DC progenitors. Such a mechanism would be similar to the enhanced recruitment of eosinophils into sites of allergic inflammation, supported by a release of eosinophilic progenitors from the BM.[10-14] Second, enhanced differentiation of freshly recruited monocytes into DCs could also lead to increased numbers of DCs being found at sites of airway inflammation.[15] In such a scenario, one would expect to find enhanced production of DC differentiation and/or growth factors within the lung. Finally, as there is continuous and high throughput migration of airway DCs from the epithelium to the draining MLNs, a small decrease of DC efflux could lead to rapid and profound accumulation of DCs within the epithelium.[5,16] To study which of these mechanisms might predominate, we have used a DC-driven mouse model of asthma.[6]

Materials and methods

Animals

All experiments were performed with 8- to 10-week-old female Balb/c (H-2^d) mice (Harlan, Zeist, The Netherlands). Mice were housed under specific pathogen-free conditions at the animal care facility at the Erasmus University Rotterdam. All of the experimental procedures used in this study were approved by the Erasmus University Committee of Animal Experiments.

Murine model of asthma

To induce sensitization to inhaled OVA, BM-derived DCs were pulsed with OVA in vitro and subsequently injected into the airways of naive mice.[6] In

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short, BM cells were cultured for 10 days in tissue culture medium (TCM) (5% fetal calf serum [FCS] from Biocell Laboratories, Rancho Dominguez, CA; RPMI 1640, gentamycine, β-mercaptoethanol, all from Gibco BRL, Paisley, Scotland) supplemented with 20 ng/mL recombinant murine (rm) granulocyte macrophage–colony-stimulating factor (GM-CSF).[17] After 9 days of culture, cells were pulsed overnight with 100 μg/mL OVA (OVA-DC) (OVA, grade V; Sigma Chemical, St Louis, MO). On day 10 of culture, cells were collected, washed, and 1×10^6 DCs were injected intratracheally in naive mice. Control mice received identical numbers of unpulsed DCs (PBS-DCs). Ten days after immunization, mice were challenged with OVA aerosol (grade III, 1% wt/vol in phosphate buffered saline [PBS], Sigma) or with PBS aerosol during a 30-minute challenge per day for 1, 3, or 7 consecutive days. In separate experiments, an additional control group consisted of naive unmanipulated mice.

Detection of airway dendritic cells in whole mounts of the trachea

At 24 hours after the last OVA challenge, animals were anesthetized and tracheal whole mounts were prepared as described earlier with a modification that the secondary antibody, used to detect rat anti–mouse major histocompatibility complex II (MHCII), was goat anti–rat $F(ab')_2$ fragments conjugated to horseradish peroxidase (Serotec, Oxford, United Kingdom).[18] The entire trachea was mounted in Entellan (Merck, Darmstadt, Germany) and viewed under a transmission light microscope equipped with Nomarski optics (Leica, Cambridge, United Kingdom).

Collection of cells and tissues

Bronchoalveolaire lavage fluid. At 24 hours after the last aerosol, groups of mice were killed by avertin overdose followed by exsanguination. Bronchoalveolaire lavage (BAL) was performed with 3 × 1 mL Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1 mM ethylenediaminetetraacetic acid (EDTA). After red blood cells (RBCs) were lysed using ammoniumchloride lysis buffer, cytospin slides were prepared and remaining cells were used for flow cytometric analysis. Supernatants of BAL fluid (BALF) and serum were stored for enzyme-linked immunosorbent assay (ELISA) quantification of GM-CSF, interleukin 6 (IL-6) (OptEIA, PharMingen, Becton Dickinson, San Diego, CA; threshold 8 pg/mL), eotaxin, and fms-like tyrosine kinase 3 ligand (Flt-3L) (R&D Systems, Abingdon, United Kingdom; threshold 5 pg/mL).

Lymph nodes. Lymph node (LN) cell suspensions were prepared by a 1-hour incubation at 37°C with 0.02 mg/mL DNAse I (Sigma Chemical) and 100 U/mL Collagenase IV (Life Technologies). RBCs were lysed and cells were passed through a 40-μm cell sieve (Becton Dickinson).

Blood. Blood was collected in heparinized tubes from the iliac artery and lysed with 20 mL RBC lysis solution for 4 minutes at 4°C.

Bone marrow. BM cells were prepared by flushing femurs and tibiae with 5 mL sterile PBS, followed by RBC lysis and passage through a 100-μm cell sieve.

Staining for major basic protein-positive eosinophils

Cytospin preparations of BALF and cultured BM were acetone fixed and blocked with 1% bovine serum albumin/PBS. Major basic protein (MBP) was detected using a rabbit anti–mouse MBP antibody (Ab) (J. J. Lee, Mayo Clinic, Scottsdale, AZ), followed by alkaline phosphatase–conjugated goat anti–rabbit Abs (Sigma) and development of signal with New Fuchsin in Tris-HCl. Slides were counterstained with Mayer haematoxilin (Merck). One investigator counted all cells.

Flow cytometric analysis on BALF, LN, and blood cells

Cells were washed in PBS containing 5% FCS and 5 mM sodium azide (FACS wash), and 1×10^6 cells were stained for 30 minutes on ice. To reduce nonspecific binding, cells were incubated with 2.4G2 blocking reagent for 15 minutes. Monoclonals used were as follows: MHCII–fluorescein isothiocyanate (MHCII-FITC) (2G9), allophycocyanin (APC)–labeled CD11c (HL3), phycoerythrin (PE)–labeled Abs against CD3 (145-2C11), B220 (RA3-6B2), and CCR3 (R&D Systems), biotin-labeled CD11b (M1/70), followed by streptavidin (SA)–PE-Cy5 (Quantum Red; Sigma). Propidium iodide (Sigma) was added for exclusion of dead cells before analysis on a FACScalibur flow cytometer using CellQuest (Becton Dickinson Immunocytometry Systems, San Jose, CA) and FlowJo software (Treestar, Costa Mesa, CA).

Flow cytometric analysis and sorting of BM cells

BM cells were stained with anti-CD31 (anti–platelet endothelial cell adhesion molecule PECAM-1; ER-MP12-bio), and anti–Ly-6C (ER-MP20-FITC; both produced in-house) followed by SA-PE or SA-PE-Cy5, allowing the discrimination of 6 distinct populations of cells.[19-22] For phenotype description the following mAbs were used: CD11b-APC, CD127-PE (IL-7Rα; SB/14), CD131-PE (JORO50), CCR3-PE, CD3-PECy5 and CD4-APC (GK1.5), CD11c-APC and B220-PECy5, or Gr1-PE (RB6-8C5). Antibodies were from PharMingen or R&D Systems.

In separate sorting experiments, 80×10^6 cells were stained with CD31-bio followed by SA-PE and Ly-6C-FITC and were sorted into $CD31^{hi}Ly\text{-}6C^{neg}$ and into $CD31^{neg}Ly\text{-}6C^{med}$ populations under sterile conditions on a FACS Vantage flow cytometer (Becton Dickinson).

Culture of $CD31^{hi}Ly\text{-}6C^{neg}$ and $CD31^{neg}Ly\text{-}6C^{med}$ populations

After sorting, cells were washed twice in TCM and cultured for 7 days at 0.25×10^6/well in 24-well plates with 40 ng GM-CSF per milliliter to induce DC differentiation. In separate experiments, cells were grown at 8×10^5 cells/well in round-bottom 96-well plates in TCM supplemented with 30% FCS and 24 ng/mL murine recombinant IL-5 (rIL-5) (PharMingen) to induce eosinophil differentiation. As a control, unsorted BM was stained and cultured under identical conditions. After 7 days, cells were analyzed for expression of MHCII-FITC, CD11c-bi (N418) in combination with CD80-PE (16-10A1), CD86-PE (GL-1), or CD40-PE (3/23), followed by SA-PECy5. For eosinophil differentiation, cytospin preparations were stained with an anti–MBP Ab as described above.

Detection of labeled DCs after adoptive transfer

BM DCs were labeled using carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), as previously described.[6] $CFSE^+$ DCs ($2\text{-}5 \times 10^6$) were transferred intratracheally into mice with established eosinophilic airway inflammation (OVA-DC/3xOVA) or into control mice (PBS-DC/3xPBS). At 48 hours after the intratracheal cell transfer, mice were killed. The number of $CD11c^+$, $CFSE^+$ DCs was determined on BALF, MLN, and inguinal LN samples.

Statistical analysis

All experiments were performed using 3 to 10 mice per group, and per time point in kinetic experiments. Comparison of means between different groups was performed with a Kruskal-Wallis test for equality among the different groups and in the case of a significant difference, the Mann-Whitney test for unpaired data was used for comparing 2 groups (SPSS 10.0 for Windows) separately. Differences were considered significant if $P < .05$.

Results

OVA exposure time-dependently induces eosinophilic airway inflammation in OVA-DC–immunized mice

Sensitization was induced by intratracheal injection of 1×10^6 OVA-pulsed DCs. As a marker for inflammation in the lungs, the total number of BAL cells was measured 24 hours after 1, 3, or 7 OVA aerosol exposures in sensitized mice (Figure 1A). The total recovery of BAL cells was not different from control mice (PBS-DC/PBS) after 1 OVA exposure, but sequentially increased 10-fold ($P = .008$) after 3 OVA exposures and 46-fold ($P = .008$) after 7 OVA exposures.

Differential analysis of the BALF cells using flow cytometry (Figure 1B) showed a significant proportional increase in lymphocytes ($CD3^+$ or $B220^+$ cells) and granulocytes (based on scatter

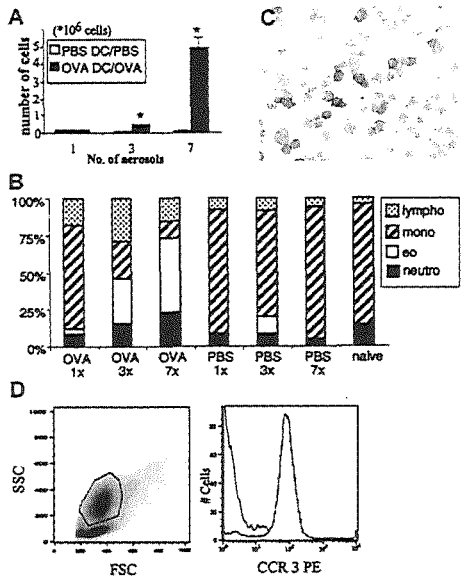

Figure 1. Effect of OVA or PBS aerosol challenge on cellular composition of BALF. Mice were immunized on day 0 with 1 × 10^6 OVA-DCs or PBS-DCs. From day 10 they were challenged daily for 30 minutes on 1, 3, or 7 consecutive days with OVA or PBS aerosols. (A) Total recovery of BALF cells as a marker of airway inflammation in response to aerosol exposures (*$P < .05$ compared with PBS-DC/PBS). Data are expressed as mean number of cells ± SEM. (B) Differential analysis of BALF cellular content based on flow cytometric analysis. The BALF composition of naive mice is included for reference. (C) Staining of BALF cytospin preparation with an Ab specific for murine MBP, identifying eosinophils in an OVA-DC immunized animal exposed 7 times to OVA aerosol. Isotype control Ab gave negative results (not shown). Original magnification, × 400. (D) Cells expressing high granularity on SSC signal (see gate) express the CCR3 receptor (filled histogram), identifying them as eosinophils. Isotype control is indicated by the open histogram.

characteristics) with a concomitant decrease in alveolar macrophages/monocytes (highly autofluorescent cells) in OVA-DC/OVA mice (Figure 2A). As the discrimination of eosinophils from other polymorphonuclear granulocytes is impossible based on scatter characteristics alone, eosinophils were further characterized as nonautofluorescent highly granular (SSC^{hi}) cells expressing intermediate levels of CD11c, and lacking expression of MHCII, B220, and CD3.[23] These highly granular cells also expressed the eotaxin receptor CCR3 (Figure 1D).[24] This method of counting eosinophils was compared with counting BALF cytospins stained with an anti–MBP Ab, yielding a highly statistically significant Pearson correlation coefficient of 0.82 ($P = .0001$) (Figure 1C). Performing 3 OVA aerosols induced an eosinophilia of 30.7 ± 6.0%, and 7 OVA aerosols induced an eosinophilia of 49.0% ± 5.1 of all BALF cells. Thus, OVA exposure in OVA-DC–immunized mice time-dependently induces eosinophilic airway inflammation.

OVA exposure leads to a massive increase in endogenous airway DCs in OVA-sensitized mice

To determine the number of DCs in the airways of PBS- and OVA-exposed mice, we have analyzed BALF cells 24 hours after the last OVA aerosol (Figure 2A). Dendritic cells were identified with multiparameter flow cytometry as nonautofluorescent CD11c^{hi}/MHCII^{hi}/B220$^-$/CD3$^-$ cells, as described previously.[3] Additional staining revealed that these cells expressed CD11b, identifying them as myeloid DCs (Figure 2D). First we verified that injected DCs could no longer be recovered from the BALF 5 days following intratracheal injection (data not shown). This eliminates the

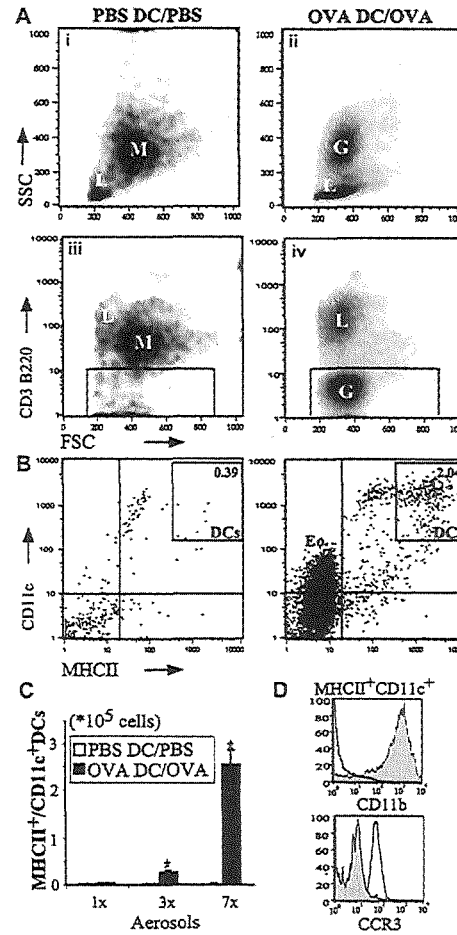

Figure 2. Effect of OVA or PBS aerosol challenge on the number of DCs in the BALF. Mice were immunized on day 0 with 1 × 10^6 OVA-DCs or PBS-DCs. From day 10 they were challenged daily for 30 minutes on 1, 3, or 7 consecutive days with OVA or PBS aerosols. (A) In OVA-DC/OVA mice (ii and iv), the FSC/SSC plot contains lymphoid cells (L), and granulocytes (G). In contrast, in PBS-DC/PBS mice (i and iii) the majority of cells are large and spontaneously autofluorescent, representing alveolar macrophages (M). A gate was set (iii and iv) on low autofluorescent cells that lacked expression of CD3 and B220. (B) Within the set gate, MHCII^{hi} CD11c^{hi} cells represent DCs, whereas CD11c^{dim} MHCII$^-$ cells represent eosinophils (Eo). In our experiments, eosinophils did not express MHCII molecules. The average percentage of MHCII^{hi} CD11c^{hi} DCs as a percentage of total cells analyzed is indicated in the plot. (C) Kinetics of increase of DCs following OVA exposure as expressed as the absolute number of MHCII^{hi} CD11c^{hi} cells within the BALF (n = 5 animals per group; *$P < .05$ compared with PBS-DC/PBS group). Data are expressed as mean number of MHCII$^+$/CD11c$^+$ DCs ± SEM. (D) Top panel: gated CD11c$^+$ MHCII$^+$ DCs are of myeloid lineage as revealed by strong staining for CD11b (filled histogram); isotype control is indicated by the open histogram. Bottom panel: gated MHCII$^+$CD11c$^+$ DCs (filled histogram) do not express the eosinophil marker CCR3, whereas gated CD11c^{dim} granular eosinophils (open histogram) clearly do.

possibility that nonendogenous DCs still remaining in the BALF could confound the counting of DCs after the aerosol challenge period (day 11 to day 17 after injection). The absolute number of DCs was elevated about 10 times in OVA-DC–immunized mice challenged with 3 OVA aerosols ($P = .008$) and increased about 100 times after 7 OVA aerosols ($P = .016$) compared with control PBS-DC/PBS mice (Figure 2B). In addition to an absolute increase in cell number, the percentage of DCs found in BALF cells was similarly increased following 3 aerosols, although not significantly ($P = .056$), and doubled after 7 aerosols ($P = .016$). In control mice the number of DCs remained at low levels, comparable to the situation in unmanipulated mice.

To determine if the increase in the number of BALF DCs was also supported by an increase in airway mucosal DCs, we visualized the DC network in tracheal whole mounts, as previously described.[18] The pattern of the MHCII staining revealed a dendritic network in the control PBS-DC/PBS mice (Figure 3A), whereas in the OVA-DC–immunized mice exposed to 3 OVA aerosols, numerous dense areas of rounded MHCII$^+$ cells lacking the typical dendritic morphology were seen (Figure 3B). Due to the fact that MHCII$^+$ cells were rounded and could also represent MHCII$^+$ B cells or eosinophils, we could not directly compare DC numbers at the tissue level, although clearly, overall MHCII staining was enhanced.

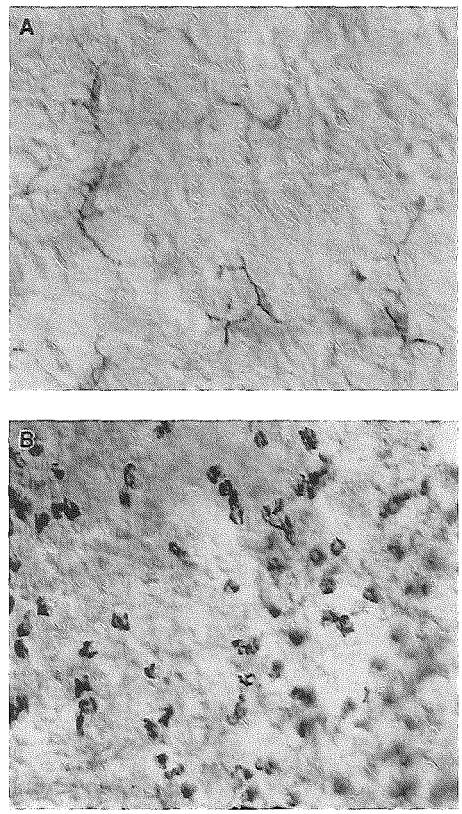

Figure 3. Effect of OVA or PBS aerosol challenge on the number of DCs in the large conducting airways. Mice were immunized with OVA-DCs or PBS-DCs and challenged daily with either OVA or PBS aerosol. Thereafter, whole mounts were prepared and stained with an Ab directed against I-A and I-E MHCII. (A) In PBS-DC/PBS-exposed animals nearly all MHCII$^+$ cells demonstrate a dendritic morphology. (B) In contrast, in OVA-DC/OVA mice the MHCII$^+$ cells are more numerous, and have a rounded appearance. They are distributed in dense clusters in the intercartilaginous area. Original magnification, × 400, Normaski optics.

OVA exposure leads to an increase in peripheral blood DCs in OVA-sensitized mice

To investigate if the accumulation of lung CD11c$^+$ CD11b$^+$ DCs was supported by recruitment from the bloodstream, the number of DCs (CD11c$^+$/MHCII$^+$/B220$^-$/CD3$^-$ cells) was determined in the blood 24 hours after the last of 3 aerosols. The percentage of MHCII$^+$ CD11c$^+$ blood DCs was significantly raised (OVA 0.81 ± 0.09% vs PBS 0.37 ± 0.03 vs naive 0.39 ± 0.03, $P < .0001$) in response to OVA challenges in OVA-DC mice (Figure 4A,C).

In the blood of the control PBS-DC/PBS–immunized mice, levels were comparable with those found in untreated animals. Additional experiments were carried out to define the CD11b$^+$ myeloid and CD11b$^-$ lymphoid population within the CD11c$^+$ MHCII$^+$ B220$^-$CD3$^-$ population of blood cells (Figure 4B-C). It appeared that the percentage of blood CD11b$^+$ myeloid DCs was significantly elevated in OVA-DC/OVA mice compared with PBS-DC/PBS mice (OVA 0.6 ± 0.066% vs PBS 0.231 ± 0.022%, $P < .0001$). In the blood of OVA-DC/OVA mice, the percentage of MHC II$^+$ CD11b$^+$ CD11c$^-$ cells (putative monocytes) was also raised significantly over the control mice (1.00 ± 0.30% vs 0.30 ± 0.03%, $P < .0001011$).

OVA exposure leads to an increase in CD31^{hi}Ly-6C^{neg} BM cells in OVA-sensitized mice

The increased presence of CD11c$^+$ DCs in the bloodstream during OVA challenge, despite the massive influx of DCs into the airways, suggested that DC output from the BM might be enhanced.

Staining of whole BM cells with the monoclonal Abs CD31 (ER-MP12) and Ly-6C (ER-MP20), gives rise to 6 distinct populations of BM cells, each with varying degrees of lineage commitment and progenitor potential (see Figure 5A).[19] This staining was used to define the cellular composition of BM in mice with or without eosinophilic airway inflammation. Performing 1, 3, or 7 OVA aerosol exposures to OVA-DC mice sequentially induced an increase in the CD31^{hi}Ly-6C^{neg} population, from 4.61 ± 0.75% of total BM at baseline up to 7.84 ± 1.25% after 3 aerosols and 11.1 ± 1.48% after 7 aerosols (Figure 5A-B). There was no increase in this population after exposure to PBS aerosols. None of the 5 other distinct populations was altered significantly by OVA aerosol in OVA-DC mice or by PBS aerosol in PBS-DC mice.

To explore whether the increase of the CD31^{hi}Ly-6C^{neg} population was due to an increase of cells with myeloid or lymphoid commitment, additional staining was performed using the myeloid differentiation marker CD11b in combination with a CD127 Ab against the IL-7Rα chain, identifying cells with lymphoid commitment.[25,26] Within the CD31^{hi}Ly-6C^{neg} subset, cells expressed CD11b or CD127 exclusively. After 4 aerosols, the percentage of CD11b$^+$ cells in this subset remained constant, whereas that of CD11b$^-$CD127$^+$ lymphoid-committed cells was 9.5 ± 1.4% in PBS-DC/PBS mice, compared with 6.1 ± 0.4% in OVA-DC/OVA mice, indicating a small but significant ($P = .004$) decrease of cells with lymphoid differentiation (potential). Cells were also stained

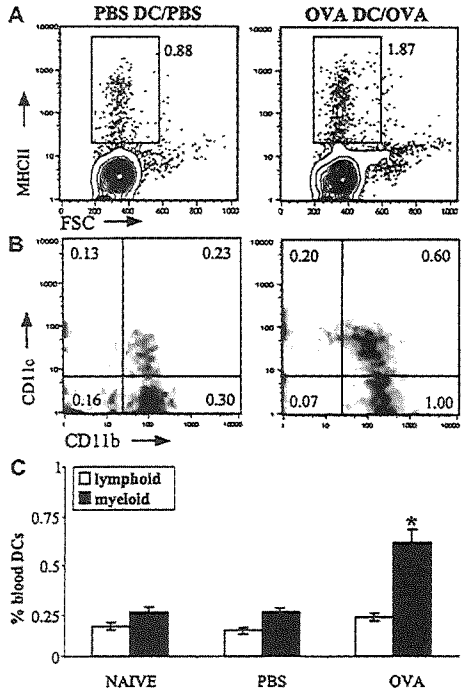

Figure 4. Effect of OVA or PBS aerosol challenge on the percentage of blood DCs. Mice were immunized with OVA-DCs or PBS-DCs and challenged daily with either OVA or PBS aerosol. (A) A gate was set on MHCII$^+$ cells within CD3$^-$B220$^-$ cells. Numbers represent the mean percentage of cells within this gate. (B) Within the MHCII$^+$ gate, CD11c$^+$ cells represent DCs. Additional staining involved CD11b to discriminate myeloid (CD11b$^+$) versus lymphoid (CD11b$^-$) DCs. Numbers indicate the mean percentage of the population as percent of total cells within lysed blood cells (n = 9 per group). (C) Quantitative summary from unmanipulated naive mice, PBS-DC/PBS animals, and OVA-DC/OVA animals (*$P < .05$ compared with the naive and PBS-DC/PBS groups). Data are expressed as mean % blood DCs ± SEM of 1 experiment and are representative of multiple experiments.

with an Ab against CD131, the common β chain of the IL-3/IL-5/GM-CSF receptor. The CD31^{hi}Ly-6C^{neg} subset had the highest expression compared with the other populations or whole BM. There was no difference in CD131 expression of the CD31^{hi}Ly-6C^{neg} subset between the PBS and the OVA exposed group.

The CD31^{hi}Ly-6C^{neg} BM subset gives rise to DCs and eosinophils under different culture conditions

As the CD31^{hi}Ly-6C^{neg} subset of cells was the only population that was increased in the BM of mice with eosinophilic airway inflammation, we examined if this subset could give rise to DCs. The CD31^{hi}Ly-6C^{neg} population was purified by flow cytometric sorting to 85% to 95% purity, and cultured in GM-CSF (40 ng/mL) (Figure 6).

After 7 days culture in the presence of GM-CSF, many colonies of proliferating cells were seen. About 77% of cells were CD11c$^+$ and more than half of these expressed MHCII, indicating maturation in culture (Figure 6C). In contrast, the CD31^{neg}Ly-6C^{med} population was sorted and cultured under the same conditions and yielded only 2.2% MHCII$^+$/CD11c$^+$cells.

Airway eosinophilia is a prominent feature of allergic airway inflammation and was also observed in our model. Therefore, we investigated whether the same CD31^{hi}Ly-6C^{neg} population contained eosinophil precursors. First, to support the concept that cells with eosinophil potential were contained within the CD31^{hi}Ly-6C^{neg} population, bone marrow subsets were stained with a monoclonal antibody against the eotaxin receptor CCR3.[24] Cells within this subset expressed CCR3 at intermediate levels (see Figure 7A). Mature granulocytes contained within the CD31^{neg}Ly-6C^{med} also contained CCR3^{hi} mature eosinophils. Next, cells in the allergen-induced enlarged CD31^{hi}Ly-6C^{neg} population were sorted

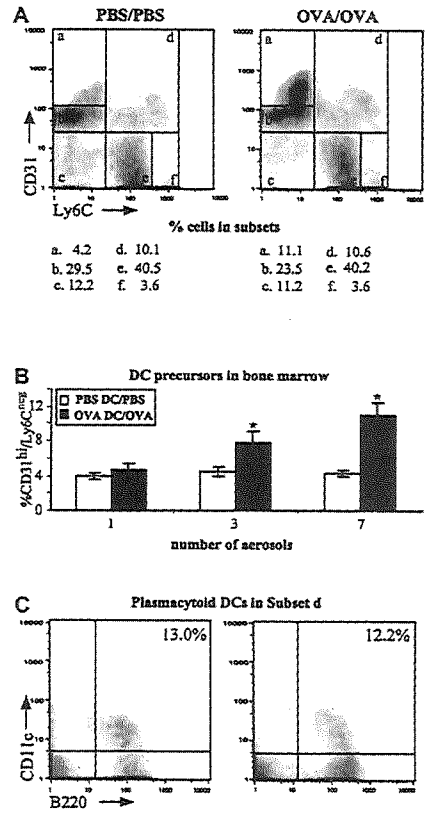

Figure 5. Effect of OVA or PBS aerosol exposure on the cellular composition of BM. OVA-DC– or PBS-DC–immunized mice were challenged with either 1 ×, 3 ×, or 7 × daily OVA or PBS aerosols. At 24 hours after the last challenge, BM was collected and stained for Ly-6C and CD31. (A) Using this combination of markers, 6 distinct populations can be identified. Morphologically, these populations consist of: (a) 70% blast cells and 25% lymphoid cells; (b) lymphoid cells; (c) erythroid cells; (d) myeloid progenitors and plasmacytoid cells; (e) granulocytes; (f) 75% monocytes and 20% myeloid progenitors.[19] Plots represent Ly-6C/CD31 staining on BM cells taken from mice exposed 7 times to PBS or OVA aerosol. There is a clear and selective increase in the CD31^{hi}Ly-6C^{neg} subset in the OVA-DC/OVA group. Percentages of each population are indicated below the FACS plots. (B) Kinetics and magnitude of increase in the CD31^{hi}Ly-6C^{neg} BM subset following OVA or PBS challenge (n = 6-8 per group per time point, * $P < .05$ compared with PBS-DC/PBS). Data are expressed as mean % DC precursors ± SEM. (C) Additional staining included CD11c and B220 to delineate plasmacytoid DCs. Plots were gated on CD31^{hi}Ly-6C^{hi} cells (population d) in PBS-DC/PBS and OVA-DC/OVA mice. Average percentage of CD11c$^+$ B220$^+$ within the gate is indicated.

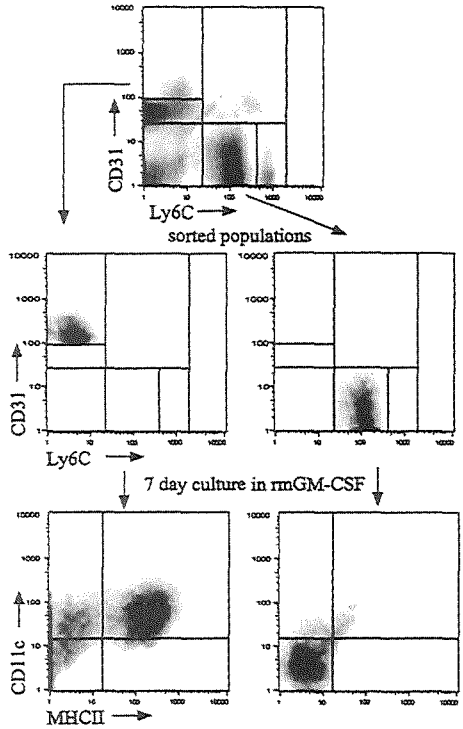

Figure 6. CD31^{hi}Ly-6C^{neg} cells give rise to DCs following culture in GM-CSF. OVA-DC– or PBS-DC–immunized mice were challenged daily for 3 days with OVA or PBS aerosols. At 24 hours after the last challenge, BM was collected and stained with CD31 and Ly-6C antibodies. Plots shown are representative of mice within the OVA-DC/OVA group. The CD31^{hi}Ly-6C^{neg} and the CD31^{neg}Ly-6C^{med} populations were purified using flow cytometric sorting (middle panels) and subsequently cultured in the presence of GM-CSF for 7 days. The lower panels represent FACS plots of cells at the end of the culture period stained for MHCII and CD11c. Immature DCs are CD11c^{+} MHCII^{-} and mature DCs are CD11c^{+} MHC II^{+}.

(about 85% to 95% pure CD31^{hi}Ly-6C^{neg} cells) and cultured in the presence of IL-5 (24 ng/mL) for 6 days (Figure 7B-C). Eosinophils were detected after 6 days by MBP staining and morphology on cytospins. The CD31^{hi}Ly-6C^{neg} subset yielded a 4-fold higher number of cells (P = .029) and a higher percentage of eosinophils compared with whole BM cultured under the same conditions (CD31^{hi}Ly-6C^{neg}: 51.2 ± 1.1% vs whole BM: 25.6 ± 2.2%, P = .029).

OVA exposure does not increase the number of plasmacytoid DCs in BM

Despite the fact that none of the other populations identified by CD31 and Ly-6C staining were percentually changed, we studied these subsets in greater detail by 4-color analysis. More specifically, the percentage of CD31^{hi}Ly-6C^{hi} cells, known to contain precursors for DCs as well as plasmacytoid DCs, was not altered by exposure to OVA.[22,27,28] The percentage of plasmacytoid CD11c^{+} B220^{+} DCs within the CD31^{hi}Ly-6C^{hi} subset was 12.2 ± 1.4% in OVA-exposed mice compared with 13.0 ± 0.8% in PBS-exposed mice after 3 aerosols (Figure 5C). However, in response to the OVA challenges, the CD31^{hi}Ly-6C^{hi} subset, expressed more CD131 compared with the control mice (data not shown).

The percentage of CD3^{+}CD4^{+} T cells (falling within the CD31^{med}Ly-6C^{neg} fraction) in total BM was significantly lower (0.16 ± 0.03%) in the OVA-DC/OVA group compared with the PBS-DC/PBS group (0.37 ± 0.07%; P = .001).

OVA exposure modifies the BALF and serum level of cytokines in OVA-sensitized mice

An increase in DCs could also be caused by increased local differentiation of DCs from monocytic precursors within inflamed tissues. To determine the presence of early growth and differentiation factors for DCs, we measured the content of the cytokines IL-6, GM-CSF, and Flt-3L in BALF 24 hours after 3 OVA aerosol exposures, when the number of DCs was significantly increased. IL-6 was significantly raised after 3 aerosols in OVA-DC/OVA mice compared with PBS-DC/PBS mice (OVA: 418.2 ± 74.6 pg/mL, PBS: 12.5 ± 1.9 pg/mL; P = .016). Ag challenge did not effect the GM-CSF level (OVA: 3.90 ± 1.50 pg/mL vs PBS: 9.45 ± 2.3 pg/mL; P = .111). The Flt-3L levels in BALF were just above the detection level of our assay, showing no detectable difference in the various groups.

In serum, levels of IL-6 (13.67 ± 3.39 pg/mL vs 2.55 ± 0.79 pg/mL; P = .016) and eotaxin (564 ± 62 pg/mL vs 282 ± 25 pg/mL; P = .008) were higher in the OVA-DC/OVA group compared with the PBS-DC/PBS group, whereas that of GM-CSF was below the detection limit.

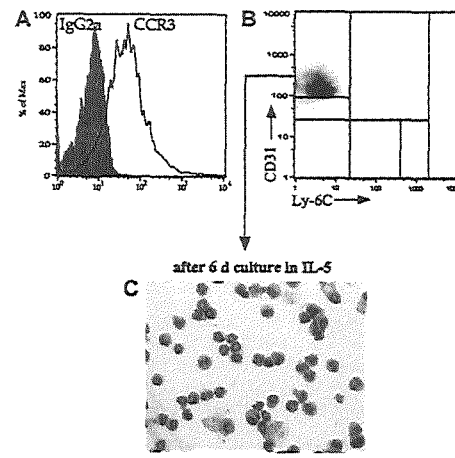

Figure 7. CD31^{hi}Ly-6C^{neg} cells give rise to eosinophils following culture in IL-5. (A) Flow cytometric staining of gated CD31^{hi}Ly-6C^{neg} cells reveals expression of the eotaxin receptor CCR3 (open histogram). Isotype control is indicated by the filled histogram. (B) CD31^{hi}Ly-6C^{neg} cells were sorted to purity using flow cytometric sorting and cultured in the presence of IL-5 for 6 days. Sorted population was obtained from bone marrow of an OVA-DC/OVA animal, exposed to 3 OVA aerosols. (C) Cultured cells have an eosinophilic cytoplasm and a donut-shaped nucleus. We next investigated whether the enhanced population of CD31^{hi}Ly-6C^{neg} cells responded differently to growth factor stimulation in mice with or without eosinophilic airway inflammation by comparing the growth of equal numbers of sorted CD31^{hi}Ly-6C^{neg} obtained from both groups. After sorting and a 7-day culture in GM-CSF there was a significant difference neither in the yield of total cells (P = .1) nor in the percentage of CD11c^{+} DCs derived from the CD31^{hi}Ly-6C^{neg} of both groups (results not shown). However, when grown in IL-5, the subset sorted from the BM of OVA-challenged animals yielded slightly more eosinophilis compared with the PBS-DC/PBS mice (51.2 ± 1.1% vs 40.5 ± 1.3%, P < .05). Cells were stained with αMBP Ab. Original magnification, × 400.

OVA exposure increases DC migration toward the draining lymph nodes in OVA-sensitized mice

In addition to the mechanisms studied above, a decreased efflux to the draining MLN could contribute to an accumulation of DCs in inflamed airways. We observed that the draining MLNs of OVA-DC/OVA mice were grossly swollen compared with nondraining nodes or MLNs of PBS-DC/PBS mice. After 3 aerosol exposures, the total number (both relative and absolute) of DCs was increased in the OVA-DC/OVA group compared with the control PBS-DC/PBS group (7.00 ± 0.53% vs 2.37 ± 0.27%; $P = .002$). This was due primarily to an increase in the CD11b$^{med/hi}$ myeloid DCs (3.5-fold increase), although the lymphoid CD11b^{-} subset was also increased following OVA challenge (Figure 8A-B). To provide further proof that migration of airway DCs was influenced by the eosinophilic airway inflammation, we injected CFSE-labeled, BM-derived, in vitro–cultured DCs intratracheally in mice with (OVA-DC/OVA group) or without (PBS-DC/PBS) eosinophilic airway inflammation. At 48 hours after injection, mice with inflamed lungs had a small, but significantly higher number of CFSE-labeled DCs in the MLNs, compared with control mice with uninflamed lungs ($9.74 \pm 1.81 \times 10^2$ vs $2.41 \pm 0.84 \times 10^2$ CFSE-labeled CD11c^{+} cells; $P = .029$; Figure 8C), demonstrating that DC efflux to the MLNs was actually enhanced in mice with eosinophilic airway inflammation. After intratracheal injection, CFSE-labeled DCs could not be detected in peripheral LN.

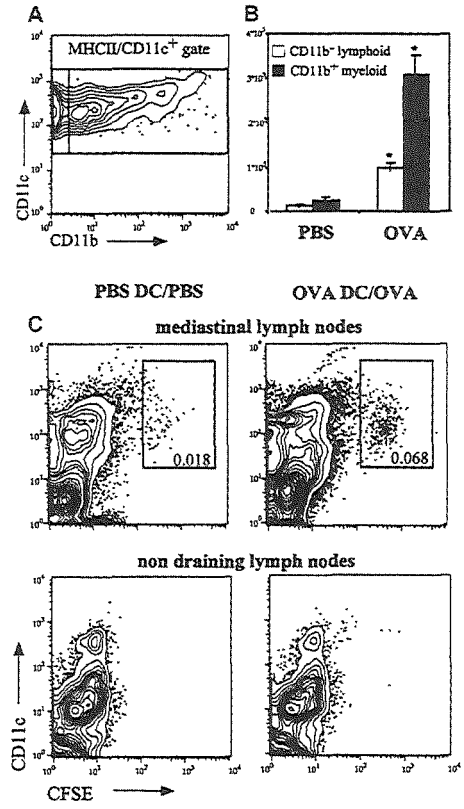

Figure 8. Effect of OVA or PBS aerosol challenge on DC subsets within the draining mediastinal LN. OVA-DC– or PBS-DC–immunized mice were challenged 3 times with either OVA or PBS aerosol. At 24 hours after the last challenge, mediastinal LN were collected, homogenized, and stained for the presence of MHCII^{+} CD11c^{+} DCs. (A) Within the MHCII^{+} population, CD11c^{+} cells were further characterized as CD11b^{+} myeloid and CD11b^{-} lymphoid DCs. (B) In OVA-DC/OVA animals, there is an absolute and relative increase in both lymphoid and myeloid DCs compared with PBS-DC/PBS mice (*$P < .05$). Data are expressed as mean number of cells ± SEM of 1 experiment representative of multiple experiments. (C) To investigate whether the observed increase in DCs in the LN was caused by enhanced migration, BM DCs were labeled with CFSE, and 2×10^6 cells were injected intratracheally into mice without (PBS) or with (OVA) eosinophilic airway inflammation, and subsequently traced in the MLN and the inguinal nondraining LN. Injected DCs can be discriminated from endogenous DCs by CFSE positivity. Numbers represent the percentage of injected DCs of total cells in the LN.

Discussion

Airway DCs have been implicated in causing the sensitization to inhaled allergens, by taking up Ag in the lung mucosa, transporting it into the draining LN and finally by inducing differentiation of Th2 effector cells that can orchestrate eosinophilic airway inflammation.[3,4,6,7] Despite these observations that airway DCs might be essential in the early steps of sensitization, less information is available on the role of DCs in stimulating memory and/or effector Th2 cells at times of repeated exposure to inhaled allergens. In this paper, we have demonstrated that the number of airway CD11c^{+} CD11b^{+} myeloid DCs is strongly increased within the airway epithelium and BALF following allergen challenge in sensitized animals. Within 3 days, an almost 10-fold expansion in the number of DCs was found following repeated OVA challenge, in parallel with an increase in CD4^{+} lymphocytes and eosinophils in the airways, reaching a 100-fold expansion at day 7. At the same time, the number of CD11c^{+} CD11b^{+} myeloid DCs in the bloodstream was increased 3-fold.

The massive increase in airway DCs and the accompanying increase in circulating blood DCs led us to investigate whether the production of DCs from the BM ("dendropoiesis") might be enhanced in mice with eosinophilic airway inflammation, to meet the enhanced demand for DCs in the inflamed lung. Numerous studies have demonstrated that the BM reacts to airway allergen challenge by increasing its output of eosinophilic precursors.[10-14] Previously, repopulation experiments in irradiated and BM-reconstituted rats have shown that airway DCs stem from a rapidly dividing precursor cell in the BM.[16] It is possible to discriminate distinct populations of BM cells using multiparameter flow cytometry.[19,22,29,30] Here, we have used the expression of Ly-6C in combination with CD31, platelet endothelial cell adhesion molecule PECAM-1, to delineate 6 discrete populations of BM cells, each with differential lineage commitment and differentiation potential.[19,22] When we stained BM cells from mice with or without eosinophilic airway inflammation, there was a striking and time-dependent increase in the population of CD31^{hi}Ly-6C^{neg} cells, whereas none of the other populations were affected by allergen challenge. In previous experiments, this population of BM cells was shown to contain cells with colony-forming unit (CFU) potential for granulocytes/monocytes, erythrocytes, megakaryocytes, and mast cells as well as cells with thymus repopulating capacity. More primitive precursors with long-term repopulating ability were found in the CD31^{med}Ly-6C^{neg} population.[29,31] In mice with eosinophilic airway inflammation, CD31^{hi}Ly-6C^{neg} cells predominantly expressed the myeloid marker CD11b, and the expression of the lymphoid

differentiation marker IL-7Rα (CD127) was slightly, but significantly, decreased, suggesting an expansion of cells with myeloid potential. When CD31^{hi}Ly-6C^{neg} cells were sorted and subsequently grown in the DC-growth factor GM-CSF, clusters were formed in liquid culture and a majority of the outgrowth of these colonies were myeloid CD11b$^+$CD11c$^+$ cells with a dendritic morphology (data not shown). In contrast, when CD31^{neg}Ly-6C^{med} cells were grown under the same conditions, no colonies were formed and hardly any CD11c$^+$ cells grew out of the cultures. When the CD31^{hi}Ly-6C^{neg} DC-precursor population was sorted from both groups of mice, and grown under the same plating density and GM-CSF concentration, there was no difference in the amount or percentage of DCs that grew from these cultures. Therefore, sensitivity to GM-CSF, either by enhanced expression of the GM-CSF receptor or by modified postreceptor events, was not enhanced in mice with eosinophilic airway inflammation. In support of this, the expression of the common β chain of the IL-3/IL-5/GM-CSF on CD31^{hi}Ly-6C^{neg} cells was not different between mice with or without eosinophilic airway inflammation.

The fact that a time-dependent increase in CD31^{hi}Ly-6C^{neg} cells with DC potential was observed in mice with massive accumulation of CD11b$^+$ CD11c$^+$ airway and blood DCs strongly suggests that the BM increased its production of DCs to meet the enhanced demand in the airways. Moreover, in mice with airway inflammation, there was no increase in the CD31^{hi}Ly-6C^{hi} population, known to contain the plasmacytoid B220$^+$ CD11c$^+$ DCs and some precursors of myeloid DCs.[22] The fact that we did not see an increase in any of these more mature DC populations following allergen challenge suggests that DC precursors leave the BM at an early stage of differentiation. One possibility that needs further investigation is that they were attracted to the airways through the action of particular chemokines. Allergic inflammation is accompanied by enhanced production of eotaxin in the airways.[32] The subset of CD31^{hi}Ly-6C$^-$ BM precursors expressed the eotaxin receptor CCR3, suggesting that these cells might be attracted into inflamed airways.

The observed increase in CD31^{hi}Ly-6C^{neg} cells in BM, without any other increase in BM populations is unique for eosinophilic airway inflammation. Bacterial infection with *Listeria monocytogenes* leads to profound changes in BM composition with a predominant time-dependent increase in CD31^{hi}Ly-6C^{hi} monocyte precursors, CD31^{neg}Ly-6C^{hi} mature monocytes, and CD31^{neg}Ly-6C^{med} granulocytes. At the same time, there was a depletion of CD31^{med}Ly-6C^{neg} lymphoid cells and more importantly, the CD31^{hi}Ly-6C^{neg} population described in this study.[19] The changes in the BM of *Listeria*-infected mice reflect an increased need for granulocytes and monocytes, which are attracted to lesions consisting of mononuclear- and neutrophil-rich cell infiltrates. However, in our model of OVA-induced airway inflammation, eosinophils are strongly recruited to the airways of challenged mice together with DCs. Not surprisingly, we were able to demonstrate that the CD31^{hi}Ly-6C^{neg} population of BM cells could also differentiate into MBP-positive eosinophils after culture in IL-5, suggesting that enhanced production of eosinophils from the BM was induced to meet the increased demand for eosinophils. This is in line with previous experiments in which mouse BM cells were cultured in the presence of IL-5 in semisolid media to asses eosinophil–colony-forming unit (CFU-Eo) potential and showing increased CFU-Eo in mice with eosinophilic airway inflammation.[11-14]

The increase in CFU-Eo in challenged mice has been attributed either to the presence of a serum factor distinct from IL-5, such as eotaxin, or to migration of IL-5–producing T cells to the BM.[13,14,33] In support of a serum factor in our system, we measured a slightly enhanced level of the DC growth factor IL-6.[34] One likely candidate that could be involved in the up-regulation of GM-CSF–responsive BM cells is eotaxin,[33] of which the serum levels were indeed doubled in mice with inflammation. Further, the CD31^{hi}Ly-6C^{neg} cells expressed the eotaxin receptor CCR3, with identical levels in allergic and nonallergic mice (data not shown). The migration of "dendropoiesis"-promoting T cells to the BM is less likely as we measured a decrease in BM CD4$^+$ T cells following allergen challenge, probably due to their migration to the lung.

Although the direct recruitment of immature blood DCs is the most likely explanation for the observed accumulation of lung DCs, one contributing mechanism could also be enhanced local proliferation of DCs from monocytic precursors in allergic lung. Although it was shown in irradiation experiments that there is little if any local self-renewal capacity for DCs in rat airways, this situation could be different under inflammatory conditions.[16] Indeed, in our experiments, there was an increase in circulating CD11c$^-$CD11b$^+$MHCII$^+$ monocytes that could be recruited to the airways and further differentiate to DCs.[15] However, when we measured the concentration of the DC differentiation factors GM-CSF and Flt-3L in the airways of allergic mice, we could not detect enhanced production.[34,35] One factor that enhances differentiation of mouse BM–derived DCs that was enhanced in BALF was IL-6.[34] It was recently shown that enhanced expression of IL-6 in the lungs of mice receiving IL-13 and interferon-γ correlates with enhanced numbers of CD11c$^+$ DCs being found in the BALF.[36] Definite proof whether locally produced IL-6, GM-CSF, or Flt-3L functionally contributes to enhanced differentiation of monocytes (precursors) to DCs in mice with airway eosinophilia awaits studies using neutralizing antibodies.

One final mechanism that could be responsible for the observed change in airway DCs in mice with airway eosinophilia would be reduced emigration of DCs. There is continuous and high throughput migration of airway DCs from the epithelium to the draining MLN and a small decrease of DC efflux could lead to rapid and profound accumulation within the epithelium.[3,5,16] To our surprise, we found that the numbers of CD11c$^+$CD11b$^+$ myeloid and CD11c$^+$CD11b$^-$ lymphoid DCs were increased strongly in the MLN of OVA-challenged mice. We believe that the increase in LN DCs was partly due to enhanced migration of lung-derived DCs into the MLN. In support of this, CFSE$^+$ CCR-7$^+$ BM DCs migrated 3 times more efficiently toward the MLNs following injection into the airways of mice with established airway eosinophilia. Reduced migration of DCs to the MLN is therefore not a mechanism that contributes to accumulation of airway DCs in our model. Enhanced migration of Ag-laden DCs to the MLN during a secondary response to inhaled Ag could prove to be necessary to stimulate the recirculating pool of nonpolarized CCR7$^+$ central memory T cells, which recirculate through the T-cell area of LN and spleen and fail to migrate into peripheral tissues.[37,38] We have shown that a proportion of divided Ag-reactive T cells remain in a nonpolarized, nonrecirculating state in the MLN after immunization with DCs.[4] It is possible that migratory DCs recruit these sessile partially activated cells into further cell division during renewed encounters with inhaled Ag.[38]

In summary, our data show that CD11b$^+$CD11c$^+$ DCs are massively attracted into the airways and draining LN upon OVA challenge in sensitized mice, a process supported by increased dendropoiesis in the BM. We have previously shown that systemic abolition of DCs in sensitized thymidine kinase transgenic mice immediately prior to secondary challenge completely suppresses eosinophilic airway inflammation, goblet cell hyperplasia, and IgE

synthesis.[18] Together, these data imply an important functional role for airway DCs not only in the induction of Th2 cells from naive precursors, but also in the maintenance of eosinophilic airway inflammation. Inhibiting the influx of DCs could prove to be a strategy for reducing airway inflammation that is typical of asthma.[8]

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Chapter 5

In vivo depletion of lung CD11c^{+} dendritic cells abrogates the cardinal features of asthma

Submitted

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Abstract

Although dendritic cells (DC) play an important role in sensitization to inhaled allergens, the exact role of these cells in ongoing Th2-mediated eosinophilic airway inflammation underlying bronchial asthma is currently unknown. Here, we show in an ovalbumin-driven mouse model of asthma that DCs interact with $CD4^+$ T cells in zones of eosinophilic inflammation around bronchi and blood vessels. Administration of diphtheria toxin (DT) to the airways of CD11c-DT receptor transgenic mice, conditionally depleted $CD11c^+$ lung DCs. When lung DCs were depleted during OVA challenge in OVA-sensitized mice, all cardinal features of asthma such as eosinophilic airway inflammation, goblet cell hyperplasia and bronchial hyperreactivity were inhibited. In the absence of DCs, endogenous $CD4^+$ Th2 cells or adoptively transferred Th2 cells did not produce the Th2 effector cytokines IL-4, IL-5 and IL-13 in response to OVA challenge. Finally, by administering DCs to the airways of sensitized mice we show that DCs are sufficient to induce the full asthmatic phenotype. These observations identify lung DCs as key proinflammatory cells that are necessary and sufficient for T cell stimulation during ongoing airway inflammation. Interfering with the function of lung DCs could be a novel strategy to treat established asthma.

Introduction

Allergic asthma is a chronic inflammatory disease of the airways associated with a predominant Th2 response to inhaled allergens leading to airway infiltration by eosinophils and mast cells, goblet cell hyperplasia, and bronchial hyperreactivity[1]. Despite significant progress in our understanding of disease pathophysiology and ways to treat the disease, prevalence continues to rise, particularly in western societies. As effector Th2 lymphocytes are intimately involved in controlling the various aspects of the disease, elucidating the mechanisms by which these cells are activated in response to allergen challenge should lead to novel ways of treatment[2].

Dendritic cells (DCs) are important antigen presenting cells in the immune system[3]. DCs are mainly recognized for their extraordinary capacity to induce primary immune responses to pathogens, allergens, alloantigens and self antigens through their potential to process antigen onto MHC molecules and provision of costimulation to naïve T cells[4]. In the lungs, airway DCs form a network in the bronchial epithelium, capture inhaled antigen (Ag) and migrate to the mediastinal lymph nodes (MLN) where Ag is presented to recirculating naive $CD4^+$ and $CD8^+$ T cells[5-9]. Not surprisingly, antigen presentation by airway DCs is at the basis of the Th2 sensitization process that occurs in patients with allergy and animals exposed to ovalbumin (OVA) antigen[10-15]. Despite these known functions of DCs on primary immune responses and allergic sensitization, it is less clear if airway DCs are also necessary for the presentation of allergen to resting memory Th2 and/or effector Th2 cells during a secondary immune response. In favor of DCs, it is known both in humans with asthma[16, 17] and from murine asthma models[18], that the number and activation state of lung DCs is elevated during secondary immune challenge with allergens. This suggests that DCs are functionally involved in presenting allergens to T cells and thus control airway inflammation[10, 12, 19, 20]. Against a role for DCs, argues the notion that previously primed T cells might need less costimulation in comparison to naïve T cells, suggesting that any peripheral APC expressing MHCII could potentially stimulate effector Th2 cells[21-23].

To examine more clearly the need for lung DCs in the induction and maintenance of effector Th2 responses to allergen challenge, we used CD11c-Diphteria Toxin Receptor transgenic mice[24](CD11c-DTR Tg), which allow the inducible, short term ablation of lung

$CD11c^+$ DCs through airway administration of diphtheria toxin (DT). Our data show that in the absence of DCs, allergen challenge fails to elicit effector cytokine release by adoptively transferred primed Th2 cells or primed Th2 cells in fully sensitized mice. DC depletion during challenge abolished the cardinal features of asthma such as eosinophilic inflammation, goblet cell hyperplasia and bronchial hyperreactivity. Supporting a crucial role for lung DCs in causing airway pathology, we furthermore demonstrate the intrinsic capacity of DCs to induce an eosinophilic airway inflammation upon transfer to the lung of sensitized mice. These studies indicate that lung DCs are necessary and sufficient antigen presenting cells for primed Th2 cells involved in the development and maintenance of asthmatic airway inflammation.

Materials and Methods

Animals

Wild type Balb/c, NOD-SCID Balb/c (Harlan, Zeist, the Netherlands) and CD11c-DTR transgenic Balb/c mice were housed under specific pathogen-free conditions at the animal care facility of Erasmus MC and experiments were approved by the ethical committee of animal experiments. The generation and screening of CD11c-DTR transgenic mice has previously been reported[24].

Depletion of lung DCs in CD11c-DTR transgenic mice by treatment with diphtheria toxin

We have previously reported on the selective depletion of $CD11c^+$ DCs in mice carrying transgenic expression of the monkey diphtheria toxin receptor (DTR) under control of the murine CD11c promoter[24]. In this study, the mice were treated intratracheally with 50 ng DT (Calbiochem, La Jolla, CA) in 80 µl of PBS and numbers of $CD11c^+$ $MHCII^+$ DCs were measured using flow cytometry in homogenized lung draining LN, spleen and in BALF at 48 h after DT injection. LN and spleen were homogenized following collagenase and DNAse I treatment as described[7].

Culture and antigen pulsing of bone marrow DCs

Bone marrow cells were collected from naïve mice, depleted of red blood cells using ammonium chloride and grown in RPMI 1640 culture medium containing 5% fetal calf serum (FCS, Sigma) and rmGM-CSF (kindly provided by Dr K. Thielemans, Free University

Brussels, Belgium) for 9 days as described[25]. At day 9, they were pulsed *in vitro* overnight with 100 μg/ml OVA (Ovalbumin, Worthington Biochemical Corp., Lakewood, NJ; LPS contamination <29 EU/mg LPS by LAL assay) (OVA-DC) or sham-pulsed with PBS (PBS-DC).

Murine model of asthma using dendritic cells

To induce sensitization to inhaled OVA, $1x10^6$ BM-derived OVA-DCs or control PBS-DCs were injected intratracheally into the airways of naive anaesthetized mice, as previously described[8]. Ten days after i.t. immunization, mice were challenged with OVA aerosol (1% w/vol in PBS, Sigma) during a daily 30 min challenge on 3-6 consecutive days. To address their functional role in generating airway inflammation, $CD11c^+$ DCs were depleted before or during OVA challenge.

In a first series of experiments, CD11c-DTR Tg mice and non-transgenic littermates were sensitized to OVA at day 0. At day 10, all mice received an i.t injection of 50 ng DT to deplete DCs, and were subsequently challenged with 3 daily OVA or control PBS aerosols from day 11-13. Mice were sacrificed at day 14.

In another series of experiments to address the role of DCs in already established inflammation, CD11c-DTR tg mice and non-transgenic littermates were sensitized to OVA at d0 and subsequently challenged with 3 OVA aerosols on day 10-12. On day 13, all mice received an i.t. injection of 50 ng DT. On day 14-16 mice were further aerosolized with OVA or as a control with PBS. Mice were sacrificed at day 17.

As CD11c is also expressed on alveolar macrophages and activated $CD8^+$ cells and weakly on eosinophils[7,18,24], we performed adoptive transfer reconstitution experiments with wild type cells at the time of DT treatment in CD11c-DTR Tg mice. Alveolar macrophages were obtained from pooled BAL fluid of naïve Balb/c mice, eosinophils were sorted from pooled BAL fluid of OVA-exposed Balb/c mice as described[26] and $CD8^+$ cells were purified using negative depletion beads from the spleen and lymph nodes of naive BALB/c mice ($CD8\alpha^+$T cell Isolation kit, Miltenyi Biotec (Sanquin, Amsterdam, The Netherlands).

Asthma model using only adoptive transfer of DCs

In separate experiments, OVA aerosol challenge was compared with challenge with OVA-pulsed DCs to test the intrinsic capacity of DCs to induce airway inflammation. For this

purpose, mice were first sensitized to OVA by an intratracheal injection of OVA-pulsed DCs. Subsequently, they received two intratracheal injections of $1x10^6$ OVA-DCs at day 10 and day 12. Alternatively, mice were aerosolized with OVA at day 10-13. Animals were sacrificed 24 h after the last aerosol or 48 h after the last DC injection. To address the role of T and B cells in mice receiving multiple DC injections, experiments were repeated in NOD-SCID Balb/c mice.

Adoptive transfer of OVA-specific Th2 cells

In some experiments, naïve mice received adoptive transfer of *in vitro* differentiated Th2 cells obtained from DO11.10 OVA-TCR transgenic mice. In brief, DO11.10 LN cells were stimulated with OVA for 6 days in the presence of 10 ng/ml IL-4, 10 μg/ml anti-IFNγ, 1 μg/ml anti-IL-12 and 5 μg/ml OVA peptide. Th2 differentiation was confirmed by quantitative PCR through a 360-fold increase in mRNA for IL-4, 892-fold increase in IL-5, 608-fold in IL-13 while mRNA for IFNγ was unaffected, compared with unstimulated DO11.10 T cells. After 6 days, cells were washed and $3x10^6$ were injected i.v. into naïve CD11c-DTR Tg mice treated or not with DT to deplete DCs. Mice were challenged with daily OVA aerosols for the subsequent 4 days. Th2 cytokine production by lung draining lymph nodes was determined 24h after the last aerosol challenge.

Collection and analysis of cells and tissues

Bronchoalveolar Lavage Fluid (BALF): Twenty-four hour after the last aerosol, BAL was performed with 3x 1 ml of Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1 mM EDTA. After red blood cell lysis using ammonium chloride lysis buffer, cells were stained with MHCII-FITC (2G9), CD11c-APC (HL3), CCR3-PE (R&D systems, Abingdon, UK), CD3-CyChr (145-2C11) and B220-CyChr (RA3-6B2). 2.4.G2 was used to prevent non-specific binding. The cellular composition of BALF cells was determined using flowcytometry as described[26] on a FACScalibur flow cytometer using CellQuest (Becton and Dickinson Immunocytometry Systems, San Jose, CA) and FlowJo software (Treestar, Costa Mesa, CA).

Lymph nodes: LN cell suspensions were plated in 96 well round bottom plates at a density of $2x10^5$ cells per well and were restimulated for 4 days with 10 μg/ml OVA. After 4 days, supernatants were harvested and stored at -20°C until quantification of IL-4, IL-5, IFN-γ

(OptEIA, PharMingen, Becton Dickinson, San Diego, CA) and IL-13 (R&D systems, Abingdon, UK) by ELISA.

Airway histology: After BAL was performed, 1 ml of OCT (1/1 vol/vol in PBS) was gently infused through the lavage needle. After lungs were snap-frozen in liquid nitrogen, they were stored at –80°C before 6 µm sections were cut and stained with Hematoxylin-Eosin or with Periodic Acid Schiff's Reagent (Sigma) and examined by light microscopy for histological changes. For immunohistochemical identification of DCs and T cells, lung sections were fixed in acetone and endogenic peroxidase was blocked with 0.1%SoAz/0.01%H2O2 in PBS for 30', blocked with NGS and NRS, incubated with hamster anti-CD11c followed by goat anti-Armenian Hamster-PO (Jackson Immunoresearch, West Grove, PA, USA), to stain for CD4, sections were incubated with rat anti mouse CD4-FITC (Pharmingen), followed by rabbit anti-FITC-AP (DAKO (ITK, Uithoorn, The Netherlands). Signal was developed with respectively AEC and Fast Blue.

Measurement of airway hyperreactivity using body plethysmography

AHR responses were assessed by metacholine-induced airflow obstruction in conscious mice placed in a single chamber whole-body plethysmograph (Emka Technologies, Paris, France) as described previously[27]. Twenty four hour after the last aerosol challenge, non-specific responsiveness was measured by exposing mice to aerosolized PBS, to set a baseline value, followed by increasing concentrations of aerosolized metacholine (Sigma; 1.5625, 3.125, 6.25, 12.5 and 25 mg/ml in PBS for 3 min) using ultrasonic nebulizers. PenH (enhanced pause) values were measured during 3 minutes following each metacholine aerosol. The average PenH values were expressed for each MCh concentration as the percentage increase over baseline PenH values measured after PBS exposure.

Statistical analysis

All experiments were performed using 5-10 mice per group. Comparison of means between different groups was performed with a Kruskal-Wallis test for equality and in case of a significant difference the Mann-Whitney U test for unpaired data was used for comparing two groups (SPSS 11.0 for Windows) separately. Differences were considered significant if $P < 0.05$. To compare histology sections, we ordered sections based on presence of goblet cell hyperplasia, perivascular and peribronchial eosinophilic infiltrates and parenchymal

inflammatory cells. By assigning each section a number, starting with the least inflamed section assigning 1, the scores per group were compared with a Kruskal-Wallis and in case of significance with Mann-Whitney U.

Results

Dendritic cells colocalize with T cells in peribronchial eosinophilic inflammation

We have previously reported in a mouse model of asthma that the number of DCs increases up to 100 fold in the bronchoalveolar lavage (BAL) fluid following OVA challenge in OVA-sensitized mice, but have not studied the functional implications of this increase[18]. To determine whether DCs interact locally with $CD4^+$ T cells at the site of inflammation, we stained cryosections of lungs of OVA-sensitized and challenged mice for the presence of $CD11c^+$ DCs and $CD4^+$ T cells. Histological analysis revealed clusters of T cells and DCs in eosinophil rich peribronchial and perivascular infiltrates (Fig 1A) and in the surrounding parenchyma. A larger magnification showed that multiple large $CD11c^+$ cells were also present in the alveolar compartment, frequently surrounded by small $CD4^+$ cells (Fig 1B).

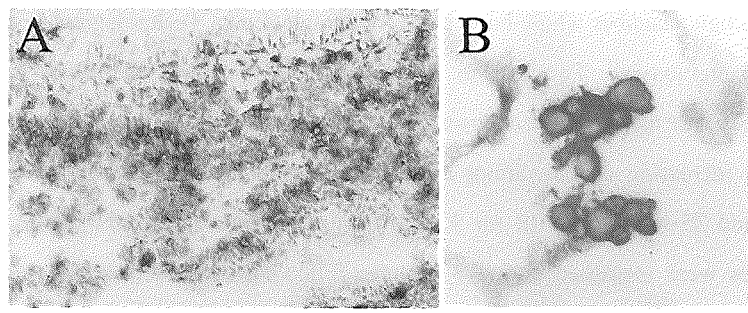

Figure 1. Interaction of CD11c+ DCs and CD4+ T cells in peribronchial inflammatory infiltrate. *Balb/c mice were OVA-sensitized and challenged to induce an eosinophilic airway inflammation. (A) 1 day after the last aerosol, peribronchial infiltrates were analyzed for the presence of $CD4^+$ T cells (blue) and $CD11c^+$ DCs (red) in frozen lung sections. (B) At a larger magnification, it is shown that DCs and T cells are interacting in the peribronchial infiltrates as well as in the lung parenchyma.*

Intratracheal injection with Diphtheria Toxin depletes airway DCs in CD11c-DTR Tg mice

The close interaction between DCs and $CD4^+$ T cells within areas of inflammation suggested that DCs were necessary for stimulating antigen-specific primed T cells. To investigate the

functional role of this interaction between DCs and T cells, we used CD11c-DTR Tg Balb/c mice in which administration of DT allows a conditional ablation of $CD11c^+$ cells[24]. CD11c is highly expressed on DCs in the mouse, as well as on alveolar macrophages and some activated $CD8^+$ T cells. FACS analysis revealed that intratracheal (i.t.) injection of 50 ng DT in naïve CD11c-DTR Tg mice depleted local $CD11c^+$ $MHCII^+$ DCs in BALF, lung tissue (data not shown) and mediastinal LNs but also in spleen (Fig 2A, B, C). DT also depleted $CD11c^+$, $MHCII^{int}$ highly autofluorescent alveolar macrophages in naïve mice (Fig 2A). Next, we determined immunohistologically on lung sections of OVA-sensitized and -challenged mice that i.t. injection of 50 ng DT during an ongoing inflammation, depleted DCs in CD11c-DTR Tg mice (Fig 2D) but not in non-transgenic (WT) littermates (Fig 2E).

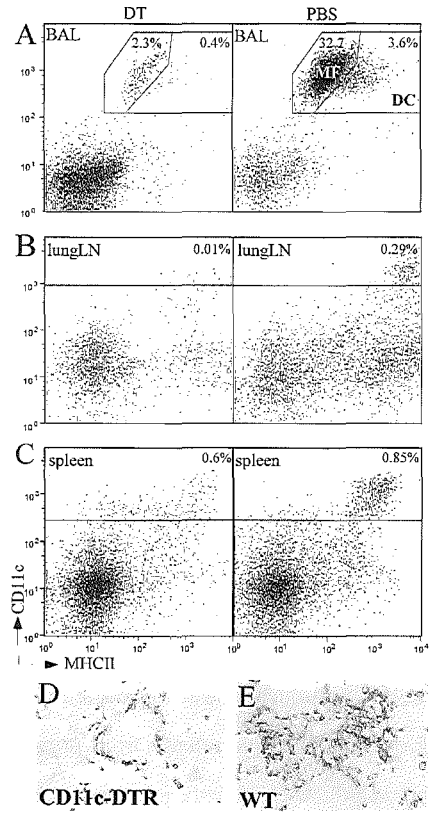

Figure 2. DT treatment depletes $CD11c^+$DCs in CD11c-DTR Tg mice. *Naïve CD11c-DTR Tg mice were i.t. injected with 50 ng DT to deplete DCs or only PBS (0 ng DT). (A,B,C) 48 h after instillation, BALF cells (A), lung draining lymph nodes (B) and spleen (C) were analyzed for the presence of $CD11c^+MHCII^+$ DCs by FACS. (D,E) OVA-sensitized mice received 3 OVA aerosols and were then treated with DT and subsequently challenged with OVA aerosols for three more days. Lung sections stained for $CD4^+$ (blue) and $CD11c^+$(red) cells showed that i.t. administration of 50 ng DT in mice with an ongoing airway inflammation depleted $CD11c^+$ cells in the peribronchial infiltrates of CD11c-DTR Tg mice (D), while in non-transgenic mice (E) DT did not effect $CD11c^+$ DCs.*

Conditional depletion of airway DCs in sensitized animals inhibits the immune response to allergen challenge

Having established this model of conditional DC depletion, we studied the response to OVA challenge in OVA-sensitized mice in the presence or absence of DCs. CD11c-DTR Tg and WT mice were sensitized to OVA on day 0 by giving an i.t. injection of OVA-pulsed bone marrow-derived DCs. At day 10, both groups of mice were injected i.t with 50 ng DT, and were subsequently challenged with 3 daily OVA aerosols from day 11-13. Analysis of BALF cells of mice that were depleted of DCs (CD11c-DTR Tg mice treated with DT will be referred to as Tg DC^-) before challenge with OVA aerosols revealed a significantly lower number of total BALF cells and eosinophils compared with mice, which were not DC-depleted (Non-Transgenic littermates treated with DT are referred to as nTg DC^+)(Fig 3A,B). In concordance, histological analysis of lung sections of Tg DC^- mice did not show peribronchial and perivascular inflammatory infiltrates as seen in nTg DC^+ mice (Data not shown). To measure the induction of bronchial hyperresponsiveness, mice were exposed to increasing doses of metacholine. After OVA challenge, nTg DC^+ mice had an increase in PenH over baseline after exposure to 6.25, 12.5 and 25 mg/ml metacholine compared with nTg DC^+ mice challenged with PBS aerosol challenge, illustrating the induction of AHR by OVA challenge in OVA-sensitized mice. In contrast, after OVA challenge, Tg DC^- mice had an AHR curve that followed the curve seen in Tg DC^- and nTg DC^+ mice that were OVA-sensitized but were challenged with PBS (Fig 3C).

Next, we determined whether the absence of these cardinal features of asthma in OVA-sensitized and challenged animals which were depleted of DCs was associated with a lack to generate effector function in primed Th2 cells. Indeed, *in vitro* restimulation of lung draining lymph node cells with OVA showed that DC depletion before allergen challenge greatly diminished the IL-4, IL-5 and IL-13 production, while IFNγ production was unaffected (Fig 3D). Supporting an essential role for DCs in eliciting effector cytokine release from primed Th2 cells, we performed adoptive transfer experiments in which previously primed *in vitro* generated OVA-specific Th2 cells from D011.10 mice were injected intravenously into naïve Tg DC^- (CD11c-DTR Tg treated with DT simultaneously with Th2 transfer) and Tg DC^+ (CD11c-DTR Tg treated with PBS simultaneously with Th2 transfer) and nTg DC^+ mice

(non-transgenic treated with DT simultaneously with Th2 transfer). As described, aerosol OVA challenge leads to Th2 cytokine production by these adoptively transferred Th2 cells in mice with DCs. However cytokine production was impaired in recipient mice that were depleted of DCs (Fig 3E).

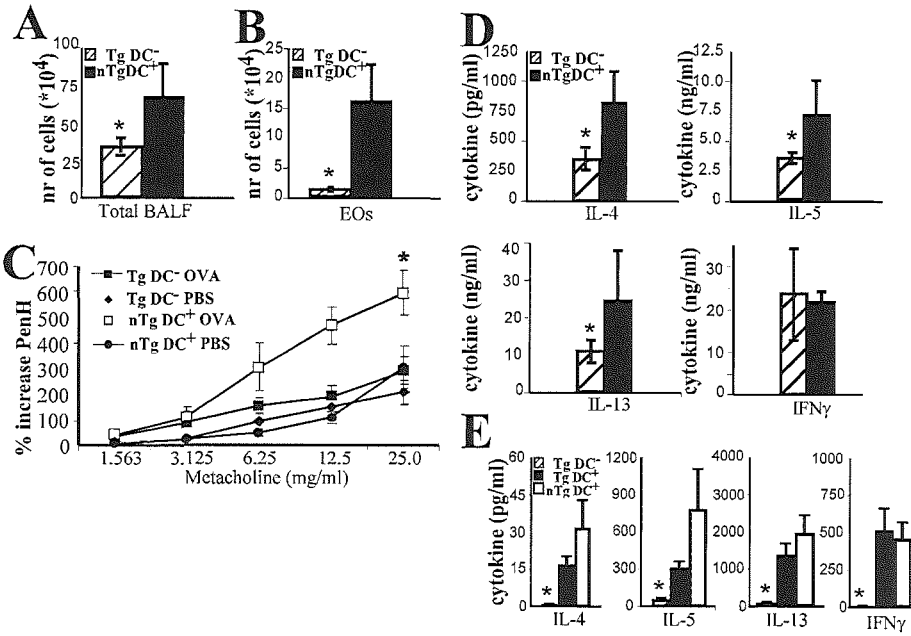

Figure 3. DC requirement for induction of secondary immune responses. *Mice were OVA-sensitized with OVA at day 0, received a DT i.t. injection at day 10 and received a daily 30 min aerosol at day 11,12,13. At day 14, mice were sacrificed. (A) BALF cells were analyzed for total BALF cell numbers and (B) the number of eosinophils 1 day after the last aerosol. (C) AHR was measured in CD11c-DTR Tg mice and nTg littermates that were sensitized with OVA (alum) and challenged with 3 OVA aerosols or as a control with PBS aerosols. 24 hours before the first OVA aerosol all mice were treated with DT. Tg DC⁻/OVA: OVA-challenged CD11c-DTR tg; nTg DC⁺/OVA: OVA-challenged nTg mice; Tg DC⁻/PBS, PBS-challenged CD11c-DTR Tg; nTg DC⁺/PBS: PBS-challenged non-tg CD11c-DTR.(D) Th2 cytokine production by lung draining lymph nodes in Tg DC⁻ and nTg DC⁺ mice. (E) A cohort of in vitro obtained memory Th2 were adoptively transferred (i.v.) into CD11c-DTR (Tg DC⁻) and non-Tg mice (nTg DC⁺), simultaneously with an i.t. injection of DT. As an additional control, one group of CD11c-DTR Tg mice was treated with PBS instead of DT (Tg DC⁺) before adoptive transfer. 24 hours after the adoptive transfer, mice were challenged with OVA aerosols for four days. Lung draining LN were restimulated ex vivo with OVA and IL-4, IL-5 IL-13 and IFNγ levels were determined in supernatant by ELISA. (*p<0.05 vs Tg DC$^{+/+}$ and nTg DC$^{+/+}$)*

The failure of CD11c-DTR mice to develop eosinophilia or BHR was not due to an intrinsic failure in Th2 responsiveness in these mice as OVA-sensitized and challenged CD11c-DTR Tg mice treated with PBS instead of DT before challenge developed similar airway

eosinophilia (%BALF eosinophils of total; 25.0±6.6% vs 27.9±3.2%), AHR and Th2 cytokine production compared with non-transgenic littermates (data not shown).

Because CD11c is also expressed by alveolar macrophages and weakly by activated $CD8^+$ T cells and eosinophils, we performed adoptive transfer reconstitution experiments in which DT-treated CD11c-DTR Tg mice received intratracheally $2x10^5$ alveolar macrophages, $1x10^6$ eosinophils or intravenously $7.5x10^6$ $CD8^+$ T cells. These reconstitution experiments were not able to restore the response to OVA-challenge in Tg DC^-, illustrating that depletion of these cell types was not causing the absence of inflammation in DT treated mice (Fig 4).

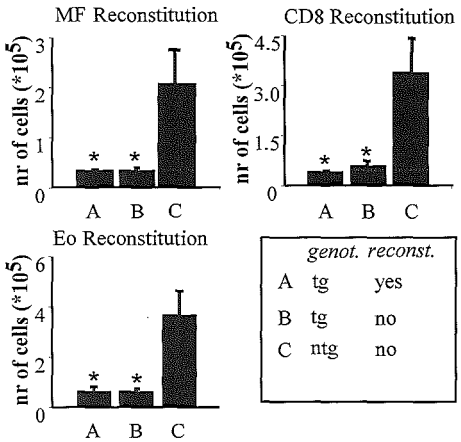

Figure 4. Reconstitution with Macrophages, $CD8^+$ cells or Eosinophils does not restore inflammation
All mice were sensitized with OVA pulsed DCs and ten days later challenged with a daily OVA aerosol for three consecutive days. One day before the first OVA aerosol, all mice were treated with DT. DT treated CD11c-DTR transgenic mice (group A) were reconstituted with Macrophages, $CD8^+$ cells or eosinophils. Eosinophil counts in BAL of this group was compared to transgenic mice (group B) and nontransgenic littermates (group C) treated with DT, which did not receive a cohort of cells.($p<0.05$ vs nTg mice treated with DT)*

DC depletion during ongoing Th2 inflammation diminishes cardinal features of asthma

Allergen induced features of asthma were almost completely abolished when lung DCs were depleted immediately before challenge, illustrating that lung DCs are necessary for generating effector function in previously primed T cells. Next, we determined the role of lung DCs during an ongoing inflammation in which effector Th2 cells are already present in the lung and eosinophilia is established. CD11c-DTR Tg or non-Tg mice were sensitized to OVA and challenged at day 10-12 with a daily OVA aerosol[8]. On day 13, when both groups had an eosinophilic airway inflammation, DT was given intratracheally to both groups to generate nTg DC^+ and Tg DC^- mice. Mice were subsequently challenged at day 14-16 with a daily OVA aerosol. One additional group of CD11c-DTR Tg mice received PBS aerosols at day 14-16 after DC depletion (referred to as Tg DC^-/PBS) to determine the resolution of airway

inflammation when allergen challenge was stopped. BALF analysis revealed that in Tg DC$^-$/OVA mice a lower number of total BALF cells and eosinophils were recovered compared with nTg DC$^+$/OVA mice (Fig 5A,B: 0.18 x10^6 vs 1.0x10^6 eosinophils). The recovered number of total BALF cells and eosinophils in Tg DC$^-$/OVA mice was similar to the numbers recovered in Tg DC$^-$/PBS, suggesting that OVA was not presented by other APCs. In support, IL-4, IL-5 and IL-13 cytokine production by lung draining LNs of Tg DC$^-$/OVA mice was decreased compared with nTg DC$^+$ littermates. Also LN of TgDC$^-$/PBS-challenged mice produced lower, although not significantly, levels of IL-4, IL-5 and IL-13 (Fig 5C). Histological analysis revealed significantly less inflammatory peribronchial and perivascular infiltrates (Fig 5D : $P \leq 0.032$) and goblet cell hyperplasia in Tg DC$^-$/OVA mice (Fig 5D) and Tg DC$^-$/PBS (Fig 5E) compared with nTg DC$^+$/OVA mice (Fig 5F).

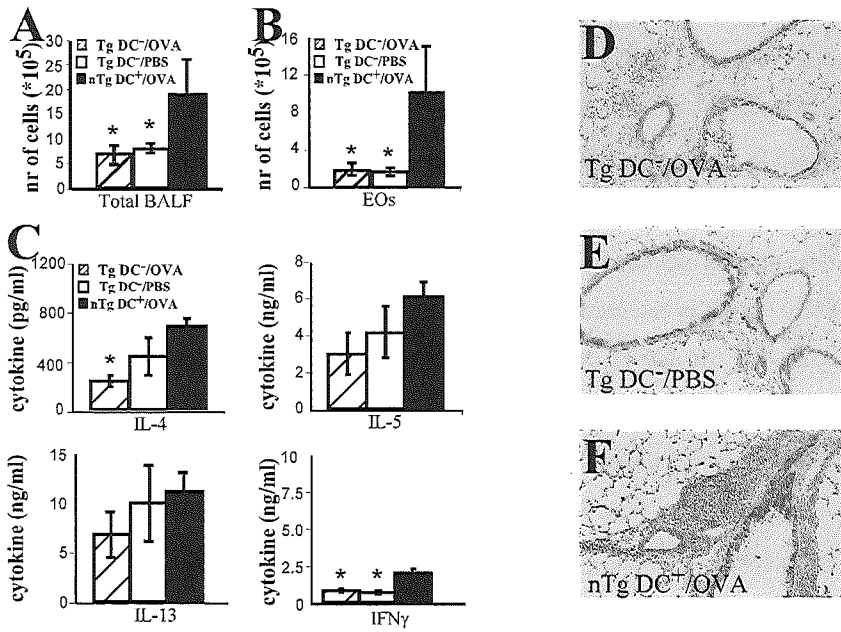

Figure 5. DC requirement for maintaining eosinophilic airway inflammation. *CD11c-DTR Tg (Tg DC$^-$/OVA) and non-Tg mice (nTgDC$^+$/OVA) were OVA-sensitized at day 0, received a daily 30 min aerosol at day 11,12,13. At day 14, mice received DT, at day 15,16,17 mice were challenged daily with a 30 min OVA. One group of CD11c-DTR Tg mice (Tg DC/PBS) was challenged with PBS aerosol after DC depletion. BALF cells were analyzed for (A) total BALF numbers and (B) the number of eosinophils.(C) Th2 cytokine production by MLN after ex vivo restimulation with OVA was measured in supernatant by ELISA. (D, E, F) Histological sections of Tg DC$^-$/OVA, Tg DC$^-$/PBS and nTg DC$^+$/OVA mice stained with PAS reagent.(*p<0.05 vs DC$^{+/+}$/OVA)*

Adoptive transfer of DCs is sufficient to induce all asthmatic features in sensitized mice

The above data showed, through transgenic depletion of $CD11c^+$ cells that DCs are required for secondary immune responses to antigen leading to asthmatic reactions. To demonstrate furthermore the involvement of DCs in secondary immune responses, we investigated whether DCs have an intrinsic capacity to induce a secondary immune response in sensitized animals.

Mice were sensitized by i.t. injection of $1x10^6$ OVA-pulsed DCs (OVA-DCs). On days 10 and 12, mice were challenged i.t. with OVA-DCs. As a positive control for the efficiency of sensitization, some sensitized mice received 4 daily OVA or PBS aerosols for 30 min, starting at day 10. On day 14, The total recovery of BALF cells in mice receiving a 'DC challenge' (referred to as DC/DC mice) or 'OVA aerosol challenge' (referred to as DC/OVA mice) were both more than 20 times higher than in mice receiving a 'PBS aerosol challenge' (referred to as DC/PBS mice) (Fig 6A: DC/DC: $7.1x10^5 \pm 1.1x10^5$ vs DC/ OVA: $9.6x10^5 \pm 3.3x10^5$ vs DC/PBS: $3.1x10^4 \pm 5.9x10^3$ (P=0.032). Differential analysis of the BALF cells showed a significant increase in eosinophils in DC/DC mice and DC/OVA mice compared to DC/PBS mice (Fig 6B). (DC/DC: 41.5±2.7% vs DC/OVA: 49.1±2.2% vs DC/PBS: 1.0%±0.4%). The minimum number of DCs required to induce a secondary immune response in DC-sensitized mice (by $1x10^6$ DCs i.t.) was determined by injecting $0.5x10^6$, $0.5x10^5$ or $1x10^4$ OVA DCs ten days after sensitisation. At day 12, both the BALF cellular count (Fig 6D, left panel) and eosinophil count (Fig 6D, right panel) decreased with lower doses of DCs given and challenge with $1x10^4$ DCs did no longer induce airway eosinophilia. Histological analysis of lung tissue from DC/DC-challenged mice (Fig 6E) and DC/OVA aerosol-challenged animals (Fig 6F) revealed intense peribronchial and perivascular eosinophilic infiltrates, of similar intensity inboth groups. Epithelial changes of goblet cell hyperplasia were readily seen in DC/DC and DC/OVA aerosol-challenged mice. As expected, DC/PBS aerosolised mice did not develop eosinophilic airway inflammation (Fig 6G). DC-challenged mice and OVA aerosol-challenged mice both had an increase in PenH compared with PBS aerosol-challenged mice (Fig 6H). Unexpectedly, when unpulsed DCs were used to sensitize or as a challenge, a secondary immune response was induced at a similar degree as with OVA-pulsed DCs.

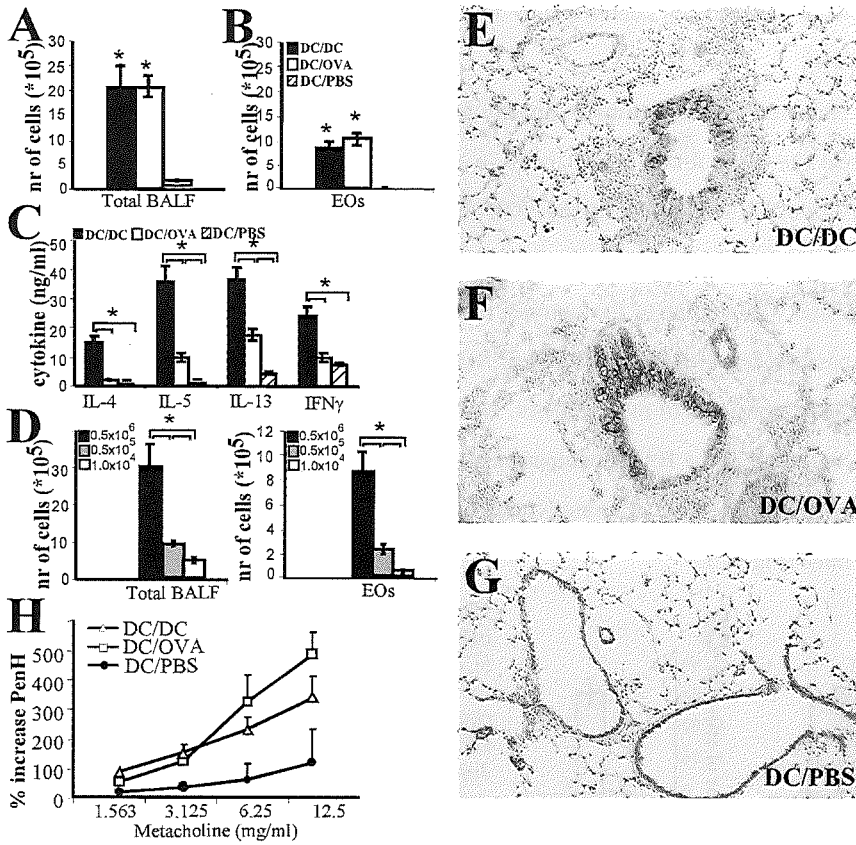

Figure 6. Adoptive transfer of DCs is sufficient to induce all asthmatic features in sensitized mice. *(A, B) Mice were OVA DC-sensitized at day 0, received at day 10 and day 12 an i.t. injection of OVA DCs (black bars) or received at day 10-13 a daily OVA aerosol(white bars) or PBS aerosols (shaded bars). At day 14, total BALF cells numbers and eosinophil numbers were determined by FACS.(*p<0.05 vs PBS) (C) Th2 cytokine production by MLN was determined after ex vivo restimulation with OVA. IL-4, IL-5, IL-13 and IFN-γ were measured in supernatant by ELISA. (D) Total BALF numbers(left panel) and BALF eosinophils(right panel) were determined by FACS after challenge with $5x10^5$ or $5x10^4$ or $1x10^4$ DCs. (F,E,G) Histological lung sections of mice challenged with OVA DCs (DC/DC), OVA aerosols (DC/OVA) and PBS aerosols (DC/PBS).(H) AHR was measured in DC/DC, DC/OVA and DC/PBS mice.*

Taking into consideration that DCs used for sensitization and challenge were both cultured in FCS, it is very likely that FCS 'delivered' antigen(s) against which sensitization was induced. To explore this, we challenged mice with splenic DCs obtained from Flt-3L-treated mice in a serum free manner[28]. Again, both OVA-pulsed and unpulsed splenic DCs were both capable of inducing a similar degree of airway inflammation in OVA-sensitized mice, as indicated by

total BALF and eosinophil cell counts and by Th2 cytokine production by draining LN T cells (data not shown).

As DCs are endowed with an intrinsic capacity to induce eosinophilic airway inflammation, we determined the need for T and B cells in the inflammatory response by performing adoptive transfer of DCs in DC-sensitized NOD-SCID mice lacking T and B cells. Forty eight hours after DC challenge in DC-sensitized mice, total number of BALF cells recovered from NOD-SCID mice was similar to the number recovered in WT mice (Fig 7A). Analysis of cellular composition revealed that in BALF of NOD-SCID mice eosinophils were completely absent and mostly consisted of macrophages. As expected, in wild type Balb/c's, eosinophils were readily recruited to the BALF compartment in response to DC challenge (Fig 7B). Th2 cytokines were produced in the draining lymph nodes of wild type Balb/c mice but not in the NOD-SCID mice (Fig 7C).

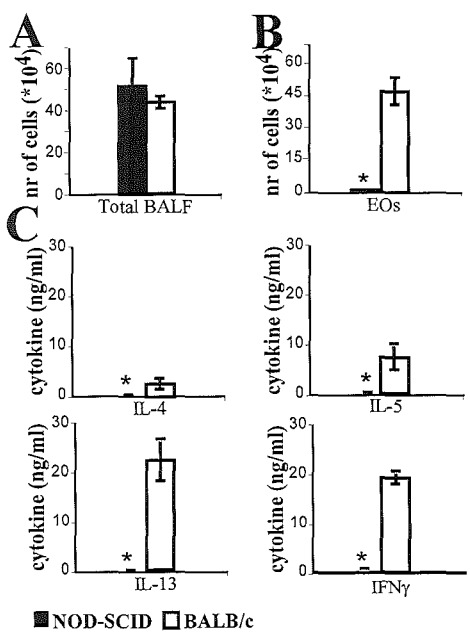

Figure 7. DC challenge induced airway inflammation is T and/or B cell-mediated. *NOD-SCID mice (black bars) and BALB/c (white bars) were sensitized with OVA-pulsed DCs and challenged at day 10 with an i.t. injection with OVA DCs. Mice were sacrificed at day 12. (A) Total BALF cells numbers were counted and (B) eosinophil numbers were determined.(C) Th2 cytokine production by lung draining lymph nodes of NOD-SCID and BALB/c mice after ex vivo restimulation with OVA.*

Discussion

Chronic airway inflammation in allergic asthma is controlled by primed Th2 lymphocytes that release effector cytokines in response to recognition of inhaled allergens. Generation of effector function in primed Th2 cells after inhalation of allergen is responsible for the cardinal features of asthma such as eosinophilic airway inflammation (IL-4 and IL-5), goblet cell hyperplasia and mucus hypersecretion (IL-4 and IL-13), and bronchial hyperreactivity (IL-4 and IL-13) eventually leading to airway narrowing and remodeling. Although it is generally accepted that Th2 cells and their secreted products are important in controlling the disease, little is known about their activation in response to allergen inhalation. For optimal activation, T lymphocytes need to recognize antigen in the context of MHC molecules (signal 1) and need additional costimulation (signal 2) classically provided by members of the B7 superfamily[29,30]. Professional APCs are capable of providing both signals and are therefore essential for optimal T cell division and differentiation into cytokine-producing effector cells[31]. Dendritic cells are believed to be the most important APCs in the lung in steady-state[3,12,15] and are mainly known for their potential to induce the activation of naïve T cells in the draining mediastinal lymph nodes leading to anti-microbial, antitumoral and allergic T cell responses[9,32]. However, from the perspective of T cell-mediated diseases such as atopic asthma, an important question is whether lung DCs are also responsible for the re-activation of allergen-specific primed T cells at times of allergen challenge, and therefore would constitute a target for therapeutic intervention. Evidence is accumulating that dendritic cells are indeed responsible for the reactivation of these primed Th2 cells upon allergen challenge (reviewed in[12]). *In vitro*, monocyte-derived dendritic cells from house dust mite (HDM)-sensitive patients have the potential to activate primed allergen-specific T cells and preferentially induce Th2 effector cytokine release[33]. In humanized severe combined immunodeficiency (huSCID) mice, the adoptive transfer of HDM-pulsed DCs to the airways of HDM-allergic PBMC-reconstituted mice led to more severe eosinophilic airway inflammation, accompanied by higher secretion of human effector Th2 cytokines IL-4 and IL-5[34]. Allergen challenge with relevant allergen to the airways of allergic asthmatics leads to the recruitment of monocytes from the bloodstream to the airways and stable asthmatics have higher numbers of DCs lining the airways[16,17]. Similarly, it was recently shown that OVA

allergen challenge in OVA-sensitized mice and rats increased the number of myeloid DCs in the airway mucosa, interstitial and bronchoalveolar compartment, at a time point that $CD4^+$ T cells were also accumulating in the lungs[10,18,35].

These data suggest in an indirect way that airway DCs might be involved in the functional restimulation of previously primed Th2 cells. In this study, we have directly addressed this issue by studying the role of $CD11c^+$ lung DCs in a well established mouse model for asthma induced by inhalation of OVA in OVA-sensitized mice. First, we confirmed that $CD11c^+$ DCs accumulated within sites of eosinophilic airway inflammation and clustered with $CD4^+$ T cells. We have previously shown that myeloid DCs accumulate within the airway mucosa and bronchoalveolar lavage fluid of OVA-challenged mice, in a dose dependent way[18]. Now we show that $CD11c^+$ DCs also accumulate within the submucosa of the airways and the perivascular space at areas of intense eosinophilic inflammation, forming multiple contacts with $CD4^+$ T cells. The consequences of this interaction with T cells have been studied elegantly by Huh et al. in a rat model of asthma. After a single OVA aerosol challenge in OVA-sensitized rats, airway DCs accumulated in the airway mucosa and submucosa and formed contacts with primed T cells in turn inducing the upregulation of CD86 on otherwise immature airway DCs[10]. Therefore, these authors hypothesized that contact with primed T cells in the lungs upon antigen recognition turns immature DCs from an antigen uptake to an antigen-presenting mode leading to local antigen presentation by DCs to mucosal T cells. Interestingly, this effect was restricted to airway DCs and did not occur in interstitial DCs resident in peripheral lung[10]. The upregulation of DC numbers was only very transient reaching a maximum at 2h post challenge in contrast to our model where prolonged increases in DC, $CD4^+$ T cell and eosinophil numbers are seen[8,18]. The most likely explanation is that we used repeated challenge with OVA aerosol leading to continuous immune stimulation in contrast to the single OVA aerosol given by Huh et al. As intense local interactions between primed $CD4^+$ T cells and DCs are a feature of eosinophilic airway inflammation across species, we next studied whether effector $CD4^+$ T cells interacted functionally with DCs within the airway mucosa and whether this interaction was critical for the cardinal features of asthma seen after allergen challenge. We addressed this question by using a novel system of conditional depletion of $CD11c^+$ cells expressing a Diphtheria Toxin (DT) receptor transgene

under the control of the CD11c promoter[24]. These mice allow $CD11c^+$ DCs to be rapidly and conditionally depleted by administration of DT. Systemic administration of DT to these mice leads to the rapid reduction in splenic and lymph node DCs, and concomitantly to an absence of the primary cytotoxic T cell responses to Listeria infection or experimental administration of protein antigens[20]. Here we adopted this strategy and administered the DT locally via intratracheal injection, leading to a loss of $CD11c^+$ cells in the lung tissues and draining mediastinal nodes. We cannot explain at present by what mechanism the $CD11c^+$ DCs were depleted from the mediastinal nodes, but we speculate that they were reduced because of the loss of import of freshly migrating DCs from the mucosa. It has indeed been shown that airway mucosal DCs have a very short half-life in the mucosa and continuously migrate to the nodes even in non-inflammatory conditions[7,36,37]. Alternatively, DT might have reached the LN directly from the bronchial lumen via the afferent lymphatics, causing local damage to resident LN DCs.

Using this unique system, we conditionally depleted $CD11c^+$ DCs from the lung immediately prior to OVA challenge in OVA-sensitized mice and saw that the cardinal features of asthma such as eosinophilic airway inflammation, goblet cell hyperplasia and bronchial hyperreactivity to metacholine failed to develop. As these features critically depend on $CD4^+$ T cells and their secreted products, the most likely explanation was that effector function was not induced in the absence of DCs. In support of this theory, we saw that in the absence of DCs, *ex vivo* isolated $CD4^+$ cells produced greatly diminished amounts of IL-4, IL-5 and IL-13. We then adoptively transferred *in vitro* primed $CD4^+$ Th2 cells from DO11.10 TCR transgenic T cells in DC depleted mice. Previous data have shown that such *in vitro* differentiated Th2 cells mimic *in vivo* generated memory T cells[38,39] and can be used to transfer passively the Th2 reactivity to OVA aerosol[40]. However, when Th2 cells were adoptively transferred in mice that were depleted of DCs, no Th2 effector cytokines IL-4, IL-5 and IL-13 were produced by the T cells in the draining lymph nodes of the lung, whereas they readily did in the presence of lung DCs.

These data of depletion of $CD11c^+$ DCs immediately prior to antigen challenge to the lung support our previous work in which we systemically depleted all myeloid DCs from the lungs, lymph nodes, bone marrow and spleen of HIV-LTR promoter driven thymidine kinase

transgenic (TK-TG) mice prior to allergen challenge. In TK-TG mice, conditional depletion of DCs took considerably more time as it depended on killing of dividing DC precursors and had to be performed prior to T cell activation as the HIV-LTR promoter used to drive transgene expression was also activated in effector T cells, a problem not encountered in the CD11c DTR Tg mice. We next questioned therefore what would be the role of lung DCs during ongoing inflammation at a time when fully activated effector $CD4^+$ Th2 cells are already causing all the features of asthma. When $CD11c^+$ DCs were depleted in the middle of the OVA aerosol period, all the salient features of asthma were strongly diminished, and effector cytokine secretion was strongly reduced. We observed that eosinophilia actually resolved in the wake of ongoing allergen exposure when DCs were depleted from the airways. It thus seems that effector Th2 responses *in vivo* in the lung continuously depend on antigen presenting cells. One possible explanation would be that effector T cells *in vivo* remain dependent on costimulation in contrast to their *in vitro* counterparts[21,41]. Indeed, numerous models of asthma have demonstrated that blocking the interaction of costimulatory molecules of the B7 superfamily (CD80, CD86, ICOS-L) or TNF-R family (OX40L) can reduce the features of asthma, even when given during challenge in sensitized mice and even when given together with *in vitro* primed Th2 cells[42-46]. As lung DCs are the predominant cell type expressing these molecules, an absence of DCs might have the same effect as blocking these costimulatory molecules. An alternative explanation would be that lung DCs are essential for producing Th2 selective chemokines. It was indeed very elegantly shown by Vermaelen et al. that lung $CD11c^+$ DCs within eosinophilic airway inflammation in the lung produce high amounts of the Th2 selective chemokine TARC, acting on $CCR4^+$ Th2 cells[19]. This is not restricted to murine DCs as human DCs obtained from HDM-allergic donors also secrete the Th2-selective chemokines TARC and MDC[47]. Therefore, depletion of lung DCs might lead to an 'unfavourable' chemokine gradient, failing to attract primed Th2 cells to the lung.

Together, these data show that $CD11c^+$ DCs are necessary for mounting the secondary response to OVA challenge in previously sensitized mice through the induction of effector function in primed Th2 cells. However, CD11c is also expressed on a subset of alveolar macrophages[48] and indeed, DT depleted nearly all macrophages in the bronchoalveolar compartment of the DTR Tg mice. Could the observed findings have been caused by a

depletion of alveolar macrophages? We think this is unlikely because of several reasons. (i) We performed reconstitution experiments in which DTR Tg mice were reconstituted intratracheally with wild type alveolar macrophages, insensitive to DT. In these experiments, the DT treatment had the same suppressing effect on the airway inflammation suggesting that macrophage depletion was not causing the defect in effector T cell generation. (ii) Studies by Huh et al. and our own studies show a predominant interaction between $CD4^+$ T cells and DCs in the submucosa of the airways, at distant sites from alveolar macrophages, making a functional contribution of these cells less likely. (iii) Previous studies in mice in which alveolar macrophages were depleted using clodronate-filled liposomes have seen an actual enhancement of airway inflammation and T cell reactivity in the lung[49]. This is explained by the normally suppressive influence of alveolar macrophages on activation of primed T cells and DCs in the lung. The fact that airway inflammation was severely suppressed in the combined absence of $CD11c^+$ DCs and $CD11c^+$ alveolar macrophages attests to the crucial role that $CD11c^+$ DCs play during the secondary response.

As the CD11c marker is also expressed weakly on activated $CD8^+$ T cells[50], we performed adoptive transfer reconstitution experiments of wild type $CD8^+$ T cells to DT-treated mice, again failing to restore the eosinophilic airway inflammation and Th2 cytokine production. Again, the role of $CD8^+$ T cells in mouse models of asthma is not very predominant as $CD8^+$-deficient and $ß_2$-microglobulin-deficient mice[49,51-53] develop normal airway eosinophilia in mouse models of asthma and some authors even suggested a suppressive role for $CD8^+$ T cells in asthma[54, 55]. Eosinophils express very low levels of $CD11c$[26], and thus are likely to be less sensitive to DT compared with DCs. We cannot completely exclude that DT would target eosinophils directly. However, treatment with DT reduced not only eosinophilia but also effector Th2 cytokine secretion, goblet cell hyperplasia and BHR, strongly suggesting that the main effect of DT treatment was on $CD11c^+$ DCs, leading to a failure of T cell activation. In support, intratracheal reconstitution with wild type eosinophils did not restore eosinophilia in the BAL compartment nor the secretion of effector Th2 cytokines.

The above data using conditional depletion of DCs imply an important functional role for DCs in mounting and maintaining the effector response to inhaled antigen in sensitized mice and are in line with the previous data from others showing the presence of DCs and T cells

within sites of acute or chronic eosinophilic airway inflammation[10, 56]. Although DCs seem essential APCs *in vivo* in these experiments, we looked for additional proof that DCs are endowed with an intrinsic potential to induce eosinophilic airway inflammation in sensitized mice. Therefore, we adoptively transferred bone marrow-derived OVA-pulsed $CD11c^+$ DCs into mice that received prior OVA-DCs for sensitization and in doing so induced all the cardinal asthma features such as eosinophilic airway inflammation, goblet cell hyperplasia, bronchial hyperreactivity and Th2 cytokine production. The degree of inflammation seen was identical to the inflammation induced by OVA aerosol challenge, thus demonstrating that DCs are sufficient to induce the full asthmatic phenotype. Surprisingly, repetitive but not single adoptive transfer of DCs that were not pulsed with OVA also induced the Th2 response. As it has been shown that antigens contained within FCS can act as a Th2 immunogen in the lung[57], we excluded this possibility by purifying DCs in an FCS free manner from the spleen and still observed the induction of eosinophilia. As this response did not occur in SCID mice devoid of lymphocytes, an active T cell-mediated immune response is most likely. Two explanations for the induced eosinophilia can be given. (i) DCs induce a Th2 response to some unidentified antigen contained within the FCS containing medium and synthetic HY medium used for isolating the splenic DCs or (ii) DCs induce an immune response to an unidentified self-antigen normally not presented in the lung. It is striking to note that in patients with intrinsic asthma, there is no sensitization to known allergens, despite the fact that this disease is almost identical to atopic asthma. Our findings in this mouse model could be the start of a more detailed study of airway DC function in patients with intrinsic asthma and open up the possibility that Th2 responses in intrinsic asthma are directed to self or environmental antigens presented by airway DCs.

In conclusion, our study shows that lung DCs are functionally required and sufficient for mounting and maintaining an effector Th2 response to allergen challenge in sensitized mice leading to all the features of asthma. This study therefore has profound implications for the design of novel therapies aimed at interfering with airway inflammation. Strategies that target the airway DC have a high chance of being successful in inhibiting the cardinal features of asthma.

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Chapter 6

Essential role of dendritic cell CD80/CD86 costimulation in the induction but not reactivation of Th2 effector responses in a mouse model of asthma

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Abstract

Background: Airway dendritic cells (DC) are crucial for the generation of Th2 cells from naïve T cells during sensitization and for reactivation of primed Th2 cells upon allergen challenge in mouse models of asthma. It is unknown if CD80/CD86 costimulation is necessary during both phases of the response, as primed T cells rely less on costimulatory molecules compared with naïve T cells.

Objective: To study the contribution of CD80/CD86 costimulatory molecules on DCs during sensitization or challenge in a mouse model of asthma.

Methods: To address the role of costimulation during sensitization, naïve Balb/c mice received an intratracheal injection of ovalbumin (OVA)-pulsed DCs obtained from the bone marrow of wild type or CD80/CD86$^{-/-}$ mice and were subsequently challenged with OVA aerosol. To address the role of costimulation during challenge, OVA-sensitized mice received OVA-pulsed WT or CD80/86$^{-/-}$ DCs without OVA aerosol.

Results: WT DCs induced the proliferation and effector Th2 differentiation of naïve OVA-specific T cells whereas CD80/CD86$^{-/-}$ DCs induced only proliferation. Not surprisingly, WT but not CD80/CD86$^{-/-}$ DCs induced sensitization to OVA in naïve mice. In contrast, in OVA-sensitized mice, intratracheal injection of CD80/CD86$^{-/-}$ OVA-pulsed DCs led to eosinophilic airway inflammation, goblet cell hyperplasia and effector Th2 cytokine production that was not different from injection with WT OVA-DCs, even when inducible costimulator ICOS was blocked or CTLA4-Ig was given.

Conclusion: CD80/CD86 costimulation on DCs is only necessary during priming of naïve T cells into Th2 cells, but not during restimulation of previously primed Th2 cells in the challenge phase.

Introduction

Asthma is a Th2-mediated disease, in which airway dendritic cells (DCs) play an important role[1, 2]. A prominent role was shown for airway DCs during the sensitization to inhaled allergen but also during the challenge phase, in which previously primed Th2 cells are reactivated in the lung to become effector Th2 cells[3-6].

The ways by which DCs activate naïve or primed T cells are incompletely understood. DCs interact with T cells through multiple cell surface interactions, some of which only serve to approximate both cell types, others leading to T cell activation. The latter molecules are called costimulatory molecules, the most studied of which is CD80/CD86 interacting with CD28 and CTLA-4[7, 8]. It is generally accepted that this pathway is absolutely crucial for naïve T cell activation and differentiation. However, it is still controversial if CD28 signaling is absolutely essential for generating primary immune responses *in vivo*[9-14]. The precise role of CD80/86 costimulation in the reactivation of primed T cells in a secondary immune response is even more complex. Primed T cells are less dependent on CD80/CD86 costimulation[15,16]. In some disease models however, blocking CD80/CD86 costimulation by treatment with CTLA4-Ig fusion proteins led to reduced memory T cell reactivation[17]. Recent reports have also shown that mucosal DCs interacting with primed T cells in the lung upregulate CD86 in mice with eosinophilic airway inflammation[5], in contrast to the low expression of CD86 on airway DCs found in the steady state[18]. These results suggest that CD80/86 costimulation on DCs might be involved in local reactivation of memory T cells in the lung, leading to eosinophilic airway inflammation. In this paper, we have studied the requirements of CD80/CD86 costimulation for the induction of primary and secondary immune responses to allergen in the lung. To address specifically the role of these molecules on DCs only, we used a model of asthma in which DCs were the only cells lacking expression of CD80/CD86, avoiding interference with CD80/86-CD28 interaction between other cells. In addition, we compartmentalized the immunization by administering DCs in the airways in contrast to other models, which use systemic sensitization routes. Our results suggest that CD80/86 costimulation provided by DCs in the lung is mainly required for generating effector T cell responses during sensitization, but is redundant during reactivation of primed T cells in the challenge phase.

These findings have important implications for the design of immunotherapeutic strategies in asthma that aim to interfere with costimulation.

Methods

Animals

All experiments were performed with 8-10 weeks old female Balb/c, DO11.10 Balb/c and CD80/CD86$^{-/-}$ Balb/c and C57Bl/6 mice (kindly provided by Dr. M. Oosterwegel, Utrecht University, The Netherlands and by B. Salomon, Pitié Salpétrière, Paris, France)[19]. Mice were housed under specific pathogen-free conditions. All of the experimental procedures were approved by an ethical committee of Animal Experiments.

Generation and antigen pulsing of bone-marrow-derived DCs

DCs were grown from bone marrow cells as described earlier[4]. On day 9 of culture, cells were pulsed overnight with 100 μg/ml of LPS-low ovalbumin (OVA DC) (Worthington Biochemical Corporation, Lakewood, NJ, USA; LPS contamination of less than 2.9 EU/ml). At day 10, cells were washed and any free OVA was removed.

Generation of eosinophilic airway inflammation using DCs

To induce sensitization to OVA, 1x10^6 OVA-DCs or control unpulsed DCs were injected into the airways of anaesthetized naive mice at day 0 as described in earlier experiments[4,20]. To address the role of costimulation during sensitization, DCs were obtained from a/ WT or b/ CD80/CD86$^{-/-}$ Balb/c mice. Ten days later, mice were challenged with a 30 min OVA aerosol (grade III, 1% w/vol in Phosphate Buffered Saline (PBS), (Sigma Chemical Co., MO, USA) on three consecutive days.

To address the role of costimulation during challenge, sensitized mice received an intratracheal injection of 1x10^6 OVA-DCs on day 11 after sensitization, in the absence of any OVA aerosol exposure, and were sacrificed two days later ('DC challenge' protocol). DC-sensitized mice were challenged with a) WT DC, b) CD80/86$^{-/-}$DC or c) CD80/86$^{-/-}$DC in combination with an i.v. injection of 250 μg CTLA4-Ig (BD Biosciences Pharmingen, San Diego, CA) or as a control 250 μg mouse IgG2a or d) CD80/86$^{-/-}$DC in combination with a daily (day 11 and 12) i.p injection of 100 μg rat anti-murine ICOS (12A8)[21] or as a control 100 μg rat IgG.

Antigen presentation assay to OVA TCR transgenic T cells *in vivo*

Cell suspensions of pooled peripheral lymph nodes (LN) from naïve OVA TCR Tg DO11.10 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR), as previously described[22]. $10x10^6$ $CFSE^+$ T cells were transferred i.v. into naive mice. Twenty-four hours later, either $1x10^6$ OVA pulsed WT or $CD80/86^{-/-}$ DCs were transferred intratracheally. Seventy-two hours after DC transfer, lung draining LN were analyzed for the proliferation of CFSE-labeled OVA TCR Tg T cells. As a control, non-lung draining LN (brachial) were used. Transferred OVA TCR transgenic $CD4^+$ T cells were recognized as $CD4^+CFSE^+$ cells expressing the clonotypic TCR recognized by the monoclonal antibody KJ1.26 (Caltag, Burlingame CA). Propidium iodide (PI, Sigma) was used to exclude dead cells. To prevent non-specific binding to Fc receptors 2.4.G2 blocking reagent was used (ATCC, Mannassas). Division plots were obtained with a FACScalibur flow cytometer (Becton and Dickinson Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo software (Treestar, Costa Mesa, CA).

Collection and analysis of cells and tissues

Bronchoalveolar Lavage Fluid (BALF): Twenty-four hours after the last aerosol challenge or 48 hour after DC challenge in sensitized mice, BAL was performed as described[4]. The number of eosinophils in BALF cells was determined by FACS analysis[23] after staining with the following monoclonal antibodies: FITC-MHCII (2G9), PE-CCR3 (R&D systems, Abingdon, UK), CyChrome-CD3 (145-2C11) and -B220 (RA3-6B2) and APC-CD11c (HL3), all in combination with 2.4.G2 blocking reagent. This method was used to determine the percentage of eosinophils without other contaminating BALF cells.

For *LN restimulation*, cells were dispersed through a 40 μm cell sieve (Becton Dickinson), and plated in 96 well round bottom plates at a density of $2x10^5$ cells per well and were restimulated for 4 days with 10 μg/ml OVA. After 4 days, levels of IL-4, IL-5, IL-10, IFN-γ (OptEIA, BD Biosciences Pharmingen) and IL-13 (R&D systems, Abingdon, UK) were measured in supernatant by ELISA.

Airway histology: lungs were infused with OCT (1/1 vol/vol in PBS) and were snap-frozen in liquid nitrogen. 3 μm cryo-sections were stained with Hematoxylin-Eosin or with Periodic Acid and Schiff's Reagent (Sigma) to detect goblet cells.

Statistical analysis

Comparison of means between different groups was performed with the Mann-Whitney U test for unpaired data (SPSS 10.0 for Windows). All experiments were performed using 6-8 mice per group. Differences were considered significant if $p<0.05$. Histology sections were assigned a score for the degree of inflammation based on perivascular and peribronchial infiltrates, goblet cell hyperplasia, and inflammatory cells in parenchyma, in this order of importance. The scores per group were compared with a Kruskal-Wallis and in case of significance, pair-wise with Mann-Whitney U. Differences were considered significant if $p<0.05$.

Results

Phenotype of injected DC

DCs were cultured from bone marrow of CD80/86$^{-/-}$ mice following an earlier described culture method[24]. Wc compared CD80/86$^{-/-}$ DCs with wild type (WT) DCs by FACS analysis. Both CD80/86$^{-/-}$ and WT DCs co-expressed MHCII and the DC marker CD11c. As expected, CD11c^{+}/MHCII^{+} cells grown out of WT donor expressed 97% CD80 and 87% CD86 compared with only 1.4% and 0.4% respectively in CD80/86$^{-/-}$ mice, representing background immunofluorescence (Fig 1). Bone marrow DC cultures of WT donors and CD80/86$^{-/-}$ donors yielded similar numbers of viable CD11c^{+}/MHCII^{+} cells (resp. 73x10^{6} vs 60x10^{6} per donor).

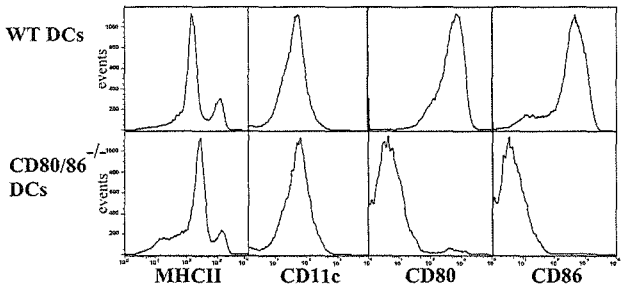

Figure 1. Phenotype of injected DCs. *WT DCs (top panels) and CD80/86$^{-/-}$ DCs (lower panels) were stained for expression of MHCII, CD11c, CD80 and CD86.*

Role of CD80 and CD86 costimulation by DCs for inducing proliferation and Th2 differentiation of naïve T cells

Mice were sensitized by an intratracheal injection of either OVA pulsed WT DCs or OVA pulsed CD80/86$^{-/-}$ DCs. To visualize naïve T cell proliferation, mice received 10x10^{6} CFSE-

labeled OVA TCR transgenic T cells i.v. one day before DC injection. Flowcytometry was used to track cell division of the transferred $CFSE^+/CD4^+$/OVA TCR Tg T cells in draining lymph nodes of the lung. $CD80/86^{-/-}$ DCs induced up to 6 divisions in OVA specific naïve T cells and WT DCs induced up to 8 divisions. $CD80/86^{-/-}$ DCs recruited significantly less cells into cell division (p=0.002). In $CD80/86^{-/-}$ DC-sensitized mice, proliferating OVA-specific T cells divided on average 2.13 times compared to 2.81 times in OVA WT DC-sensitized mice. (Fig 2A). No division was seen in non-draining LN (brachial) or in mice sensitized with unpulsed DCs.

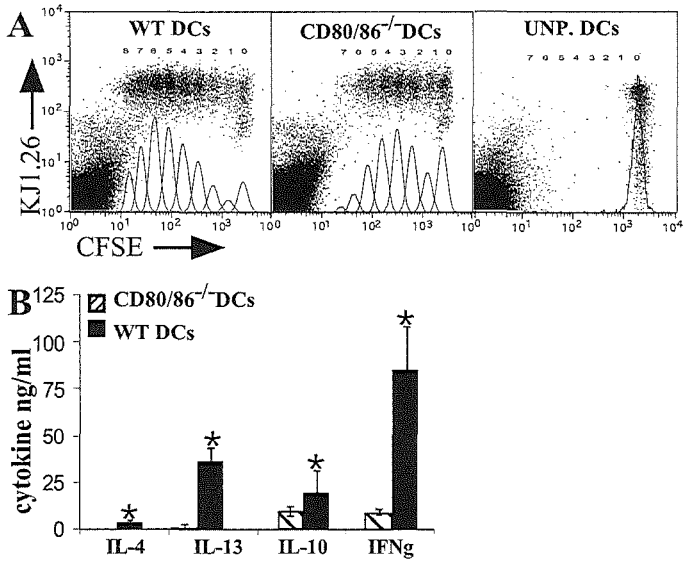

Figure 2. Proliferation and Th2 differentiation of naive T cells induced by sensitization with $CD80/86^{-/-}$ DCs compared with WT DCs. *Mice were sensitized by an intratracheal injection of either OVA pulsed wild type (WT) DCs (A; left panel) or OVA pulsed $CD80/86^{-/-}$ DCs (A; mid panel) or unpulsed DCs (A; right panel), which received one day earlier $10x10^6$ CFSE-labeled OVA TCR transgenic T cells i.v. At 72 hours, lung draining lymph nodes of $CD80/86^{-/-}$ DCs or WT DC-sensitized mice were collected and $2.5x10^5$ $CFSE^+/CD4^+$/OVA TCR Tg T cells were analyzed for the number of induced divisions. (B) Remaining of the lung draining lymph node cells were restimulated in vitro with OVA at fixed concentrations for 96 hours and supernatants were analyzed for IL-4, IL-13, IL-10 and IFNγ levels. (*P<.05 in comparison with WT DCs).*

We investigated whether sensitization with DCs lacking CD80/86 impaired the differentiation of divided cells into effector cells. We determined cytokine production by T cells in the lung draining LN after restimulation with OVA *ex vivo*. Despite displaying only a small reduced proliferation, T cells of mice sensitized with $CD80/86^{-/-}$ DCs produced dramatically lower

levels of IL-4, IL-13 and IFNγ compared to the mice that received OVA WT DCs (p=0.002, Fig 2B). Also less IL-10 was produced after sensitization with CD80/86$^{-/-}$ DCs, compared with mice sensitized with OVA WT DCs (p=0.002).

Role of CD80 and CD86 costimulation by DCs during sensitization on development of eosinophilic airway inflammation in response to inhaled OVA

The lower production of Th2 cytokines by OVA-specific T cells after injection of CD80/CD86-deficient DCs, suggested that differentiation of T cells into effector cells was suppressed. This was investigated by challenging OVA-DC or OVA-CD80/86$^{-/-}$ DC immunized mice with OVA aerosols. In these experiments, no OVA-TCR Tg T cells were given. Mice were sensitized by an intratracheal injection of OVA-CD80/86$^{-/-}$ or OVA-WT DCs. Unpulsed DCs were used as a negative control. From day 10-12, mice were challenged with OVA aerosols. Analysis of BALF cells revealed that OVA aerosol challenge in mice sensitized with CD80/86$^{-/-}$ DCs led to a significantly lower number of total cells (Fig 3A, p=0.02) and eosinophils (Fig 3B, p=0.002) compared to mice sensitized with WT DCs. Although airway inflammation was severely reduced in mice immunized with CD80/CD86$^{-/-}$ OVA-DCs, it was still higher compared with the baseline level seen in mice immunized with unpulsed DCs (Fig 3A,B).

We next determined the type and magnitude of cytokine production of T cells in the lung-draining LN in response to the inhaled allergen. In agreement with the BALF data, Th2 cytokine production of IL-4, IL-5, IL-13 and IL-10 (p=0.002) were significantly lower when mice were immunized with CD80/86$^{-/-}$ DCs compared with OVA pulsed WT DCs (Fig 3C). Mice which were sensitized with unpulsed WT DCs had lower IL-13 levels than mice sensitized with CD80/86$^{-/-}$ DCs ($p \leq 0.026$). Sensitization with CD80/86$^{-/-}$ DCs induced similar IFNγ production compared with sensitization with OVA-pulsed or PBS-pulsed WT DCs. Histological analysis of lungs of mice sensitized with OVA-pulsed WT DCs revealed intense inflammatory perivascular and peribronchial infiltrates and goblet cell hyperplasia (Fig 3D) after OVA challenge. Lungs of mice sensitized with CD80/86$^{-/-}$ DCs (Fig 3E) revealed less inflammatory perivascular and peribronchial infiltrates, compared with mice sensitized with WT OVA-DCs . As expected, eosinophilic airway inflammation and epithelial changes were almost completely absent in mice immunized with unpulsed WT DCs (Fig 3F).

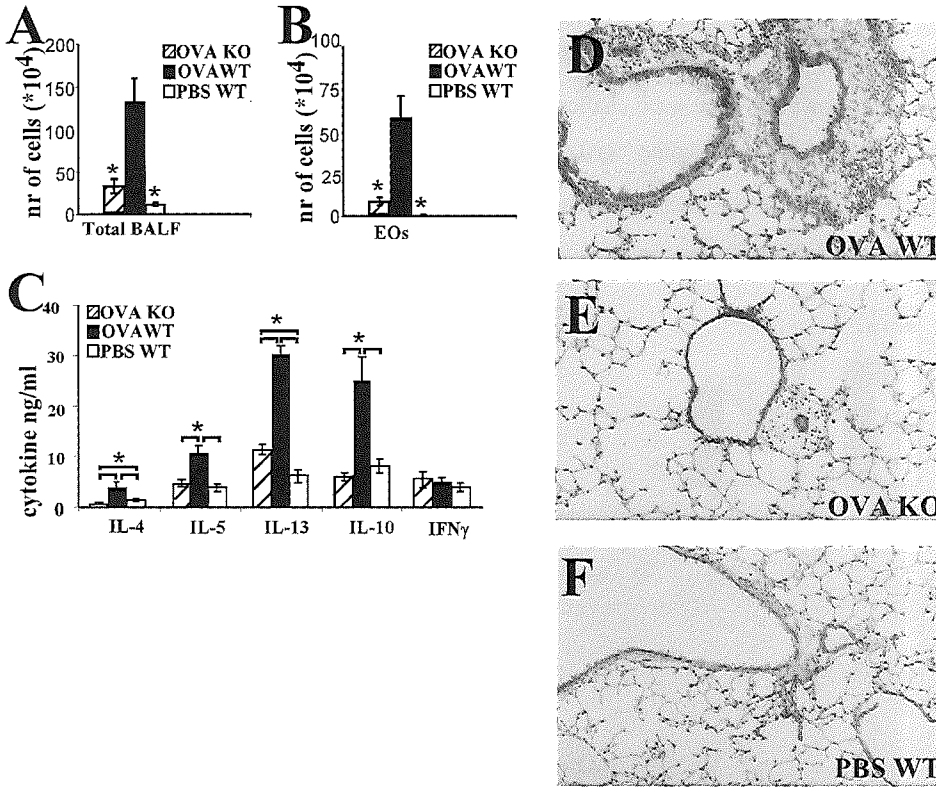

Figure 3. Lack of induction of eosinophilic airway inflammation in response to inhaled allergen in CD80/86$^{-/-}$ DC-sensitized mice. *Mice were sensitized by an intratracheal injection at d0 of either OVA pulsed WT DCs (OVA WT), OVA pulsed CD80/86$^{-/-}$ DCs (OVA KO) or as a control unpulsed WT DCs (PBS WT) and challenged at d10-12 with OVA aerosols. 24 Hours after the last OVA aerosol, total cell numbers (A) and number of eosinophils (EOs) (B) in BALF were determined (*P<.05 in comparison with OVA WT DCs). (C) Draining lymph node cells of the lung were restimulated in vitro for 96 hours with OVA and supernatants were analyzed for IL-4, IL-5, IL-13, IL-10 and IFNγ levels (*P<.05). Sections of lungs from OVA aerosol-challenged mice sensitized with OVA pulsed WT DC (D), OVA pulsed CD80/86$^{-/-}$ DC (E), and as a control PBS pulsed WT DCs (F). Lungs were collected 24 hours after the last OVA aerosol challenge and stained with Periodic Acid and Schiff's reagent.*

Role of CD80/86 costimulation by the antigen presenting DCs during antigen challenge in sensitized mice

To determine the role of CD80/86 costimulation provided by DCs during the effector phase, we used a newly developed model in which sensitized mice are challenged intratracheally with injected DCs, instead of the more commonly used challenge with three OVA aerosols. This protocol induces a secondary immune response in mice resulting in all the hallmarks of asthma, including eosinophilic airway inflammation, Th2 cytokine production, high IgE

serum level and an enhanced AHR to metacholine. This response was T cell-dependent, as applying this protocol to SCID-mice did not induce eosinophilia (Chapter 5).

Ovalbumin-sensitized mice were challenged by an intratracheal injection of $1x10^6$ WT OVA-DCs (WT/WT) or with CD80/86$^{-/-}$ OVA-DCs (WT/KO). As shown in figure 4A, total number of BALF cells and BALF eosinophil counts were similar after challenge with either OVA WT DCs or CD80/86$^{-/-}$ DCs.

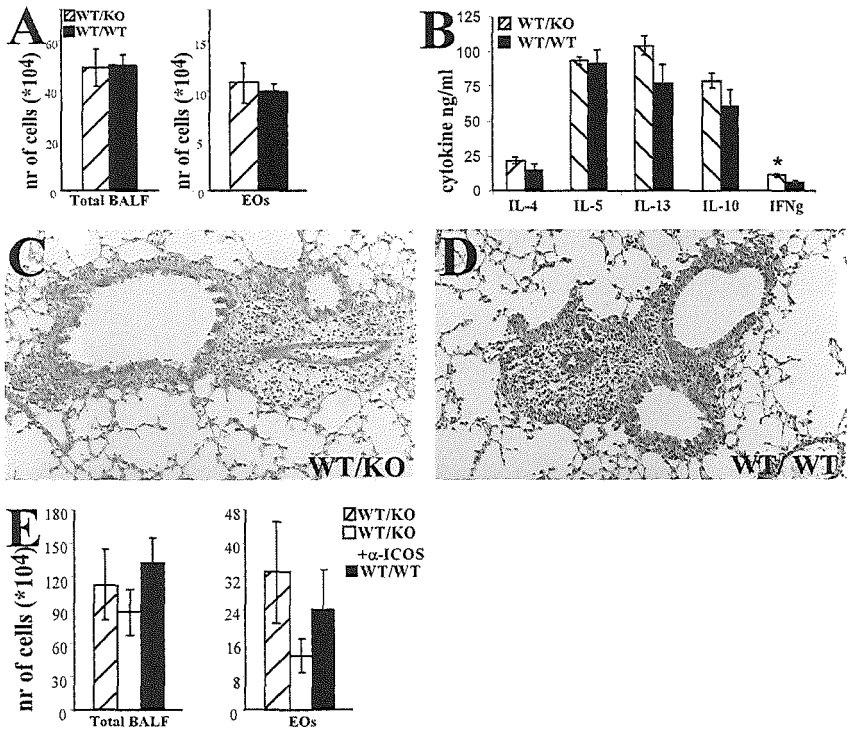

Figure 4. Induction of eosinophilic airway inflammation in response to challenge with CD80/86$^{-/-}$ DC in WT DC-sensitized mice. *Mice were sensitized by an intratracheal injection at d0 with OVA pulsed WT DCs and challenged at d11 with either an intracheal injection of OVA pulsed CD80/86$^{-/-}$ DC (WT/KO) or WT DCs (WT/WT). (A) At d13, total cell numbers and number of eosinophils in BALF were determined. (B) Draining lymph node cells of the lung were restimulated in vitro for 96 hours with OVA and supernatants were analyzed for IL-4, IL-5, IL-13, IL-10 and IFNγ levels (*P<.05). (C/D) Lungs were embedded in histowax until staining with hematoxylin/eosin. (E) C57Bl/6 mice were sensitized with OVA pulsed WT DCs and challenged with either OVA pulsed CD80/86$^{-/-}$ DC (WT/KO), CD80/86$^{-/-}$ DC in combination with α-ICOS (WT/KO + α-ICOS), or WT DCs (WT/WT). At d13, total cell numbers and number of eosinophils in BALF were determined.*

Also cytokine production of IL-4, IL-5, IL-13 and IL-10 by lymphocytes of the lung-draining LN after restimulation with antigen was not significantly different between both groups (Fig 4B) except for a higher IFNγ production (p=0.009) by CD80/86$^{-/-}$ DCs immunized mice.

Analysis of airway histology revealed similar levels of peribronchial and perivascular inflammatory infiltrates and goblet cell hyperplasia in mice challenged with CD80/86$^{-/-}$ DC or WT DCs (Fig 4C,D).To determine whether bystander cells provided supplementary CD80/CD86 costimulation during antigen presentation induced by CD80/CD86$^{-/-}$ DCs, we blocked CD80 and CD86 molecules by administering CTLA4-Ig to sensitized mice, 24 hours before we injected OVA pulsed CD80/86$^{-/-}$ DCs. The number of recovered eosinophils from BALF after CTLA4-Ig treatment was not significantly different from control mice treated with mouse IgG2a ($3.8x10^4$ vs $5.8x10^4$, p=0.7).

As we found that CD80/86 absence did not affect the potential of DCs to induce effector responses in primed mice, we questioned whether costimulation through B7-Rp1 interacting with ICOS would be responsible for residual costimulatory capacity in the absence of CD80/86. These experiments were performed in C57Bl/6 mice. Mice were treated with anti-ICOS or control rat IgG blocking antibody at the time of injection with CD80/CD86$^{-/-}$ DCs and 24 h later. One day after the last anti-ICOS injection, analysis of BALF cells revealed a lower, although not statistically significant, number of BALF eosinophils recovered from mice challenged with CD80/86$^{-/-}$DCs in combination with ICOS blockade compared with mice challenged with CD80/86$^{-/-}$DCs only or with wild type DCs (Fig 4E).

Discussion

We previously showed the involvement of DCs during sensitization and challenge in a murine model for asthma[3,4,20,22]. As activation of naïve T cells and primed T cells depends differentially on provision of costimulatory molecules, it was of interest to study if CD80/CD86 costimulation provided by DCs was necessary for induction of Th2 effector responses in the lung and whether there would be a difference in the need for costimulation during the sensitization phase (i.e. when naïve T cells become primed) compared with the challenge phase (when primed T cells become effector T cells).

For activation of naïve T cells *in vitro*, strong costimulatory signals are required. However, it has been less clear whether the CD80/86–CD28 interaction is essential for inducing primary immune responses *in vivo*. Several reports in CD28$^{-/-}$ mice show dependence on CD28 for generation of effective immune responses[9-11], while others show that these mice mount an

equally effective immune response compared with their WT littermates in models of allograft rejection[12], graft versus host disease[13], and generation of antiviral immunity.[14] These differences in outcome might be due to differences in the type of APCs that induce the T cell response or differences in the degree of maturity that is induced in the APCs. In our study, CD80/86 costimulation provided by DCs was required for an efficient sensitization to OVA, illustrating that DC maturation was necessary for induction of a fully activated T cell response. Although CD80/86$^{-/-}$ DCs were able to induce strong proliferation in naïve OVA specific T cells, these T cells produced virtually no IL-4, IL-13, IL-10 and IFNγ, indicating that no potent effectors were induced. Although T cell division is often measured as a read-out of T cell activation, many attention has recently been paid to the uncoupling of naive T cell proliferation from differentiation to effector T cells[11, 25-30]. A new concept has emerged from the observation that cells receiving a sub-optimal antigen and/or costimulatory stimulus proliferated as strongly as fully activated T cells, but subsequently die by neglect. The term launched by Lanzavecchia's group is 'unfit T cells', representing the idea that T cells stimulated *in vitro* under suboptimal conditions show some signs of activation such as division, but do not develop into pro-inflammatory effector cells and are unable to survive when the amount of antigen becomes limiting[31,32]. Many studies have demonstrated that adoptively transferred TCR-transgenic T cells can proliferate *in vivo* in response to harmless free peptide or to peripheral self antigen but without differentiation to effector function, and even leading to deletional tolerance[22,29,33-35]. In these experiments, using harmless (self) antigens, DC maturation is not induced due to a failure of activation of innate immune receptors on DCs[35, 36]. Following this concept, CD80/86$^{-/-}$ DCs presenting antigen in our studies might resemble immature DCs that do not engage T cell contact of sufficient strength, leading to generation of 'unfit' T cells, which proliferate in response to allergen but do not differentiate into effector T cells. In support of the 'unfit' T cell concept, rechallenge of CD80/86$^{-/-}$ DC immunized mice with antigen did not induce airway inflammation in contrast to mice sensitized with wild type DCs. The partially activated T cells that were induced by CD80/86$^{-/-}$ DCs *in vivo* could not be rescued into effector function by a second injection of wild type DCs, illustrating that they were either deleted or rendered anergic (unpublished

observations). Further T cell tracking experiments can elucidate the fate of these 'unfit' T cells *in vivo*.

The above data imply that DC maturation in the form of CD80/CD86 expression is important for differentiation of naïve T cells into primed Th2 cells in the lung in the process of sensitization. Whether DC maturation and CD80/CD86 expression is also important for activation of already primed T cells in the lung is currently unknown. *In vitro*, primed T cells need less costimulatory molecules compared with naïve T cells for full activation[15,16] and therefore could be activated by less mature DCs or even less professional APCs such as B cells, macrophages and epithelial cells expressing MHC. In support, studies using CD80/86$^{-/-}$ splenic APCs, showed that memory T cells required less costimulation than naïve cells for cytokine production and proliferation in response to Ag encounter[15,16]. Recent papers have shown that CD86 expression is rapidly triggered on airway mucosal DCs after OVA inhalation in sensitized animals[5] and on BAL fluid DCs during ongoing eosinophilic airway inflammation[18]. When isolated from the lung, airway mucosal DCs upregulate CD86 after coculture with effector T cells and not after coculture with naive T cells[5]. It was therefore proposed that Th memory cells mediated this activation, suggesting a role for CD80/86 costimulation during secondary immune response. In our studies we addressed directly through adoptive transfer of DCs whether CD80/86 costimulation was absolutely necessary during the reactivation of primed T cells in the lung at times of allergen challenge, and found that CD80/CD86 expression is redundant. The observation that CD86 is upregulated on DCs during allergen challenge *in vivo* however still suggests some functional role of CD86. Some studies in mouse models of allergy showed that blocking B7-CD28 ligation by CTLA4-Ig could partially or totally inhibit secondary immune responses[21,37-39,40,41]. Other studies showed however that inhibition was not possible using CTLA4-Ig during challenge, particularly when mice were very strongly sensitized by intraperitoneal injection of OVA in alum or when primed Th2 cells were adoptively transferred[42,45]. An explanation for our findings that CD80/CD86 is of less importance when adoptively transferred DCs restimulate primed T cells in the lung would be that other endogenous cells provide CD80/86 costimulation during antigen presentation by transferred CD80/CD86$^{-/-}$ DCs or that antigen would be transferred from injected to endogenous DCs. To exclude this possibility, we treated mice with CTLA4-

Ig before challenge with CD80/86$^{-/-}$ DCs. Mice treated with CTLA4-Ig had a similar number of recovered BALF eosinophils compared with untreated mice, making this mechanism unlikely.

It is therefore likely that other costimulatory molecules besides CD80/CD86 are involved in activating T cells in secondary immune responses. Several molecules on the surface of the DC, like B7RP-1 (also known as ICOSL), OX-40L, 4-1BBL, CD40, and ICAM-1 have been reported to have costimulatory capacity and can be responsible for the induced reaction in the absence of CD80/CD86[45]; a likely candidate in this context is B7RP-1, induced on mature DCs in lung draining lymph nodes[46]. Blockade of ICOS-ICOS-L using an ICOS-Ig fusion protein was able to completely suppress asthmatic features induced by adoptive transfer of OVA-specific Th2 cells to the airways of mice[43]. When we blocked ICOS-ICOSL interaction with an anti-ICOS antibody during CD80/86$^{-/-}$ DC challenge in sensitized mice, this did not reduce significantly airway eosinophilia, although there was a trend towards reduced airway eosinophilia. One explanation for the poor efficacy of ICOS in this model in contrast to previous models would be the recent observation that DCs down regulate the expression of B7RP-1 during eosinophilic airway inflammation[18]. Another costimulatory pathway able to compensate for the lack of CD80/86/B7RP-1 costimulation on DCs, would be OX-40L. OX-40 (CD134), a member of the TNF-R family, is a major regulator of anti-apoptotic proteins such as Bcl-xL and Bcl-2 and strongly promotes the survival of antigen-activated primary CD4^{+} T cells. In addition, OX-40 is preferentially expressed by memory Th2 cells. Blocking OX40-OX-40L interaction impaired all features of asthma induced by adoptive transfer of OVA-specific Th2 cells[47]. Follow-up experiments involving *in-vivo* blockade of OX-40-OX-40L will demonstrate whether this molecule is responsible for the residual stimulation of primed Th2 cells in the combined absence of CD80/86 and ICOSL.

In conclusion, we have shown the dependence on DC-derived CD80/86 costimulation during primary immune responses *in-vivo* in the lung. In the absence of CD80/86 costimulation OVA specific T cells were driven into proliferation but this did not lead to differentiation into effector T cells, illustrating the essential role of CD28 in Th2 differentiation. Secondary immune responses induced by DCs were not dependent on costimulation provided by B7

family members. These results have implications for inhibition of costimulatory molecules as a target for primary prevention strategies in asthma[48]. It will have to be elucidated which costimulatory molecules on DCs or other APCs are absolutely required for a secondary immune response before a strategy of costimulatory blockade can be envisaged as a therapy for established asthma.

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Chapter 7

Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells

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Airway Eosinophils Accumulate in the Mediastinal Lymph Nodes but Lack Antigen-Presenting Potential for Naive T Cells[1]

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Asthma is characterized by infiltration of the airway wall with eosinophils. Although eosinophils are considered to be effector cells, recent studies have reported their ability to activate primed Th2 cells. In this study, we investigated whether eosinophils are capable of presenting Ag to unprimed T cells in draining lymph nodes (DLN) of the lung and compared this capacity with professional dendritic cells (DC). During development of eosinophilic airway inflammation in OVA-sensitized and challenged mice, $CCR3^+$ eosinophils accumulated in the DLN. To study their function, eosinophils were isolated from the bronchoalveolar lavage fluid of mice by sorting on $CCR3^+B220^-CD3^-CD11c^{dim}$ low autofluorescent cells, avoiding contamination with other APCs, and were intratracheally injected into mice that previously received CFSE-labeled OVA TCR-transgenic T cells. Eosinophils did not induce divisions of T cells in the DLN, whereas DC induced on average 3.7 divisions in 45.7% of T cells. To circumvent the need for Ag processing or migration in vivo, eosinophils were pulsed with OVA peptide and were still not able to induce T cell priming in vitro, whereas DC induced vigorous proliferation. This lack of Ag-presenting ability was explained by the very weak expression of MHC class II on fresh eosinophils, despite expression of the costimulatory molecules CD80 and ICAM-1. This investigation does not support any role for airway eosinophils as APCs to naive T cells, despite their migration to the DLN at times of allergen exposure. DC are clearly superior in activating T cells in the DLN of the lung. *The Journal of Immunology,* **2003, 171: 3372–3378.**

Airway mucosal eosinophilia is one of the hallmarks of allergic asthma. Airway eosinophilia is controlled by allergen-specific Th2 cells. In response to Ag presentation by dendritic cells (DCs),[3] Th2 cells release several inflammatory cytokines inducing adhesion molecules on endothelium and recruiting eosinophils to the inflamed airways (1). Eosinophils are considered to be terminal effector cells (1–3). By releasing numerous proinflammatory mediators and cytokines as well as cationic proteins, they damage lung epithelium and account for many of the histopathologic abnormalities of asthma (4). However, studies showing expression of MHC class II (MHCII) and the costimulatory molecules CD80 and CD86 by eosinophils (5–10) suggested a possible function as APCs. Ag presentation by eosinophils is getting more and more consideration. Murine eosinophils derived from bronchoalveolar lavage (BAL) fluid of sensitized and challenged mice were reported to stimulate sensitized Th2 cells in vivo, although stimulation was read out ex vivo (7). Eosinophils derived from the peritoneal cavity of IL-5-transgenic (Tg) mice were capable of sensitizing mice after repeated i.p. injections, although the mechanism of T cell priming was not investigated in a direct manner (8).

As yet no studies have addressed the question whether eosinophils obtained from the bronchoalveolar compartment of inflamed lungs are able to directly activate Ag-specific naive T cells. Therefore, we investigated the Ag-presenting potential of eosinophils for naive T cells in vitro and in vivo and compared it with Ag presentation by professional Ag-presenting DCs. Bronchoalveolar lavage fluid (BALF) eosinophils from allergic lungs were isolated from the lungs of mice with experimental murine asthma using a new flow cytometric sorting method, based on scatter characteristics and staining for the eotaxin receptor CCR3, at the same time avoiding contamination with other BALF APCs such as macrophages, DCs, and B cells (11, 12). To detect T cell priming in vivo and in vitro, T cell proliferation was studied in OVA TCR Tg T cells (DO11.10 T cells) labeled with the mitosis-sensitive dye CFSE. Our data show that although eosinophils accumulate in the draining lymph nodes during eosinophilic airway inflammation and are able to induce some proliferation in effector T cells in vitro, they are not capable of inducing T cell proliferation in OVA-specific naive T cells.

Materials and Methods

Animals

All experiments were performed with 8- to 10-wk-old female BALB/c ($H\text{-}2^d$) mice (Harlan, Zeist, The Netherlands) and DO11.10 mice (Erasmus Medical Center, Rotterdam, The Netherlands). Mice were housed under specific pathogen-free conditions at the animal care facility at the Erasmus University Rotterdam. All of the experimental procedures used in this study were approved by the Erasmus University Committee of Animal Experiments.

Generation of eosinophilic airway inflammation

To induce sensitization to inhaled OVA, bone marrow-derived DCs were pulsed with OVA in vitro and subsequently injected into the airways of naive mice (11). In short, bone marrow cells were cultured for 10 days in tissue culture medium (5% FCS; Biocell Laboratories, Rancho Dominguez, CA), RPMI 1640, gentamicin, 2-ME (all from Life Technologies, Paisley, U.K.) supplemented with 20 ng/ml recombinant murine GM-CSF (13). After 9 days of culture, cells were pulsed overnight with 100 μg/ml OVA (OVA-DC) (OVA, Worthington Biochemical, Lakewood, NJ). On day 10 of culture, cells were collected, washed, and 1×10^6 DCs were injected

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[3] Abbreviations used in this paper: Dc, dendritic cell; MHCII, MHC class II; BAL, bronchoalveolar lavage; BALF, BAL fluid; i.t., intratracheal; Tg, transgenic; PI, propidium iodide.

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intratracheal (i.t.) in naive mice (11), followed 10 days later by a booster of 10 μg OVA absorbed in 1 mg alum adjuvant i.p. injection. Ten days after boosting, mice were challenged four times with a daily 30-min OVA aerosol (grade III, 1% w/v in PBS; Sigma-Aldrich, St. Louis, MO).

Flow cytometric analysis on BALF and lymph node eosinophils

Twenty-four hours after the last aerosol, mice were sacrificed by avertin overdose followed by exsanguination. BAL was performed with 3 × 1 ml of Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1 mM EDTA. RBCs were lysed using ammonium chloride lysis buffer. BALF cells and cell suspensions of lung draining and nondraining lymph nodes were stained with the following monoclonals: MHCII-FITC (2G9), allophycocyanin-labeled CD11c (HL3), and CyChrome-labeled Abs against CD3 (145-2C11) and B220 (RA3-6B2) and PE-labeled CD80, CD86, ICAM-1 (BD PharMingen, San Diego, CA), or CCR3 (83101.111; R&D Systems, Abingdon, U.K.). To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used. Eosinophils were recognized as nonautofluorescent highly granular (SSC^{high}) cells expressing the eotaxin receptor CCR3, intermediate levels of CD11c, and very low or lacking expression of MHCII, B220, and CD3. In indicated experiments, propidium iodide (PI; Sigma-Aldrich) was used to determine viability of the cells.

Purification and Ag pulsing of airway eosinophils

Eosinophils were purified from BALF under sterile conditions on a FACS DIVA flow cytometer (BD Biosciences, Mountain View, CA). BALF cells were stained as described above and eosinophils were recognized and sorted as nonautofluorescent highly granular (SSC^{high}) cells expressing the eotaxin receptor CCR3, intermediate levels of CD11c, and lacking expression of B220 and CD3. Purity of >96% was determined by H&E staining on cytospins of sorted eosinophils. Four hundred cells were counted per cytospin. In some experiments, eosinophils were pulsed ex vivo with 10 μg/ml $OVA_{323-339}$ peptide (Ansynth Service, Roosendaal, The Netherlands) for 45 min at 37°C or cultured overnight with or without GM-CSF (2 ng/ml) in the presence of $OVA_{323-339}$ peptide. The Ag-presenting capacity of eosinophils was compared with that of well-known professional APCs, bone marrow-derived OVA-pulsed DCs grown in GM-CSF as previously described (11–13).

Ag presentation assay to OVA TCR Tg T cells in vivo

Cell suspensions were made of pooled peripheral lymph nodes (cervical, mediastinal, brachial, axillary, inguinal, and mesenteric) from DO11.10 mice and were labeled with the mitosis-sensitive dye CFSE (Molecular Probes, Eugene, OR) as previously described (14). $CFSE^+$ T cells (10 $\times 10^6$) were transferred i.v. into naive mice. Twenty-four hours after T cell adoptive transfer, either 1 × 10^6 purified eosinophils, 1 × 10^6 OVA peptide-pulsed eosinophils, or 1 × 10^6 OVA-pulsed DCs (11, 12) were transferred i.t. or 2 × 10^6 OVA peptide-pulsed eosinophils i.p. Seventy-two hours after immunization, lung draining lymph nodes of recipient mice were analyzed for the proliferation of CFSE-labeled OVA TCR Tg T cells on a FACSCalibur flow cytometer using FlowJo software (Treestar, Costa Mesa, CA). As a control, brachial lymph nodes were also harvested to compare division of T cells in lymph nodes not draining the lung. Transferred OVA TCR Tg $CD4^+$ T cells were recognized as $CFSE^+$/$Kj1.26^+$(MM7504; Caltag Laboratories, Burlingame CA)/$CD4^+$ cells; PI (Sigma-Aldrich) was used to exclude dead cells. To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used.

Ag presentation assay to OVA TCR Tg T cells in vitro

Eosinophils, bone marrow DCs, and OVA TCR Tg T cell suspensions were prepared as described above. To obtain unstimulated T cells, DO11.10 lymph node cell suspensions were depleted from MHCII, B220, CD11b-positive cells by Dynal beads (Dynal, Etten-Leur, The Netherlands) to prevent stimulation by endogenous APCs. To obtain effector T cells, DO11.10 lymph node suspensions were cocultured with OVA-pulsed DCs for 2 wk; fresh DCs were added after 1 wk. After 2 wk, $CD4^+$ cells were isolated using a negative CD4 T cell isolation kit for the auto-MACS (Miltenyi Biotec, Gladbach, Germany) with a purity of 99%, of which 75% had an effector T cell phenotype ($CD62L^{low}$/$CD44^{high}$). The kit was used according to the manufacturer's instructions. Cells were rested overnight in 50 U/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ) before coculture with eosinophils. Fifty thousand $CFSE^+$ unstimulated or effector T cells were cocultured with 1 × 10^3, 1 × 10^4, or 1 × 10^5 either unpulsed or peptide-pulsed eosinophils in comparison to 1 × 10^3, 1 × 10^4, or 1 × 10^5 bone marrow-derived OVA-pulsed or unpulsed DCs. Seventy-two hours later, stimulation of naive T cells or effector T cells was analyzed by staining cocultures with Kj1.26-PE and CD4-APC; PI was used to exclude dead cells. To prevent a specific binding to FcR, 2.4.G2 blocking reagent was used.

Results

OVA exposure induces accumulation of eosinophils in BALF and lung draining lymph nodes of OVA DC-immunized mice

Sensitization was induced by i.t. injection of 1 × 10^6 OVA-pulsed DCs, followed by a booster of OVA/alum i.p. 10 days later. Sensitized mice were challenged with four OVA aerosols. Mice were sacrificed 24 h after the last challenge. Differential analysis of BALF cells showed a distinct increase in eosinophils. Using flow cytometry, several markers were used to determine eosinophils in BALF. Eosinophils were characterized as nonautofluorescent highly granular (SSC^{high}) cells expressing intermediate levels of CD11c and lacking expression of B220 and CD3, as described previously (12) (see also Fig. 3). These highly granular cells also expressed the eotaxin receptor CCR3 (15). Average percentage of eosinophils in BALF after challenge was 40–70%. In addition to the induced eosinophilic airway inflammation, also other hallmarks were induced as goblet hyperplasia and Th2 cytokine production by draining lymph nodes (data not shown).

To investigate whether airway inflammation increased the number of eosinophils in the draining lymph nodes of the lung, where APCs present Ag to naive T cells, lymph nodes were collected, homogenized, and analyzed for the presence of $CCR3^+$ eosinophils. Draining lymph nodes of the inflamed lungs showed an increased number of eosinophils compared with lymph nodes not draining the lungs and to lung draining lymph nodes from naive animals (3.6 × 10^4 vs, respectively, 246 (p = 0.008) and 52 (p = 0.008; Fig. 1).

To determine whether eosinophils in the bronchoalveolar compartment are expressing the molecules needed to prime T cells,

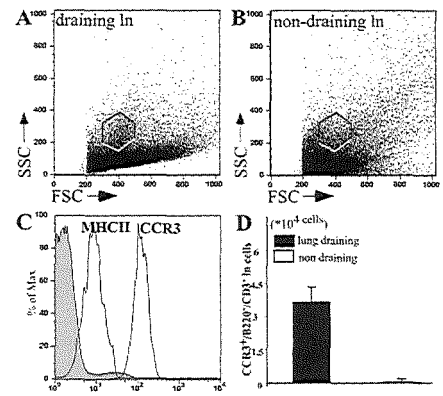

FIGURE 1. Accumulation of $CCR3^+$ eosinophils in draining lymph nodes of the lung. Mice were immunized on day 0 with 1 × 10^6 OVA-pulsed DCs and received an OVA/alum booster injection at day 10. At days 20–23, they were challenged daily for 30 min with OVA aerosols. *A* and *B*, Ungated forward scatter (FSC)/side scatter (SSC) plots of cell suspensions of draining or nondraining lung lymph nodes of OVA-sensitized and challenged mice showing the presence of highly granular (SSC^{high}) cells. A gate was set on these cells (*C*). Within the same gate, these granular cells expressed CCR3 and very weakly MHCII (open histograms). Staining by isotype control IgG2a is indicated by the filled histogram. *D*, $CCR3^+MHCII^{low}$ eosinophils accumulated in draining lymph nodes of the lung and not in nondraining lymph nodes.

eosinophils from allergically inflamed lungs were examined for the expression of MHCII, costimulatory molecules CD80 and CD86, and ICAM-1. Eosinophils showed a very weak expression of MHCII and a distinct expression of CD80 and ICAM-1, but had a minimal expression of CD86 (Fig. 2)

Intratracheal injection of BALF eosinophils does not prime naive OVA TCR Tg T cells in vivo

As eosinophils accumulated in the draining lymph nodes at times of eosinophilic inflammation and expressed the molecules necessary for T cell interaction, we speculated that they might be presenting Ag to OVA-specific T cells. To investigate this further, eosinophils were purified from BALF of sensitized and challenged mice based on characteristics described above. Using this method, a distinction could be made between eosinophils and other (possible) APCs in BALF (Fig. 3) e.g., B cells (Fig. 3*E*, $B220^{high}$ $MHCII^{high}$), macrophages (Fig. 3*F*, large autofluorescent cells), and DCs (Fig. 3*D*, nonautofluorescent $CD11c^{high}MHCII^{high}$ cells) (12). Purity of sort was determined by immunocytochemical staining on cytospins of sorted eosinophils identifying eosinophils by their donut-shaped nucleus and eosin staining of cytoplasm (Fig. 4*B*). After sorting, a purity of >96% was achieved based on flow cytometry and differential cell counts on cytospins (Fig. 4). Purified in vivo-pulsed eosinophils were injected into the trachea of naive mice. Viability of these transferred cells was determined by flow cytometry 3 days after instillation. A distinct $CCR3^+$ eosinophil population could be observed in the BAL compartment, which was PI negative, indicative of viable cells. (20.4% eosinophils of total BAL cells vs 0.7% after OVA-pulsed DC instillation and 0.5% after unpulsed DC instillation) (Fig. 5). Mice received a cohort of CFSE plus OVA TCR Tg T cells by i.v. injection 1 day before i.t. injection of purified in vivo-pulsed eosinophils. BALF eosinophils from allergic mice did not induce divisions of naive T cells in contrast to OVA-pulsed DCs, which induced vigorous T cell division (Fig. 6*A*).

The lack of T cell division after injecting eosinophils obtained from OVA-induced airway inflammation could be due to insufficient uptake of OVA by eosinophils in vivo. To investigate whether eosinophils were able to present processed OVA peptide to naive OVA TCR Tg T cells in vivo, bypassing the need for Ag uptake and processing, eosinophils were pulsed in vitro with OVA peptide after sorting. Intratracheal injection of in vitro-pulsed eosinophils in mice that received $CFSE^+$ T cells i.v. did not induce divisions (Fig. 6*B*). The absence of T cell division was not due to a lack of responsiveness of our T cells as i.t. injection of DCs induced an average of 3.7 divisions in 45.7% of the T cells.

To exclude the possibility that eosinophils from the BALF were incapable of priming naive T cells because of their inability to migrate to the draining lymph nodes, 2×10^6 peptide-pulsed eosinophils were injected i.p. because i.p. injection of cells leads to localization of cells in the thoracic lymph nodes by peritoneal drainage (16). Eosinophils still did not induce divisions of T cells when injected i.p. (Fig. 6*C*).

BALF eosinophils do not prime naive OVA TCR Tg T cells in vitro in contrast to effector T cells

To investigate the Ag-presenting capacity of eosinophils in direct contact with OVA TCR Tg T cells, 5×10^4 $CFSE^+$ $MHCII^-$ $B220^-CD11b^-$ unstimulated T cells were cocultured with 1×10^3, 1×10^4, or 1×10^5 fresh in vivo-pulsed BALF eosinophils or in vitro OVA-pulsed eosinophils (Fig. 7*B*, *i-vi*). After 72 h, no

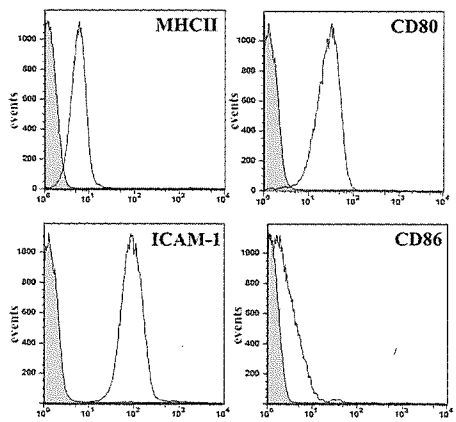

FIGURE 2. Expression of molecules associated with Ag presentation on BALF eosinophils. Eosinophils were recognized as highly granular, low autofluorescent cells that lacked expression of CD3 and B220. Staining revealed a very low expression of MHCII, a distinct expression of CD80 and ICAM-1, and a minimal CD86 expression. Staining by isotype control Abs is expressed as filled histograms.

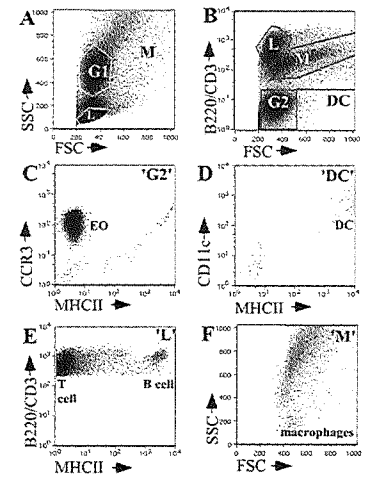

FIGURE 3. Isolation of BALF eosinophils without contamination of other APCs. BALF cells of OVA-sensitized and challenged mice were stained to isolate eosinophils (EO) using flow cytometry. *A*, The forward scatter (FSC)/side scatter (SSC) plot of ungated BALF cells contains granulocytes (G1) and lymphocytes (L) and macrophages (M). *B*, By staining with B220 and CD3, several BALF cell populations could be discriminated. Gates were set on these populations and analyzed further by additional staining or by scatter characteristics. Used gates are displayed in the *upper right corner*. *C*, To isolate eosinophils, a gate was set on low autofluorescent cells that lacked expression of CD3 and B220 (G2). Within this gate, CCR3 expression made it possible to distinguish eosinophils. BALF eosinophils expressed MHCII very weakly. *D*, Other APCs could be identified as $MHCII^{high}CD11c^{high}$ cells representing DCs (*B* and *D*), whereas B cells can be identified in the L gate from T cells as $MHCII^{high}$ (*B* and *E*), and alveolar macrophages (M) as large and spontaneously autofluorescent cells (*B* and *F*).

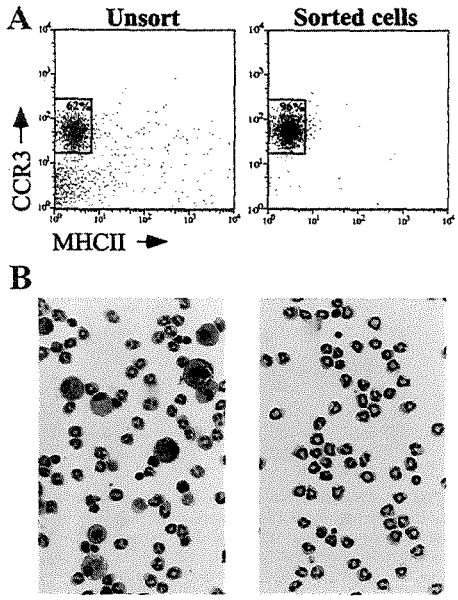

FIGURE 4. Sorting eosinophils from BALF. Cells were sorted as $CCR3^+$, low autofluorescent $CD3^-B220^-$ cells as described in Fig. 3. *A, Left,* MHCII vs CCR3 on ungated cells before sorting. *Right,* MHCII vs CCR3 on ungated cells after sorting. *B,* Cytospins of unsorted and sorted populations were analyzed for the number of eosinophils identified by an eosinophilic cytoplasm and a donut-shaped nucleus. *Left,* Sixty-five percent of cells were identified as eosinophils before sort; *right,* 96% of cells were identified as eosinophils after sort.

divisions of T cells were induced. In contrast, OVA-pulsed DCs induced a vigorous proliferation of 80% of T cells with an average of 3.8 divisions. (Fig. 7*A*).

Eosinophils are reported to present Ag to already sensitized T cells (7, 8). To investigate whether BALF eosinophils do have the capacity of stimulating sensitized T cells, 1×10^3, 1×10^4, or 1×10^5 fresh in vivo-pulsed BALF eosinophils or in vitro OVA-pulsed eosinophils were cocultured with effector $CD62L^{low}CD44^{high}$ OVA TCR Tg T cells that were obtained by in vitro stimulation by

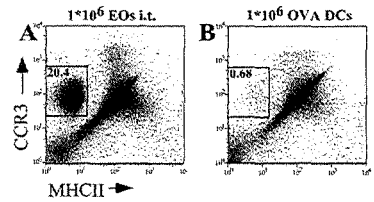

FIGURE 5. Viability of eosinophils from BALF after i.t. injection. Sorted eosinophils were injected i.t. in naive mice and 3 days later, lack of PI uptake by BALF eosinophils was determined as a marker for viability. *A,* Eosinophils were identified as $CCR3^{high}MHCII^{low}$ cells in the granulocyte gate and were all low in PI uptake, indicative of viable cells. By contrast, mice that received 1×10^6 OVA-DCs had no eosinophils in the BALF (*B*) EO, eosinophil.

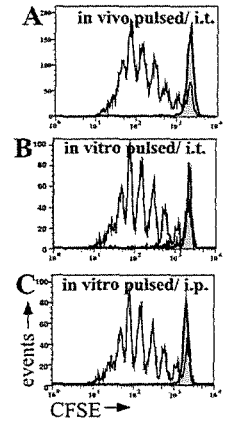

FIGURE 6. Ag presentation assay in vivo. Ag-presenting capacity of eosinophils was investigated by injecting eosinophils i.t. in mice that received CFSE-labeled OVA TCR Tg T cells i.v. 1 day earlier. Division of T cells induced by eosinophils was determined by FACS analysis (filled histograms). OVA-pulsed bone marrow-derived DCs were used as control APCs (open histograms). *A,* Eosinophils exposed to OVA in vivo by aerosols were not capable of inducing proliferation of naive T cells. *B,* Exposing eosinophils in vitro to OVA peptide, avoiding the need for Ag capture and processing, still did not induce priming capability for naive T cells in eosinophils. *C,* Intraperitoneal injection of OVA peptide-exposed eosinophils did not prime naive T cells. Plots shown are representative of multiple experiments with four to eight mice per group.

OVA-pulsed DCs. Both in vivo- and ex vivo-pulsed eosinophils were able to induce some proliferation in effector T cells but not as vigorous as that induced by OVA-pulsed DCs (Fig. 8*B*: resp. 1 APC: 5 T cells: 16.9 and 16.1% compared with 88.5% by DCs, 2 APC: 1 T cell: 31.4 and 37.8% compared with 70% by DCs). Experiments with or without adding 5 ng/ml GM-CSF to the culture medium yielded similar proliferation results (data not shown). T cells cultured without APCs did not divide (Fig. 8).

Expression of molecules associated with Ag presentation on BALF eosinophils after stimulation with GM-CSF

Since naive T cell priming was not properly induced by freshly sorted eosinophils in vitro or in vivo, eosinophils were incubated overnight with GM-CSF to enhance Ag-presenting capacities as shown by others. We sorted $CCR3^+$ eosinophils from lavage fluid of sensitized and challenged mice and incubated these cells for 1 day with GM-CSF before culture with CFSE-labeled OVA TCR Tg T cells in the presence or absence of in vitro OVA peptide pulsing. Eosinophils cultured in GM-CSF did not differ in MHCII staining compared with eosinophils cultured in medium and accordingly failed to induce T cell division readily observed following stimulation with DCs (data not shown). No difference in viability between eosinophils cultured with or without GM-CSF was observed, both conditions yielded ~99% PI^- viable eosinophils after culture with T cells (data not shown).

Discussion

For several years, it has been described that eosinophils are able to express MHCII and costimulatory molecules and are able to migrate to the draining lymph node T cell area, suggesting that they

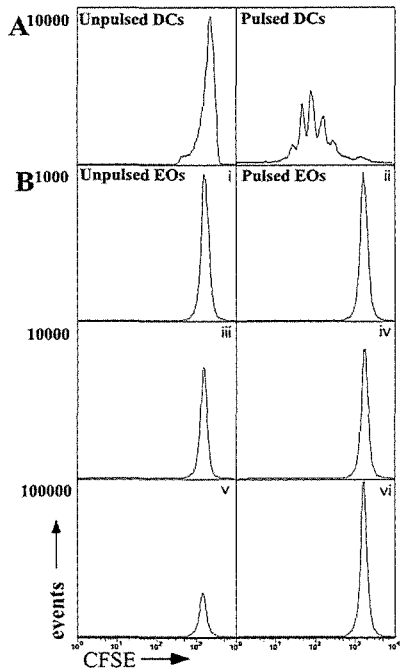

FIGURE 7. In vitro eosinophil Ag-presenting capacity to naive T cells (*B*). In vivo OVA aerosol exposed (indicated by unpulsed: *i*, *iii*, and *v*)- and in vitro OVA peptide-exposed eosinophils (indicated by pulsed: *ii*, *iv*, and *vi*) were cocultured with 50,000 naive T cells. Eosinophils (EOs) were added in different concentrations to naive T cells: 1×10^3,1×10^4, or 1×10^5. With up to two eosinophils per naive T cells, there was still no proliferation of naive T cells (*v* and *vi*). *A*, Bone marrow-derived DCs induced in 80% of T cells an average of 3.8 divisions only when DC were pulsed with OVA.

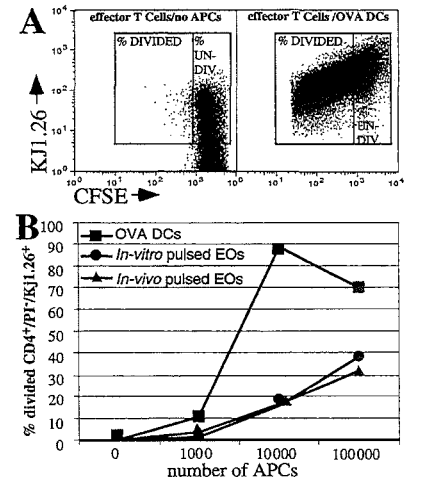

FIGURE 8. In vitro eosinophil Ag-presenting capacity to effector T cells Effector $CD62L^{low}CD44^{high}$ OVA TCR Tg T cells (5×10^4 cells/well) obtained in vitro by OVA DC stimulation of naive OVA-specific T cells were cocultured with 0, 1×10^3, 1×10^4, or 1×10^5 freshly isolated eosinophils (EOs) from BALF of OVA-sensitized and challenged mice. *A*, *Left*, A gate was set around $Kj1.26^+$ T cells cultured without APCs after gating on PI^-CD4^+cells to define undivided T cells. *Right*, Divided T cells were defined as $Kj1.26^+$ cells with low CFSE content. The limit to define undivided T cells was set on the CFSE content of unstimulated T cells. *B*, Eosinophils were either pulsed in vivo by OVA aerosols or ex vivo by OVA peptide pulsing followed by vigorous washing. Ex vivo- and in vivo-pulsed eosinophils were both able to induce some proliferation at a ratio of one or two eosinophils per effector T cells but induced proliferation in a lesser degree than OVA-pulsed DCs. Effector T cells cultured without any APCs did not divide.

might be endowed with Ag-presenting capacity. In our experiments, an accumulation of $CCR3^+$ eosinophils in draining lymph nodes of the lung was indeed observed at a time point when eosinophils were accumulating in the allergically inflamed lung, suggesting that eosinophils were migrating from the lung to the lymph nodes where T cells are recirculating. These findings are consistent with earlier reports by other groups and indeed suggest some Ag-presenting function (7–10). In our study, in addition to the presence of eosinophils at the site of T cell encounter, BALF eosinophils weakly expressed MHCII and expressed high levels of costimulatory molecules CD80 and ICAM-1. Functional studies have been performed to investigate Ag presentation of BALF eosinophils, but these studies focused mainly on the stimulation of polyclonal primed T cells and did not directly address T cell activation in vivo (7, 8).

This stimulation of primed T cells by eosinophils led us to investigate whether eosinophils obtained from an inflammatory site were capable of sensitizing naive Ag-specific T cells in vivo. We isolated eosinophils from the bronchoalveolar compartment of the inflamed lungs of OVA-sensitized and challenged mice (12, 14). Isolated eosinophils that were exposed to OVA Ag by aerosol exposures in vivo were introduced in the airways of naive mice that received CFSE-labeled OVA TCR Tg T cells i.v. 1 day earlier. Eosinophils injected into the airway lumen have been shown to reach the T cell area of the draining nodes of the lung (7). The adoptive transfer of CFSE-labeled Ag-specific T cells enabled us to investigate the Ag-presenting capacity of eosinophils to unprimed Ag-specific T cells in the draining lymph nodes of the lung by visualizing the number of T cell divisions in vivo (14). This system is extremely sensitive in detecting the presence of APCs. After 3 days, there was no induction of proliferation of T cells in the draining lymph nodes of the lung while OVA-pulsed DCs, injected i.t. as a positive control, induced proliferation in 45.7% of T cells, some cells reaching six to seven divisions. The absence of T cell priming was not due to the death of transferred eosinophils, as 20.4% of total BALF cells were viable eosinophils. One possible explanation for the lack of T cell priming could be that eosinophils from the BALF were not capable of capturing and processing sufficient OVA. To bypass the need for Ag uptake and processing, eosinophils were pulsed with OVA peptide in vitro. Intratracheally injecting in vitro-pulsed eosinophils still did not induce any divisions of OVA TCR Tg T cells. Still, these findings cannot exclude that eosinophils might have some Ag-presenting function. Isolated BALF eosinophils injected into the trachea had to migrate from the trachea through the epithelial barrier toward draining lymph nodes to sensitize naive T cells. Since the migration potential of eosinophils might be lower in noninflamed lungs,

an alternative route of injection was chosen in our studies. Material injected in the peritoneal cavity is drained nonspecifically to the thoracic lymph nodes (16). Therefore, we injected sorted OVA peptide-pulsed eosinophils i.p. to allow eosinophils to reach the draining lymph nodes, but still observed no divisions in Ag-specific T cells.

Finally, to exclude that the number of eosinophils encountering naive T cells was not sufficient to induce priming and to exclude that absence of migration was causing the absence of T cell activation, T cells were cultured in vitro with sorted eosinophils in a very high stimulator to the responder setting. In vitro data were in accordance with in vivo data, showing no Ag presentation despite these permissive conditions of naive T cell activation. In contrast, a proliferation of naive T cells by OVA-pulsed DCs was almost 80% of T cells in vitro (compared with 45.7% of T cells in vivo) at very low stimulator:responder ratios. To verify whether these eosinophils were capable of some Ag presentation, eosinophils were also cocultured with in vitro-obtained effector OVA-specific T cells. These eosinophils were capable of stimulating effector T cells, in accordance with other studies (7), although less strongly than proliferation induced by professional Ag-presenting DCs. Together these data suggest that freshly isolated eosinophils from the BAL compartment have no evident Ag-presenting capacity for naive T cells, whereas professional Ag-presenting DCs are clearly able to activate unstimulated T cells.

What then might be the explanation for this lack of APC function to naive T cells while at the same time eosinophils induced some proliferation in primed T cells? In accordance with other studies, the isolated BALF eosinophils used in this study expressed CD80 and ICAM-1 involved in T cell costimulation (7). However, they consistently expressed MHCII at very low levels in the bronchoalveolar compartment where the lung encounters inhaled Ag and did not up-regulate MHCII following their migration to the draining nodes or after overnight culture with or without GM-CSF. Naive T cells have more stringent requirements for signal 1 and signal 2 compared with effector T cells, which might explain the difference.

There are some conflicting data about MHCII expression by eosinophils. In humans, expression of the molecules for T and B cell interactions was only reported on eosinophils in allergen-challenged airways, but not on activated blood eosinophils (6, 17, 18). An explanation for this phenomenon could be that cytokines secreted by allergic lung cells could up-regulate MHCII expression on eosinophils. Several cytokines like IFN-γ, IL-3, and GM-CSF secreted by inflammatory cells are reported to induce MHCII expression in vitro (5, 17, 19). In support of this, eosinophils from the peritoneal cavity of IL-5 Tg mice that were purified using Percoll gradients and adherence steps were reported to have no MHCII expression, except when cultured with GM-CSF (19). Others showed that BALF eosinophils isolated using Percoll gradients and adherence culture weakly expressed MHCII, probably because of GM-CSF release in vivo (7). This is unlikely in our studies, as we have shown in previous experiments that GM-CSF levels in BAL are below detection limit (12). Similarly, eosinophils from BALF isolated based on forward and side scatter characteristics and light polarization were reported to have a low expression of MHCII, although at higher levels than on the eosinophils described in this study (8). In all of these studies, eosinophils were first purified using extensive protocols, before MHCII staining was performed, suggesting that MHCII might have been up-regulated ex vivo. In our hands, fresh eosinophils in lavage fluid, identified based on scatter characteristics and CCR3 staining, had a very weak MHCII expression that was only able to stimulate effector T cells in very high stimulator:responder ratios. Culture of highly purified BALF eosinophils, specifically in GM-CSF, did not enhance MHCII expression (data not shown). Perhaps the isolation method can attribute for the difference found in MHCII expression.

Others have demonstrated T cell stimulation of primed polyclonal T cells by peritoneal eosinophils and alveolar eosinophils in vitro. Proliferation of primed T cells in vitro was only induced by eosinophils stimulated with GM-CSF and not by directly isolated eosinophils (7, 19). In this study, we confirmed these findings using OVA-specific TCR Tg T cells and showed that freshly isolated BALF eosinophils were able to induce some proliferation in effector T cells but less strongly compared with professional DCs (Fig. 8).

We took great care to isolate eosinophils without contamination of other APCs. We developed a new more accurate purification method. By using flow cytometric scatter characteristics, multiple positive and negative markers, and taking advantage of the autofluorescence of macrophages, it was possible to sort $CCR3^+$ eosinophils while avoiding B cells, T cells, macrophages, and DCs, preventing contamination with these APCs (12, 15). In this study, Ag presentation of $CCR3^+$ purified eosinophils from the BALF to naive T cells was investigated directly in vitro and in vivo with a very sensitive readout, using naive OVA TCR Tg T cells labeled with CFSE, showing no direct Ag presentation. One other study has also investigated the potential of eosinophils to induce priming in a naive setting. In this study, eosinophils from the peritoneal cavity of IL-5 Tg mice were capable of sensitizing mice when injected repeatedly in the peritoneal cavity. After subsequent challenge with OVA aerosols, eosinophilia could be observed, and thus it was concluded that eosinophils induce T cell responses to OVA (8). These studies did not directly address when and how T cell priming occurred however. One possibility could be that repeated injection of Ag-carrying eosinophils led to uptake of Ag by endogenous professional APCs such as DCs. DCs have been shown to capture Ag from apoptotic cells, leading to Ag presentation to naive CD4 and CD8 T cells (20). Moreover, injected IL-5 Tg eosinophils in the peritoneum reach the thoracic lymph nodes by nonspecific drainage (16) or specific migration, introducing a source of IL-5 to the site of challenge. This higher level of IL-5 production in the lung could have lowered the threshold for development of eosinophilic airway inflammation induced by eosinophil immunization and/or subsequent OVA challenge, as Il-5 is known as an important chemoattractant and differentiation factor for eosinophils (1).

If eosinophils do not seem to activate naive T cells in the draining nodes, what then might be the role of eosinophil accumulation in the lung draining lymph nodes of allergic mice? One possibility would be that they stimulate already primed T cells (7, 8) and not naive T cells. It has been shown that the requirements for naive T cell activation are more stringent than for primed T cells, and indeed the very low MHCII expression level in our model did not induce proliferation in naive T cells but allowed induction of some proliferation of effector T cells, although not as strongly as professional DCs. Alternatively, eosinophils might indirectly effect Ag presentation by DCs by secreting cytokines and mediators. Studies that have used anti-IL-5 to eliminate eosinophils systemically from the body have however observed no obvious change in the levels of Th2 cytokine secretion in draining nodes or effector site, arguing against an absolute requirement for eosinophils in the stimulation of primed Th2 cells (21).

Together these data imply that although eosinophils accumulate in the draining nodes at times of eosinophilic airway inflammation and do express CD80, ICAM-1, and very low levels of MHCII,

they do not present Ag to naive T cells. These results do not diminish their evident role as effector cells in asthma. Further elucidation of the most important aspects of eosinophil biology in ongoing airway inflammation is necessary to develop an effective intervention therapy targeted to these cells.

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Chapter 8

General Discussion and Conclusion

This Thesis

Dendritic cells (DCs) are generally accepted to be involved in the induction of the primary immune response by provision of signal I (interaction of MHC bound allergen and the TCR), and of signal II (costimulation) to T cells and their potential to migrate to the draining lymph nodes (**Chapter 1**)[1].

In this thesis, we specifically addressed whether DCs have a role beyond sensitization. To elucidate the role of dendritic cells during a secondary immune response to inhaled antigen, we used a DC driven murine model for asthma[2]. Balb/c mice were sensitized to OVA by an intratracheal injection of OVA pulsed bone marrow-derived DCs. Ten days post-sensitization, mice were challenged with OVA aerosols inducing an eosinophilic airway inflammation as shown by histological analysis of lungs revealing peribronchial and perivascular inflammatory infiltrates, goblet cell hyperplasia, increased numbers of eosinophils in bronchoalveolar lavage fluid and Th2 cytokine production by draining lymph nodes of the lung. This murine model of asthma with its advantages and disadvantages is described in detail in **Chapter 2.**

In **Chapter 3,** a new flow cytometric method was first described to study more carefully the different inflammatory cells in the bronchoalveolar lavage fluid. The degree and type of airway inflammation is often studied by determination of cellular composition of bronchoalveolar lavage fluid cells. Especially the number of eosinophils in BALF is often used as an indicator for the type of immune response induced and correlates very well to the degree of inflammation in the airways. The differentiation of total BALF cells into macrophages, lymphocytes, eosinophils and neutrophils is generally determined by morphological analysis of BALF cell cytospins stained with May-Grünwald Giemsa. We developed a new flow cytometric analysis in which we were able to identify eosinophils, dendritic cells, B cells, T cells, macrophages and neutrophils in one staining. This new flowcytometric method proved to be a time saving, investigator independent, and a more detailed method for general use in experimental asthma research compared with morphological analysis of cells on cytospins. In addition, identification of cells based on fluorescently labeled antibodies, provides a tool to isolate pure populations from BALF for further investigation (Chapter 7). This new method was used throughout the thesis to determine and isolate various populations of BALF cells, with a special emphasis on antigen presenting cells.

In **Chapter 4**, we showed for the first time that the number of dendritic cells in the bronchoalveolar compartment was increased in OVA-sensitized and challenged mice, in an

allergen dose-dependent way. The increased presence of DCs at the luminal side could imply an enhanced antigen uptake and subsequent migration to draining lymph nodes. Migration patterns of DCs from the tracheal lumen to the draining lymph nodes of the lung indeed revealed an enhanced and faster migration kinetic during inflammation compared with migration of DCs during steady-state. Therefore, a reduced migration of DCs to the draining nodes was not the reason for the observed increase in tissue DCs during allergen challenge.

The number of DC precursor $CD11b^+$ monocytes was also increased in peripheral blood of OVA-sensitized and challenged mice compared with control mice, which indicated an increased output of the bone marrow of dendritic cell progenitors analogous to the described increase in eosinophil progenitor output during allergen induced airway inflammation[3-6]. To investigate this possibility, we stained bone marrow with antibodies for CD31 (PECAM-1) and Ly-6C, known to distinguish between 6 discrete populations in bone marrow, each with different lineage commitment and differentiation potential[7]. Mice with an eosinophilic airway inflammation showed a dose dependent increase in the population of $CD31^{hi}Ly\text{-}6C^{lo}$ early myeloid progenitor cells in bone marrow. After purification, this population was shown to contain cells with potential to develop in dendritic cells after culture in GM-CSF but also into eosinophils after culture in IL-5. These results suggested that a serum factor was secreted from cells at the site of airway inflammation and that this factor had a systemic action. It is noteworthy that the eotaxin level was doubled in serum of mice with an airway inflammation and that $CD31^{hi}Ly\text{-}6C^{lo}$ cells expressed the eotaxin receptor CCR3, suggesting that eotaxin could be a candidate serum factor for enhancing output of bone marrow progenitors. It has indeed been shown that eotaxin acts as a granulocyte-macrophage colony forming unit enhancer[8]. Future studies, which have been initiated in our laboratory will study the functional role of CCR3 on enhancing the output of BM precursors, by using small molecular weight CCR3 antagonist drugs.

We also observed that during allergic airway inflammation, there was an increase in mature circulating $CD11b^+CD11c^+$ DCs in the bloodstream. We do not know at present whether these cells are derived from the airway inflamed tissues or whether they represent DCs that have matured locally in the bloodstream as a result of exposure to circulating maturation factors. In contrast, allergen challenge of human asthmatics leads to a reduction in circulating DCs during acute allergen challenge[9], suggesting that mature DCs are recruited from the bloodstream to the lung. The difference could be that we observed increased numbers of DCs after repetitive days of inflammation, whereas human studies used fairly acute challenge models.

In **Chapter 5**, we investigated the functional role of the dendritic cell increase seen during the effector phase of the immune response to inhaled allergen challenge. First we noticed that DCs formed clusters with memory CD4 T cells within the lung itself in areas of intense eosinophilic airway inflammation. This already strongly suggested to us that these cells were functionally interacting and that DCs were necessary for generating effector function in the primed T cells. To investigate the role of DCs during the activation phase of eosinophilic airway inflammation, we depleted lung dendritic cells in sensitized mice before OVA aerosol challenge. We used CD11c Diphtheria toxin receptor (DTR) transgenic mice in which $CD11c^{+}$ airway dendritic cells can be depleted for about 48 hours by exposing the mice intratracheally to diphtheria toxin[10]. In transgenic mice, DC depletion by diphtheria toxin treatment decreased the number of BALF eosinophils, eosinophilic airway inflammation, Th2 cytokine production and bronchial hyperreactivity compared with non-transgenic littermates. This suggested that dendritic cells are required for proper activation of memory T cells and for consecutive induction of an effector T cell response to inhaled antigen, leading to an eosinophilic airway inflammation. In addition, these experiments suggested that other antigen presenting cells present in the lung like B cells, macrophages and epithelial cells were not capable of compensating the absence of DCs.

From *in-vitro* experiments, it has been shown that effector T cells are less dependent on costimulation[11]. This would imply that during an ongoing inflammation when effector T cells are present, other APCs providing less costimulation than DCs are sufficient to stimulate allergen-primed T cells. To investigate the requirement of DCs *in-vivo*, we depleted DCs in an ongoing inflammation when effector T cells are already present and fully functional in the airway. DC depletion in animals with an already established eosinophilic airway inflammation, decreased the number of eosinophils, Th2 cytokine production, inflammatory peribronchial and perivascular infiltrates and mucus production by goblet cells in response to inhaled allergen compared with sham depleted mice. In the absence of DCs, allergen was not presented by other APCs present in the lung as the airway inflammation that was observed when depleting DCs during ongoing allergen challenge fell back to the level of airway inflammation when mice were challenged with saline, containing no allergen. In support of this, we could not measure any boosting of Th2 responses when DCs were depleted during allergen challenge. Also adoptively transferred *in vitro* primed Th2 cells did not produce effector cytokines in response to *in vivo* allergen challenge in the absence of DCs.

In mice with an eosinophilic airway inflammation in which antigen exposure was ceased after a few days of exposure, clearance of eosinophilic airway inflammation was faster in the

presence of DCs compared with the situation when DCs were depleted (unpublished results). During the induction of an immune response to inhaled allergen, there are recent studies showing that DCs can instruct differentiation of naïve T cells into regulatory T cells[12-14], and can stimulate the function and expansion of regulatory T cells[12, 15]. This suggested a regulatory function of DCs during the resolution of inflammation. It is speculative to explain this difference in clearance of eosinophilic airway inflammation through the induction of regulatory T cells by residual DCs. An alternative explanation might be that effector T cells die by neglect when the amount of antigen becomes limiting and that this process is stimulated when DCs are still present[16]. Another explanation could be a disturbed balance between subtypes of airway DCs, in which one subset (e.g. myeloid DCs) has a stimulatory capacity and another (plasmacytoid) a suppressive capacity by either inducing apoptosis of effector T cells or induction of regulatory T cells[17]. During allergen challenge, the stimulatory subset might prevail while during resolution of inflammation, the regulatory subset takes over leading to faster resolution. Following this theory, depletion of this regulatory subset in our experiments might have led to a sustained airway inflammation.

In the second part of chapter 5, we investigated whether DCs have the intrinsic capacity to induce a secondary immune response in sensitized mice. Bone marrow-derived DCs were intratracheally injected in sensitized mice. Dendritic cells were capable of inducing an eosinophilic airway inflammation as shown by histological analysis of lungs, and the increase of the number of BALF eosinophils. In DC-challenged mice, an enhanced Th2 cytokine production of lung draining lymph nodes was noticed, leading to bronchial hyperreactivity and to goblet cell hyperplasia, phenomena clearly associated with the Th2 cytokine IL-13. DCs did not induce airway eosinophilia by themselves but needed lymphocytes for this process, as exemplified by the absence of the response in SCID mice without T and B cells.

The capacity of DCs to induce a secondary immune response in sensitized mice offers the opportunity to modify DCs to investigate factors provided by DCs for their importance for inducing a secondary immune response without effecting other involved inflammatory cells. This new model allowed us to address the controversial matter of discussion about the requirement of costimulation by DCs for activating memory and effector T cells. Previous *in vitro* studies showed that effector T cells need less CD80/86 costimulation[18]. However, we reported in Chapter 4 and 5 that DCs seem to be important during a secondary immune response *in vivo*, which suggests that also memory and effector T cells need APCs which can provide costimulation. Therefore, we investigated the *in-vivo* requirement of CD80/86 costimulation provided by DCs for mounting secondary immune responses.

In **Chapter 6,** we used CD80/86$^{-/-}$ bone marrow-derived DCs to investigate the importance of CD80/86 costimulation for inducing a secondary immune response *in-vivo*. In contrast to the observed requirement for CD80/86 costimulation to prime naïve T cells, injection of CD80/86$^{-/-}$ DCs in wild type sensitized mice led to a severe eosinophilic airway inflammation, similar to the one induced by wild type DCs. Costimulation by ligation of CD80/86 on DCs seems therefore not to be important for inducing a secondary immune response. We examined the possibility that antigen was taken up by endogenous DCs[19, 20], and that this was the reason for the observed inflammation. However, when we blocked CD80/86 expression on endogenous cells by injecting CTLA4-Ig during challenge with CD80/86$^{-/-}$DCs, a similar number of eosinophils was recovered from BALF with or without injecting CTLA4-Ig.

However, it is likely that other costimulatory molecules provided by dendritic cells are important during the secondary immune response. The CD28 family member molecule ICOS is expressed on antigen-stimulated T cells, making ICOS-L a likely candidate molecule for providing costimulation[21-23]. However, blockade of ICOS by administering ICOS-Ig simultaneously with challenge with CD80/86$^{-/-}$ DCs decreased the number of eosinophils in the BALF, although not significantly, suggesting that ICOS-ICOS-L interaction is not fully compensating for CD80/86-CD28 interaction. Another likely candidate would be the TNF-R family member OX-40L. OX-40 (CD134) is preferentially expressed by memory Th2 cells[24]. OX-40 is a major regulator of anti-apoptotic proteins such as Bcl-2 and Bcl-xL and strongly promotes the survival of antigen-activated primary CD4^{+} T cells[25]. Blocking OX-40 before antigen challenge revealed the dependence on OX-40 for all aspects of lung inflammation driven by Th2 memory T cells[24].

Further experiments will have to elucidate which costimulatory signals provided by DCs are involved in the secondary immune response. Although dendritic cells seem to be very important for secondary immune responses, perhaps also other APCs can contribute to the allergen presentation.

In **Chapter 7,** we addressed the question whether eosinophils are able to present antigen in the lung. Eosinophils are generally thought of as effector cells, responsible for releasing cationic proteins which are toxic to the intruding microorganism but also damage the epithelium[26]. Recent studies showed MHCII and costimulatory molecules CD80 and CD86 expression on eosinophils[27,28], suggesting an antigen presenting role for eosinophils. In support, some mouse models showed that eosinophils induce proliferation in effector Th2 cells[29] or were able to sensitize mice to allergen[30]. However, the eosinophil purification method used in the studies mentioned above did not exclude contamination with professional

APCs such as B cells, DCs and macrophages. Nonetheless, taking into account the large number of attracted eosinophils in the lungs during an ongoing inflammation, the antigen presenting capacity of eosinophils had to be investigated before we could state that dendritic cells are the most important APCs during inflammation. In mice with allergen induced airway inflammation, eosinophils were recruited to the draining lymph nodes and expressed CD80 and ICAM-1, suggesting that eosinophils indeed might be endowed with antigen presenting capacity. However, MHCII was only marginally expressed. To determine the antigen presenting capacity of airway eosinophils, we isolated BALF eosinophils of mice with an allergen induced airway inflammation using the method described in Chapter 3 to avoid contamination with other APCs present in the BALF. Eosinophils were intratracheally injected in mice that previously received CFSE-labeled unstimulated OVA TCR transgenic T cells. Eosinophils did not induce divisions of T cells in the lung draining lymph nodes in contrast to DCs. To circumvent the need for antigen processing or migration *in vivo*, eosinophils were pulsed with OVA peptide but were still not able to induce T cell priming *in vitro*, whereas DCs induced vigorous proliferation. In contrast, eosinophils were able to induce some proliferation in *in-vitro* obtained effector T cells, although not as strongly as professional DCs. The role of eosinophils in the draining lymph nodes is not clear. We cannot exclude that the eosinophil population in the draining lymph nodes is functionally different from the BALF eosinophils and is capable of presenting antigen to naïve T cells. Possibly, draining lymph node eosinophils might contribute indirectly to T cell activation induced by DCs, by secreting cytokines and mediators.

In conclusion, the work presented in chapters 3-7 illustrates the involvement of dendritic cells in inducing and maintaining a secondary immune response in the lung. According to Koch's postulates modified to this pathology, three criteria are required to determine if a cell plays a crucial role in the pathogenesis of a disease. In this modern version[31], these postulates state that to prove causality between a cell and a disease, the number of cells has to be increased during the disease, introduction of the cell in a healthy animal has to cause the disease and removing the cell will reduce severity of the disease. In Chapter 4 and 5, we have demonstrated that increased numbers of DCs are present in the airways in close proximity to T cells (postulate 1), which is supported by an increased production of dendritic cell progenitors in bone marrow. In Chapter 5, we have demonstrated that introduction of allergen pulsed dendritic cells in already sensitized animals is sufficient to induce an eosinophilic airway inflammation in the lung (postulate 2). In chapter 5, we also showed that depletion of

dendritic cells in sensitized mice as well as in mice with a full blown eosinophilic airway inflammation, decreased the development of cardinal features of asthma such as Th2-mediated eosinophilic airway inflammation, bronchial hyperreactivity and goblet cell hyperplasia. (postulate 3). All criteria have been met to conclude that dendritic cells have a functional contribution to the establishment and maintenance of the eosinophilic airway inflammation. In Chapter 7, we rejected the hypothesis that eosinophils are able to present antigen and therefore in the asthmatic lung could compete with dendritic cells as antigen presenting cell. Comparison of BALF eosinophils and bone marrow-derived dendritic cells in stimulating OVA specific T cells revealed that dendritic cells are superior in antigen presentation to both naïve and effector T cells.

Interaction of dendritic cells with other cell types contributing to the maintenance of eosinophilic airway inflammation in asthma

This thesis has focused mainly on the role of one cell type, the dendritic cell as antigen presenting cell, in the induction and maintenance of allergen induced eosinophilic airway inflammation. Obviously, this cell does not operate independently but is part of a complex interaction of different cell types that are involved through the secretion of cytokines and mediators in the maintenance of eosinophilic inflammation. Indeed, mast cells, epithelial cells, eosinophils, basophils, fibroblasts, smooth muscle cells and macrophages have all been proposed to play an essential role in asthmatic inflammation (see Chapter 2). The following paragraph will discuss the way in which dendritic cells can be influenced by several other 'players of the game', leading to a possible mechanism by which dendritic cells might maintain chronic airway inflammation.

In the airway, dendritic cells reside in close proximity to epithelial cells[32,33]. The close proximity of airway epithelial cells and dendritic cells suggest that these cells interact and that epithelial cells are a likely candidate for influencing DC responses (See Fig 1). The epithelium constitutes the interface between the internal milieu and the external environment, and as such, it is the first point of contact for inhaled antigens. The major function of the epithelium was once thought to be primarily that of a physical barrier. However, there is more and more evidence that epithelial cells are directly involved in the outcome of several immune responses by secretion of inflammatory cytokines and growth factors[34,35]. Dendritic cells can be influenced by epithelial cells in different ways[36] (See Fig 1). Recent studies have shown that airway epithelium can play a direct role in the recruitment of immature dendritic cells to the mucosal site during inflammation[37]. Presence of two characteristic Th2 cytokines IL-4 and

IL-13 in culture, induced CCL-20 (MIP-3α) secretion by bronchial epithelium[38]. CCL-20 (MIP-3α) is the ligand for CCR6, which is selectively expressed by some immature DCs[38]. Expression of this chemokine by the epithelium could facilitate the migration of immature DCs to the inflamed airways, which can be important for maintaining inflammation. This finding could explain the increased number of dendritic cells in epithelium in patients with asthma[39,40]. Dendritic cells can present antigen derived from the epithelium in such a way that a Th1 or Th2 response is induced[41]. The polarization is believed to be determined at least partly by the cytokine environment[42]. In addition to the increased recruitment of immature DCs, airway epithelium cells can also contribute to this cytokine microenvironment.

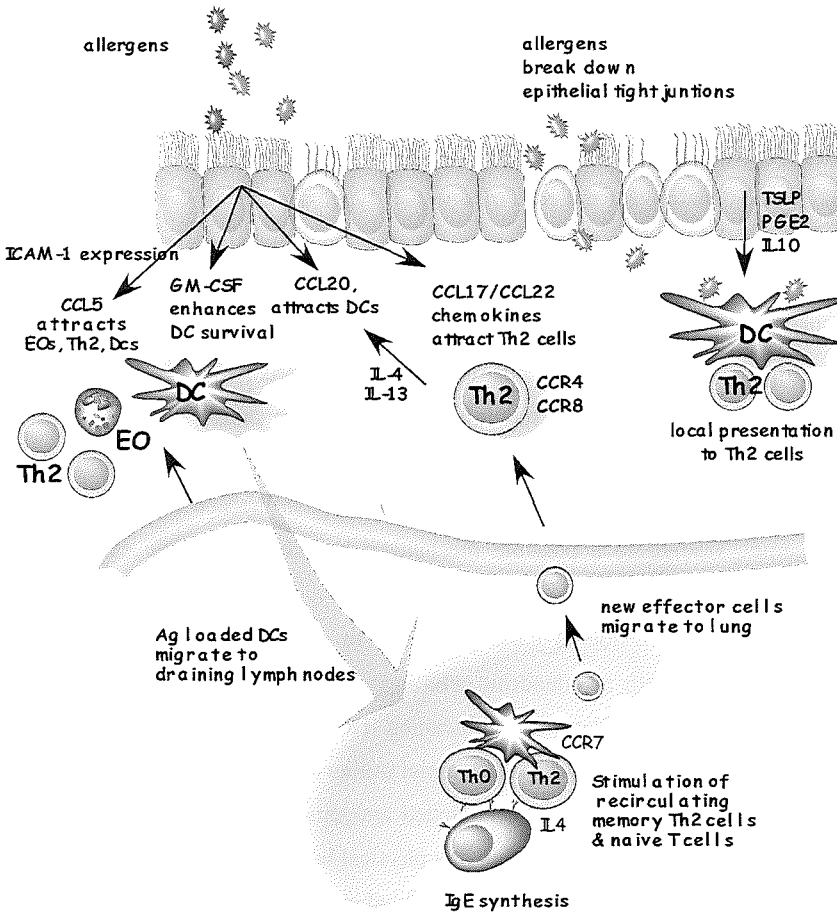

Figure 1. Influence of epithelial cells on dendritic cells

It is therefore not surprising that epithelial cells of individuals with asthma have a different cytokine secretion pattern compared with healthy controls. It has been shown that cultured bronchial epithelial cells of asthma patients constitutively released significantly larger amounts of IL-8, GM-CSF (granulocyte macrophage-colony-stimulating factor) and expressed more ICAM-1 (intercellular adhesion molecule) than epithelial cells of subjects without asthma[43]. Given the potency of GM-CSF in promoting maturation of cells from the monocyte/macrophage lineage into antigen presenting DCs and in activation of dendritic cells[44], it is conceivable that the observed number of DCs in epithelium of asthmatic patients[39,40] may have arisen through a similar mechanism. ICAM-1 expression by epithelial cells facilitates the transmigration of dendritic cells from the blood into the airway-lining surface. Moreover, RANTES (CCL5; Regulated upon Activation, Normal T cell Expressed and Secreted) was only released by bronchial epithelial cells of patients with asthma in response to diesel exhaust particles[43]. RANTES attracts eosinophils[45], Th2 cells and immature DCs, the 'key players' of the eosinophilic airway inflammation. Another study showed that epithelial cells of asthma patients released larger amounts of IL-8, GM-CSF and TGF-β in response to IL-4 and IL-13 and major house dust mite allergen Der p 1, suggesting that activated epithelial cells of asthma patients contribute to the maintenance of inflammation[46]. The influence of expression of cell adhesion molecules expressed by epithelial cells (such as E-Cadherin) on the retention of dendritic cells in the airways is less clear. In contrast, Langerhans cells reside for long time periods in epithelia before migrating to T-cell-rich areas of regional lymph nodes[47]. If this mechanism would be enhanced during eosinophilic airway inflammation, it might contribute to the increased accumulation of DCs.

In asthma, common aero-allergens can induce a Th2 immune response. Th2 cells orchestrate the induction of airway inflammation by secretion of inflammatory mediators, which results in the influx of eosinophils, a cell type which is normally absent in the mucosa. Lee and colleagues discussed the role of eosinophils in the context of T cells as a coconspirator or as an independent trigger of allergic respiratory pathology[48]. Human eosinophils mediate effects from at least 5 independent mechanisms that allow the eosinophil to modulate the intensity of pulmonary inflammation (See Fig 2), as well as elicit the cell death and loss of structural integrity of the mucosa, leading to pulmonary dysfunction. Some mechanisms directly influence dendritic cells or indirectly by modulation of intensity of pulmonary inflammation. First, eosinophils are able to influence polarization of DCs by secreting Th2 cytokines[49]. Second, activated eosinophils generate and release copious amounts of reactive oxygen intermediates, including super oxide anion, hydrogen peroxide, and hydroxy radicals, known

to induce DC maturation[50,51]. Taking into account that oxidative stress can induce upregulation of IL-8 and TNF-α of human DCs, the release of granule proteins can contribute to the innate immune response by inducing the release of inflammatory cytokines by DCs[52]. Third, recruited eosinophils are a source of small-molecule lipid mediators of inflammation. In particular, studies have shown that eosinophils generate cysteinyl leukotrienes, 5-HETE, PGE2 and platelet activating factor. The presence of PGE2 at a site of inflammation can induce maturation of DCs that have both a reduced capacity to release IL-12 and a tendency toward Th2 polarization[53-55]. Fourth, eosinophils secrete a series of degradative enzymes which may have significant effects on lung structure, including pulmonary surfactant activity[56-58].

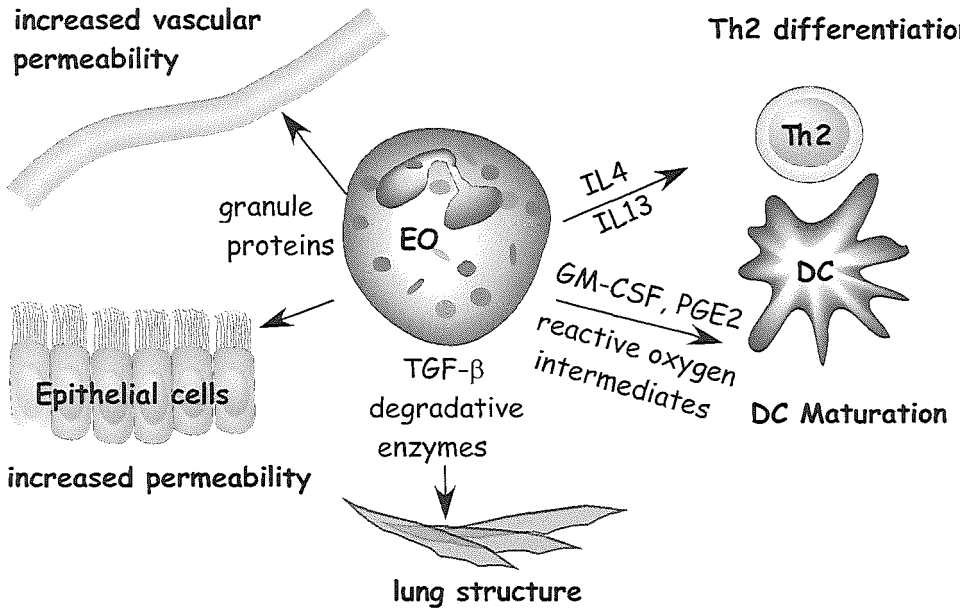

Figure 2. Eosinophils influence the severity of airway inflammation in several ways

Fifth, the release of toxic granule proteins by activated eosinophils recruited to the lung has been suggested as the major mode by which these cells execute effector functions[59]. These eosinophil secondary granule proteins are toxic to epithelial cells in the concentrations found in respiratory secretions from asthmatic patients[60] and may increase vascular permeability[61]. By damaging the epithelial barrier, it is not unlikely that the antigen load of the airways increases because of the increased permeability to inhaled antigens. Besides the effector cell function, eosinophils have been suggested to be able to present antigen to T cells[29, 30, 62]. It has been shown that eosinophils migrate from the airway mucosa to the draining lymph nodes of

the lung during eosinophilic airway inflammation[29,63]. In addition, expression of MHCII and costimulatory molecules would able them to activate antigen specific T cells[27,28]. However, murine eosinophils from the BALF compartment were not able to activate unstimulated OVA TCR tg T cells (Chapter 7). The expression of these molecules could still lead to T cell interaction and possibly resulting in signaling cascades within the eosinophil that potentially affect downstream activities, including the recruitment, activation, and execution of other effector functions. However, there is still a possibility that eosinophils that are residing in the draining lymph nodes do have antigen presenting capacities, in contrast to their BALF counterparts. The precise role of eosinophils in inducing T cells activation of the lung therefore remains unclear. In our opinion, it is not a major player in activating Th2 cells. In support, the first trials that used monoclonal antibodies against IL-5 did reduce the number of peripheral blood and bronchial mucosal eosinophils but did not alter the distribution and activation status of T cells[64] and failed to inhibit allergen induced bronchoconstriction and hyperresponsiveness[65].

In allergic responses mediated by IgE, mast cells are considered to be major players. Mast cells orchestrate the early response to allergen that occurs within minutes after inhalation of allergen by degranulation of several mediators after cross linking of allergen on their membrane bound IgE. Human biopsy studies have shown that an elevated number of mast cells within the airway smooth muscle is associated with airway hyperreactivity[66]. Mast cell protease 1 staining on lung sections of mice with an allergen induced airway inflammation revealed an increase in the number of mast cells compared to sham-sensitized animals (unpublished results, A. KleinJan). It is reported that AHR was inhibited in mast cell-deficient mice after allergen challenge[67]. In human asthma, mast cells are thought to be very important[68]. Binding of allergen to IgE on mast cells triggers the release of several mediators, inducing bronchoconstriction. It is proposed that human mast cells are involved in inducing maturation and functional activation of dendritic cells, by secretion of histamine and of exosomes.

Histamine is one of the most abundantly secreted mast cell mediators. Histamine can directly act on immature DCs by the H2 receptor, profoundly altering their T cell polarizing capacity[69]. Presence of histamine during culture increased IL-10 and decreased IL-12 production by LPS-matured DCs. Naïve T cells that were activated by these dendritic cells differentiated toward Th2 cells as compared with T cells activated by DCs that had matured in the absence of histamine[69,70]. It has been shown that histamine can upregulate CD86 and increase the cytokine production by immature dendritic cells. However, histamine-treated

DCs do not have a similar phenotype like mature DCs, as they do not upregulate CCR7. Histamine may therefore participate locally in T cell stimulation by inducing DCs capable of antigen presentation[71] and Th2 differentiation. Other factors released by mast cells inhibit DC maturation. Our laboratory has recently described that PGD2, released by mast cells upon cross linking of the FcεRI, suppressed DC migration. PGD2 immobilized lung DCs in the airway mucosa[72]. In vitro, PGD2 agonists suppressed the expression of costimulatory molecules and IL-12 by DCs, leading to the formation of Treg cells upon interaction with naïve T cells (unpublished data H. Hammad). Given the multiple facets of their interaction, the true role of mast cell activation on DC function *in vivo* remains to be determined.

Bone marrow-derived mast cells secrete exosomes, like several eukaryotic cells do. Exosomes are vesicles that are heterogeneous in shape and size, released from the lumen of multivesicular bodies into the extracellular environment. Endocytosed antigens accumulate in exosomes. Exosome-associated antigens are shown to have a higher stimulating capacity than free antigens, possibly by the expression of hsp60 and hsc70 on exosomes, two molecules known for their adjuvant activity on immune responses. Mast cell and DC exosomes are able to deliver antigenic peptides to APCs propably by transfer of antigen/hsc70 complexes to host DCs, leading to an allergen specific immune response, in contrast to exosomes derived from B cells and macrophages[73]. How these exosomes contribute to DC function in allergic inflammation is currently unknown, but will be the subject of further study in our lab.

There is some recent evidence that not only the function of mast cells is IgE-mediated. Novak and al, have recently shown that also DCs have IgE-mediated functions. Dendritic cells of atopic and non-atopic donors differed in the expression of the high affinity receptor for IgE (FcεRI). FcεRI is strongly upregulated on APCs from atopic donors which suggests involvement in the pathophysiology of atopic diseases. *In vitro* experiments demonstrated that IgE treated DCs from atopics revealed an enhanced T cell stimulatory capacity[74], whereas others have shown that IgE triggering leads to upregulation of indoleamine 2,3 dioxygenase (IDO) gene transcription by DCs. IDO is an enzyme involved in the catabolism of tryptophan and is involved in the suppression of T cell responses by inducing cell death mediated by tryptophan catabolites[75].

A role for dendritic cell in chronic asthma?

Besides the reversible allergen-induced airway inflammation and the bronchospasm there are also structural changes induced in the lungs of patients suffering from chronic asthma, collectively referred to as airway remodeling. It is clear from pathological investigations that

structural alterations exist in the asthmatic airway, even at very early stage of disease[76]. Mathematical modeling studies have provided evidence that these alterations contribute to the symptoms and physiologic dysregulation seen in asthma. Remodeling is a dynamic process that involves the balance of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metallo proteinases (TIMPs). Abnormalities in MMP-9 production, a protease involved in the remodeling of the extracellular matrix, have been observed in asthma patients leading to increased levels of MMP-9 in the subepithelial basement membrane and in serum[77, 78]. In a murine model for asthma, it was shown that MMP-9 is involved in the migration of DCs to the mucosal lumen where they encounter antigen[79]. How exactly collagen or extracellular disposition of MMP9 might influence DC function is currently unknown. It is possible that ligation of integrin receptors on DCs by extracellular matrix components leads to alterations in DC function. Alternatively, some matrix degradation products such as heparan sulphate have been shown to induce DC maturation[80]. If structural remodeling of the airways influences DC function, this might be the explanation why some allergic asthmatic reactions become chronic, despite avoidance of allergens.

Local stimulation of memory T cells by dendritic cells at the mucosa

Effector inflammatory cells are recruited to the mucosal site during a secondary immune response to inhaled allergen. Therefore it is very interesting to investigate at which location in the airways previously primed T cells are activated. In contrast, it is generally known that naïve T cells are predominantly activated in the draining lymph nodes by mature DCs, which have picked up antigen in the periphery. For previously primed T cells, it is not known whether activation occurs locally at the site of deposition of inhaled allergen in the respiratory tree, or at distal locations such as the draining lymph nodes.

There is accumulating evidence that the immunological interaction between DCs and T cells is not restricted to the draining lymph nodes but that effector T cells can also be activated in the non-lymphoid tissue of the lung. In sensitized mice, $CCR7^+$ central memory T cells (T_{cm}) recirculate through the lymphoid tissues and a $CCR7^-$subset (T_{em}) migrates to the lung, where the first encounter with antigen took place. In case of a second encounter with the same antigen, local memory T cells (T_{em}) can be activated by antigen presenting resident dendritic cells after recognition of the antigen but these locally activated T cells are not able to divide. At the same time, dendritic cells migrate to the draining lymph nodes to activate recirculating memory $CCR7^+$ T_{CM} cells to proliferate and differentiate into new waves of effector Th2 cells, which can migrate to the site of antigen exposure[81]. In view of these different fates of T cell

activation, it is likely that there are several subsets of dendritic cells, each with a different chemokine receptor expression and migration pattern, responsible for activating the effector memory T cells and central memory T cells.

In support of a local T cell activation, several studies have shown that immature DCs upregulated CD86 after interaction with memory T cells in the lung[82, 83]. It is possible that upregulation of CD86 is a sign of an enhanced DC maturation induced by memory T cells. In this way, mature antigen-bearing DCs, which have upregulated several chemokine receptors like CCR7, will reach the draining lymph nodes faster in comparison with the situation in which memory T cells are absent. This mechanism could contribute to the enhanced migration of DCs which we observed during inflammation (Chapter 4). Our data using conditional knock-out of lung DCs only furthermore demonstrated that local DCs are crucial for mounting an effector Th2 response in the lung and for causing all the features of asthma. Similarly, previous studies have shown that systemic depletion of DCs using a ganciclovir dependent transgenic models also abolished airway inflammation in challenged and sensitized mice[84].

Despite the fact that Huh et al. and Vermaelen et al. found temporary upregulation of CD86 on DCs obtained from mice with eosinophilic airway inflammation[82, 83], we could not observe a functional role for either CD80 and CD86 costimulation provided by DCs in the local stimulation of primed T cells (Chapter 6). Future studies will therefore elucidate the mechanisms by which DCs stimulate primed T cells locally in the lung.

Some unresolved mysteries about the etiology of asthma

The above described interactions of dendritic cells with other inflammatory cells and local activation of memory T cells still does not explain the persistent character of airway inflammation in asthma. There are some intriguing factors concerning the immunological basis for asthma.

First, allergic asthma patients have IgE against one or more antigens from a more or less defined set of allergens and not to random antigens. It is therefore likely that there must be a link between common aeroallergens such as animal dander, house dust mite and cockroach emanations, latex components, pollen grains and mold spores. Parasitic antigens, such as tropomyosins and gluthathione S-transferases, have their allergenic homologs in house dust mite (HDM)[85, 86] and would use the same mechanism to induce an immune response. Nonetheless, until now no common trait among aeroallergens has been discovered.

Secondly, these antigens do not elicite a strong Th2 response in every individual. However, it is not clear which endogenous factor(s) in the immune system are responsible for inducing this excessive immune response. There is some intriguing evidence that dendritic cells from allergic patients are phenotypically different from healthy persons. Monocyte-derived DCs obtained from HDM allergic patients were uniquely sensitive to the enzymatic activity of Der p 1, one of the major allergens of HDM, leading to the preferential induction of Th2 responses by these Der p 1 treated DCs[87].

Thirdly, the massive increase in asthma prevalence the last 3 decades, which cannot be explained by a simultaneous increase in allergens or by genetic influences, is indicative of another environmental factor. It has been proposed that conditions of improved hygiene, such as those occurring in the westernalized world, lead to less infectious pressure from the environment on the immune system. There is preliminary evidence that lack of infectious stimuli leads to a relative inefficiency of regulatory T cell induction[86]. Recently, many studies investigating immune disorders, have concentrated on finding a disturbance in the number or function of regulatory T cells[88]. Also in asthma research the importance of regulatory T cells has received attention of several groups[89]. The specific mechanisms by which the regulatory cells function or the specific characteristics of these cells are still being investigated. Several promising $CD4^+$ regulatory/suppressor T cell subsets have been identified but further studies will have to reveal their role in the pathogenesis of asthma. In this regard, it is striking that immature DCs induce regulatory T cells[90]. One explanation for chronic ongoing asthma could therefore be that local inflammation and tissue remodeling leads to continuous maturation of DCs, so that Treg cells with anti-inflammatory capacity can no longer be induced or maintained[83].

In conclusion, the data presented in this thesis supports the hypothesis that dendritic cells contribute to the induction and maintenance of the secondary immune response to inhaled allergen. However, the next step will be to identify targets for DC directed therapy. Therefore, it will be crucial to elucidate the mechanisms by which dendritic cells maintain the chronic airway inflammation.

MAIN FINDINGS

Dendritic cells seem to play an important role in the secondary immune response to inhaled allergen as supported by the following findings described in this thesis:

- The number of dendritic cells in the airways is increased during a secondary immune response to inhaled allergen, this increase is supported by an enhanced dendritic cell progenitor production in the bone marrow (Chapter 4).
-Administration of dendritic cells induces asthmatic features in sensitized mice. (Chapter 5).
-Removal of dendritic cells during antigen challenge abolishes the cardinal features of asthma (Chapter 5).
-The function of DCs in asthma is dependent on CD80/86 during priming, but not during challenge (Chapter 6).
-Eosinophils are recruited to the draining lymph nodes of the lung during challenge, but lack significant antigen presenting capacity to activate naïve T cells(Chapter 7)

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Summary

Asthma is a chronic inflammatory disease characterized by the clinicopathologic symptoms of intermittent and reversible airway obstruction, enhanced mucus production, chronic bronchial inflammation with eosinophils, and bronchial smooth muscle cell hypertrophy.

In the Netherlands, the prevalence of asthma has increased tremendously in the last three decades. One of the most important predisposing factors for asthma is allergy as indicated by the strong association between serum IgE and the risk for asthma. It is currently believed that a dysregulated cellular (primed T cells) and humoral (Immunoglobulin (Ig)E antibodies) immune response to allergen is the basis for atopy. However before T cells become activated, they have to recognize antigen. Allergen is presented by antigen presenting cells. Animal studies have demonstrated that dendritic cells are the most important antigen presenting cell in the lungs. Dendritic cells (DCs) are generally accepted to be involved in the induction of the primary immune response by provision of signal I (interaction of MHC bound allergen and the TCR), and of signal II (costimulation) to T cells and their potential to migrate to the draining lymph nodes (**Chapter 1**)[1].

In this thesis, we specifically addressed whether DCs have a role beyond sensitization. In **Chapter 2,** a general introduction is given about the immunological basis of asthma, the role of dendritic cells in asthma and the use of murine asthma models.

In **Chapter 3,** a new flow cytometric method was first described to study more carefully the different inflammatory cells in the bronchoalveolar lavage fluid. The degree and type of airway inflammation is often studied by determination of cellular composition of bronchoalveolar lavage fluid cells. Especially the number of eosinophils in BALF is often used as an indicator for the type of immune response induced and correlates very well to the degree of inflammation in the airways. The differentiation of total BALF cells into monocytes, lymphocytes, eosinophils and neutrophils is generally determined by morphological analysis of BALF cell cytospins stained with May-Grünwald Giemsa. We developed a new flow cytometric analysis in which we were able to identify eosinophils, dendritic cells, B cells, T cells, macrophages and neutrophils in one staining. This new flow cytometric method proved to be a time saving, investigator independent, and a more detailed method for general use in experimental asthma research compared with morphological analysis of cells on cytospins.

In **Chapter 4**, we showed that in OVA-sensitized and challenged mice, the number of dendritic cells in the bronchoalveolar compartment was increased in an allergen dose-

dependent way. The increased number of DCs at the luminal side could imply an enhanced antigen uptake and subsequent migration to draining lymph nodes. Migration patterns of DCs from the tracheal lumen to the draining lymph nodes of the lung indeed revealed an enhanced and faster migration kinetic during inflammation compared with migration of DCs during steady-state. Therefore, a reduced migration of DCs to the draining nodes was not the reason for the observed increase in airway DCs during allergen challenge.

The number of DC precursor $CD11b^+$ monocytes was also increased in peripheral blood of OVA-sensitized and challenged mice compared with control mice, which indicated an increased output of the bone marrow of dendritic cell progenitors analogous to the described increase in eosinophil progenitor output during allergen-induced airway inflammation. To investigate this possibility, we stained bone marrow with antibodies for CD31 (PECAM-1) and Ly-6C, known to distinguish between 6 discrete populations in bone marrow, each with different lineage commitment and differentiation potential. Mice with an eosinophilic airway inflammation showed a dose dependent increase in the population of $CD31^{hi}Ly\text{-}6C^{lo}$ early progenitor cells in bone marrow. After purification, this population was shown to contain cells with potential to develop in dendritic cells after culture in GM-CSF but also into eosinophils after culture in IL-5. We also observed that during allergic airway inflammation, there was an increase in mature circulating $CD11b^+CD11c^+$ DCs in the bloodstream. We do not know at present whether these cells are derived from the airway inflamed tissues or whether they represent DCs that have matured locally in the bloodstream as a result of exposure to circulating maturation factors.

In **Chapter 5**, we investigated the functional role of the dendritic cell increase seen during the effector phase of the immune response to inhaled allergen challenge. We depleted lung dendritic cells in sensitized mice before OVA aerosol challenge. We used CD11c Diphtheria toxin receptor (DTR) transgenic mice in which $CD11c^+$ airway dendritic cells can be depleted for about 48 hours by exposing the mice intratracheally to diphtheria toxin. In transgenic mice, DC depletion by diphtheria toxin treatment decreased the number of BALF eosinophils, eosinophilic airway inflammation, Th2 cytokine production and bronchial hyperreactivity compared with non-transgenic littermates. This suggested that dendritic cells are required for proper activation of memory T cells and for consecutive induction of an effector T cell response to inhaled antigen, leading to an eosinophilic airway inflammation. In addition, these experiments suggested that other antigen presenting cells present in the lung like B cells, macrophages and epithelial cells were not capable of compensating the absence of DCs.

From *in-vitro* experiments, it has been shown that effector T cells are less dependent on costimulation. This would imply that during an ongoing inflammation when effector T cells are present, other APCs providing less costimulation than DCs are sufficient to stimulate allergen-primed T cells. To investigate the requirement of DCs *in-vivo*, we depleted DCs in an ongoing inflammation when effector T cells are already present and fully functional in the airways. DC depletion in animals with an already established eosinophilic airway inflammation, decreased the number of eosinophils, Th2 cytokine production, inflammatory peribronchial and perivascular infiltrates and mucus production by goblet cells in response to inhaled allergen compared with sham depleted mice. To determine whether other APCs were able to compensate for the DCs, we continued the OVA challenge after DC depletion or treated the mice with a PBS challenge. Interestingly, there was no difference in the number of BALF eosinophils, Th2 cytokine production and peribronchial and perivascular inflammatory infiltrates. Together these data show that DCs are also involved in the maintenance of the secondary immune response and that other present APCs are not sufficient to compensate for the lack of DCs.

In the second part of chapter 5, we investigated whether DCs have the intrinsic capacity to induce a secondary immune response in sensitized mice. Bone marrow-derived DCs were intratracheally injected in sensitized mice. Dendritic cells were capable of inducing an eosinophilic airway inflammation as shown by histological analysis of lungs, and the increase of the number of BALF eosinophils. In DC-challenged mice, an enhanced Th2 cytokine production of lung draining lymph nodes was noticed, leading to bronchial hyperreactivity and to goblet cell hyperplasia, phenomena clearly associated with the Th2 cytokine IL-13. DCs did not induce airway eosinophilia by themselves but needed lymphocytes for this process, as exemplified by the absence of the response in SCID mice without T and B cells.

In **Chapter 6,** we investigated which factors on DCs were involved in the induction of a secondary immune response. We used the model described in chapter 5 in which sensitized mice are challenged with dendritic cells. We used CD80/86$^{-/-}$ bone marrow-derived DCs to investigate the importance of CD80/86 costimulation for inducing a secondary immune response *in-vivo*. In contrast to the observed requirement for CD80/86 costimulation to prime naïve T cells, injection of CD80/86$^{-/-}$ DCs in wild type sensitized mice led to a severe eosinophilic airway inflammation, similar to the one induced by wild type DCs. Costimulation by ligation of CD80/86 on DCs seems therefore not to be important for inducing a secondary immune response. We examined the possibility that antigen was taken up by endogenous DCs, and that this was the reason for the observed inflammation. However,

when we blocked CD80/86 expression on endogenous cells by injecting CTLA4-Ig during challenge with CD80/86$^{-/-}$DCs, a similar number of eosinophils was recovered from BALF with or without injecting CTLA4-Ig.

However, it is likely that other costimulatory molecules provided by dendritic cells are important during the secondary immune response. The CD28 family member molecule ICOS is expressed on antigen-stimulated T cells, making ICOS-L a likely candidate molecule for providing costimulation[21-23]. However, blockade of ICOS by administering ICOS-Ig simultaneously with challenge with CD80/86$^{-/-}$ DCs decreased the number of eosinophils in the BALF, although not significantly, suggesting that ICOS-ICOS-L interaction can not compensate for the lack of CD80/86-CD28 interaction. Further experiments will have to elucidate which costimulatory signals provided by DCs are involved in the secondary immune response. Although, dendritic cells seem to be very important for secondary immune responses, perhaps also other APCs can contribute to the allergen presentation.

In **Chapter 7,** we addressed the question whether eosinophils are able to present antigen in the lung. To determine the antigen presenting capacity of airway eosinophils, we isolated BALF eosinophils of mice with an allergen induced airway inflammation using the method described in Chapter 3, to avoid contamination with other APCs present in the BALF. Eosinophils were intratracheally injected in mice that previously received CFSE-labeled unstimulated OVA TCR transgenic T cells. Eosinophils did not induce divisions of T cells in the lung draining lymph nodes in contrast to DCs. To circumvent the need for antigen processing or migration *in vivo*, eosinophils were pulsed with OVA peptide but were still not able to induce T cell priming *in vitro*, whereas DCs induced vigorous proliferation. In contrast, eosinophils were able to induce some proliferation in *in-vitro* obtained effector T cells, although not as strongly as professional DCs. The role of eosinophils in the draining lymph nodes is not clear. We cannot exclude that the eosinophil population in the draining lymph nodes is functionally different from the BALF eosinophils and is capable of presenting antigen to naïve T cells. Possibly, draining lymph node eosinophils might contribute indirectly to T cell activation induced by DCs, by secreting cytokines and mediators.

In conclusion, the work presented in chapters 3-7 illustrates the involvement of dendritic cells in inducing and maintaining a secondary immune response in the lung. According to Koch's postulates modified to this pathology, three criteria are required to determine if a cell plays a crucial role in the pathogenesis of a disease. In this modern version, these postulates state that to prove causality between a cell and a disease, the number of cells has to be increased during

the disease, introduction of the cell in a healthy animal has to cause the disease and removing the cell will reduce severity of the disease. In Chapter 4 and 5, we have demonstrated that increased numbers of DCs are present in the airways in close proximity to T cells (postulate 1), which is supported by an increased production of dendritic cell progenitors in bone marrow. In Chapter 5, we have demonstrated that introduction of allergen pulsed dendritic cells in already sensitized animals is sufficient to induce an eosinophilic airway inflammation in the lung (postulate 2). In chapter 5, we also showed that depletion of dendritic cells in sensitized mice as well as in mice with a full blown eosinophilic airway inflammation, decreased the development of cardinal features of asthma such as Th2-mediated eosinophilic airway inflammation, bronchial hyperreactivity and goblet cell hyperplasia. (postulate 3). All criteria have been met to conclude that dendritic cells have a functional contribution to the establishment and maintenance of the eosinophilic airway inflammation. In Chapter 6, we demonstrated that CD80/86 costimulation provided by DCs is not required to activate memory T cells. In Chapter 7, we rejected the hypothesis that eosinophils are able to present antigen and therefore in the asthmatic lung could compete with dendritic cells as antigen presenting cell. Comparison of BALF eosinophils and bone marrow-derived dendritic cells in stimulating OVA specific T cells revealed that dendritic cells are superior in antigen presentation to both naïve and effector T cells.

Nederlandse Samenvatting

Astma is een chronische luchtwegziekte die gekenmerkt wordt door benauwdheid, piepen en hoesten. Deze symptomen worden grotendeels veroorzaakt door een vernauwing van de luchtwegen. Een verhoogde luchtweggevoeligheid voor provocerende stimuli en verhoogde slijmproductie door de slijmbekercellen zijn twee van de belangrijkste oorzaken voor de vernauwing. De laatste drie decennia zijn de ideeën over het ontstaan van astma substantieel veranderd. Door de vooruitgang die geboekt is in het ontwikkelen van invasieve en niet-invasieve methoden werd het mogelijk de pathogenese van astma nader te bestuderen.

In een gesensitiseerd persoon kan een bronchiale allergeen expositie leiden tot acute benauwdheid. Op cellulair niveau verbindt het ingeademde allergeen de gebonden IgE moleculen op een mestcel. Mestcellen scheiden na stimulatie, verschillende voorgevormde mediatoren uit die vervolgens het gladde spierweefsel rond de luchtwegen laat samentrekken, bloedvaten laten verwijden, en een verhoogde lekkage van plasma naar de luchtwegen veroorzaakt. Deze vorm van IgE gemedieerde allergie wordt ook type I immediate hypersensitivity genoemd. In ongeveer 50% van de blootgestelde personen vindt ook een late immuunreactie plaats. Deze wordt gekarakteriseerd door een infiltratie van het longweefsel met mononucleaire cellen, T cellen en eosinofielen. Deze reactie wordt vooral geleid door type 2 helper T cellen die de verschillende cytokines IL-4, IL-5, IL-9, IL-10, IL-13, IFNγ uitscheiden. Deze cytokines zijn betrokken bij de aantrekking en activatie van ontstekingscellen, IgE synthesis, goblet cel hyperplasie en bronchiale hyperreactiviteit. Deze reactie leidt opnieuw tot benauwdheid.

T cellen scheiden pas hun cytokines uit nadat er contact is tussen het allergeen en de specifieke T cel receptor. Allergenen worden door antigeen presenterende cellen aangeboden. Deze cellen hebben naast een MHCII complex waarop het allergeen gepresenteerd wordt ook costimulatoire moleculen die een tweede signaal afgeven. Zonder dit tweede signaal raken niet eerder gestimuleerde T cellen niet geactiveerd. Echter, voor reeds geactiveerde cellen is dit minder duidelijk. *In vitro* proeven hebben namelijk laten zien dat 'effector' T cellen geen costimulatie nodig hebben om te prolifereren. Aangezien de allergeen specifieke T cellen van een astma patient reeds geactiveerd zijn, is het daarom van groot belang om inzicht te krijgen in de factoren die deze cellen activeren. Een belangrijke vraag in dit kader is de betrokkenheid van antigeen presenterende cellen. In de long komen verschillende antigeen presenterende cellen voor waaronder macrofagen, B cellen, dendritische cellen en mogelijk ook

eosinofielen. Uit eerder proefdieronderzoek is echter gebleken dat de dendritische cel de belangrijkste allergeen presenterende cel in de long is.

In dit proefschrift worden de onderzoeken beschreven die de rol van de dendritische cel als antigeen presenterende cel in de secundaire immuun respons tegen ingeademd allergeen hebben onderzocht.

In **Hoofdstuk 1** wordt een algemene inleiding gegeven waarin de doelstellingen van het onderzoek en de opzet van het gebruikte diermodel wordt beschreven. We gebruikten het eerder ontwikkelde astma model waarin muizen tegen een allergeen worden gesensitiseerd door een injectie van allergeen gepulsde dendritische cellen in de luchtpijp. Een blootstelling aan hetzelfde allergeen in de vorm van een aerosol induceert een Th2 gemedieerde immuunrespons wat vervolgens leidt tot een eosinofiele luchtwegontsteking, slijmbekercel hyperplasie en een verhoogde Th2 cytokine productie door de drainerende lymfeklieren van de long. In dit model hebben we de rol van de dendritische cel tijdens de secundaire immuunrespons tegen ingeademd allergeen bestudeerd.

In **Hoofdstuk 2** wordt een algemene introductie gegeven over de immunologische basis van astma, de rol van dendritische cellen in astma en het gebruik van astma-diermodellen.

Hoofdstuk 3 beschrijft een nieuwe methode om de cellulaire compositie van bronchoalveolaire lavage vloeistof te bepalen. In astma onderzoek in muizenmodellen wordt dit vaak gebruikt als parameter voor de mate van eosinofiele luchtwegontsteking. In het algemeen wordt de cellulaire samenstelling van BALF bepaald op histologisch gekleurde cytospins waarbij gekeken wordt naar de morfologische karakteristieken van de verschillende celpopulaties. We hebben een flowcytometrische methode ontwikkeld waarbij in één kleuring eosinofielen, macrofagen, neutrofielen, dendritische cellen, B cellen en T cellen onderscheiden kunnen worden. De identificatie van de verschillende celpopulaties met behulp van de flowcytometer is gebaseerd op celgrootte, verstrooiing van licht door de cel en de expressie van specifieke markers.

Hoofdstuk 4 laat zien dat het aantal dendritische cellen toeneemt in de luchtwegen tijdens een secundaire immuunrespons tegen ingeademd allergeen. De simultane significante toename van de migratie van dendritische cellen naar de drainerende lymfeklieren wijst op een verhoogde toevoer van allergenen naar naive T cellen en centrale memory T cellen. Dit mechanisme zou als een positief feedbackmechanisme kunnen werken die op deze manier de ontsteking in stand houdt. Tevens is het aantal DCs in het bloed groter, dit suggereert dat de

uitgave van DC (-precursors) uit het beenmerg groter is. Analyse van het beenmerg toonde inderdaad aan dat 1 populatie cellen significant toenam ten op zichte van de andere populaties. Deze cellen bleken in staat te zijn om uit te groeien tot dendritische cellen.

In **Hoofdstuk 5** is de functionele rol van DCs bepaald door in allergeen gesensitiseerde muizen de luchtweg-DCs te depleteren en vervolgens de muizen bloot te stellen aan allergenen via een aerosol. In dieren die niet gedepleteerd waren, leidde de allergeen blootstelling tot een eosinofiele luchtwegontsteking met slijmbekercel hyperplasie, verhoogde Th2 cytokine productie en een verhoogde bronchiale hyperreactiviteit. In dieren die gedepleteerd waren van hun luchtweg DCs bleven al deze kenmerken achterwege. Ook een bestaande ontsteking kon geremd worden door het depleteren van DCs. Dit is een sterke aanwijzing voor de betrokkenheid van DCs in de secundaire immuunrespons. In hetzelfde hoofdstuk onderzochten we of DCs voldoende intrinsieke capaciteit hebben om een secundaire immuunrespons te induceren. Een intratracheale injectie van OVA presenterende DCs in dieren die gesensitiseerd waren voor OVA bleek voldoende te zijn om een secundaire immuunrespons te induceren. Deze secundaire immuunrespons leidde tot een eosinofiele luchtwegontsteking, Th2 cytokine productie en bronchiale hyperreactiviteit.

In **Hoofdstuk 6** onderzochten we welke factoren op de DC belangrijk zijn voor het induceren van een secundaire immuunrespons. Hiervoor gebruikten we het in hoofdstuk 5 beschreven model waarin we gesensitiseerde muizen intratracheaal injecteren met allergeen gepulsde dendritische cellen. We gebruikten DCs die geen CD80 en CD86 tot expressie brachten. In dit model bleek dat antigeen presentatie door DCs zonder CD80/86 een vergelijkbare immuunrespons opleverde als na injectie van $CD80/86^{+/+}$ DCs. Deze *in-vivo* resultaten zijn in overeenstemming met andere *in-vitro* studies die aantoonden dat eerder met antigeen gestimuleerde T cellen minder costimulatie door CD80/CD86 nodig hebben. Het is daarom interessant om te onderzoeken welke costimulatoire moleculen op DCs wel betrokken zijn bij het induceren van een secundaire immuunrespons.

In **Hoofdstuk 7** onderzochten we de antigeen presenterende capaciteit van eosinofielen uit de BAL vloeistof. Eosinofielen uit de BAL vloeistof bleken niet in staat om naieve T cellen aan te zetten tot proliferatie zowel *in vitro* als *in vivo*. We hebben rekening gehouden met het wellicht ontbrekende vermogen om allergeen op te nemen en te verwerken tot allergene peptiden, met het ontbreken van een actief migratie patroon naar de long drainerende lymfeklieren en een mogelijk te lage ratio tussen T cellen en eosinofielen. Echter deze factoren konden uitgesloten worden als oorzaak voor het onvermogen van eosinofielen om antigeen te presenteren aan naïeve T cellen.

Kortom: deze studies laten zien dat de dendritische cel een belangrijke rol speelt tijdens het ontstaan en voortduren van de eosinofiele luchtwegontsteking. Echter het vermogen om CD80/86 costimulatie te kunnen leveren lijkt hiervoor niet belangrijk te zijn. Deze studies kunnen een bijdrage leveren aan een beter begrip van de chronische luchtwegontsteking die kenmerkend is voor astma en wellicht uiteindelijk leiden tot de ontwikkeling van een DC gerichte astma therapie. Toekomstige studies moeten echter uitwijzen welke factoren, geleverd door dendritische cellen, hier een belangrijke rol bij spelen.

List of abbreviations

Ab	Antibody
Ag	Antigen
AHR	Airway Hyperreactivity
APC	Antigen Presenting Cell
BALF	Broncho-alveolar Lavage Fluid
BHR	Bronchial Hyperreactivity
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CFSE	Carboxy Fluorescein Diacetate Succinimidylester
CFU	Colony Forming Unit
d	Day
DC	Dendritic cell
DT	Diphtheria Toxin
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Scanner
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FLT-3L	Flt-3 ligand
FSC	Forward Scatter
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HDM	House Dust Mite
HRP	Horseradish Peroxidase
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

i.p.	intraperitoneal
i.t.	intratracheal
i.v.	intravenous
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MCP	Monocyte Chemotatic Protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NKT	Natural Killer T cell
nTg	Non-transgenic
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECy5	Phycoerythrin-cychrome 5
RANTES	Regulated on activation, normal T cell expressed, and secreted
SD	Standard deviation
SEM	Standard Error of the Mean
SSC	Side scatter
TCR	T cell receptor
Tg	Transgenic
Th	T Helper
WT	Wild type

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Curriculum Vitae

The author of this thesis was born on August 30, 1976 in Leiderdorp, The Netherlands. After finishing high school in 1994, a supplementary certificate for Physics was obtained at the summer school program at the James Boswell Institute in Utrecht. From September 1994-1999 the author studied Human Nutrition and Health at Wageningen University. Training periods were performed at the department of gastroenterology at the Radboud University Hospital Nijmegen, Department Toxicology at the Wageningen University, at the GGD in Ede and at TNO Nutrition and Food Research Zeist. From 1999-2004 the author is employed as PhD student at the department of Pulmonary Medicine and Critical Care Medicine at the Erasmus Medical Center in Rotterdam. The work leading to this thesis was performed under supervision of Prof.dr. Henk C. Hoogsteden and dr. Bart N. Lambrecht.

List of Publications

1. van Rijt, L. S. and B. N. Lambrecht (2001). "Role of dendritic cells and Th2 lymphocytes in asthma: lessons from eosinophilic airway inflammation in the mouse." *Microsc Res Tech 53(4): 256-72.*

2. van Rijt, L. S., Prins, J. B., Leenen, P. J., Thielemans, K., de Vries, V. C., Hoogsteden, H. C., Lambrecht, B. N.(2002). "Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma." *Blood 100(10): 3663-71.*

3. Van Rijt, L. S., N. Vos, Hijdra, D., De Vries, V. C., Hoogsteden, H. C., Lambrecht, B. N.(2003). "Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells." *J Immunol 171(7): 3372-8.*

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5. van Rijt, L.S., H. Kuipers, N. Vos, D. Hijdra, H.C. Hoogsteden and B.N. Lambrecht (2004). "A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma" *J Immunol Methods: In press.*

6. van Rijt, L.S., N. Vos, M. Willart, A. KleinJan, A. Coyle, H.C. Hoogsteden, B.N. Lambrecht (2004). "Essential role of dendritic cell CD80/CD86 costimulation in the induction but not reactivation of Th2 effector responses in a mouse model of asthma" *J Allergy Clin Immunol: In press.*

7. van Rijt, L.S., S. Jung, A. KleinJan, N.Vos, M. Willart, C. Duez, H.C. Hoogsteden, B.N. Lambrecht "In vivo depletion of lung $CD11c^+$ dendritic cells abrogates the cardinal features of asthma" *Submitted.*