Dendritic Cells of Vital Importance for Immune Regulation in the Lung

Hendrik Jan de Heer

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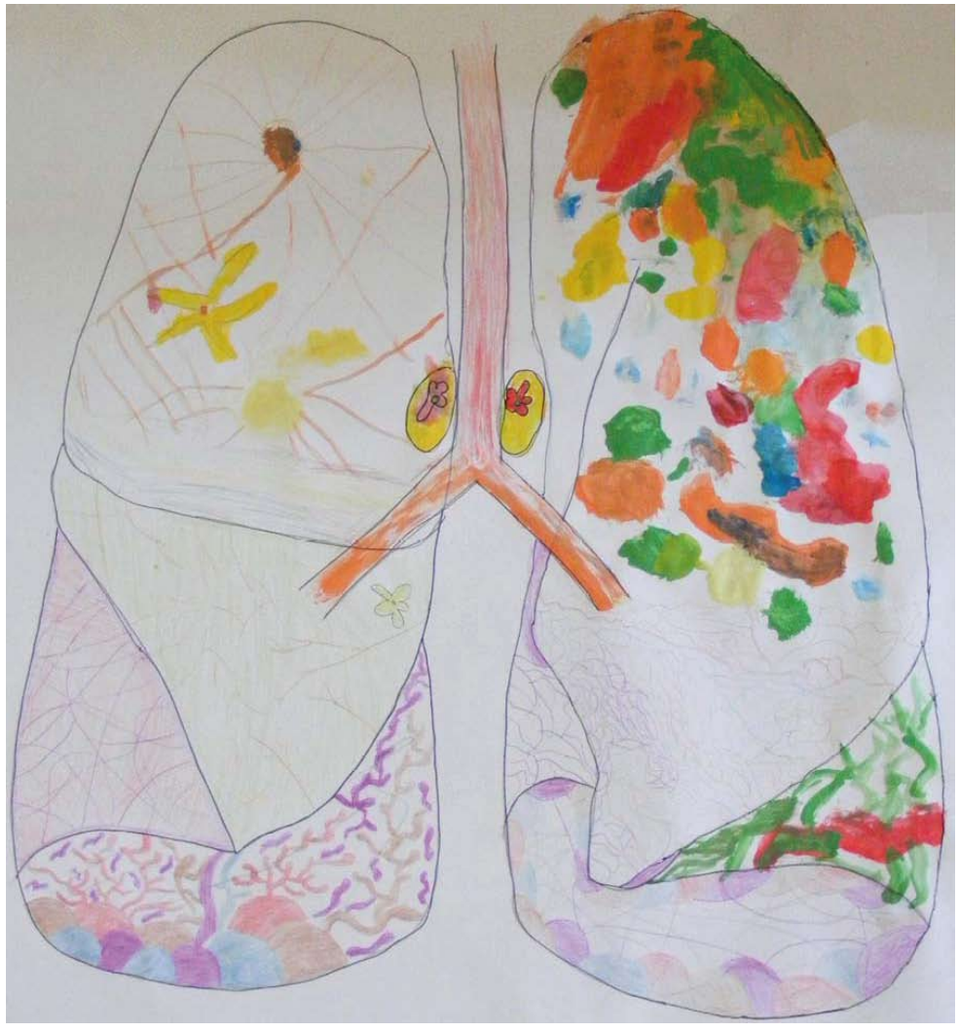

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Dendritische cellen van vitaal belang
voor immuun regulatie in de long

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Voor mijn vrouw Rachel en
mijn kinderen: Jonathan, Elise, Daniel en Isabel

Voor al jullie warmte en liefde

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Chapter 1

General Approach and Aim of the Thesis

Dendritic cells in the lung

Dendritic cells (DCs) are known to play a pivotal role in the induction of a primary and secondary immune response in the lung (1) (see chapter 2 for a full theoretical introduction on DC subsets). By taking up antigen under steady state and inflammatory conditions from the tissue where they reside, DCs can subsequently migrating to draining lymph nodes (LNs). Here they can present antigen bound peptides on either MHC class I and/or II to CD8+ or CD4+ T cells respectively. These DCs induce either tolerance or immunity, depending on the type of stimulus that they received during residence in the periphery (2). Recently it has become clear that there are different subtypes of DCs, with a major division between conventional DC (cDC), plasmacytoid DC (pDC), and an inflammatory type DC (iDC). The cDC is found in steady state conditions in the central lymphoid organs and some peripheral tissues like lung, gut and skin. The pDC was known to be a major interferon producing cell type, but later on also proved to have antigen presenting cell (APC) qualities under certain conditions. The iDCs derive from monocytes under conditions of inflammation and can be generated in large quantities from human monocytes, and mouse bone marrow cells (3). Although this emerging concept of DC subsets is now well accepted for central lymphoid organs and skin, relatively little is known about the precise control mechanisms of lung DC function and possible regulation by different DC subsets. Studying the precise regulation of DC function on the lung could however be key to understanding many immune mediated diseases of the lung such as asthma, sarcoidosis, hypersensitivity pneumonitis or other interstitial diseases of unknown origin.

In this thesis, we specially addressed the functional contribution of different DC subsets, the importance of their maturation state and type of innate stimulation on the regulation of the pulmonary immune response, and how this can influence the decision between tolerance or immunity, health or disease. Furthermore the role of these DC subsets was investigated in two important anatomical compartments of the lung namely the conducting airways and the peripheral vascular compartment. The importance of the degree of maturity of iDCs was studied, attempting to modulate DC functions using a Toll-like receptor (TLR) agonist in vitro, or anti-inflammatory compounds.

To address these issues, we have used several animal models of disease, to put our findings into a relevant context. To examine the role of cDCs and pDCs in immunity and tolerance induction especially in the conducting airways of the lung, well-established murine models of eosinophilic airway inflammation and tolerance were used, that have been mostly used to study the pathogenesis of asthma. For the sake of clarity, these models are introduced here first, so that the reader has an overview for the remaining part of the thesis.

1. In the **classical model of alum-induced asthma**, sensitization is induced by injecting mice with Ovalbumin (OVA) admixed with the Th2 adjuvant aluminum hydroxide (OVA-Alum) intraperitoneally (i.p.). Mice are challenged 10 to 14 days later with OVA aerosols generating asthma (figure 1). Recently it has been shown by Kool et al. in our group that the sensitization to OVA in this model is driven by monocyte-derived iDCs that are attracted to the site of i.p. injection via release of uric acid and triggering of the NALP3 inflammasome (4)

2. In an **adoptive transfer model of DCs**, bone-marrow derived GM-CSF cultured iDCs are pulsed with OVA and injected intratracheally (i.t.). The injection of these iDCs leads to Th2 priming, while challenging of these mice is done by OVA aerosols 10 days later. Again, this protocol leads to induction of asthmatic features (5). This model was used to study the effect on the degree of maturity of GM-CSF derived iDC on the potential to induce effector T cell responses in the lungs. DC maturation was modified via anti-inflammatory compounds.

In both models, sensitization, followed by aerosol challenge leads to typical features of asthma such as perivascular and bronchial airway inflammation, eosinophilia, antigen specific IgE antibody production, goblet cell hyperplasia, bronchial hyperreactivity (BHR) and T helper 2 (Th2) cell cytokine production (e.g. IL-4, IL-5, IL-13) (Figure 1).

3. **Models of tolerance**. To address the issue whether DC subsets are involved in mediating tolerance to inhaled antigen, we have used a classical model of inhalation tolerance, initially developed by Holt et al. In these experiments, mice are first exposed to a single high dose of LPS-free OVA antigen intratracheally. Subsequently, mice are exposed to OVA aerosol. This does not lead to airway inflammation however, as no adjuvant was used. Others have shown that this response can be turned into Th2 immunity by adding TLR agonists to the harmless OVA (6). Others argue that this does not probe for tolerance, but could also signify that a 'null event' (i.e. the antigen was not recognized in the first place) is turned into immunity. To really probe for tolerance induction, mice need to be immunized again via the i.p. route with OVA/alum adjuvant, followed by OVA aerosol challenge 10 days later (like in the classical asthma model). In the case of succesful tolerization, the

OVA/alum model no longer leads to asthmatic inflammation (7). These models were employed to study the contribution of pDCs and cDCs on tolerance and immunity in the lung.

4. Models to address the function of DCs in **various anatomical compartments**. We studied the role of DCs in a compartment of the lung that is vital for the gas exchange, the interstitial compartment. It is currently unknown if this compartment also acts as a site of immune induction, similar to the respiratory mucosa. Therefore we have used a system that delivers a particulate-antigen via intravenous injection, to the vascular bed of the lung in close proximity to the interstitial compartment (see figure 1). By contrasting this with our other models in which antigen is given via i.t. injection (models 1-3) we can draw important conclusions about the effects of tissue residence on DC functional subspecializations.
5. Finally, throughout our work, we have also employed models to conditionally deplete DC subsets. In one strategy, we have employed CD11c-diphteria toxin receptor (CD11cDTR) transgenic mice in which $CD11c^{hi}$ cells (predominantly the iDCs and the cDCs, as well as alveolar macrophages) can be depleted by i.t. or systemic administration of diphterea toxin (DT) (8). To prove that any of the depleted cell types is responsible for the observed phenotype, these depleted mice can be reconstituted with wild type APCs, like monocytes (9, 10). In some experiments, we have also used monoclonal antibodies like Gr1 (Ly6C/G) and 120G8 to selectively deplete DC subsets or their precursors (11).

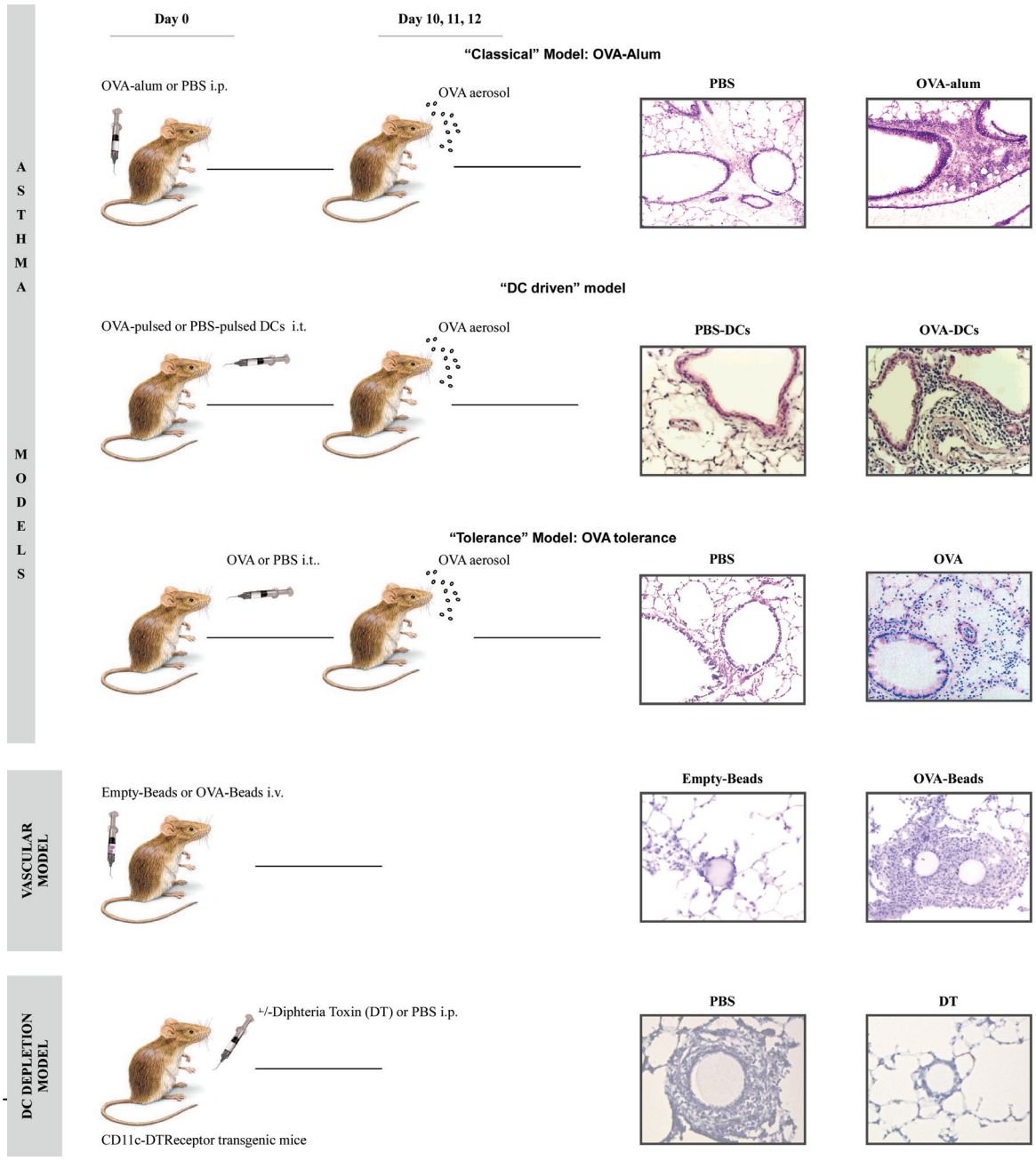

Figure 1. Mouse Models used to investigate the role of DC subsets in the lung

RESEARCH QUESTIONS

To determine the role of different DC subsets, their maturation and type of innate stimuli, as well as their anatomical location on the control of pulmonary immunity, the following research questions were addressed in this thesis using murine models for asthma, and peripheral vascular lung inflammation:

1. Do different subsets of DCs (cDCs, pDCs and iDCs) play specific roles in tolerance and immunity in the lung? (Chapters 3 and 7, theoretically introduced in chapter 2)
2. Is the degree of maturation/activation of iDCs and cDCs important to induce immunity and tolerance in the lung? (Chapters 4, 5, and 7, theoretically introduced in chapter 2)
3. Can the type of stimulation encountered by iDCs influence the subsequent immune response? (Chapters 6 and 7)
4. Is there a mechanism by which DCs sense interstitial or intravascular antigens in the lung, and what is the outcome of such encounters? (Chapter 7)

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Chapter 2

Introduction: Dendritic cell subsets and immune regulation in the lung

(Adaptation from: Dendritic Cell Subsets and Immune Regulation in the Lung. Seminars in Immunology 2005: 17: 295-30)

De Heer, H.J., Hammad, H., Kool, M., Lambrecht, B.N.

Introduction

The lung is continuously exposed to inhaled particles, microbes and harmless antigens to which either immunity or tolerance is induced. Dendritic cells (DCs) are mainly recognized for their extraordinary capacity to induce a primary immune response in the lung. Recent evidence suggests that particular subsets of DCs are essential in the decision between immunity or tolerance. Moreover, DCs play an essential role during secondary immune responses in the lung, where they control the inflammatory reaction. These novel concepts are of particular interest in understanding the pathogenesis of asthma, a disorder of aberrant immune reactivity to inhaled harmless allergens.

Numerous environmental pathogens, particulate matter, allergens and harmless antigens are present in the air we breathe. Although most of these particles will be held up in the upper airways, the lung is one of the most challenged organs of the body. The function of the lung is the vital exchange of oxygen and carbon dioxide and for this a delicate gas exchange mechanism has developed. Moreover, the outside air is separated from the bloodstream in lung capillaries only by one layer of alveolar epithelial cells, two basement membranes and a layer of endothelial cells. Therefore access to the bloodstream is easy and the lung has to defend itself from infection, yet at the same time keep inflammation in check, as this might damage the gas exchange apparatus vital to life. Therefore, the usual functional outcome of harmless antigen encounter in the lung is ignorance or tolerance. Yet, when faced with pathogens, the immune defence mechanisms of the lung can generate a protective immune response. Many cells of the innate and adaptive immune system play an important role in the induction of inhalation tolerance or immunity. DCs are crucial in regulating the immune response by bridging

innate and adaptive immunity. Signals from the type of antigen and the response of the innate immune response to it are translated by DCs into a signal that can be read by the cells of the adaptive immune response leading to an optimal response for a particular insult. Together, these signals consist of provision of a particular density of peptide-MHC, the expression of costimulatory or T helper (Th) polarizing cell surface molecules and the expression of soluble cytokines and chemokines that polarize T cells or enhance their survival (Figure 1).

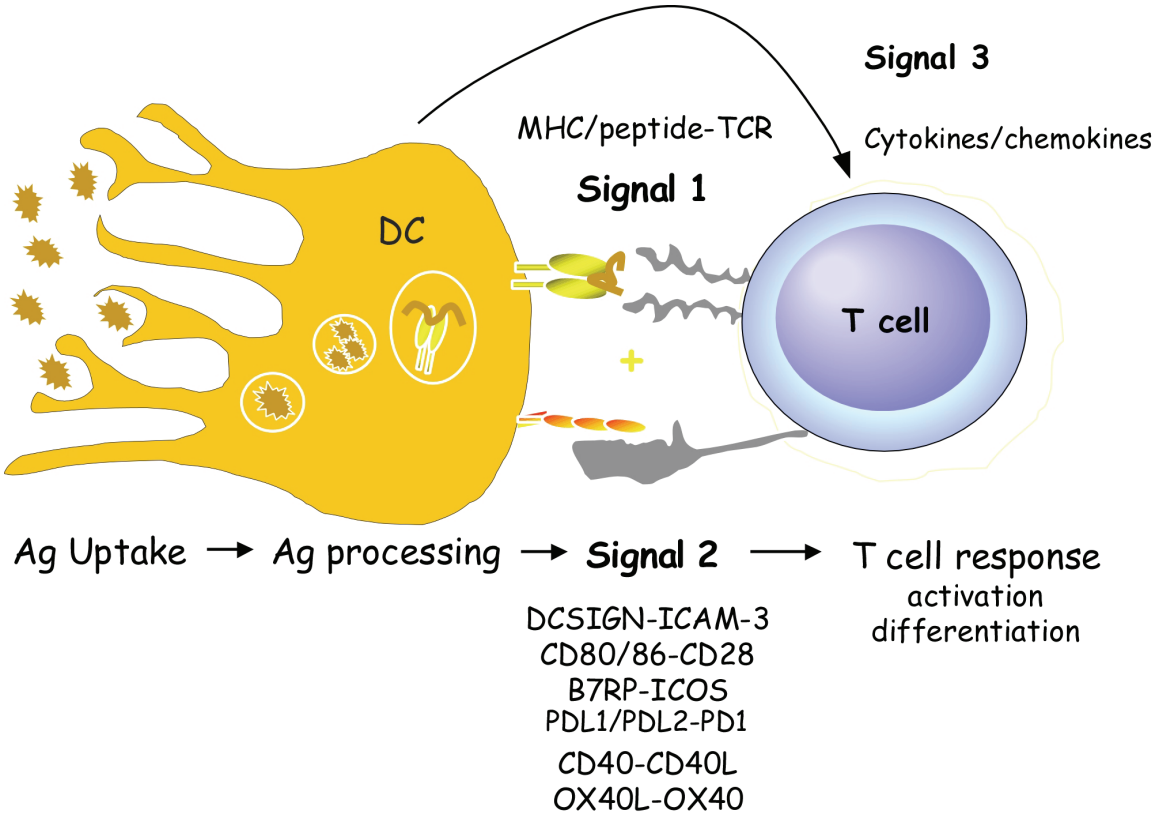

Figure 1. Dendritic cell induced naïve CD4 T cell activation. Upon maturation and antigen uptake, DCs can activate naïve T cells to differentiate and proliferate in the T cell area of draining lymph nodes. Three important signals are known to play pivotal roles in the proper activation and proliferation of these naïve T cells. Signal 1 consist of intracellular degenerated peptides presented on MHC class II molecules on DCs to naïve $CD4^+$ T cell receptor (TCR) T cells that can specifically recognize the presented peptide-MHC class II combination like a key in a lock. Signal 1 is not enough for complete activation, this is mediated by signal 2 which consist of upregulated co-stimulatory molecules on the cell surface of mature DCs Several DC-T cell interacting molecules are shown. Furthermore signal 3 is provided by cytokines and chemokines produced by e.g. these maturated (see also Figure 4) DCs which further determine the type of T cell development (e.g. Th1, Th2, or Th17). Lack of maturation of cDCs can lead to regulatory T induction (Figure 4).

At the same time, DCs also control the function and expansion of regulatory T (Treg) cells that tightly control overzealous inflammatory T cell responses. Although controversial, it has been suggested over recent years that particular functions of DCs such as tolerance or immunity or Th1/Th2 differentiation might be a specialized function of defined subtypes of DCs (1, 2). Others have refuted this idea and have claimed that DCs are very versatile cells, and can virtually induce any type of response depending on the need of the moment (3). Here, the different subsets of lung DCs and their role in the maintenance of tolerance or the induction of immunity will be addressed.

Dendritic cell subsets in mice and humans

In mice, up to five $CD11c^{high}$ DC populations are consistently found in lymphoid tissues. The spleen contains three subsets of conventional (cDC): the $CD4^{-}CD8^{+}$, the $CD4^{+}CD8^{-}$ and the $CD4^{-}CD8^{-}$ DCs (4). The $CD8^{+}$ DCs are located in the T cell area and the $CD8^{-}$ DCs are in the marginal zone. However, $CD8\alpha^{-}$ DCs can migrate to the T cell area after stimulation with bacterial compounds (5, 6). $CD4^{-}CD8^{+}$ DCs express the endocytic marker DEC205 while lacking expression of the myeloid marker CD11b, and are also found in small numbers in lymph nodes (LNs). They appear to be the main type of DCs in the thymus (7). Peripheral LNs contain two extra subsets of DCs which are not present in the spleen: $CD4^{-}CD8^{-}CD11b^{+}$ DCs are thought to be an immigrated form of tissue interstitial DCs, and another subset only found in skin draining LNs and representing immigrated Langerhans cells, expressing high levels of DEC205 and Langerin, and intermediate levels of CD8α (8). Although it was believed for a while that most of these subsets derived either from myeloid or lymphoid progenitors (9), recent data suggest that

both Common Myeloid Progenitors (CMP) and Common Lymphoid Progenitors (CLP) can give rise to all these DC subsets (10, 11), indicating that DC development seems to be an instructional program (12). Still others have suggested that there might be a dedicated circulating precursor for all DC lineages. Because of this, the denominator myeloid or lymphoid is best abandoned and most authors now refer to these 5 subsets of DCs found in central lymphoid organs as cDCs (13).

In recent years a $CD11c^{dim}$ murine plasmacytoid DC (pDC) population has been characterized and is found in all lymphoid tissue and bone marrow. These cells are the main source of both interferon-α (IFN-α) and IFN-β when stimulated with bacterial CpG or by viral infection. The early progenitors of pDCs have been described within the bone marrow hematopoietic cells, and express Ly6-C and CD31 (14-16). This pDC population has been ignored for a long time because of its expression of the B cell marker B220 and of the granulocyte marker Gr-1 (17). pDCs differ from cDCs by their expression of high levels of CD45RA, 120G8, and 440C, and their low expression of CD11c and MHC class II (18-20). In contrast to freshly isolated pDCs, which have low expression of CD8α in a proportion of cells, activated pDCs have high expression of CD8α (18, 21).

Under conditions of inflammation, monocytes can give rise to inflammatory type DCs (iDCs). $CCR2^{+}$ monocytes can be the immediate precursors to so-called inflammatory $CD11b^{hi}$ $CD11c^{hi}$ DCs, that still express high levels of Ly6C as a remnant of their monocytic descent (22). Under some conditions, these DCs have been called TIP-DCs for TNF-producing iNOS-producing DCs (23). In the mouse, two subtypes of monocytes are found, one expressing high levels of the fractalkine receptor CX3CR and low levels of Ly6C, the other one expressing intermediate levels of CX3CR and high levels of Ly6C

and CCR2 (24), and it is clear now that both subsets of monocytes can give rise to DCs, under particular conditions (25).

In contrast to studies on mice DC subtypes, very few investigators have studied **human** DCs directly isolated from lymphoid tissues. A major problem in comparing human with mouse DCs is the lack of expression of CD8 on human DCs. DCs isolated from spleen and tonsils show heterogeneity in the expression of CD4, CD11b and CD11c, indicating a level of complexity resembling mouse splenic DCs (26). Similarly, human blood DCs are heterogeneous in their expression of a variety of markers, but many of these reflect differences in the maturation or activation states of DCs rather than separate lineages (27). Most of the knowledge about human DCs has come not from the direct isolation of DCs from tissues or blood, but rather from their development *in vitro* from precursors. Three different precursors can be used to generate human DCs in vitro: (i) $CD34^+$ hematopoietic cells can give rise to E-cadherin$^+$ langerin$^+$ Langerhans cells when cultured in the presence of particular cytokines (28), (ii) blood monocytes can give rise to $CD11c^+$ HLA-DR^{lo} $CD1a^+$ DCs when cultured with GM-CSF and IL-4 (29), and (iii) pDCs (can be generated in vitro on OPG9 cells transfected with the notch ligand jagged-1, and in the presence of IL-7 and Flt3L (30). These human pDCs were discovered well before their mouse counterparts and are characterized by their unique phenotype IL-3 receptor $(CD123)^+$ BDCA-2^+ $CD4^+$ L-selectin$^+$ $CD11c^-$ lineage markers (2).

It is clear now that different subsets of DCs perform different tasks, some subsets of cDCs being better at crosspresentation of antigen to CD8 cells on MHCI molecules and others better at presenting endocytosed antigen to CD4 T cells on MHCII molecules (31-34). These differences might relate to intrinsic differences between DC subsets in

expression of endocytic receptors that target their cargo to well defined intracellular processing compartments (31) or to intrinsic differences in the expression levels of MHC processing machinery proteins in various subsets (33).

Phenotype of lung DCs

Dendritic cells are distributed throughout the whole lung and can be found in every compartment including the conducting airways, lung parenchyma, alveolar space, visceral pleura, and the pulmonary vascular bed (Figure 2) (35-40). However, the different subsets of lung DCs are differentially distributed. In studying DC phenotype, one needs to realize that most studies have used enzyme digestion of whole lung to study DC phenotype across species, obviously leading to a mixing of conducting and small airways with alveolar cells and lung interstitial cells, considerably confusing the literature (41).

In mice, all DC subsets express the integrin CD11c and DC subsets are further defined based on the expression of the marker CD11b, and on their anatomical location in the lungs (for example, conducting airways, lung parenchyma, alveolar compartment or pleura). The trachea and further branches of large conducting airways have a well-developed network of intraepithelial DCs, even in steady-state conditions. Here $CD11c^{high}$ DCs form a dense network underneath and within the epithelium with dendrite projections towards the lumen to sample for environmental antigens. These cells resemble skin Langerhans cells, and have been shown to express langerin and CD103 but not CD11b (42, 43). In the submucosa of the conducting airways, $CD103^{-}$

$CD11b^{+}CD11c^{+}$ cDCs can be found, and these cells are particularly suited for priming and restimulating effector $CD4^{+}$ T cells in the lung (44, 45).

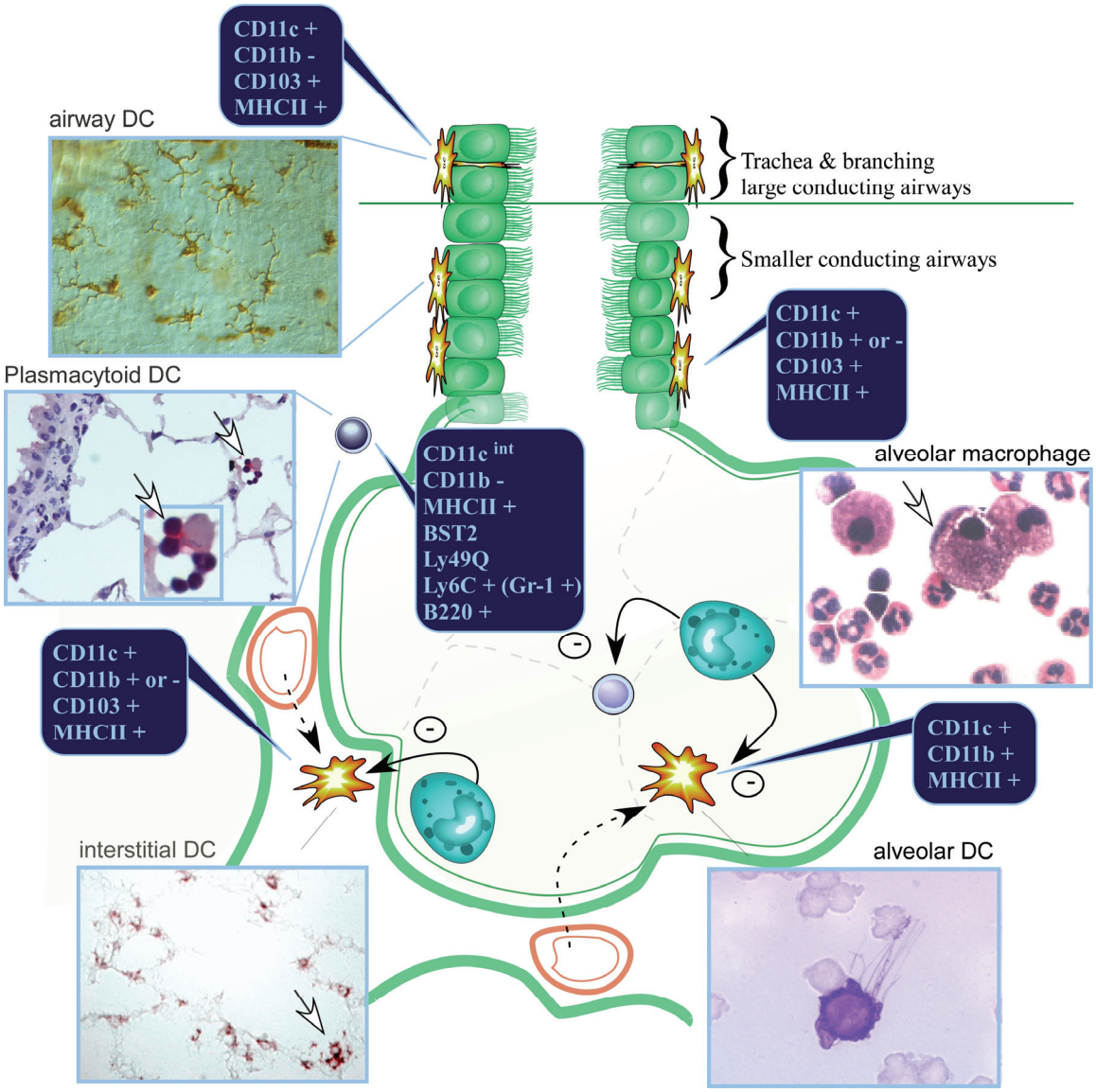

Figure 2. Distribution of dendritic cells in respiratory tract and lung. Airway dendritic cells (DCs) are located as a network immediately above and beneath the basement membrane, in between basal epithelial cells, they are $CD11c^{+}$, express langerin and CD103 but are CD11b negative. Interstitial DCs (stained for CD11c) are composed of a bone marrow stromal antigen 2 (BST2, recognized by monoclonal antibodies mPDCA-1 or 120G8) and B220+ Ly6C+ (Gr-1+) Ly49Q+ $CD11c^{dim}$ plasmacytoid DC subset and a B220- conventional DC subset being either CD11b+ or CD11b-. Their function can be suppressed by alveolar macrophages. Alveolar DCs can consistently be recovered by bronchoalveolar lavage in humans, rats and mice, particularly when inflammation is induced. The function of these cells can similarly be suppressed by alveolar macrophages. Alveolar macrophages can also directly suppress the function of T cells in the lung interstitium and alveolar compartment. Alveolar DCs form a minor fraction of alveolar cells, but they can expand rapidly during ongoing Th2 immune responses.

The lung interstitium, which is accessible by enzymatic digestion, also contains $CD11b^+$ and $CD11b^-$ cDCs that access the alveolar lumen (46-48). These cells are immature as assessed by the low expression of the costimulatory molecules CD40, CD80, CD86 (41, 45, 49-52) and by the high expression of several receptors for inflammatory chemokines and endocytic receptors (53). Although $CD8\alpha^+$ DCs can be found in the LNs draining the lung after intranasal infection of mice with viruses (54), no expression of this marker has been demonstrated on DCs freshly isolated from the lung. However, $CD8\alpha^+$ DCs can be obtained after overnight culture of $CD8\alpha^-$ lung DCs (51, 55). It is generally believed that interstitial lung DCs have a long half life of about 10 days, whereas conducting airway DCs have a half life of a couple of days (36). It should be noted that this population of cells also comprises DCs that line the small intrapulmonary bronchioles, as well as those that line the vessel walls. Intravascular DCs are also highly enriched in the lung capillaries and most authors studying lung DCs try to eliminate this subset prior to enzymatic digestion of the lung by rinsing the right heart circulation via the pulmonary artery (40).

In the nearby alveolar lumen, autofluorescent $CD11b^{lo}$ $CD11c^{hi}$ alveolar macrophages and alveolar $CD11b^{hi}$ $CD11c^{hi}$ DCs can be found. One needs to be particularly careful not to confuse $CD11b^{lo}$ $CD11c^{hi}$ alveolar macrophages with $CD11b^{lo}$ cDCs, as macrophages can profoundly suppress functional characteristics of lung DCs (56). Therefore, protocols that solely rely on purification of lung DCs by CD11c magnetic beads are confounded. We and others commonly use the characteristic autofluorescence of alveolar macrophages to discriminate the two when performing flow-cytometry based sorting experiments on lung DCs (45, 51, 52). Some data in the mouse suggest a particularly

critical role for alveolar DCs in retaining antigens for a prolonged time. These DCs are positive for F4/80, CD11c and CD11b, and capture airborne antigens and maintain the capacity to activate specific T cells long after antigen exposure (57). In steady state, these cells comprise a minor fraction of alveolar cells, but they expand considerably in the lungs with ongoing Th2 immune responses (57, 58). In mice, alveolar DCs also express the transcription factor, RunX3 conferring susceptibility to tumor growth factor β (TGFβ) regulation (59).

Lung pDCs in steady state only represent a minor population of $CD11c^{int}$ cells that also express bone marrow stromal antigen-2 (BST2, recognized by moAb 120G8 and mPDCA-1), Siglec-H, Ly6C, and B220 (49, 60, 61) and can be found in large conducting airways and lung interstitium (48).

Under inflammatory conditions, things become even more complicated. Inflammatory stimuli like TLR ligands, virus or bacterial infection, or environmental exposure to pollutants like cigarette smoke or ozone trigger the production of chemokines that attract monocytes and other inflammatory cells to the lungs in a CCR2 and/or CCR5 dependent manner (22, 42, 62-64). These $CCR2^{+}$ monocytes can be the immediate precursors to so-called inflammatory $CD11b^{hi}$ $CD11c^{hi}$ DCs, that still express high levels of Ly6C as a remnant of their monocytic descent (22). Under some conditions, these DCs have been called TIP-DCs for TNF-producing iNOS-producing DCs (23), and in some conditions lung iDCs have been shown to produce iNOS (22). These inflammatory DCs closely resemble the steady state $CD11b^{+}$ cDCs and therefore some authors have suggested that cDCs 'mature' or alter phenotype or even proliferate under inflammatory conditions (65, 66). A second confounder when studying DC subsets under inflammatory conditions,

particularly when high levels of IFNα are being produced during viral infection, is the fact that BST2 and B220 are also induced on inflammatory type $CD11b^{+}$ DCs (20), considerably confounding the discrimination beween pDCs and cDCs if the characteristics of size and expression of Siglec-H are not taken into account (GeurtsvanKessel, unpublished). This has led to the misinterpretation that pDCs can differentiate to cDCs and vice versa, or that pDCs become myeloid-like (62, 67, 68). Finally, a recently described population of NK cells with high MHCII and intermediate CD11c expression was discovered and named interferon-producing killer dendritic cell (IKDC) (69). It was recently shown that this subset is also found inside the lungs following inflammatory stimuli (GeurtsvanKessel, unpublished). As these IKDCs have a $CD11c^{dim}$, $B220^{dim}$, $MHCII^{dim}$ $CD11b^{-}$, $CD19^{-}$, $CD3^{-}$ phenotype, it is easy to confuse this subset with pDCs if one does not use Siglec-H or a specific NK marker like NK1.1 or NKp46 to discriminate the two.

In humans, very few studies have addressed the presence of DCs in conducting airways. Irregularly shaped cells with a marker pattern comparable to that of blood-derived DC (positive for DR, DQ, L25, RFD1, CD1c, and CD68) were predominantly observed in the epithelium and subepithelial tissue of the bronch(iol)us and in the bronchus-associated lymphoid tissue. In the epithelium, only approximately 30% of these cells were positive for CD1a (OKT6) and demonstrated Birbeck granules typical of Langerhans cells. In the subepithelial tissue, DCs formed characteristic small clusters with T cells (39, 70, 71). The precise origin of conducting airway DCs (in humans) is unknown. In humans, DCs of the mucosal aiway express CCR6 (72), a receptor for the MIP-3α a chemokine produced abundantly by bronchial epithelial cells (73). For a long time, it was thought

that pDCs were absent from the human lung as several groups have failed to detect them (70). The finding that pDCs were virtually absent from the lower conducting airways contrasted with previous findings in upper airway allergy (74). Recently however, one group has been able to identify a population of pDCs in enzymatic lung digests of humans (75). Like their counterparts in the blood, lung pDCs are characterized by the expression of CD123 and BDCA-2. However, the anatomical location of human pDCs in the lung has not yet been characterized.

In humans, alveolar DCs are low autofluorescent cells expressing CD11c and HLA-DR, and are very immature as indicated by their low expression of costimulatory molecules and CD83 (71).

Function of lung DCs in primary immune responses to inhaled antigen

Immature DCs are distributed throughout the lung and are at the focal control point determining the induction of pulmonary immunity or tolerance (76-78). Airway DCs form a dense network in the lung ideally placed to sample inhaled antigens and these cells migrate to draining lymph nodes (LNs) to stimulate naive T cells (Figure 3). Just as in the gut, airway DCs extend long dendrites to the lumen of the airways, forming bud-like extensions at the border of the air interface (79). Within a few hours after inhalation, airway cDCs and pDCs have taken up fluorescently labeled antigen within the draining mediastinal LNs (49, 52, 80). After 24 hours, both cDCs and pDCs in the mediastinal LNs contain antigen inside vesicles of the cytoplasm. What is unclear at present is whether pDCs take up antigen in the periphery of the lung and subsequently migrate to the nodes, or whether antigen is being transported to them by migratory cDCs or even a

specific subset of CD8α$^-$ CD11b$^-$ migratory DCs recently described by Belz et al. and GeurtsVanKessel (42, 54). Transport of immunogenic material from one non-migratory DC to another is certainly a possibility, as we and others saw that CD8α$^+$ DCs injected into the lung induce an immune response in the mediastinal node without migrating into it (81, 82).

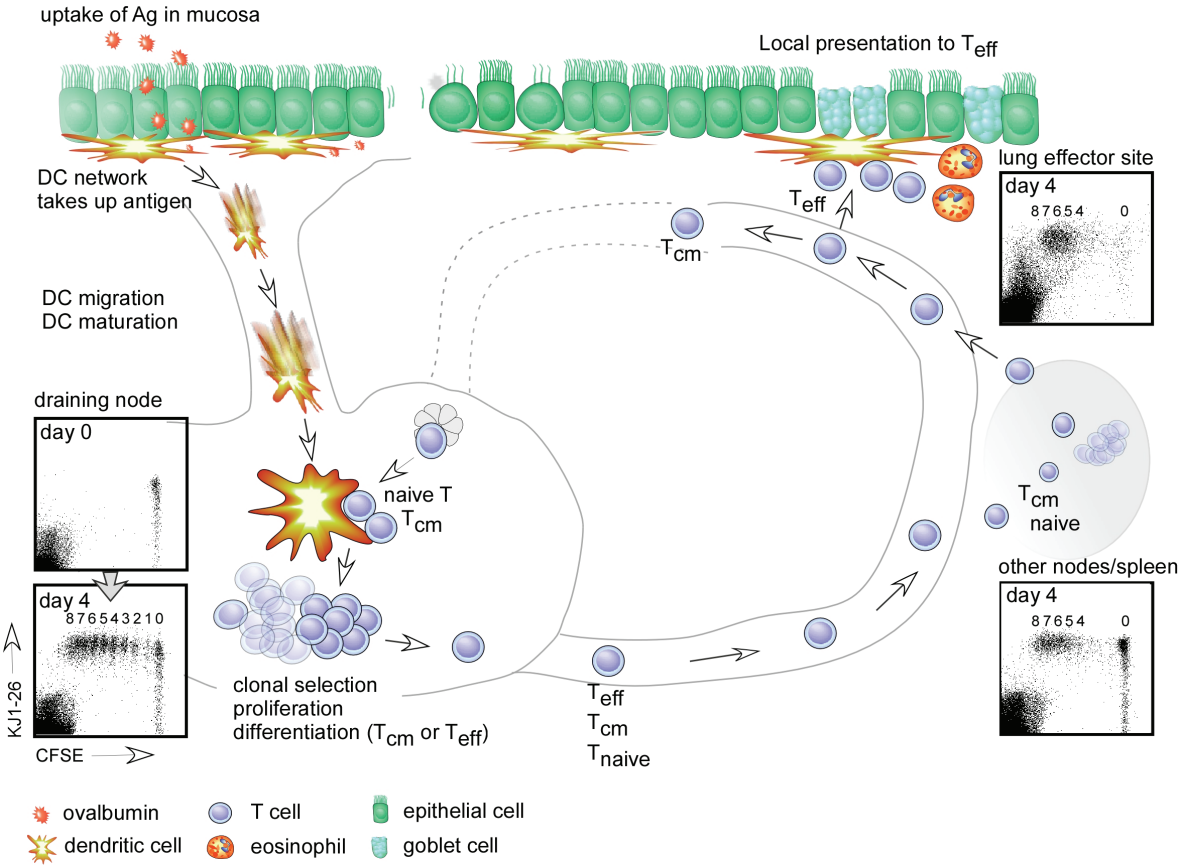

Figure 3. Overview of DCs and CD4+ T-cell migration during primary and secondary immune responses. Antigen is taken up by dendritic cells (DCs) across the mucosal impermeable barrier. Mucosal DCs continuously migrate from the lungs to the T-cell area of mediastinal lymph nodes (MLNs). In the presence of inflammation, this process is amplified, increasing the possibility that pathogenic substances will be presented to recirculating naïve T cells (T_{naive}) or central memory T (T_{CM}) cells. At the same time, DC maturation will be fully induced. When mature DCs arrive in the MLNs, they select specific T cells from the polyclonal repertoire of cells that migrates through the high endothelial venules and T-cell area. Within 4 days, this will lead to clonal expansion of antigen-specific T cells (**BOX 1**). When a T cell has acquired a certain threshold number of divisions, it will leave the MLN, to become either a T_{CM} cell or an effector T cell (T_{eff}). This is where migration pathways separate, and consequently the anatomical requirements for reactivation diverge. The T_{CM} cells will extravasate in other non-draining nodes and spleen, and will eventually accumulate in the spleen over time. Reactivation of these cells will ,therefore, only occur in central lymphoid organs. By contrast, effector T cells will extravasate in peripheral sites of inflammation, including the lung when the original inflammation is still present. In contrast to naive T cells, which are excluded from lung tissues, these effector T cells can be stimulated by local airway DCs to mediate their effector function. In this scenario, alternative antigen-presenting cells might be eosinophils or even epithelial cells, expressing MHC molecules.

Under steady state conditions, cDCs continuously migrate to draining LNs and present either (self)-auto antigens or harmless antigen in a tolerogenic form (83). Once they have reached the draining LNs, cDCs express intermediate levels of costimulatory molecules and MHC II. Under most conditions, DC migration is linked to partial activation, and even in the absence of infection, the majority of epithelial derived DCs arrive in the mediastinal nodes in a partially mature $CD86^+$ $CD40^+$ state (84).

BOX 1. Measurement of dendritic cell induced naïve T cell expansion by Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Under steady state conditions naïve CD4 and CD8 antigen specific T cells recirculate from lymph node (LN) to LN but in very low numbers. To follow the fate of naïve antigen specific T cells in antigen (e.g. Ovalbumin, OVA) sensitized wilde type (WT) mice in vivo, adoptive transfer of purified CFSE labeled OVA specific T cell receptor (TCR) transgenic T cells (e.g. 10.10^6) i.v. into WT mice can be used. For example when WT mice receive OVA antigen i.t. on day 0, and 1 or 2 days prior to that they received CFSE stained OVA TCR transgenic naïve CD4+ T cells i.v., the primary immune response that follows can be examined. As shown in Figure 2, on day 0, just after priming WT mice with OVA i.t., the draining MLNs of the lung shows one undivided population in a FACS dot plot whereby MLN cells are stained with KJ1-26-PE (an OVA TCR specific antibody) depicted on the y-axis and CFSE staining (intensity) depicted on the X-axis. DCs lining the conducting airways take up the OVA antigen and migrate to draining MLNs of the lung (this takes place within 24 hours). On day 4 after the OVA i.t., antigen specific TCR transgenic T cells have divided upon stimulation by DCs that have migrated and processed the OVA into MHC-II OVA peptide expressed on their cell membrane, that can be recognized by the LN circulating naïve CFSE labeled CD4+ OVA specific T cells. As shown every division of these OVA specific T cells can be followed because of the log-scale on the X-axis, whereby the 0 shows the non-divided naïve OVA specific and non-specific CFSE labeled T cells and 1 stands for OVA specific CD4+ T cells that have divided once out of the first non-divided OVA-specific T cells pool. Because of the log-scale and the fact that CFSE labeled cells that divided from one into two cells, whereby the later ones contain 50% of the CFSE staining each, every division can be followed, untill 8 divisions in this case. Most interestingly non (lymph) draining places of the lung such as inguinal LNs, spleen, but also the lung effector site show non divided (group 0) and 4 times divided OVA specific CD4+ T cells which is not only prove that priming of naïve CD4+ antigen specific T cells takes place in draining MLNs of the lung after antigen sensitization of the lung, but also that several divisions (e.g. 4) must take place before T_{eff} and T_{cm} leave the draining MLNs and circulate to other LNs and secondary lymphoid organs as well as the effector site where the antigen was first encountered (in this case the conducting airways of the lung).

For more details see the excellent review on this matter by Fazekas de St Groth, B. et al. Immunol Cell Biol. 1999, 77:530

Recent studies have suggested that lung DCs mediate protective immunity to inhaled antigens only when properly activated by innate immune system activating immune signals, acting through Toll like receptors (TLRs) or other pattern recognition receptors. It is unclear at present whether this occurs through activation of resident DCs by TLRs or via the recruitment of novel subsets of inflammatory type DCs (85). Virtually all subsets of DCs serve as sentinels in peripheral tissues, alert the immune system of the presence of pathogens, and initiate rapid immune responses. In the airways of rodents, infection with respiratory viruses (influenza A, parainfluenza, and Sendai virus), bacteria (*Mycobacterium tuberculosis*) or fungi (*Aspergillus fumigatus*) induce a rapid but

transient migration of mucosal DCs to draining LNs accompanied with their maturation (increased co-stimulatory expression CD40, CD80, CD86 and MHC II expression) (86-90). Under inflammatory conditions such as those provided by LPS or virus infection, the expansion of T cells induced by myeloid DCs leads to the generation of effector cells in the mediastinal nodes (42, 87, 91). In a very elegant paper, it was shown recently that virus infection of the lung also induces the formation of organized lymphoid tissue in the lung interstitium, closely associated with the airways and accompanying blood vessels. These structures contain bona fide DCs, T cells, B cells and even demonstrate germinal center reactions where class switching and affinity maturation occur during the primary immune response. The exact mechanisms by which these structures are formed, or how they sample antigen from the conducting airways is currently unknown. The same structures are seen in patients with end stage obstructive lung disease caused by smoking (COPD) and in this disease, latent infection with adenovirus or some other pathogen might be the trigger for formation (92). The signals that determine the type of response after encountering a pathogen in the lung are delivered by DCs in the LN (Figure 4 and Box 2).

Sporri and Reis e Sousa recently suggested that DC maturation and provision of peptide-MHC and costimulation to T cells is sufficient to induce T cell proliferation but not necessarily sufficient to generate effector cells (93). Additional signals are required. Cytokines are dominant signals that determine the quality and quantity of an effector immune response. During generation of an efficient effector immune response, DCs also have to overcome suppression by Treg cells, and the dominant way by which they seem to do this is by producing IL-6, that releases the suppression by naturally occurring Tregs

(94). Certain pathogens or pathogen-derived products induce the direct secretion of Th1 polarizing cytokines by DCs. Alternatively, NK cells reacting to pathogens can be an important source of initial IFN-γ for developing a strong Th1 response. Although it was recently shown that DCs in skin-draining lymph nodes recruit and activate NK cells as a source of IFN-γ, this pathway has not yet been demonstrated in the lung, a normally Th2 biased compartment (95). Stumbles et al. and Dodge et al. showed that resting respiratory tract DCs mainly induced Th2 responses, unless instructed by a strong TLR signal to induce the Th1 polarizing cytokine IL-12 (50).

Also in the lung, the type of immune response induced depends on the strength of the activating innate immune system stimulus. Elegant studies by Eisenbarth showed that low level TLR4 agonists prime DCs to induce a Th2 response, by inducing their full maturation, yet not their production of IL-12 (91). High level LPS administration induced high level IL-12. IL-12 seems to be a dominant cytokine for Th1 responses in the lung, yet the LPS induced Th1 response induced by myeloid DCs in the lung was not dependent on IL-12 (96). Although interleukin 12 may be redundant for some Th1 inducing stimuli, it is certainly sufficient as retroviral overexpression of this cytokine in myeloid DCs in the lung induced strongly polarized Th1 responses (97).

A role for dendritic cells in effector responses in the lung

There is increasing evidence that particular DC subsets also play a role in chronic immune responses, beyond the sensitization phase. For the lung, this is particularly

relevant to asthma, a Th2 lymphocyte mediated disease characterized by effector Th2 responses to inhaled harmless allergens.

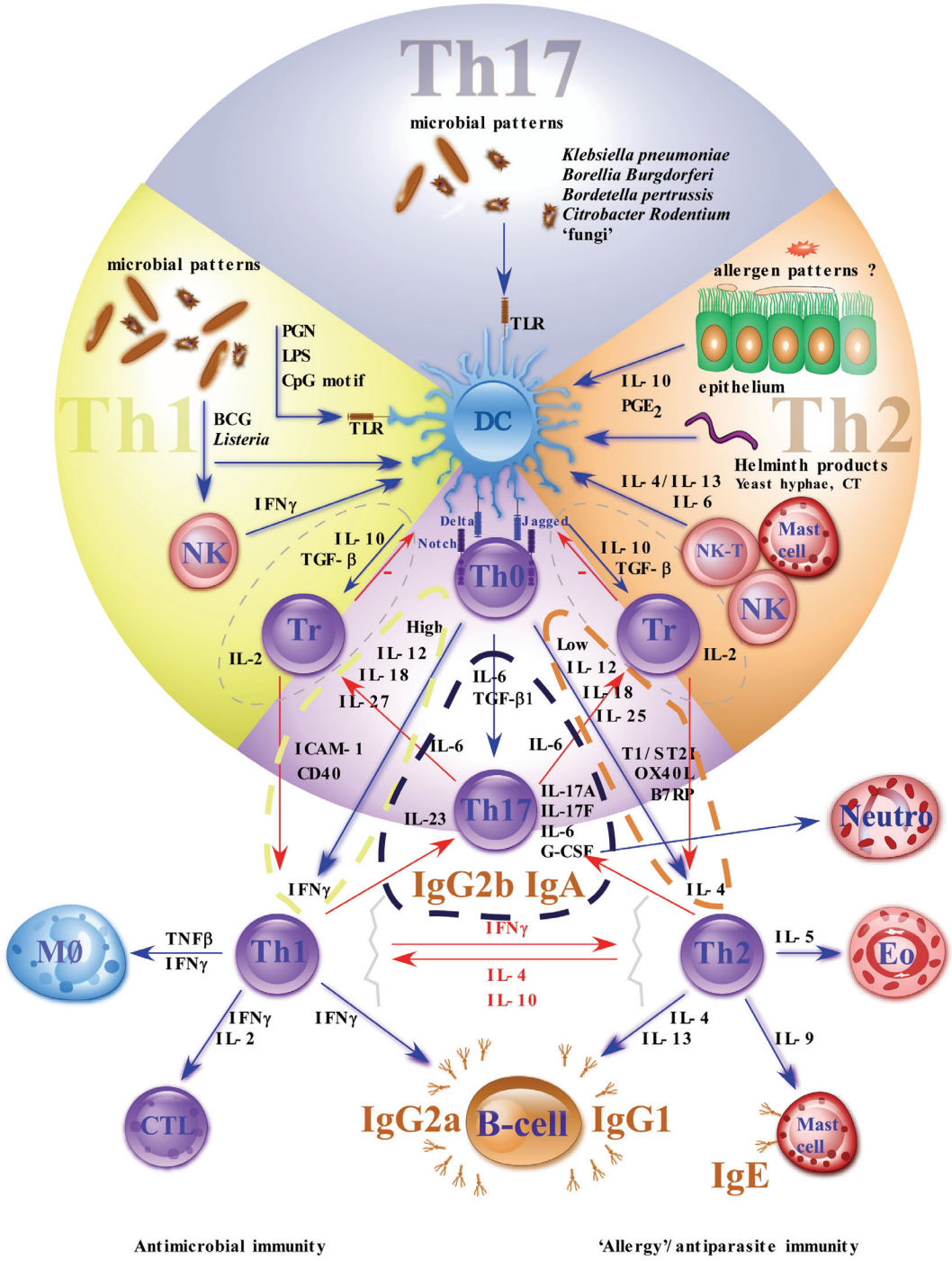

Figure 4. Dendritic cell mediated naïve CD4+ T cell skewing in lung draining lymph nodes.
Dendritic cells (DCs) express extracellular and intracellular receptors, such as Toll-like receptors and nucleotide-binding oligomerization domain (NOD) receptors that recognize pathogen-associated molecular patterns (PAMPs) contained in microbial motifs, as well as a wide variety of C-type lectin receptors that discriminate glycosylation patterns on self versus non-self proteins. In a vast network of interactions between encountered antigens (self and non-self, harmful and harmless proteins) and local cell types such as epithelial, vascular, and different types of cells of the immune system, DCs induce different types of stimulation of naïve $CD4^+$ and $CD8^+$ (not shown) T cells, primarily in the T cell area of draining lymph nodes.

Box 2. Dendritic cell mediated naïve CD4+ T cell skewing in lung draining lymph nodes. In the last decade more and more is known about how naïve CD4 T cells are induced to become Th1, Th2, Tregs, and most recently Th17 cells with specific immune functions (see for more extensive reviews Weaver, C.T. et al. Immunity 2006: 24, 677-688 and Veldhoen, M. & Stockinger, Trends Immunol. 2006: 27, 358-361):

Type of cell:	Induction by cytokines /receptors	Development and/or homeostatis induced by:	Development and/or homeostatis blocked by:	Function
Th1	IFN-γ	STAT1	GATA-3	Th1 develop mainly in T cell areas in peripheral lymphoid tissue by cDCs that migrated to this area and present antigens on MHC-II molecules in combination with maturation (co-stimulatory molecule expression). Th1 cells play an important role in clearance of intracellular present pathogens such as *mycoplasm* and *mycobacterium tuberculosis*, thru activation of MØ, CTL and IgG2a induced production by B cells (see Figure 4)
	IFN-α	T-bet	IL-4	
	IL-12	HIx	TGF-β	
	IL-18	STAT4		
	IL-27			
	Delta			
	ICAM-1			
	CD40			
Th2	IL-4	STAT6	IFN-γ	Th2 develop mainly in T cell areas in peripheral lymphoid tissue by cDCs that migrated to this area and present antigens on MHC-II molecules in combination with maturation (co-stimulatory molecule expression). Th2 cells play an important role in clearance of parasites (such as helminth and schistosoma), thru activation of Mast cells, Eosinophils, IgG1 and IgE production by B cells (see Figure 4)
	Low IL-12	GATA-3	TGF-β	
	Low IL-18	cMAF		
	Low IL-25			
	T1/ST2L			
	OX40L			
	B7RP			
Th17	IL-6	RORgammat	IFN-γ	Th2 develop mainly in T cell areas in peripheral lymphoid tissue by cDCs that migrated to this area and present antigens on MHC-II molecules in combination with maturation (co-stimulatory molecule expression) and produce IL-6 and TGF-b1. Th17 play an important role in the protection against extracellular bacteria (such as *Klebsiella pneumoniae*, *Borerelia burgdorferi*, *Bordetella pertussis*) and fungi. They were discovered in there role in auto-immune diseases such as EAE and CIA.
	TGF-β1		IFN-α	
	IL-23		IL-4	
	IL-17A			
	IL-17F			
	IL-21			
Natural Tregs	High-aff-TCR	Foxp3	IL-6	Develop intrathymically expressing Foxp3 and CD4, and are mostly present in T cell zones of secondary lymphoid tissues where they preempt effector T cell development from naïve precursors, thereby terminating (auto-reactive) effector CD4 T cell development before it begins by cell-cell contact.
	High-aff-TCR	IL-2	IL-1	
	CTLA4	CD25		
	GCN2	TGF-β1		
		pDCs, immDCs		
aTregs	TGF-β	Foxp3	IL-6	Develop from naïve $CD4^+$ $Foxp3^-$ T cell precursors in peripheral lymphoid tissues, where they may develop in parallel to effector T cells and track them to sites of inflammation to diminish T effector cell-driven inflammation by TGF-β or IL-10 (Tr1) production as pathogen-associated antigens are cleared.
	CD80/CD86	TGF-β	Smad7	
		ImmDCs, aTreg		
aTregs/Tr1	IL-10	ImmDCs	IL-6	
		aTreg/Tr1		

We and others showed that there is a 80-fold increase in the number of inflammatory $CD11b^+$ DCs in the airway mucosa and in the bronchoalveolar lavage fluid of mice and rats with experimentally induced asthma (45, 58). These DCs form intense clusters with

$CD4^+$ Th2 cells, leading to the local maturation of DCs (41, 45). GM-CSF-cultured DCs (most closely resembling the phenotype of inflammatory DCs) are by themselves capable of inducing all the features of asthma when given to the airways of sensitized mice (98). Moreover, the administration of bone marrow-derived OVA-pulsed myeloid $CD8\alpha^-$ DCs, but not of $CD8\alpha^+$ DCs, into the airways of mice could lead to a strong Th2 response associated with all cardinal features of asthma upon rechallenge of the mice with OVA (81, 99). It was also demonstrated that the intratracheal injection of human monocyte-derived cDCs obtained from house dust mite allergic patients and pulsed with the allergen Der p 1 into Severe Combined Immune Deficiency syndrome (SCID) mice led to an enhancement of the Th2-dependent allergic inflammation (100). Finally, by using CD11c-diphteria toxin receptor transgenic mice, in which airway cDCs can be depleted by the intratracheal administration of diphteria toxin, we showed that airway $CD11c^+$ DCs, were essential for maintaining the characteristic features of asthma such as eosinophilia, mucus cell hyperplasia and bronchial hyperreactivity (45). In the absence of $CD11c^+$ DCs, Th2 polarized lymphocytes failed to produce effector cytokines and induce asthma, illustrating that airway DCs are functionally required for effector function of Th2 cells. Interestingly, the function of DCs in established airway inflammation seems to be regulated by Tregs expressing TGFβ. Mice lacking the transcription factor RunX3 can spontaneously develop asthma features (59), and show a strong increase in the number of alveolar $CD11b^+$ DCs (cDCs or iDCs). These DCs display a mature phenotype (increased expression of MHC II and OX40-Ligand, a costimulatory molecule shown to play a critical role in the development of allergic lung inflammation (101)) and have an increased capacity to stimulate T cells. Moreover, these RunX3-/- DCs are able to mount

inflammatory responses to otherwise harmless inhaled antigens, possibly through their lack of responsiveness to locally secreted TGF-β (59), a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties.

Therapeutic implications of studying DC subsets and mechanisms by which these get activated.

If DCs are so crucial in mounting and maintaining immune responses to inhaled allergen, then interfering with their function could constitute a new form of treatment for allergic diseases. In addition, pharmacological modification of DCs might reset the balance of the allergic immune response in favour of the development of regulatory T cells and therefore lead to a more long-lasting effect on the natural course of allergic disease. Steroids are currently the cornerstone of anti-inflammatory treatment in allergic disease. Inhaled steroids reduce the number of DCs in the lungs and nasal tissues of patients with allergic asthma and allergic rhinitis, respectively (102). Steroids also induce the activation of indoleamine 2,3-dioxygenase (IDO) in pDCs in a glucocorticoid-induced TNF-receptor-related protein ligand (GITRL)-dependent manner, thereby broadly suppressing pro-inflammatory responses (103). Recently, several new molecules have been identified that can alter DC function in allergic inflammation and therefore could be possible therapeutic targets. Many of these compounds were first discovered by their potential to interfere with DC‑driven TH2-cell sensitization. The sphingosine 1‑phosphate receptor antagonist FTY720 is currently used in clinical trials for the treatment of multiple sclerosis and transplant rejection. When administered locally in the

lungs of mice with established inflammation, it strongly reduced inflammation by suppressing the T‑cell stimulatory capacity and migratory behaviour of lung DCs, without the lymphopenia that is commonly observed when the drug is given orally (104). FTY720 inhibited the potential of iDCs to form stable synapses with naive antigenspecific T cells as well as effector TH2 cells, providing a possible explanation as to how these drugs might work to inhibit allergic inflammation.

Selective agonists of particular receptors for members of the prostaglandin family might also suppress DC function. Prostaglandin D2 (PGD2) has pleiotropic effects in the immune system, owing to its activity on the DP1 and CRTH2 (also known as DP2) receptors, which are widely expressed on immune cells. The DP1 agonist BW245C strongly suppressed the spontaneous migration of lung cDCs to the mediastinal lymph nodes (MLNs) (80).

More importantly, BW245C suppressed airway inflammation and bronchial hyper-reactivity when given to allergic mice by inhibiting the maturation of lung cDCs.

In the presence of BW245C, DCs induced the formation of induced forkhead box P3 (FOXP3)+ regulatory T cells from FOXP3– antigen-specific T cells (105). A very similar mechanism was described for inhaled iloprost, a prostacyclin analogue that acts on the I prostanoid receptor expressed by lung cDCs (104, 106). As the number and activation status of lung CD11b+ iDCs during secondary challenge seem crucial for controlling allergic inflammation, studying the factors that control recruitment, survival or egress of DCs from the lung during allergic inflammation will be important, as this might reveal new therapeutic targets. Eicosanoid lipid mediators, such as prostaglandins and leukotrienes can also influence the migration of lung DCs (105). More detailed

information on the interactions between DC subsets, other inflammatory cells and epithelial cells will undoubtedly lead to the discovery of more potentially interesting drugs. In this regard, blocking the interaction of thymic stromal lymphopoietin (TSLP) and GM‑CSF with its respective receptor with small-molecule inhibitors or blocking antibodies might prove very useful (85).

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Chapter 3

Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen

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Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen

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Abstract

Tolerance is the usual outcome of inhalation of harmless antigen, yet T helper (Th) type 2 cell sensitization to inhaled allergens induced by dendritic cells (DCs) is common in atopic asthma. Here, we show that both myeloid (m) and plasmacytoid (p) DCs take up inhaled antigen in the lung and present it in an immunogenic or tolerogenic form to draining node T cells. Strikingly, depletion of pDCs during inhalation of normally inert antigen led to immunoglobulin E sensitization, airway eosinophilia, goblet cell hyperplasia, and Th2 cell cytokine production, cardinal features of asthma. Furthermore, adoptive transfer of pDCs before sensitization prevented disease in a mouse asthma model. On a functional level, pDCs did not induce T cell division but suppressed the generation of effector T cells induced by mDCs. These studies show that pDCs provide intrinsic protection against inflammatory responses to harmless antigen. Therapies exploiting pDC function might be clinically effective in preventing the development of asthma.

Key words: asthma • plasmacytoid dendritic cells • tolerance • mucosal immunity • regulatory T cell

Introduction

Asthma is an increasingly common disease that remains poorly understood and difficult to manage. Its incidence has doubled in westernized countries in the last two decades and worldwide costs are estimated to exceed those from tuberculosis and HIV/AIDS combined (1), necessitating a way to prevent this disorder. Asthma is a Th2 lymphocyte–mediated inflammatory airway disease characterized by airway eosinophilia, increased mucus production by goblet cells, and structural remodeling of the airway wall. This leads to variable airway obstruction and to bronchial hyperresponsiveness to nonspecific stimuli. In allergic asthma, the presence of high levels of allergen specific IgE are a reflection of an aberrant Th2 cell immune response to common inhaled environmental allergens such as house dust mite or pollen allergen (2). This process of Th2 cell sensitization to inhaled allergens occurs at a very young age and is influenced by environmental factors such as childhood infections and environmental exposure to microbial compounds (3). It is currently unknown how exposure to harmless inhaled antigen such as allergen leads to prolonged Th2 cell sensitization in individuals with allergy, as respiratory exposure to harmless antigen is a tolerogenic event (4). Recent evidence shows that airway DCs are at the focal control point determining the induction of pulmonary immunity or tolerance (5–8). Airway DCs form a dense network in the lung ideally placed to sample antigens and migrate to draining LNs to stimulate naive T cells (9–12). Airway DCs play a central role not only in initiating specific Th2 cell immune responses leading to experimental asthma (13, 14), but they also restimulate effector cells during ongoing airway inflammation (8, 15–17). Less is known about the tolerogenic capacity of airway DCs. The immune response leading to inhalation tolerance is accompanied by a considerable degree of primary T cell division in draining cervical and mediastinal LNs (MLNs) and therefore it is likely that it also involves antigen presentation by professional APCs (4, 18–21). In support of this theory, DCs obtained from lung draining LNs of tolerized mice were able to induce T cell unresponsiveness ex

vivo and transfer tolerance to naive mice, in a process that required active T cell costimulation through either CD86 or ICOS-L (3, 5, 21). Despite the occurrence of inhalational tolerance, some authors have witnessed sensitization to inhaled inert antigen, particularly when signals activating the innate immune system were coadministered (14, 22). As inhalation of harmless antigen induces sensitization or tolerance in a process controlled by lung DCs, we hypothesized that a more detailed study on the functional outcome of antigen presentation by subsets of DCs in the lung might provide insight into the decision governing tolerance or immunity. We found that particular subsets of DCs were able to take up and transport antigen in the lung. Whereas myeloid DCs (mDCs) were important for generating T cell division and priming, plasmacytoid DCs (pDCs) suppressed T cell effector generation. Strikingly, in the absence of pDCs, exposure to harmless antigen led to Th2 cell sensitization and to features of asthma.

Materials and Methods

Mice. 6–8-wk-old BALB/c mice were purchased from Harlan. OVA-TCR transgenic mice (DO11.10) on a BALB/c background were bred at the Erasmus Medical Center. All experiments were performed according to institutional guidelines of the animal ethics committee at Erasmus Medical Center.

Isolation of Bronchoalveolar, Lung, and LN Cells. After anesthesia with 2.5% avertin, mice were bled and bronchoalveolar lavage (BAL) was performed using 3 x 1 ml warm PBS containing 0.1 mM EDTA through a canula placed in the trachea (10). To obtain single lung cell suspensions, lungs were perfused with 20 ml PBS through the right ventricle, minced using iridectomy scissors, and digested with collagenase III and DNase I, as described previously (11). For obtaining single cell suspensions from LNs, MLNs were excised, minced, and digested as described above. After blocking the reaction with excess EDTA, cells (>95% viability) were washed and stained for flow cytometry.

Flow Cytometry and Sorting. All staining reactions were performed at 4°C. First, cells were incubated with 2.4G2 Fc receptor Ab to reduce nonspecific binding. Dead cells and debris were excluded using propidium iodide. To detect DCs, single cells were stained with APC-labeled anti-CD11c (HL3) and FITC-labeled anti–Gr-1 (RB6-8C5) Abs. The phenotype of $CD11c^{int}$ $Gr\text{-}1^{+}$ (pDCs) and $CD11c^{hi}Gr\text{-}1^{-}$ (mDCs) cells was determined by using PE-labeled anti-B220 (RA3-6B2), CD8α (Ly-2), CD19 (ID3), CD4 (RM4-5), CD40 (3/23), CD80 (16-10A1), CD86 (GL-1), CD54 (3E2), CD62L (MEL14), CD24 (M1/69), MHC II (2G9), CD45 RB (16A), and pan NK (DX5) Abs (all from BD Biosciences), and anti–PD-L1 (MIH5) and anti–PD-L2 (Ty25; eBioscience). In experiments where FITC-OVA uptake was studied, DCs were detected by PE-labeled anti–Gr-1 and APClabeled anti-CD11c Abs.

In some experiments, total lung and LN cells were sorted based on CD11c-APC and Gr-1-FITC staining using a FACSDiVa® flow cytometer (BD Biosciences). Cytospins of the different sorted lung cell populations were stained with May-Grunwald Giemsa.

Confocal Microscopy. Confocal analysis was performed on 6-μm cryostat sections of perfused lungs stained with anti–Gr-1 FITC and anti–B220-PE to detect pDCs. Sections were analyzed on a confocal laser microscope (LSM-510; Carl Zeiss MicroImaging, Inc.). To detect intracellular uptake of FITC-OVA, cytospins of $CD11c^{int}$ $Gr\text{-}1^{+}$ cells sorted from MLNs taken 36 h after FITC-OVA administration were stained with anti–B220-PE, and FITC signal was observed using confocal laser microscopy.

Functional Activity of DC Subsets. To detect the antigen uptake capacity of DC subsets, mice received an intratracheal (i.t.) injection of 800 μg FITC-OVA (screened for low LPS content; Molecular Probes) in a volume of 80 μl PBS. Draining MLNs were excised 36 h later and analyzed by flow cytometry. To study antigen-presenting capacity of DC subsets ex vivo, mice first received an i.t. injection of 800 μg OVA (LPS content, 20 pg/mg OVA; Seikagaku). Next, $CD11c^{int}$ $Gr\text{-}1^{+}$ $B220^{+}$ (pDCs) and $CD11c^{+}$ $Gr\text{-}1^{-}$ $B220^{-}$ cells (mDCs) were sorted from the MLNs 36 h later and cocultured with purified CD4 (purity, > 95% using magnetic bead purification; Miltenyi Biotec) naive carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled DO11.10 TCR transgenic T cells at a 1:10 ratio. The labeling of T cells with CFSE was essentially as described previously (10). The stimulatory capacities of lung DC subsets were compared with those of in vitro OVA-pulsed pDCs or mDCs grown from BM cultures in the presence of Flt3L or GM-CSF, respectively. Generation of BM DCs was essentially as described previously (23). 4 d later, proliferation of $CD4^{+}$ $KJ1\text{-}26^{+}$ T cells was determined by flow cytometry. As IFN-α production by pDCs is a defining characteristic of these cells, we cultured sorted $CD11c^{int}$ $Gr\text{-}1^{+}$ and $CD11c^{hi}$ $Gr\text{-}1^{-}$ DCs in the presence or absence of 2 μM CpG-oligodeoxynucleotides (5-TGACTGTG-AACGTTCCGAGATGA-3; Sigma-Aldrich). Supernatants were collected 24 h later and analyzed by ELISA for the presence of IFN-α (PBL Biomedical Laboratory, Inc.).

Immune Response to Inhaled OVA in the Presence or Absence of pDCs. Next, we studied the immune response to harmless inhaled OVA in mice with or without pDCs. For depletion of pDCs, 250 μg/ml depleting anti–Gr-1 or control isotype Abs were given on 4 consecutive days (24), starting 1 d before i.t. priming with 800 μg LPS^{low} OVA (LPS contamination of 2.9 ng/mg protein; Worthington) or, to avoid confounding effects of LPS, with 800 μg LPS-free OVA (LPS, 20 pg/mg protein; Seikagaku). In separate experiments, mice received four i.p. injections of pDC-selective depleting 120G8 Ab (50 μl ascites/day; provided by C. Asselin-Paturel, Schering-Plough, Dardilly, France; reference 25). On day 4, the depletion of $CD11c^{int-}$ $Gr\text{-}1^{+}$ cells was evaluated on total lung cells and MLNs. Next, we addressed whether mice were sensitized to inhaled OVA by rechallenging with OVA aerosol. After a wash out period of 20 d, mice received 3 OVA aerosols (10 mg/ml in PBS, generated using a jet nebulizer) of 30 min on 3 consecutive days. 24 h later, BAL fluid was taken and analyzed by flow cytometry as described previously (26). In brief, eosinophils were characterized as $CCR3^{+}$ SSC^{hi} cells, neutrophils as $CCR3^{-}$ SSC^{hi} cells, lymphocytes as $B220^{+}$ / $CD3^{+}$ FSC^{lo} cells, and macrophages as large autofluorescent cells. To measure cytokine levels, MLN cells were plated in roundbottom 96-well plates (10^6 cells/ml) and restimulated with 10 μg/ml OVA for 4 d. Production of IL-4, IL-5, IL-10, IL-13, and IFN-γ was assayed on supernatants by ELISA (BD Biosciences) as described previously (12). Levels of OVA-specific IgE were measured by ELISA as described previously (13). In addition, lungs were resected and embedded in HistoWax (Intertiles). 4-mm sections were stained with May-Grunwald Giemsa.

To study primary T cell activation in the lung in the presence or absence of pDCs, mice received an i.v. injection of 10 x 10^6 CFSE-labeled DO11.10 T cells day -1 before injection of 0, 8, 80, and 800 μg LPS-free OVA (day 0). From day -1 to 2, the mice received an i.p. injection of anti–Gr-1 Abs or control isotype Abs (250 μg/day). T cell responses were analyzed in the draining MLNs at day 4 by observing CFSE division profiles of live $KJ1\text{-}26^{+}$ $CD4^{+}$ T cells. Cytokine levels were measured as described above.

Induction of Regulatory T (T reg) Cells by Lung pDCs. To detect induction of T reg cells, we used a method recently described by Martin et al. (27). First, 800 μg LPS-free OVA was injected i.t. and $CD11c^{int}$ $Gr\text{-}1^{+}$ $B220^{+}$ cells were sorted from the MLNs 36 h later. Sorted DCs were cultured with naive DO11.10 T cells for 3 d at a 1:10 ratio. In the second phase, pDC-stimulated T cells were replated and cultured at 5 x 10^5 cells per well in the presence of 1 ng/ml mouse IL-2 (R&D Systems) for an additional 7 d without OVA. The T reg cell assay was performed by culturing freshly purified DO11.10 $CD4^{+}$ T cells (10^5 per well) with irradiated BALB/c splenocytes (10^5 per well) in 96-well plates with 10 μg/ml $OVA_{323\text{-}339}$ peptide in the presence or absence of pDC-stimulated T cells (10^5 per well). Cell proliferation was assessed after 48 h by [^{3}H]thymidine (1 μCi/well) uptake in a 16-h pulse.

Adoptive Transfer of pDCs into the Lung. $CD11c^{+}$ $CD11b^{-}$ $B220^{+}$ pDCs were sorted from Flt3L-cultured BM as described previously using a FACSDiVa® flow cytometer (23, 28). 100 μg/ml OVA was added during the last 24 h of culture. 4 x 10^5 OVA-pulsed or -unpulsed pDCs were injected i.v. three times on alternate days. 1 wk later, mice received an i.p. injection of OVA-alum (10 μg OVA adsorbed to 1 mg aluminiumhydroxide; Sigma-Aldrich; reference 15). Mice were rechallenged three times with OVA aerosols 10 d later, as described above. 24 h after the last aerosol exposure, BAL fluid was performed and cell differential counts were analyzed by flow cytometry (26).

Statistical Analysis. For all experiments, the difference between groups was calculated using the Mann-Whitney U test for unpaired data. Differences were considered significant when $P < 0.05$.

Online Supplemental Material. Data showing the phenotype of DC subsets and IFN-a production by DCs from the lung (Fig. S1) and the induction of tolerance by a single i.t. injection of OVA (Fig. S2) are available at: http://www.jem.org/cgi/content/full/jem.20040035/DC1, and below.

Results

Different Subtypes of DCs Are Present in the Lung. In humans and mice, there are two major subsets of DCs, called mDCs and pDCs. pDCs are known to be present in hematopoietic organs (29, 30), blood (31), and lymphoid organs (32–35), and play a critical role in mounting the protective immune response to virus infection by producing large amounts of IFN-a (31, 32, 34). Although lung mDCs have been described in detail, it is not known if lung also contains pDCs or what the function of these cells might be. To analyze the subsets and numbers of DCs present in the lung of naive mice, whole lungs were digested and stained for Gr-1 and CD11c, markers known to distinguish between mDCs ($CD11c^{hi}$ $Gr\text{-}1^{-}$) and pDCs ($CD11c^{int}$ $Gr\text{-}1^{-}$) in the spleen and LNs (35). Several populations of lung leukocytes could be discriminated by their expression of Gr-1 and CD11c (Fig. 1, A and B). When

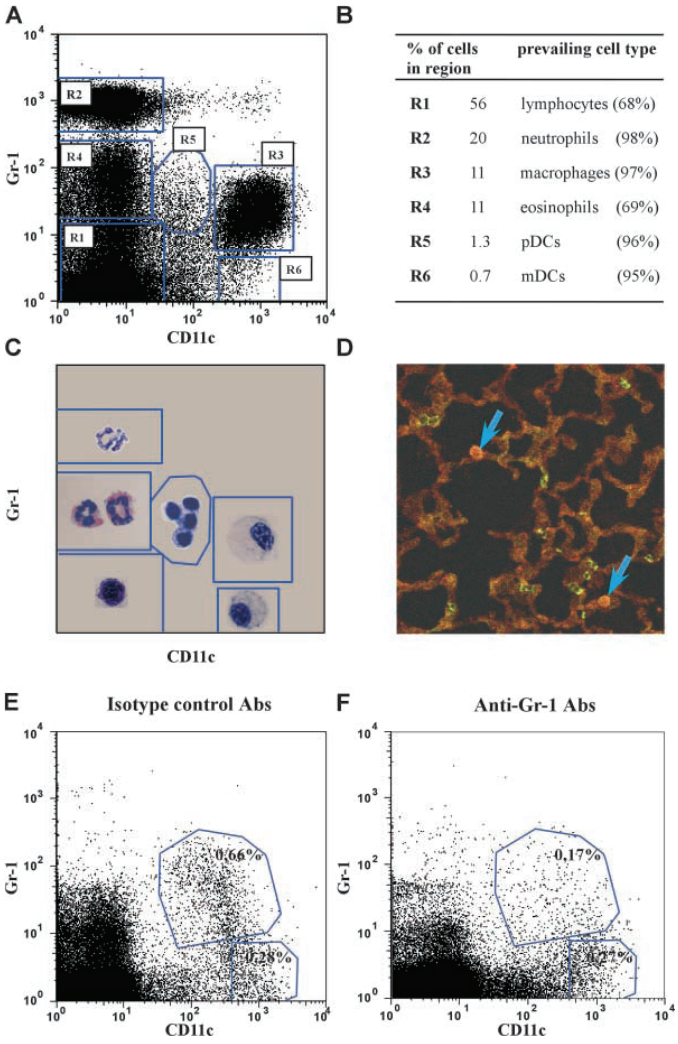

Figure 1. Identification of pDCs in the lung. (A and B) After digestion of the lungs, cells were stained for Gr-1 and CD11c expression, gated on live cells, and analyzed by flow cytometry. At least six different leukocyte populations were identified (regions R1–R6). (C) All leukocyte populations were sorted on Gr-1 and CD11c expression and stained with May-Grunwald Giemsa. Cytospins showed typical morphology of lymphocytes (R1), neutrophils (R2), macrophages (R3), eosinophils (R4), pDCs (R5), and mDCs (R6). Magnification, 100. (D) Confocal microscopy of frozen lung section stained with anti–Gr-1 FITC (green) and anti–B220-PE (red) Abs. Double positive colocalization signal appears orange (blue arrows). (E) Digested MLNs of naive mice were stained for Gr-1 and CD11c expression and gated on live cells. $CD11c^{int}$ $Gr\text{-}1^{+}$ pDCs and $CD11c^{hi}$ $Gr\text{-}1^{-}$ mDCs are shown. (F) MLNs of mice receiving anti–Gr-1 Abs for 4 d were digested and stained as described above. Depletion of $CD11c^{int}$ $Gr\text{-}1^{+}$ pDCs but not $CD11c^{hi}$ $Gr\text{-}1^{-}$ mDCs is shown.

sorted, the different populations showed typical morphology of lymphocytes, neutrophils, macrophages, eosinophils, pDCs, and mDCs (Fig. 1 C). At least 50% of the pDCs gated in region R5 were $B220^{+}$ (27, 35) and expressed $CD45RB^{+}$ (34), yet none expressed the classical B cell markers or the "lymphoid" marker CD8α, expressed on various subsets of spleen DCs (reference 36; Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20040035/DC1, and below). Furthermore, pDCs had a more immature phenotype than mDCs as assessed by their lower expression of costimulatory molecules and MHC II. On the contrary, the inhibitory B7 family costimulatory molecule PD-L1 was highly expressed on pDCs compared with mDCs (mean fluorescence intensity: 409.5 ± 42.5 and 328.5 ± 44.5, respectively; Fig. S1 B).

Confocal microscopy of lung tissues of naive mice confirmed that $B220^{+}$ $Gr\text{-}1^{+}$ pDCs are a major subset of DCs that are mainly located in the lung interstitium (Fig. 1 D).

Only $CD11c^{int}$ $Gr\text{-}1^{+}$ cells were able to produce IFN-α in response to CpG-oligodeoxynucleotides (Fig. S1 C), a defining functional characteristic of pDCs (24, 31). As a positive control, BM-derived pDCs grown in Flt3L produced very similar levels of IFN-α, whereas lung mDCs or BM-derived mDCs did not. As expected, $CD11c^{int}$ $Gr\text{-}1^{+}$ pDCs were also found in the MLNs of naive mice as described previously by others (reference 24; Fig. 1 E).

Lung pDCs Prevent Sensitization to Inhaled Harmless Antigen. Next, we questioned if pDCs influence the decision between tolerance or immunity to inert antigens in the lung (5). In our protocols using commercially available OVA screened to contain no or very low levels of LPS endotoxin, a single i.t. injection of OVA did not lead to sensitization or to Th2 cell–associated eosinophilic airway

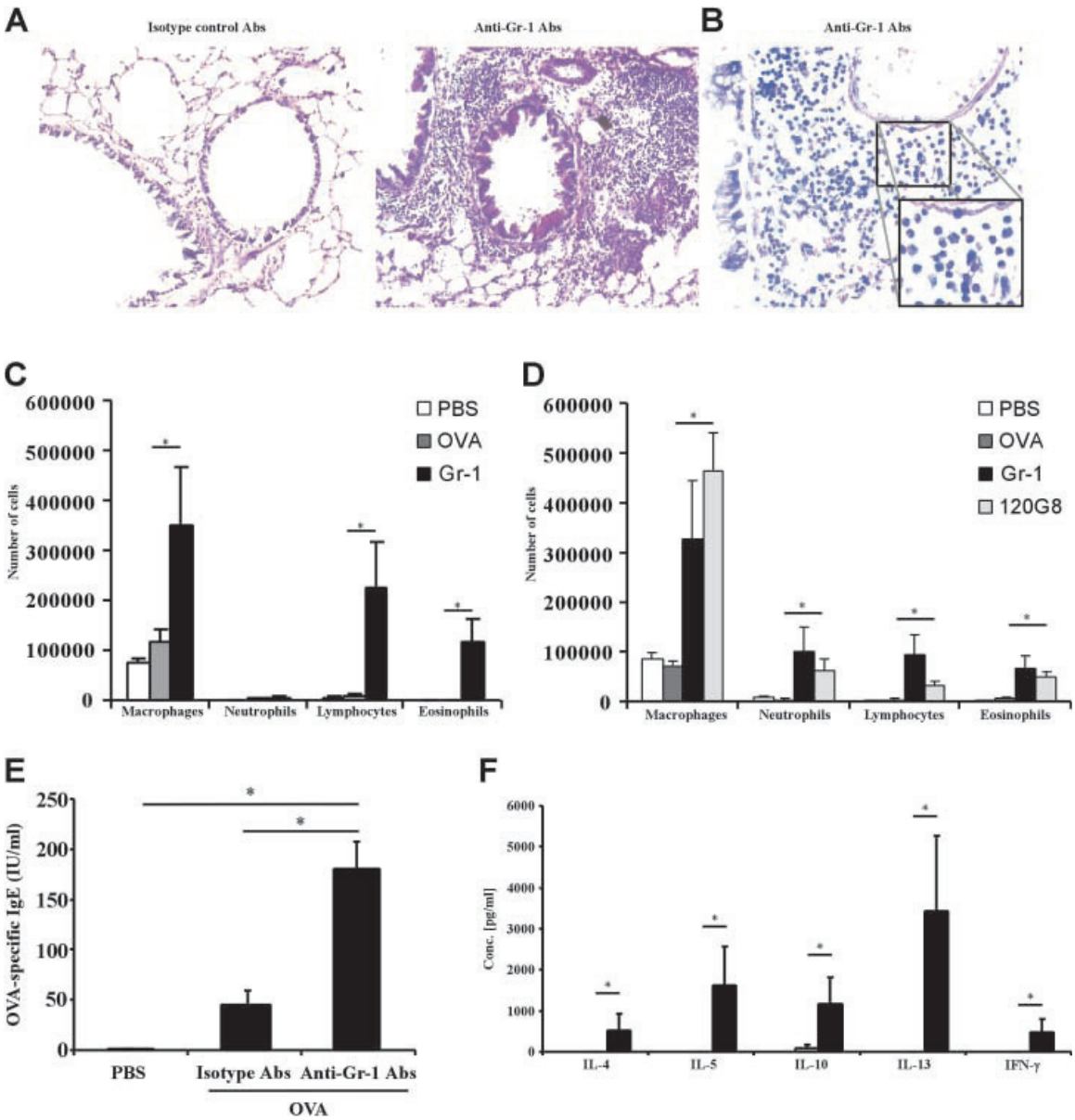

Figure 2. Role of pDCs in asthma. (A) On day 0, mice received an i.t. injection of LPS_{low} OVA. From days -1 to 2, mice were injected i.p. with anti–Gr-1 Abs or isotype control Abs. 20 d later, mice were exposed to three OVA aerosols. Lungs of mice depleted or not of pDCs were stained with periodic acid-Schiff. (B) Lungs were also stained with May-Grunwald Giemsa to detect eosinophils. (C) BAL fluid of mice injected with anti-Gr1 Abs (solid bars) or with isotype control Abs (shaded bars), and of naive mice (open bars) was analyzed by flow cytometry. Data are shown as mean ± SEM. *, $P < 0.05$. (D) Experiments were repeated using LPS-free OVA and the depleting Ab 120G8, given at the same time points as for Gr-1. (E) Sera from mice depleted or not from pDCs were analyzed for the presence of OVA-specific IgE. Results are shown as mean ± SEM. *, $P < 0.05$. (F) MLN cells collected from mice injected with anti-Gr1 Abs (solid bars) or with isotype control Abs (shaded bars), and of naive mice (open bars) were plated and restimulated in vitro for 4 d with OVA. Supernatants were assayed for IL-4, IL-5, IL-10, IL-13, and IFN-γ production. Data are shown as mean ± SEM. *, $P < 0.05$.

inflammation upon repeated challenge with OVA aerosol, and even lead to suppression of airway inflammation in a classical alum-adjuvant–driven animal model of asthma, a defining characteristic of true immunological tolerance (Fig. 2 A and Fig. S2, A and B, available at http://www.jem.org/cgi/content/full/jem.20040035/DC1, and below). To specifically address the role of pDCs in this process, pDCs were depleted using anti–Gr-1 Abs. Flow cytometric staining on draining MLNs (Fig. 1 F) confirmed that this Ab depleted pDCs but not mDCs as described previously by others (24). However, in the lung, pDCs were not as strongly depleted as in the MLNs (unpublished data). pDCs were depleted during a first exposure to OVA antigen given by i.t. injection.

When challenged with OVA aerosols 3 wk later, control mice, as expected (14), did not develop features of asthma (Fig. 2 A). In contrast, pDCdepleted mice developed cardinal features of asthma as shown by the eosinophilic inflammation around bronchiand blood vessels, the occurrence of goblet cell hyperplasia (Fig. 2, A and B), eosinophilia in the bronchoalveolar compartment (Fig. 2 C), and the presence of OVA-specific IgE in the serum (Fig. 2 E). These characteristics of asthma in pDC-depleted mice were associated with a substantial increase in Th2 cell cytokine production in MLNs (Fig. 2 F). These experiments were performed with OVA containing 2.9 ng LPS/mg OVA protein, potentially confounding our study (14). Moreover, the Gr-1 Ab can also deplete neutrophils or other Gr-1_ cells. Therefore, the experiments were also performed with LPS-free OVA using a pDCselective depleting Ab 120G8. Under these conditions, exposure to LPS-free OVA also led to sensitization to inhaled OVA (Fig. 2 D). These observations are strong evidence that lung pDCs suppress sensitization to otherwise harmless tolerogenic protein antigens.

Functional Activities of Lung pDCs. Next, we examined how lung pDCs might influence the immune response to inhaled antigens. Up to now, it is uncertain whether pDCs can take up whole antigens (besides viruses) for processing and presentation. Therefore, FITC-labeled OVA was given

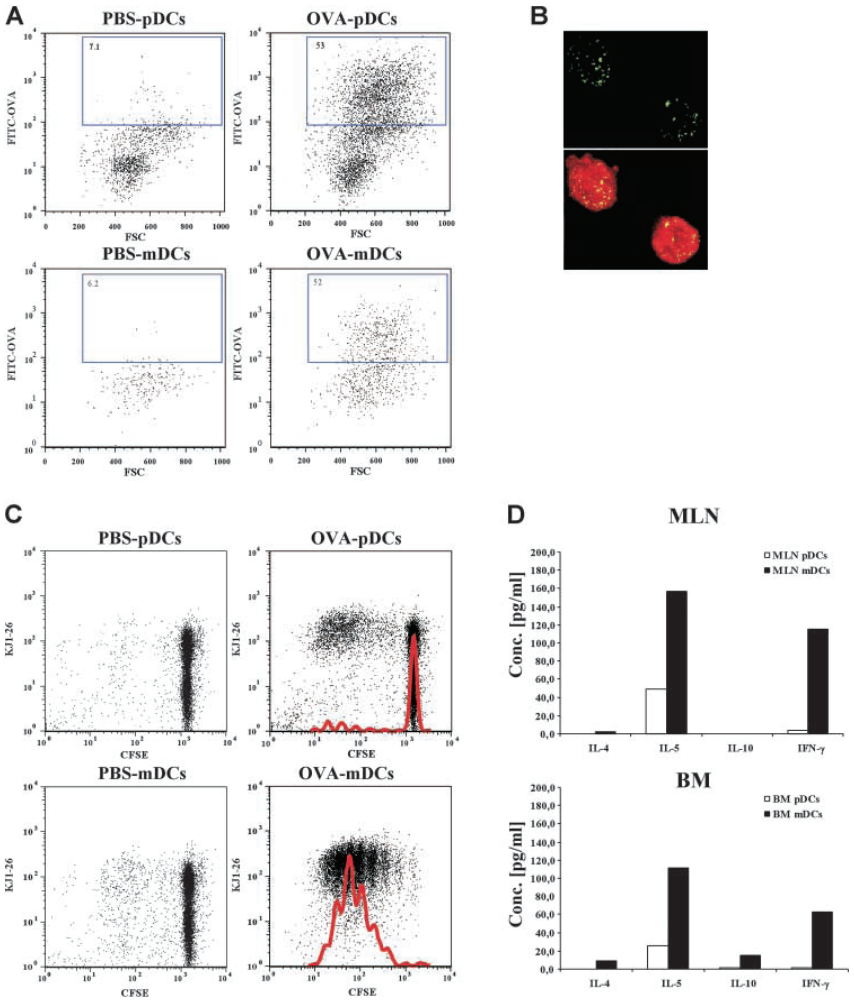

Figure 3. pDCs transport OVA from the lung to the draining LNs but fail to prime naive T cells in vitro. (A) Mice were injected i.t. with FITC-OVA. 2 d later, MLNs were analyzed for the presence of OVA-loaded pDCs (Gr-1$^+$ CD11c^{int}) and mDCs (Gr-1$^-$ CD11c^{hi}). (B) Confocal analysis of FITC-OVA uptake by pDCs. Mice were injected i.t. with FITC-OVA. 2 d later, cytospins of CD11c^{int} Gr-1$^+$ FITC-OVA$^+$ cells sorted from MLNs were prepared and stained with anti-B220 PE Abs. FITC$^+$ cells (green, top) are also positive for B220 (red, bottom) (C) Mice were injected i.t. with unlabeled OVA. 2 d later, pDCs (gated for Gr-1$^+$ CD11c^{int}) and mDCs (gated for Gr-1$^-$ CD11c^{hi}) were sorted from MLNs and cocultured with purified CFSE-labeled CD4$^+$ T cells from DO11.10 mice. 4 d later, proliferation of OVA-specific T cells was determined. (D) Cytokine production by T cells in vitro stimulated with pDCs. OVA-pulsed pDCs and mDCs were sorted from MLNs (top) or from the BM (bottom), and cocultured for 4 d with purified CD4$^+$ T cells from DO11.10 mice. Cytokines were assayed in the supernatants.

i.t. (11). pDCs as well as mDCs were able to take up FITC-OVA locally in the lung (unpublished data). Strikingly, after migration to the draining MLNs 36 h later, only mDCs and pDCs were FITC-OVA$^+$. The number of FITC-OVA$^+$ pDCs in the MLNs was higher than FITC-OVA$^+$ mDCs (Fig. 3 A). At this time point, >50% of the pDCs and mDCs present in the MLNs were positive for FITC-OVA. Moreover, as shown by confocal microscopy, FITC-OVA was present inside vacuoles of B220$^+$

pDCs (Fig. 3 B). As both FITC-OVA–loaded pDCs and mDCs were present in MLNs, their capacity to stimulate naive TCR-transgenic OVA-specific T cells ex vivo was compared. Only mDCs loaded with OVA in vivo were able to induce a strong proliferation (78.5% OVA-specific T cells had divided in response to mDCs vs. only 2.37% with pDCs; Fig. 3 C). Moreover, OVA-loaded mDCs obtained from MLNs or derived from the BM induced production of both Th1 and Th2 cell cytokines by T cells, whereas pDCs from the lung and BM did not produce cytokine levels above the level of unstimulated T cells (Fig. 3 D).

pDCs Suppress the Generation of Effector Cells by mDCs In Vivo. Next, we examined the contribution of pDCs to T cell division induced by various doses of LPS-free OVA inhalation by depleting with anti–Gr-1 Abs (37). As the direct examination of naive T cell activation is impossible due to the low precursor frequency, we adoptively transferred naive OVA-specific T cells from DO11.10 TCR transgenic mice. As shown previously (12), without OVA inhalation, DO11.10 T cells did not divide, whereas after inhalation of increasing doses of OVA, vigorous proliferation occurred 4 d later in OVA-specific CD4_ T cells of the MLNs (Fig. 4 A). T cell proliferation was of similar magnitude in the presence or absence of pDCs regardless of the dose of OVA used (Fig. 4 A), consistent with the in vitro observation that pDCs by themselves were unable to induce T cell proliferation. Although no apparent difference was detected in terms of T cell proliferation between mice depleted of pDCs or not, there was a significant increase in the amount of effector cytokines (IL-5, IL-10, IL-13, and IFN-γ) produced by proliferating T cells taken from immunized pDC-depleted animals (Fig. 4 B, 800-μg dose). This suggests that lung pDCs suppress the generation of effector function in naive T cells, which proliferate in response to Ag presentation by mDCs, and provide a mechanism for the occurrence of sensitization in the absence of pDCs.

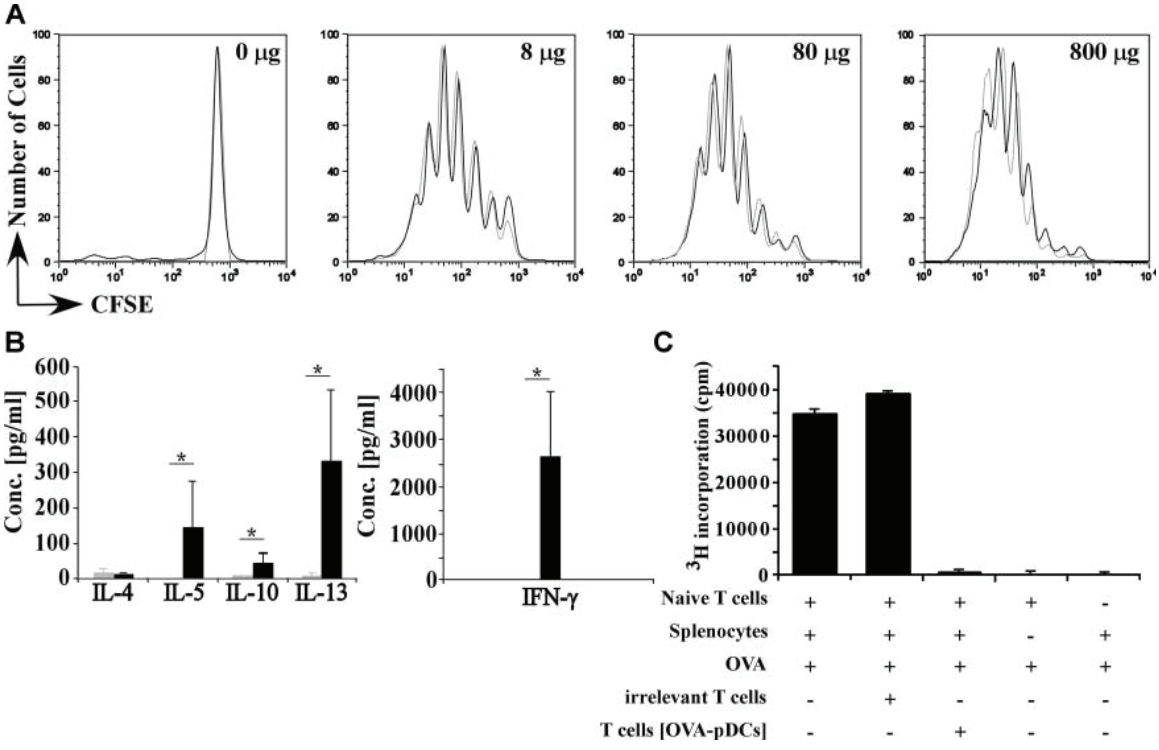

Figure 4. pDCs down-regulate the immune response in vivo without affecting T cell division. On day _1, mice were injected i.v. with CFSE-labeled T cells from DO11.10 mice. On day 0, they received an i.t. injection of different doses of LPS-free OVA (0, 8, 80, and 800 μg). (A) From days -1 to 2, mice were injected i.p. with anti–Gr-1 Abs (gray line) or isotype control Abs (black line), and proliferation of CFSE-labeled OVA-specific T cells was determined at day 4 in MLNs. (B) MLN cells were plated for 4 d and supernatants were assayed for cytokine production from mice injected with anti-Gr1 Abs (solid bars) or isotype control Abs (shaded bars). Data are mean ± SEM. (C) T reg cell assay. Proliferation of OVA-specific T cells after a 2-d coculture with syngeneic splenocytes and OVA peptide in the presence of T cells previously stimulated with OVA-pulsed pDCs (T cells [OVA-pDCs]) or irrelevant BALB/c T cells. Cell proliferation was assessed by [^{3}H]thymidine (1 μCi/well) uptake in a 16-h pulse. Data are mean ± SEM for triplicate cultures.

pDCs Induce the Generation of Suppressive T Cells In Vitro. Next, we investigated whether pDCs could be endowed with a tolerogenic potential by inducing T cell unresponsiveness and the differentiation of T reg cells. T cells that were first stimulated in vitro with pDCs obtained from the

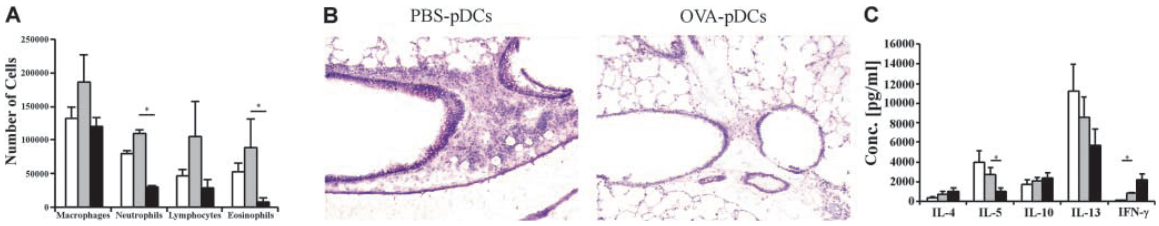

Figure 5. Adoptive transfer of pDCs protects against asthma development. pDCs were sorted from Flt3L-cultured BM, pulsed (solid bars) or not (shaded bars) with OVA, and injected i.v. three times on alternate days. As a control, some mice were not injected with pDCs (open bars). 1 wk later, all mice were immunized with OVA-alum i.p. and rechallenged three times with OVA aerosols 10 d later. (A) BAL fluid was analyzed by flow cytometry as in Fig 2. Data are mean ± SEM. *, $P < 0.05$. (B) Lungs from mice injected with OVA-pulsed or -unpulsed pDCs were stained with May-Grunwald Giemsa. (C) MLN cells were plated for 4 d in the presence of 10 μg/ml OVA. Supernatants were assayed for the presence of cytokines. Data are mean ± SEM. *, $P < 0.05$.

MLNs of OVA-exposed mice strongly reduced the proliferation of freshly purified naive T cells from DO11.10 mice induced by OVA peptide–pulsed splenocyte APCs. This was not due to dilution or to competition for IL-2, as polyclonal wild-type BALB/c T cells added to naive DO11.10 T cells and splenocytes in the same proportion did not lead to reduced proliferation (Fig. 4 C). In conclusion, our data suggest that pDCs induced the differentiation of T reg cells capable of suppressing antigen specific T cell proliferation.

Adoptive Transfer of pDCs Prevents Development of Asthma. Together, these data show that lung pDCs provide an intrinsic mechanism to suppress inflammation in response to inhalation of harmless antigen, and therefore might provide a mechanism to prevent the occurrence of sensitization. To more directly demonstrate this intrinsic tolerogenic capacity of pDCs, BM-derived OVA-pulsed pDCs (29) were injected three times i.v. before subjecting mice to a fully immunogenic asthma protocol onsisting of sensitization by i.p. injection of OVA in alum, followed by OVA aerosol challenge (38). As expected, mice receiving no pDCs or unpulsed pDCs uniformly developed eosinophilic airway inflammation and goblet cell hyperplasia (Fig. 5, A and B). In contrast, administration of OVA-pulsed pDCs before OVA-alum sensitization was able to strongly inhibit the aerosol-induced BAL eosinophilia (Fig. 5 A), airway inflammation (Fig. 5 B), and the production of Th2 cell cytokines by MLN T cells (Fig. 5 C). Although we did not use lung pDCs because of technical difficulty in\ obtaining sufficient cells, these data support the idea that the intrinsic tolerogenic capacity of pDCs can be exploited to prevent the onset of asthma.

Discussion

By showing the presence and antigen uptake of pDCs in the lung, by demonstrating direct priming to antigen in the absence of pDCs, and by demonstrating transfer of the tolerizing effect through injection of pDCs, we believe that pDCs have an essential immunoregulatory role in the lung that protects against development of airway inflammation to harmless antigens.

To our knowledge, this is the first description of pDCs in the lung of mice. These cells demonstrate intermediate expression of CD11c, express Gr-1, B220, and CD45RB, and they produce large amounts of IFN-α after stimulation with CpG motifs. These cells take up inhaled FITC-OVA protein and migrate to the MLNs draining the lung carrying their fluorescent cargo inside vacuoles. We believe that these cells are most likely related to the pDCs also described in the draining LNs and spleen of mice (35), and are distinct from the $CD45RB^+$ DCs recently described by Wakkach et al. (39) by their expression of Gr-1. We are currently studying if these $CD45RB^+$ $Gr\text{-}1^+$ DCs are also present in the lung.

In the absence of pDCs during primary OVA exposure to the lung, sensitization was induced, which led to features of Th2 cell–associated asthma after inhaling harmless antigen. This is a remarkable observation, as primary exposure to inhaled OVA in our system usually leads to inhalational tolerance, even when mice are immunized with OVA in alum (Fig. S2; references 18–21). For our studies in which we depleted pDCs, we have used Gr-1 Abs that also deplete neutrophils (24). We do not believe that absence of neutrophils had a predominant effect on T cell priming, as Gr-1^+ neutrophils did not carry Ag to the node. However, to more directly prove a role for pDCs as tolerogenic cells, we used another, more selective pDC-depleting Ab, 120G8, and found identical results, arguing against a predominant role for the neutrophil (25). Moreover, we performed adoptive transfer experiments before a fully immunogenic asthma protocol. Adoptive transfer of OVA-pulsed BM-derived pDCs (BM-pDCs) before induction of asthma completely inhibited airway inflammation. This response required the presence of OVA, as unpulsed pDCs did not suppress airway inflammation. Remarkably, under the same conditions, BM-mDCs did not exert this effect and even strongly enhanced the cardinal features of asthma (unpublished data and 13).

Next, we performed experiments to elucidate the mechanism by which absence of pDCs might lead to sensitization. It is known that inhalational tolerance to harmless antigen is accompanied by a significant amount of primary T cell activation in the draining nodes, and therefore it was suggested that depletion of pDCs might influence T cell division. To our surprise, however, we noticed no difference in the primary division of naive T cells when the outcome was either immunity (i.e., in the case of depletion of pDCs) or tolerance (treatment with control Abs), illustrating that the strength of primary T cell division does not predict the functional outcome of a T cell response. Moreover, removing pDCs during OVA inhalation resulted in higher levels of effector Th2 (IL-5, IL-10, IL-13) and Th1 cell cytokines (IFN-γ) being produced by T cells in the draining MLNs. Indirectly, these data show that the mDC subset is the predominant APC responsible for inducing naive T cell proliferation in the draining nodes. The uncoupling of T cell proliferation from generation of effector potential and from differentiation into memory cells is emerging as a new concept in T cell biology (4, 40). Lanzavecchia and Sallusto (41) recently proposed that T cell stimulation of insufficient strength or duration leads to proliferation of T cells that are "unfit" to respond to T cell survival signals and do not produce effector cytokines. The fact that in our experiments T cell proliferation was accompanied by proinflammatory effector T cell generation only when pDCs were depleted, suggests that pDCs are inefficient APCs that compete with immunogenic mDCs for antigen presentation, thus effectively reducing the overall strength or duration of T cell stimulation. From our and previous studies there is evidence that pDCs are weak APCs. Only when pDCs were pulsed with various doses of OVA peptide in vitro, could either Th1 or Th2 cell responses be induced (23). The fact that lung mDCs and pDCs take up fluorescent OVA does not imply that both cells provide an identical TCR ligand density, as there might be significant differences in antigen processing and presentation efficiency (32). Alternatively, pDCs in the lung and MLNs are immature, expressing low levels of MHC II and costimulatory molecules, yet high levels of the inhibitory B7 family member PD-L1 molecule, known to suppress T cell activation through interaction with PD-1 (42). This profile of high inhibitory and low costimulatory molecule expression together with the poor T cell stimulatory capacity ex vivo, point out to the "tolerogenic" potential of pDCs in the lung. It remains to be demonstrated whether the depletion of pDCs during priming would also lead to longer survival of responding T cells as would be predicted from the "T cell fitness" model (40, 41). Alternative explanations could be that immature lung pDCs are important for generating T reg cells after exposure of harmless antigen, as proposed in vitro by others (27, 29, 39, 43) and also shown indirectly ex vivo here (Fig. 4 C). In this scenario, in the absence of pDCs, T reg cells would no longer be induced from naive T cells or expanded from a pool of naturally occurring OVA-responsive $CD4^+$ $CD25^+$ regulatory cells (44, 45). The exact phenotype and function of these T reg cells induced by lung pDCs remains to be determined.

Concomitant exposure of harmless antigen with signals that activate innate immune responses such as viral infection, bacterial LPS, or GM-CSF, lead to a break in inhalational tolerance presumably through activation of mDCs (4, 7, 14, 46). Supporting this idea, injection of mature mDCs but not pDCs indeed leads to Th2 cell sensitization in the lung (unpublished data and 13). We observed induction of Th2 cell sensitization in response to LPS-free OVA inhalation, which was shown by us

(Fig. S2) and by others (47) to lead to tolerance and did not induce any form of mDC activation (unpublished data). Therefore, we believe that modulation of the intrinsic tolerogenic function of pDCs represents a second mechanism by which "adjuvant factors" lead to Th2 cell sensitization to harmless antigens. In this context, there is a known association between respiratory viral infections such as respiratory syncytial virus at a young age and the development of asthma (48). As viruses strongly interact with pDCs, we propose that inhibition of the normal tolerogenic activities of lung pDCs by viral infection could be the mechanism behind this association. Viral infections induce maturation of pDCs in vivo and this might explain the breaking of inhalational tolerance (37). Some "adjuvants" might also alter the ratio between immunogenic mDCs and pDCs in the lung and thus influence the decision between immunity and tolerance. This was nicely shown in the case of adenoviral transfection of the lung with GM-CSF, a predominant growth factor for mDCs but not pDCs, which led to sensitization (46). It will be interesting to study if increasing pDCs would have the opposite effect. It is striking that administration of CpG motifs to the lung leads to strong accumulation of lung pDCs (unpublished data) and to protection from asthma through unclear mechanisms not mediated by Th1 cell cytokines (49).

The discovery that pDCs influence the generation of T cell effector responses has extensive implications for our understanding of immunoregulation in a wide range of T cell–mediated diseases. Inhibition of the tolerogenic capacity of pDCs could thus offer an explanation why some viral infections lead to a break in peripheral self-tolerance in a number of autoimmune diseases such as diabetes (50, 51). In light of our findings, it will be very interesting to see whether the function of lung pDCs in humans with allergy or asthma would also be reduced, thus favoring Th2 cell sensitization to inhaled antigen (52). One recent report showed that the number of circulating pDCs in children with atopic asthma was reduced compared with healthy controls (53).

In conclusion, we have identified a role for endogenous pDCs in the protection against asthmatic reactions to harmless antigens by demonstrating that asthma develops in the absence of pDCs. Therefore, aberrant function of pDCs might contribute to allergic sensitization. Therapies targeted at amplifying the intrinsic tolerogenic capacity of lung pDCs may limit the development of asthma.

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Supplemental Material

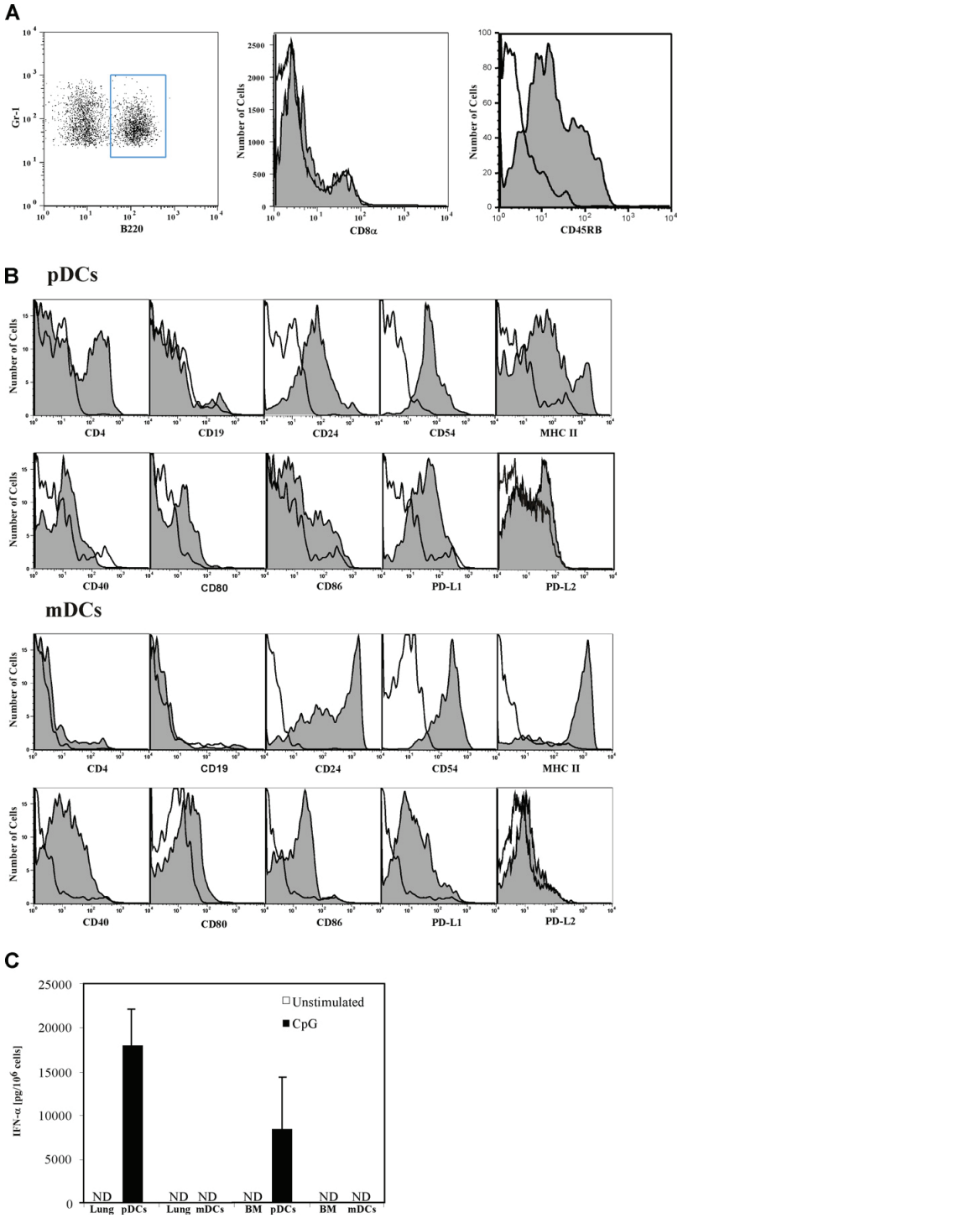

Figure S1. Phenotype of DC subsets and IFN-α production by pDCs from the lung. (A) Gr-1$^+$ CD11c^{int} cells (region R5 in Fig. 1) were analyzed for the expression of B220, CD8α and CD45RB. (B) pDCs (Gr-1$^+$ CD11c^{int}) and mDCs (Gr-1$^-$ CD11c^{hi}) were analyzed for the expression of CD4, CD19, CD24, CD54, MHC II, CD40, CD80, CD86, PD-L1, and PD-L2 (filled histograms). Open histograms show isotype-matched Abs. (C) pDCs (Gr-1$^+$ CD11c^{int}) and mDCs (Gr-1$^-$ CD11c^{hi}) were sorted from the lung or from Flt3L bone marrow (BM) cultures, and stimulated for 24 h with CpG-oligodeoxynucleotides. Supernatants were assayed for the presence of IFN-α. Data are shown as mean ± SEM. ND, not detectable.

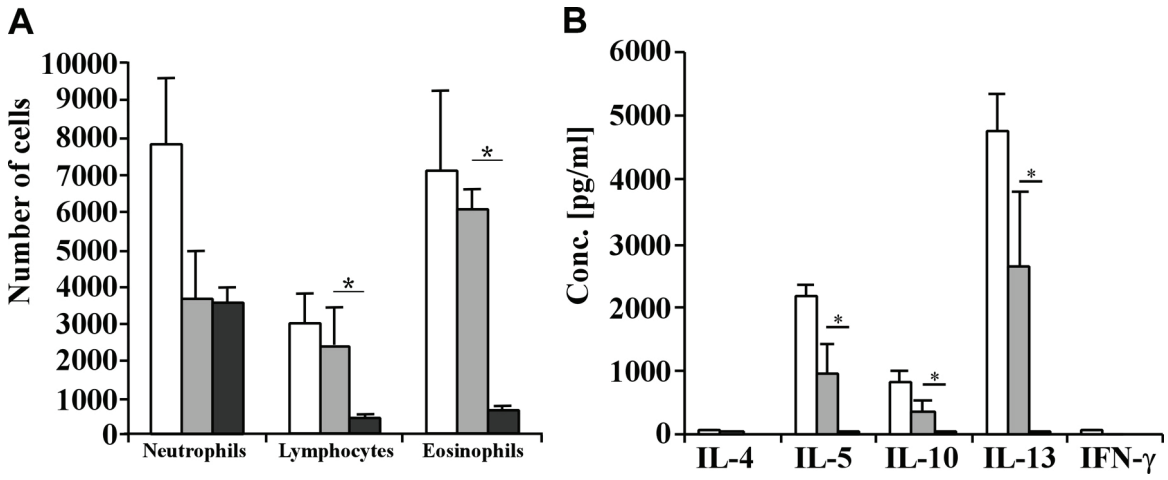

Figure S2. Induction of tolerance by a single intratracheal injection of OVA. Naive BALB/c mice received an i.t. injection of 800 µg LPS-free OVA (black bars) or as a control, PBS (gray bars). Some mice did not receive any injection (open bars). 1 wk later, the mice were immunized with either OVA-alum (10 µg OVA adsorbed to 1 mg aluminium hydroxide) or alum i.p. 10 d later, mice were challenged with three daily 30-min OVA aerosols (10 mg/ml in PBS, generated using a jet nebulizer). (A) After 24 h, BAL fluid was taken and total and differential cell counts were analyzed by flow cytometry Data are mean ± SEM. *, $P < 0.05$. (B) MLN cells were plated for 4 d in the presence of 10 µg/ml OVA. Supernatants were assayed for the presence of cytokines. Data are mean ± SEM. *, $P < 0.05$.

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Chapter 4

Activation of Peroxisome Proliferator-Activated Receptor-γ in Dendritic Cells Inhibits the Development of Eosinophilic Airway Inflammation in A Mouse Model of Asthma

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Activation of Peroxisome Proliferator-Activated Receptor-γ in Dendritic Cells Inhibits the Development of Eosinophilic Airway Inflammation in a Mouse Model of Asthma

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Peroxisome proliferator-activated receptors (PPARs) are activated by an array of polyunsaturated fatty acid derivatives, oxidized fatty acids, and phospholipids and are proposed to be important modulators of immune and inflammatory responses. Recently, we showed that activation of PPAR-γ alters the maturation process of dendritic cells (DCs), the most potent antigen-presenting cells. In the present report, we investigated the possibility that, by targeting DCs, PPAR-γ activation may be involved in the regulation of the pulmonary immune response to allergens. Using a model of sensitization, based on the intratracheal transfer of ovalbumin (OVA)-pulsed DCs, we show that rosiglitazone, a selective PPAR-γ agonist, reduces the proliferation of Ag-specific T cells in the draining mediastinal lymph nodes but, surprisingly enough, dramatically increases the production of the immunoregulatory cytokine interleukin (IL)-10 by T cells, as compared to control mice sensitized with OVA-pulsed DCs. After aerosol challenge, the recruitment of eosinophils in the bronchoalveolar lavage fluids was strongly reduced compared to control mice. Finally, T cells from the mediastinal lymph nodes produced higher amounts of IL-10 and interferon-g. Inhibition of IL-10 activity with anti-IL-10R antibodies partly restored the inflammation. The specificity of the phenomenon was confirmed by treating OVA-pulsed DCs with ciglitazone, another PPAR-γ agonist, and by using GW9662, a PPAR-γ antagonist. Our data suggest that PPAR-γ activation prevents induction of Th2-dependent eosinophilic airway inflammation and might contribute to immune homeostasis in the lung. *(Am J Pathol 2004, 164:263–271)*

Dendritic cells (DCs) are powerful antigen-presenting cells with a unique capacity to stimulate naïve T cells.[1] In the airways, immature lung DCs are ideally placed to sample inhaled antigens.[2] After they acquire antigens in the periphery, DCs migrate to the draining lymph nodes (LNs) where they localize in the T cell rich area and initiate immune responses. However, some evidence also suggests that tissue-resident DCs take up tissue antigens, migrate to the afferent LNs even in the absence of inflammatory conditions,[3] and contribute to the maintenance of tolerance.[4,5]

The signals that control DC migration from the periphery to the LNs are not fully understood. During inflammation, it is known that tumor necrosis factor-α and/or interleukin (IL)-1β provoke the departure of DCs from the periphery to the draining LNs by affecting the expression of adhesion molecules and chemokine receptors (particularly CCR7), by increasing the synthesis of metalloproteinases and by stimulating actin-dependent movements.[6-10] In addition to inflammatory cytokines and chemokines, other factors such as fatty acid derivatives also play a critical role in DC migration. For instance, LTC_4 and PGE_2, two major lipoxygenase and cyclooxygenase products of arachidonic acid, have been shown to promote the chemokine-driven DC migration.[11,12] On the other hand, by activating the D prostanoid receptor 1 (DP1), PGD_2 has an opposite action by preventing the departure of DCs from the skin and from the lung to the draining LNs.[13,14] PGD_2 is further metabolized into the cyclopentenone prostaglandin 15-deoxy-Δ(12,14)-prostaglandin-J_2(15d-PGJ_2), a peroxisome proliferator-activated receptor (PPAR)-γ agonist. PPARs are nuclear eicosanoid receptor transcription factors that regulate adipocyte differentiation and metabolism.[15] Three PPAR isoforms (α, β, and γ) have been identified. PPAR-γ is expressed in adipocytes, in the vascular wall,[16] and in cells of the immune system such as monocytes/macrophages,[17,18] B and T cells,[19,20] and DCs.[21,22] Moreover, it has been proposed that PPAR-γ may possess anti-inflammatory properties.[23-25] We have recently shown that PPAR-γ, a fatty acid-activated nuclear receptor, inhibits the tumor necrosis factor-α-induced migration of epidermal Langerhans cells and reduces the spontaneous migration of lung DCs.[13,26] These findings have encouraged different laboratories to investigate the role of PPAR-γ as therapeutic

targets for immune-mediated diseases. PPAR-γ agonists such as the thiazolidinedione class of anti-diabetic drugs have been shown to suppress experimental 2,4,6-trinitro-benzene sulfonic acid (TNBS) induced colitis and experimental allergic encephalitis.[27-29] Very recently, an effect of PPAR-γ agonists on lung allergic responses has also been observed.[30,31]

In this study, using a model of sensitization based on the intratracheal transfer of ovalbumin (OVA)-pulsed DCs,[32.33] we show that the PPAR-γ agonist rosiglitazone (RSG) inhibits the migration of antigen-loaded DCs in the mediastinal lymph nodes (MLNs) and reduces, in a quantitative and qualitative manner, the T-cell response in the MLNs. After challenge, a marked shift in the immune response [increased interferon (IFN)-γ and IL-10] was observed in the MLNs and was accompanied by a significant decrease in airway eosinophilia. These data suggest that activation of PPAR-γ in lung DCs might be important in the regulation of airway inflammatory diseases such as asthma.

Materials and Methods

Reagents and Antibodies

Reagents

OVA was from Worthington Biochemical Corp (Lakewood, NJ). At the dose we used in our experiments, the endotoxin level of OVA measured by a limulus-amebocyte lysate assay (Biowhittaker, Verviers, Belgium) was <0.001 μg. RSG was kindly provided by Dr. A. Bril (Glaxo Smithkline, Rennes). The PPAR-γ agonist ciglitazone and the PPAR-γ antagonist GW9662 were from Cayman (Ann Arbor, MI). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR). The fluorescein isothiocyanate (FITC)-conjugated anti-IAd/I-Ed (M5/114.5.2), Cy-chrome-labeled anti-CD3 (145.2C11), and anti-CD45R (RA3-6B2), and APC-labeled anti-CD4 (RM4-5) and anti-CD11c (HL3) were all from Pharmingen (Heidelberg, Germany). The phycoerythrin (PE)-conjugated anti-CCR3 and KJ1-26 (clonotypic OVA-TCR) were from R&D (Abingdon, UK) and Caltag Laboratories (Burlingame, CA). The polyclonal rabbit anti-PPAR-γ was from Calbiochem (San Diego, CA) and the secondary FITC-labeled goat anti-rabbit was from Nordic Laboratories (Aalborg, Denmark). The CCL19-Fc was a gift from J. Cyster (University of San Francisco, San Francisco, CA) and the secondary FITClabeled anti-human IgG Fab fragments were from Jackson Immunoresearch Laboratories (West Grove, PA).

Mice

BALB/c mice (6 to 8 weeks old) were purchased from Harlan (Zeist, The Netherlands). OVA-TCR transgenic mice (DO11.10) on a BALB/c background were bred at the Erasmus University (Rotterdam, The Netherlands).[34] All experiments were performed according to institutional guidelines of the animal ethics committee at Erasmus Medical Center.

Generation and Antigen Pulsing of Bone Marrow DCs

DCs were prepared as previously described.[35] Briefly, bone marrow was flushed with RPMI 1640 (Invitrogen, Paisley, UK) from femurs and tibiae of BALB/c mice. Cells were washed, enumerated, and plated in bacteriological 100-mm-diameter Petri dishes. Cell-culture medium (TCM) was RPMI 1640 supplemented with gentamicin (60 μg/ml), 2-mercaptoethanol (5 x 10^{-5} mol/L) and 5% fetal calf serum (Biocell Laboratories). At day 0 of the culture, the cells were seeded at a concentration of 2 x10^6/dish in medium containing rm GM-CSF (200 IU/ml) (kindly provided by K. Thielemans, University of Brussels, Belgium). At day 3, TCM containing 200 IU/ml rm GM-CSF was added. At days 6 and 8, half of the medium was collected, centrifuged, and the pellet was resuspended in TCM containing 200 IU/ml of recombinant murine GM-CSF.

At day 9 of the culture, DCs were pulsed overnight with 100 μg/ml of OVA containing the vehicle (dimethyl sulfoxide) in which RSG was resuspended (OVA-DCs), or with medium alone as a control. To stimulate PPAR-γ, some plates were also treated with 10 μmol/L of RSG (RSG/OVA-DCs) or 10 μmol/L of ciglitazone added 30 minutes before addition of OVA. To confirm the specificity of the phenomenon, in some experiments, we also pretreated DCs with 10 μmol/L of the PPAR-γ antagonist GW9662 for 30 minutes

before the addition of RSG. After antigen pulsing overnight, nonadherent DCs were collected, washed to remove free OVA or RSG, and resuspended in phosphate-buffered saline (PBS) at a concentration of 12.5 x 10^6 cells/ml. The viability of the DCs after treatment with the agonists or with the antagonist was >99% as assessed by trypan blue exclusion. The phenotype of DCs was determined by staining for 30 minutes with CD11c-APC, MHCII-FITC, in combination with CD40-PE, CD80-PE, and CD86-PE dissolved in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide. To detect CCR7 expression, DCs were first stained with CCL19-Fc for 30 minutes, washed, and incubated for another 30 minutes with anti-human IgG. DCs were washed and analyzed by flow cytometry on a FACScalibur (BD).

Intratracheal Injection of DCs

Mice were anesthetized with avertin (2% v/v in PBS) and 80 µl of the cell suspension (1 x 10^6 DCs) was instilled through the opening vocal cords.

Migration of DCs

At day 10 of the culture, unpulsed DCs and OVA-pulsed DCs treated or not with RSG were collected, washed, and labeled with CFSE as previously described.[33] One million DCs were instilled into the trachea of naïve BALB/c mice. Twenty-four hours later, MLNs were collected and minced using scissors. The LNs were then incubated for 1 hour at 37°C in RPMI 1640 containing 5% fetal calf serum, 1 mg/ml collagenase type 2 (Worthington), and 0.02 mg/ml DNase I (Sigma, Zwijndrecht, The Netherlands), according to a modified protocol.[36] LN cells were resuspended in PBS containing 10 mmol/L of ethylenediaminetetraacetic acid and centrifuged. $CFSE^+$ DCs were detected by flow cytometry on a FACScalibur. Cell viability was determined by trypan blue and was >95%.

Effect of RSG on the Activation of OVA-Specific Naïve T Cells in a Primary Immune Response

Because the frequency of OVA-specific T cells is very low in immunized animals, the primary activation of a naïve T cell is difficult to detect. To avoid this problem, a detectable number of naïve T cells purified from DO11.10 mice were adoptively transferred into BALB/c mice. Briefly, LNs and spleen were collected from DO11.10 mice and smashed. After red blood cell lysis, cells were labeled with CFSE. Cells were enumerated and dead cells, stained for trypan blue, were excluded. Cells (10 x 10^6) were injected intravenously in the lateral tail vein of BALB/c mice (day -2). On day 0, the mice received an intratracheal injection of OVA-DC, RSG/OVA-DC, or control unpulsed DCs. On day 4, MLNs were collected, homogenized, and stained for the presence of $KJ1\text{-}26^+$ $CD4^+$ reactive OVA-specific T cells. Some of the LN cells (200,000 cells/well in triplicates) were resuspended in RPMI 1640 containing 5% fetal calf serum and antibiotics and placed in 96-well plates. Four days later, supernatants were harvested and analyzed for the presence of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-10) cytokines by enzyme-linked immunosorbent assay (BD Pharmingen).

Effect of PPAR-γ Agonists on the Potential of DCs to Prime for Eosinophilic Airway Inflammation

On day 0, BALB/c mice were injected intratracheally with unpulsed DCs, OVA-DCs treated or not with ciglitazone (ciglitazone/OVA-DCs), or with RSG (RSG/OVA-DCs). To confirm the specificity of the phenomenon, RSG/OVADCs were also treated or not with the antagonist GW9662 (GW9662/RSG/OVA-DCs). In some experiments, mice received 250 µg of blocking anti-IL-10R antibodies or control antibodies (BD) 1 day before secondary challenge. From days 10 to 13, mice were exposed to 30-minute OVA aerosols (Grade III, Sigma). Mice were sacrificed 24 hours after the last aerosol. Bronchoalveolar lavage (BAL) was performed with 3 x 1 ml of Ca^{2+} - and Mg^{2+} - free HBSS (Invitrogen) supplemented with 0.1 mmol/L of sodium ethylenediaminetetraacetic acid. The BAL fluid was centrifuged; the cells were resuspended in HBSS, and enumerated in a hemocytometer. After washing, cells were stained for 30 minutes with anti-I-Ad/I-Ed FITC (macrophages), anti-CCR3 PE (eosinophils), anti-CD3 cy-chrome, anti-

B220 cy-chrome (T and B cells, respectively), and anti-CD11c APC (macrophages) in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide. Cells were washed and analyzed by flow cytometry as previously described.[37]

Airway Histology

After BAL was performed, 1 ml of fixative was gently infused through the catheter. The lungs were resected and embedded in paraffin. Four-µm sections were performed and stained with May-Grunwald Giemsa.

Cytokine Measurements in MLNs in a Secondary Response

MLNs were removed, homogenized, and resuspended in RPMI 1640 containing 5% fetal calf serum and antibiotics before enumeration. *Ex vivo* production of cytokines by T cells collected from the MLNs was measured after restimulation of 2 x 10^6 cells/ml with 10 µg/ml of OVA for 4 days.

Statistical Analysis

For all experiments, the difference between the groups was calculated using the Mann-Whitney *U*-test for unpaired data. Differences were considered significant if *P* was <0.05.

Results

We have previously established a model of adoptive transfer of bone marrow-derived DCs (BM-DCs) pulsed with OVA into the trachea of naïve mice. This model generates a primary OVA-specific immune response in the lung draining MLNs[33] that leads to Th2 priming. When mice are challenged with OVA aerosols, a Th2-dependent eosinophilic airway inflammation develops.[32] This model was used to test the effect of RSG.

RSG Impairs OVA-Induced BM-DCs Maturation

We have previously shown that mouse spleen DCs taken from Flt-3L-treated mice express the PPAR-γ.[21] By staining permeabilized DCs with a polyclonal rabbit anti-PPAR-γ antibody, we confirmed that BM-DCs also express PPAR-γ (Figure 1). We next investigated whether RSG, a PPAR-γ agonist, could alter the OVA induced maturation of BM-DCs. Compared to unpulsed DCs, the expression of the co-stimulatory molecules CD40, CD80, and CD86, but also of MHC II was increased in OVA-DCs (100 µg OVA/ml), a phenomenon probably caused by trace amounts of LPS in commercially available OVA. However, PPAR-γ activation by RSG treatment did not modify the expression of these markers on the cells (data not shown). Compared to

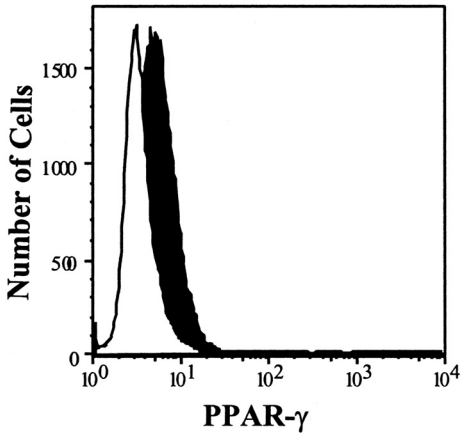

Figure 1. PPAR-γ expression in BM-DCs. DCs were fixed, permeabilized, and stained for PPAR-γ expression (**black histogram**). **White histogram** represents fluorochrome-matched isotype control mAbs.

unpulsed DCs, OVA DCs express a higher amount of CCR7, as assessed by CCL19-Fc binding (Figure 2). However, compared to untreated OVA-pulsed DCs, CCR7 expression was down-regulated in cells treated with RSG. These data suggest that PPAR-γ activation alters DC maturation by affecting the expression of CCR7, a chemokine receptor involved in DC emigration, but not the synthesis of costimulatory molecules and MHC II.

Activation of PPAR-γ Inhibits the Migration of OVA-DCs in Vivo

Because the treatment of OVA-pulsed DCs with RSG decreased the expression of CCR7, we next hypothesized that PPAR-γ activation might affect the migratory capacities of DCs to the MLNs. As shown in Figure 3, when injected into the trachea, CFSE-labeled OVA-DCs could be detected in the MLNs 24 hours after the instillation.[32,37] However, the treatment of OVA-DCs with RSG reduced their capacity to reach the MLNs, as compared to mice injected with OVA-DCs. These data were confirmed *in vitro* in chemotaxis assays performed in transwells, where the migration of OVA-DCs treated with RSG at the dose of 10 µmol/L in response to the CCR7 ligand MIP-3β was highly reduced as compared to OVA-DCs. Treatment of OVA-DCs with 1 µmol/L of RSG did not modify the migration as compared to untreated OVA-DCs (data not shown). These data suggest that the activation of PPAR-γ interferes with the CCR7-mediated migratory capacities of DCs. Because only the dose of 10 µmol/L of RSG altered DC migration *in vitro*, all further experiments were performed with this single dose of RSG.

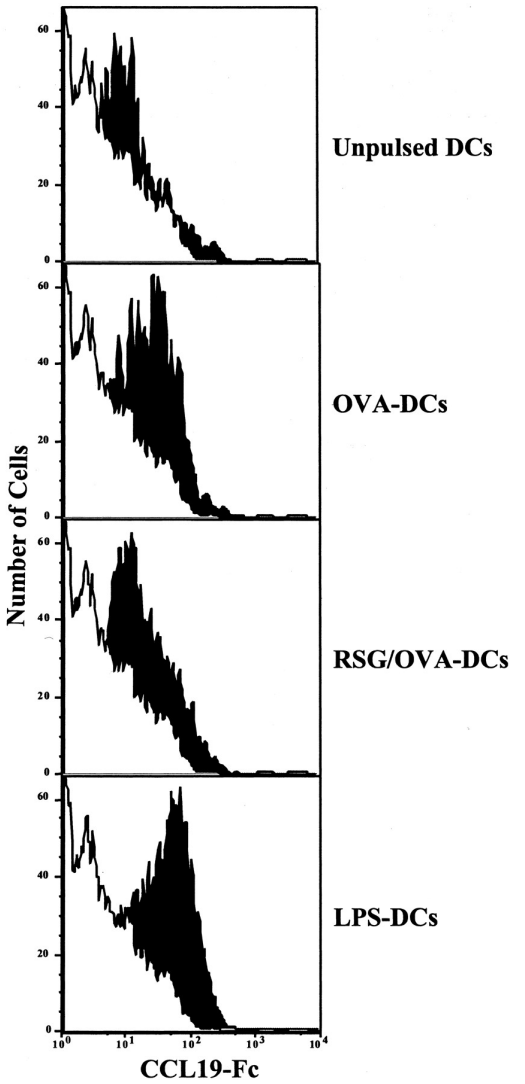

Figure 2. Effect of RSG treatment on CCR7 expression by DCs. BM-DCs were pulsed or not overnight with 100 µg/ml of OVA in the presence or in the absence of 10 µmol/L of RSG. LPS at the dose of 500 ng/ml was also used as a positive control. Cells were incubated with CCL19-Fc for 30 minutes before addition of PE-labeled anti-human IgG (**black histograms**). **White histogram** represents fluorochrome-matched isotype control mAbs.

Activation of PPAR-γ Affects T-Cell Proliferation and Differentiation in the MLNs

As PPAR-γ activation affects the migration of OVA-pulsed DCs to the MLNs, we next investigated whether it could also impact the activation of naïve T cell by DCs within the MLNs. To this end, naïve T cells from DO11.10 mice were labeled with CFSE and adoptively transferred, on day -2, into BALB/c mice. On day 0, these mice received an intratracheal administration of OVA-DCs, RSG/OVA-DCs, or unpulsed DCs. Flow cytometry was used to track cell division of CFSE-labeled T cells in MLNs. Figure 4 shows that 4 days after transfer of OVA-DCs, some transgenic T cells had already undergone seven divisions. In mice that received RSG/OVA-DC, some T cells also reached the seventh division peak, but the total number of naïve T cells

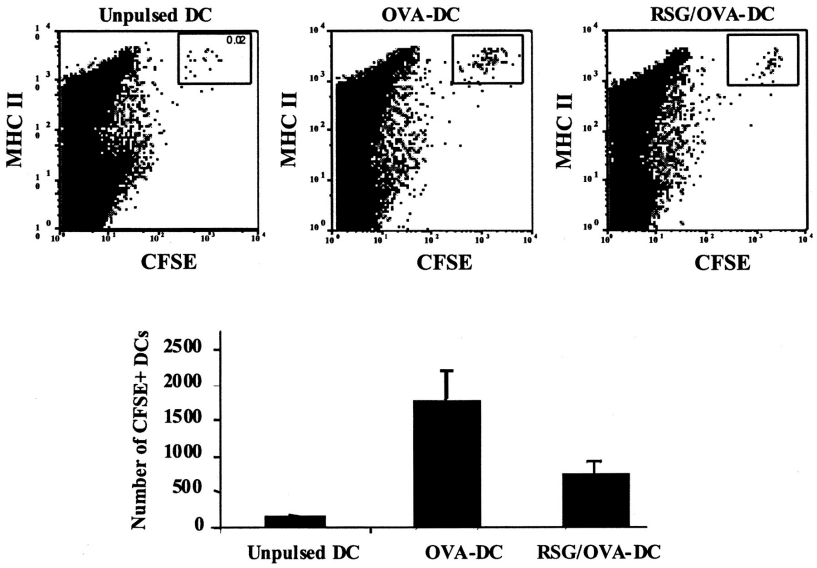

Figure 3. RSG treatment inhibits the migration of OVA-pulsed DCs. BM-DCs were pulsed or not with OVA overnight in the presence or in the absence of RSG (RSG/OVA-DCs). The next day, DCs were labeled with CFSE and 1 x 10^6 OVA-DCs, RSG/OVA-DCs, or unpulsed DCs were instilled into the trachea of naïve BALB/c mice. Twenty-four hours later, the presence of migrating CFSE-labeled DCs was investigated in the MLNs. **Top**: Plots show one representative mouse of four. The histogram represents the mean number of $CFSE^+$ DCs ± SEM from five mice per group.

activated and recruited into divisions was lower as compared to the group immunized with OVA-DCs. As expected, in mice immunized with unpulsed DCs, naïve T cells failed to divide (data not shown).

As PPAR-γ activation impacts the activation of OVA-specific naïve T cells in the MLNs, we next investigated whether the cytokine profile of T cells obtained from the MLNs was also changed. At day 4, cells from MLNs of mice that received OVA-DC, RSG/OVA-DCs, or unpulsed DCs were plated at 37°C for another 4-day period in the absence of OVA. The supernatants were then collected and assayed for the presence of IL-4, IL-5, IL-10, and IFN-γ. As expected, in mice that received OVA-DCs, T cells produced more IL-4, IL-5, IL-10, and IFN-γ than the cells of mice that were injected with unpulsed DCs (Figure 5). However, when mice were instilled with RSG/OVADCs, the production of IL-4, IL-5, and IFN-γ was identical but the level of the immunoregulatory cytokine IL-10 was dramatically enhanced (2.9-fold increase) as compared to the mice immunized with OVA-DCs. These results show that activation of PPAR-γ affects T-cell activation and modifies the nature of the primary immune response in the MLNs.

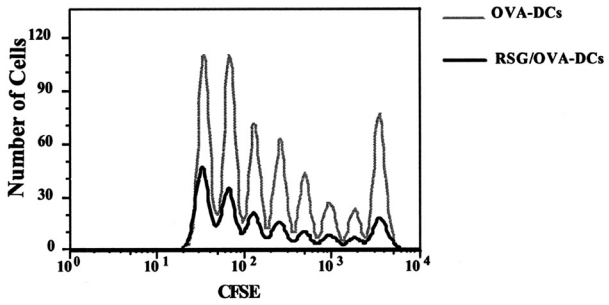

Figure 4. RSG impairs T-cell proliferation in the MLNs. On day -2, BALB/c mice received a cohort of CFSE labeled OVA-specific naı¨ve T cells. On day 0, mice were administered intratracheally with OVA-DCs, RSG/OVA-DCs, or with unpulsed DCs. Four days later, the proliferation of $CD4^+$ KJ1–26^+ $CFSE^+$ T cells was analyzed by flow cytometry. Results show one representative experiment of 10 to 12 mice per group. *, $P < 0.05$.

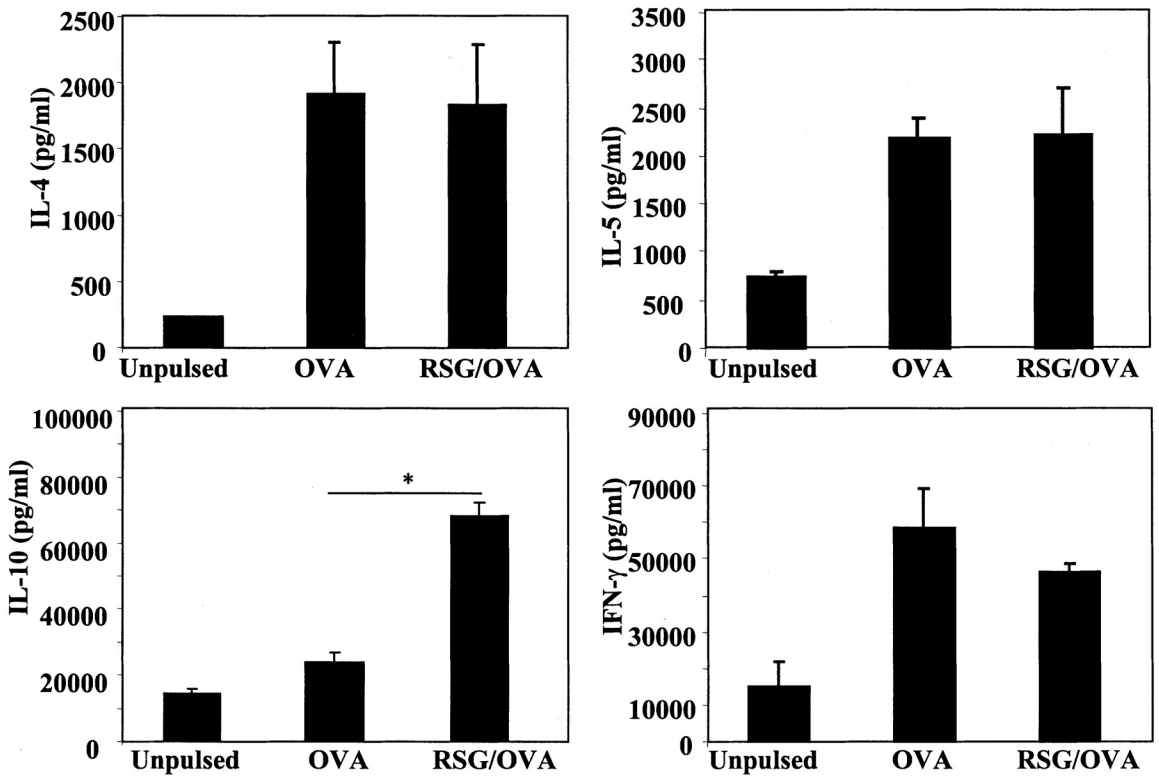

Figure 5. RSG-treated DCs modify the pattern of cytokine production of naï¨ve T cells. On day -2, BALB/c mice received a cohort of CFSE-labeled OVA-specific naïve T cells. On day 0, mice were administered intratracheally with OVA-DCs, RSG/OVA-DCs, or with unpulsed DCs. Four days later, T cells from MLNs were collected and incubated for another 4-day period at 37°C. Supernatants were harvested and assayed for the presence of IL-4, IL-5, IL-10, and IFN-γ. Results are represented as mean ± SEM from 10 to 12 mice per group. *, $P < 0.05$.

Activation of PPAR-γ Reduces Eosinophilic Inflammation Induced by DCs

As PPAR-γ activation in DCs induced a T-cell response in the MLNs with maintained levels of IL-4, IL-5, and IFN-γ, but increased levels of IL-10, we next studied whether PPAR-γ stimulation in DCs could modify their inherent capacity to prime for eosinophilic airway inflammation on secondary challenge to OVA aerosol. For this purpose, mice were immunized as described before and re-exposed to OVA aerosols 10 days later.[32,37] BAL fluids were collected 24 hours after the last OVA aerosol exposure. As expected, in mice that received unpulsed DCs, only a few inflammatory cells, mainly macrophages, were observed in the BAL (Figure 6). In contrast, in mice immunized with OVA-DCs, a strong cell recruitment occurred as confirmed by the high number of lymphocytes and eosinophils in the BAL fluid of these mice. However, when mice received OVA-DCs treated with RSG (RSG/OVA-DCs) or with ciglitazone, another PPAR-γ agonist (ciglitazone/OVA-DCs), the number of inflammatory cells including lymphocytes and eosinophils was significantly reduced as compared to mice immunized with OVA-DCs. To confirm the specificity of the phenomenon, in some experiments, mice also received RSG/OVA-DCs pretreated with the PPAR-γ antagonist GW9662. In mice that received GW9662-treated DCs, the eosinophilia was restored and was comparable to the one present in mice that received OVA-DCs. Because RSG-treated DCs induced a T-cell response with high levels of IL-10, the importance of this cytokine in the establishment of airway eosinophilia was investigated. For this purpose, some mice that received RSG/OVA-DCs on day 0 were injected intraperitoneally with 250 μg of blocking anti-IL-10Rα antibodies 1 day before the first aerosol exposure. As shown in Figure 6, the pretreatment of mice with anti-IL-10Rα could partially restore the BAL eosinophilia as compared to mice injected with RSG/OVA-DCs, suggesting that the effects induced by RSG-treated DCs were partly mediated through an IL-10-dependent mechanism.

Because the activation of PPAR-γ reduced the number of inflammatory cells in the BAL, we next looked at the inflammation at the tissue level. Histological analysis of the lungs of mice injected with OVA-DCs and re-exposed to OVA aerosols revealed strong perivascular and peribronchial inflammatory lesions (Figure 7) composed mainly of eosinophils and mononuclear cells. These changes were absent from the lungs of mice injected with unpulsed DCs (data not shown).[32] Interestingly, in the lungs of mice injected with RSG/OVA-

DCs, less inflammatory cells were present around the bronchi or around the vessels of the lungs compared to mice immunized with OVA-DCs.

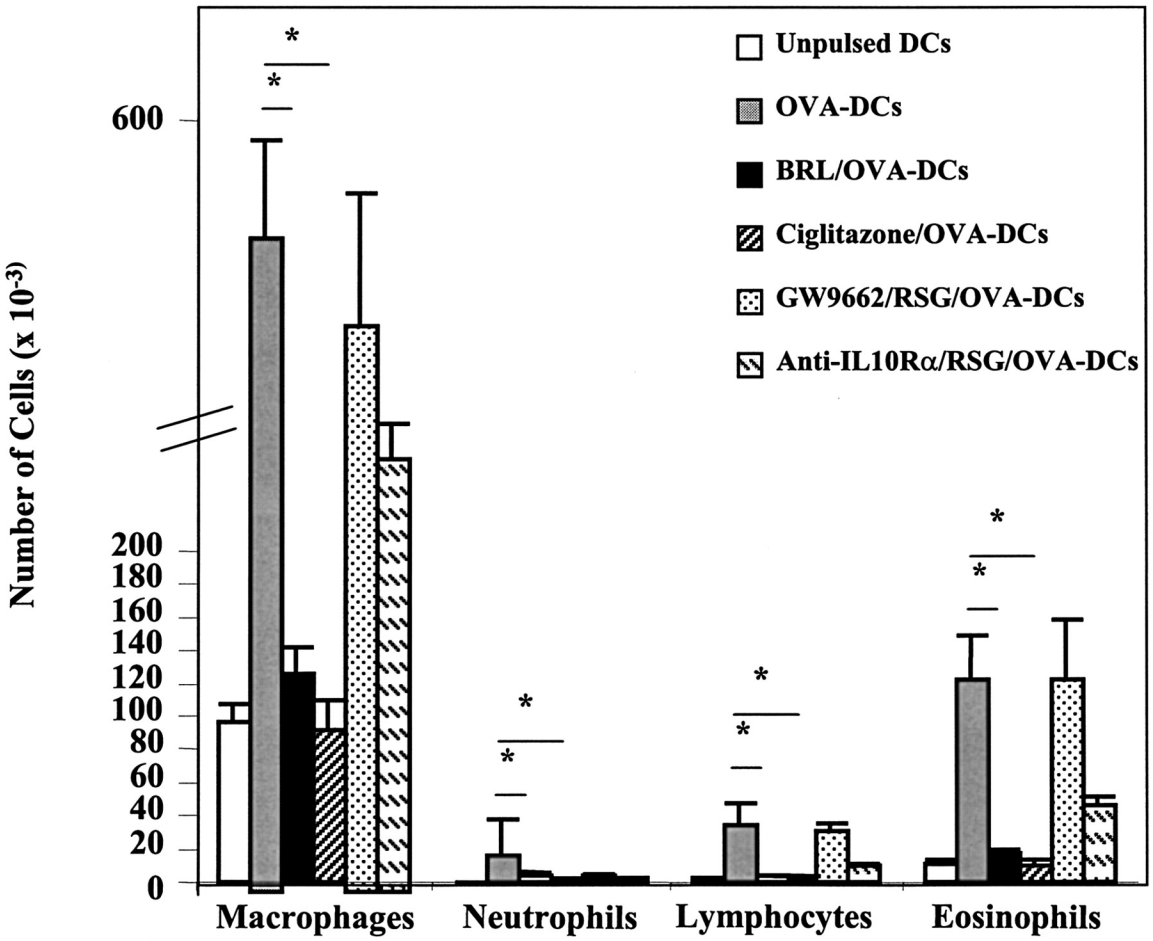

Figure 6. RSG-treated DCs modify the cellular composition of BAL. On day 0, mice received an intratracheal injection of OVA-DCs, ciglitazone/OVADCs, RSG/OVA-DCs treated with the PPAR-γ antagonist GW9662 (GW9662/RSG/OVA-DCs), or unpulsed DCs. From days 10 to 13, mice were exposed to OVA aerosols. To test the role of IL-10 in RSG-induced effects, some mice that received RSG/OVA-DCs on day 0 were injected with anti-IL-10Rα (IL-10Rα/RSG/OVA-DCs) 24 hours before aerosol exposure. Twenty-four hours after the last aerosol, BAL was performed. Results are expressed as mean ± SEM from 8 to 10 mice per group. *, $P < 0.05$.

Activation of PPAR-γ Impairs Cytokine Production in MLNs

To study the mechanism of inhibition of eosinophilic airway inflammation, we quantified the production of cytokines by T cells from MLNs. To this end, MLNs were collected 24 hours after the last aerosol exposure, homogenized, and cells were restimulated *in vitro* for 4 days with OVA. Supernatants were harvested and assayed for the presence of Th1/Th2 cytokines. In mice that received an intratracheal injection of OVA-pulsed DCs, the restimulation of MLNs with OVA led to a strong Th2 response with an up-regulation of the production of IL-4 and IL-5, as compared to control mice that received unpulsed DCs (Figure 8). Interestingly, in mice injected with RSG/OVA-DCs, the levels of IL-4 and IL-5 produced by MLN T cells were similar to those observed in the OVA-DC group. However, the levels of IL-10 but also of IFN-γ were higher in mice injected with RSG/OVA-DCs than in mice that received OVA-DCs.

Discussion

Migration of DCs from the periphery to the draining LN is a critical step for the induction of immunity. However, the signals controlling the migration of DCs to the nodes and controlling immunity are still poorly understood. Recently the role of lipid mediators such as polyunsaturated fatty acids, leukotrienes, and prostaglandins has received a lot of attention as inflammatory mediators with immunomodulatory potential on DCs and T-cell activation. Many of these lipid mediators such as the α-linoleic acid, γ-linoleic acid, arachidonic acid, 9-hydroxyoctadeca-9Z, 11E-dienoci acid (9-HODE), 15d-PGJ$_2$ are agonists of PPAR-γ, a nuclear receptor widely expressed on cells of the immune system, including DCs. Here we show that PPAR-γ activation by a selective agonist, namely RSG, impairs the emigration of intratracheally injected BM-DCs from the lung to the MLNs, probably by reducing the expression of the CCR7 receptor necessary for migration to the draining nodes. In addition, RSG-treated DCs failed to respond to the CCR7 ligands CCL19 and CCL21 in a chemotaxis assay performed *in vitro* (data not shown). PPAR-γ activation has also been reported to affect human DC maturation by reducing different costimulatory molecules,[22,38] although in our hands, no effect on the expression of CD40, CD80, or CD86 was observed (data not shown). Differences in the mode of stimulation or in the origin of DCs might explain this discrepancy.[21]

In the next step, we then investigated whether the intratracheal injection of OVA-DCs treated with RSG could affect the proliferation of adoptively transferred naïve OVA-specific T cells. In mice injected with RSG/OVA-DCs, the number of cell divisions was not affected but the number of OVA-specific T cells recruited into divisions was decreased as compared to mice instilled with OVA-DCs. Therefore, treatment of DCs with RSG does not alter the antigen capture capability nor the capacity to activate naïve T cells, but merely reduces the strength of the induced T-cell response. The lower expression of CCR7 on DCs treated with RSG might limit the number of antigen-presenting cells reaching the thoracic nodes, which might, in turn, affect the outcome of the primary immune response as already reported in *plt* mice[39] or CCR7-deficient mice.[40] Interestingly, the pattern of cytokines produced by the T cells activated by RSG/OVA-DCs was

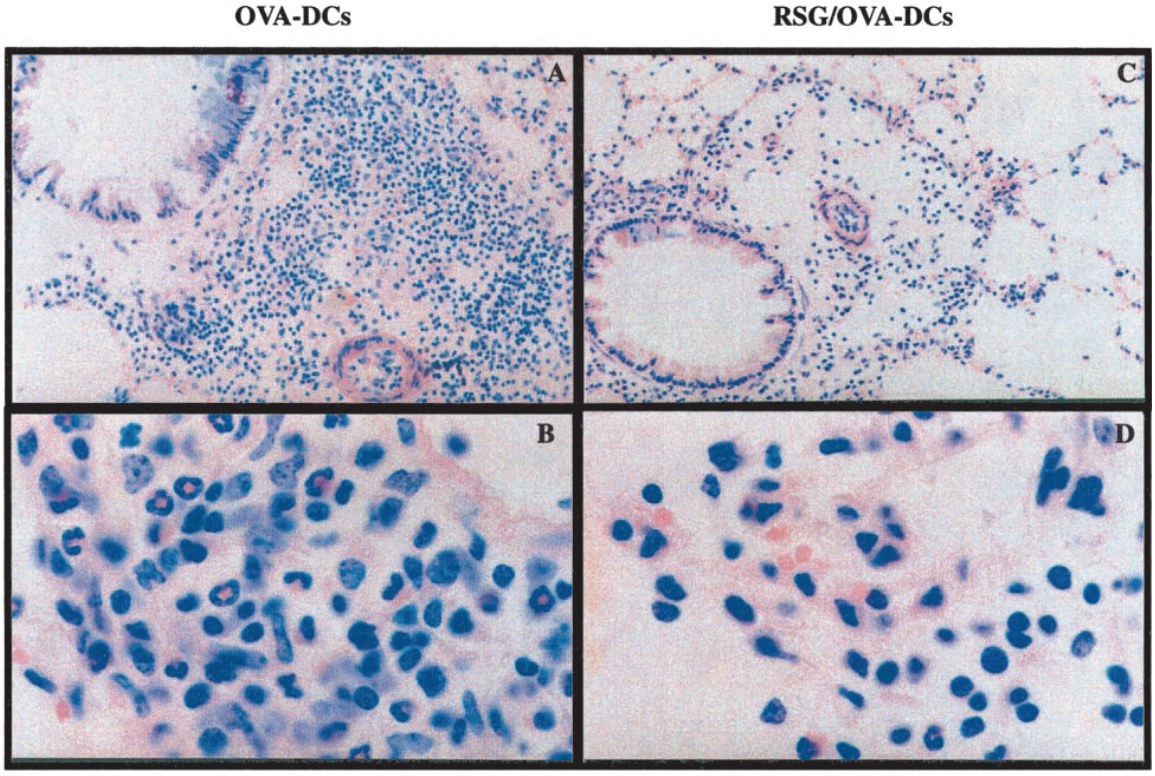

Figure 7. RSG-treated DCs reduce the pulmonary inflammatory response in the lung. On day 0, mice received an intratracheal injection of OVA-DCs, RSG/OVA-DCs, or unpulsed DCs. From days 10 to 13, mice were exposed to OVA aerosols. Four-μm sections of the lungs were stained with May-Grunwald Giemsa. Mice that received OVA-DCs revealed strong perivascular and peribronchial inflammatory lesions composed mainly of eosinophils and mononuclear cells. These features were strongly reduced in the lungs of mice injected with RSG/OVA-DCs. Original magnifications: x20 (**A**, **C**); x100 (**B**, **D**).

different from the one induced by OVA-DCs. As compared to mice injected with OVA-DC, the production of the immunoregulatory cytokine IL-10 was dramatically increased in mice that received RSG/OVA-DCs, whereas the levels of IL-4, IL-5, and IFN-γ were similar in both groups. These results are in discrepancy with another study in which we showed that RSG induced a general decrease in the cytokine production induced by FITC-OVA.[26] In the latter study, RSG was introduced directly into the trachea of mice and therefore one cannot rule out an indirect effect of RSG on airway DCs. Here, RSG was given *in vitro* to DCs and washed away before instillation. The effects induced might strictly be because of direct modifications of

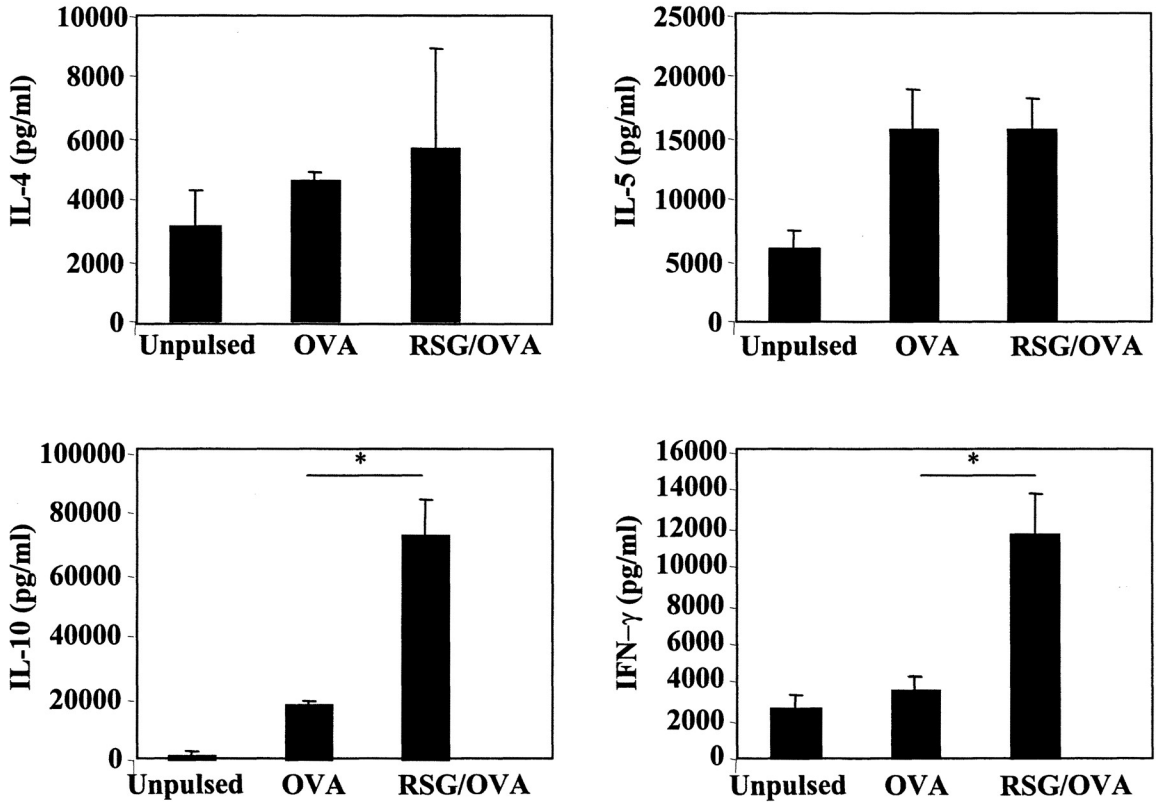

Figure 8. RSG-treated DCs modify the pattern of cytokine production of T cells after re-exposure of the mice to OVA aerosols. On day 0, mice received an intratracheal injection of OVA-DCs, RSG/OVA-DCs, or unpulsed DCs. From days 10 to 13, mice were exposed to OVA aerosols. Twenty-four hours after the last aerosol, cells from MLNs were collected and restimulated for 4 days in the presence of OVA. The supernatants were harvested and assayed for the presence of IL-5, IL-10, and IFN-γ. Results show the mean ± SEM from 8 to 10 mice per group. *, $P < 0.05$.

DC functions by PPAR-γ agonists. We have previously shown that OVA-pulsed BM-DCs injected into the trachea lead to Th2 immunity that primes for the development of eosinophilic airway inflammation on rechallenge with OVA aerosols.[32,27] When the mice were instilled with RSG/OVA-DCs and exposed to OVA aerosols 10 days later, T cells from the LN restimulated *in vitro* with OVA produced the same amounts of IL-4 and IL-5 but higher levels of IL-10 but also of IFN-γ as compared to mice that received untreated OVA-DCs. In mice that received RSG-treated DCs, the number of eosinophils in the BAL and lung inflammation was dramatically reduced as compared to mice injected with OVA-DCs. As the levels of IL-5 during the secondary immune response are not affected by the treatment of DCs with RSG, we can hypothesize that the decreased eosinophilia might be because of the high levels of IL-10 produced in these mice. IL-10 by accelerating the eosinophil death[41] may play an important regulatory role in airway allergic responses as already reported.[42,43] Our data suggest that RSG-treated DCs can induce the generation of a population of IL-10- and IFN-γ-producing T cells that could suppress some of the features of asthma. These T cells have similarities with Tr1 cells that also secrete high levels of IL-10 and IFN-γ.[44] More recently, it was shown that such regulatory T cells could also inhibit Th2-specific responses *in vivo*.[45,46] However, further experiments aimed at characterizing phenotypically and functionally the T cells in MLNs of mice injected with RSG/OVA-DCs remain to be performed. The production of IL-10 induced by RSG-treated DCs was physiologically relevant as inhibition of signaling through IL-10R with blocking antibodies partially restored inflammation.

Thus, from our data, it seems that RSG-treated DCs can improve the features of asthma when given during the sensitization phase. To test the effect of RSG on an ongoing Th2 response, some mice were injected with OVA-DCs and fed or not with RSG 30 minutes before each OVA aerosol. RSG failed to reduce eosinophilic airway inflammation but inhibited lymphocytic and neutrophilic inflammation (data not shown). These results are in contradiction with those from Trifilieff and colleagues[31] in which they show that another PPAR-γ agonist (GI262570) could down-regulate eosinophilic airway inflammation. The reasons of such differences between our results might be because of the route of administration of each compound (gavage *versus* intranasally).

It remains to be proven whether endogenous activators of PPAR-γ such as polyunsaturated fatty acids, eicosanoids, and cyclopentenone prostaglandins (PGs) contribute to down-regulation of sensitization to inhaled allergens. Alveolar macrophages have been shown to suppress the activation of T cells in the lung through inhibitory effects on airway and interstitial DCs, mediated by nitric oxide, transforming growth factor-β, IL-10, or IL-1RA.[47] Alveolar macrophages are a rich source of 15d-PGJ_2, a major PGD_2 metabolite, which could downregulate DC function under steady-state conditions through PPAR-γ activation. Similarly, at the resolution of inflammation, there is high-level expression of the inducible COX-2 enzyme, generating mainly PGD_2 rather than PGE_2.[48] It is likely that metabolism of PGD_2 would subsequently dampen the immune response by ligation of PPAR-γ in DCs. Finally, there is an association between altered levels of plasma polyunsaturated fatty acids and the risk to develop atopy, although it remains to be proven that PPAR-γ stimulation of DCs would be implicated as a mechanism to reduce sensitization to inhaled Allergens.[49] The immunomodulatory capacity of PPAR-γ agonists to inhibit sensitization to inhaled allergens clearly deserves more attention.

This is another example of the extraordinary capacity of PPAR-γ agonists to serve as anti-inflammatory compounds.[25,27-29] It was previous hypothesized that PPAR-γ agonists might be good tools for therapeutic intervention in asthma, because of their inhibiting effects on chemokine release by bronchial epithelial cells.[50] Our data suggest that they have additional suppressing effects on airway DCs, known to be important in sensitization to inhaled antigen and for maintaining established eosinophilic airway inflammation.[51]

Acknowledgment

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Chapter 5

Prostaglandin D_2 Inhibits Airway Dendritic Cell Migration and Function in Steady State Conditions by Selective Activation of the D Prostanoid Receptor 1

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Prostaglandin D_2 Inhibits Airway Dendritic Cell Migration and Function in Steady State Conditions by Selective Activation of the D Prostanoid Receptor 1

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Abbreviations used in this paper: COX, cyclooxygenase; DC, dendritic cell; DP, D prostanoid receptor; LN, lymph node; LTC_4 leukotriene C_4.

PGD_2 is the major mediator released by mast cells during allergic responses, and it acts through two different receptors, the D prostanoid receptor 1 (DP1) and DP2, also known as CRTH2. Recently, it has been shown that PGD_2 inhibits the migration of epidermal Langerhans cells to the skin draining lymph nodes (LNs) and affects the subsequent cutaneous inflammatory reaction. However, the role of PGD_2 in the pulmonary immune response remains unclear. Here, we show that the intratracheal instillation of FITC-OVA together with PGD_2 inhibits the migration of $FITC^+$ lung DC to draining LNs. This process is mimicked by the DP1 agonist BW245C, but not by the DP2 agonist DK-PGD_2. The ligation of DP1 inhibits the migration of FITC-OVA^+ DCs only temporarily, but still inhibits the proliferation of adoptively transferred, OVA-specific, CFSE-labeled, naive T cells in draining LNs. These T cells produced lower amounts of the T cell cytokines IL-4, IL-10, and IFN-γ compared with T cells from mice that received FITC-OVA alone. Taken together, our data suggest that the activation of DP receptor by PGD_2 may represent a pathway to control airway DC migration and to limit the activation of T cells in the LNs under steady state conditions, possibly contributing to homeostasis in the lung. *The Journal of Immunology,* 2003, 171: 3936–3940.

Prostaglandins are small lipid molecules that play an important role in the modulation of inflammatory diseases. They are produced from arachidonic acid, which is converted to PGH_2, a common precursor of several PGs, by the cyclooxygenase enzymes COX-1 and COX-2. PGH_2 can then be converted into a series of PGs, including PGE_2 and PGD_2 (1). PGD_2 binds two receptors: the D prostanoid receptor 1 (DP1) (2) and DP2, also known as CRTH2 (3), which is preferentially expressed on Th2 cells, eosinophils, and basophils. PGD_2 has been associated with the development of pulmonary inflammatory diseases such as asthma (4). The major sources of PGD_2 include activated mast cells (5), Th2 cells (6), and dendritic cells (DCs) (7).

DCs are APCs that can initiate immune responses after they have captured Ags in peripheral tissues and have migrated to the T cell area in draining lymph nodes (LNs). The mechanisms that control DC migration from the periphery to the LNs are not completely understood. Chemokines and the pattern of chemokine receptors expressed by DCs seem to play a crucial role. However, more recently other factors, such as lipid mediators, also play a critical role in the migration of DCs from the periphery to the LNs.

Leukotriene C_4 (LTC_4) and PGE_2 promote the migration of DCs from the skin to the LNs (8, 9), whereas PGD_2 has the opposite effect and prevents the departure of DCs from epidermis to draining LNs (10).

Here we show that the migration of airway DCs to the thoracic LNs in steady state conditions is mediated through DP1, not DP2, activation. Moreover, administration of the DP1 agonist BW245C reduces the proliferation of Ag-specific T cells and the cytokine production by LN cells.

Materials and Methods

Reagents and Abs

PGD_2, BW245C, DK-PGD_2 (13,14-dihydro-15-keto-PGD_2), and PGD_2 were purchased from Cayman Chemicals (Ann Arbor, MI). FITC-labeled OVA and CFSE were obtained from Molecular Probes (Eugene, OR). Collagenase type II was purchased from Worthington Biochemical Corp. (Lakewood, NJ). DNase I was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The PE conjugated anti-I-Ad/I-Ed (M5/114.5.2) was obtained from BD PharMingen (Heidelberg, Germany). The PE-conjugated KJ1-26 (clonotypic OVA-TCR) was purchased from Caltag Laboratories (Burlingame, CA). The allophycocyanin-labeled anti-CD4 (RM4-5) and anti-CD11c (HL3) were obtained from BD PharMingen. The endotoxin level of FITC-OVA determined by a *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD) was <0.001 μg which was previously reported not to affect DCs (11).

Mice

BALB/c mice (6–8 wk old) were purchased from Harlan (Zeist, The Netherlands). OVA-TCR transgenic mice (DO11.10) on a BALB/c background were bred at Erasmus University (Rotterdam, The Netherlands).

Intratracheal administration of reagents

Mice were anesthetized by i.p. injection of avertin. Eighty microliters of FITC-OVA (10 mg/ml), with or without BW245C, DK-PGD_2 or PGD_2, was administered intratracheally under direct vision through the opening vocal cords using a 18-gauge polyurethane catheter connected to the outlet of a micropipette as previously described (12). Control mice received 80 µl of PBS/DMSO.

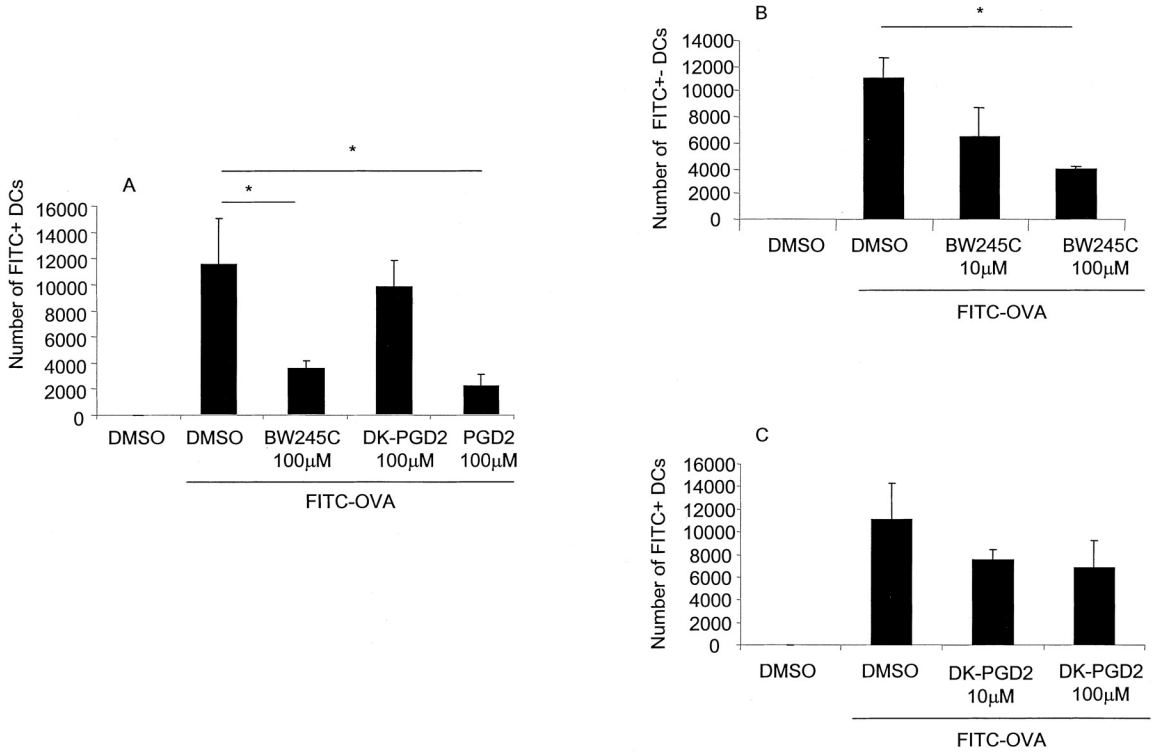

FIGURE 1. Effects of PGD_2, BW245C, and DK-PGD_2 on lung DC migration to the thoracic draining LNs. On day 0, mice were instilled intratracheally with 10 mg/ml FITC-OVA with or without different doses of PGD_2, BW245C (*A* and *B*), and DK-PGD_2 (*A* and *C*). On day 2, the presence of $FITC^+$ migrating DCs in thoracic draining LNs was analyzed by flow cytometry. Results are shown as the mean ± SD and are representative of 8–12 mice/group.

Preparation of single-cell suspension from LNs

At several time points following FITC-OVA instillation, animals were killed by an overdose of avertin. Thoracic draining LN cells and lung cells were obtained as previously described (13). Briefly, thoracic LNs or lungs were digested for 1 h at 37°C in RPMI 1640 containing 5% FCS, 1 mg/ml of collagenase II, and 2 IU/ml of DNase I. The reaction was stopped by addition of PBS containing 10 mM EDTA. LN cells (>95% viability) were washed, stained with anti-MHC class II and anti-CD11c Abs, and analyzed by flow cytometry for FITC positivity. Dead cells and debris were excluded using propidium iodide.

Effect of BW245C on T cell proliferation in thoracic LNs

Because the frequency of OVA-specific T cells is very low in immunized animal, naive T cells purified from DO11.10 mice were adoptively transferred into BALB/c mice. Briefly, LNs and spleen were harvested from DO11.10 mice and homogenized, and after RBC lysis, cell suspensions were labeled with CFSE as previously described (14). On day -2, 10 x 10^6 live cells were injected i.v. in the lateral tail vein of each mouse (day -2). On day 0, mice received an intratracheal injection of FITC-OVA (0.1, 1, or 10 mg/ml) with or without 100 µM BW245C (final concentration, 8 nmol). On day 4, thoracic LNs were collected and stained for the presence of $KJ1\text{-}26^+$ $CD4^+$ OVA-specific T cells. Some of the LN cells (2 x 10^5 cells/well in triplicate) were resuspended in RPMI 1640

containing 5% FCS and antibiotics and placed in round-bottom, 96-well plates. Four days later, supernatants were harvested and analyzed for the presence of IL-4, IL-10, and IFN-γ (BD PharMingen).

Statistical analysis

For all experiments the difference between the various groups was calculated using the Mann-Whitney *U* test for unpaired data. Differences were considered significant at $p < 0.05$.

Results

PGD_2 impairs lung DC migration to draining LNs through DP1

As shown in Fig. 1*A*, migrating $MHCII^+/CD11c^+/FITC^+$ DCs are detected in the thoracic LNs 2 days after the instillation of FITCOVA. The OVA induced migration of DCs was strongly inhibited by PGD_2. To identify which of the PGD_2 receptors (DP1 or DP2) was involved in reducing DC migration to the LNs, FITC-OVA was injected intratracheally together with the DP1 agonist BW245C or the DP2 agonist DK-PGD_2. BW245C dose-dependently inhibited the migration of lung DCs to thoracic LNs (BW245C/FITC; Fig. 1, *A* and *B*). Interestingly, the migration of lung DCs was not affected by DK-PGD_2 (Fig. 1, *A* and *C*), suggesting that the migration of lung DCs to the nodes was mediated mainly through DP1, not DP2. As the maximal effect was obtained with a dose of 100 μM BW245C, additional experiments were performed with this dose. Moreover, to exclude a possible toxic effect of BW245C on lung DCs, total lungs were digested and stained for $MHCII^+$ $CD11c^+$ DCs. The total number of DCs detected in the lungs of animals that were instilled with FITC-OVA alone and that in animals given BW245C/FITC-OVA was not significantly different (52,520 ± 16,678 and 74,080 ± 8,120, respectively; Fig. 2), indicating that the effect induced by BW245C was not due to cell death, but, rather, to the immobilization of DCs in the airways.

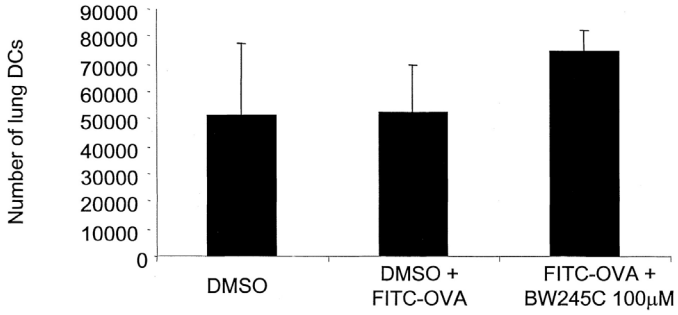

FIGURE 2. Effect of BW245C on lung DCs. On day 0, mice were instilled intratracheally with 10 mg/ml FITC-OVA with or without BW245C. On day 2, lungs were enzymatically digested and stained for the presence of $FITC^+$ MHC II^+ $CD11c^+$ DCs. Results are shown as the mean ± SD and are representative of 10–12 mice/group.

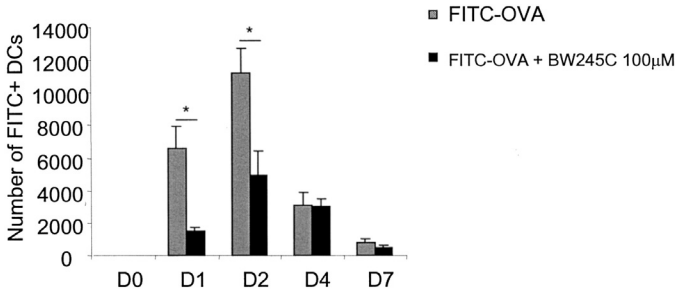

FIGURE 3. Kinetics of action of BW245C on lung DC migration to draining LNs. On day 0, mice were instilled intratracheally with 10 mg/ml FITC-OVA with or without 100 μM BW245C. On days 1, 2, 4, and 7 following instillation, thoracic LNs were digested and stained for the presence of $FITC^+$ MHC II^+ $CD11c^+$ DCs. Results are shown as the mean ± SD and are representative of 10 mice/group.

BW245C temporarily blocks DC migration in vivo

As the selective activation of DP1 impaired lung DC migration, we studied how long a single injection of BW245C would affect the migration of endogenous DCs to the thoracic LNs. For this purpose, BALB/c mice were injected intratracheally with 10 mg/ml of FITC-OVA with or without 100 μM BW245C. Mice were killed 1, 2, 4, or 7 days later. Compared with mice that received FITCOVA alone, the number of migrating $FITC^+$ DCs was reduced up to day 2 in mice that received an instillation of BW245C. However, no difference was observed in the number of $FITC^+$ DCs

reaching draining LNs at 4 or 7 days in mice that received either FITC-OVA alone or FITC-OVA containing BW245C (Fig. 3).

BW245C impairs OVA-specific T cell proliferation in thoracic LNs

As BW245C inhibited the migration of endogenous lung DCs, we next hypothesized that it could also impact T cell activation in thoracic LNs. To test this we first set up an experiment in which BALB/c mice were adoptively transferred with cells from DO11.10 mice on day -2 and were injected with increasing doses of FITC-OVA (0.1, 1, and 10 mg/ml) with or without 100 μM BW245C on day 0. Mice were killed on day 4, and cell divisions were evaluated by flow cytometry. As expected, in mice that received the higher dose of FITC-OVA (10 mg/ml), T cells had undergone seven divisions (Fig. 4). Interestingly, the less FITC-OVA the mice received, the fewer T cells entered into divisions. BW245C reduced the number of T cells entering into division without affecting the number of cell divisions independently from the dose of FITC-OVA. However, the effect of BW245C on T cell proliferation appeared to be dependent on the dose of FITC-OVA. The stronger effect was obtained with the lower concentration of FITC-OVA (0.1 mg/ml). We chose this dose of OVA to perform the following experiment.

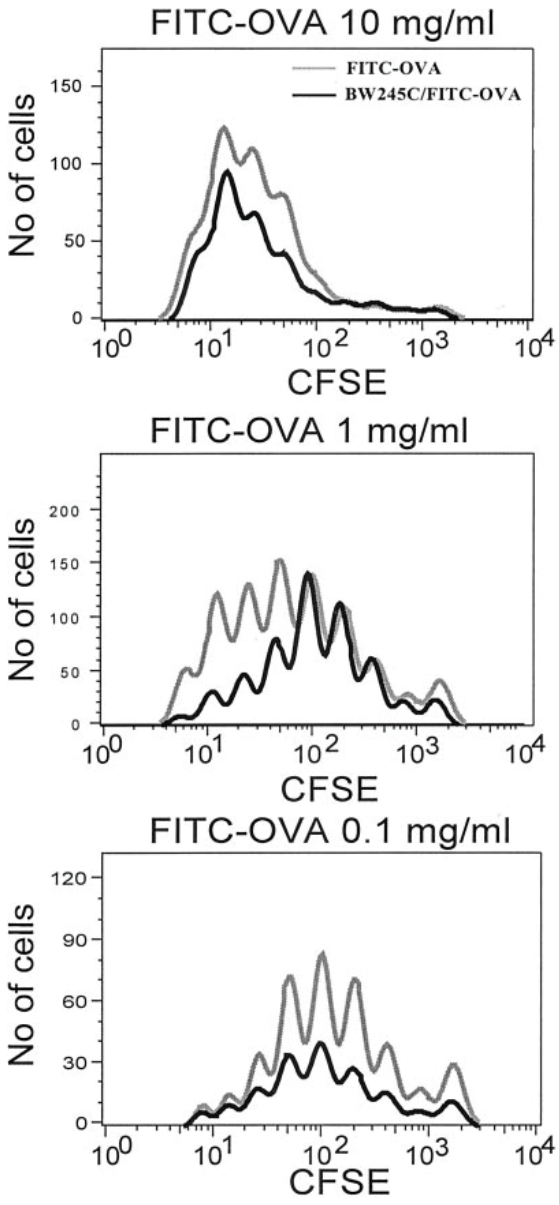

FIGURE 4. Effect of BW245C on DC-induced T cell proliferation in draining LNs. On day -2, mice were injected i.v. with OVA-specific naive T cells from DO11.10 mice. On day 0, mice were instilled intratracheally with increasing doses of FITC-OVA (0.1, 1, and 10 mg/ml; **grey line**) in the absence or the presence of 100 μM BW245C (**black line**). On day 4, the proliferation of KJ1–26$^+$ CD4$^+$ CFSE$^+$ T cells was analyzed by flow cytometry. Results show one representative experiment with 10–12 mice/group.

BW245C reduces cytokine production by draining LN T cells

We next investigated whether the administration of BW245C could affect FITC-OVA-induced cytokine production by thoracic LN T cells. In these experiments, 4 days following the injection of FITC-OVA with or without BW245C, thoracic LN cells were collected and cultured for 4 days in the absence of exogenous OVA. Supernatants were then tested for the presence of IL-4, IL-10, and IFN-γ. As shown in Fig. 5, compared with levels in mice that received FITC-OVA alone, treatment with BW245C reduced the amounts of all cytokines tested.

Discussion

The migration of DCs from the periphery to draining LNs is a key step leading to the initiation of immune responses or to tolerance, depending on their maturation stage (15, 16). The molecular events that induce or control DC migration have been the purpose of extensive research in the past few years. The factors involved in the migration of Langerhans cells, which are known to have low turnover (17), have been widely studied. The presence of inflammatory cytokines, such as TNF-α or IL-1β, in their microenvironment induces the departure of Langerhans cells (18). More recently, it has been shown that some products of arachidonic acid (LTC_4 and PGE_2) could up-regulate the chemokine-driven migration of DCs (8, 9, 19). However little is known about the migration of DCs from other organs, such as the lung. In this study we show that airway DCs migrate very efficiently to thoracic LNs following intratracheal injection of FITC-OVA, as previously reported (20). Interestingly, the OVA-induced migration was inhibited by BW245C, a selective agonist for DP1, and not by the DP2 agonist, DK-PGD_2. DP1 activation has been reported to inhibit Langerhans cell

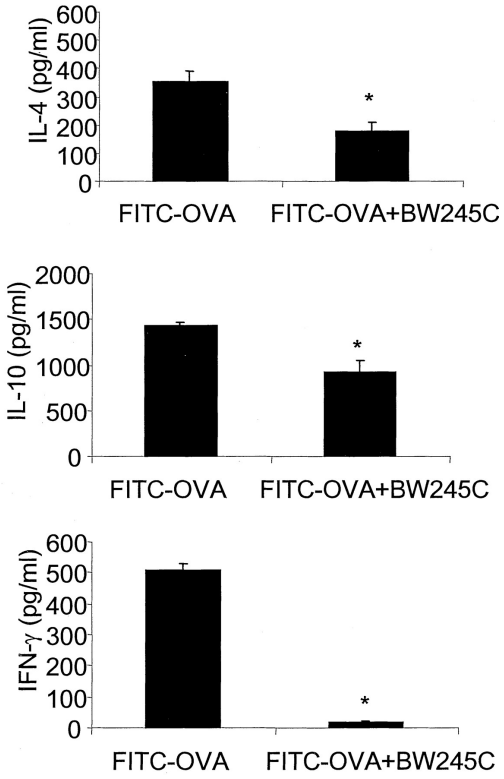

FIGURE 5. Effect of BW245C on cytokine production by T cells of the LNs. On day -2, mice were injected i.v. with OVA-specific naive T cells from DO11.10 mice. On day 0, mice were instilled intratracheally with FITC-OVA (0.1 mg/ml) in the absence or the presence of 100 μM BW245C. On day 4, LN cells were collected and cultured in 96-well plates for 4 days. The presence of IL-4, IL-10, and IFN-γ in the supernatants was analyzed by ELISA. Results are shown as the mean ± SD and are representative of 10–12 mice/group.

migration in a model of parasite infection (10). However, to our knowledge, this is the first report showing the inhibition of DC migration with a high turnover (17) and under steady state conditions. The direct effect of BW245C on lung DCs remains to be determined. Lung DCs are difficult to purify in sufficient amounts to perform functional studies. However, we have evidence that in vitro treatment of bone marrow-derived DCs with BW245C reduces their potential to migrate from the bronchoalveolar lavage fluid compartment to the lung draining LNs in vivo (our unpublished observations and Ref. 14). However, an indirect effect of the agonist on airway DCs cannot be ruled out in this study, as BW245C was administered into the trachea of mice.

As the migration of airway OVA-loaded DCs reaching the LNs was less strong and delayed over time by BW245C treatment, we looked at the effect of BW245C administration on T cell activation. In mice that received only FITC-OVA, T cells from the LNs had already undergone eight divisions by day 4 following instillation. These data are in agreement with those from other groups showing that under steady state conditions, DCs reaching the LNs show high levels of Ag presentation to specific T cells (21). However, when mice received BW245C together with FITC-OVA, the number of T cells recruited into divisions was lower, but the number of divisions undergone by some T cells was not affected. Moreover, the levels of all cytokines produced by T cells of BW245C treated mice were reduced compared with those in mice instilled with only FITC-OVA. This suggests that BW245C can impair the primary activation of T cells by DCs within draining LNs. The lower T cell response observed in BW245C-treated mice may be explained 1) by the lower number of DCs reaching the LNs or 2) by the fact that BW245C could limit the maturation of airway DCs by affecting the levels of costimulatory molecules or/and the expression of the chemokine receptor CCR7 whose ligands (CCL19/CCL21) are known to direct mature DCs to draining LNs (22). The unresponsiveness of DCs to the ligands of CCR7 may subsequently affect the primary immune response, as previously reported in *plt* mice (23) or CCR7-deficient mice (24).

Our data show for the first time that PGD_2, through selective activation of DP1, can reduce the migration of lung DCs. Moreover, T cell activation within the thoracic draining LNs was also significantly reduced. This is another example of interference of DC migration by lipid mediators. Regulation of DC migration by PGD_2 or its metabolites might have a physiological meaning. PGD_2 is produced by mast cells and APCs, such as alveolar macrophages. Under steady state conditions, macrophages might secrete PGD_2 to suppress DC and T cell activation (25). During inflammation, PGE_2 and LTC_4 are produced and induce differentiation and maturation of tissue-resident DCs (8, 19). Under these conditions, the anti-inflammatory effects of PGD_2 are overridden by the proinflammatory effects of PGE_2, LTC_4, and proinflammatory cytokines. Also, at the resolution of inflammation, COX-2 enzyme mainly generates PGD_2, rather than PGE_2 (26). The release of PGD_2 might suppress DC migration to prevent further immune stimulation, thus contributing to the resolution of inflammation. Additional experiments will be necessary to understand the differential effects of PGs on DC functions. These data may have important consequences to improve the treatments of lung diseases such as asthma, where the migration of DCs to the thoracic LNs is known to induce or enhance the Ag-specific Th2 response (12, 14, 27, 28).

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Chapter 6

Proinflammatory Bacterial Peptidoglycan as a Cofactor for the Development of Central Nervous System Autoimmune Disease

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Proinflammatory Bacterial Peptidoglycan as a Cofactor for the Development of Central Nervous System Autoimmune Disease

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Upon stimulation by microbial products through TLR, dendritic cells (DC) acquire the capacity to prime naive T cells and to initiate a proinflammatory immune response. Recently, we have shown that APC within the CNS of multiple sclerosis (MS) patients contain peptidoglycan (PGN), a major cell wall component of Gram-positive bacteria, which signals through TLR and NOD. In this study, we report that *Staphylococcus aureus* PGN as a single component can support the induction of experimental autoimmune encephalomyelitis (EAE) in mice, an animal model for MS. Mice immunized with an encephalitogenic myelin oligodendrocyte glycoprotein peptide in IFA did not develop EAE. In contrast, addition of PGN to the emulsion was sufficient for priming of autoreactive Th1 cells and development of EAE. In vitro studies demonstrate that PGN stimulates DC-mediated processes, reflected by increased Ag uptake, DC maturation, Th1 cell expansion, activation, and proinflammatory cytokine production. These data indicate that PGN-mediated interactions result in proinflammatory stimulation of Ag-specific effector functions, which are important in the development of EAE. These PGN-mediated processes may occur both within the peripheral lymph nodes as well as in the CNS and likely involve recognition by TLR on DC. Thus, PGN may provide a physiological trigger of DC maturation, and in this way disrupt the normal tolerance to self Ag. As such, PGN signaling pathways may serve as novel targets for the treatment of MS. *The Journal of Immunology,* 2005, 174: 808–816.

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Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; DC, dendritic cell; MØ, macrophage; MHC-II, MHC class II; PGN, peptidoglycan; sPGN, soluble PGN; iPGN, insoluble PGN; NAMLAA, *N*-acetylmuramyl-L-alanine amidase; PGRP-L, PGN recognition protein-L; MOG, myelin oligodendrocyte glycoprotein; TCM, cell culture medium; DAP, diaminopimelic acid.

Multiple sclerosis (MS) is considered to be a chronic autoimmune disease of the CNS of unknown etiology critically driven by $CD4^+$ Th1 cells. However, the presence of autoreactive T cells is clearly not the single determining factor for the development of MS, because myelin-reactive T cells are also found in healthy individuals (1, 2). This implies that other factors play an important role in the initiation and perpetuation of pathological autoantigen-specific immune processes. Bacteria and viruses are prominent cofactors that have been implicated in autoimmune disease initiation and persistence (3). As such, it has been shown that MS relapses are frequently associated with antecedent infections (4, 5), which in some cases are bacterial (6). In experimental autoimmune encephalomyelitis (EAE), an animal model for MS, a strong adjuvant containing killed whole *Mycobacterium tuberculosis* (CFA) is required for disease induction. When IFA emulsion is being used, animals will not develop EAE (7, 8). Microbial components, such as CpG-DNA and LPS, are physiological stimuli of EAE development via innate receptors on APC (9 –11). These data indicate that noninfectious microbial components contribute to disease development in EAE and likely in MS. Peptidoglycan (PGN) is an important bacterial cell wall component implicated in chronic inflammation (12). We have previously demonstrated that PGN isolated from sterile human spleen stimulates T cell proliferation and cytokine production (13). Furthermore, we have shown that phagocytic cells may distribute PGN to sites of chronic inflammation. At these sites, PGN-containing dendritic cells (DC) and macrophages (MØ) are present in high numbers and express costimulatory molecules and cytokines, as revealed by extensive in situ analysis of MS brain tissue (14) and rheumatoid arthritis synovial tissue (15, 16). In conclusion, these data support the concept that biologically active PGN can be transported by APC to lymphoid tissues and sites of chronic inflammation. Therefore, we hypothesized that PGN acts as a supporting factor of autoimmune processes in MS. As a mechanism of action, PGN can activate the innate immune system through binding of extracellular and intracellular receptors (17–19). Extracellular PGN has been claimed to bind TLR2 in association with CD14, expressed on the cell surface of APC. Some internalized TLR ligands can also engage TLR in phagosomes (20). In addition, two new intracellular receptors for PGN fragments, NOD1/2 (CARD4/15) have been described recently (21, 22). Although high numbers of immunocompetent PGN-containing APC were found in MS brain tissue by in situ analysis, a functional role for PGN in the pathogenesis of MS remains to be elucidated. Therefore, we assessed the role

of PGN in the development of mouse EAE. To achieve this, mice were immunized with autoantigen emulsified in IFA supplemented with PGN. We used PGN derived from *Staphylococcus aureus*, which does not contain the motif required for NOD1 ligation (22), but contains the motif necessary for NOD2 ligation (23, 24). Mice were assessed for EAE development, and draining lymph nodes were analyzed. for the presence of PGN-containing cells. Because DC play a prominent role in linking the innate and adaptive immune response, we assessed the functional role of PGN in Ag uptake, DC maturation, and T cell polarization. Taken together, our data show that proinflammatory PGN stimulates the development of autoimmune-mediated processes in EAE.

Materials and Methods

Mice

Female C57BL/6 mice (8–12 wk old) and BALB/c mice (6–10 wk old were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHC class II (MHC-II)-restricted, TCR transgenic (OT-II) mice, backcrossed on the C57BL/6 background, were bred within the facility. All animal experiments were performed with approval of the Erasmus University MC committee (Rotterdam, The Netherlands) for animal ethics. Mice were housed under specified pathogen-free conditions and received water and food ad libitum. Paralyzed mice with EAE scores >2.5 were afforded easier access to food and water.

Peptides, Abs, and PGN

Myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) was a kind gift from Dr. U. Günthert (Basel University, Basel, Switzerland). Soluble PGN (sPGN) was prepared from *S. aureus* by gel-permeation chromatography (25), and insoluble PGN (iPGN) was prepared with standard methods. The LPS content of both PGN fractions was rigorously checked and was <13 pg of LPS per milligram of PGN as measured by *Limulus* amebocyte lysate assay, which are background values. Absence of LPS was further confirmed by in vitro culture with mouse bone marrow-derived DC. Purification and identification of the biologically active components in the sPGN fractions are currently being performed, and might reveal other TLR2 agonists as recently described by Travassos et al. (26).

PGN of all bacterial species is composed of two alternating sugar residues, *N*-acetyl glucosamine and *N*-acetyl muramic acid, forming glycan strands. These strands are connected via stem peptides that differ from one bacterial species to another, and that have the sequence L-alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine in *S. aureus*. In addition, PGN from *S. aureus* contains pentaglycine bridges. The complete PGN network is a highly complex ordered network, the detailed structure of which is a subject of debate to this day (27). The two main hydrolyzing enzymes capable of PGN degradation are lysozyme and *N*-acetylmuramyl-L-alanine amidase (NAMLAA), which is identical with PGN recognition protein-L (PGRP-L) (28, 29). Lysozyme hydrolyzes the bond between the two sugar moieties, whereas NAMLAA/PGRP-L hydrolyzes the lactate bond between *N*-acetyl muramic acid and the amino group of the first L-alanine.

Induction and clinical evaluation of EAE

C57BL/6 mice were immunized s.c., at four sites (axillar and inguinal), with 200 µg of MOG_{35-55} in 0.1 ml of PBS emulsified in an equal volume of CFA containing 200 µg of *M. tuberculosis* (H37/Ra; Difco Laboratories). Additionally, mice were injected i.p. with 200 ng of *Bordetella pertussis* toxin in 0.2 ml of PBS on days 0 and 2 after immunization (Sigma-Aldrich). Mice were weighed and scored for clinical signs of EAE daily according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE.

Histology

The immunohistochemical techniques used in this study have previously been described in detail (30, 31). In brief, frozen sections of 6 µm were thaw-mounted on gelatin-coated glass slides and stored overnight in a humidified atmosphere. Sections were air-dried for 1 h and fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Then the slides were air-dried for 10 min, washed with PBS, and incubated with predetermined optimal dilutions of reagents overnight at 4°C in a humidified atmosphere. Secondary and tertiary reagents were applied and incubated for 1 h at room temperature. Between each incubation, slides were washed twice with PBS/0.05% Tween 20. HRP converted 3-amino-9-ethyl-carbazole into a bright red precipitate upon incubation for 10 min. Incubation of slides with naphthol-AS-MX phosphate (Sigma-Aldrich) and fast blue BB base (Sigma-Aldrich) for 30 min at 37°C resulted in a blue precipitate indicating alkaline phosphatase activity. As a negative control, the primary Ab was omitted. Nuclei were counterstained by hematoxylin.

Four to 7 wk after immunization, mice were euthanized using CO_2, and brain tissues were snap frozen in liquid nitrogen and stored at -80°C. To determine the cellular infiltration in the CNS, immunohistochemical stainings were performed on frozen sections with anti-mouse B220 (RA-3-6B2; BD Pharmingen), anti-human cross-reactive to mouse CD3 (DakoCytomation), and anti-mouse MHC-II (M5/114). For CD3, donkey anti-rabbit Igbiotin (Amersham Biosciences) was used as a conjugate. For B220 and MHC-II, rabbit anti-rat IgG-biotin (DakoCytomation) was used as a conjugate. As a tertiary step, slides were incubated with ABComplex-HRP (DakoCytomation).

To assess the presence of PGN within the draining lymph nodes, we isolated axillary and inguinal lymph nodes at 4 and 96 h after immunization. Lymph nodes were snap frozen in liquid nitrogen and stored at -80°C. A double-staining procedure was used to determine which cell types contain PGN. PGN-containing cells were detected with mAb 15704-biotin (Gentaur Molecular Products) raised against *S. aureus* PGN, followed by streptavidin-FITC (BD Biosciences). DC were detected by incubation with anti-mouse CD11c-PE (HL3; BD Biosciences).

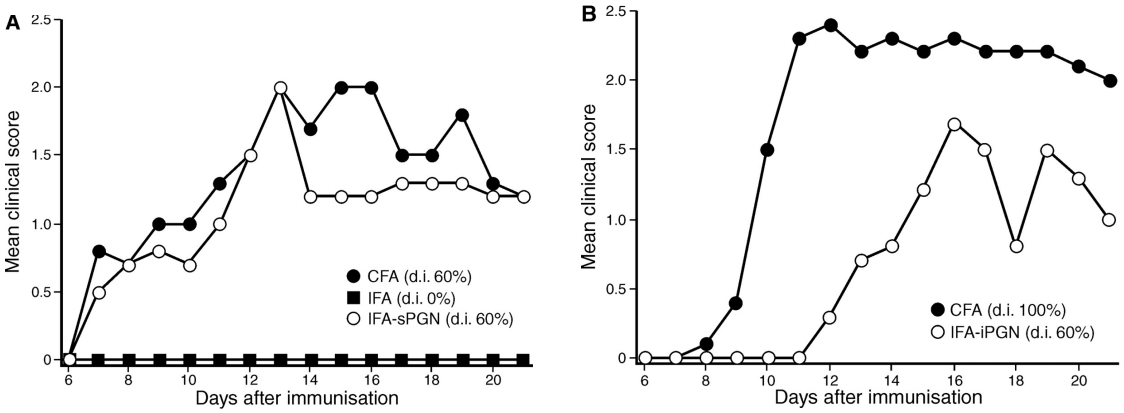

FIGURE 1. Both sPGN and iPGN are potent adjuvants for the induction of EAE. *A* and *B*, In two separate experiments, C57BL/6 mice were immunized with MOG_{35-55} emulsified in CFA (●). In *A*, mice were immunized with MOG_{35-55} in IFA (■), or IFA-25 μg sPGN (○). In *B*, mice were immunized with IFA-400 μg iPGN (○). Mice were weighed and scored for clinical signs of EAE daily. Five animals were used for each group. Graphs show mean clinical scores of animals that developed EAE. d.i., Disease incidence.

Uptake of OVA by DC

DC were prepared as previously described (32). Briefly, bone marrow was flushed with RPMI 1640 (Invitrogen Life Technologies) from femurs and tibiae of C57BL/6 mice. Cells were washed, enumerated, and plated in bacteriological 100-mm diameter petri dishes. Cell culture medium (TCM) was RPMI 1640 supplemented with gentamicin (60 μg/ml; Invitrogen Life Technologies), 2-ME (5×10^5 mol/L; Sigma-Aldrich) and 5% (v/v) FCS (Biocell Laboratories). At day 0 of the culture, the cells were seeded at a concentration of 2×10^6/dish in medium containing recombinant mouse GM-CSF (200 IU/ml; kindly provided by K. Thielemans (University of Brussels, Brussels, Belgium)). At day 3, TCM containing 200 IU/ml recombinant mouse GM-CSF was added. At days 6 and 8, half of the medium was collected and centrifuged, and the pellet was resuspended in TCM containing 200 IU/ml recombinant mouse GM-CSF. At day 9, 100 μl TCM containing 1×10^5 DC were plated in round-bottom 96-well plates for 30 min at 4 or 37°C. DC were pulsed with different concentrations (0, 0.01, 0.1, 1 mg/ml) of OVA-FITC (Molecular Probes). The endotoxin level of FITC-OVA determined by a *Limulus* amebocyte lysate assay (BioWhittaker) was <0.001 μg, which was previously reported not to affect DCs (33). DC were stimulated with 10 μg/ml sPGN or iPGN. Cells were stained for 30 min at 4°C with anti-CD11c-allophycocyanin (HL3; BD Biosciences) dissolved in PBS containing 0.5% BSA and 0.01% sodium azide. Uptake of OVA-FITC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

Table I. *Clinical parameters of EAE induced by sPGN or iPGN*

Adjuvant	PGN (μg/animal)	Disease Incidence (%) $n = 5$	Onset Day[a]	Maximum Score[a]	Cumulative Score[a] (day 20)
CFA	n.a.[b]	60	8.7 ± 2.9	2.0	20.2 ± 8.8
IFA	0	0	0	0	0
IFA-sPGN	25	60	7.0	2.0	15.8 ± 8.8
	250	0	0	0	0
CFA[c]	n.a.	100	9.5 ± 1.6	2.7 ± 0.5	24.0 ± 4.6
IFA-iPGN	25	0	0	0	0
	200	0	0	0	0
	250	40	12.0	2.0	11.0 ± 4.2
	400	60	13.7 ± 1.5	1.7 ± 0.6	9.8 ± 6.7
	600	0	0	0	0

[a] Represented values are from animals that developed EAE.
[b] n.a., Not applicable.
[c] Mean values of two separate experiments.

DC maturation assay

At day 9 of the culture, DC were pulsed overnight with either 100 ng/ml LPS, or 10 μg/ml sPGN or iPGN. To exclude LPS contamination of the PGN preparation, polymyxin B was added to the culture. Cells were centrifuged, and supernatants were collected for cytokine analysis (IL-6, IL-10, IL-12p70, and TNF-α). The maturation state of DC was determined by staining for 30 min with mAbs anti-CD11c-allophycocyanin, anti-MHCII-FITC (M5/114.5.2), in combination with anti-CD40-PE (3/23), anti-CD80-PE (16-

10A1), and anti-CD86-PE (GL-1) (all obtained from BD Biosciences) diluted in PBS containing 0.5% BSA and 0.01% sodium azide. Maturation of DC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

OVA-specific T cell proliferation assay

DC were obtained from bone marrow culture as described above. At day 9 of culture, DC were plated in 24-well plates containing 2 ml of TCM, and pulsed with 10 mg/ml OVA-Worthington (Biochemical) for 24 h. At the dose we used in our experiments, the endotoxin level of OVA measured by a *Limulus* amebocyte lysate assay (BioWhittaker) was <0.001 μg. Simultaneously, DC were stimulated with 100 ng/ml LPS (*Escherichia coli*, strain O26:B6; Sigma-Aldrich), 10 μg/ml sPGN, or iPGN. OT-II cells were purified as described previously (32). Briefly, lymph nodes and spleens were collected from OT-II mice. After RBC lysis, cells were labeled with CFSE. Cells were enumerated, and dead cells, stained for trypan blue, were excluded. After 24 h, DC were washed and 1 x 10^4 DC were cocultured with 1 x 10^5 CFSE-labeled OVA TCR transgenic $CD4^+$ OT-II T cells in round-bottom 96-well plates containing 200 μl of TCM. Cells were centrifuged, and supernatants were harvested and stored at -20°C for cytokine analysis. Cells were resuspended and incubated with anti-Vα2-biotin and Vβ5.1/5.2-PE TCR mAbs (eBioscience) for 30 min at 4°C. Biotinylated anti Vα2 was detected using streptavidin-allophycocyanin (BD Biosciences). Percentages of proliferating OVA-specific $CD4^+$ T cells were determined by gating on cells that were double positive for Vα2 and Vβ5.1/5.2. Dead cells and debris were excluded using propidium iodide.

$MOG_{35–55}$-specific T cell proliferation assay

For evaluation of MOG_{35-55}-specific lymph node cell proliferation, mice were immunized with MOG_{35-55} in adjuvant. Mice were sacrificed, and draining inguinal, brachial, and axillar lymph nodes were isolated at 33 days after immunization. Lymph node cells were cultured for 4 days in 96-well plates in 200 μl of RPMI 1640 supplemented with 10% heatinactivated FCS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Cells (4 x 10^5/well) were cultured at 37°C and 5% CO_2 in the presence or absence of 10 μg/ml MOG_{35-55}. After 72 h of culture, 1 μCi of [^{3}H]thymidine (Amersham Biosciences) was added for 16 h.

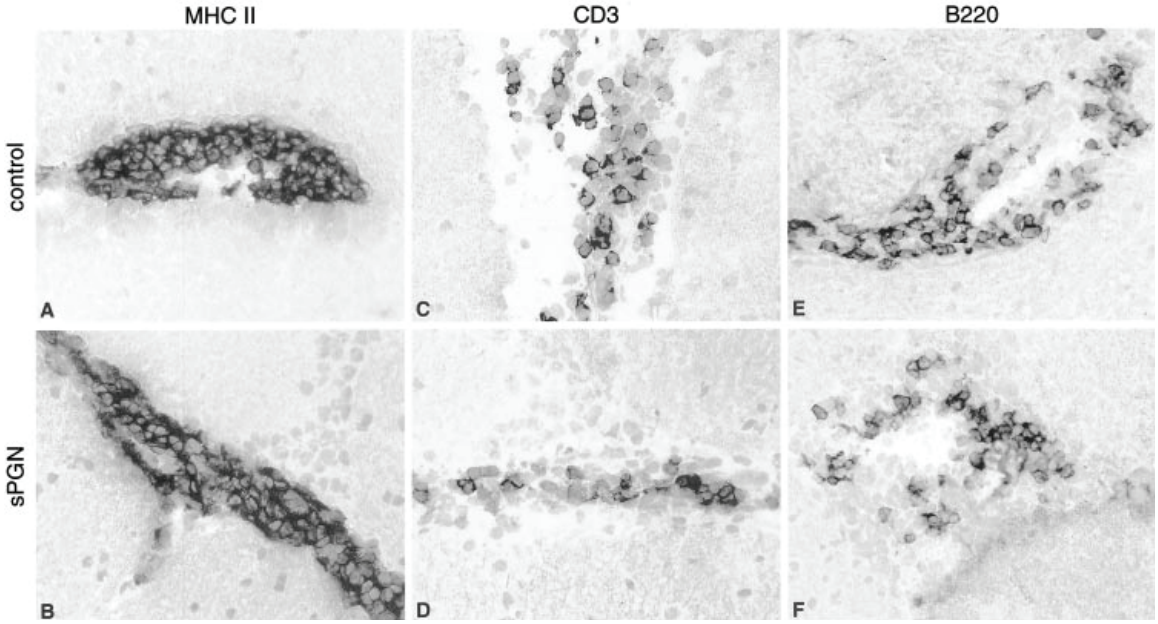

FIGURE 2. Immunization with PGN-containing adjuvant induces classical EAE histopathology. Brain tissues were isolated between 4 and 7 wk after immunization. Frozen brain sections were stained with hematoxylin in combination with MHC-II (*A* and *B*), CD3 (*C* and *D*), and B220 (*E* and *F*).

Incorporation of [3H]thymidine was measured in triplicate using a filtermat harvester and a beta plate counter (PerkinElmer). Supernatants from MOG_{35-55} proliferation assays were harvested 96 h after culture, centrifuged, and stored for further cytokine analysis at -20°C.

Cytokine measurement by ELISA

According to the manufacturer's instructions, concentrations of IL-4, IL-10, IL12-p70, IFN-γ, and TNF-α were determined by using OptEIA ELISA kits (BD Biosciences).

Table II. *Histological analysis of infiltrates in EAE brain tissue*

Adjuvant	PGN (μg/animal)	Number[a]	Size[b]
CFA	n.a.[c]	>10	+
	n.a.	>10	+
	n.a.	4–10	+
	n.a.	4–10	+
	n.a.	>10	+
IFA-sPGN	25	>10	+
	25	4–10	+
	25	>10	+
IFA-iPGN	250	4–10	−/+
	250	4–10	+
	400	1–3	−/+
	400	1–3	−/+
	400	4–10	−/+

[a] Number, Number of inflammatory foci.
[b] Infiltrate size, −, no infiltrating cells; −/+, 1–5 cells; +, 6–20 cells.
[c] n.a., Not applicable.

Statistical evaluations

Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney *U* test was used to analyze differences in cytokine production and T cell proliferation. A value of $p < 0.05$ was considered statistically significant.

Results

Both sPGN and iPGN are potent adjuvants for the induction of EAE. PGN is a known proinflammatory stimulator of the innate immune response, but very little is known about the possible role of PGN in the development of MS and EAE. To determine whether PGN by itself is a sufficient proinflammatory stimulus for the development of EAE, we added highly purified sPGN or iPGN from *S. aureus* to an emulsion of IFA and MOG_{35-55}. In three independent experiments, standard induction of EAE by immunization of mice with MOG_{35-55} in CFA resulted in an EAE incidence of 60–100%, with a normal EAE course (Fig. 1 and Table I). Mice immunized with MOG_{35-55} in IFA, as a negative control, did not develop EAE, as expected. In contrast, addition of sPGN or iPGN to IFA/MOG_{35-55} induced EAE with a disease incidence from 40 to 60%. Different dosages of sPGN and iPGN were used for EAE induction. High dosages of 250 μg of sPGN and 600 μg of iPGN did not induce EAE (Table I). Importantly, however, lower PGN dosages induced EAE with an incidence of 40–60%. The lowest effective dose for EAE induction with sPGN (25 μg/animal) was 10 times lower than with iPGN, in keeping with the higher biological activity described for sPGN previously. These data demonstrate that PGN is a potent adjuvant and can substitute whole *M. tuberculosis* in the induction for EAE.

PGN-adjuvant induces classical EAE histopathology

Brain tissue was examined by histology to assess whether the signs of paralysis of mice immunized with PGN containing adjuvant are associated with a characteristic EAE histopathology. In brain tissue of animals that developed EAE, infiltrates were found containing $CD3^+$ T cells, $B220^+$ B cells, and MHC-II^+ APC (Fig. 2). A comparable number of infiltrates and infiltrating cells were detected in EAE mice injected with CFA and IFA-sPGN, whereas the infiltrate size and number of infiltrating cells in IFA-iPGN-injected EAE mice was slightly lower (Table II). These data confirm that paralysis induced with PGN-containing adjuvant is associated with a characteristic EAE histopathology.

PGN-containing adjuvant promotes MOG_{35-55}-specific Th1 cell polarization

It has been well established that EAE induction by immunization with an encephalitogenic Ag in CFA results in the generation of autoantigen-specific $CD4^+$ IFN-γ-producing T cells (34). To address whether the PGN component of bacteria is able to generate MOG_{35-55}-specific Th1 cells, we determined MOG_{35-55}-specific T cell proliferation and IFN-γ, IL-4, and IL-10 production at day 33 after immunization. Lymph node cells from mice immunized with MOG_{35-55} in CFA proliferated dose dependently and produced IFN-γ upon in vitro restimulation with MOG_{35-55} (Fig. 3). Mice immunized with MOG_{35-55} in IFA also showed Ag-specific proliferation, but only low amounts of IFN-γ were produced. Addition of sPGN to the immunization mixture resulted in increased Agspecific proliferation and IFN-γ production compared with immunization with IFA/ MOG_{35-55}. IL-4 and IL-10 were undetectable in any of the groups.

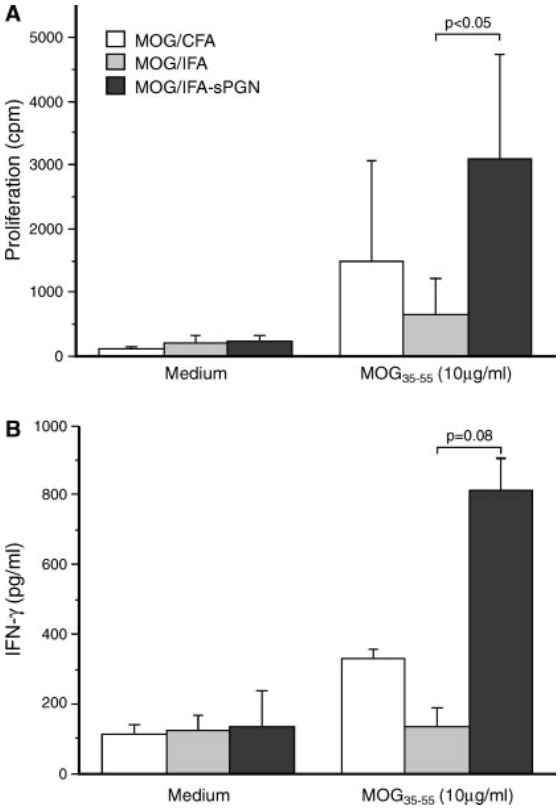

FIGURE 3. Immunization with PGN-containing adjuvant stimulates the development of MOG_{35-55}-specific Th1 cells. Draining lymph nodes from five individual mice per group were harvested at day 33 after immunization. *A*, Cells were stimulated in vitro with 10 µg/ml MOG_{35-55}, and proliferation was measured after 96 h of culture by [^{3}H]thymidine incorporation. *B*, Supernatants were harvested after 96 h of culture and measured by ELISA for IFN-γ content. Mean values per group ± SD are shown.

These data show that PGN creates an inflammatory environment. In combination with autoantigen presentation, PGN can effectively induce autoantigen-specific Th1 cells.

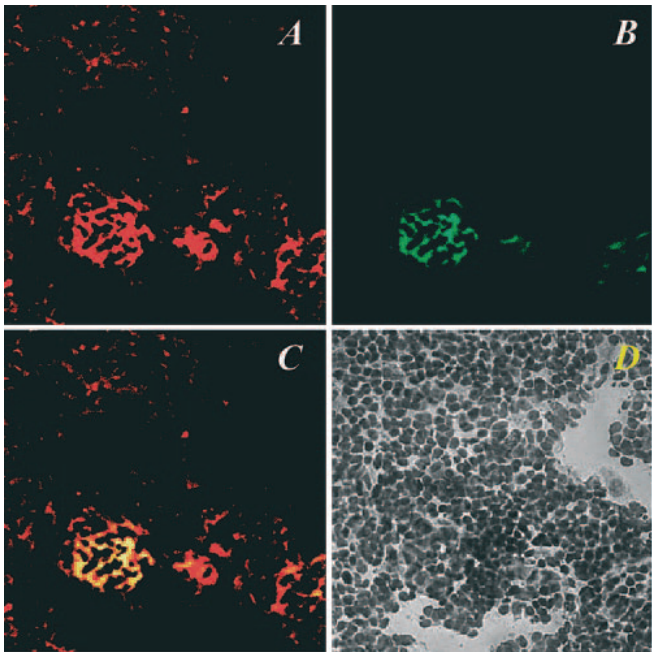

FIGURE 4. PGN is redistributed from the site of immunization to DC in draining lymph nodes. Draining lymph nodes were isolated at 4 h after s.c. immunization with MOG_{35-55} in IFA-sPGN. Frozen sections were stained by immunofluorescence for DC (CD11c in red, *A*) and for PGN (green, *B*). Overlay in *C* demonstrates clusters of DC containing PG. *D* is a lightmicroscopic image of the same area.

PGN is redistributed from the site of immunization to the draining lymph nodes

To determine whether PGN is transported from the site of immunization to secondary lymphoid organs during EAE development, we injected PGN in combination with IFA and MOG_{35-55} and isolated draining lymph nodes at different time points after immunization. Two mice per group were injected with either 25 μg of sPGN or 400 μg of iPGN in IFA/MOG_{35-55} emulsions, dosages of PGN optimal for EAE induction. Two naive mice were used as negative controls. Axillary, brachial, and inguinal lymph nodes were isolated and frozen at 4 and 96 h after immunization. Per mouse, four lymph nodes were analyzed for the presence of PGNcontaining cells. In naive mice, individual PGN-containing cells were occasionally found in three of eight lymph nodes (38%), in agreement with previous observations (35, 36). In contrast, we detected several clusters of PGN-containing cells in >90% of the draining lymph nodes from mice immunized with sPGN and iPGN. The number of PGN-containing clusters did not vary be between the time points (data not shown). These data indicate that APC can take up and transport PGN from the site of immunization to the draining lymph nodes within hours. Double-labeling experiments identified cells in the PGN-containing clusters as DC expressing CD11c (Fig. 4). PGN-containing clusters may thus contribute to the development of a proinflammatory environment to help generate MOG_{35-55}-specific Th1 cells.

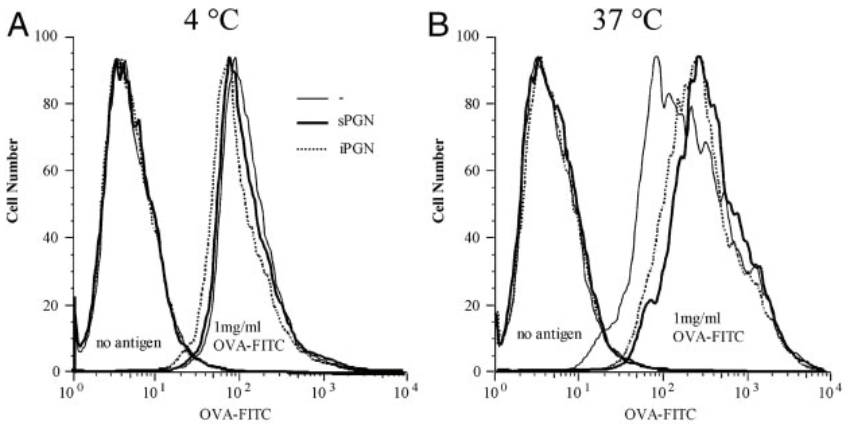

FIGURE 5. Stimulation of DC by PGN increases protein uptake. Immature DC were cultured in the absence or presence of OVAFITC. DC were either not stimulated (thin lines) or stimulated with sPGN (thick lines) or iPGN (dotted lines). Uptake of OVAFITC was determined at 4°C (*A*) or 37°C (*B*) after 30 min of incubation. Histograms represent the number of CD11c-gated cells.

Stimulation of DC by PGN increases protein uptake and induces DC maturation

At the site of immunization, PGN can be recognized by different cell types, i.e., DC, MØ, and granulocytes. Because PGN was mainly observed in DC in the lymph nodes, we assessed whether PGN stimulates protein uptake, cell maturation, T cell stimulation, and polarization by this APC subset crucial for priming of T cells. For these experiments, immature mouse bone marrow-derived DC were used. To examine the effects of PGN on protein uptake, DC were incubated with OVA conjugated to FITC in the presence or absence of 10 μg/ml PGN at 4 or 37°C. Different concentrations of OVA-FITC (0, 0.01, 0.1, and 1 mg/ml) were applied, and uptake was determined by flow cytometry. Incubation at 4°C showed a dose-dependent passive uptake of OVA-FITC (data not shown). Addition of PGN did not alter the uptake of OVA-FITC at 4°C (Fig. 5*A*). When cells were cultured at physiological temperature (37°C), both sPGN and iPGN stimulated the uptake of OVA-FITC (Fig. 5*B*).

To assess the effects of PGN on DC maturation, we stimulated DC with sPGN or iPGN. As a positive control, DC were stimulated with LPS. Maturation of DC was determined by the expression of MHC-II, CD40, CD80, and CD86, and by the induction of several cytokines. Both sPGN and iPGN stimulated expression of MHCII, CD40, CD80, and CD80. Stimulation of DC with sPGN efficiently induced expression of MHC-II, CD40, CD80 and CD86 to a similar or higher level as compared with LPS stimulation. These molecules were also induced by iPGN, to a similar or lower level compared with LPS (Fig. 6*A*). Both sPGN and iPGN induced production of several cytokines, but with a different pattern of production. Whereas sPGN induced production of all proinflammatory cytokines measured, iPGN induced TNF-α, but not IL-6 and IL-12 (Fig. 6*B*). IL-10 was not produced under these conditions (data not shown). As predicted, addition of the LPS antagonist polymyxin B effectively inhibited LPS-induced maturation marker expression and cytokine production (Fig. 6*B*), whereas no inhibition was seen when cells were stimulated with PGN, demonstrating that the effects of PGN were not the result of LPS contamination. Taken together, these data demonstrate that both forms of PGN can stimulate protein uptake and induce DC maturation in vitro.

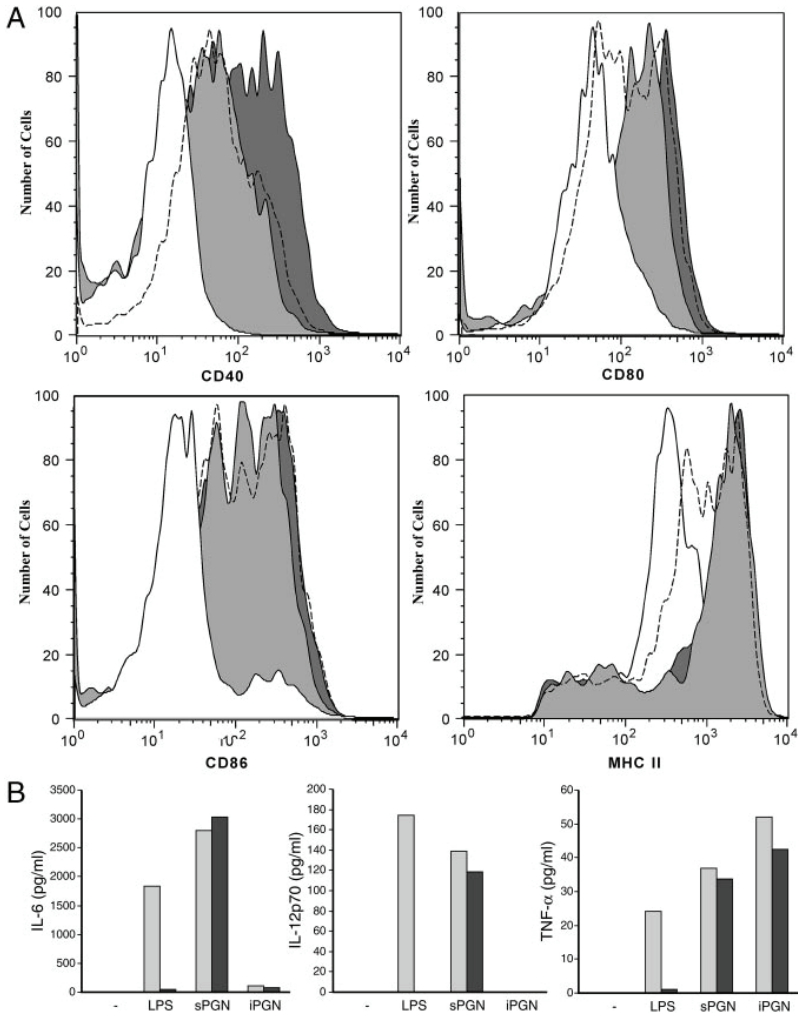

FIGURE 6. PGN induces maturation of bone marrow-derived DC. Immature DC were either not stimulated (white histogram, closed line) or stimulated with LPS (white histograms, dotted line), sPGN, or iPGN (light-gray and dark-gray histograms, respectively).
A, Maturation of DC was determined by measuring expression of MHC-II, CD40, CD80, and CD86 by flow cytometry. Histograms represent the number of CD11cgated cells.
B, Supernatants were harvested, and production of IL-6, IL-12p70, and TNF-α was determined by ELISA. Black bars represent cells that were incubated with polymyxin B to exclude LPS contamination.

PGN-OVA-pulsed DC stimulate T cell proliferation and Th1 cell polarization

After Ag uptake and migration of DC to the draining lymph nodes, mature DC will prime naive T cells to proliferate and differentiate along a certain pathway, depending on the nature of the stimulus. To address whether sPGN and iPGN are able to stimulate Agspecific T cell proliferation and Th1 cell polarization, we used an in vitro model system. Bone marrow-derived DC were cultured with OVA in combination with LPS, sPGN, or iPGN. After 24 h, the DC were incubated with CFSE-labeled naive $OVA_{323-339}$ TCR transgenic $CD4^+$ T cells. OVA-pulsed DC that were stimulated by LPS, sPGN, or iPGN, induced an increase in the OVA-specific T cell proliferation compared with unstimulated cells (Fig. 7, *B–E*). Furthermore, both forms of PGN induced significantly increased levels of IFN-γ compared with both nonstimulated and LPS-stimulated DC (Fig. 7*F*). Only low levels of IL-4 and IL-10 were detected in all OVA-pulsed DC-T cell cultures (data not shown). In summary, these data show that PGN stimulates Ag-specific T cell proliferation and Th1 cell development.

Discussion

Bacterial and viral components stimulate innate immune responses that potentially contribute to the development and persistence of autoimmune disease (3, 5). Although it is very difficult to demonstrate mechanistic links between microbial agents and MS, it has been shown epidemiologically that antecedent infections are associated with an increased risk of relapse development (4, 5). Components of bacterial agents may function as physiological adjuvants and stimulate autoantigenspecific adaptive immune responses. In this study, we show that PGN, a large bacterial cell wall component, can act as an environmental factor promoting CNS autoimmune disease development via innate immunity.

PGN from a vast array of different bacterial species can be derived from different anatomical sites, including all mucosa permanently exposed to the outside world. During bacterial infection, PGN can be released, either from

bacterial cells upon replication or upon uptake and processing by APC. In the absence of infections, the major bacterial load is located at mucosal sites. At these sites, i.e., the gut, DC may sample bacteria through the intestinal epithelium and subsequently migrate to secondary lymphoid organs (37, 38). Even under normal circumstances, some PGN can be detected in the blood circulation, within the liver, and within lymphoid tissues, reflecting physiologic processes dealing with exposure to bacterial components (13, 39 – 45).

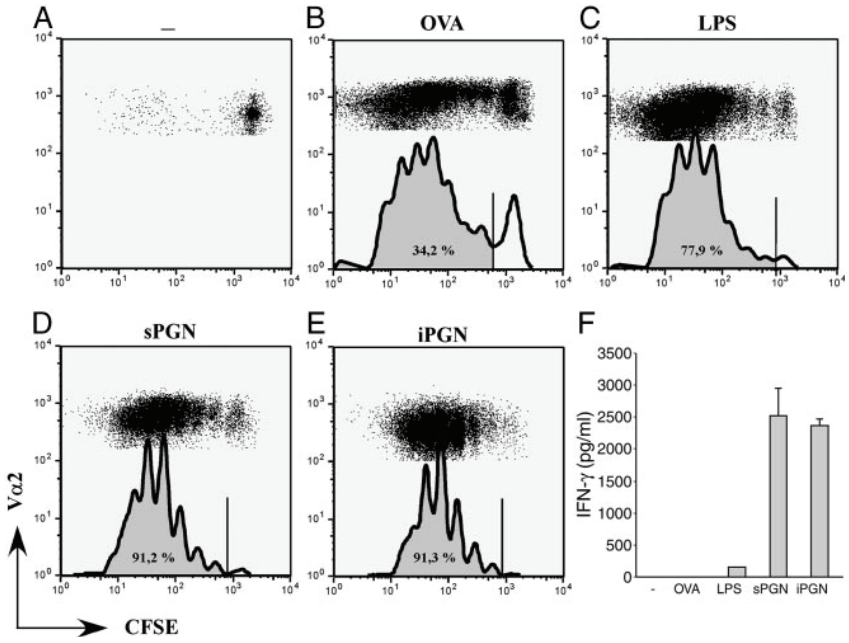

FIGURE 7. PGN stimulates Ag-specific T cell proliferation and Th1 cell polarization. Immature DC were not stimulated (*A*) or stimulated with OVA (*B*) combined with LPS (*C*), sPGN (*D*), or iPGN (*E*) for 24 h. $OVA_{323-339}$ TCR transgenic $CD4^+$ T cells were incubated with all DC populations. *A–E*, After 6 days of culture, $OVA_{323-339}$-specific T cell proliferation was assessed by flow cytometry using CFSE. *F*, Supernatants were harvested, and production of IFN-γ was determined by ELISA. Percentages indicate the number of divided $OVA_{323-339}$ $CD4^+$ T cells. Histograms represent the number of cell divisions.

We therefore hypothesized that bacterial PGN can contribute to disease development and progression in MS and EAE in the absence of infection or bacterial replication. To functionally test this hypothesis, we determined whether EAE could be induced by immunization of mice with an encephalitogenic peptide (MOG_{35-55}) emulsified in IFA supplemented with *S. aureus* PGN, asking whether PGN by itself is a sufficient proinflammatory cofactor for induction of EAE.

For our studies, we used two different types of *S. aureus* PGN. We observed functional differences between sPGN and iPGN in vivo as well as in vitro. Although EAE could be induced with both types of PGN, the effective dose for EAE induction was 10-fold lower for sPGN compared with iPGN. In vitro stimulation of DC with iPGN only induced TNF-α production, whereas sPGN could induce IL-6 and IL-12 in addition to TNF-α. These data implicate that partially degraded PGN is more effective in exerting proinflammatory effects than iPGN.

High dosages of both sPGN and iPGN were not able to induce EAE. The reasons for this are not clear but may be related to dose-dependent induction of Th1 vs Th2 responses by administration of TLR ligands in vivo (33). Furthermore, it has been shown that apoptosis of MØ is induced by PGN, as described by Fukui et al. (46). As recently shown, TLR engagement triggers DC apoptosis through up-regulation of the proapoptotic Bcl-2 family member Bim. In this way, both innate and acquired immune system signals cooperate to determine DC lifespan, and hence also the longevity of T-DC interaction (47, 48).

L-Lysine or diaminopimelic acid (DAP) at position 3 of the stem peptide are important structural components of PGN. DAP-type PGN is present in all Gram-negative bacteria, whereas most Grampositive bacteria contain lysine-type PGN (49). The intracellular PGN-sensing molecules NOD1/2 discriminate between lysine-type and DAP-type PGN (50). NOD2 is a general sensor for both Grampositive and Gram-negative bacteria, through the recognition of muramyl dipeptide, the minimal motif found in all PGNs (23, 24). In contrast to NOD2, NOD1 specifically recognizes DAP-type PGN (21, 22). Bacteria containing L-lysine can induce chronic arthritis, whereas bacteria with other peptides at this position are nonarthritogenic (51). Several other studies have also identified the presence of L-lysine as typical for proinflammatory PGN (52–54). Considering the arthritogenic effects of L-lysine at position 3 of the stem peptide, one

might expect similar findings in EAE development. Indeed, we have now shown that EAE can be induced with lysine-type *S. aureus*-derived PGN as adjuvant.

Already at 4 h after immunization, PGN-containing clusters could be detected in the draining lymph nodes. These clusters persisted at least for 7 days after immunization. Others have also shown the persistence of PG-containing components until at least 63 days after injection of PGN-polysaccharides by muramic acid detection, a specific component of PGN, through gas chromatography and mass spectrometry (40). PGN degradation is mainly dependent on the enzymes lysozyme and NAMLAA/PGRP-L (55). Recently, it was found that the PGRP-L is an amidase (28, 29) and is identical with NAMLAA, which we have characterized in detail previously (55, 56). In contrast to granulocytes, MØ and DC lack NAMLAA/PGRP-L, which may result in incomplete or delayed PGN degradation and persistence of intracellular PGN. This may explain why DC in MS brain tissue and in EAE lymph node tissues contain PGN. The fact that PGN is observed both in lymph nodes and in the CNS is important, because PGN may act as a physiological proinflammatory adjuvant by stimulating autoantigen-specific immune responses. Indeed, in the current study, we could demonstrate that PGN-containing adjuvant enhanced MOG_{35-55}-specific lymph node cell proliferation and IFN-γ production at 4 wk after immunization.

DC recognize specific motifs on pathogens by expressing a diverse repertoire of pathogen-associated recognition receptors. Upon bacteria-induced stimulation, DC mature, migrate to the circulation, and prime the adaptive arm of the immune response by initiating the development of effector T cells in secondary lymphoid organs (57). Because we detected PGN within DC in MS brain, rheumatoid arthritis synovium, and EAE lymphoid tissue, we addressed the functional role for PGN in EAE development by in vitro simulation of the different processes involving DC that occur during immunization for EAE development.

Early events after immunization include uptake of adjuvant and Ag by DC, inducing their maturation. These processes can be accelerated by whole *M. tuberculosis*-containing adjuvant as has been demonstrated in vivo (7). Also bacterial components like CpG-DNA are able to increase Ag uptake, provided that the Ag is covalently linked to CpG-DNA (58–60). In concordance with these studies, we now demonstrate that PGN increases Ag uptake by DC.

Upon stimulation with TLR ligands or cytokines, DC will mature and migrate to secondary lymphoid tissues to initiate T cell activation. It has been demonstrated that *S. aureus* PGN may serve as a maturation signal for murine DC, by inducing the expression of CD86 and MHC-II, and the production of MIP-2 and proinflammatory cytokines (61, 62). Also, human monocytes secrete several chemokines (IL-8, MIP-1α) and proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-12) after PGN stimulation, as shown by cDNA array analysis (63). In agreement with these studies, we now show that both forms of *S. aureus*-derived PGN induce DC maturation, as demonstrated by enhanced expression of MHC-II and costimulatory molecules, and production of proinflammatory cytokines. These proinflammatory cytokines, induced upon TLR stimulation, can exert direct effects on T cell polarization (i.e., IL-12) (64, 65) and T cell regulation (i.e., IL-6) (66) and have been implicated in the pathogenesis of MS as well as EAE (67– 69).

Classical EAE induction is dependent on the presence of autoantigen and proinflammatory stimuli from adjuvant components (whole *M. tuberculosis*). A combination of both factors will result in the development of autoantigen-specific $CD4^+$ Th1 cells within the secondary lymphoid organs. Not much is known about the effect of PGN on T cell proliferation and differentiation. A recent report proposed that Th cell polarization is dependent on differential TLR ligation (70). Our in vitro studies demonstrate that OVA-pulsed DC did induce T cell proliferation but did not induce Th1 cell development. However, when OVA-pulsed DC were coincubated with PGN, DC were able to induce OVA specific Th1 cell development, which confirms previous reports (71). In addition, PGN-stimulated OVA-DC increased the expansion of OVAspecific T cells, compared with OVA-pulsed DC alone. These data provide evidence that PGN stimulates DC-mediated processes and serves as a potent proinflammatory trigger for the development of autoantigen specific Th1 cells.

In this study, we show that the TLR-NOD2 ligand *S. aureus* PGN acts as a proinflammatory factor that is involved in the development of autoimmune disease of the CNS. The contribution of PGN on disease development is likely dependent on DC-mediated processes, because PGN is located within DC in MS brain tissue and mouse EAE lymph node tissue. We have demonstrated that PGN is able to potentiate different DC-mediated processes such as Ag uptake, DC maturation, and subsequent induction and stimulation of autoantigen-specific Th1 cell development. Targeting PGN receptors or their respective signaling pathways may well become a novel therapeutic strategy for the treatment of autoimmune disease in humans.

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Chapter 7

Venous embolic antigens are filtered in the lung vascular bed and presented by monocyte-derived lung dendritic cells in the mediastinal lymph nodes

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Venous embolic antigens are filtered in the lung vascular bed and presented by monocyte-derived lung dendritic cells in the mediastinal lymph nodes

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Abstract

The bloodstream is an important route of dissemination of invading pathogens like bacteria, fungi, viruses and helminthic parasites. Most of these pathogens will be filtered by the spleen or liver sinusoids, and presented to the immune system by dendritic cells (DCs) that probe these filters for the presence of foreign antigen. However, large pathogens (> than 20μm) are mostly trapped in the lung capillary filter. It has never been demonstrated that antigen trapped in this vast capillary area can be presented to cells of the immune system. Here, using a model of embolism of large particulate antigen (in the form of OVA-coated sepharose beads) in the lung capillaries, we show that antigen is presented and crosspresented to CD4 and CD8 T cells in the mediastinal lymph nodes (MLNs) by monocyte-derived DCs. Dividing T cells return to the lungs and only a short lived infiltrate consisting of T cells and DCs is seen, even when Toll-like receptor (TLR)

stimulation is given and full DC maturation is induced. These findings show that the lung capillary filter represents the third filter for bloodborne antigens especially large bloodborne antigens that clogg the capillaries. Immune responses at this site are hardwired to protect the delicate gas exchange mechanism in the lung interstitium.

Introduction:

Dendritic cells (DCs) are the most important antigen presenting cells for mounting a primary immune response to foreign antigens that invade the various barriers of the body such as the skin, gastrointestinal and respiratory mucosae. Here, DCs have been well studied and their function is to take up antigen across the lining barrier, integrate signals on the pathogenicity of the antigen, and migrate via the afferent lymph to the regional draining lymph nodes (LNs). In the T cell area of these draining LNs, DCs induce a tailor-made immune response that is optimal to clear the pathogen in the best possible way, while avoiding damage to self (1). Another portal of entry, but also a portal of dissemination of invading pathogens, is the bloodstream. Pathogens that reach the bloodstream, either through direct puncture through the skin, or invasiveness through mucosal surfaces such as the nasopharynx or lung are easily carried throughout the body. It is most often assumed that these antigens will be filtered by the splenic micro architecture, particularly the splenic marginal zone, and subsequently presented by splenic marginal zone DCs to naïve T cells, for induction of a primary protective immune response (2, 3). For pathogens that replicate and invade the bloodstream via the gastrointestinal tract, the portal circulation can lead to filtering in the liver blood sinusoids and antigen presentation by DCs in liver draining LNs (4). A third, often

neglected mechanism might exist in the lungs to filter blood borne pathogens, particularly when the particulate antigen or pathogen is bigger than 20 μm in size. Seventy five procent of the total circulating blood volume in humans is found in the venous system and the lung's micro vascular bed is the first set of small vessels through which the blood flows. The pulmonary vascular system with its small diameter arterioles (20-500 μm diameter) and capillaries (<10 μm diameter) forms an extensive meshwork that receives the complete cardiac output of blood (for comparison, the spleen receives only 5% of cardiac output or 200-300 ml/min). Large particles that circulate in the bloodstream (called emboli in the medical literature) are very efficiently trapped by this system. Emboli of foreign material occur in intravenous drug users leading to drug abuser's lung or talc granulomatosis. Sometimes, embolic material is infectious, such as when it is derived from infected venous thrombi or right sided endocarditis, leading to formation of multiple infectious foci in the lung. It is also known that parasites or parasite eggs that gain access to the venous system as part of their replicative cycle end up in the lung arterioles and capillaries, leading to formation of lung granulomas or transient pulmonary inflammatory infiltrates (called Löffler's syndrome). It is currently unknown whether antigens or pathogens that clogg the capillary lung filter would be presented to the immune system in a way that leads to protective immunity, analogous to the splenic or liver filter system. The lung capillaries are situated in the lung interstitium, where a well developed network of interstitial DCs and macrophages is present. These antigen presenting cells have generally been regarded as sessile cells that only stimulate already primed T cells, e.g. during pulmonary delayed-type hypersensitivity (DTH) reactions and

granuloma formation (5-8). DCs are also found to line the larger vessel wall and could probe the luminal contents for antigens (Ag) (9).

Here, we have carefully studied whether and where intravenously injected large particulate antigens, in the form of large, > 40μm ovalbumin (OVA) coated Sepharose beads, are presented to the immune system. Our studies revealed a hitherto unexplored potential of $CD11c^+$ DCs or their immediate monocytic precursors to sample the lung capillary contents and present antigen to naïve T cells in the mediastinal lymph nodes (MLNs). Primed T cells subsequently return to the lung interstitium where they surround the antigen leading to short lived inflammatory lesions. Antigen presentation and inflammatory lesions were abolished in mice in which $CD11c^+$ DCs or their monocytic precursors were conditionally depleted.

Results:

Embolic large particulate antigen is presented and cross presented exclusively in the mediastinal lymph nodes draining the lung.

It is currently unknown whether the lung capillary filter would allow antigen sampling and thus act as a site of immune induction following intravenous (i.v.) injection of particulate antigen. To study this in detail, we injected into the tail vein large sepharose beads coupled or not to $OVA_{323-339}$ peptide (coded respectively as OB and EB), the immuno-dominant MHCII-restricted peptide of OVA. To allow detection of primary T cell activation and division, mice received 2 days earlier a cohort of CFSE-labeled $CD4^+$ OVA TCR transgenic DO11.10 T cells (Fig. 1A), recognizing the $OVA_{323-339}$ peptide in the context of $I\text{-}A^d$ (MHCII).

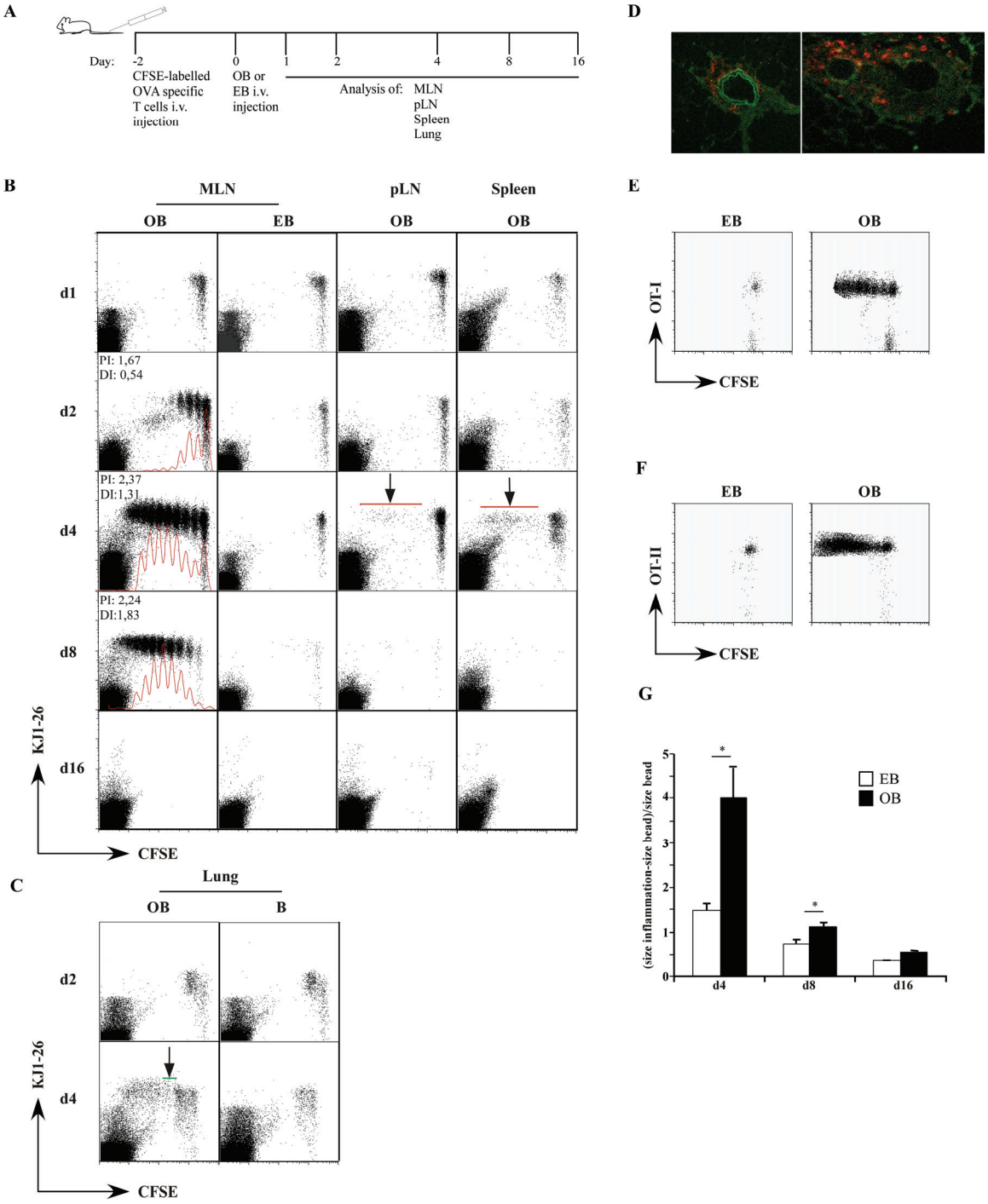

Figure 1: Embolic large particulate antigen in the lung is presented in draining mediastinal lymph nodes.
(A) Experimental setup: Balb/c and C57Bl/6 were injected with OVA specific CFSE labeled TCR transgenic T cells 2 days before particulate antigen (either $OVA_{323-339}$ peptide, LPS free Ovalbumin, or control glycine) intravenous injection. At 1, 2, 4, 8, and 16 days after injection of particulate antigen mice were sacrificed and MLNs, pLNs, spleen, and lungs were analyzed for OVA specific TCR transgenic T cells response. (B) Response of OVA specific CFSE labeled $CD4^+$ DO11.10 T cells after injection of $OVA_{323-339}$ peptide coated beads (OB) or "empty" control beads (EB) at indicated days. MLNs, pLNs, spleens were analysed for OVA specific T cell division by expression of $CD4^+$ OVA specific transgenic T cell receptor with KJ1-26-PE (y-axis), live (PI^-) gated, and CFSE positivity (x-axis). No priming occurred in spleens, and pLNs as indicated by the gap in first three divisions (arrow). (C) Response of OVA specific CFSE labeled CD4+ DO11.10 T cells in the lung after injection of OB or EB, two and four days earlier. As shown no priming occurred in the lung at day two and four after injection of particulate antigen as indicated by the gap in divisions (arrow). (D) Confocal imaging of frozen section of lungs stained with the OVA specific TCR specific marker KJ1-26-PE, at day 4 after injection of

particulate antigen. OVA specific effector T cells could be found leaving the endothelium (left) and being part of the inflammation formation around the particulate antigen (right). (E) Whole particulate antigen is presented and cross-presented in draining MLNs of the lung. At day –2 OVA specific transgenic CD4+ CFSE labeled OT-I, were injected i.v., at day 0, Ovalbumin (OVA, LPS free) coated beads (OB) or Empty beads (EB) were injected i.v., and at day 4, MLNs were analyzed for division of OVA specific T cells. Priming of CFSE (X-axis) OT-I T cells was analyzed as described above, for analysis of OT-I priming, cells were gated on CD8 and anti-Vβ5.1/5.2-PE positivity (Y-axis). (F) Priming of CFSE labeled CD4+ OT-II OVA specific T cells in MLNs, 4 days after OVA beads injection, cells were gated for CD4 positivity and anti-Vβ5.1/5.2-PE positivity (Y-axis). (G) Size of inflammation surrounding control "empty" beads (EB) or $OVA_{323-339}$ peptide coated beads (OB) in the lung decreases quickly as shown by image analysis measuring at days 4, 8 and 16 after particulate antigen injection.

Because of their size (±40-150μm), i.v. injected beads are specifically retained in the vascular bed of the lung (while no beads could be found in cross-sections of total spleens and MLNs, data not shown).

Proliferation and localization of T cell proliferation, as measured by sequential halving of CSFE intensity with each round of T cell division, was measured over time in different anatomical compartments, including the lung, draining MLN, peripheral LN (pLN) and spleen, up until 16 days following injection of beads. Proliferation of naïve OVA specific T cells only occurred in draining MLNs of the lung, starting 2 days after injection of OVA-beads, but not following injection of uncoated beads (Fig. 1B). No divisions were observed in spleen or non-draining peripheral LNs signifying that no free or particle bound antigen leaked beyond the lung capillary filter. In contrast to an earlier report showing that antigen presentation can occur *in situ* in the lung of lymphotoxin (LT)-α knock-out mice (10), remarkably no T cell divisions occurred on day 2 in lung tissues, nor on later time points (Fig. 1C). When the T cell response was followed over time (Fig. 1B), it became evident that by day 4 following injection of OVA coated beads, divided $CD4^+$ T cells also appeared in the non-draining nodes, as well as the spleen. These T cells had divided at least 3-4 times and expressed high levels of CD44, while having down regulated the early activation marker CD69, consistent with an activated phenotype (data not shown). Strikingly, these divided cells could also be traced back to the lungs (by flow

cytometry Fig. 1C), where they were found surrounding the injected OVA beads (by confocal imaging and immunostaining for DO11.10 TCR, Fig 1D), effectively leading to inflammatory lesions in the lung interstitium. Some DO11.10 T cells were seen in immediate proximity of lung vessels in the vicinity of OVA-coated beads, suggesting a specific recruitment mechanism at this site. If the immune response was evaluated even later (day 8 and day 16), it was clear that divided $CD4^+$ T cells could no longer be traced back in the LNs, spleen or lung, suggesting that they were deleted or their numbers had declined to a level below the threshold of detection by flow cytometry.

As the above findings certainly suggest that there is a mechanism of immune induction for antigen that is trapped in the lung capillaries, these experiments were not conclusive as to whether antigen would also be processed, as beads were coated with preprocessed OVA peptide. We therefore also studied whether whole Ovalbumin (OVA) protein coupled to sepharose beads would be processed and presented to CD4 and crosspresented to CD8 T cells. As MHCI restricted TCR Tg Balb/c mice do not exist, we therefore turned to the C57Bl/6 background, in which both CD4 (OTII) and CD8 (OTI) TCR Tg mice exist (11). For this, C57Bl/6 mice received MHCI-restricted OT-I or MHCII-restricted OT-II OVA TCR Tg T cells. In both circumstances we observed T cell divisions occurring in the MLNs, 4 days after injection of OVA protein-coupled beads (Fig. 1E and F). Again, injection of OVA coated beads did not lead to T cell divisions occurring outside the draining area of the lung.

In the lungs, we followed the size of the inflammatory lesions surrounding $OVA_{323-339}$-coated or uncoated beads. By day 4 of the response, the inflammatory lesions (as measured by the average surface area of the inflammation minus the surface area of the

bead divided by the bead surface area, see *Materials and Methods*) was greatly enhanced in mice receiving OVA-coated beads compared with uncoated beads. However by day 8-16, all inflammation surrounding the beads had disappeared, illustrating that the lung inflammatory lesions are very transient (Fig. 1G).

Monocyte-derived DCs accumulate around particulate antigen in the lung interstitium

As the previous data demonstrated unexpectedly that i.v. injected embolic antigenic material was exclusively processed and presented in MLNs without particulate antigens being found in these nodes, we next addressed the question which APC would be important for taking up the antigen and migrating to the draining nodes. Twenty four hours after injection of uncoated or $OVA_{323-339}$ coated beads, we detected a population of $CD11c^+$ cells around the injected beads, irrespective of whether beads were coated with OVA-peptide (Fig. 2A) or not (data not shown). Also, MHC class II^+ cells started to appear around these beads around this time period (Fig. 2A). Double staining revealed these cells to be $CD11c^+$ and MHC class II^+ double positive, strongly suggesting they were DCs (Fig. 2A). When followed over time, the number of $MHCII^+CD11c^+$ cells was increased at day 4 following injection of OVA-coated beads compared with injection of uncoated beads, a time point when inflammatory lesions and accumulation of $CD4^+$ T cells were also maximal (Fig. 2B). Later in the response, CD11c+ DCs were still present around beads, but gradually their numbers decreased, with faster kinetics if the beads were not coated with antigen. To check for the presence of myeloid cells (like monocytes, neutrophils and some DC subsets), we also stained for the presence of $CD11b^+$ cells.

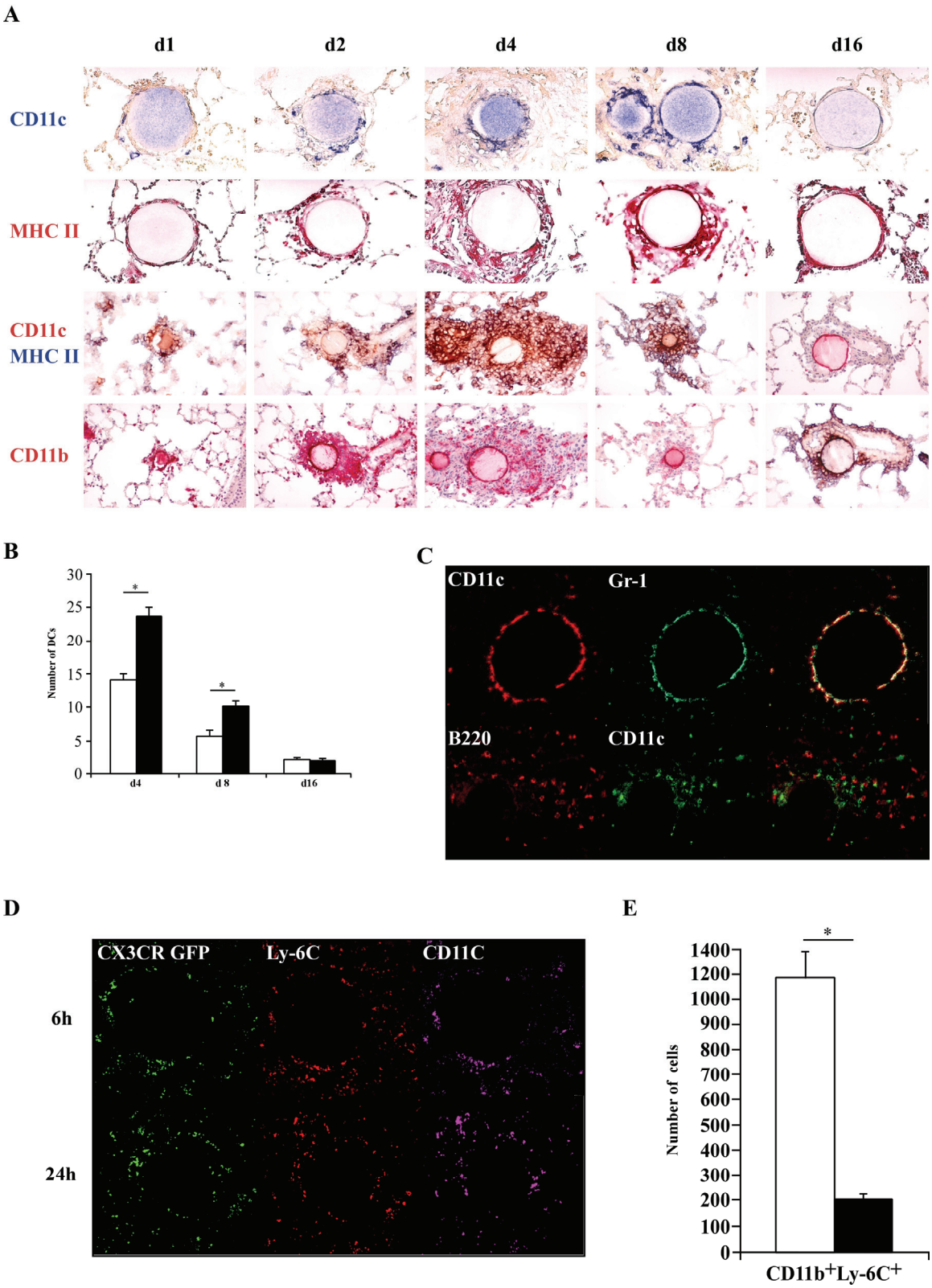

Figure 2: CD11c+ MHC-II+ CX3CR+ Ly-6C+ dendritic cells surround particulate antigen within 24h.

(A) 3 µm lung sections at 1, 2, 4, 8, and 16 days after injection of particulate antigen (OB) were single and double stained for CD11c and MHC class II expression to identify the appearance of DCs around the particulate antigen. Furthermore inflammation cells surrounding OB were stained for CD11b to further characterize the DCs. (B) Absolute numbers of DCs surrounding OB and EB

(double stained for $CD11c^+$ and MHC^+) were counted for n=4 mice per day per group, results are shown as mean of $CD11C^+MHCII^+$ DCs of 30 separate inflammations surrounding the beads ± SEM. *, P<0.05. (C) Confocal microscopy of frozen lung sections double stained with anti-CD11c-PE (red) and anti Gr-1 FITC (green) top, and anti-B220-PE (red) and CD11c-FITC (green) bottom. (D) Presence of monocyte derived DCs being CX3CR-GFP (green), Ly-6C, CD11c positive surrounding lung OB as early as 6 hours (top) and 24 hours (bottom) after OB i.v. injection. (E) Absolute number of $CD11b^+Ly\text{-}6C^+$ cells in peripheral blood two days after OB injection (white bar) or non injected wild type Balb/C mice, n = 4 mice per group. Data are shown as mean ± SEM. *, P < 0.05.

We found CD11b positive cells to be abundantly present around beads, particularly when they were coated with OVA-peptide (Fig 2A, lower panels).

The CD11c integrin is expressed on different cell types in the mouse but is mainly restricted to $CD11b^+$ and $CD11b^-$ conventional lung DCs, inflammatory type $CD11b^+$DCs and $CD11b^-$ alveolar macrophage in the lung (12-16). Lung plasmacytoid DCs (pDCs) are characterized by intermediate expression of CD11c, and expression of Gr-1 (Ly6G/C), B220 and the specific marker BST2 (recognized by the moAb 120G8) (14, 17, 18). To analyze which subset of $CD11c^+$ DC was recruited to particulate antigen, frozen sections were stained with CD11c and Gr-1 (recognizing Ly6C/G), or B220. Remarkably $CD11c^+$ cells surrounding beads were $Gr\text{-}1^+$ but lacked expression of the B220 marker, normally expressed on the surface of pDCs (Fig. 2C). We also stained for pDCs by using the pDC-specific Ab 120G8, and this staining confirmed that Gr1+ CD11c+ DCs were not pDCs (data not shown).

The expression of Gr1 (Ly6G/C) on lung $CD11c^+$ cells surrounding beads was unexpected, as this marker is classically not found on lung DCs in steady state (13). Gr1 also recognizes the Ly6C antigen, expressed on a subset of monocyte-derived inflammatory DCs (19-22). In the mouse, circulating monocytes can be discriminated into $Ly6C^+$ $CX3CR^{int}$ $CD11b^+$ monocytes and $Ly6C^-$ $CX3CR^{hi}$ $CD11b^+$ monocytes (20). Several groups have now shown that both types of monocytes are immediate circulating precursors for lung DCs but not steady state lymphoid tissue cDCs (20, 23-25).

To test the presence and differentiation of monocytes around injected beads at earlier time points, we injected CX3CR^{GFP} mice with OVA-beads (26). In these CXCR^{GFP} mice, all monocytes can be identified using confocal imaging studies. As shown in Figure 2D, 6 h after injection of OVA-coated beads, there was already an accumulation of CX3CR^{GFP} positive monocytes that also expressed Ly6C and the CD11c dendritic cell marker. To see if bead injection was associated with a systemic alteration in circulating blood monocytes, the absolute numbers of circulating CD11b^{+} Ly6C^{+} monocytes in peripheral blood of bead-injected mice were enumerated using an adapted form of absolute cell counting with StemKit®. As shown in Figure 2E, we found a significant decline in the absolute number of live (PI^{-}) gated CD11b^{+} Ly-6C^{+} monocytes present in whole peripheral blood two days after i.v. injection of beads compared to non injected mice.

Monocyte-derived DCs are necessary and sufficient for antigen presentation of intravenously injected embolic antigen in draining mediastinal lymph nodes

Our histological data suggested that early after bead injection, Ag was seen mainly by Ly6C^{+} monocytes that acquired an 'inflammatory' monocyte-derived DC phenotype. To address more directly the functional role of these monocytes in causing T cell proliferation following i.v. injection of embolic antigen, we performed depletion experiments in which monocytes were depleted using the depleting Gr1 (Ly6C/G) antibody (25). Consistent with a predominant role for Ly6C^{+} monocytes as precursors for DCs in this response, we found that treatment with Gr1 antibody two days prior to bead injection led to a considerable delay in the onset of T cell division occurring in mice injected with OVA-coated beads (Fig. 3A). At day 4, mediastinal T cell division was

severely reduced in Gr1 treated mice (Fig 3A, lower panels) compared with isotype treated mice (upper panels), almost down to the level of uncoated bead injected mice (not shown).

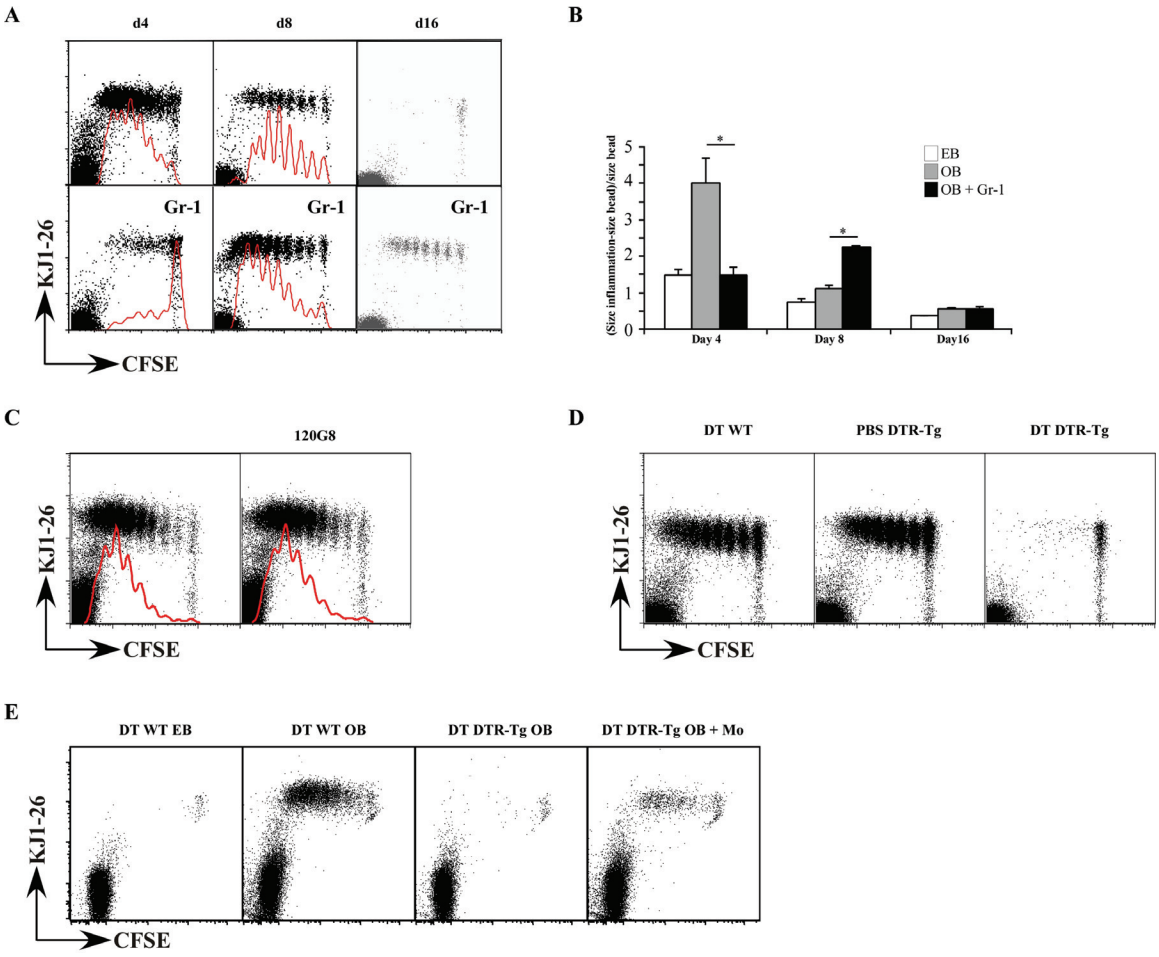

Figure 3: Monocyte-derived CD11c$^+$ dendritic cells are essential for embolic antigen inflammation formation and priming of naïve CD4$^+$ in lung draining MLNs.
(A) Comparison of CFSE labeled OVA specific CD4$^+$ DO11.10 divisions in draining MLNs, in mice treated or not with Gr-1 i.p. at day 0 and injected with OB, measured at days 4, 8 and 16. (B) Measurement of inflammation size around beads in the lungs of mice receiving either empty-beads (EB, white bars), $OVA_{323-339}$ coated beads (OB, gray bars) or Gr-1 (at day -2) and OB (black bars) at day 0. Data are shown as mean (n=4 mice/group) ± SEM. *, $P < 0.05$. (C) Comparison of CFSE labeled OVA specific CD4$^+$ DO11.10 divisions in draining MLNs, in mice treated or not with 120G8 (for pDC depletion) i.p. at day 0 and injected with OB, measured at days 4. (D) Establishing the importance of CD11c$^+$ DCs in antigen presentation in lung draining MLNs after embolic antigen presentation in the peripheral lung. By depleting CD11c positive cells with DT i.p. injection on day 0 in DTR-Tg mice expressing DTR on CD11c$^+$ cells, hardly any division in draining lung MLNs could be found of CFSE labeled DO11.10 OVA specific CD4$^+$ T cells 4 days after OB injection on day 0, while normal divisions were seen at that same time point (day 4) in wild type (WT) mice receiving OB and DT or DTR-Tg mice receiving OB and PBS i.p. on day 0. (E) Wild type or CD11c/DTR-Tg mice received CFSE specific OVA specific CD4$^+$ T cells at day -2, with DT at day 0, with either EB (WT) at day 0, or OB (WT) at day 0, OB at day 0 (DTR-Tg mice) with or without 250 000 bone-marrow purified (>99%) monocyted (DTR-Tg mice). CD4$^+$ OVA specific T cell divisions in these groups is shown in draining MLNs at day 4 after EB or OB injection. As shown DTR-Tg mice depleted of CD11c$^+$ by DT show reconstitution of divisions by monocyte i.v. injection at day 0.

By day 8 of the response, there was partial recovery of this response in that T cell division was higher in monocyte-depleted mice compared with isotype-treated mice, and comparable in strength to the response seen at day 4 in isotype treated mice. At day 16 of the response, a time point when all T cells are normally deleted in this response (see Fig. 1B), we still observed divided T cells in the mice depleted of monocytes. These findings are most consistent with a delayed Ag presentation in monocyte-deprived mice. The formation of inflammatory lesions around injected OVA-coated beads was also significantly suppressed and delayed untill day 8 following injection of beads in mice depleted of Gr-1$^+$ monocytes compared to the control isotype treated mice which had the maximum inflammatory lesions at day 4 (Fig. 3B). The Gr1 antibody has also been used to deplete pDCs from the lungs of mice (14, 27). To address the specific contribution of pDCs in this response, we also treated mice with the depleting antibody 120G8, recognizing the more pDC restricted antigen bone marrow stromal antigen-2 (18). The treatment with 120G8 around injection of OVA-coated beads did not affect the degree of T cell proliferation in draining MLNs at day 4 of the response, compared with isotype treated mice (Fig. 3C).

Recently, a mouse model of conditional depletion of CD11c$^+$ DCs has become available (28). These mice express the human Diphteria Toxin Receptor (DTR) under the control of the murine CD11c promotor, allowing the DT induced conditional depletion of all CD11c$^+$ cDCs, while leaving pDCs largely unaffected (29). To study if the recruited monocyte-derived CD11c$^+$ cells were indeed responsible for Ag presentation in the MLN, we administered DT intraperitoneally (i.p.) to mice that received DO11.10 T cells and subsequently injected with i.v. OVA-coated beads. DC depletion was performed at the

day of beads injection. As seen in Figure 3D, T cell proliferation at day 4 of the response in the MLNs of CD11c-DTR Tg mice given DT was dramatically reduced to the level seen in mice given uncoated beads. However, wild type mice given DT (or CD11c-DTR Tg mice given PBS, data not shown) still mounted a normal response to bead injection. The reduction of T cell proliferation in DC-depleted mice was accompanied by a severe reduction in the size of the inflammatory lesions surrounding the OVA-coated beads at day 4 following injection, and similar to the reduction seen in monocyte-depleted mice (data not shown).

As these experiments in monocyte-depleted and DC-depleted mice clearly demonstrated that these cells were necessary for immune induction in the lung capillary filter, we next questioned if monocytes would also be sufficient. For this purpose, $Ly6C^{hi}$ monocytes were sorted from the bone marrow (based on expression of CD11b, Ly6C and lack of expression of CD31) and adoptively transferred intravenously in CD11c DTR Tg mice given DT around the time of bead injection. As seen in Figure 3E, restoration of OVA specific $CD4^{+}$ T cell divisions could be accomplished by injection bone marrow purified $Ly6C^{hi}$ monocytes. These data therefore show that venous embolic antigens are filtered in the lung vascular bed and presented by monocyte-derived lung dendritic cells in the MLNs.

Induction of maturation of monocyte-derived DCs by microbial stimuli and trimeric CD40L increases effector potential of T cells without affecting T cell division.

In all our experiments, we observed that inflammatory lesions around the OVA-coated beads eventually resolved by day 8 of the response. This could be due to the fact that

OVA-coated beads are seen as relatively harmless antigens, leading to a failure of maturation in monocyte-derived $CD11c^+$ DCs.

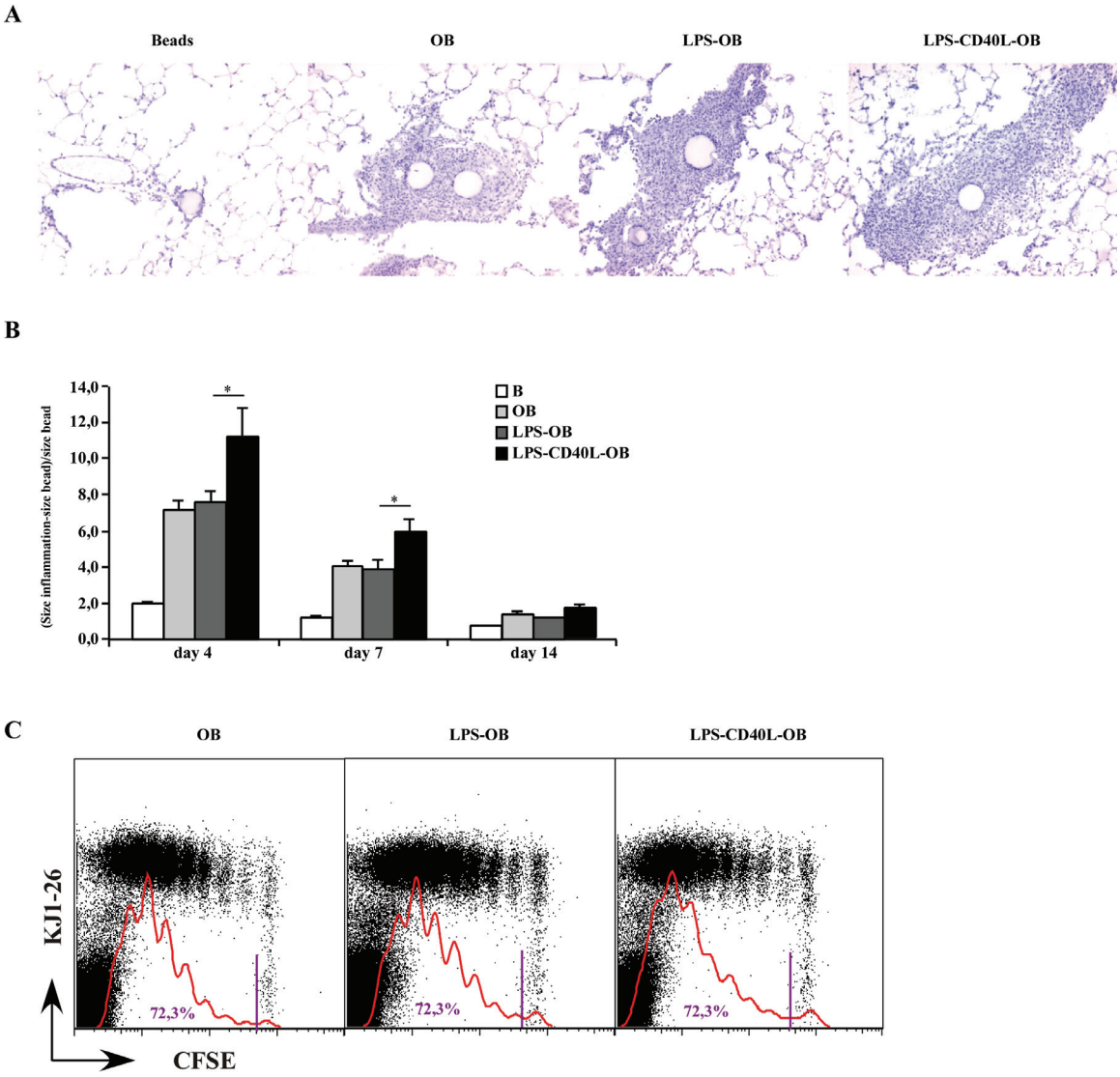

Figure 4: Maturation induction by CD40L of embolic antigen surrounding DCs in the lung cause amplified and longer lasting inflammation formation.
(A) Mice received OVA-specific $CD4^+$ DO11.10 T cells at day -2, and either "empty-beads" (EB), Ovalbumin-beads (OB), OB "co-coated" with LPS (LPS-OB), or OB "co-coated" with LPS and trimeric CD40L (LPS-CD40L-OB) at day 0, 4 days later inflammation surrounding the beads are shown in these groups. (B) Size of inflammation surrounding the beads in the lungs in these groups is shown at day 4, 8 and 16. Data are shown as mean (n=4 mice/group) ± SEM. *, $P < 0.05$. (C) Representative flow cytometry analyzes of (n=4, per group) OVA specific $CD4^+$ T cell divisions in draining lung MLNs at day 4 are shown.

To address this point, mice were injected with OVA-coated beads that were also coated or not with LPS or LPS combined with trimeric sCD40L or uncoated beads (EB).

Trimeric CD40L is an effective agonist of CD40 on DCs and is known to induce DC maturation, particularly when a concomitant TLR agonist such as endotoxin is administered (30).

In mice receiving OB coated in combination with trimeric CD40L and LPS, there was a strong increase in the size of inflammatory lesions around the OVA-coated beads (Fig. 4A). LPS "co-coating" of OB by itself was insufficient to obtain this effect. This enhancing effect was maintained until day 16 after injection of beads, although inflammatory lesions became much smaller compared with those seen on day 4 and day 8 (Fig. 4B). Provision of these DC maturing stimuli did not affect the strength of T cell divisions in the MLN at day 4 after OB injection (Fig 4C).

Discussion:

In this paper, using a system of traceable Ag reactive T cells specific to ovalbumin, we report that the lung capillary filter allows the effective filtering of large embolic antigens followed by uptake of the antigen by monocyte-derived $CD11c^+$ DCs and antigen presentation to $CD4^+$ and $CD8^+$ T cells in the MLNs. Primed T cells subsequently return to the lung and together with DCs form short lived inflammatory lesions that are ultimately cleared. This response clearly depends on monocytes and $CD11c^{hi}$ DCs as it is eliminated in mice with a conditional deletion of $CD11c^+$ cells and depleted of Gr1 positive cells, and restored by adoptive transfer of $Ly6C^{hi}$ monocytes (28). These data therefore identify a third filtering network for bloodborne antigens, in addition to the spleen and liver, that is specialized for immune induction towards large embolic material.

Strikingly, early after injection of embolic material, there was an accumulation $CD11c^{+}MHCII^{+}$ DCs surrounding the injected beads of which the origin can be debated. One possibility is that the resident interstitial DCs of the lung that can be found in alveolar septa would migrate to the occluded vessels and sample the vessel content. Numerous studies in mouse and rat have indeed demonstrated that alveolar septa contain DCs in close proximity to small diameter arterioles and alveolar capillaries (8, 31, 32). However, these interstitial DCs have classically been described as sessile cells that fail to migrate to draining LNs and would merely restimulate already primed T cells during pulmonary DTH-like reactions (33, 34). Another possible explanation would be that DCs accumulated on the injected beads from within the vessel lumen. Pulmonary vessels already contain a large marginating pool of DC precursors, possibly hardwired for antigen recognition at this site. This has been suggested by Schneeberger's group, who demonstrated that the blood canulated from the lungs of rats contained much more DC-precursors compared with vena cava canulated blood, and these cells obtained DC potential after culture in appropriate cytokines (35). Whether this sequence of DC-differentiation from vascular precursors would occur in our system remains to be shown.

Although all of the above senarios are not mutually exclusive, we favor a third potential source for bead associated $CD11c^{+}$ DCs. Antigen presentation in our system of i.v. embolization occurred as the result of Ag recognition by $Gr1^{+}$ (Ly6C/G) $CD11c^{+}$ cells that accumulated as early as 6h following bead injection. These cells most likely represented monocyte derived DCs, and not pDCs as they lacked expression of 120G8. The evidence for this comes from the co-expression of the fractalkine (CX3CR) receptor, Ly6C and CD11c on these cells and the fact that T cell proliferation as well as

development of inflammatory lesions in the lung were significantly reduced when mice were depleted of Gr1 (Ly6G/C) positive cells, but not by depletion of 120G8 positive cells. In previous work, $Ly6C^{+}$ monocytes have been shown to be precursors for inflammatory type DCs, thus acquiring APC function and expression of the CD11c marker and most likely represent the immediate precursor to "nature's adjuvant, the immunogenic DC (36-40). In the mouse, circulating monocytes can be discriminated into $Ly6C^{+}$ $CX3CR^{int}$ $CD11b^{+}$ inflammatory monocytes and $Ly6C^{-}$ $CX3CR^{hi}$ $CD11b^{+}$ monocytes (20). Several groups have now shown that both types of monocytes are immediate circulating precursors for lung DCs but not steady state lymphoid tissue DCs (20, 23-25). Strikingly, inflammatory type monocytes express the CCR2 receptor for monocyte chemotactic protein-1. Although we have not measured the level of this chemokine, others using a injection model of Schistosoma or PPD-coated sepharose beads could measure an early production of this chemokine by lung structural cells (41). Supporting a crucial role for inflammatory monocytes in early granuloma formation, CCR2 deficient mice had a defect in early (day1-2) granuloma formation. It will be interesting to study whether these CCR2 deficient mice also have a reduced DC accumulation around beads and delayed Ag presentation in our model. This could be a possibility as CCR2 was shown to be crucial for DC recruitment to the other lung compartment, the airway mucosa, under inflammatory conditions (42).

One striking observation of this and other models of embolization such as injection of *Schistosoma* soluble antigen-coated or *Mycobacterium* purified protein derivate (PPD) antigen-coated sepharose beads was that inflammatory lesions around injected embolic material were very short lived (7, 43). One explanation would be that in our model,

monocyte-derived $CD11c^+$ DCs would not get the proper activation status to induce full blown effector cells, thus leading to a program of deletional proliferation of T cells. Arguing against this possibility is the fact that injection of CD40 and LPS, two known activators of the DC system (30), enhanced the size of the inflammatory lesions, but eventually these were still cleared from the lung. Also, models in which PPD-coated beads or *Schistosoma* eggs are injected would lead to full DC activation, but still these 'granulomata' are cleared eventually (7). Such a system to clear the lung interstitial tissue of overt inflammatory lesions makes evolutionary sense as this is the site of vital gas exchange (44). Several mechanisms in the lung, such as the suppressive function of the nearby alveolar macrophage with its secretion of anti-inflammatory factors such as TGF-β, IL1-RA, IL-10 and prostaglandins might keep inflammation in check by down regulating the antigen-presenting capacity of DCs (7, 8, 45). Alternatively, early fibrosis occurring around injected beads could effectively shield off the antigen, so that it is further ignored by the immune system eventually leading to apoptosis of T cells (41).

In conclusion, we have provided evidence for a highly active antigen sampling mechanism for embolic material trapped in the small vessels of the lung. It is likely that this system developed during evolution to allow the immune system to effectively recognize large particulate antigens acquiring access to the venous blood by invasion, such as parasite eggs or worms, or following direct (traumatic) access to the bloodstream. When such antigens would be retained in the lungs without a possibility for Ag recognition, this could lead to a very effective way of pathogen immune subversion, as the spleen or liver, two other major sites of immune induction against blood particulate antigens, cannot access these large antigens and the pathogen could mutate during

residence in the lung. It will be interesting to study whether certain pathogens take advantage of this system by blocking DC migration to the mediastinal nodes. It could also be that allergenic cells disrupted as a group of cells from freshly transplanted vascularized organs are presented via this route, as these vascularized grafts lack a direct connection with afferent lymph and the lung capillaries are the first small vessel filter encountered. Clearly, this highly effective pathway of immune induction in the lung interstitium deserves further study.

Materials and Methods:

Mice.

Female Balb/c (H-2^d) mice 6 to 8-week-old were purchased from Harlan (Zeist, The Netherlands). Ovalbumin-TCR transgenic mice (DO11.10) on a Balb/c background, OT-II and OT-I Ovalbumin-TCR transgenic mice on a C57BL/6 background, CD11c-DTR transgenic mice on a Balb/c background were bred at the Erasmus University (Rotterdam, The Netherlands). CX3CR-GFP on a C57BL/6 background (kind gift from S. Jung). 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.

Reagents and Antibodies

$OVA_{323-339}$ peptide was obtained from Ansynth Service B.V. (Roosendaal, The Netherlands). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, Oregon). FITC-labeled anti-Gr-1 (RB6-8C5), PE-labeled KJ1-26 (clonotypic OVA-TCR) was from Caltag Laboratories (Burlingame, CA), PE-Labeled anti-B220 (RA3-6B2), CD8α (Ly-2), Vb5.1/5.2, The APC-labeled anti-CD4 (RM4-5), all from BD Biosciences). 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).

Intravascular embolic lung inflammation induction

For inflammation formation in the vascular bed of the lung, wild type Balb/c mice received intravenously (i.v.) in the lateral tail vein 1.10^4 OVA-coated (OB) or non-coated (glycine, EB) (CNBr)-activated Sepharose 4B beads on day 0. Beads were prepared as previously described (43). Because of low OVA TCR specific naïve T cell frequency in these wild type Balb/c mice, 10 x 10^6 live CFSE labeled OVA TCR transgenic naïve DO11.10 T cells were given i.v. 2 days before injection of the beads (day –2). Briefly, to prepare these T cells, LNs and the spleen were harvested from DO11.10 mice, homogenized and after red cell lysis, cell suspensions were labeled with CFSE as previously described (14).

Detection of the primary T cell response to OVA-bead injection i.v.

OVA-specific TCR Tg T cells were collected from the lymphoid organs of naïve 4-6 wk old DO11.10, OT-I or OT-II mice and stained with CFSE (Molecular Probes, Eugene, Oregon), as previously described (14). 10 x 10^6 cells were injected i.v. in the lateral tail vein of Balb/c or C57BL/6 mice (day -2). On day 0, mice received an i.v. injection of 10 x 10^4 OVA-beads (OB) or "empty"-beads (EB). On days 1, 2, 4, 8 and 16 MLNs, pLNs (axillary), spleens and lungs were removed, and individual cell suspensions were prepared as previously described (14).

In experiments to address the functional role of DCs in inflammation formation around beads, $CD11c^+$ cells were depleted by injecting 100 ng of DT i.p. in CD11c-DTR Tg mice (28). In experiments to address the functional role of pDCs in inflammation formation around beads, pDCs were depleted by 120G8 antibody injection (provided by

C. Asselin-Paturel, Shering-Plough, Dardilly, France). For monocyte depletion anti-Gr-1 i.p. injection was used.

In experiments to address if monocytes could restore the inflammation around beads in the DT-treated CD11c-DTR Tg mice, 3.5 x 10^5 $CD11b^+Ly\text{-}6C^+CD31^-$ monocytes sorted from bone marrow of Balb/c mice were injected i.v. on day 0 after OB or EB injection.

Confocal microscopy.

Confocal analysis was performed on 6-µm cryostat sections of lungs stained with anti-Gr-1 FITC, anti-B220-PE, Ly-6C, CD11c, 120G8 coated with Quatum dots (MicroProbes). Sections were analyzed on a confocal laser microsope (LSM-510; Carl Zeiss MicroImaging, Inc.).

Immunohistochemistry and inflammation surrounding beads size analysis

For immunohistochemistry analysis of the inflammation 6-µm cryostat sections of lungs stained with CD11c, CD11b, MHCII. For inflammation size, hematoxylin counterstained lungs where analyzed with an image analysis system (Quantimed, Leica, Rijswijk, The Netherlands) whereby 30 beads per mouse were measured: dividing the surface size of the bead (μm^2) by the surface size of inflammation and bead together (μm^2).

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Chapter 8

General Discussion and Conclusion

Summary of this thesis

Dendritic cells (DCs) are known to play a pivotal role in the induction of a primary and secondary immune response in the lung (1). By taking up antigen under steady state and inflammatory conditions from the tissue where they reside and subsequently migrating and presenting antigen bound peptides on MHC class I and/or II in draining lymph nodes (LNs) to CD8+ or CD4+ T cells respectively, DCs induce either tolerance or immunity (2).

In this thesis, we specially addressed the role of different DC subsets, the influence of maturation and type of stimulation, and the difference between DC subsets at different anatomical locations in inflammatory and tolerance induction. Therefore we used different mouse models for asthma and a vascular disease model of the lung, as well as different compounds that could influence DC stimulation and maturation as described in detail in **Chapter 1**.

In **Chapter 2,** we gave an overview of the literature on the involvement of DC subsets in immune regulation in the lung during steady state and inflammatory conditions. The two large family subsets of DCs are present in the lung in steady state, namely conventional DCs (cDCs) and plasmacytoid DCs (pDCs) and they play specific roles in the immune responses in the airways. Under inflammatory conditions, monocytes can furthermore give rise to inflammatory DCs (iDCs). The orchestration of tolerance by pDCs through induction of regulatory T cells (Tregs) in the mediastinal lymph nodes (MLNs) and the induction of effector Th2 responses by cDCs and/or iDCs via the induction of effector and memory $CD4^+$ and $CD8^+$ T cells were studied mainly in the mouse model for asthma. Furthermore therapeutic interventions using these DC subsets were highlighted.

In **Chapter 3,** We analyzed the capacity of pDCs to prevent asthma features during exposure of harmless inhaled antigen. The experiments described in this chapter were designed to elucidate the mechanisms that were involved during the process of inhalation tolerance, a state of unresponsiveness to inhaled antigens. We found for the first time that pDCs are present in the interstitium and mucosal airways in mice, and can be recognized as being $CD11c^{int}$ $B220^+$ $Gr\text{-}1^+$ ($Ly\text{-}6c^+$) BST2 positive (see Chapter 2).

We found that by depleting mice of pDCs at the time of sensitization, mice subsequently developed asthma features after challenge with OVA aerosols. Furthermore we found that both pDCs and cDCs take up inhaled OVA in the lung and present it either in a tolerogenic form or immunogenic form respectively. As we found that pDCs were able to take up OVA-FITC and $FITC^+$ pDCs could be found after 24h in MLNs after intra-tracheal (i.t.) injection of OVA-FITC. Yet when purified OVA^+-pDCs from these MLNs were cocultured with naive CFSE labeled OVA specific TCR transgenic T cells, hardly any divisions or cytokine production was observed, this in sharp contrast to OVA^+ mDCs sorted from the same MLNs which induced strong OVA specific $CD4^+$ T cell proliferation and production of cytokines (IL-5 and IFN-γ). Indirectly indicating that priming of naive $CD4^+$ T cells is mostly restricted to cDCs. We also found that asthmatic reactions could be prevented in a mouse model of asthma by the adoptive transfer of OVA pulsed pDCs just before challenge. Therefore we can conclude that pDCs not only play a pivotal role in the prevention of asthmatic reactions but could also be exploited in therapies aimed at preventing asthmatic reactions.

In **Chapter 4.** we investigated the role of peroxisome proliferator-activated receptor-γ (PPAR-γ) in DCs in a mouse model for asthma. We used our well established murine model of asthma in which Balb/c mice were sensitized by an i.t. injection of OVA-pulsed bone marrow-derived BM-DCs treated or not with the selective PPAR-γ agonist rosiglitazone (RSG). The BM-DCs most closely resemble the iDCs. Ten days later, mice were challenged with OVA aerosols for three consecutive days. Mice receiving untreated OVA-DCs developed eosinophilic airway inflammation as shown by the presence of peribronchial and perivascular infiltrates and goblet hyperplasia, an increased influx of eosinophils in bronchoalveolar lavage fluid (BALF) and Th2 cytokine production in draining MLNs. We show that RSG treated DCs not only induced a reduction in the proliferation of Ag-specific T cells in the draining MLNs of the lung but also an increase in the production of the immunoregulatory cytokine interleukin (IL-)10 by these T cells, as compared to control mice sensitized with OVA-pulsed DCs. Futhermore all the asthma features were strongly reduced in mice receiving RSG/OVA-pulsed DCs compared to mice control mice receiving OVA-pulsed DCs. Notably the anti-IL-10R antibodies into

RSG/OVA-DC treated animals compared to mice treated with the isotype control antibody partly restored the inflammation in the former group. By using ciglitazone, another PPAR-γ agonist, and by using GW9662, a PPAR-γ antagonist we could confirm that PPAR-γ interferes with the capacity of DCs to induce Th2 sensitization thus providing another important possibility of a therapy aimed at reducing asthmatic reactions by targeting iDCs function.

In **Chapter 5**, we determined the role of Prostaglandin D_2 (PGD_2) in DC migration and function under steady state conditions in the lung. Because it had been shown that PGD_2 inhibits the migration of epidermal Langerhans cells to the skin draining LNs and altered the subsequent cutaneous inflammatory reaction, we wanted to investigate the role of this mediator in the lung in the absence of inflammation.
Under steady state conditions, harmless antigen such as Ovalbumin (OVA), when introduced into the airways of mice, can be taken up by these ideally situated conducting airways cDCs, and presented the antigen to naïve $CD4^+$ T cells in the draining MLNs. We found that i.t. instillation of PGD2 and OVA-FITC inhibited the migration of $FITC^+$ DCs to draining MLNs. This effect was mimicked by the selective DP1 agonist BW245C and not by the CRTH2 agonist DK-PGD2. The proliferation and production of inflammatory cytokines (such as IL-4, IL-10, and IFN-γ) by OVA-specific CFSE-labeled, naïve T cells in draining MLNs was also diminished in mice receiving OVA combined with BW245C, compared to mice receiving OVA. This might indicate the important role of PGD_2, a lipid mediator, in suppressing cDC and T cell activation under steady state conditions (in the absence of danger signals).

In **Chapter 6**, we investigated the effect of bacterial peptidoglycan (PGN), a Toll-like receptor (TLR) 2 agonist on iDCs. DCs can recognize pathogen-associated molecular patterns (PAMPs) that are contained in microbial motifs through their expression of extracellular and intracellular receptors, such as TLR receptors and nucleotide-binding oligomerization domain (NOD) receptors, and a wide variety of C-type lectin receptors that can discriminate between self versus non-self proteins on the basis of glycosylation patterns.

We found that PGN leads to enhanced uptake of OVA, maturation of iDCs (grown from the bone marrow in GM-CSF) and to an increased iDC induced Th1 reponses in vitro. Furthermore we found that the administration of an encephalitogenic myelin oligodendrocyte glycoprotein peptide combined with PGN leads to priming of autoreactive Th1 cells and the development of experimental autoimmune encephalomyelitis (EAE) in mice. The injected PGN could be found in LN within clustered DCs, and can be present for a long period of time, altering DC function. These findings indicate the role of TLR stimulation of DCs in vitro and in vivo and the subsequent preferred T cell skewing by these DCs during priming or challenge with normally harmless antigens such as OVA in the lung. This is important as recently it has been shown that viral infection (3) and certain bacterial infection (4) of the lung leads to enhanced asthmatic reactions.

In **Chapter 7**, we studied the role of DCs in the alveolar compartment of lung that is vital for the gas exchange. Up to now little is know about the subsets and the role of DCs in this compartment. Therefore we used a system in which we delivered a particulate antigen via the intravenous route in the vascular bed of the lung in the vicinity of the alveolar compartment. We found that $CD11c^{+}$ $Gr\text{-}1^{+}$ monocyte-derived DCs not only induce priming of naïve OVA specific T cells in this compartment of the lung in vivo in draining MLNs, but also that there is only a transient inflammation around the beads in the lung. By adding Lipopolysaccharide (LPS, a major component of the outher membrane of Gram-negative bacteria) and anti-CD40L (an important co-stimulatory molecule on T cells) to the particulate-antigen we were able to induce stronger lung inflammatory lesions that lasted for a longer period of time. Indicating that although tolerance can be broken in the alveolar compartment, this lasts only temporarily to ensure the vital gas exchange.

Discussion

Based on the observations and experiments described in Chapters 3-7, we propose that different subsets of DCs have import roles in the induction and maintenance of immune responses in the lung leading to either eosinophilic airway inflammation or interstitial inflammation in the lung or tolerance induction. Activation seems to be mostly dependent on (inflammatory/conventional) $CD11b^+$ DCs and their possible TLR induced maturation leading to an inflammatory response, whereas tolerance induction would be induced by either immature cDCs or pDCs that require different PAMPs to be activated and mature. However in the lung interstitium, even monocyte-derived iDCs still induce only short lived inflammatory lesions, illustrating that anatomical context also determines the eventual outcome of a DC driven immune response.

The findings in this thesis lead to the following answers to the research questions stated at the beginning of this thesis **(chapter 1):**

<u>Research question 1 and 2 :</u> Is tolerance or immunity induction in the lung a property of specialized DC subsets, and is the degree of maturation/activation of cDCs important to induce immunity of tolerance in the lung?

Even if DCs can induce priming to inhaled antigen, still the predominant outcome of inhalation of harmless antigens is tolerance. This is shown best for the model antigen ovalbumin (OVA). When given to the airways of naïve mice via aerosolization, nasal droplet aspiration or intratracheal injection, it renders mice tolerant to a subsequent immunization with OVA in adjuvant, and effectively inhibits the development of airway inflammation, a feature of true immunological tolerance (5-7). Although DCs carrying harmless OVA antigen induce a vigorous proliferation in naive antigen-specific T cells, the outcome of the response is tolerance and not immunity (6, 8, 9). One possible explanation is that the (self) antigens encountered by airway DCs under non-

inflammatory conditions cannot induce a full activation of cDCs sufficient to induce T cell effector function, and therefore do not serve as 'effector DCs" (10). Partially mature DCs would then induce an abortive immune response, either leading to the generation of regulatory T cells (11-13) or would lead to generation of unfit T cells that fail to reach the threshold for survival and are finally deleted (14). Furthermore, we believe that proper known environmental triggers for Th2 sensitization in the lung, that lead to a break in inhalation tolerance, such as LPS, viral infection, diesel smoke exposure or cigarette smoke exposure can also lead to recruitment of inflammatory type DCs from monocytes in the bloodstream, and these might be the real vehicles of immunity. In this regard, Kool recently demonstrated that alum, the best known Th2 sensitizer also works via this mechanism (15).

In **chapters 4 and 5** we found evidence for the fact that keeping DCs in an immature state promotes tolerance. In later work, Hammad et al. found that these immature DCs promoted tolerance via induction of $Foxp3^+$ Tregs (16). A recent study showed that mediastinal $CD8\alpha^-$ mDCs of OVA-tolerized mice produce high levels of IL-10 and induce the generation of a protective population of Th2-like regulatory T cells expressing both Foxp3 and GATA-3 (7). This response required the presence of surface ICOS-L and CD86 (17, 18). Another paper from the same group demonstrated that the transfer of splenic $CD8\alpha^+$ cDCs but not of $CD8\alpha^-$ cDCs of mice immunized with OVA and heat-killed Listeria could protect against asthma development through the induction of Th1-like regulatory cells (19). These regulatory cells produced both IL-10 and IFN-γ, express T-bet and Foxp3, indicating that the Th1-like regulatory cell population generated is different from Tr1 cells (13) or from in vitro-generated regulatory T cells (20). Although

the CD8α$^+$ DC subset has not yet been described in the lungs, the phenotype most closely resembling this cell type is the immature cDC, that can be either CD11b$^-$ or CD11b$^+$. In total, these findings suggest that a spectrum of adaptive regulatory T cell types can be generated in the draining LNs of the lung, depending on the type of antigen encountered and the subset of DCs involved. Some bacteria have taken advantage of this DC-induced regulatory T cell induction to their own advantage. Mycobacteria and *Bordetella pertussis* bind to DC-SIGN on the surface of monocyte derived DCs, leading to a decrease in the expression of CD80, CD83, and CD86. At the same time, lung DCs increase their production of IL-10 and promote the generation of regulatory T cells (21, 22), leading to a dampening of efficient effector T cell response in the lung. Following exposure to lipid mediators such as PPAR-γ agonists or prostaglandins that keep cDCs or monocyte derived iDCs in a persistently immature state, these DCs lose their capacity to prime for eosinophilic airway inflammation, and induce a protective response in the lung (23, 24).

In **chapter 3** we found that pDCs in the lung are able to take up harmless antigen, migrate to draining LNs to induce tolerance through the induction of regulatory T cells (6). Strikingly, the depletion of pDCs with either depleting anti-Gr-1 antibodies or with 120G8 (25) led to a break of inhalational tolerance to this harmless antigen and induced a very strong Th2-dependent pulmonary inflammatory reaction, showing that pDCs provide intrinsic protection against inflammatory responses to harmless antigen in the lung. The tolerogenic properties of pDCs have been described before. A couple of studies have shown that human pDCs can induce CD4$^+$CD25$^+$ T regulatory cells. In the mouse, freshly isolated antigen-pulsed spleen pDCs induce minimal proliferation and no

cytokine polarization in antigen-specific T cell receptor–transgenic T cells (26, 27). These T cells do not proliferate even if exogenous IL-2 is added but they acquire regulatory activity and suppress antigen-specific T cell proliferation (27). In **chapter 3** we also showed that the adoptive transfer of OVA-pulsed pDCs could completely suppress the development of cardinal features of asthma (6), likely through the generation of regulatory T cells. A possible explanation for the tolerogenic potential of pDCs might be that pDCs can produce indoleamine 2,3-dioxygenase (IDO), which has a strong inhibitory activity on T cell proliferation, (28) and inhibits inflammatory airway disease (29). Interestingly, IDO expression has been demonstrated recently in pulmonary $CD11c^+$ cells (30) although the exact cell type involved has not clearly been identified. Another explanation to the tolerogenic properties of pDCs is related to their immature phenotype, as it has been demonstrated that immature DCs can induce regulatory T cells (11-13).

In contrast to immature pDCs, activated pDCs can augment cell surface expression of MHC class II and costimulatory molecules, increasing their T cell stimulatory ability and become immunogenic. When pDCs are exposed *in vivo* to bacterial CpG motifs, to influenza or to cytomegalovirus, they become capable of priming $CD8^+$ T cells and induce efficient cytotoxic responses (31-33). However, recent data form Liu et al. showed that CpG activated pDCs still induce Tregs via an ICOSL-dependend pathway. From these data, it seems that activated pDCs can present antigens and induce considerable expansion of T cell populations, although less efficiently than cDCs. One mechanism recently raised and that could explain why pDCs can prime T cells is their capacity to differentiate into a cDC phenotype upon viral stimulation. These data were observed only when very immature bone marrow-derived pDCs and not more differentiated splenic

pDCs were used. However, whether this really happens in peripheral organs such as the lung remains to be addressed. It is tempting to speculate anyway that this conversion of pDCs of tolerogenic to immunogenic cells could be an explanation why infections with respiratory viruses lead to a break in inhalational tolerance and are often associated with an enhanced allergic responses to harmless antigens (34-36).

Research question 3 : Can the type of stimulation encountered by different DCs influence the subsequent immune response

DCs express extracellular and intracellular receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors that recognize pathogen-associated molecular patterns (PAMPs) contained in microbial motifs, as well as a wide variety of C-type lectin receptors that discriminate glycosylation patterns on self versus non-self proteins. Recognition of PAMPs by DCs normally leads to their maturation and migration from the peripheral organ to the draining LN and activation and differentiation of naïve T cells to effector T cells (**Chapter 2**). It is well known that harmless antigen such as Ovalbumin needs a second maturation signal for DCs by a PAMP to lead to cardinal features of asthma, such as a certain amount of LPS (TLR4) stimulation (37). This TLR4 activation is MyD88 dependent (38) which is also the case in the "classical model for asthma" where monocyte-derived DCs are activated by alum through the uric acid MyD88 dependent pathway (15). We found that stimulation and subsequent maturation of cDCs by peptidoglycan (PGN, a TLR2 agonist), leads to similar naïve CD4 T cell differentiation (**Chapter 6**), although we now know, to Th17 cytokine producing $CD4^+$ T cells, which play a pivotal role in the development of EAE in mice and humans (39, 40). It has since been shown that also in real life EAE lesions, central

nervous system iDCs preferentially process myelin derived antigen to CD4 T cells that subsequently differentiate into pathogenic Th17 cells (41). Our findings of TLR2 driven development of Th17 responses could also be of importance for understanding lung immunity, as it is now clear that Th17 cells play an important role in the protection against extracellular bacteria (such as *Klebsiella pneumoniae*, *Borrelia burgdorferi*, *Bordetella pertussis*) and fungi, and IL17 has also been implicated in several models of asthma (42-44). In this thesis, we have not performed enough experiments to address the question whether different subsets of lung DCs react differentially to different innate immune triggers.

Research question 4 : Is there a mechanism by which DCs sense interstitial or intravascular antigens in the lung, and what is the outcome of such encounters?

Little is know about the role of DCs in the interstitial compartment of the lung, where the vital gas-exchange takes place. We were able to show for the first time that monocyte-derived iDCs take up vascular encountered embolic peripheral lung particulate antigen and present this to naïve $CD4^+$ T cells in the draining MLNs of the lung (**Chapter 7**). Although maturation of these DCs by stimulating them locally with LPS and CD40L together with OVA antigen, leads to induced inflammation in this model, this is also short lived, favouring the vital gas-exchange. As depletion of pDCs did not change the priming and division of naïve CD4 T cell in draining MLNs in this model (Chapter 7), it is of interest to find out which DC subset or other cells such as macrophages are responsible for tolerance induction in this compartment. One striking observation of this and other models of embolization such as injection of *Schistosoma* soluble antigen-coated or *Mycobacterium* purified protein derivate (PPD) antigen-coated sepharose beads was that

inflammatory lesions around injected embolic material were very short lived (45, 46). One explanation would be that in our model, monocyte-derived $CD11c^+$ DCs would not get the proper activation status to induce full blown effector cells, thus leading to a program of deletional proliferation of T cells. Arguing against this possibility is the fact that injection of CD40 and LPS, two known activators of the DC system (47), enhanced the size of the inflammatory lesions, but eventually these were still cleared from the lung. Also, models in which PPD-coated beads or *Schistosoma* eggs are injected would lead to full DC activation, but still these 'granulomata' are cleared eventually (45). Such a system to clear the lung interstitial tissue of overt inflammatory lesions makes evolutionary sense as this is the site of vital gas exchange (48). Several mechanisms in the lung, such as the suppressive function of the nearby alveolar macrophage with its secretion of anti-inflammatory factors such as TGFβ, IL1-RA, IL-10 and prostaglandins might keep inflammation in check by down regulating the antigen-presenting capacity of DCs (45, 49, 50). Alternatively, early fibrosis occurring around injected beads could effectively shield off the antigen, so that it is further ignored by the immune system eventually leading to apoptosis of T cells (51).

In conclusion, we have provided evidence for a highly active antigen sampling mechanism for embolic material trapped in the small vessels of the lung. It is likely that this system developed during evolution to allow the immune system to effectively recognize large particulate antigens acquiring access to the venous blood by invasion, such as parasite eggs or worms, or following direct (traumatic) access to the bloodstream. When such antigens would be retained in the lungs without a possibility for Ag recognition, this could lead to a very effective way of pathogen immune subversion, as

the spleen or liver, two other major sites of immune induction against blood particulate antigens, cannot access these large antigens. It will be interesting to study whether certain pathogens take advantage of this system by blocking DC migration to the mediastinal nodes. It could also be that allergenic cells disrupted as a group of cells from freshly transplanted vascularized organs are presented via this route, as these vascularized grafts lack a direct connection with afferent lymph and the lung capillaries are the first small vessel filter encountered. Clearly, this highly effective pathway of immune induction in the lung interstitium deserves further study.

General conclusion from this thesis:

A model is emerging whereby the control of tolerance and immunity in the lung is controlled at multiple levels. In baseline conditions, self or harmless antigens are presented by partially mature cDCs leading to an abortive proliferation of Ag specific T cells. At the same time, pDCs suppress the generation of effector function from these dividing T cells. Concomitantly, immature cDCs and pDCs induce the generation of Treg cells capable of suppressing airway inflammation. Under inflammatory conditions such as microbial infection, exposure to environmental sensitizers like cigarette smoke, diesel exhaust particles and ozone, additional subsets of monocyte-derived inflammatory DCs are attracted and these iDCs become effector DCs that express a full array of costimulatory molecules and cytokines, leading to effector Th cells, and releasing the brakes of Treg control (Figure 1). Certain microbial stimuli also activate pDCs so that the latter turn into immunogenic rather than tolerogenic APCs. Activated pDCs, through release of IFNα might even enhance cDC or iDC function. This new conceptual framework encompassing pDCs as tolerogenic cells predicts that the best adjuvants will enhance cDC and/or iDC immunogenic function, and at the same time abolishing pDC function or turning pDCs into mature cells. As a very nice illustration of this new paradigm, cholera toxin, a model mucosal adjuvant depletes pDCs from the lymphoid organs, possibly explaining how it works as an adjuvant. (Kellsal B, Keystone symposium; and (52)). Understanding the precise contribution of these different subsets in the induction of immunity and tolerance in mice and humans will be necessary to find new therapeutic strategies to prevent the development of airway diseases and has implications for mucosal vaccine design.

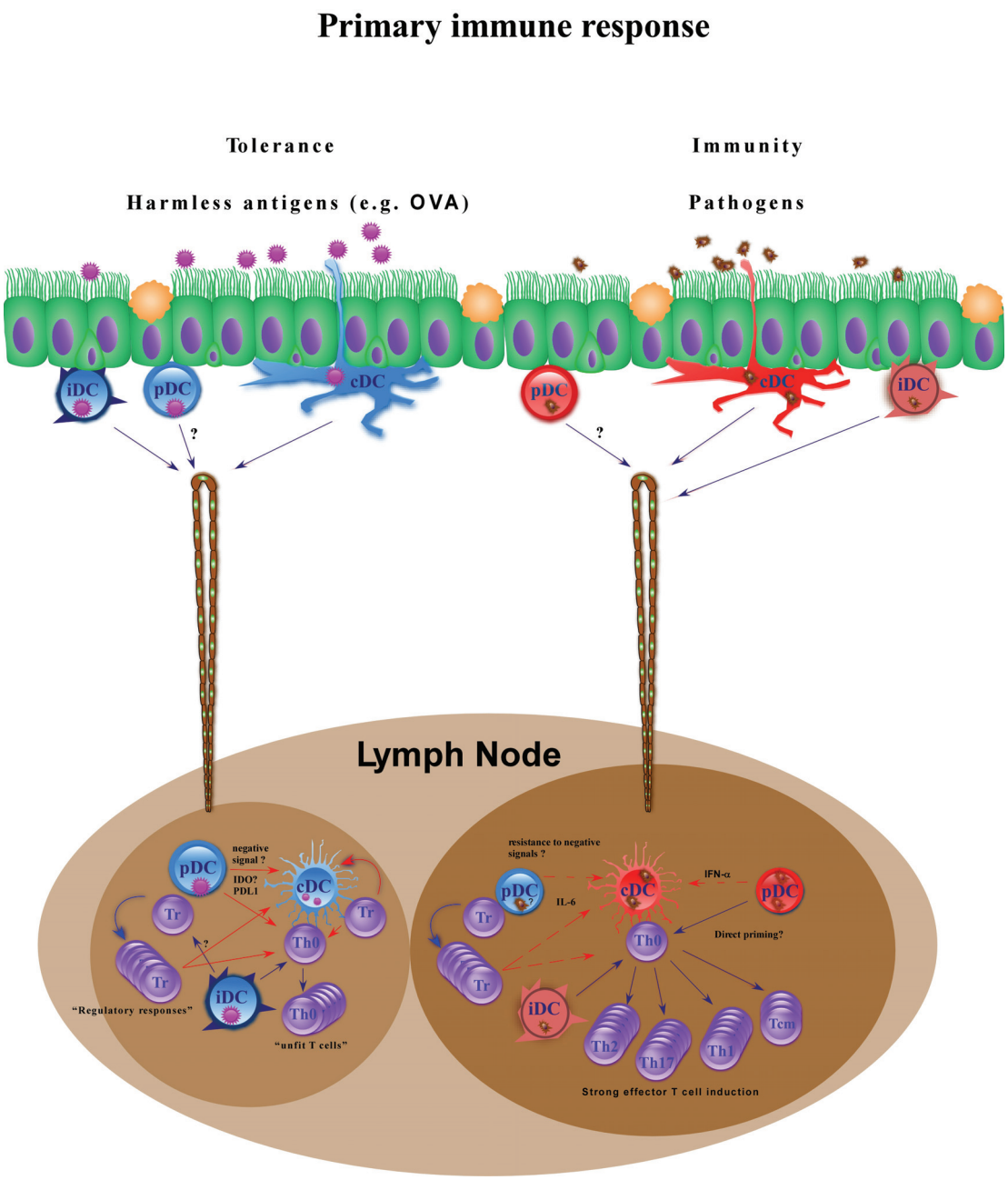

Figure 1. Immune regulation by cDCs and pDCs in the lung. Under steady state conditions (left), in the absence of accompanying danger signals in the lung, inhaled antigens are picked up by cDCs, iDCs and pDCs, which take the antigen (Ag) to the mediastinal lymph nodes. Here, partially mature cDCs induce a short lived boost of division in Ag-specific T cells, but these T cells fail to differentiate into effector cells and die. Some T cells might also differentiate into Treg cells. Plasmacytoid DCs in the draining node influence the generation of T effector cells from dividing T cells, probably by giving negative signals (IDO, PDL-1) to T cells and cDCs. At the same time they also generate Treg cells. Under inflammatory conditions (right), cDCs and iDCs now arrive in the draining node as fully mature cells, resistant to Treg suppression. Ag-specific T cells again undergo proliferation, but this time generating effector cells. On the other hand, pDCs might also acquire a mature phenotype and prime Ag specific T cells to become effector cells as well.

MAIN FINDINGS:

Different dendritic cells subsets such as pDCs, cDCs and iDCs play an important role in tolerance and immunity induction in the lung, whereby the degree of maturation and stimulation of the DC type and location in the lung induce different types of immune responses. This is supported by the following findings described in this thesis:

- pDCs induce tolerance by T regulatory cell induction during primary exposure of the lung with harmless ovalbumin antigen, while cDCs prime and induce differentiation of Th2 cells with cardinal features of asthma in the absence of pDCs (Chapter 3)
- Treatment of iDCs with compounds that alter their state of maturation can abolish cardinal features of astma (Chapters 4 and 5)
- Activation of iDCs with Toll-like receptor (TLR) agonists induces maturation during harmless antigen uptake and distinct type of differentiation of $CD4^+$ naïve T cells by these DCs (Chapters 6 and 7)
- monocyte-derived iDCs take up particulate-antigen in the lung capillaries and induce naïve $CD4^+$ T cell proliferation in draining mediastinal lymph nodes (Chapter 7), leading to only very transient inflammatory lesions in the lung, to protect the delicate gas exchange mechanism of the lung.

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Een belangrijke taak van de longen in een menselijk lichaam is ervoor te zorgen dat rode bloedcellen (erytrocyten) voorzien worden van zuurstof (O_2). De long bestaat uit grote geleidende luchtwegen die vertakken in uiteindelijk kleine luchtwegen en uitlopen in kleine longblaasjes (alveoli). Het hart pompt in een paar minuten al het zuurstofarm bloed dat zich in het lichaam bevindt via de longslagader door de longen. De erytrocyten met zuurstofarm bloed komen via de longslagader bij de alveoli en kunnen daar zuurstof opnemen. Dit kan doordat er slechts een zeer dunne wand is tussen de alveoli waar de ingeademde lucht met zuurstof zich bevindt en de erytrocyten in de haarvaatjes (kleine en zeer dunne bloedvaten, die capillairen worden genoemd). De zuurstofrijke erytrocyten komen via de longvenen terug in het hart, waar ze door de linker kamer het gehele lichaam ingepompt worden via de grote lichaamsslagader (aorta). Zuurstof wordt in het lichaam via een stelsel van capillairen afgegeven aan allerlei cellen (spieren, darmcellen, huidcellen, hersencellen, etc.), die het gebruiken om energie te genereren via verbranding van m.n. suiker (glucose). Naast het opnemen van zuurstof, geven erytrocyten afvalproducten af, in de vorm van kooldioxide (CO_2), die bij de energieverbranding vrijkomen uit de cellen, in dezelfde capillairen van de long. Deze CO_2 ademen wij uit. Deze gasuitwisseling van zuurstof en kooldioxide is een van de belangrijkste functies van de long.
Omdat het bloed in de capillairen van de long zo dicht in aanraking komt met de buitenlucht, die naast gassen ook allerlei andere stoffen, partikels en ziekteverwekkers (pathogenen) als virussen kan bevatten, heeft het lichaam gedurende de evolutie allerlei beschermingsmechanismen ontwikkeld. Allereerst ademen wij meestal door de neus, hetgeen er voor zorgt dat lucht wordt opgewarmd en dat vele kleine deeltjes neerslaan in de keel. Deze deeltjes slikken wij in en komen via de maag (waar het maagzuur vele indringers doodt) in het spijsverteringskanaal terecht. Verder zorgt slijmproductie (sputum) tezamen met een trilhaarsysteem, die zich in de geleidende luchtwegen bevinden, ervoor dat de meeste deeltjes en ziekteverwekkers neerslaan, voordat deze de alveoli kunnen bereiken. De trilhaartjes bewegen het slijm naar de keel waar het doorgeslikt kan worden en weer in de maag terecht komt.
Daarnaast is er in de luchtwegen en long een uitgebreid netwerk van afweercellen en stoffen, die ervoor zorgt dat pathogenen (bijv. bacteriën en virussen) direct worden opgeruimd of als dat niet meteen lukt door specifieke cellen van het afweersysteem worden aangevallen en verwijderd.
De eerste lijn van afweercellen die pathogenen opruimen, behoort tot de natuurlijke afweer (innate immunity). Tot deze afweercellen behoren o.a. macrofagen (letterlijk: grote eters). Deze kunnen direct bacteriën opnemen (fagocyteren) en daarna doden. Een tweede lijn bestaat uit een specifiek afweersysteem (specific immunity) van cellen. Een belangrijke cel van dit systeem is de dendritische cel (DC, meervoud DCs). DCs bevinden zich in het gehele lichaam. Wat doen DCs? Als u gevaccineerd wordt komen via een injectie in de huid en spier meestal dode deeltjes (antigenen) bacterie of virus (afhankelijk van het type vaccinatie) in het lichaam. Op de plaats waar ingespoten wordt zitten DCs of worden door de reactie die ontstaat, aangetrokken. DCs nemen de deeltjes in hun cel op en migreren dan via lymfevaten naar een lokale lymfeklier. De lymfeklier is te beschouwen als de vergaderzaal van het specifieke immuunsysteem. Hier presenteert de DC stukjes van de opgenomen deeltjes op zijn celoppervlak aan m.n. T cellen die het specifieke stukje (bijvoorbeeld) bacterie kunnen herkennen. De T cellen gaan zich delen

(zo hard soms dat de lymfeklier/knoop opzet) en kunnen bijvoorbeeld B cellen aanzetten tot het maken van specifieke antistoffen. Ook kunnen sommige specifieke T cellen, door vanuit de lymfeklier te gaan naar de plaats van infectie in het lichaam, daar besmette cellen doden. Bijna alle typen infectie kunnen zo herkend worden, wat de afweerreactie specifiek maakt (er worden specifieke antistoffen en T cellen ontwikkeld). Daarnaast blijven er in het lichaam geheugencellen (memory B en T cellen) die, wanneer er in het lichaam een zelfde infectie met bijvoorbeeld een specifieke bacterie plaatsvindt, er voor zorgen dat deze sneller (in kortere tijd) en efficiënter wordt opgeruimd. De dirigent van deze specifieke afweer is de DC.
In het lichaam bevinden zich verschillende soorten van DCs die een eigen functie en naam hebben. De conventionele DC (cDC) bevindt zich onder vaste (rustige) omstandigheden in centrale lymfoide organen (lymfeklieren/milt) en in sommige perifere organen als de long, darm en huid. De plasmacytoide DC (pDC) produceert grote hoeveelheden interferon-α (wat cellen beschermt tegen virus infectie), maar kan ook onder bepaalde condities antigen presenteren en daarmee T cellen stimuleren. De inflammatoire DCs (iDCs) zijn afkomstig van monocytaire (monocyten) voorlopers uit het bloed gedurende met name inflammatoire (ontstekings-) reacties.
In dit proefschrift hebben wij onderzocht wat de rol is van deze verschillende DC subgroepen in de long. Hierbij hebben wij gekeken naar de invloed van maturatie en stimulatie en het verschil tussen DC subgroepen in verschillende anatomische lokaties van de long gedurende ontsteking en tolerantie inductie. Hiervoor hebben wij verschillende muismodellen voor astma en vaatziekten van de long gebruikt, als ook verschillende stoffen die van invloed zijn op DC stimulatie en maturatie zoals beschreven in hoofdstuk 1.
Hoofdstuk 2 geeft een overzicht van de literatuur over de betrokkenheid van DC subgroepen in immuunregulatie van de long gedurende normale en inflammatoire condities. De twee subgroepen van cDCs en pDCs spelen tijdens normale (rustige) omstandigheden een specifieke rol in de immuunreactie van de luchtwegen. Tijdens inflammatoire reacties kunnen monocyten uit het bloed iDCs genereren die in de long komen. De rol van pDCs in tolerantie inductie en cDCs en iDCs in inflammatie inductie werd voornamelijk in het muismodel voor astma bestudeerd. Verder werden therapeutische interventies, gebruik makende van de DC subgroepen, toegelicht.
In hoofdstuk 3 onderzochten wij de capaciteit van pDCs om eigenschappen van astma te voorkomen gedurende onschadelijke inademing van antigen. Wij vonden voor het eerst de aanwezigheid van pDCs in de long. Door deze pDCs te depleteren (weg te halen) tijdens het geven van onschadelijk kippeneiwit (Ovalbumin, OVA), vonden wij dat muizen kenmerken van astma ontwikkelden tijdens blootstelling 10 dagen later aan OVA via de luchtwegen. Verder vonden wij dat in een muizenmodel voor astma, het injecteren van met OVA geladen pDC voor de blootstelling aan OVA 10 dagen later, astmatische reacties kon voorkomen. Wij vonden dat het vermoedelijk mechanisme waarmee pDCs astma voorkomen, veroorzaakt wordt doordat pDCs (OVA) specifieke T regulatoroire (Tregs) cellen kunnen aanzetten die de immuunreactie onderdrukken en aldus tolerantie induceren. In Hoofdstuk 4 onderzochten wij de rol van een peroxisoom proliferatie-activatie receptor-γ (PPAR-γ) op DCs in een muismodel voor astma. iDCs, gekweekt uit voorlopers uit het beenmerg, kunnen worden opgeladen met OVA. Zoals in hoofdstuk 1 beschreven kunnen deze OVA-iDCs astmatische reacties induceren wanneer zij gegeven

worden in de longen van muizen als deze 10 dagen later weer via de luchtwegen blootgesteld worden aan OVA. Wij vonden dat als deze OVA-iDCs behandeld worden met PPAR-γ astmatische reacties voorkomen konden worden. Wij toonden ook aan dat deze PPAR-γ behandelde OVA-iDCs in staat waren om IL-10 producerende Tregs te induceren, welke bekend staan om hun tolerantie inducerende kwaliteiten. Door de IL-10 productie te blokkeren met antistoffen zagen wij dat de astmareacties terugkwamen in muizen behandeld met PPAR-γ/OVA-iDCs.

In hoofdstuk 5 bestudeerden wij de rol van Prostaglandine D_2 (PGD_2) in DC migratie gedurende rustige condities (steady state conditions) in de long. Gedurende deze regelmatige normale condities kunnen onschadelijke stoffen als OVA worden opgenomen door luchtweg cDCs en gepresenteerd aan naïve $CD4^+$ T cellen in drainerende lymfeklieren in het mediastinum (ruimte tussen de longen). Wij vonden dat het geven van PGD_2 en OVA in de luchtwegen van muizen de migratie van de cDCs naar deze lymfeklieren verminderde. Waarbij de stimulatie en productie van inflammatoire $CD4^+$ T cellen in deze lymfeklieren ook verminderd was t.o.v. het geven van alleen OVA. In een latere studie door Hamida Hammad et al. bleek dat deze cDCs specifieke Tregs kunnen induceren die astmatische reacties in het muizenmodel voor astma kunnen onderdrukken.

In Hoofdstuk 6 bestudeerden wij het effect van bacteriële peptidoglycaan (PGN) op iDC maturatie. Hierbij bleek dat het geven van PGN tezamen met OVA aan iDCs, gekweekt uit het beenmerg, leidt tot een hogere opname van OVA en inductie van specifieke inflammatoire T cellen dan iDCs die alleen OVA krijgen. Dit is van belang omdat in onze studie PGN, gegeven in combinatie met myeline eiwit (hetgeen om zenuwen in zenuwschede zit), encefalitis kan induceren in een experimenteel autoimmuun muizenmodel (EAE). Recent is aangetoond dat deze vorming van EAE afhankelijk is van een subgroep van T cellen, zogeheten T helper (Th) 17 cellen. Deze Th17 cellen zijn ook van groot belang voor de afweer tegen allerlei bacterie infecties in de long (zie ook hoofdstuk 2).

In Hoofdstuk 7 hebben wij de rol van DCs in de perifere longruimte bestudeerd door het geven van in verhouding grote ronde bolletjes (beads), opgeladen met OVA, in de ader van een muis. Deze OVA-beads lopen vast in de kleinere slagaders (arteriolen) van de long dichtbij de alveolaire ruimte waar de gasuitwisseling plaatsvindt. Wij vonden dat DCs afkomstig uit monocyten (iDCs) uit het bloed de OVA kunnen opnemen en presenteren aan T cellen in drainerende mediastinale lymfeklieren. De inflammatie die rond de beads ontstaat kon voorkomen worden door DCs specifiek te depleteren. Waarbij het teruggeven van OVA-iDCs (gekweekt uit het beenmerg) in dit model de inflammatie weer kon induceren. Verder toonden wij aan dat stoffen, die DCs stimuleren tot maturatie (LPS en CD40L), gekoppeld aan beads naast de OVA, leiden tot grotere en langer durende inflammatie rond de beads ten opzichte van alleen OVA-beads. Opmerkelijk hierbij is dat de inflammatie toch redelijk snel verdwijnt (na zo'n twee weken) ten behoeve van de van vitaal belang zijnde gasuitwisseling.

In hoofdstuk 8 komen wij ten slotte tot de volgende conclusies wat betreft de rol van verschillende DC subgroepen in de long:

- pDCs induceren tolerantie door Treg inductie tijdens primaire expositie van de long aan onschadelijk ovalbumine antigen, terwijl cDCs Th2 cellen stimuleren

en aanzetten, die belangrijke eigenschappen van astma in de afwezigheid van pDCs kunnen induceren (Hoofdstuk 3)

- Behandeling van iDCs met stoffen die hun staat van maturatie veranderen, kunnen typische kenmerken van astma voorkomen (Hoofdstukken 4 en 5)
- Behandeling van iDCs met Toll-like receptor (TLR) agonisten induceert maturatie gedurende onschadelijke antigen opname en typische differentiatie van naïve $CD4^+$ T cellen door deze DCs (Hoofdstukken 6 en 7)
- iDCs gevormd uit monocyten nemen antigen van partikels op in de longcapillairen en induceren naïve $CD4^+$ T cel proliferatie in drainerende mediastinale lymfeklieren (Hoofdstuk 7), wat leidt tot een tijdelijke inflammatie laesies in de long, om de gevoelige gasuitwisseling aldaar te beschermen.

Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cell
BALF	Broncho-alveolar Lavage Fluid
BHR	Bronchial Hyperreactivity
BM	Bone marrow
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CFSE	Carboxy Fluorescein Diacetate Succinimidylester
d	Day
DC	Dendritic cell
DT	Diphteria Toxin
DTH	delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flueorescence Activated Cell Scanner
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FLT-3L	Flt-3 ligand
FSC	Forward Scatter
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
i.t.	intratracheal
i.v.	intravenous
GM-CSF	granulocyte monocyte colony stimulating factor
LPS	Lipopolysaccharide
LN	Lymph Node
mAb	Monoclonal Antibody
MHC	Major histocompatibility complex
MLN	Mediastinal lymph node
NOD	Nucleotide-binding oligomerization domain
nTg	Non-transgenic
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECy5	Phycoerythrin-cychrome 5
PGD	Prostaglandin D
PPD	Purified protein derivative
RSG	Rosiglitazone

SD	Standard deviation
SEM	Standard Error of the Mean
SSC	Side scatter
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T Helper
TLR	Toll like receptor
Treg	Regulatory T
WT	Wild type

Dankwoord

Nu u aan het einde gekomen bent van de dingen beschreven in dit proefschrift, wil ik u hier graag laten delen in de grote schare van mensen die mij hebben geholpen en gesteund om dit alles tot stand te brengen. Zonder hun hulp en aanmoedigingen was het nooit gelukt.
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Hendrik Jan

P.S.: Geniet van het leven maar bovenal van elkaar.

Curriculum Vitae

De auteur werd geboren op 23 januari 1970 te Spijkenisse. Na het behalen van het V.W.O. diploma in 1991, vervulde hij in 1992/1993 zijn militaire dienstplicht. Hierna ving hij in 1993 aan met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens deze studie werd (afstudeer-) onderzoek gedaan op de afdeling Immunologie van de Erasmus Universiteit. Na het afleggen van het artsexamen in 2001, werkte hij als agio op de afdeling longziekten (hoofd prof.dr. H.C. Hoogsteden), waarna van 2002 tot en met 2004 zijn promotieonderzoek in het laboratorium van de longziekten onder leiding van prof. dr. B.N. Lambrecht plaatsvond. De vooropleiding Interne Geneeskunde vond plaats in het Albert Schweitzer ziekenhuis te Dordrecht (opleider dr. A.C.M. van Vliet) in 2005 en 2006. Vanaf zomer 2008 is de auteur werkzaam geweest bij de Alarmcentrale (Eurocross) te Noordwijk en vanaf september 2008 is hij werkzaam als arts psychiatrie bij de Bavo Europoort te Rotterdam.

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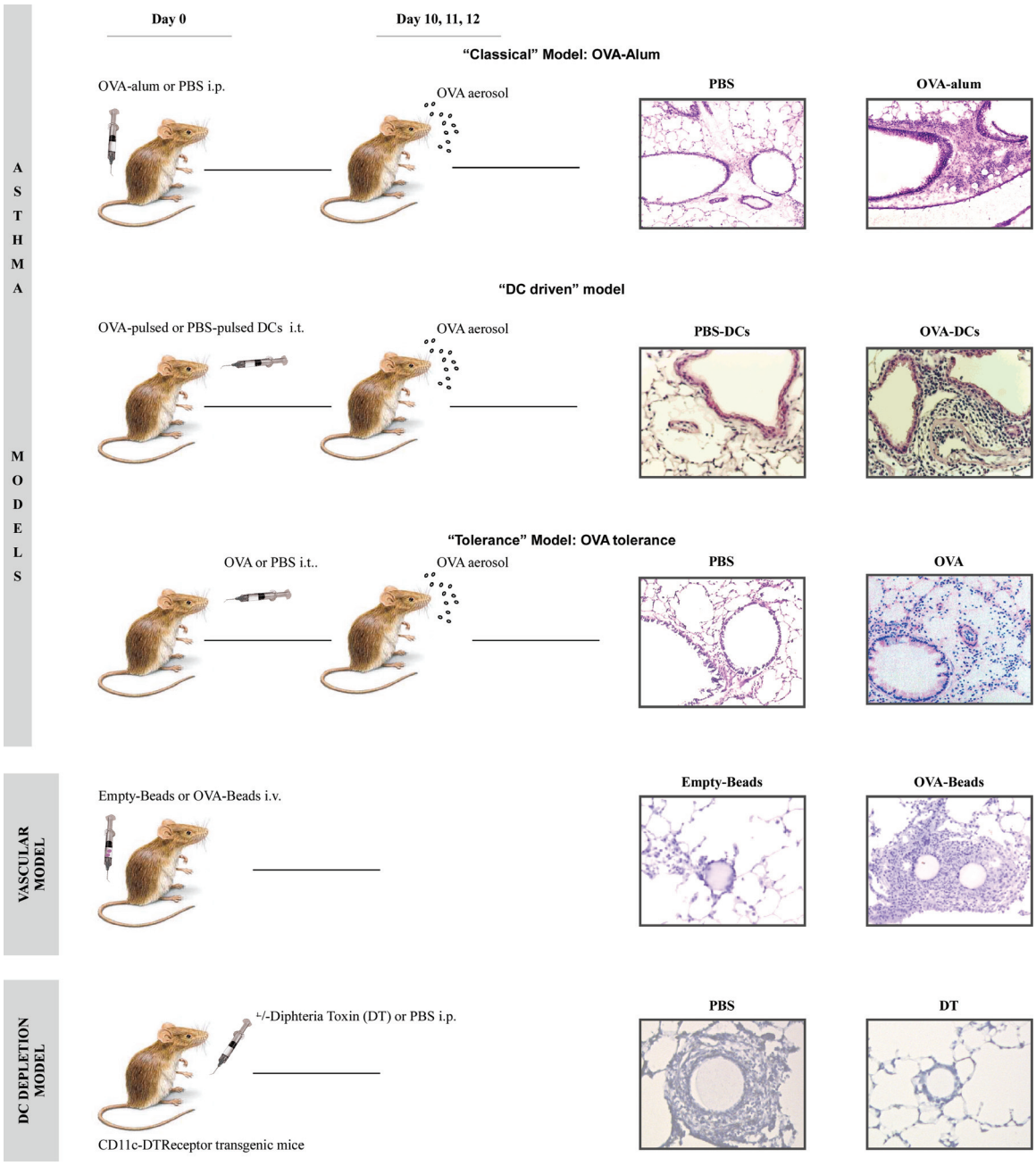
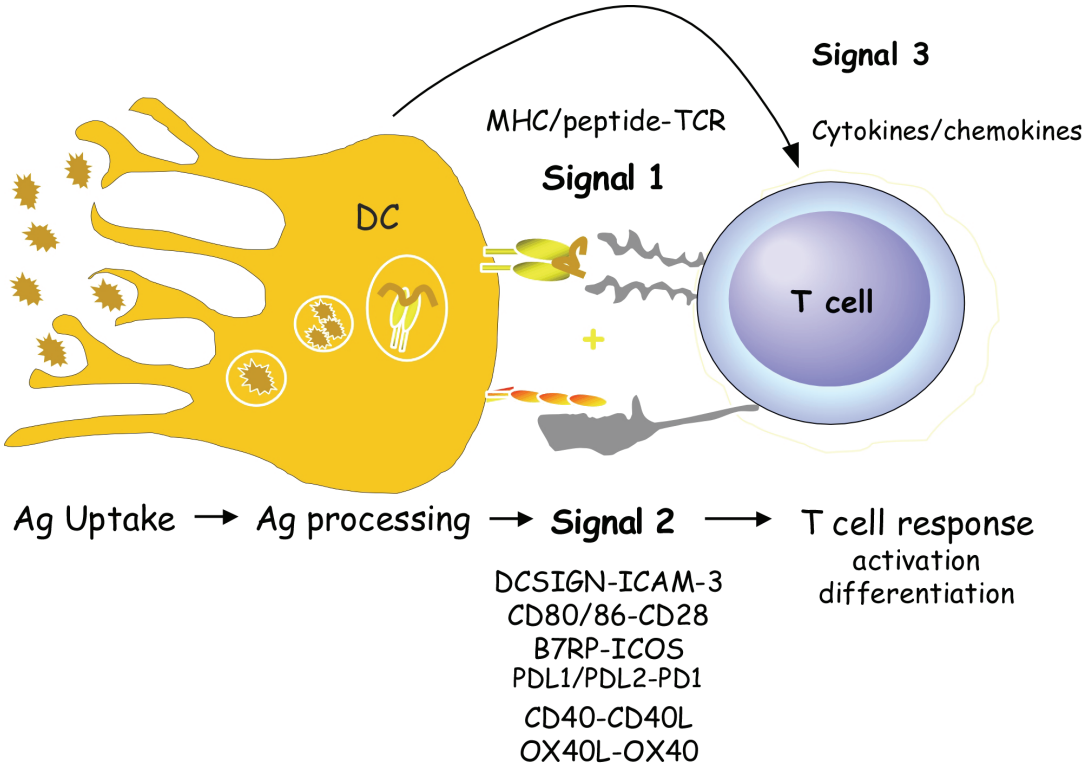
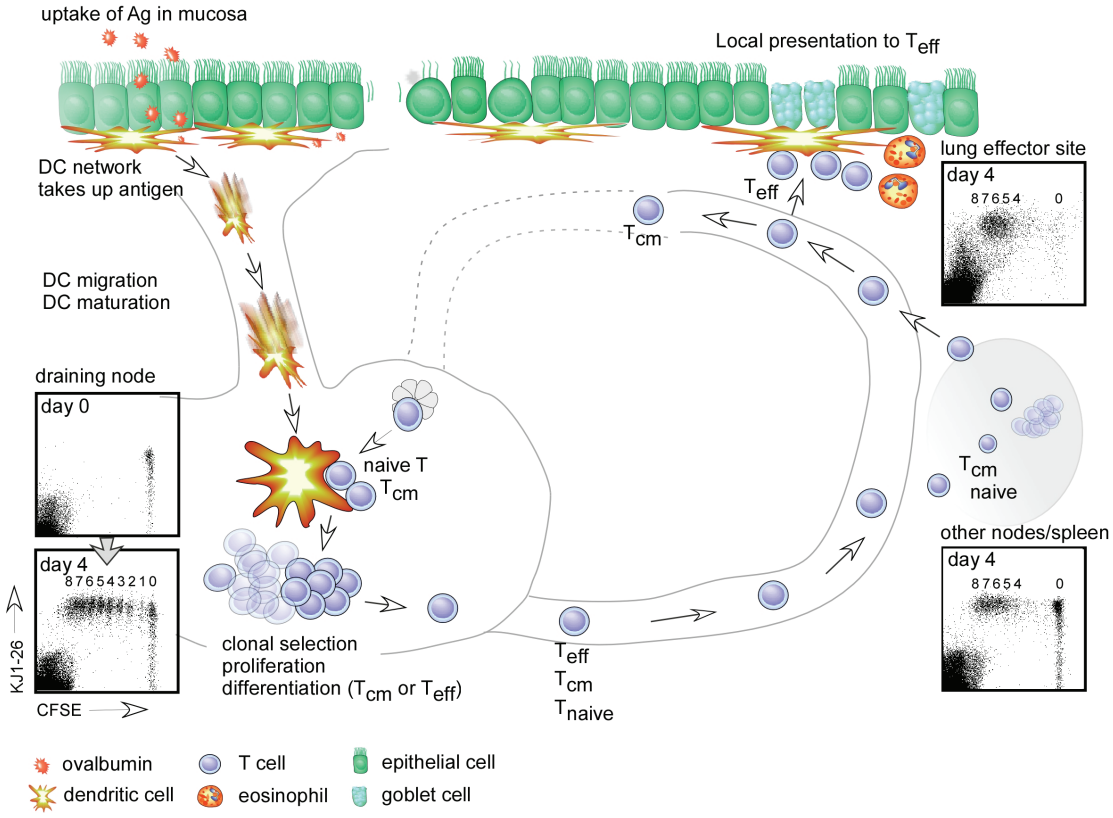
Color Figures



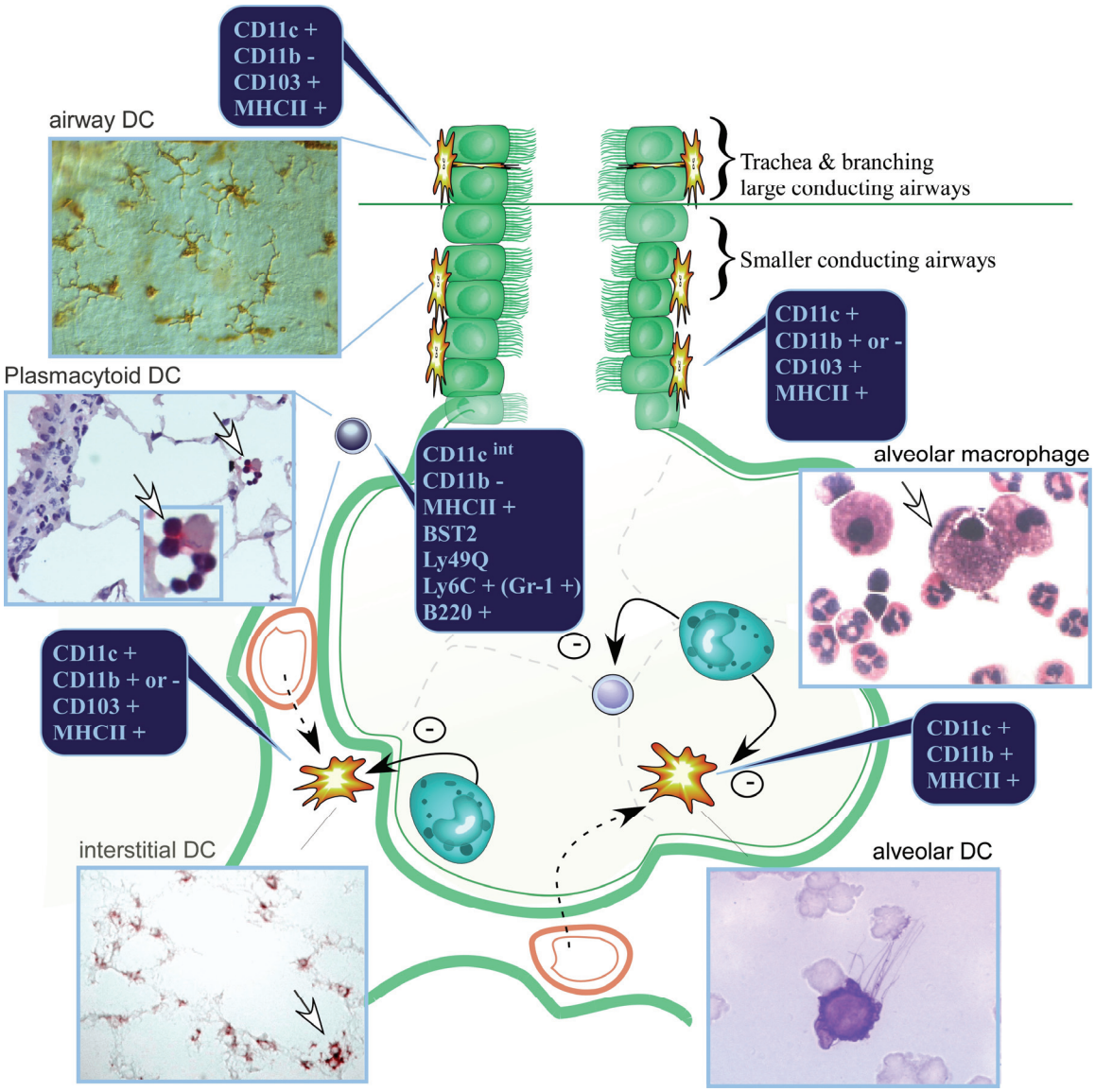
Chapter 1, figure 1



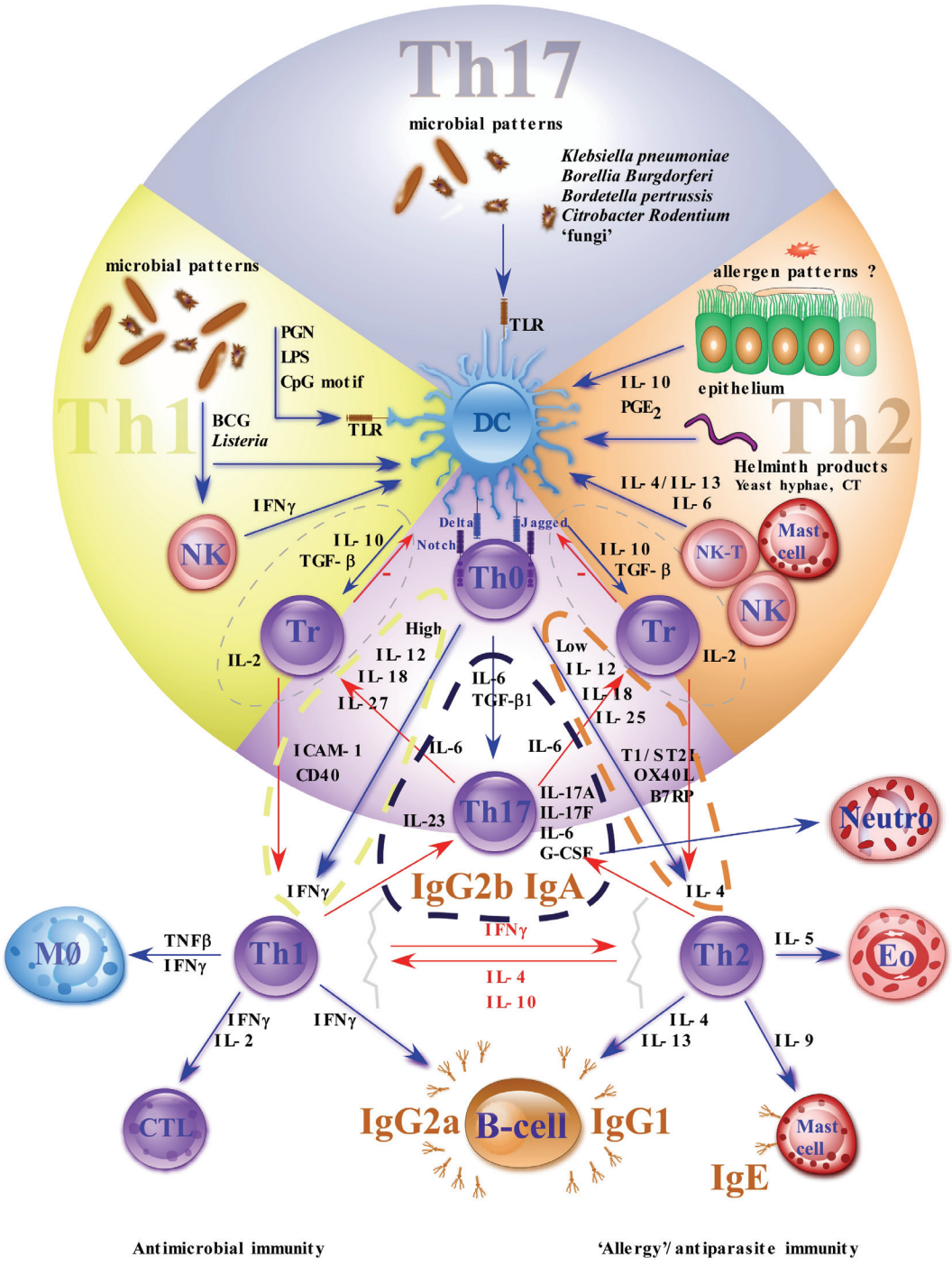
Chapter 2, figure 1



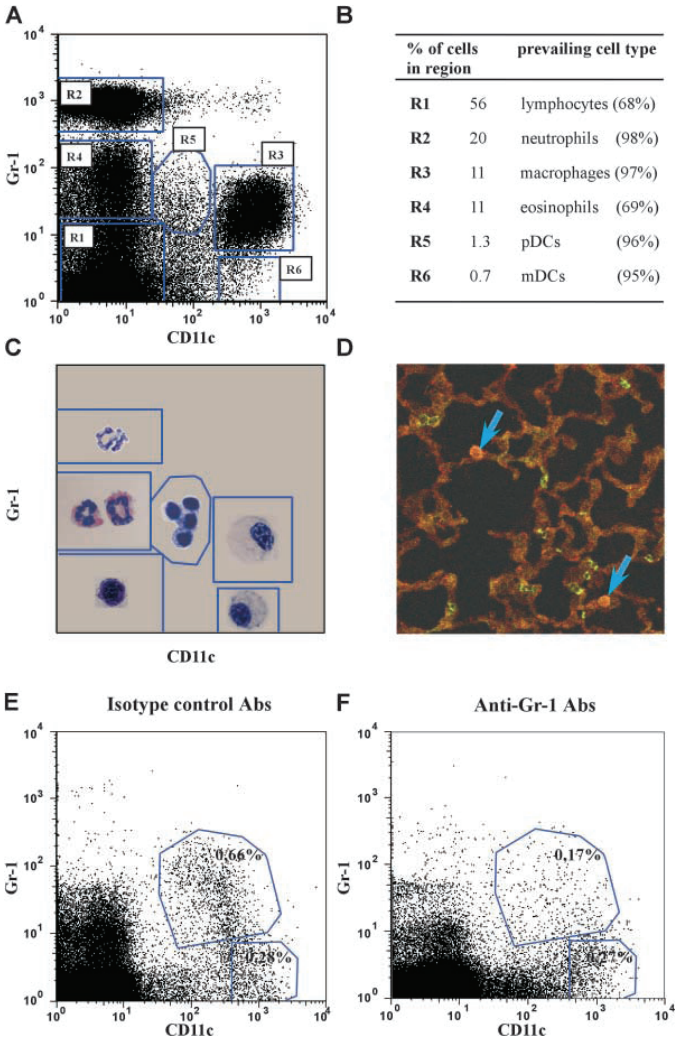
Chapter 2, figure 3



Chapter 2, figure 2

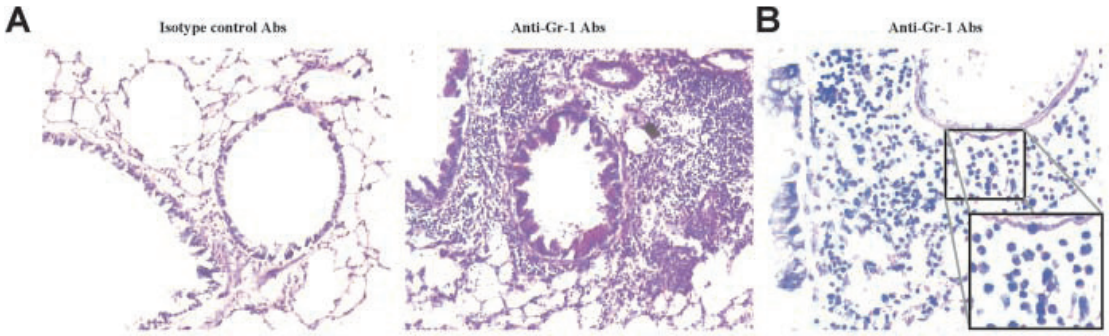


Chapter 2, figure 4

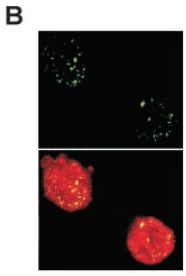


% of cells in region		prevailing cell type
R1	56	lymphocytes (68%)
R2	20	neutrophils (98%)
R3	11	macrophages (97%)
R4	11	eosinophils (69%)
R5	1.3	pDCs (96%)
R6	0.7	mDCs (95%)

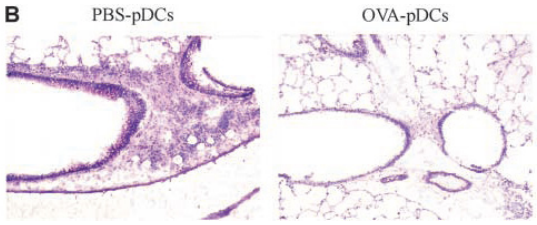
Chapter 3, figure 1



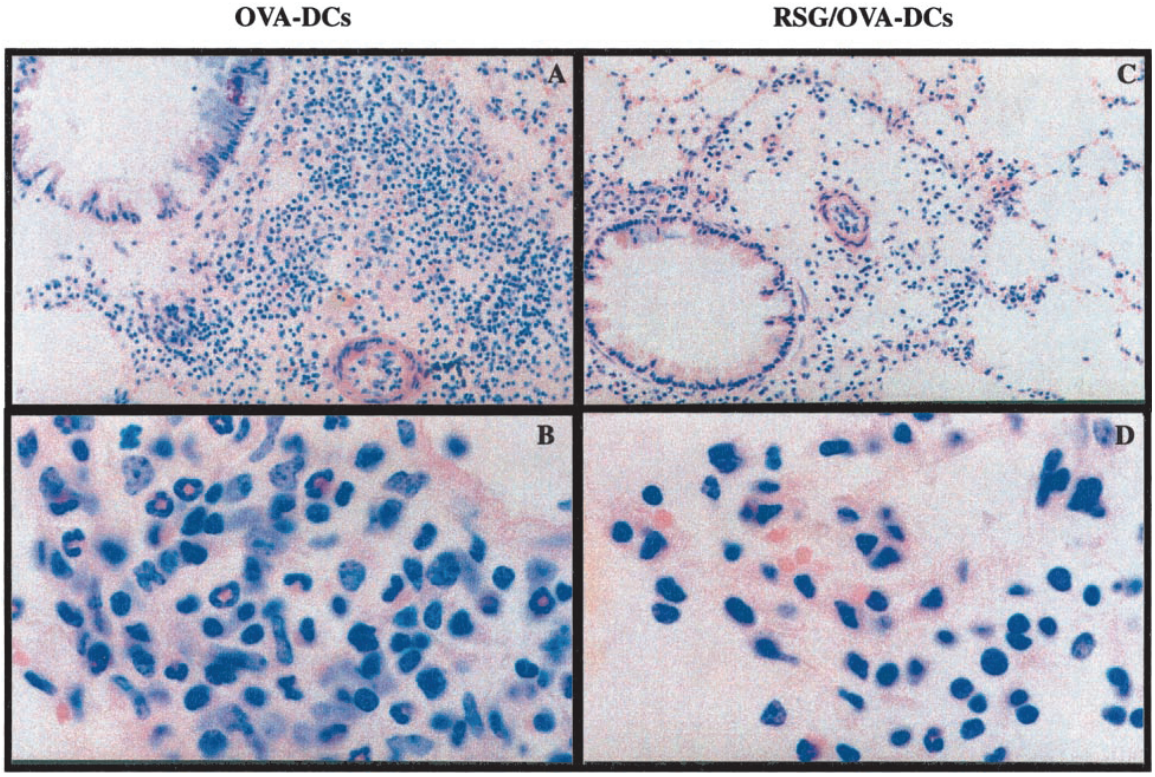
Chapter 3, figure 2



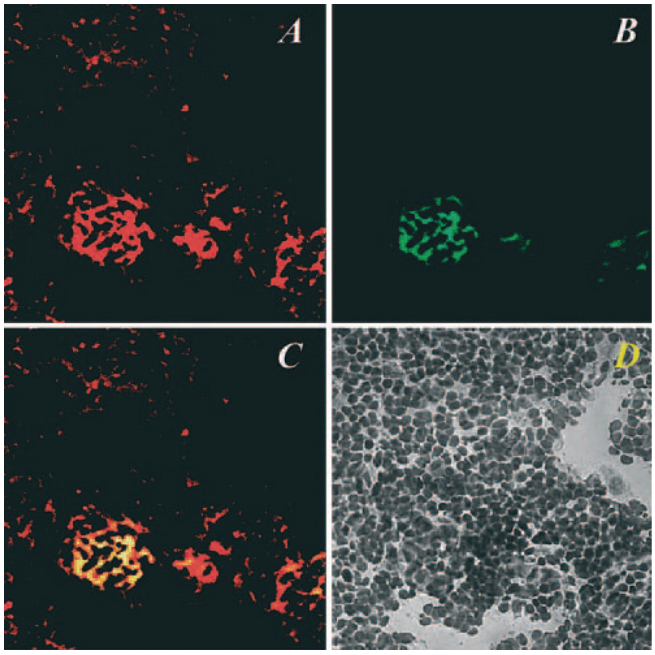
Chapter 3, figure 3



Chapter 3, figure 5

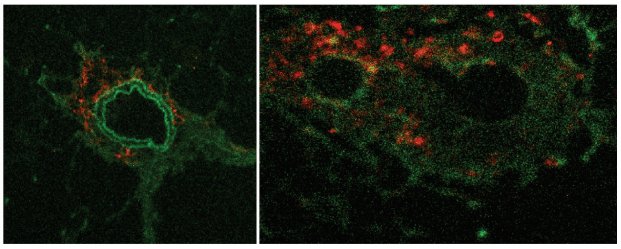


Chapter 4, figure 7

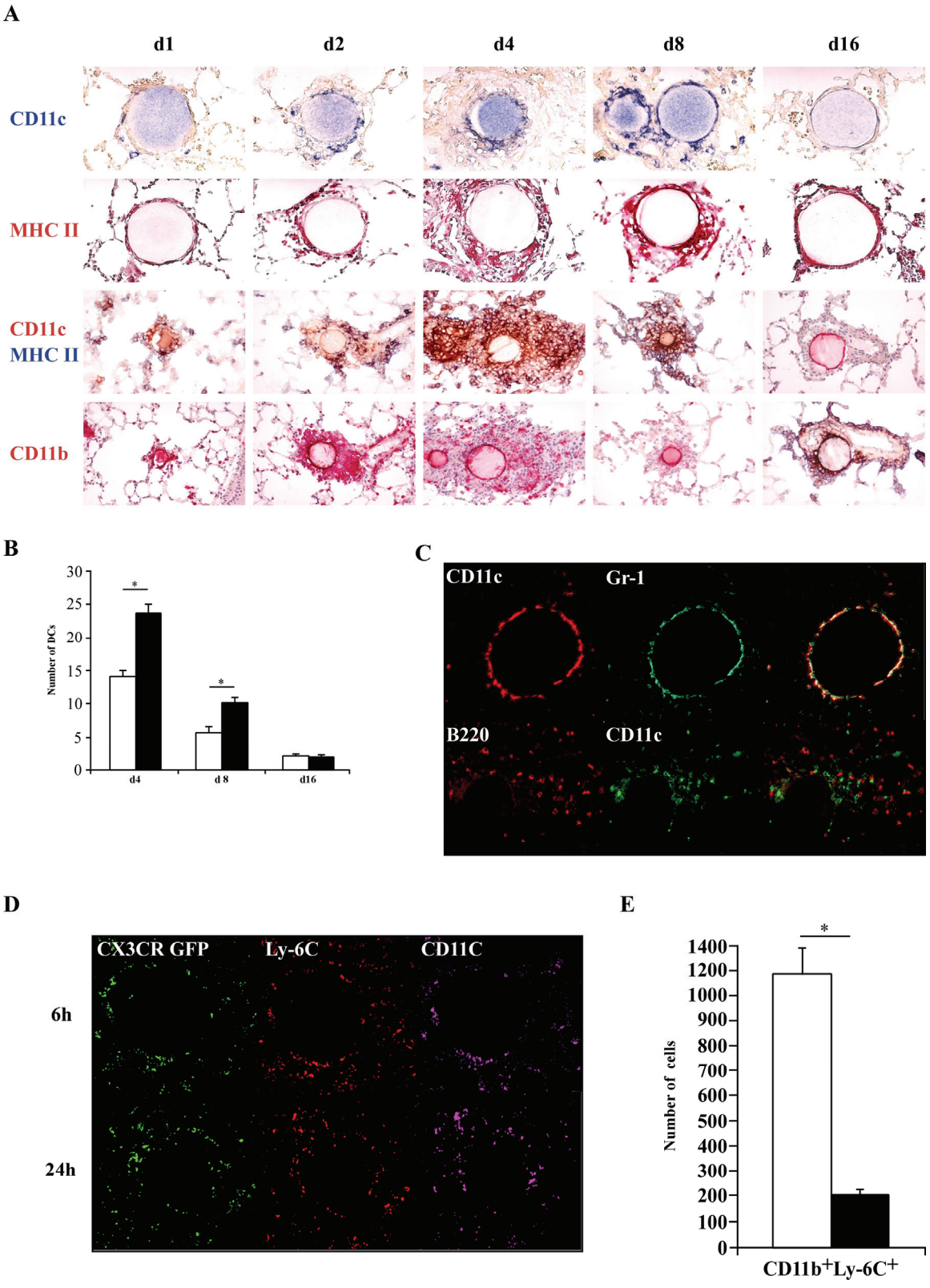


Chapter 6, figure 4

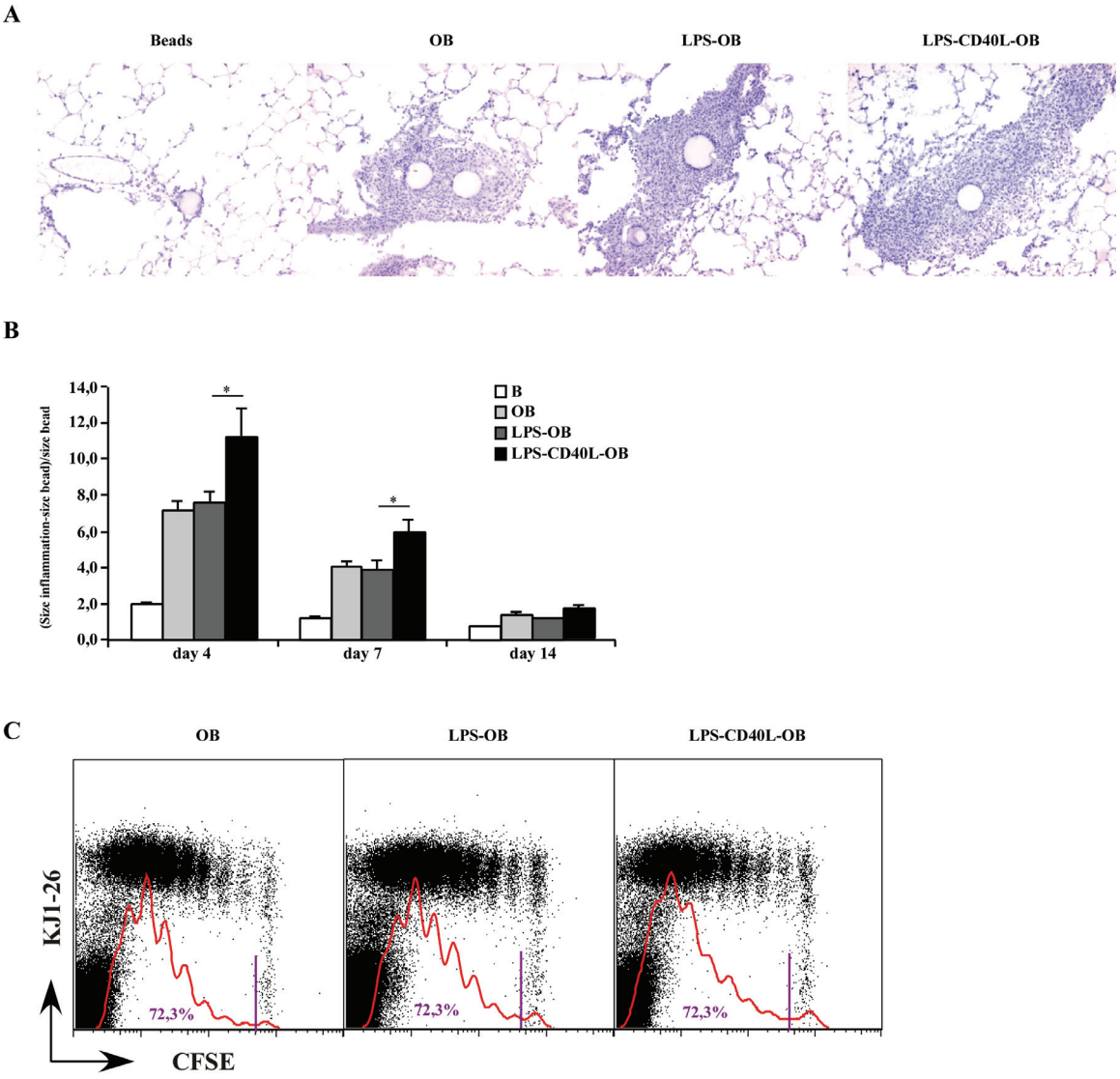
D



Chapter 7, figure 1

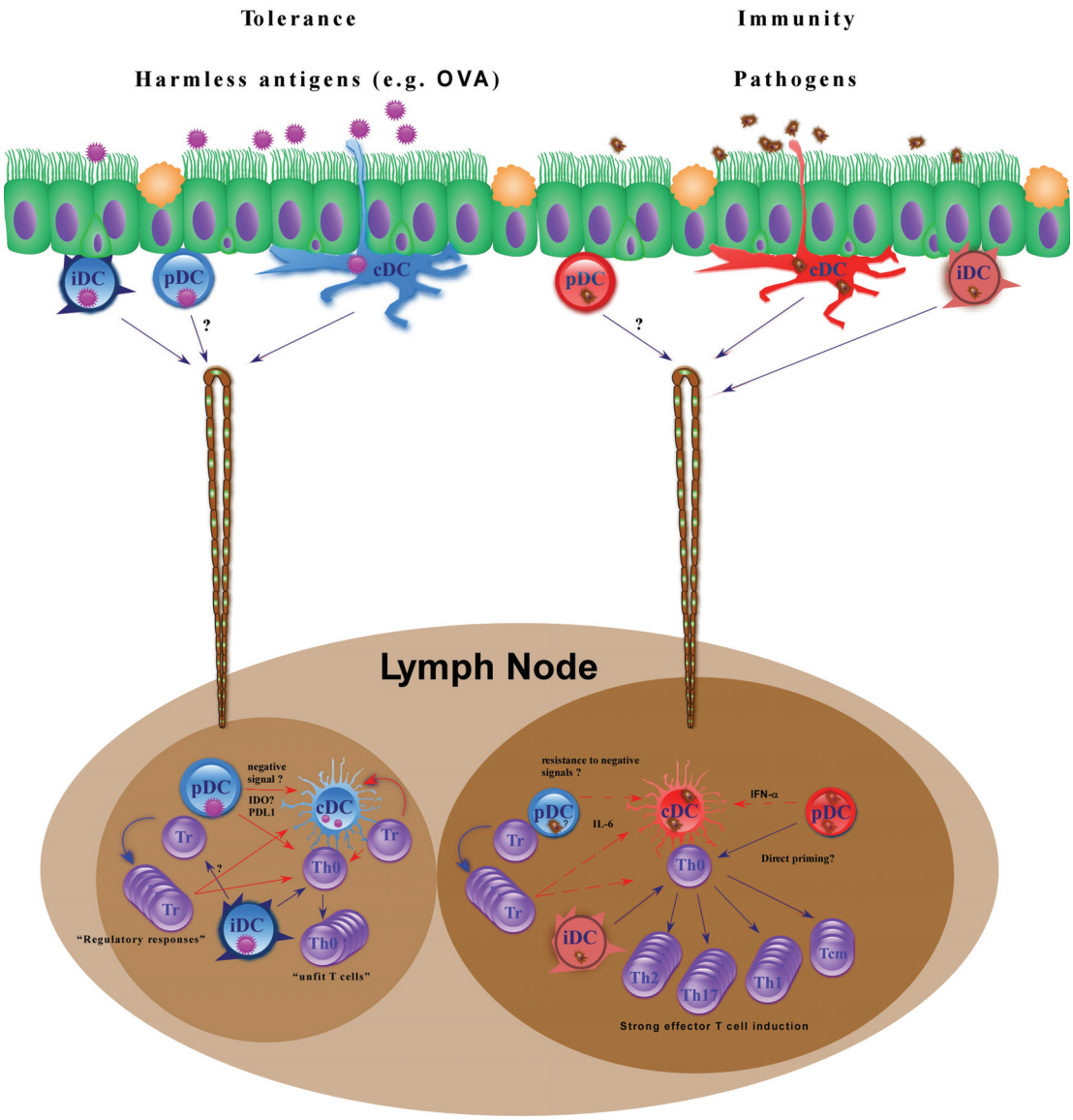


Chapter 7, figure 2



Chapter 7, figure 4

Primary immune response



Chapter 8, figure 1