Dendritic cells: Cause and Cure of Asthma?

A feasibility study of dendritic cell based
asthma immunotherapy in mice

Harmjan Kuipers

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Dendritische cellen: reden en redding voor astma?

Een haalbaarheidsstudie in muizen naar het gebruik van
dendritische cellen als immunotherapie voor astma

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Introduction

1 Dendritic cells

Thirty years after their modern description [1], dendritic cells (DCs) have emerged as the principal cell type involved in naïve T cell activation and differentiation, effectively representing the controlling arm of the immune system [2]. DCs are bone marrow-derived leukocytes that are sparsely distributed in peripheral tissues. The appreciation for the central role of this cell type in initiation and direction of adaptive immune responses was unraveled by some key discoveries in DC biology. First, it was recognized that DCs roughly exist in two states; a resting state in the peripheral tissues, that continuously samples the environment for pathogens, and an activated cell state in the draining lymph nodes (LNs), optimal to activate lymphocytes. Once DCs are activated they downregulate their antigen uptake and processing machinery, thereby 'fixing' the antigen repertoire from the pathogen on their cell surface, which in turn ensures antigen presentation in the lymph nodes that faithfully represents the antigen repertoire derived from the site of antigen acquisition.
A major breakthrough was the identification of toll-like receptors (TLRs) as a major group of pattern recognition receptors (PRRs) which are responsible for pathogen recognition by DCs [3, 4], which will be discussed in more detail below.
Secondly, with the realization of the central role of DCs in regulation of the adaptive immune responses came the insight that DCs are also an important element in the maintenance of peripheral tolerance. It is currently believed that presentation of self-antigen by resting DCs may foster anergy or apoptosis of autoreactive T cells. In addition, these immature DCs may also promote the expansion and/or functionality of regulatory T cells [5-7].
Third, it should be mentioned that these advances in DC biology may not have been possible without the simultaneous development of techniques to generate large amounts of these otherwise rare cells [8-11].

1.1 DC subsets

Since the first description of DCs by Steinman and colleagues [1], a bewildering number of DC subsets have been described in humans as well as mouse [12, 13]. Several factors have added to the complexity of this field. First, considerable heterogeneity among different studies exist in the antibody panels used to characterize DC subsets, especially in the mouse where the ready availability of tissues and reagents has led to numerous studies into this subject. In humans, the lack of expression of CD8α by any human DC subset makes comparison with mouse DC subsets difficult [12]. Furthermore, it is still a matter of debate whether the different phenotypes described represent different activation states or that the distinguishable DCs subsets represent separate developmental lineages [14-18].

1.1.1 Mature murine DC subsets

Despite the use of different antibody panels, several common markers have been used in most studies so far, enabling some comparison between the described subsets and has led to the identification of six distinct subsets[12].
Five subsets express high levels of CD11c, three of which are present in the

spleen and can be subdivided into $CD8\alpha^+CD4^-CD11b^-CD205^+$ lymphoid DCs, $CD8\alpha^-CD4^+CD11b^+CD205^-$ myeloid DCs and another subset of myeloid DCs which is $CD8\alpha^-CD4^-CD11b^+CD205^-$ (also referred to as tissue interstitial DC). Two additional populations are present in lymph nodes. A $CD8\alpha^-CD4^-CD11b^+$ subset that has upregulated CD205, believed to be the mature form of the tissue interstitial DC, and only in skin-draining LNs, $CD8\alpha^{low}CD4^{low}CD11b^+$ DCs expressing langerin, which are believed to be the mature form of Langerhans cells [19, 20].
The DC subset expressing low to intermediate levels of CD11c and MHC class II and in addition stains positive for Ly-6G/C (GR-1) and B220 is called the plasmacytoid DC and was only recently describecd in the mouse [21].
Finally, several in vitro culture methods have been described to generate DCs from murine bone marrow, using GM-CSF and/or Flt-3L as the major differentiation stimuli [8, 9, 22].

1.1.2 Mature human DCs subsets

Compared to mouse DC studies, relatively few studies have been performed on human DCs freshly isolated from tissues. Therefore, most of the data acquired about human DC subsets is derived from several in vitro culture systems [12]. Four different mature subsets have been identified so far. Analogous to the murine homologue, langerhans DC have been described, expressing in addition to CD11c also langerin, E-cadherin and contain Birbeck granules in their cytoplasm. Interstitial, or 'dermal' DC, are a second lineage which are $CD11c^+$, and can be further identified by CD68 and the coagulation factor XIIIa. Blood monocytes are the precursor cells for monocyte-derived DCs, termed DC1, and are generated during a six-day culture period in the presence of GM-CSF and IL-4 [11]. These cells are positive for CD11c and upon maturation induced by proinflammatory cytokines such as TNF-α or microbial products (e.g. LPS), acquire a $CD14^-$, $CD83^+$, $CD86^+$, $MHCII^{hi}$ phenotype. The final subset described in humans is the plasmacytoid subset, termed pDC2, which is characterized by low levels of CD11c expression and the only DC subset to known to express IL-3Rα (CD123) [23, 24]. Recently, antibodies against certain proteins present on the cell surface of DCs subsets have been raised, with BDCA-2 appearing to be highly specific for plasmacytoid DCs [25].

1.2 DC activation, migration and T cell activation

In all nonlymphoid tissues, DCs are in a immature state (alternatively named 'resting' or quiescent) and continuously sample the environment via three major mechanisms; receptor-mediated endocytosis, macropinocytosis and phagocytosis. Receptor-mediated uptake is accomplished via a myriad of receptors, including C-type lectin receptors (such as langerin, DC-SIGN, dectin, DEC-205) or the mannose receptor for glycoproteins, and Fc-receptors for immune complexes [26, 27].
A key aspect of DCs is their migration towards draining lymph nodes upon activation by pathogen derived signals. This activation process compromises a highly coordinate series of steps, including upregulation of costimulatory molecules, a switch in chemokine receptor expression, secretion of cytokines and chemokines and increased expression of antigen-loaded MHC molecules on the cell surface.

The importance of these innate immune system signals for directing adaptive immunity was first proposed by Charles Janeway, in an article proposing a hypothesis for the need to use adjuvants to get effective immune responses [28]. It was subsequently shown that Toll-like receptors (TLRs) were a major group of receptors responsible for sensing pathogens (in the form of pathogen associated molecular patterns (PAMPs)) in the environment and initiating the adaptive immune response [29, 30]. The TLR family currently comprises of 11 members. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface and seem to specialize in recognizing products unique to microbes. TLR 3, 7, 8 and 9 detect different forms of nucleic acids [4, 31]. In the latter case, the recognition of ligands for this group of receptors is not only mediated by the molecular nature of the PAMP, but also by the location of the TLRs, in intracellular compartments, preventing activation of these TLRs by host nucleic acids. DCs express many of the known TLRs, with distinct expression patterns between different DC subsets, especially in human DCs [4]. It has been speculated that TLR expression profile correlates with the main DC subset function. For example, human plasmacytoid DCs is the sole DC subset expressing TLR9, which recognizes hypomethylated double-stranded DNA sequences (CpG motifs), in line with their efficient detection of bacterial and viral infections.

TLR mediated signaling leads to downregulation of the chemokine receptor CCR6 and upregulation of CCR7 [32], governing the migration to the draining lymph nodes. During this transition phase DCs also undergo a maturation program which consists of upregulation of costimulatory molecules, notably B7-1 (CD80) and B7-2 (CD86), but also CD40, OX40L, 4-1BBL, SLAM and high levels of MHC-peptide expression [33].

In the lymph node, DCs migrate to T cell areas and produce certain chemokines that attract naïve (MIP-3ß/CCL19) as well as memory T cells (MDC/CCL22 TARC/CCL17) [34], thereby increasing the likelihood of interaction with antigen-specific T cells. The initial contact between DCs and T cells is antigen independent and results in partial activation of the T cell, necessary to mediate TCR recognition of peptide-MHC complexes on the DC surface [35]. Successful interaction between TCR and peptide-MHC complexes lead to the formation of immunological synapses; concentrations of activated TCRs surrounded by co-stimulatory molecules, cytokine receptors, signal transduction molecules and integrins [36, 37]. Similarly, DCs concentrate peptide-MHC and costimulatory molecules toward the site of T cell engagement [38]. Recent advances in the field of optical imaging, such as two-photon microscopy, permit visualization of this process in which three phases can be distinguished. The first phase consists of serial brief encounters between the T cell and DCs. The second phase, 8 hours after the initial start of the T cell activation and lasts for 12 hours, is characterized by prolonged stable DC-T cell interaction and coincides with the start of T cell IL-2 and IFN-γ production. During the third phase, T cells resumed their rapid motility and brief DC contacts and this phase correlates with the onset of T cell proliferation [39, 40]. The second phase seems of particular importance for generation of a effector T cell response, as immunization with tolerizing DCs is characterized by the absence of this phase [39]. The lifespan of the activated DC is regulated by innate (TLRs) and acquired immunity (CD40-CD40L and TRANCE-TRANCE-L pathways)

which induce a Bcl-XL survival pathway and a molecular timer consisting of the anti-apoptotic protein Bcl-2 and the pro-apoptotic molecule Bim. The ratio of Bim to Bcl-2 gradually increased during a four day culture period, eventually leading to DC death [41].

2 CD4+ T cells

2.1 Th1 & Th2 subsets

A major breakthrough was the classification of CD4+ T cells into T helper type 1 (Th1) and T helper type 2 (Th2) subsets based on cytokine secretion in vitro, which correlated with effector function [42, 43]. Th1 cells are necessary for immune responses against intracellular pathogens, promoting the production of opsonizing and complement-binding antibodies by plasma cells and activate macrophages and neutrophils. Immune responses against helminths are dependent on Th2 cells, switching antibody production towards neutralizing IgG antibodies as well as IgE and lead to activation of eosinophils [43].
The prototypic cytokine for Th1 cells is IFN- γ . Besides increasing phagocytic activity, IFN- γ also supports the development of cytotoxic CD8+ T cells that are required for killing virus-infected host cells. In contrast, Th2 cells produce mainly IL-4, IL-5 and IL-13. In particular IL-4 is important, generating an autocrine feedback loop for further Th2 development and essential for IgE antibody production by plasma cells [43] Another important feature of these two CD4+ T cell subsets is the ability to cross-regulate each others generation and function, although most evidence for this phenomenon is derived from in vitro experiments [43].
Although first identified by differences in cytokine production patterns, many more differentially expressed molecules have been described nowadays (Table 1), some of which will be discussed in this section.

2.2 Regulatory T cells (Tregs) subset

A third group of CD4+ T cells, with immunosuppressing functions, has made a comeback and is now the focus of intense research [44]. The regulatory T cells (Tregs), or suppressor T cell, subset can be further subdivived in 'naturally occurring' Tregs and inducible Tregs [7, 45]. Naturally occurring Tregs represent 5-10% of the CD4+ T cell lymphocyte pool in healthy adult humans and mice and are thought to be important for keeping autoreactive T cells in check that have escaped negative selection in the thymus, but they have also been shown to downregulate Leishmania major mediated immune responses [46] and allergy [47]. These cells are phenotypically characterized by surface expression of the IL-2Rα chain (CD25) [48], and also express the forkhead/winged helix transcription factor Foxp3 [49]. In addition, several markers have been described that were implicated to be molecules used by these CD4+CD25+ T cells to exert their suppressive function, such as CTLA-4 [50] and GITR [51]. Of note, except for foxp3, none of the phenotypic markers described so far (see table 1) is unique to regulatory T cells, limiting their description and isolation. IL-10 and TGF-ß are important effector molecules, suppressing disease pathology in experimental models of colitis as well as allergic and autoimmune diseases [52-54], but mecha-

nisms requiring cell-cell contact have also been reported [55], presumably via membrane-bound TGF-ß [56, 57].
Another subset of regulatory T cells is not selected for in the thymus during T cell maturation, but is induced in the periphery during infection or can be induced by tolerance-inducing antigen administration regimens [7, 58]. Alternatively, this cell population can be derived from *in vitro* culture systems [59]. No characteristic stable cell-surface markers have been described for this population, although the costimulatory molecule ICOS has been reported to be expressed on this subset [53] They are currently best described by their cytokine signature consisting of IL-10 (Tr1 cells) or TGF-ß (Th3 cells) secretion [7]. It should be emphasized that both cytokines are not uniquely produced by this cell type, but that the classification of regulatory T cells is dependent on the cytokine secretion profile. For example, Th2 cells also produce IL-10, but in less quantities while producing large amounts of IL-4.
Furthermore, due to the lack of reliable (cell-surface) markers it is at present unclear what, if any, relationship there is between the natural and inducible Treg lineages.
Regulatory T cells target multiple cell types of the immune system. Tregs inhibit proliferation of and cytokine production by naïve $CD4^+$ T cells as well as Th1 or Th2 effector cells *in vitro* and *in vivo* [46, 60], and have also been shown to inhibit activation of $CD8^+$ T cells *in vitro* and *in vivo* [61, 62]. Indirect effects of IL-10 may also account for T cell inhibition, as IL-10 also acts on DCs by means of downregulating inflammatory cytokine production and MHC-peptide complexes on the cell surface, resulting in decreased effector T cell activation whilst promoting the generation of regulatory T cells [7]. Finally, a direct suppressive effect, independent of T cells, on cells of the innate immune system has also been described [63].

Table 1: Molecules used to discriminate between CD4⁺ T cell subets

Th1	Th2	Tregs*
	secreted mediators	
IFN-γ	IL-4	IL-10
TNF-ß	IL-5	TGF-ß
	IL-9	
	IL-13	
	cell surface molecules	
CXCR3	T1/ST2	LAG-3
CCR5	CXCR4	GITR
TIM-3	CRTH2	CD25‡
IL-12Rß	CCR3	CD38
TRANCE	CCR4	CD62L
CD94	IFN-γ-R	CTLA-4
	ICOS†	$\alpha_E\beta_7$
	CCR8	ICOS†
	intracellular	
SOCS-5	c-MAF	Foxp3
T-bet	SOCS-3	
HLX	GATA-3	
	NFATc	

*: includes all described Treg populations
†: stable expression on Th2 cells, also on subset of inducible Tregs
‡: expressed on naïve naturally occurring Tregs, as well as activated CD4⁺ T cells

3 Lineage decisions of CD4⁺ T cells

Naïve CD4⁺ T cells polarize towards Th1/Th2 effector cells or regulatory T cells, dependent on the type of immune response that needs to be mounted in order to eradicate the pathogen. In recent years many details have been elucidated about the molecular processes involved in Th1/Th2 lineage decision but much less is known about Tregs development

3.1 Th1 development

Th1 polarization is initiated in the presence of TCR signaling by IFN- γ mediated, STAT-1 dependent, expression of an essential transcription factor, T-bet. [64, 65]. This initial IFN- γ is provided by cells of the innate immune system, in particular NK cells which migrate to the lymph nodes under influence of mature DCs [66] The significance of T-bet is underscored by the observation that mice deficient in T-bet succumb to Leishmania major infection, a prototypic Th1 pathogen [64]. This activation of T-bet sets a cascade of events in motion. It induces expression of IFN- γ , and in addition leads to epigenetic remodeling of the ifng locus by rearrangement of chromatin regions allowing active gene transcription. Simultaneously, IL-12Rß2 gene expression is induced and its own expression stabilized, either by intrinsic positive feedback loop or in an autocrine fashion via IFN- γ production [67]. In effector Th1 cells, IFN- γ production is rapidly upregulated via two independent signaling pathways; TCR signaling or (IL-12) cytokine stimulation [65]. The latter pathway largely depends on STAT-4 signaling, although residual IFN- γ production has been noted in Stat4$^{-/-}$ animals [68]. In the absence of IL-12 however, no effective immune response can be generated against Th1 pathogens [43]. Recently, IL-27 has also been implicated in Th1 differentiation,

although subsequent studies have also revealed an suppressive role for this cytokine [69, 70]. In addition, various non-cytokine instructive signals have been identified. The LFA-1-ICAM-1 pathway has been reported to favor Th1 development, [71], as well as members of the notch ligand family Delta [72].

3.2 Th2 development

A cardinal transcription factor, GATA-3, is involved in Th2 cell commitment [73]. Analogous to Th1 instruction, the best known and studied signal for Th2 development is a cytokine, IL-4. IL-4 induces phosphorylation of STAT6, which in turn increases GATA-3 expression [74]. In combination with TCR-mediated signals, GATA-3 remodels the chomosome region containing the Th2 cytokine cluster (consisting of IL-4, IL-5 and IL-13) [65, 75]. The remodeling of the Th2 cytokine cluster leads to a loop formation that brings a cis-acting enhancer sequence, the locus control region (LCR), in close distance of the promoters of the Th2 cytokine cluster genes [76]. GATA-3 not only induces IL-4 transcription, but also inhibits the expression of IL-12Rβ_2, thereby suppressing IL-12-mediated Th1 development [74].
Upon TCR triggering of terminally differentiated Th2 cells, acute Th2 cytokine transcription is mediated via Th2-specific (c-MAF, GATA-3) or general (NFAT family members, AP-1) transcription factors [65].
A remaining paradox was the apparent need for IL-4 to induce GATA-3 expression, which in turn initiated IL-4 production. Although various cell types of the innate immune system, such as basophils, mast cells and NKT cells produce IL-4, Th2 polarization appears to be independent of these sources [77]. It might be that the absence of activation of the innate immune system removes inhibition of Th2-cell development by IFN-γ and IL-12, which allows Th2-cell development to be driven by positive feedback through IL-4 and GATA-3 [65]. Alternatively, several groups have reported that the notch ligand family Jagged has Th2 polarization capabilities, inducing GATA-3 independent of STAT6/IL-4 signaling [72, 78].

3.3 Treg development

Virtually nothing is known about molecular processes involved in regulatory T cell development. It is only known that $CD4^+CD25^+$ Treg development is dependent on Foxp3, a member of the forkhead transcription-factor family[49]. The importance of this gene for Treg development is illustrated by the (auto)immune disorders humans or mice with mutations in this gene acquire due to a loss of regulatory T cells [79, 80]

4 DC mediated $CD4^+$ T cell polarization

It has been well established that DCs have a pivotal role in the differentiation of naïve $CD4^+$ T cells into Th1 and Th2 cells and evidence is accumulating that DCs are also able to direct the development of Tregs [2, 6]. The final outcome of the differentiation process is determined by several factors, with the type of pathogen and the subset of DC stimulated being the major determinants. Figure 1 provides an overview of (microbial) stimuli that influence DC phenotype and

DC-derived mediators that subsequently modulate Th cell differentiation.

4.1 DC polarization stimuli

A myriad of molecules have been described that have the capability to direct helper T cell polarization via modulation of the DC phenotype (signal 3 hypothesis; [81]). One of the first molecules reported for its potent Th1 polarizing ability was E.coli LPS [82]. Numerous molecules have been added to this Th1 list, including, CpG motifs, poly I:C and undefined extracts of pathogens such as T. gondii and the unicellular form of several fungi [6, 83, 84].
In comparison to the number of known Th1-driving compounds, the number of selectively Th2-inducing molecules is relatively small, but this could be a consequence of the original hypothesis that TLR-triggering unequivocally led to Th1 polarization, thereby biasing the compound screening towards Th1 compounds. S. mansoni egg extract (SEA) and a glycoprotein of a filarial nematode (ES-62) are among the best described Th2-inducing compounds [83, 85]. Furthermore, Cholera toxin and LPS of the gram-negative bacteria P. gingivalis have been reported, although the latter remains controversial [83, 86, 87]. Interestingly, DC have also the capability to discriminate between different forms of the same pathogens. As mentioned before, Candida yeasts or Aspergillus condida evoke a Th1 promoting DC phenotype, while the hyphae form of the same organism induce Th2 promoting DCs [84, 88]. As a consequence of the relatively recent (renewed) interest in regulatory T cells (Treg), microbial products that polarize DCs towards a Treg-inducing phenotype are beginning to be identified. Well documented molecules are filamentous haemagglutinin (FHA) and adenylate cyclase toxin (CyaA), derived from B. pertussis, Cholera toxin ß-subunit, S. mansoni lysophosphatidylserine and hepatitis C virus glycoprotein NS4 (all reviewed in [7]).
An indirect, but potentially important source of DC polarization stimuli induced by pathogens originates from tissue or innate immune cells at the site of inflammation. IFN-γ produced by NK cells supports type 1- inducing DCs, while mast cell-derived histamine and PGD2, and TSLP produced by epithelial cells, drive Th2 effector response via their effect on DC phenotype [6]. Analogue to factors driving Th1/Th2 DC phenotypes, IL-10 and TGF-ß from the environment stimulate the formation of DC with regulatory functions [6]. The importance of tissue derived stimuli induced by pathogens was also underscored by the 'danger signal' theory proposed by Matzinger who stated that endogenous signals derived from pathogen-induced necrotic cells indirectly activate DCs, as opposed to Janeway's direct activation of DC by microbial signals [28, 89]. Acceptance of this model was hampered by the lack of mediators, although recently molecules that fulfill this role have been proposed [90].

4.2 DC-derived effector molecules

Various DC-derived molecules with Th-polarizing capacities have been identified. One of the most documented is IL-12, a powerful inducer of effector Th1 cells [91]. More recently, IL-23 and IL-27 which are closely related to IL-12, have also been implicated to drive Th1 differentiation [70, 91]. IL-18 also has a Th1-promoting effect, amplifying the effects of IL-12, but not capable of Th1 differentiation by itself [91]. Another group of important in Th1-driving factors humans,

but not in mice, are type I IFNs (e.g. IFN-α/ß) In both man and mice, type I IFN production is strongly associated with virus infection and may be crucial in the development of protective Th1 immunity [6]. Several cell-surface molecules have also been shown to have a Th1 polarizing effect, among them ICAM-1 and the notch-ligand family Delta [71, 72].
It was once postulated that MHC-peptide (signal 1) and costimulation (signal 2) signaling in the absence of Th1 inducing PAMP signal transduction (MYD88), leads to Th2 differentiation by default [92, 93]. However, careful examination of immune responses in mice deficient for IL-12 or immune responses generated through indirectly activated DCs do not confirm this hypothesis [94, 95] Furthermore, a number of molecules implicated in Th2 differentiation have been identified up till now, arguing against this hypothesis, although it should be emphasized that no clear-cut picture has emerged yet as seen with Th1 driving cytokines such as IL-12. Some recently identified secreted mediators with Th2 inducing properties include CCL2 (MCP-1), IL-25, IL-6 [84, 96], while IL-18 has been reported to be able to drive Th2 development in the absence of IL-12 [97]. Cell-surface molecules with Th2-driving properties are OX40L and Jagged family members [6, 72]. Finally, DC-derived molecules that are important in Treg induction are IL-10 and TGF-ß (in the absence of IL-12) [6, 7], and one study also reported a role for the ICOS ligand B7-RP1 [53].
Besides identified distinct signal molecules, a poorly defined term, called 'maturation' seems also important in T helper cell differentiation, in particular for Treg induction. The maturation status is commonly defined by the expression levels of costimulatory markers CD40, CD80 and CD86. High levels of all these molecules support Th1 induction . In agreement with this finding, IL-23 has been shown to enhance DC maturation and might via this route contribute to Th1 polarization [98]. It should be emphasized however, that other molecules besides the well-known DC maturation markers such as described here seem important. Reis e Sousa elegantly demonstrated that only direct activation of DCs with PAMPs resulted in IL-12 production and Th1 effector cell generation, while indirect activation of antigen-presenting DCs does result in a similar activation status, but these DCs are not capable of generating bona fide Th1 cells.[95]
Low levels of MHCII and costimulatory molecules, in particular CD40L,have been associated with Treg induction [6, 7, 99]. Although nowadays the definition of regulatory DCs has been subtly refined from 'immature' towards 'quiescent' DCs, intended to indicate a specialized subset of mature DC, there is still evidence that some pathogens block DC maturation, leading to Treg development in order to protect itself of detrimental effects of the immune response [6]. Other evidence of the relationship between maturation state of DCs and tolerance induction was acquired with elegant mouse models, targeting antigen specifically to DCs and assess the immune response under steady state or inflammatory conditions. The results unequivocally showed that under non-inflammatory responses tolerance against the antigen was observed, while under inflammatory conditions a vigorous response ensued [100, 101].

4.3 DC subsets & CD4+ T helper priming.

Traditionally, it was thought that the different human DC subsets had fixed instructive capacities, hence the DC1/DC2 nomenclature [102]. Studies on lymphoid and myeloid DC populations in mouse confirmed the different priming capabilities [103, 104]. However, a conceptual problem arose from this model; how could different DC subsets discriminate between pathogens when simultaneously present at the site of infection and ensure a proper type of immune response? The identification of TLRs as the main family of PRRs could not entirely solve this theoretical flaw, as the distribution pattern of TLR members show partial overlap between different DC subsets [4]. Indeed, in recent years it has become increasingly clear that DC subsets exhibit flexibility with regard to their CD4+ T cell polarization capacity, based on microbial stimuli and local environmental factors [83, 105, 106]. Despite this plasticity, some DC subtypes seem to have a different functional bias. For example, freshly isolated human pDCs are the only group expressing TLR9, providing the ability to respond to viral DNA sequences, which is in line with the reported massive production of type 1 IFNs by these subset after viral infections [21].

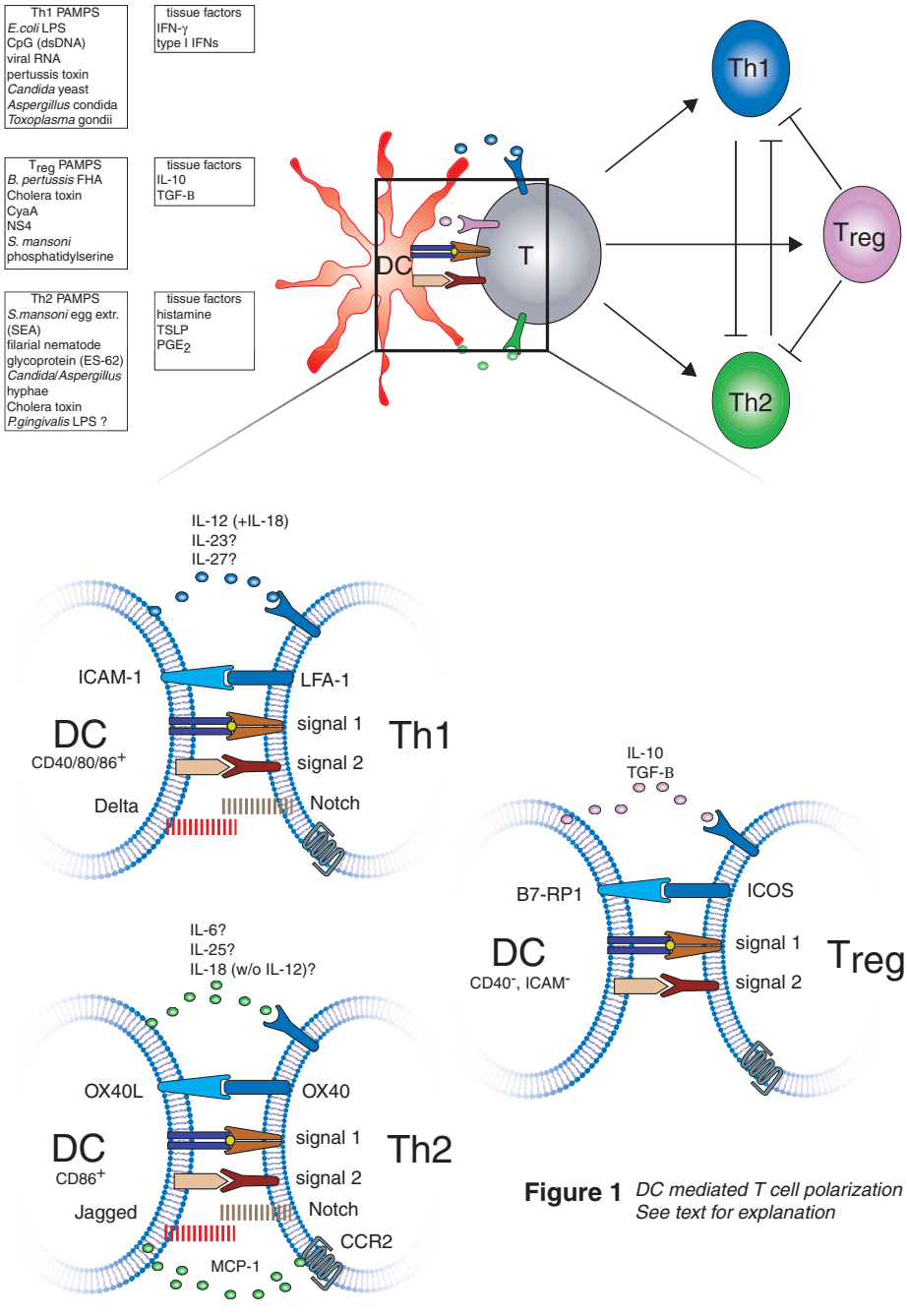


Figure 1 *DC mediated T cell polarization*
See text for explanation

5 Atopic asthma

Asthma is a chronic inflammatory disease of the airways, characterized by intermittent airway narrowing and variable symptoms of chest tightness, wheeze and shortness of breath. Asthma can roughly be divided in two groups; intrinsic (non-atopic) asthma and allergic (atopic) asthma, which will be the focus of this thesis. Atopy and allergy are used almost synonymously in English-speaking literature, and are now defined as the propensity to develop immediate-type hypersensitivity reactions to common environmental proteins, known as allergens [107], although formally allergic reactions are only atopic when the observed immune response is IgE-mediated.
The word 'atopic' is derived from the Greek 'atopos', meaning 'strange, out of place'. Sadly, this is not the case for asthma nowadays, as incidence and prevalence of atopic asthma continue to rise in the First World countries and developing Second World countries. For example in the USA, the overall prevalence rate of asthma is about 5-8% and currently affects about 20 million persons including approximately 4.8 million children [108]. In Bangalore, India the prevalence of asthma in the pediatric population has risen from 9 % in 1979 to 29.5% in 1999 and it is forecasted that the asthmatic population in total India will reach 250 million by 2013. Likewise, in the same timeframe, the estimated population affected by asthma in China will be around 150 million [109]. In the Netherlands, prevalence has increased to around 3% in 1999 [110]. Apart from the reduced quality of live, asthma also places a large burden on the healthcare system with respect to costs. In the USA, expenses have risen form an estimated US$6.2 billion in 1990 to US$ 12.7 billion in 1998 [111].

5.1 Pathology of asthma

The tell-tale symptoms of asthma, shortness of breath, coughing and wheezing, are caused by airway obstruction and airway hyperresponsiveness (AHR). AHR, which is defined as an increased bronchoconstrictor response to nonspecific stimuli such as smoke, cold air, exercise, amongst others [112]. These characteristics of the asthmatic airways are in turn the result of inflammation and airway remodeling.
On a microscopic level, pulmonary inflammation is manifested by the infiltration of the airway wall with mononuclear cells, mostly $CD4^+$ T cells, and eosinophils but depending on the severity, also plasma cells, neutrophils, macrophages, dendritic cells, and mast cells have been shown to be increased. In the airway lumen, goblet cell hyperplasia leads to increased mucus production and this mucus is mixed with activated macrophages, lymphocytes, eosinophils and shed epithelial cells [113, 114]. Further evidence of this inflammatory response is the presence of proinflammatory cytokines an chemokines [115].
Structural changes to the airways, collectively referred to as airway remodeling, is another hallmark of asthma. The accumulation of these changes lead to increased thickness of the airway wall, varying between 10% to 300%. Besides the infiltration of inflammatory cells, mucous gland hypertrophy, smooth muscle cell hyperplasia and metaplasia of airway epithelium into mucus-secreting cells also contribute to the increase in thickness. In addition there is collagen deposition

(types I, III and V) beneath the basal membrane, leading to pseudo-thickening of the basement membrane [116].
In a temporal manner, the asthmatic reaction can be divided into an early phase and a late phase. The early phase reaction is maximal at 15-30 min after allergen inhalation and generally resolves within 1-2 hours. This phase is characterized by mast cell degranulation, leading to bronchoconstriction, vascular leakage and mucus production, which will be discussed in more detail below. In approximately 50% of patients, this reaction is followed by the so-called late phase, peaking at 6-12 hr after allergen challenge and usually resolves within a few days. During this phase, a massive infiltration of the airway wall with eosinophils and lymphocytes is observed [116].

5.2 The etiology of asthma

5.2.1 Genetic factors

Although family and twin studies have provided evidence that asthma is a heritable disorder, identification of susceptibility genes has been hampered by several factors. First, asthma is a multigenic disorder and evidence is accumulating that genetic heterogeneity exists across populations, i.e. a similar phenotype is produced by different genes. Second, the linkage of specific genes to asthma is difficult due to the lack of objective clinical benchmarks of the disease. For this reason objective, quantitative traits such as total IgE, AHR and skin prick tests have been used as surrogate markers for asthma. Using these markers to define asthma bears the risk that genes involved in asthma pathogenesis but not underlying the intermediate phenotypes remain unidentified. For example, atopy is one of the strongest risk factors for asthma, but it alone is not sufficient to induce asthma, as many atopic individuals do not have asthmatic symptoms [117].
Although several approaches can be taken to identify asthma-susceptibility genes, they are all combinations of two basic methods; genome-wide screens and the candidate-gene approach. The genome-wide screening involves linkage analysis of markers that span the entire genome to map all loci with detectable effects on a phenotype of interest. The main advantage of this method is its unbiased nature, which may more reliably identify susceptibility genes, in particular genes that were not previously implicated in asthma pathogenesis. However, due to the large number of independent variables, this study setup might suffer from lack of statistical power which in turn necessitates large study cohorts. In addition, the regions identified with this method are generally broad chromosomal regions containing many candidate genes, requiring labour-and cost intensive fine mapping to identify the particular gene. Genome-wide screens published so far have confirmed the linkage of regions to the asthma phenotype known to contain genes that are involved in the pathogenesis of asthma such as the cytokine cluster located at the 5q chromosome region [117].
The candidate-gene approach analyses the linkage of polymorphisms in genes suspected to be involved in asthma pathogenesis to a particular phenotype. The rationale to select a particular gene is based on its biological activity (e.g. IL-13) or on previous associations of the region with the trait of interest. An overwhelming number of candidate genes has been proposed and studied, many of them

located in the 5q chromosome region where the cytokines genes are located, believed to play a principal role in the immunological processes underpinning atopic asthma [117].
A major recent breakthrough in asthma genetics is the progression from broad linkage regions to individual genes. In the last four years, six asthma-susceptibility genes have been identified. In 2002, the Adam33 gene was identified [118]. Adam33 is a member of a family of genes encoding membrane-anchored zinc-dependent metalloproteinase which are implicated in cell-cell interactions, cytokine activation and cell signaling, but it's exact function is not known. Using similar approaches, dpp10, phf11 and gpra genes were identified [119-121]. Additionally, using congenic or a combination of genetic and genomic approaches, two new susceptibility genes, tim1 and C5, have been identified using murine models of asthma [122, 123]. Although functional studies are awaited to confirm the role of these genes, the fact that none of them was previously implicated to be part of known pathways associated with asthma pathogenesis underscores the strength of hypothesis-independent genome-wide screening.

5.2.2 Environmental factors

Although it is undisputed that atopic asthma has a heritable component, the dramatic increase in prevalence of the disease seen over the past decades in the Western World occurs too fast to be explainable by a shift in genetic makeup. Therefore, the focus has been put on environmental factors. Although the presence of allergens is the primary prerequisite for development of atopic asthma, a strong correlation between in the intensity of exposure to such allergens and the development of allergic disease has yet to be found [124], resulting in alternative hypotheses.
The hypothesis that has gained most attention originated from the observation that an inverse relationship existed between the risk of atopic sensitization and family size; 'the hygiene theory' [125]. Similar correlations have been reported for other indicators of childhood infection, such as day-care attendance, low parental social-economic status and growing up on a farm [124]. However, although this hygiene hypothesis suggests that protection from allergy is associated with an increase in microbial exposure, direct evidence is scarce. The most promising data in support of this theory is the discovery of a tim-1 gene polymorphism that also acts as cellular receptor for hepatitis A virus. Patients carrying this particular polymorphism showed a correlation between hepatitis A virus seropositivity and protection against atopy [126]. Indirect epidemiological evidence that advocates this relationship is provided by a study among Italian military recruits that revealed a similar significant relationship between the presence of antibodies to hepatitis A and various measures of atopy [127]. Other proposed protective stimuli such as measles infection, BCG vaccination or commensal gut flora have been under intensive scientific scrutiny, without yielding any convincing candidate that may be responsible for atopy protection [124].
The immune system of neonatal humans is thought to have a Th2 bias which gradually diminishes during the first 2 years of life in non-allergic individuals, but increases in allergic infants [128]. The hygiene hypothesis is explained from an immunological point of view that a Th1 stimulus is needed to avoid development

of atopy. However, several lines of epidemiological evidence argue against this Th1 switch hypothesis. First of all, not only the prevalence of allergic diseases has increased, also Th1-associated autoimmune diseases have also risen over the same time period [129]. Furthermore, populations with high rates of helminth infections, which induce strong Th2 responses, are also protected from allergic diseases [130]. It was shown by the authors that IL-10 was responsible for this protective effect. The important role for IL-10 was further underscored by recent findings that IL-10 protein levels in the BAL fluid of asthmatics are markedly lower compared to controls and an IL-10 promoter polymorphism more common in asthmatic individuals has been described as well [131, 132]. studies in mouse models support a protective role for IL-10 [133-135], although paradoxically IL-10 is necessary for AHR induction [136].
Based on these data a refined immunological explanation of the hygiene hypothesis was formulated, the 'counter-regulatory' hypothesis [124], which postulates that infections or colorizations with microbes during childhood results in upregulation of IL-10 production which subsequently protects against the development of atopic diseases in susceptible individuals. Although it is unlikely that IL-10 is solely responsible for this protection, evidence is accumulating that regulatory $CD4^+$ T cells are the principal source of IL-10. Strong microbial stimuli applied to the airways of rodents induce IL-10 producing regulatory $CD4^+$ T cells [60, 137]. Subsequent studies in humans found lower numbers of IL-10-producing regulatory T cells in atopic individuals compared to non-atopic controls [138], as well as evidence for reduced function of these cells in atopic individuals [47].
The differentiation of regulatory T cells in turns seems to be dependent on IL-10 as well, at least in rodents were it is produced by pulmonary DC exposed to strong microbial stimuli [60, 139]. Clinical data in agreement with this hypothesis is provided by the observation that monocyte-derived DCs from atopic children produce less IL-10 upon LPS stimulation [140].

6 Immunological basis of asthma

Despite the fact that it has been known for almost 100 years that eosinophilic infiltrates are present in the airways of asthmatic patients [141], it was not until the advent of use of flexible, fiberoptic bronchoscopy and biopsies that inflammation was regarded to be the cause of asthma. In addition, it was also known for a long time that asthma was linked with high IgE serum levels, but the reason for this was not understood. In this section, we will discuss the various cell types of the immune system with respect to their function in the pathology of asthma. Figure 2 depicts a schematic overview of some of major immunological pathways involved during distinct phase of the immune response.

6.1 DCs

An adaptive immune response against any antigen, including aeroallergens, starts when antigen is provided in the right cellular context (mature APCs) to T-and/or B cells. DCs have emerged as the principal APC in priming naïve T cells, including T cells involved in atopic asthma [2]. The important role of these cells in allergy is mainly derived from animal studies. Adoptive transfer of antigen-

pulsed DC into the airways is able to sensitize mice and rats for development of eosinophilic airway inflammation [142, 143], while conditional depletion of DCs before allergen challenge abrogates airway inflammation development [144, 145]. In these models, airway DCs are rapidly recruited to the mucosal sites of inflammation during pulmonary inflammation [146, 147], and the quantity of myeloid dendritic cell precursors in the bone marrow expands [147]. These data derived from murine models are also supported by observations taken from the airways of atopic asthma patients, which reveal an active recruitment and increased number of myeloid DCs in the mucosa accompanied by a concomitant decrease in numbers of myeloid DC in blood [114, 148, 149].
In the lung, aeroallergens first have to cross the lung epithelium to gain access to DCs lining the respiratory tract, Many allergens, such as derp1, the principal allergen of house dust mite, possess endogenous proteinase activity capable of breakdown of tight junctions of the epithelium [150]. Moreover, in the intestine, DCs are able to extend their cellular processes (dendrites) to the luminal side of the epithelium without disrupting epithelial integrity [151]. This phenomenon has not yet been formally demonstrated for DCs lining the respiratory tract, but evidence in support of this mechanism is illustrated by the reduced airway inflammation in mice deficient for matrix metalloproteinase 9, which is necessary for DC-mediated epithelium rearrangement [152, 153].
DCs, activated by either exposure to allergen or pathogens, migrate with accelerated kinetics to the draining lymph nodes [154, 155] possibly due to upregulation of CCR7, CXCR4 and CCR8, while simultaneously downregulating CCR5 and CCR6, as is the case for skin derived DCs [156-158], although not formally shown for lung DCs.

6.2 Th2 cells

It was found that both IgE production and specific recruitment of allergy associated immune cells was dependent on Th2 cells, putting these cells into the spotlight of asthma research. Th2 cells are now generally believed to initiate an perpetuate disease. Upon arrival in the draining lymph nodes of the lung, DCs migrate to the T cell areas and present antigen to naïve T lymphocytes, which in the case of allergic airway inflammation will differentiate into Th2 cells .
So what evidence comprises the claim that $CD4^+$ Th2 cells are the main orchestrators of allergic airway inflammation? First of all, Th2 cells are present in the airways of asthmatics [159] and possess an activated phenotype [160, 161]. Transcripts and protein of the Th2-associated cytokines IL-4, IL-5 and IL-13 are elevated in BAL fluid, BAL cells and airway biopsies [159, 162, 163]. At the molecular level, the Th2 phenotype of $CD4^+$ T cells is confirmed by GATA-3, which is expressed at high levels in $CD4^+$ T cells isolated from the airways of asthmatic patients [164], whereas the Th1 master switch T-bet is reduced [165]. This circumstantial evidence has been strongly supported with data from animal studies. Mice deficient in IL-4 or STAT6 are not able to mount an allergic airway response [166, 167], while mice overexpression of Th2 cytokines in the airways such as IL-4, IL-5 and IL-13 exhibited characteristic features of asthma [168-170]. Mice expressing a dominant negative form of GATA-3 in T cells do not develop eosinophilic airway inflammation [171], while mice deficient in T-bet spontaneously

develop asthma-like symptoms [165].
As mentioned before, the current paradigm of Th subsets is based mainly on their cytokine production, which is also their principal effector mechanism. Here we will briefly discuss the role of the different cytokines produced with regard to asthma.

6.2.1 IL-4

IL-4 is especially important during the sensitization phase, as mice deficient for IL-4 or any component of the IL-4 signaling pathway exhibit greatly reduced Th2 responses and airway eosinophilia [26, 166, 167]. Its principal role is mediating B cell isotype switching to IgE. However, it also has some functions in the effector phase of asthma, and is involved in goblet cell hyperplasia, induces chemokine production by activated respiratory epithelium and upregulates VCAM-1 on endothelial cells, thereby facilitating VLA-4 mediated eosinophil transmigration [26].

6.2.2 IL-5

The most important role of IL-5 in asthma is driving the differentiation of bone marrow progenitors into eosinophils [172]. In the absence of IL-5, lung tissue and BAL eosinophils are not increased in response to Th2 activation [173, 174]. Furthermore, IL-5, in collaboration with eotaxin, recruits eosinophils from the bone marrow during allergic inflammation [175].

6.2.3 IL-13

IL-13 is an essential cytokine for AHR development. Although it was first thought that IL-4 and IL-13 could have a complimentary roles in AHR induction [176], further research with IL-4/IL-13 double knockout mouse firmly established that IL-13 is necessary and sufficient for AHR [177]. Mucus hypersecretion has also shown to be dependent on IL-13 [178, 179].

6.2.4 IL-25

A recently described cytokine with potent Th2 inducing properties produced by Th2 cells is IL-25. IL-25 induces Th2-type cytokine production by accessory cells that are MHC class II^{high}, CD11c^{dull}, and lineage negative [180]. Mice given intranasal IL-25 developed marked eosinophilia in the BAL and lung tissue, epithelial cell hyperplasia, increased mucus secretion, and airway hyperreactivity [181].

6.3 B cells

Primed Th2 cells will subsequently migrate to the B cell follicles due to acquisition of the CXCR5 receptor [182]. Allergen-specific B cells will be activated upon allergen encounter and initiate immunoglobulin production. Two distinct signals are essential for isotype class switching towards IgE: IL-4/IL-13 and CD40-CD40L signaling [26]. In addition, the B7RP-1/ICOS pathway has also been shown to be important for IgE class switching [183].

6.4 Mast cells

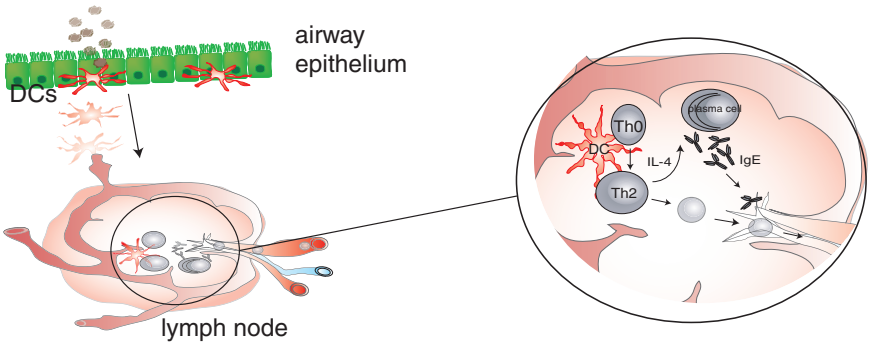
Mast cells are present in large numbers in the mucosa, submucosa and alveolar walls [184]. They are also seen to infiltrate smooth muscle layers and this infiltration is deemed essential for bronchial airway hyperreactivity [185]. Mast cells express the high affinity receptor for IgE, FcεR1 and will bind allergen-specific IgE (sensitization). Re-exposure to the same antigen (challenge) will crosslink IgE on the mast cell surface leading to the release of preformed mediators such as histamines, neutral proteases (predominantly tryptase and chymase), leukotrienes and prostaglandins.
These inflammatory components typically have a short half-life and are held responsible for the early phase reaction which consists of bronchoconstriction and increased mucus secretion, leading to the general feeling of chest tightness and breathing difficulties. This phase usually resolves in a few hours and is followed by the late phase reactions; recruitment and activation of other effector cells such as Th2 lymphocytes, basophils and eosinophils. Chemokines, cytokines and leukotrienes produced by activated mast cells are primarily responsible for this late phase recruitment [26].

6.5 Eosinophils

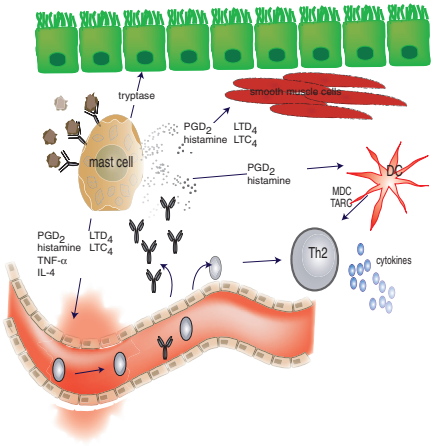
One of the most prominent features of this disease is the presence of eosinophils in the airways of asthmatics, but their role in the pathophysiology is a matter of debate. In favor of a direct role are the observations that: 1) the number of activated eosinophils closely correlates with severity of disease [161], 2) eosinophils produce mediators that damage airway epithelium (e.g. eosinophil peroxidase, major basic protein) or have bronchomodulatory properties (e.g. leukotrienes) and 3) reducing the number of eosinophils mildly ameliorates the asthma symptoms [26]. However, in murine models pulmonary eosinophilia has often been separable of disease pathology. Neutralization of IL-13 reduced AHR and goblet cell hyperplasia, without affecting eosinophilia [186], while IL-5 deficiency has yielded conflicting results in both human and murine studies [173, 187-189]. Recently, two new transgenic mice strains have been constructed that are devoid of eosinophils [190]. Although results from these two mice strains are not totally consistent, possibly due to the way eosinophil eradication is achieved or difference in background strains, preliminary results point again to an essential role for eosinophils in airway remodeling but not in AHR or mucus production.

6.6 Animal models of asthma

Animal models have been, and are, invaluable to study the patholgenesis of asthma. Whereas human studies show associations between pathophysiological characteristics of the disease and biological systems such as the immune system, animal models offer the possibility to study cause and effect. For example, mice deficient in IL-4 do not develop eosinophilic airway inflammation [191], establishing the crucial role of this cytokine for induction of asthma. Most animal models have evolved around the mice, due to the availability of a plethora of reagents, i.e. monoclonal antibodies, cytokines, chemokines. In addition, the technique to generate transgenic mice (either deletion of a gene, called 'knock-



Early phase



Late phase

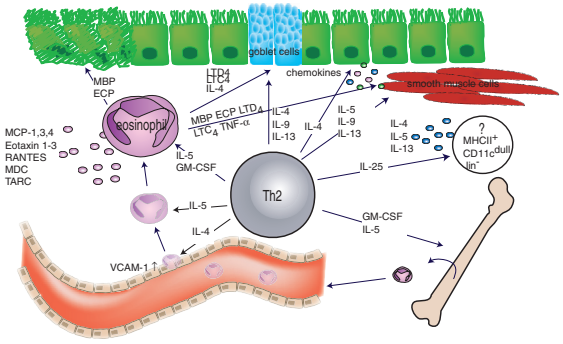


Figure 2 *Immunology of eosinophilic airway inflammation. See text for explanation*

out', or insertion of a gene) is more efficient in mice than other animal species. In spite of the universal choice for the mouse as experimental species, there is a bewildering variety in strains, antigens, adjuvants, route of sensitization and challenge, number of sensitizations and challenges, and the time frame used. A comprehensive review of the literature has distilled the minimum requirements necessary for the induction of eosinophilic airway inflammation characteristics, consisting of usage of the Balb/c strain, primed with two systemic antigen doses in adjuvant followed by multiple airway allergen challenges [192].
These murine models mimic many features of human asthma, including pulmonary tissue and BAL eosinophilia, AHR, Th2 cytokine production and antigen-specific IgE production. However, some pathological observations of asthma in humans are missing in murine models, questioning the relevance of these models to study the mechanisms behind asthma. Most notably are the lack of plasma extravasation and mucosal edema [193], and eosinophils fail to localize in the airway epithelium and do not degranulate [194], although a recent study did report degranulation of eosinophils in the airway lumen [195]. In addition, most mouse models are relatively short-term (up to two weeks) thus modeling the acute phase inflammation reactions, but not chronic events such as airway remodeling. Experimental setups using controlled exposure to low concentrations of aerosolized antigen for long periods of time have succeeded to elicit some of the features of airway wall remodeling, although persistency of the characteristics is debatable [196, 197].
Despite the drawbacks, mouse models have provided functional evidence for many cells and mediators identified from clinical studies and likely will be of paramount importance to screen for and test new therapeutic compounds.

7 Therapy

7.1 Current therapy

Current asthma therapy is highly effective in controlling symptoms of asthma, although the mechanisms of action are incompletely understood. Many patients are treated according to international agreed guidelines [198], which are based on the use of inhaled corticosteroids to control inflammation as first-line therapy, in combination with long-acting $ß_2$ agonists (a bronchodilator), and antileukotrienes to fine-tune symptom control, For short term relief, short-acting $ß_2$ agonists are prescribed [26]. Although current treatment is adequate and safe, corticosteroids do not affect TGF-ß levels and collagen deposition, important for remodeling events [199] It is therefore still unknown if inhaled steroids alter the natural history of its disease.
Furthermore, although there is no doubt that the benefits of current corticosteroid therapy vastly outweigh the potential risk, some concerns remain, in particular about long-term effects as a trend is observed towards earlier use of corticosteroids among children. Some known corticosteroid associated side-effects include adrenal suppression, increased risk of osteoporosis and fracture, ocular problems, such as ocular hypertension and posterior subcapsular cataracts, and skin effects (thinning and bruising) [200].

7.2 Immunotherapy

A commonly accepted definition of immunotherapy is 'treatment of a disease with therapeutic agents that promote or inhibit immune responses'. Strictly speaking, corticosteroid treatment should be considered immunotherapy according to this classification, but due to the non-specific immunosuppressive mechanism of these agents, we define immunotherapy here as modulation of the $CD4^+$ T cell response in order to prevent or suppress an allergen-specific Th2 response.

7.2.1 Counterbalancing the Th2 response

One of the first approaches to specifically target the Th2 nature of eosinophilic airway inflammation consisted of either stimulation of Th1 development, (based on the observation that Th1 effector cytokines (in particular IFN-γ) antagonize the development and function of Th2 cells [43]), or direct administration of the Th1 effector cytokine IFN-γ.

In experimental mouse models, treatment with IFN-γ during sensitization or challenge showed promising results [201, 202]. Clinical trials with inhaled or subcutaneously administered IFN-γ however, disappointingly showed no beneficial effects on symptom scores or lung function [203, 204]. A more indirect way to establish an allergen-specific Th1 response uses IL-12, which directs the differentiation of naïve $CD4^+$ T cells strongly towards Th1. Similar to IFN-γ, treatment of eosinophilic airway inflammation with recombinant IL-12 in mouse models showed beneficial effects, even when given during the challenge phase [205]. Unfortunately, a clinical trial with recombinant IL-12 given subcutaneously to mild asthmatics showed no signs of lung function improvement despite a reduction in blood and sputum eosinophils [206]. Moreover, there were serious side-effects reported in this trial and others, including deaths [206, 207]. Along similar lines, Th1 promoting immunotherapies have been studied using bacteria (e.g. BCG) or CpG motifs with comparable results (for a review see [205] and [26]).

Apart from the effectiveness of the strategies described above, the question remains whether induction of an allergen-specific Th1 response has any adverse effects. Studies using adoptive transfer of Th1 cells in mice with eosinophilic airway inflammation report conflicting results, with the majority of studies showing lack of suppression or even enhancement of inflammation [129, 192]. Furthermore, it was shown that IFN-γ prevented the migration of eosinophils into the airway lumen but not to the lung parenchyma [208], providing an explanation for the observed decrease in BAL eosinophilia whilst histological examination showed similar numbers of eosinophils in the lung parenchyma of the adoptive transfer experiments mentioned above. These mouse model data corroborate with an IFN-γ inhalation safety study, revealing an increase in lymphocytes in the BAL of healthy volunteers [209].

To summarize, despite the numerous possibilities to direct the allergen-specific Th2 response towards Th1, the results of experiments performed so far raise considerable doubt whether this strategy should be further pursued as an intervention strategy. Nonetheless, this therapy may still hold prophylactic value, as shown in chapter 4.

7.2.2 Suppressing the Th-2 response

The advancements in understanding the immunological processes underlying the pathological features of asthma has led to strategies targeting Th2-specific effector molecules. Depletion of IL-5 with two different monoclonal antibodies against IL-5 resulted in a dramatic reduction of circulating eosinophils, but did not alter asthma symptoms or AHR, fuelling the debate about the exact role of eosinophils in asthma etiology. Antibodies or soluble receptor antagonists intervening with IL-4 or IL-13 signaling are also at various stages of development or in clinical trial [210]. Antibodies against IgE prevent binding of free IgE to its receptors, thereby preventing basophil and mast cell degranulation. Indeed, clinical trials have shown reductions in serum free IgE levels up to 1% of baseline levels. More importantly, clinical trials have shown to reduce both the EAR and LAR and symptoms of IgE-mediated allergy. Clinical studies have shown that the patients who benefit most from omalizumab therapy are those at high risk of exacerbations, those with poorly controlled and/or severe asthma, and those with IgE-mediated comorbidities. Based on these studies, it is expected that anti-IgE will be introduced as an (very expensive) add-on therapeutic [211]

7.2.3 Allergen immunotherapy

Allergen immunotherapy is discussed here as a separate entity because the therapy is based on the observation that administering gradually increasing quantities of antigen sometimes leads to unresponsiveness to that particular antigen via only partially understood mechanisms, which may depend on the type of antigen (venoms or inhalation allergens) and route of sensitization. In vitro analysis of cytokine production by PBMCs from patients after treatment showed evidence for either both a Th2 to Th1 shift and induction of Treg cells [212]. The efficacy of immunotherapy for the management of asthma is controversial. Studies assessing the relationship between childhood immunotherapy and the development of asthma later in life suggest some preventive effect [213, 214]. Immunotherapy for patients with established asthma also showed some benefits, in particular in reduction of allergen-specific AHR [215]. However, at present allergen immunotherapy is not recommended medical practice, due to the limited efficacy, the risk for severe side effects (anaphylaxis) and the availability of safer and more effective pharmacological alternatives [215, 216].

7.3 Somatic gene therapy

Somatic gene therapy is the introduction of new genetic material into the non-germline cells of an individual for therapeutic purposes. It has some distinct pharmacological advantages over the more classical (protein) therapies. First, classical therapeuticals (e.g cytokines) are delivered systemically or semi-systemically (e.g. aerosolisation) and will form a concentration gradient towards the affected tissue, which might be too low to ensure long-lasting therapeutic effect. Therefore, multiple doses need to be administrated at relatively high concentrations, increasing the risk of intolerable side-effects. By contrast, depending on the delivery system used, gene therapy that is aimed at the affected tissue can achieve high concentrations of therapeutic agent locally, reducing the risk of

side-effects and reducing the number of treatments. Second, gene expression can be regulated. Several regulatory expression systems have been developed, either pharmacological based (e.g. the 'tet-on' and 'tet-off' systems; [217]) or regulated by pathophysiological processes; a recent example of this last category is a construct consisting of the human IL-1ß enhancer with the IL-6 promoter region which drives gene expression under inflammatory conditions in a mouse model of arthritis [218]. A more thorough review of the subject is provided by Chernajovsky *et al.* [219].

Efficient gene delivery is the key to successful gene therapy. Gene therapy can broadly be divided in *in vivo* gene therapy, with administration of the vector directly to patients, or *ex vivo* strategies. *Ex vivo* treatment consist of removing cells from patients, which are subsequently genetically modified *in vitro* to encode for a therapeutic protein before their return to the patient. *Ex vivo* treatment can further be divided based on the migration properties of the modified cells; mobile (e.g. DCs) or immobile cells (e.g. pancreatic islets cells). Several vector systems have been developed for gene delivery, which differ in features such as immunogenicity and the size of insert that can be cloned. The choice of vector is dependent on the followed strategy, and some common vector systems have been summarized in table 2.

7.3.1 Gene therapy for asthma

Gene therapy approaches targeting the pathophysiology of asthma are still in an experimental stage and have been focused on either modulation of the inflammatory response or direct targeting of mechanisms responsible for the symptoms such as mucus production and bronchoconstriction.

Similar strategies aimed at shifting the Th2 response towards Th1 as previously discussed at the immunotherapy section have been used for asthma gene therapy, including transduction of airway epithelium with vectors encoding for IL-12, IFN-γ and IL-18 [202, 220-224]. The use of cytokine genes (which act in a paracrine fashion) and adenoviral constructs (having a natural tropism for airway epithelia) in the majority of studies enhances the biological efficacy of these regimens. However, the conceptual problems behind this strategy, whether a Th2 to Th1 shift is beneficial to asthma pathology, remains. A genetic approach to suppress the Th2 response has also been tested with an adeno-associated vector (AAV) expressing an IL-4Rα antagonist blocking IL-4 and IL-13-mediated signaling. Treatment with this vector, either intravenously or applied locally in the airways, led to reduced inflammation, mucus production and AHR [225]. Finally, suppressing both Th1 and Th2 responses by adoptive transfer of IL-10 or TGF-ß engineered allergen-specific T cells is an alternative strategy [135, 226] , although clinical use is not foreseeable in the near future, due to the requirement for antigen characterization and the inherent risks associated with retroviral vectors as have been used in these studies [227].

Gene therapy aiming at the mechanisms responsible for asthma associated symptoms are also being investigated. Preliminary data suggests that adenoviral transduction of a $ß_2$-adrenergic receptor in the bronchial epithelium of normal mice attenuates methacholine-induced bronchospasm [228]. Targeting *gob5*, encoding a Ca^{2+}-dependent chloride channel, with antisense RNA attenuated mu-

cus production and AHR in a mouse model of eosinophilic airway inflammation [229]

Table 2: features of the common vectors used for gene therapy

vector	host cell	vector genome	transgene capacity	immunogenicity	genomic integration	duration of expression	target cell	advantages	disadvantages
Gutless adenovirus	human	dsDNA	37 kb	low	no	At least 1 year	Dividing and quiescent	Good for long-term expression	Difficult to produce
Adeno associated virus (AAV)	human	ssDNA	4 kb	low	rare	Up to 1 year	Dividing and quiescent	Good for long-term expression	Insert size is small; 30% of human population has pre-existing Abs
Herpes simplex virus	human	dsDNA	35 kb	high	no	At least 6 months	Dividing and quiescent	Can express multiple genes; mainly neurotropic in vivo	Induces cellular toxicity and inflammation
Retrovirus	mouse	RNA	7 kb	low	yes	For the life of the cell	dividing	Has ex vivo applications	Can cause insertional effects
Lentivirus	Human	RNA	7 kb	low	Yes	For the life of the cell	Dividing and quiescent	Can be produced at high titres	Can cause insertional effects

8 Aim and outline of this thesis

Asthma is reaching epidemic proportions in the developed countries, having a negative impact on the quality of life and places an increasing burden on health-care costs. The sharp increase in incidence during the last decades remains an enigma, yet the relative short time span of increasing prevalence points toward a large contribution of environmental factors.

Although current treatment of asthma with inhaled corticosteroids and long-acting $ß_2$ agonist is adequate and safe, concerns remain of the long-term effects of corticosteriods, in particular in light of the decreasing age at which therapy starts for infants. Furthermore, current therapy does not seem to inhibit TGF-ß levels and collagen deposition, important for airway remodeling which in turn contributes to a great extent to airway hyperresponsiveness.

In essence, atopic asthma is the manifestation of an underlying aberrant Th2-dependent immune response to innocuous airborne allergens. The requirement for a Th2-dependent immune response implies the involvement of antigen presenting cells for priming and recall responses to allergen. Dendritic cells (DCs) have emerged as the principal cell type for activation and differentiation of naïve $CD4^+$ T cells towards the distinct subsets. Moreover, deletion of DCs during the primary and secondary immune response of eosinophilic airway inflammation in animal models of asthma revealed an absolute requirement for this cell type during both phases. **Chapter 2** provides an up-to-date review of current research topics in DC biology, in the context of asthma. However, less is known about modulation via DCs of the Th2 response in eosinophilic airway inflammation.

In this thesis, we investigated the effects of DC-driven modulation of the Th2 response in a murine model of eosinophilic airway inflammation based on priming with bonemarrow derived DCs. Furthermore, the unsurpassed capacity of DCs to direct the differentiation of $CD4^+$ T cells, in combination with its requirement for initiating and maintaining eosinophilic airway inflammation, provide potential new strategies for immunotherapy, aimed at modulating the asthma-associated $CD4^+$ T cell response.

A well documented alternative route of $CD4^+$ T cell differentiation is polarization towards the Th1 subset, which antagonizes function and development of Th2 cells. DC-derived IL-12 is an important factor driving Th1 development and it has been postulated that decreased IL-12 production capacity of DCs is a key determinant of allergic sensitization. Several stimuli have been described that promote IL-12 production by DCs, including endotoxins such as LPS. Further support for the role of IL-12 in allergic disorders was the discovery of an inverse correlation between endotoxin exposure and atopic sensitization, leading to the 'hygiene hypothesis'. Indeed, administration of LPS in animal models of asthma was capable of inhibiting eosinophilic airway inflammation development, but these studies did not examine the cellular targets of the administered LPS. In **chapter 3** we investigated whether this LPS-mediated inhibition of Th2 priming was dependent on IL-12 production by DCs.

A more direct approach to study the effects of IL-12 production by DCs on $CD4^+$ T cell differentiation and subsequent eosinophilic airway inflammation was taken by immunizing with DCs retrovirally transduced with the genes encoding for IL-12. In addition, this approach served as a proof of concept for DC mediated immunotherapy for eosinophilic airway inflammation. Despite the fact that immune deviation towards Th1 nowadays is not regarded to be the optimal treatment strategy for allergic airway inflammation, the well documented functions of IL-12 makes it a good candidate to test the feasibility of DC immunotherapy in eosinophilic airway inflammation. The results of this work are described in **chapter 4**.

Another strategy to suppress or prevent eosinophilic airway inflammation is direct inhibition of the associated Th2 response. Recently, new members of the B7 family of costimulatory molecules with inhibitory functions have been identified. Two of these, PD-L1 (B7-H1) and PD-L2 (B7-DC) share the same receptor, expressed on activated T and B cells. This receptor, PD-1, attenuates TCR-mediated T cell activation upon ligation. The use of cell surface bound molecules for immunotherapy has the advantage that only T cells specific for the antigen are inhibited, minimizing unspecific inhibition of bystander T cells. We therefore analyzed the suppressive capacity of PD-L1 and PD-L2 transduced DCs in our model of eosinophilic airway inflammation (**chapter 5**).

A previously unacknowledged direction of signaling has recently been described for several costimulatory pathways, termed 'reverse signaling'. It has been found that both CTLA-4 and CD28 are capable of transmitting signals into DCs, resulting in an inhibitory or stimulatory DC phenotype, respectively. To address whether PD-1 can act in a similar fashion, we analyzed the effects of soluble PD-1 on DC-T cell interaction as well as on each individual cell type. Moreover, we tested whether this reagent had suppressive properties in our mouse model of eosinophilic airway inflammation (**chapter 6**).

In the last chapter, **chapter 7**, the main findings of the previous chapters are summarized and discussed, followed by a review of the pros and cons of the different strategies currently pursued for DC-mediated immunotherapy against allergic disorders such as asthma.

9 References

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2

The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma

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Abstract

Dendritic cells are essential for Th2 differentiation of naïve CD4+ T cells in response to aeroallergens, and in recent years it has been well established that these cells play a pivotal role in the initiation phase of allergic asthma. Dendritic cells are also crucial for maintaining eosinophilic airway inflammation by controlling the recruitment and activation of primed Th2 cells in the lung. A picture is emerging wherein the balance of pathogenic Th2 cells and regulatory T cells is tuned by dendritic cells not only at the initiation but also at the effector stage of the allergic immune response.

Introduction

Allergic asthma is a chronic disease of the airways characterized by eosinophilic inflammation, mucus hypersecretion, and bronchial hyperreactivity, triggered by inhalation of environmental allergens, and eventually leading to structural abnormalities of the lung. T helper type 2 (Th2) cells are crucial for causing disease, by producing key cytokines like IL-4, IL-5 and IL-13 [1]. Dendritic cells (DCs) are essential for priming and Th2 differentiation of naïve $CD4^+$ T cells towards aeroallergens, and in recent years it has been well established that these cells play a pivotal role in the initiation phase of allergic asthma [2]. Upon antigen encounter, respiratory tract DCs migrate to the draining mediastinal lymph nodes where they control the activation and differentiation of antigen specific T cells. Activated effector T cells will migrate back to the site of inflammation and will be (re)stimulated locally by activated DCs. (Figure 1) In this review, we will focus on recent advances in our knowledge of these distinct phases, and how these can be regulated by $CD4^+$ regulatory T (Treg) cells.

Dendritic cell activation and migration

DCs form a network in the airway epithelium that continuously samples the environment for antigen. They migrate to the draining lymph nodes of the lung, also under non-inflammatory conditions [3-5]. This steady state migration of DCs to the mediastinal nodes necessitates continuous replenishment from progenitor cells. It is now clear that blood $Gr1^{lo}$ $CX3CR^{hi}$ monocytes can serve as the immediate precursors of lung DCs in steady state conditions [6]. When DCs are activated however by allergen exposure or viral infection, the migration rate is accelerated, enabling faster transfer of information about the pathogen to the secondary lymphoid organs [7-9]. Under most conditions, DC migration is linked to 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 [5].

Whether distinct DC subsets are responsible for antigen transportation in response to different types of antigen exposure is an area of investigation. Belz and colleagues [10] reported that only a specific subset of $CD8\alpha^-$ $CD11b^-$ $CD11c^+$ DCs transported antigen to the draining nodes after viral infection of the lung, yet non-migratory $CD8\alpha^+$ $CD11c^+$ DCs also acquired the capacity to prime naïve CD8 T cells. In response to harmless ovalbumin inhalation, we demonstrated that both myeloid and plasmacytoid DCs within the mediastinal nodes had taken up antigen, while

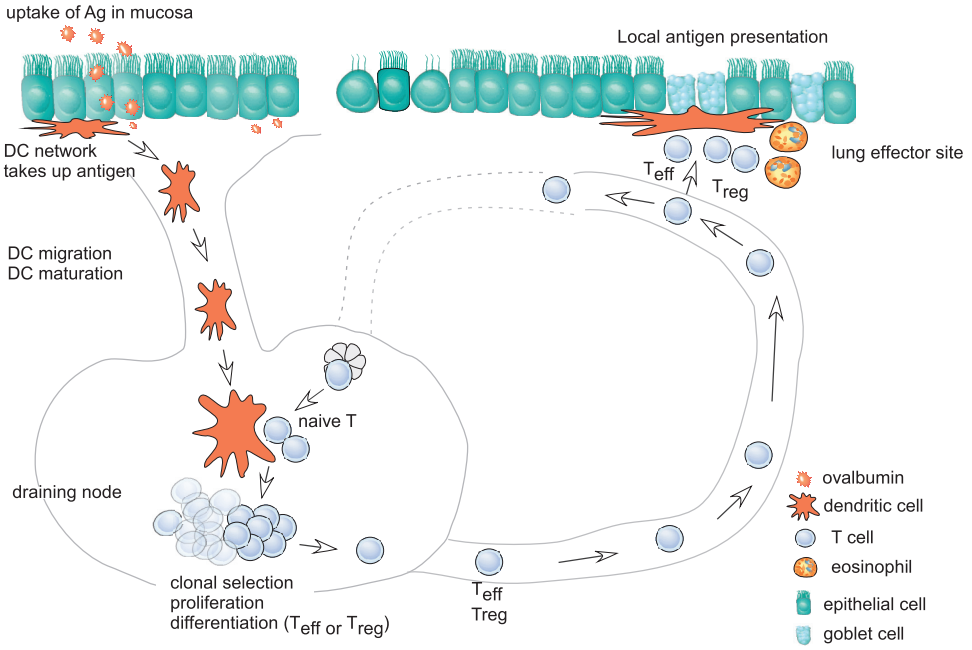

Figure 1 Function of dendritic cells in allergic airway disease
DCs take up antigen when resident in the airway mucosa, and subsequently migrate to the draining mediastinal nodes where they arrive in a mature, antigen priming mode. Depending on the nature of the antigen and the inflammatory context, antigen specific T cells are induced to differentiate into T effector cells (Th1 or Th2) or regulatory T cells (Treg). Differentiated effector T and/or memory T cells subsequently migrate back to the site of allergen exposure where they control inflammation. Here again, they are restimulated by resident airway DCs.

only the myeloid DC subset presented the antigen to naïve CD4 T cells [11]. If DC activation is a prerequisite for enhanced migration under inflammatory conditions, which signals are then responsible for DC activation? A molecular basis for DC activation has been provided with the discovery of pattern recognition receptors (PRRs). PRRs recognize conserved microbial structures, PAMPs, and signaling via PRRs lead to DC activation, defined by upregulation of MHCII and costimulatory molecules. The significance of this finding for induction of pulmonary immunity is underscored by the fact that lipopolysaccharide (LPS) is necessary for Th2 sensitization in mouse models of asthma [12, 13]. Whether other PAMPs such as peptidoglycan (TLR2/6 agonist), ds RNA (TLR3 agonist) or unmethylated CpG motifs (TLR9 agonist) can do the same remains to be shown.

The role of DCs in pulmonary tolerance

Under non-inflammatory conditions, the outcome of a pulmonary immune response to inhaled harmless antigen is tolerance [1, 2, 14]. Several mechanisms have been proposed. The most probable explanation is that partially activated DCs migrate to the lympoid organs, inducing an abortive proliferative response of 'unfit' T cells that fail to reach the threshold for cytokine survival signals, leading to death by neglect

[15]. Another possibility is the generation of regulatory T cells that actively suppress immune responses.

It was shown that, following exposure to harmless antigen, respiratory tract DCs of mice induced a regulatory T cell response, dependent on the B7RP-1 (ICOS-L) and CD86 pathway and on IL-10 secretion. Transfer of these Treg cells to naïve mice before inhaled antigen challenge inhibited inflammatory responses [16-18]. In addition to induced Treg cells, naturally occurring Treg cells, characterized by CD4 and CD25 expression, also appear capable of suppressing Th2 development and eosinophilic airway inflammation [19, 20]. Recently, these data from experimental models have been supported with clinical data. Comparison of IL-4, IL-10 and IFN-γ secretion of allergen-specific $CD4^+$ T cell populations in blood between atopic patients or healthy individuals revealed that healthy individuals have increased numbers of antigen-specific IL-10 secreting cells and decreased numbers of IL-4 secreting cells. An in vitro suppression assay showed that these IL-10 secreting $CD4^+$ T cells have suppressive activity [21]. From these studies a picture is emerging in which the balance between allergen-specific regulatory T cells and Th2 cell is decisive in the development of allergy.

It is presently unclear whether DC-mediated tolerance induction is a function of maturation state, or whether specialized DC subsets are responsible for this phenomenon. Steinman has suggested that peripheral tolerance induction is a function of immature DCs [22]. When bone marrow myeloid DCs were kept in an immature state through pre-treatment with the peroxisome proliferator activating receptor (PPAR)-γ agonist and subsequently injected into the trachea, they suppressed development of airway inflammation in an IL-10 dependent way, whereas when maturation was allowed, they induced Th2 priming [23, 24]. However, data by Umetsu et al. showed that lung DC-mediated tolerance depended upon CD86 and ICOS-L, suggesting that some degree of DC maturity was necessary for tolerance induction, consistent with the fact that mucosal tolerance is accompanied by vigorous naïve T cell division [17, 18, 25]. More and more data seem to suggest that fully mature DCs, induced in the presence of certain PAMPs such as Mycobacterium vaccae, can also induce Treg cells [26, 27].

Tolerance induction could also be the function of specialized DC subsets. A subset inducing peripheral tolerance under non-inflammatory conditions in the spleen and lymph nodes of mice might be the plasmacytoid DCs (pDCs) [28]. Recent work in our laboratory has extended these findings to the lung. We detected a $CD11c^{int}$, $GR\text{-}1^+$, $B220^+$, $CD45RB^+$, $CD11b^-$ subset of DCs in the lungs of mice, resembling the murine pDC phenotype. Strinkingly, depletion of this subset with a pDC-specific antibody prior to exposure to PAMP-free OVA resulted in a breakdown of tolerance to this harmless antigen and led to all Th2-dependent cardinal features of asthma. Conversely, transfer of antigen-laden pDC into naïve mice was able to induce tolerance in an alum-driven mouse model of asthma that normally elicits a strong pulmonary immune response [11]. It is currently unclear how pDCs induce this inhalational tolerance, but ex vivo lung pDCs had the potential to induce Tregs while at the same time inhibiting the generation of effector Th2 cells by myeloid DCs. These findings shed new light on a recent study by Hagendorens et al. [29] that observed a reduction in circulating pDCs in cord

blood of children that subsequently developed atopic wheezing compared to healthy controls. Defects in tolerogenic pDC function at an early age, such as those induced by respiratory viral infection, might therefore predispose to atopic sensitization.

The role of pulmonary DCs in priming CD4$^+$ Th2 immunity

The development of Th2-driven atopic asthma is dependent on a complex interaction between genotypic and environmental factors in the developing immune system of a child. Some recent studies have linked DC biology to known issues of asthma development. Infection with respiratory viruses may induce development and cause worsening of asthma [30, 31]. Recently, Dahl and colleagues provided a structural framework for this observation [32], by demonstrating that in an allergen-induced mouse model of inflammation, pulmonary DCs acquired a stable Th1-promoting phenotype after infection with influenza virus. Surprisingly, subsequent Th2 immune responses to harmless antigen were also increased. Adoptive transfer of pulmonary DCs, obtained after viral infection and clearance, to naïve recipients which were subsequently exposed to allergen showed that this effect was mediated by pulmonary DCs. Thus, contrary to current beliefs, there may be subsets of lung DCs that have an extended lifespan and are able to modulate unrelated immune responses in the opposite polarizing direction.

Alternatively, polarized effector T cells may modulate successive immune responses via modulation of DC function. An elegant study by Alpan *et al.* demonstrated that antigen-specific CD4$^+$ T effector cells could influence the CD4$^+$ T cell differentiation directed against an unrelated antigen by modulating DC function, but only if antigens were presented by the same DCs [33]. This DC modulating capacity of activated CD4$^+$ T cells was IL-4 and IL-10 dependent, possibly explaining why in a Th2-adoptive transfer model of asthma, Th2 priming to an unrelated novel inhaled antigen was dependent on IL-4 produced by the transferred Th2 cells [34].

Immune responses by DCs in the lung are Th2 biased, but nevertheless can be Th1 skewed when the need is there [35, 36]. In recent years a myriad of environmental instruction signals have been described that influence the Th polarizing capacity of DCs. IL-12 is considered to be the key cytokine responsible for Th1 induction, although other cytokines such as IL-23 and IL-27 appear to be important as well [37, 38]. We have shown that retroviral overexpression of IL-12 in myeloid DCs is sufficient to turn these cells into strong Th1 inducers, even in the Th2 prone milieu of the lung [39]. However, IL-12 is not necessary for Th1 development by DCs, as LPS-stimulated IL-12 p40$^{-/-}$ DCs still induce Th1 development in the lung [40]. In contrast to the signals governing Th1 development, the mechanisms for DC-driven Th2 development have remained somewhat enigmatic. According to one theory Th2 development occurs as a default in the absence of polarizing IL-12. Alternatively, some regard Th2 development as an instructive event requiring specific cytokines or cell surface molecules on DCs. Although the prototypic Th2 cytokine IL-4 is important for Th2 development *in vivo*, it is not produced by DCs directly, but can be induced in other cells by DC contact. Early sources of IL-4 (and IL-13) might be naïve T cells, eosinophils or CD1d restricted NKT cells, reacting to antigens

presented by CD1d on airway DCs [41]. Recent work form Amsen identified the cell surface Notch ligand families delta and jagged to instruct for Th1 and Th2 differentiation, respectively [42]. The expression pattern of these ligands on DCs correlated with the ability of known Th1 or Th2 inducing stimuli like cholera toxin, PGE2 or LPS to induce T cell differentiation [43]. Ectopic expression of jagged1 and delta1 skewed naïve $CD4^+$ T cells towards Th2 and Th1, respectively.

A role for DCs beyond sensitization

Dendritic cells are mainly known for their capacity to induce primary T cell activation. Evidence is accumulating that these cells also control inflammatory reactions to foreign and self antigens once T cell priming has occurred, and in this way contribute to the maintenance of T cell mediated diseases such as colitis, diabetes, and asthma.

Following aerosol challenge of Th2 sensitized mice with allergen to induce airway inflammation, there is a dramatic recruitment of myeloid DCs into the airway lumen, bronchial mucosa and lung interstitium, suggesting that DCs are functionally involved in controlling the activation of previously primed T cells [7, 8, 44]. The same pattern is seen after allergen challenge of human asthmatics, leading to an accumulation of myeloid but not plasmacytoid DCs in the airway mucosa [45, 46].

Several lines of evidence suggest that allergen-induced airway inflammation induces local DC maturation, accompanied by upregulation of CD80, CD86, ICAM-1, CD40, PDL-1, PDL-2, but not ICOS ligand (BN Lambrecht, unpublished; ref [7]). There are several possibilities by which DC maturation might be induced. First, allergen activated epithelial cells could release DC-maturation factors such as GM-CSF, defensins or TSLP. Huh et al elegantly demonstrated that airway, but not lung interstitial, DCs upregulated the co-stimulatory molecule CD86 upon contact with allergen specific T cells. These findings suggest that T cells interact locally with primed Th2 cells in the airway mucosa to generate effector function and subsequent inflammation [44]. The question whether (all) costimulatory molecules expressed by mature DCs are important for generating airway inflammation is a matter of debate. Despite the induction of CD80 and CD86 on lung DCs, we found that secondary immune responses induced by adoptive transfer of DCs to the lung were not dependent upon CD80, CD86 or ICOS-L [47]. One way by which DCs might control Th2 effector function and eosinophilic airway inflammation independently of costimulation is through recruitment to the airways of memory Th2 cells. Following allergen challenge, both mouse and human DCs produce the Th2-selective chemokines CCL17 and CCL22 [48, 49]. In elegant studies, Voehringer et al. and Kelly-Welch et al. were able to show that bone marrow derived IL-4R positive cells, putative DCs, were important for controlling the recruitment of inflammatory cells and Th2 cells into the inflamed lungs of mice, suggesting that IL-4 and/or IL-13 might be crucial for amplifying DC-driven Th2 inflammation [41, 50]

The most direct evidence for a functional role for DCs in maintaining Th2 effector responses and eosinophilic airway inflammation came from mice in which myeloid DCs were conditionally depleted using a ganciclovir suicide technique, abolishing all the cardinal features of asthma [51]. These findings have been

extended recently using CD11c-promotor driven diphteria toxin receptor transgenic mice, in which selective depletion of $CD11c^+$ APCs abolished even ongoing established inflammation and bronchial hyperreactivity by reducing Th2 effector production from primed Th2 cells (LS van Rijt and BN Lambrecht, unpublished). These data also indirectly demonstrate that other proposed antigen presenting cell types such as eosinophils, macrophages or epithelial cells cannot take over antigen presentation to primed T cells in the lung. Although eosinophils can support T cell effector function of primed T cells *in vitro*, this function is very limited compared with professional DCs. Although antigen pulsed eosinophils accumulate within the lungs and mediastinal nodes of allergic mice, they fail to strongly activate T cells [52]. One of the striking findings emerging from the proteomics and transcriptomics analyses that have been performed recently is that many proteins expressed in allergic inflammation are associated with alternative activation of macrophages, such as the Ym1 and Ym2 protein, Fizz-1, and arginase (BN Lambrecht, unpublished; refs[53, 54]). Whether alternatively activated macrophages would contribute to ongoing Th2 stimulation in the allergic lung remains to be tested.

Control of airway inflammation by regulatory T cells acting on dendritic cells

From the data described above it is becoming increasingly clear that DCs control effector T cell responses at sites of inflammation. An obvious question therefore is whether Treg cells also exert their action by regulating the function of DCs within sites of inflammation, by producing IL-10, TGF-β or through cell-cell contact. Induction of CD86 on DCs by inflammation might lead to the local expansion of Treg cells, and after a few days of inflammation, Treg numbers might outnumber pathogenic effector T cells [55]. Within sites of chronic inflammation such as colonic inflammation induced by transfer of $CD45RB^+$ cells into SCID mice, Tregs interact locally with DCs and pathogenic effector T cells and suppress effector T cell activation by downregulating OX40L [56]. This might also occur in asthma, where OX40L is critical for effector Th2 cells [57]. The chemokines produced by DCs in response to allergen recognition not only attract pathogenic Th2 cells, but also CCR4 and CCR8 expressing Tregs [49, 58].

How Tregs downregulate inflammation is a matter of debate. In one scenario, Tregs might keep DCs in an immature state, unable to induce Th immunity [59]. In a very interesting study, mice that were deficient in Runx3, a critical downstream mediator of TGF-β signaling, had lung DCs that were in a spontaneously activated and mature state, leading to Th2 mediated lung inflammation to environmental antigens [60]. It was also shown that murine natural Tregs have the capacity to induce 2,3-indoleamine deoxygenase (IDO) activity and tryptophan catabolism in myeloid DCs, thus leading to T cell suppression [61]. High level IDO expression, such as induced by treatment with CpG motifs has been shown to suppress eosinophilic airway inflammation [62].

If Treg cell suppression of allergic immune responses is mediated at the level of the DC, it will be important to find out if DCs from atopic individuals have a reduced capacity to interact with or stimulate Treg function or alternatively are resistant to Treg regulation. Inflammatory cytokines produced by DCs (e.g.

IL-6) might render effector T cells irresponsive to Treg suppression [63]. Alternatively, decreased Treg activity in atopic individual might contribute to chronic inflammation [64].

Conclusion

Dendritic cells have functions not only in the induction of allergic Th2 responses, but also during the effector response as it occurs in clinical asthma. In the coming years it will be interesting to find out how these processes are regulated by regulatory T cells. From a therapeutic perspective, the capacity of DCs to stimulate the expansion and function of antigen specific Treg cells might be exploited to treat allergic disease. Exploiting this knowledge could lead to the design of better prevention or therapeutic strategies for asthma.

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3

LPS induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells

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Abstract

The prevalence of atopic asthma, a Th2-dependent disease, is reaching epidemic proportions partly due to improved hygiene in industrialized countries. There is an inverse correlation between the level of environmental endotoxin exposure and the prevalence of atopic sensitization. As dendritic cells (DC) have been implicated in causing sensitization to inhaled antigen, we studied the effect of endotoxin on Th2 development induced by bone marrow (BM) DC *in vitro* and by intratracheal (i.t.) injection *in vivo*, with particular emphasis on the role played by the polarizing cytokine IL-12.

BM derived DC stimulated with *E. coli* LPS produced IL-12p70 for a limited period of time, after which production became refractory to further stimulation with CD40L, a phenomenon previously called 'exhaustion'. The level of IL-12 production of DC did not correlate with Th1 development, as exhausted OVA-pulsed DC were still capable of shifting the cytokine pattern of responding OVA-specific Th cells towards Th1 *in vitro* and *in vivo*. When mice were first immunized by i.t. injection of OVA-DC and subsequently challenged with OVA aerosol, prior *in vitro* stimulation of DC with LPS reduced the development of airway eosinophilia and Th2 cytokine production, typical of asthma. Most surprisingly, the capacity of LPS to reduce Th2 dependent eosinophilic airway inflammation was IL-12 independent altogether, as IL-12p40 knockout DC had a similar reduced capacity to prime for Th2 responses. These results suggest that LPS reduces sensitization to inhaled antigen by reducing DC-driven Th2 development, but that IL-12 is not necessary for this effect.

Introduction

Thelper type 1 (Th1) and type 2 (Th2) effector cells play a pivotal role in the adaptive immune response towards distinct classes of antigens [1]. Dendritic cells (DC)[3] are generally considered to be the principal antigen presenting cells involved in the generation of polarized effector cells [2]. However, the exact mechanisms by which DC induce polarized T helper responses are only incompletely understood, and many of these mechanisms are complementary. As such, the route of antigen encounter and the subtype of DC presenting the antigen can profoundly influence Th differentiation [3-6]. The type of pathogen encountered or the adjuvant used for DC activation has an even greater impact on Th cell differentiation. DC activating molecular patterns such as LPS [7], poly I:C [8] and CpG motifs [9, 10] induce Th1 polarization by signaling through pattern recognition receptors, while other products, like soluble egg Ags of the helminth *Schistosoma mansoni*, glycoproteins from the filarial nematode *Acanthocheilonema viteae*, or cholera toxin result in a skewing towards Th2 [8, 11]. Recently, yet another model of DC-induced Th cell polarization *in vitro* was proposed [12]. According to this 'kinetics of activation' model, Th cell differentiation following DC encounter with microbial compounds is time-dependent, early in the response favoring Th1 activation while later on favoring Th2 polarization.

All these different models of T cell polarization share the paradigmatic view that the type of Th response that arises correlates closely with the capacity of DC to produce polarizing cytokines such as bioactive IL-12, a dominant Th1 promoting cytokine [2, 4, 13, 14]. Indeed,

subsets of DC have differential capacity to produce IL-12 and many of the Th1-prone molecular patterns in microbes induce IL-12 production in DC [7-9, 13]. Along the same lines, the kinetics of activation model was explained by initial strong production of IL-12 by LPS-stimulated DC, after which prolonged stimulation would lead to exhaustion of IL-12 production, thus favoring Th2 development [12]. According to some models, Th2 development indeed occurs as a default pathway when DC fail to produce polarizing IL-12 [4], although the relevance of this depends on the nature of the antigen and the experimental model used [15-17].

The question how and under which microbial control mechanisms DC induce stable Th polarization is particularly relevant to atopic asthma. Atopic asthma is characterized by chronic eosinophilic airway inflammation, and occurs in individuals with strongly polarized Th2 recall responses to environmental allergen. The incidence of atopy and asthma has greatly increased over recent years, concomitant with an improved hygienic status in the industrialized world, suggesting some form of environmental control over Th2 development [18, 19]. Interestingly, it has been reported that exposure to high levels of LPS endotoxins during early childhood are correlated with a lower incidence of asthma later in life [20]. The mechanisms by which this occurs are currently unknown but systemic exposure to LPS during sensitization to inhaled antigen decreased the severity of airway inflammation in animal models of asthma [21, 22], possibly by shifting the balance of immune responsiveness towards Th1. We have previously shown in a murine model of asthma, that airway DC are essential for inducing Th2 sensitization to inhaled allergen, leading to eosinophilic airway inflammation [6, 23]. As DC are also responsive to LPS, we set out to study the effects of bacterial LPS on Th2 development induced by DC in vitro and in the airways in vivo, with a particular emphasis on the role played by the dynamic secretion of the polarizing cytokine IL-12. Our data show that LPS reduces DC-driven Th2-development in vitro and in vivo, through an IL-12-independent mechanism, resulting in reduced eosinophilic airway inflammation.

Materials and methods

Mice

Female Balb/c mice (6-10 wks old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHCII restricted, TCR transgenic (DO11.10) [24] and IL-12p40$^{-/-}$ mice crossed back on a Balb/c background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolators under specified pathogen-free conditions and experiments were performed under approval of the Erasmus MC committee for animal ethics.

Antigen

Chromatographically purified OVA was obtained from Worthington Biochemical Corp. (Lakewood, NJ). Endotoxin activity was 29 EU/mg, which corresponds to 2.9 ng LPS per mg OVA, as determined by BioWhittaker Europe (Verviers, Belgium). This residual endotoxin activity did not result in IL-12p70 production when added to bone marrow (BM) DC cultures (data not shown). LPS (Escherichia coli, strain O26:B6) was purchased from Sigma (St. Louis, MO) .

Generation and stimulation of bone marrow derived DC

BM derived DC were generated as described [25]. After RBC lysis, BM cells were resuspended at $2*10^5$ per ml in DC culture medium (DC-CM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Biocell,

Rancho Dominguez, CA), 50 µM β-ME (Sigma), 50 µg/ml gentamycin (Invitrogen) and 20 ng/ml rmGM-CSF (a kind gift from Prof. K. Thielemans, VUB, Brussels, Belgium). $2*10^6$ cells were seeded in tissue-culture grade 100 mm petri dishes (day 0). At day 3, 10 ml fresh DC-CM was added. On day 6 and day 8, 10 ml of each plate was centrifuged and resuspended in 10 ml fresh DC-CM. At day 9, cells were pulsed for 24 h with either OVA (100 µg/ml; OVA-DC), LPS (100-500 ng/ml; LPS-DC) or a combination of these two (OVALPS-DC). In some experiments, unstimulated DC (control-DC) were used as a negative control. At day 10, mature DC were harvested by gentle pipetting. In some experiments BM cells were cultured in 6 wells-plates, with cell numbers and culture volumes adjusted to surface area. At day 9, LPS (25 ng/ml) was added for 8 or 24 h, cells were washed with DC-CM, and recultured for an additional 24 h either with or without soluble trimeric CD40L (sCD40L, a generous gift of Dr. C. Maliszewski, Immunex, Seattle, WA) at 2.5 µg/ml. After 24 h, cell recovery was determined and supernatants were kept at –20 °C till further analysis.

Real-time quantitative RT-PCR

Cells were harvested at various time points and total RNA isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was generated from total RNA using random hexamers and the Omniscript Reverse Transcriptase Kit (Qiagen). Relative expression levels were determined with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and probes for murine IL-12p40, IL-12p35, CCR7 and GAPDH were obtained from Perkin Elmer (Foster City, CA). PCR conditions were 50 °C for 2 min., 95 °C for 10 min., followed by 40 cycles of 95 °C for 15 sec. and 60 °C for 1 min. PCR amplification of the housekeeping gene GAPDH was performed to control for sample loading and to allow normalization between samples. Genomic DNA and water controls were included to ensure specificity. Data are expressed as the ratio between cytokine/chemokine transcript levels and GAPDH transcript levels.

CFSE labeling & analysis of T cell division

For fluorescent cell labeling, cells were washed twice with serum-free medium, labeled with 1 µM (DC) or 5 µM (T cells) CFSE (Molecular Probes, Oss, The Netherlands) in serum-free medium for 10 min. at 37 °C, and the reaction was stopped by adding excess ice-cold DC-CM [26]. For quantification of cell division based on serial halving of CFSE intensity, algorithms provided by FlowJo software (Treestar, San Carlos, CA) were used. The number of events N under each CFSE fluorescence peak i was determined using a nonlinear least-squares fitting of a series of Gaussian functions. Once the data set had been fitted, the software derived two statistics to describe the proliferation. The proliferation index (PRI) has been defined as the average number of divisions of the cell fraction that divided (equation 1).

$$PRI = \frac{\sum_{1}^{i} \frac{i \times N_i}{2^i}}{\sum_{1}^{i} i \times N_i} \qquad (1)$$

The responder frequency (%D) has been defined as the percentage of input cells that responded to stimulation by dividing (equation 2).

$$\%D = \frac{\sum_{1}^{i} \frac{N_i}{2^i}}{\sum_{0}^{i} \frac{N_i}{2^i}} \qquad (2)$$

In addition to cell division parameters, the original T cell pool that was necessary to generate the total number of daughter cells can also be calculated and compared between treatment groups (equation 3). Therefore, this so-called 'CFSE content' is an indicator of cell death and/or recruitment of antigen-specific T cells within the lymph node.

$$CFSE\ content = \sum_{0}^{i} \frac{N_i}{2^i} \qquad (3)$$

Th cell priming in vitro

Spleen and lymph node cells were isolated from DO11.10 mice and labeled with CFSE. These cells (10^6/well) were cocultured in a 24-wells plate with OVA-DC ($5*10^4$/well), OVALPS-DC or control-DC.

After 96 h, cells were harvested and secretion levels of IL-4 and IFN-γ detected by flow cytometry.

DC migration and IL-12 staining in vivo

On day 0, mice (n=5) were anaesthetized with avertin and $2*10^6$ CFSE labeled OVA-DC or OVALPS-DC were injected intratracheally (i.t.) in a volume of 80 µl PBS using the technique of Ho and Furst [27]. After 36 h the mediastinal lymph nodes (MLN) were isolated and pooled. As a control for specific migration, the axillar lymph nodes (ALN) were resected. Lymph nodes were incubated for 60 min. at 37 °C in digestion mixture (Collagenase type II, 1 mg/ml, Worthington Biochemicals; DNAseI, 2 U/ml, Sigma), supplemented with monensin ('Golgi-Stop', BD Biosciences, Alphen a/d Rijn, The Netherlands). Frequency and IL-12p40 production of $CFSE^+$ DC were analyzed using flow cytometry.

Primary immune response following intratracheal injection of DC

On day –2, 10^7 CFSE labeled DO11.10 TCR transgenic cells were adoptively transferred i.v. into naïve Balb/c mice. On day 0, mice (n=9-10 per group) were i.t. immunized with 10^6 OVA-DC or OVALPS-DC. On day 4, mice were sacrificed and MLN, ALN and lungs were collected separately. Single cell suspensions of lymph nodes were prepared by mechanical disruption and lungs were homogenized using digestion mixture. Single cell suspensions were analyzed by flow cytometry or were restimulated ($2*10^6$ cells per ml) with 10 µg/ml OVA for 96 h, after which supernatants were harvested and assayed for IL-4, IL-5, IL-10, IL-13 and IFN-γ content.

Secondary immune response following intratracheal injection of DC

On day 0, groups of mice (n=9-10 per group) were immunized i.t. with $1*10^6$ OVA-DC or OVALPS-DC. In some experiments control animals received control-DC. From day 10 onwards, mice were exposed to OVA aerosols (1% (w/v) in PBS) for 3-4 consecutive days, 30 min. daily. Twenty-four hours after the last exposure mice were sacrificed and broncho alveolar lavage (BAL) was performed as described [6]. MLN were isolated and single-cell suspensions were cultured in vitro as described above. As a site-specific control, ALN of each group were pooled and treated identical to MLN.

Airway histology

After BAL, lungs were slowly inflated with 10% buffered formalin, the right-middle lobe excised and fixed in formalin overnight. Subsequently, lungs were embedded in paraffin, 3 µm sections were stained with hematoxylin and periodic acid-Schiff reagent (Sigma) and photographed with a Leica DM-LB microscope (Leica Microsystems, Rijswijk)

Cytokine measurements

Levels of IL-12p70, IL-4, IL-5, IL-10 and IFN-γ in culture supernatants or BAL were measured using OptEIA kits (BD Biosciences) according to manufacturer's instructions. IL-13 levels were measured using a commercially available kit from R&D Systems (Minneapolis, MN)

Flow cytometry

To reduce non-specific antibody binding, anti-FcγRII antibody (2.4G2, ATCC, Manassas, VA) was included in all cell surface stainings. To detect intracellular IL-12p40, single-cell lymph node suspensions were incubated for 3 h in the presence of monensin, followed by cell surface staining. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min. at 4 °C. (Cells were either used directly or stored overnight in FACSwash (PBS, 0.5% BSA, 0.05% NaN_3)). Cells were then resuspended in permeabilisation buffer (Perm/Wash Buffer, BD Biosciences) and stained for 30 min. at 4 °C. The following antibodies were used: anti-CD3ε-PE (145-2C11), anti-pan-NK-PE (DX5), anti-CD19-PE (1D3) in combination with anti-IL-12p40-APC (C15.6) or an isotype matched control.

To assess Th cell priming in vitro, IL-4 and IFN-γ secretion levels were measured with cytokine secretion assays (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Briefly, cell cultures were restimulated with platebound anti-CD3 (145-2C11, BD Biosciences, 1

µg/ml) and anti-CD28 (37.51, 4 µg/ml) for 4 h and subsequently labeled with the capture antibody for either IL-4 or IFN-γ. The cells were then diluted in medium and allowed to secrete cytokines for 45 min. at 37 °C, under continuous rotation. $CD4^+$ T cells secreting cytokines were identified by staining with PE-conjugated IL-4 or IFN-γ detection antibody and CD4-APC (RM4-5). Prior to acquisition, propidium iodide (PI; 0.5 µg/ml) was added for discrimination of dead cells.

To study the primary immune response in CFSE adoptive transfer experiments, cells were labeled with anti-CD4-APC in combination with the anti-clonotypic DO11.10 TCR Ab KJ1-26 [24], conjugated to PE. Dead cells were excluded by labeling with PI prior to acquisition.

Anti-CCR3-PE was used to detect eosinophils in the lung [28], together with anti-CD19-FITC (1D3), anti-CD8-PECy5 (53-6.7) and anti-CD4-APC (RM4-5) to determine the cellular composition in BAL. All fluorochrome-conjugated antibodies were purchased from BD Biosciences, except anti-CCR3-PE, which was from R&D Systems (Minneapolis, MN) and anti-clonotypic-TCR-PE (KJ1-26), which was from Caltag Laboratories (Burlingame, CA). $5*10^4$ – $1.5*10^6$ events were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Statistical analysis

Reported values are expressed as mean ± standard error of the mean (SEM), unless indicated otherwise. Statistical analyses were performed with SPSS (SPPS Inc, Chicago, IL) using a Mann-Whitney U-test. p-Values less than 0.05 were considered significant.

Results

Bone marrow derived DC express IL-12p70 encoding genes

IL-12p70 has been shown to be an important cytokine in the differentiation and polarization of $CD4^+$ Th cells towards type 1 [29, 30]. To investigate the IL-12p70 expression kinetics of murine BM derived DC in vitro, DC cultures were exposed to LPS (100 ng/ml) and relative mRNA expression levels of the subunit genes IL-12p35 and IL-12p40 were determined. Both subunit genes were upregulated after LPS stimulation, reaching a maximum around 12 h after onset of stimulation (Fig. 1A and 1B). After 24 h of stimulation, expression levels of both subunits genes dropped significantly and remained low thereafter. Although LPS mediated apoptosis of DC could be a possible explanation

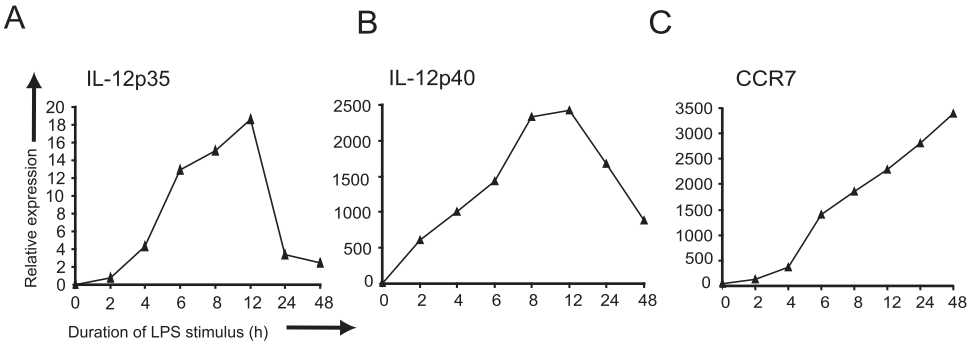


Figure 1. murine DC produce IL-12 transiently after LPS stimulation. BM derived DC cultures were stimulated with LPS (100 ng/ml) at day 9. Cells were harvested at indicated time points, total RNA isolated and converted to cDNA. mRNA expression levels of IL-12p35, (A) IL-12p40 (B) and CCR7 (C) were determined by quantitative real-time RT-PCR. mRNA levels are displayed relative to GAPDH mRNA expression levels in each sample.

for the sharp decrease in gene expression observed, the CCR7 mRNA levels increased consistently after addition of LPS (Fig. 1C), arguing against LPS mediated cell death during culture.

The kinetics of IL-12 subunit gene expression suggested that no IL-12p70 was produced beyond 24 h of LPS stimulation. To test this hypothesis, cultured DC were stimulated for 8 or 24 h with LPS, extensively washed and recultured either in medium (to assess residual IL-12p70 production) or with sCD40L (to test IL-12p70 production capacity after secondary stimulation). As shown in Fig. 2, the residual release of IL-12p70 into fresh medium was greatly reduced after LPS stimulation for 8 h and 24 h (hatched bars). Of note was the decreased production after 8 h of LPS stimulation, despite the expression of the IL-12p35 and IL-12p40 genes (Fig. 1A and 1B), still maximal at this point. However, secretion of IL-12p70 was restored by culturing the cells in a second stimulus provided by sCD40L, a surrogate marker for T cell contact. This restoration did not occur after prolonged LPS stimulation for 24 h (Fig. 2, open bars). Levels of the DC maturation markers CD40, CD80 and CD86 also increased after 24 h of LPS stimulation (data not shown). Altogether, these data show that with progression in time, LPS activated DC acquire a higher costimulatory phenotype, but exhaust their IL-12 production capacity, in agreement with previous findings with human monocyte derived DC [12, 31].

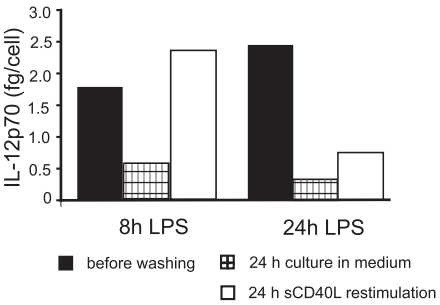
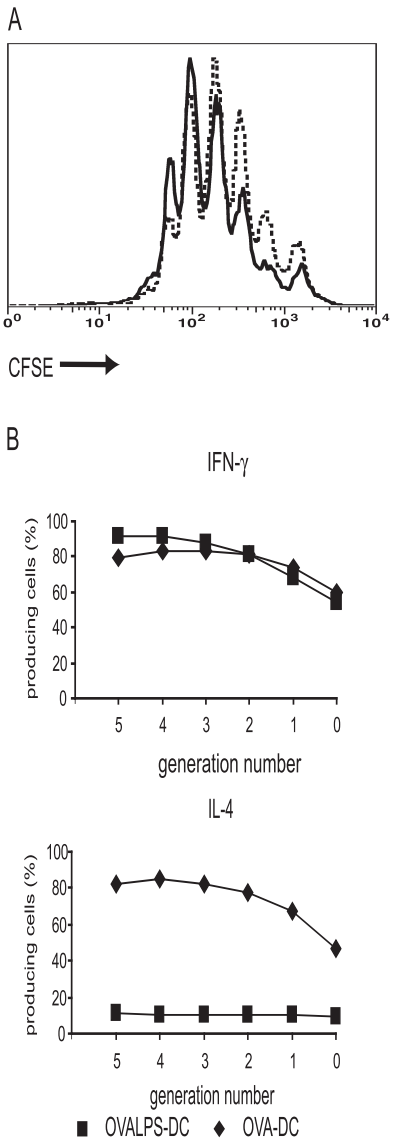


Figure 2. IL-12p70 producing capacity upon restimulation depends on duration of LPS stimulation. BM derived DC were stimulated for 8 h or 24 h with LPS (25 ng/ml). Supernatants were harvested (black bars) and cells were washed with DC-CM. DC were cultured for an additional 24 h either in DC-CM alone (hatched bars) or in the presence of sCD40L (2.5 µg/ml; open bars). Supernatants were harvested and the cell number of each condition was determined. IL-12p70 cytokine levels were measured by ELISA. The IL-12p70 levels are expressed on a per cell basis to account for differences in cell numbers. The data are representative of duplicate experiments.

Th cell activation with LPS stimulated DC result in a reduced Th2 profile in vitro

We next determined the functional consequences of LPS-induced DC maturation and exhaustion of IL-12 secretion, on Th cell polarization in vitro. OVA-pulsed-DC (OVA-DC) were pretreated or not for 24 h with LPS (OVALPS-DC) and were cocultured with naïve CFSE-labeled lymphocytes from OVA-TCR transgenic mice. After 4 days, the cell division profile (Fig. 3A) as well as IL-4 and IFN-γ secretion levels as a function of cell division number (Fig. 3B) of resulting Th effector cells were determined. LPS stimulation had no significant effect on the stimulatory capacity of DC, as the division profile of OVALPS-DC stimulated Th cells was almost similar to that of Th cells cultured with OVA-DC (Fig. 3A). This was confirmed by quantification of the cell proliferation, which revealed no major changes in the average number of divisions of the cell population that divided, hereafter referred to as proliferation index (OVA-DC: 2.52 $\pm$ 0.013, OVALPS-DC: 2.09 $\pm$ 0.023), and the responder frequency, defined as the cell population that participated in clonal expansion



(OVA-DC: 68.9 ± 0.58, OVALPS-DC: 75.3 ± 2.12). In the OVA-DC group, secretion of IL-4 increased with every cell division, reaching a plateau after 4 divisions. The use of OVALPS-DC as APC resulted in significantly decreased levels of IL-4 secretion in each generation of dividing $CD4^+$ T cells compared to the OVA-DC group (Fig. 3B). No major difference in IFN-γ secretion was observed, with a large percentage of $CD4^+$ T cells secreting IFN-γ irrespective of whether the DC were previously stimulated with LPS or not (Fig. 3B). Consistent with these single-cell secretion data, IL-4 and IFN-γ levels in the supernatant, as measured by ELISA, exhibited the same secretion profile (data not shown). The levels of the Th2 associated cytokines IL-5 and IL-13 were also decreased in the supernatant of OVALPS-DC stimulated T cell cultures (data not shown). Thus, LPS-stimulation of DC resulted in a selective decrease in Th2 cytokine secretion in responding T cells, without affecting Th1 cytokine secretion and despite the exhaustion of IL-12 production in vitro. It is unlikely that this reduced Th2 development was due to less stimulatory capacity or apoptosis of OVALPS-DC, since IFN-γ production and T cell division were similar compared with the OVA-DC group.

Figure 3. Stimulatory capacity of LPS stimulated DC on naïve Th cells and the type of effector Th cell generated. BM derived DC were pulsed with OVA protein (100 µg/ml) in the absence of presence of LPS (100 ng/ml). After 24 h incubation, cells were thoroughly washed and used to stimulate CFSE labeled Th cells. Four days later, cells were harvested and IL-4/IFN-γ secretion was measured, together with the division profile of CFSE labeled cells. A, Division profile of living (PI^-), $CD4^+$ cells, stimulated with either OVA and LPS pulsed DC (dashed line) or OVA alone (solid line). B, IL-4 and IFN-γ secretion per generation of PI^-, $CD4^+$ T cells stimulated with OVA-DC (triangles) or OVALPS-DC (squares). Data is depicted as percentage secretion above background. Background secretion levels were defined as the cytokine secretion measured without stimulation and were determined for each group and cytokine individually. Non-pulsed or DC pulsed with LPS do not result in cell division or cytokine secretion of Th cells (data not shown).

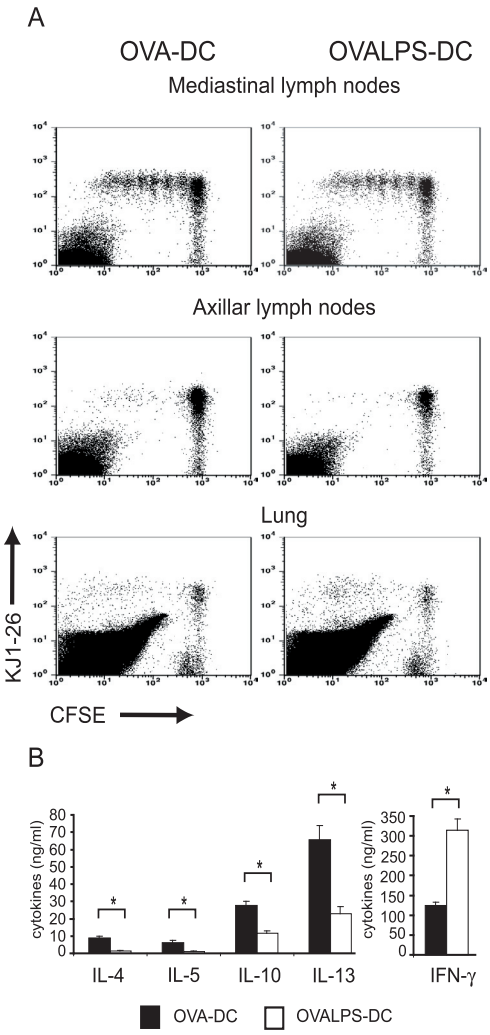


Figure 4. LPS stimulated DC have equal stimulatory capacity but a distinct T helper cell differentiation capacity. Naïve Balb/c mice received a cohort DO11.10 T cells (KJ1-26$^+$) and were subsequently immunized with either OVA pulsed DC (n=9) or OVA+LPS pulsed DC (n=10). Four days later, MLN, ALN and lungs were resected and analyzed for CFSE$^+$ T cells directly or cultured *in vitro* in the presence of OVA for 4 days. A, Frequency and cell division profile of OVA transgenic T cells in the MLN, ALN (PI$^-$, CD4$^+$) and lungs (PI$^-$) was assessed by flow cytometry. Dot plots shown are from representative mice of each group. B, Cytokine levels after *in vitro* restimulation with OVA. No cytokines could be detected in cultures of ALN (data not shown). Results are expressed as mean ± SEM from 5 mice per group. *: p< 0.05

Effect of LPS stimulation of DC on proliferation and differentiation of Ag-specific CD4$^+$ T cell response in vivo

Next, we examined the effect of LPS on the stimulatory and polarizing capacity of DC in vivo. CFSE labeled, OVA-TCR transgenic T cells were adoptively transferred to syngeneic recipient mice, which were subsequently immunized via the airways with OVA pulsed DC, either stimulated with LPS or not. After 4 days, the frequency of OVA-specific T cells in the MLN, ALN and lungs was determined. In the MLN, up to 7 generations of divided cells could be distinguished (Fig. 4A). There was a slight decrease in frequency of OVA-specific T cells in the OVALPS-DC group compared to the OVA-DC group (0.48% ± 0.06% vs. 0.36% ± 0.04%), respectively. Quantification of the cell proliferation revealed a small decrease in the proliferation index, as well as a decrease in the responder frequency (Table I). The quantification of cell division also enabled us to calculate the size of the original OVA-specific T cell population the progeny had arisen from ('CFSE content'), revealing differences in cell number at any given site as a result of recruitment, migration or cell death, independent of cell division. There was no significant decrease in the original OVA-specific T cell pool size in the OVALPS-DC group compared to the OVA-DC group, indicating that LPS stimulated DC were equally capable to recruit naïve T cells (Table I). Due to the insufficient number of OVA-specific progeny T cells, we were unable to calculate the average division number of T cells in the non-draining ALN. However, as evident from Fig. 4A, a minor population of divided cells (>3 divisions) was present. No significant differences in frequencies of the PI$^-$, KJ1-26$^+$ T cell subset could be observed

Table I: Quantification of OVA-specific $CD4^+$ T cell proliferation in the MLN

Immunization	Proliferation index[a]	Responder frequency[b]	CFSE content/ 10^5 PI^- cells[c]
OVA-DC	1.97 ± 0.04[d]	15.70 ± 0.67	237 ± 35
OVALPS-DC	1.82 ± 0.04	11.98 ± 1.30	211 ± 17

[a] defined as the average number of divisions of the $CD4^+$, $KJ1\text{-}26^+$, PI^- cell population that divided
[b] defined as the percentage of input $CD4^+$, $KJ1\text{-}26^+$, PI^- cells that responded to stimulation by dividing
[c] defined as the original T cell pool size necessary to generate the total number of daughter cells present, expressed per 10^5 living cells
[d] values represent the mean ± SEM of the data (n=4-5 animals per group)

(OVA-DC: 0.47% ± 0.02%; OVALPS-DC: 0.48% ± 0.02%). OVA-specific T cells that underwent multiple divisions were also present in the lung but the number was insufficient to quantify the average cell division (Fig. 4A). There were also no differences in the frequencies of the PI^-, $KJ1\text{-}26^+$ T cell subset in the lung (0.117% ± 0.015% versus 0.107% ± 0.004% for OVA-DC and OVALPS-DC, respectively).

To determine the consequences of LPS stimulation on Th cell polarization in vivo, we also measured the cytokine levels of ex vivo cultures of lymph node cells taken at day 4 of the primary response after i.t. injection of DC (Fig. 4B). In the OVALPS-DC immunized animals a decrease in the production of all Th2 cytokines (IL-4, IL-5, IL-10, IL-13) with a concomitant increase in IFN-γ secretion was observed when compared with OVA-DC, indicating a clear shift in balance towards Th1 cytokine secretion.

Migration and IL-12 production of LPS stimulated DC in vivo

Our in vitro experiments indicated that LPS stimulation of DC resulted in exhaustion of IL-12p70 production after 24 h, which would predict less Th1 development and more Th2 development in responding T cells. However, our in vitro and in vivo polarization experiments showed the opposite. To clarify this issue, we analyzed the expression of the IL-12p40 subunit by DC, previously used as a marker for IL-12p70 production by DC in vivo [32], after the migration of i.t. injected DC to the draining lymph nodes of the lung. After incubation with OVA and/or LPS, DC were labeled with CFSE and injected i.t. Thirty-six hours after instillation, there was a substantial increase in the percentage (Fig. 5A) and number (Fig. 5B) of OVALPS-DC migrating to the MLN compared with OVA-DC. These findings of enhanced migration are consistent with the upregulation of CCR7 gene expression and downregulation of CCR5 and CCR6 expression following LPS stimulation of BM derived DC (Fig. 1C and data not shown). The same migration trend was observed when we looked at an earlier time point (24 h; data not shown), which makes it unlikely that there is a difference in migration kinetics between the 2 groups.

In contrast to the in vitro findings, OVALPS-DC produced more IL-12p40 following migration to the draining MLN compared with OVA pulsed DC alone (Fig. 5C).

LPS stimulation of DC results in decreased development of eosinophilic airway inflammation

Despite the discrepancies between T cell polarization capacity and IL-12 secretion capacity of LPS-stimulated DC, the above data demonstrate that LPS-stimulated DC reduce Th2 development during the primary immune response. We next examined the effect of LPS activation of DC in a relevant Th2 dependent mouse model of eosinophilic airway inflammation [6]. In this model, sensitiza-

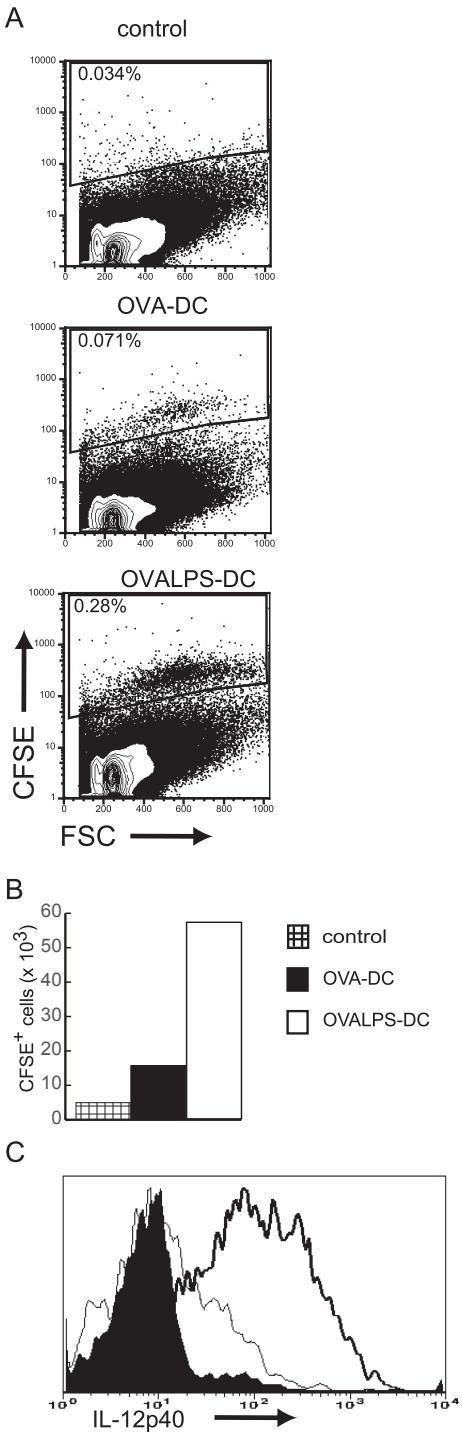


Figure 5. LPS increases migration and IL-12 production of DC *in vivo*. OVA pulsed DC (OVA-DC) and OVA+LPS pulsed DC (OVALPS-DC; both $2*10^6$/ mice) were CFSE labeled and injected i.t. (n=5 per group). Thirty-six hours later, mediastinal lymph nodes from each group were isolated, pooled and analyzed by flow cytometry for migration and IL-12 production of injected DC. As a control for specific migration, the axillar lymph nodes of the OVALPS DC were analyzed as well. Frequency (A) and total number (B) of $CFSE^+$ DC. C, IL-12p40 staining of $CFSE^+$ DC. Solid histogram; isotype-matched control. Regular line; OVA-DC group. Bold line; OVALPS-DC group. $1.5*10^6$ cells per sample were acquired. Data representative of 3 independent experiments.

tion to OVA is induced by i.t. injection of OVA-DC, followed by OVA aerosol challenge 10 days later, resulting in peribronchial and perivascular eosinophilic airway inflammation. When mice were immunized with control DC and subsequently challenged with OVA aerosol, there was no airway inflammation (data not shown and [6]). When analyzing the degree of lung inflammation, immunization with OVALPS-DC revealed only a slight decrease in total cell number in BAL fluid compared with OVA-DC (Fig. 6A). However, the cellular composition of the BAL fluid was markedly different. A significant decrease in the frequency of eosinophils was observed, accompanied by an increase in frequency of alveolar macrophages in the mice immunized with OVALPS-DC (Fig. 6A). The frequency of T cells in BAL fluid was identical in both groups. Lung histology data were in concordance with BAL data, with lower but marked cellular infiltrate and mucus secretion in the OVALPS-DC group compared to the OVA-DC group (Fig. 6D). To explain why the development of airway eosinophilia was suppressed despite similar T cell recruitment, we also measured cytokine levels in lungs and MLN. In the BAL fluid there was a decrease in the levels of IL-5 and IL-13 after immunization with

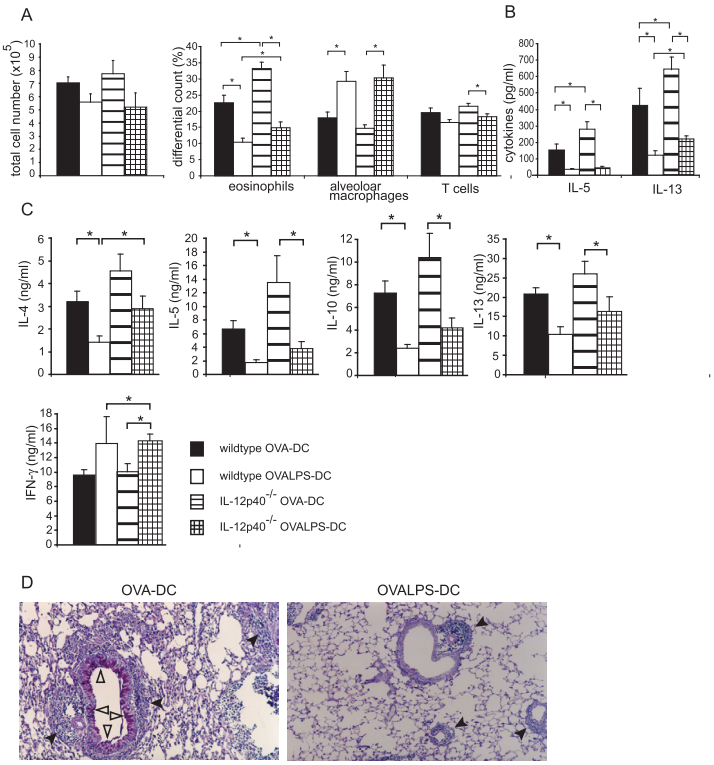


Figure 6. LPS activated DC reduce inflammation in a mouse model of eosinophilic airway inflammation independent of DC derived IL-12. On day 0, groups of mice were immunized by i.t. administration of $1*10^6$ OVA-DC or OVALPS-DC, derived from either wildtype or IL-12p40$^{-/-}$ mice. On days 10-13, mice were exposed to OVA aerosols for 30 min. daily. At 24 h after the last exposure mice were sacrificed, BAL performed and lymph nodes isolated as described in *Materials and methods.* A, Cellular composition of BAL fluid. Alveolar macrophages were characterized by their light scatter and autofluorescence properties. Eosinophils are defined by their CCR3$^+$, CD4$^-$, CD8$^-$ staining pattern. The T cell fraction consists of CD4$^+$ and CD8$^+$ cells within the appropriate light scatter gate. B, Cytokine levels in BAL fluid. C, Cytokine levels after *in vitro* restimulation of MLN cells with OVA antigen. Note that ALN cells of each group were pooled and culture in an identical manner. No cytokines could be detected in these cultures (data not shown). Results are expressed as means ± SEM from 8-10 mice per group. *: $p< 0.05$. Data are representative for 1-3 independent experiments. D, Leukocyte infiltration and PAS staining in lungs of wildtype OVA-DC or OVALPS-DC immunized mice. Solid arrowheads indicate cellular infiltrate, open arrowheads indicate mucus accumulation (PAS$^+$)

OVALPS-DC compared with OVA-DC (Fig. 6B). Th2 associated cytokine levels were also decreased in the MLN, in particular IL-4, IL-5, IL-10 and IL-13, while the levels of the Th1 cytokine IFN-γ did not significantly change following LPS stimulation of OVA-DC (Fig. 6C). These results suggest that LPS stimulated DC, while still eliciting an immune response, have reduced capacity to prime for Th2 effector cells.

LPS mediated suppression of Th2 development in vivo does not require IL-12

In an attempt to dissect the contribution of DC-derived IL-12 and recipient-derived IL-12 on Th2 effector cell generation in vivo, we first immunized wild type mice with DC derived from IL-12p40 homozygous knock-out mice. In the absence of LPS stimulation, BAL fluid analysis revealed that

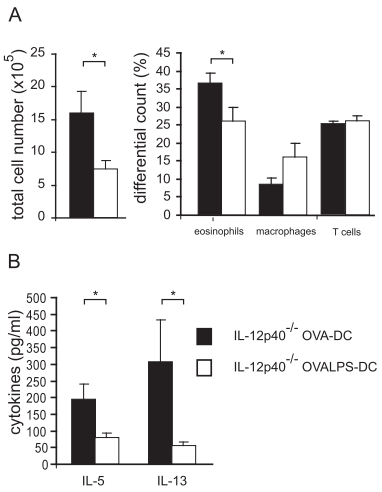


Figure 7. LPS activated DC reduce airway inflammation independently of IL-12. A similar experiment as described in *figure 6* was performed with IL12p40$^{-/-}$ mice derived DC transferred into IL12p40$^{-/-}$ recipients. A, Cellular composition of BAL fluid. B, cytokine levels in BAL fluid. Results are expressed as means ± SEM from 8 mice per group. *: p< 0.05

IL-12p40$^{-/-}$ OVA-DC induced a stronger Th2 response compared with wild type OVA-DC, as the levels of BAL fluid eosinophilia were higher (Fig. 6A) and Th2 cytokine levels in BAL fluid revealed a significant increase in the Th2 cytokines IL-5 and IL-13 in the IL-12p40$^{-/-}$ group (Fig. 6B). Restimulation of lymph node cells in vitro also showed an increase for the Th2 cytokines in the IL-12p40$^{-/-}$ group (Fig. 6C). To our surprise, LPS stimulation of IL-12p40$^{-/-}$ OVA-DC had a similar suppressive effect compared with LPS stimulation of wild type OVA-DC. In the IL-12p40$^{-/-}$ OVALPS-DC group, there was a slight decrease in total cell number accompanied by a statistical significant change in cellular composition, consisting of a decrease in the frequency of eosinophils and an increase in the frequency of alveolar macrophages compared with the IL-12p40$^{-/-}$ OVA-DC group (Fig. 6A). Additionally, the levels of IL-5 and IL-13 were significantly lower in BAL fluid of IL-12p40$^{-/-}$ OVALPS-DC mice compared with the IL-12p40$^{-/-}$ OVA-DC group (Fig. 6B). Finally, Ag specific restimulation of MLN cells in vitro revealed lower levels of the Th2 specific cytokines measured, in particular IL-5 and IL-10, accompanied by a significant increase in the levels of IFN-γ after stimulation with LPS (Fig. 6C). To rule out any role for IL-12 in Th2 differentiation in this model, we next immunized IL-12p40$^{-/-}$ mice with IL12p40$^{-/-}$-derived DC. Immunization with OVALPS DC resulted in lower total cell number, frequency of eosinophils and Th2 associated cytokines in the BAL compartment, compared to OVA-DC immunized mice (Fig. 7A & 7B). In summary, these data suggest that suppression of Th2 effector cell development by LPS stimulation of DC occurs independently of IL-12 secretion by either adoptively transferred DC or recipient-derived IL-12.

Discussion

Th cell polarization is influenced by the route of antigen exposure, the subtype of DC presenting the antigen, the dose of antigen, the genetic background of the host, and most importantly, by the nature of the antigen [2]. It is increasingly clear that different molecular patterns expressed on pathogens can fundamentally influence Th differentiation by signaling through molecular pattern recognition receptors on DC [33, 34]. The way by which these various factors are integrated by DC into a signal that can determine Th cell polarization is an area of intense research but involves the generation of a particular peptide-MHC density (signal 1), the provision of a particular combination of costimulatory molecules (signal 2) and the secretion of polarizing cytokines such as IL-

12, IL-18 and IL-27 [2, 8, 35, 36]. Many of the microbial patterns such as bacterial LPS, peptidoglycan, CpG motifs and viral double stranded RNA, induce the secretion of IL-12 by signaling through the Toll-like receptors and the MyD88 signaling pathway, thus inducing polarization towards Th1 responses [16, 34].

We have examined the role of DC-derived IL-12 in LPS-mediated suppression of Th2 responses and took into account the recently proposed mechanism by which DC exert their influence on Th cell polarization by dynamic and time-dependent secretion of IL-12p70 [12, 31]. We have shown that in vitro BM derived DC stimulated with LPS express IL-12p35 and IL-12p40 mRNA only transiently, in line with other reports [37], while the expression of other maturation markers such as the chemokine receptor CCR7 gradually increased over time. Of note, the IL-12p40 and IL-12p35 peaked at around 12 h post LPS activation, followed by a steep decline in relative expression. The capacity to produce IL-12p70 upon restimulation with a surrogate T cell contact provided by sCD40L also decreased with increasing duration of the LPS stimulus, a phenomenon previously termed 'IL-12 exhaustion'. Surprisingly, despite exhaustion of IL-12 production, LPS stimulated DC used as APC resulted in a more pronounced Th1 phenotype of Th cells in vitro, where individual T cells produced dramatically decreased levels of IL-4 and maintained levels of IFN-γ. Therefore, on a population level, the Th cell differentiation was skewed towards Th1, a finding also supported by the ELISA data. This was not due to a decreased strength of stimulation due to apoptosis of DC, as the overall degree of naïve T cell activation was similar in both groups. Our findings are in contrast with a study of Langenkamp et al. [12], which concluded that LPS-induced IL-12 exhaustion in human monocyte-derived DC contributed to enhanced Th2 and non-polarized central memory Th0 development. In this in vitro model, human naïve T cells were stimulated by the TSST-1 superantigen on syngeneic human monocyte-derived DC, and reacting T cells were expanded for 3-9 days in neutral conditions using IL-2 before analysis of intracellular cytokine content, which might explain differences in outcome. To clarify this discrepancy further, we also examined the relevance of our findings in vivo, where DC interact with naïve antigen specific T cells in the T cell area, providing a physiologically relevant stimulus for terminal functional maturation of DC function, rather than the artificial CD40L stimulation in vitro [38, 39]. To our surprise, we saw that DC that were pulsed with LPS for 24 h and subsequently injected into the lungs migrated to the draining lymph nodes and were still producing IL-12p40 36 h after injection. It is possible that in vivo, many other ligand/receptor interactions in addition to CD40/CD40L contribute to the rescue of IL-12 synthesis. As such, other members of the TNF/TNFR family have been shown to enhance the terminal differentiation of DC as they reach the T cell area [40]. Not surprisingly, also in vivo did the LPS-stimulated DC induce the proliferation and differentiation of naïve OVA-specific T cells towards Th1 cells producing mainly IFN-γ, whereas unstimulated DC induced T cells that made predominantly IL-4, IL-5, IL-10 and IL-13. Therefore, the in vivo data about Th polarization support the in vitro data and argue against the theory that IL-12 exhaustion would contribute to enhanced Th2 development [12]. Based on the observed inconsistency

between IL-12 production capacity and Th cell polarization by DC, we next studied the relevance of LPS-induced IL-12 production by DC using an established model of Th2 effector cell-driven eosinophilic airway inflammation that utilizes DC for immunization [6, 41]. As DC can be manipulated *in vitro* before injection *in vivo*, this model is very useful to address the direct effects of LPS on sensitization via the airways, without avoiding the effects of LPS on other cells in the lungs. When DC were pretreated with LPS prior to injection into the trachea, in particular the allergen-induced influx of eosinophils was markedly decreased. Cytokines associated with eosinophilic airway inflammation, IL-5 and IL-13, were also decreased in the lung. Histological findings were in agreement with the BAL data, with decreased, but not totally absent, cellular infiltrates and mucus secretion. This reduced inflammation in the lung was accompanied by a shift in the cytokine profile in the draining MLN towards a Th1 type of response, with decreased levels of Th2-associated cytokines.

It has been shown that IL-12 has the capacity to downregulate eosinophilic airway inflammation and airway hyperreactivity in mouse models of asthma, when given during the sensitization phase of the response [42-44]. It has been hypothesized that IL-12 exerts its effect via induction of a Th1 type of response that reciprocally dampens the Th2 responses involved in asthma, analogous to what has been shown in models of parasitic infection [45]. Moreover, immunization with DC that constitutively express high levels of IL-12 dramatically reduces eosinophilic airway inflammation (HK, DH and BNL, unpublished data). As LPS induced an increase in IL-12 production in DC following adoptive transfer *in vivo* we initially hypothesized that the development of eosinophilic airway inflammation was suppressed through the release of IL-12 *in vivo*. However, to our surprise, the experiments with IL-12p40 knockout mice indicated that the LPS-induced suppression of eosinophilic inflammation was independent of DC-derived IL-12. Moreover, when both the immunizing DC and the recipient mice could not produce IL-12, LPS was still capable to suppress airway inflammation. A possible explanation for this IL-12 independent effect of LPS might be that alternative pathways or cytokines were involved. Freudenberg *et al.* showed IL-12 independent IFN-γ production by Gram-negative bacteria via STAT4 activation with type I IFN and IL-18 [46]. Other papers that reviewed the role of IL-12 in Th cell differentiation also found that Th1 cells did develop in the absence of IL-12 [16, 47]. In both cases however, IL-12 did amplify the Th1-dependent immune response. The recently described cytokine IL-27 may also be important as it induces IFN-γ production by naïve T cells and is produced by APC. Importantly, its expression is upregulated upon activation by LPS and mice deficient for its receptor TCCR have impaired Th1 responses [36, 48]. Another novel candidate cytokine expressed by DC is IL-23, which shares the p40 subunit with IL-12 [49]. Since the IL-12-deficient mice carry an inactivated p40 subunit, the Th1 polarizing capacity of the DC in these experiments can not be attributed to this cytokine. One finding that also appeared from our studies using IL-12p40 knockout mice was the enhanced Th2 response that was induced in the absence of IL-12 production by DC. It is less established how DC can polarize Th2 responses as there is very little if any evidence that they can produce the Th2 skewing cy-

tokine IL-4 [2]. Perhaps the simplest model for Th2 polarization would be one in which Th cells default to the Th2 pathway in the absence of IL-12 secretion by DC. Cholera toxin, IL-10 and PGE_2 indeed induce Th2 development by suppressing the production of IL-12 in DC *in vitro* [4, 50]. However, in the absence of IL-12, $CD4^+$ T cell responses to the intracellular pathogens *Toxoplasma gondii* and *Mycobacterium avium* and to the parasite *Trichinella spiralis* fail to default to the Th2 pathway *in vivo* [16, 17]. Therefore the exact contribution of DC-derived IL-12 to Th cell polarization is unclear and depends on the nature of the antigen and the experimental model used [4, 8, 16, 17]. It has however been suggested that deficient production of IL-12 by APC of allergic donors might be a key determinant of allergic sensitization [51, 52]. In accordance with this it was recently shown that polymorphisms in the IL-12p40 promotor gene, leading to lower levels of produced IL-12, are clearly associated with the development of atopic sensitization [53], a finding here supported by our data in the mouse model.

The findings in our study that LPS stimulation of DC during the priming alters the outcome of Th responses has implications for understanding the development of atopic sensitization. Studies of the effect of LPS on asthma appear to be complex. Epidemiological data has established a correlation between high endotoxin levels on farms and reduced likelihood of children living on these farms to develop asthma [20], while other studies found that endotoxins augment the severity of asthma once it is established [54, 55]. The effect of LPS in animal models of asthma has also been conflicting, with most studies assigning a protective role to LPS [21, 22, 56], while others do not [57], probably depending on the time and route of antigen and/or LPS administration. One study implicated that outcome of sensitization is dependent on the dose of LPS present during priming, with high doses favoring a Th1 response and low doses a Th2 response [58]. However, the majority of studies that administer LPS before or during sensitization show a decrease in airway inflammation, although it is not exactly clear if this is due to an increase in Th1 effector function. Other bacterial factors that suppress development of Th2-associated sensitization and eosinophilic airway inflammation such as bacterial CpG motifs have also been shown to exert their effects independently of IL-12 and even IFN-γ, suggesting that they do not work by inducing a counter regulatory Th1 population [59]. A recent epidemiological study in German children correlated current levels of LPS exposure in mattress covers with risk of atopic sensitization and diseases and found that increasing levels of LPS exposure led to reduced occurrence of atopic diseases, but also to lower levels of IL-12 production in polyclonally stimulated PBMCs, again arguing against a dominant role for IL-12 production in mediating the effects of LPS on sensitization [60].. We are currently addressing if LPS-stimulated DC reduce eosinophilic airway inflammation by inducing a particular population of regulatory T cells, which have been shown to be involved in tolerance induction in the lung [61]. Indeed, other bacterial motifs such as present in *Mycobacterium vaccae* suppress eosinophilia by such a mechanism [62].

To summarize, we have shown that LPS stimulation of DC suppresses the Th2 dependent development of eosinophilic airway inflammation independently of IL-12. In addition, the IL-12 expression levels of DC could not predict their

polarizing capacity in vitro and in vivo, arguing against the recently proposed pathway of kinetics of activation, in which LPS-induced exhaustion of IL-12 secretion determines Th cell polarization.

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4

Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization

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Abstract

It has been postulated that low level IL-12 production of antigen presenting cells is associated with the risk of developing atopic asthma. To study the relationship between IL-12 production capacity of dendritic cells (DCs) and development of T helper (Th) type 2 responses in the lung, we genetically engineered DCs to constutively overexpress bioactive IL-12. Retrovirally mediated overexpression of IL-12 in DCs strongly polarized naïve ovalbumin (OVA)-specific $CD4^+$ T cells towards Th1 effector cells in vitro. After intratracheal injection, OVA-pulsed IL-12 overexpressing DCs failed to induce Th2 responses in vivo and no longer primed mice for Th2 dependent eosinophilic airway inflammation upon OVA aerosol challenge, readily observed in mice immunized with sham-transfected OVA pulsed DCs. Analysis of a panel of cytokines and chemokines in the lung demonstrated that the lack of Th2 sensitization was accompanied by increased production of the Th1 cytokine IFN-γ, chemokines induced by IFN-γ and of the immunoregulatory cytokine IL-10. When Th2 priming was induced using OVA/alum prior to intratracheal DC administration, DCs constitutively expressing IL-12 were no longer capable of preventing eosinophilic airway inflammation, and even enhanced it. These data directly show that high level expression of IL-12 in DCs prevents the development of Th2 sensitization. Enhancing IL-12 production in DCs should be seen as a primary prevention strategy for atopic disorders. Enhancing IL-12 production in DCs is less likely to be of benefit in already Th2 sensitized individuals.

Introduction

Asthma is an inflammatory disease of the airways leading to significant morbidity and mortality. With advances in the understanding of the molecular and cellular mechanisms involved in the asthmatic response, researchers have identified specific mediators that may be targeted to control the inflammatory state of asthma. The Th2 hypothesis proposes that the inflammation in asthma arises from an imbalance between the two CD4+ T lymphocyte subsets, Th1 and Th2. Th2 cells release many cytokines that have been shown to regulate the inflammatory response, while it has been suggested that the Th1 cytokines counteract this response [1]. As Th2 cells are the main orchestrators of allergic airway inflammation, elucidating how these cells arise from their precursors is an area of intense research [2]. Airway dendritic cells play a crucial role in this process as they pick up allergen in the lung and transport it to the draining lymph nodes (LN) where they prime naïve $CD4^+$ T cells to differentiate into Th1, Th2 or regulatory T cells [3-9]. The precise mechanisms by which DCs determine Th polarization in the lung are currently unknown. It was suggested that the lung tissue microenvironment is Th2-prone, although the exact explanation for this observation is unclear [10-12]. Several factors besides tissue environment influence Th cell differentiation through effects on DCs, in particular the type and dose of antigen, and the presence of polarizing microbial motifs acting through pattern recognition receptors [13-16]. Integration of these different stimuli in DCs leads to the expression of MHC-peptide and costimulatory molecules, and to the release of cytokines and chemokines, resulting in a polarization signal for na-

ïve $CD4^+$ T cells [2, 13]. A key cytokine produced by DCs involved in Th polarization is IL-12, which polarizes naïve CD4 T cells towards the Th1 end of the spectrum [17, 18]. It has been suggested that lung DCs in a resting state produce little bioactive IL-12p70, which might explain the Th2 prone state of the lung [5, 12].

Based on several observations, a reduced capacity of DCs to provide pro Th1 signals, such as IL-12, has been implicated in the development of Th2 responses to inhaled allergens. Firstly, DCs from atopic individuals and their immediate blood monocyte precursors produce less IL-12p70 compared with non-atopic controls [19, 20]. Secondly, sensitization to inhaled allergens occurs predominantly before the age of 12, when the capacity to produce IL-12 is severely reduced compared with adults, potentially explaining the Th2 bias at a young age [21, 22]. In children at risk for allergic diseases, low IL-12 production by circulating DCs in the neonatal period is associated with stronger Th2 responses to inhaled and food allergens [23]. Thirdly, polymorphisms in the IL-12B gene promoter leading to lower levels of IL-12 production are associated with an enhanced severity of atopic asthma in children [24]. Finally, certain diseases characterized by high level production of IL-12 such as multiple sclerosis are associated with a reduced risk of developing allergic diseases [25, 26].

Together, these papers suggest that low IL-12 levels in DCs are associated with the risk of developing Th2 immunity to allergens, whereas high levels of IL-12 production seem to offer protection from allergic diseases. In this paper, we have directly addressed this issue by retrovirally overexpressing IL-12 p35 and p40 in DCs that were subsequently used to sensitize mice to inhaled antigens. For this purpose we used a previously established model of eosinophilic airway inflammation that utilizes ex-vivo generated bone marrow DCs injected in the airways to prime naïve $CD4^+$ T cells [4, 27]. Overexpression of IL-12 in DCs (IL12-DC) strongly reduced Th2 sensitization to inhaled antigen and abolished subsequent eosinophilic airway inflammation, by skewing the response towards strong Th1 immunity. However, DCs overexpressing IL-12 were not capable of preventing eosinophilic airway inflammation in animals sensitized prior to IL12-DC instillation, and even enhanced eosinophilic airway inflammation. These data directly show that high level expression of IL-12 in DCs can prevent the development of Th2 sensitization and therefore should be seen as a primary prevention strategy for atopic disorders. Enhancing IL-12 production in DCs is less likely to be beneficial in already sensitized individuals.

Material & Methods

Mice

Female Balb/c mice (6-10 wks old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHCII restricted, TCR transgenic (DO11.10) mice [28] were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolators under specified pathogen-free conditions and experiments were performed under approval of the Erasmus MC committee for animal ethics.

Retroviral vectors and generation of retroviral particles

The retroviral construct secreting bioactive IL-12p70 (pMFG-IL12) was generated by cloning of the murine IL-12 subunits p35 and p40 into the moloney murine leukemia virus derived backbone MFG [29]. The p35 gene was cloned by PCR from pcDNA1Amp-p35 (a kind gift from Dr. T. Gajew-

ski) with the forward primer 5'-cccatgggtcaatcacgctacctcc-3' and the reverse primer 5'-cccctcaggcggagctcagatagccc-3'. This PCR product was cloned in pCR2.1 and completely sequenced. The p35 fragment was digested with NcoI and EcoRV and cloned into NcoI-SmaI restricted pBlue-IRES (obtained by transfer of the EcoRI-BamHI IRES fragment). The HindIII-XhoI p40 fragment from pcDNA1Amp-p40 (also a gift from Dr. T. Gajewski) and the XhoI-BamHI IRES-p35 fragment were cloned in a three-fragment ligation in pEE14 HindIII-BamHI. The entire IL-12 (p40-IRES-p35) gene was excised with BamHI and cloned in pMFG (a gift from Dr. O. Danos). A retroviral vector not expressing a gene was used to control for virus-specific effects (pMFG-S). Retroviral particles were produced by transient transfection as described [30], with the minor modification that the medium consisted of DC culture medium (DC-CM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Biocell, Rancho Dominguez, CA), 50 µM β-ME (Sigma, St Louis, MO) and 50 µg/ml gentamycin (Invitrogen).

Generation and retroviral transduction of bone marrow derived DCs

Bone marrow (BM) derived DCs were generated as described [30], with some minor modifications. After red blood cell lysis, BM cells were incubated for 30 min with a panel of monoclonal antibodies consisting of anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD45R (RA3-6B2) anti-Ly6-G (RB6.8C5) and anti-I-$A^{b,d,q}$/I-$E^{d,k}$ (M5/114) to deplete lineage positive cells. All the Abs used were purchased from BD Biosciences (San Diego, CA), except anti-I-$A^{b,d,q}$/I-$E^{d,k}$ which was produced in-house. Ab-labeled cells were incubated with sheep anti-rat Ab-coated magnetic beads (Dynal, Oslo, Norway) at a cell-to-bead ratio of 1:4. Lineage-negative cells were plated in 24-well culture plates (10^6 cells/ml; 1 ml/well) in DC-CM supplemented with 20 ng/ml recombinant mouse GM-CSF (produced in-house) and 10 ng/ml recombinant human Flt3-L, (a generous gift of Dr. C. Maliszewski, Amgen, Seattle, WA). After overnight incubation, the cells were replated in 24-well culture plates (5 x 10^5 cells/well in 1 ml). On days 2, 3, and 4 the medium was removed and replaced with 1 ml viral transfection supernatant containing 8 µg/ml polybrene (Sigma). DCs transduced with MFG-IL12 containing supernatant are hereafter designated IL12-DC, MFG-S transduced DCs are hereafter named control-DC. The cells were transduced during centrifugation of the 24-well plates for 2 h at 2500 rpm at 32 °C, after which the supernatant was replaced with DC-CM supplemented with 10 ng/ml recombinant human Flt3-L and 20 ng/ml recombinant mouse GM-CSF. At day 7 and 9, the medium was refreshed with DC-CM supplemented with 20 ng/ml recombinant mouse GM-CSF. At day 10, cells were pulsed with OVA protein (100 µg/ml). This batch of OVA contained low levels of LPS (29 endotoxin units per mg; Worthington Biochemical Corp, Lakewood, NJ) and did not induce IL-12 production in purified BM-DCs (data not shown). After 24 h, DCs were harvested by gentle pipetting and washed three times with PBS. Expression and secretion of IL-12p70 was confirmed with a commercial available ELISA (BD Biosciences).

Effect of IL-12 overexpression in DCs on T cell polarization in vitro and in vivo

Spleen and LN cells were obtained from DO11.10 mice and untouched $CD4^+$ T cells were isolated by negative depletion with a commercially available panel of biotin-conjugated antibodies, followed by labeling with anti-biotin MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting population was typically > 95% $CD4^+$. Cells were labeled with CFSE as described previously [8, 27]. 5 x 105 cells were cultured with OVA-pulsed, MFG-IL12 or control virus transduced DCs, starting at a DC to T cell ratio of 1:10 up to 1:160 in 48-wells plates. After 96 h, cells and supernatant were harvested and OVA-specific T cell proliferation and cytokine levels were determined.

To examine the primary immune response in vivo, 10^7 CFSE labeled transgenic $CD4^+$ T cells specific for OVA323-339 were adoptively transferred i.v. into naive Balb/c mice on day -2. On day 0, mice (n=8-10 per group) were intratracheally (i.t.) immunized with 10^6 OVA-pulsed IL12-DC or OVA-pulsed control-DC. On day 4, mice were sacrificed and mediastinal LN (MLN) and axillary LN (ALN)

were collected separately. Single cell suspensions of LNs were prepared by mechanical disruption and analyzed by flow cytometry or were restimulated (2 x 10^6 cells per ml) with 10 µg/ml OVA for 96 h, after which supernatants were harvested and assayed for IL-4, IL-5, IL-10, IL-13 and IFN- γ content.

Effect of IL-12 overexpression in DCs on the potential to induce asthma

Groups of mice (n=4-10 per group) were immunized i.t. on day 0 with 1 x 10^6 IL12-DC or control-DC pulsed overnight with OVA. From day 10 onwards, mice were exposed to OVA aerosols (1% (w/v) in PBS generated through jet nebulizers) for 3-4 consecutive days, 30 min daily as previously mentioned [8]. Twenty-four hours after the last exposure mice were sacrificed and bronchoalveolar lavage (BAL) was performed as described [31]. In one experiment analyzing gene expression levels of cytokines, chemokines and chemokine receptors, lungs were excised, snap-frozen in liquid nitrogen and stored at –80 °C until processing for RNA analysis.

In a second experiment, the effect of IL-12 overexpressing DCs on the development of asthma in already sensitized mice was studied. Therefore, groups of mice (n=5-10 per group) were first immunized i.p. with 10 µg OVA emulsified in 1 mg aluminum hydroxide (Sigma), a protocol previously shown to induce strong Th2 priming for OVA [31]. Ten days later, control-DC and IL12-DC, either pulsed or not with OVA, were i.t. injected. At day 20, 21 and 22, mice were challenged with OVA aerosol as described above, and after 24 h the degree of airway inflammation was analyzed.

Airway histology

In some experiments, after BAL lungs were slowly inflated with a 1:1 (v/v) mixture of PBS and OCT compound, excised, snap-frozen in liquid nitrogen and stored at –80 °C until further processing. 7 µm sections were cut, subsequently stained with hematoxylin and periodic acid-Schiff reagent (PAS; Sigma) and photographed with a Leica DM-LB microscope (Leica Microsystems, Rijswijk, The Netherlands).

Flow cytometry

To reduce non-specific antibody binding, anti-FcγRII antibody (2.4G2, ATCC, Manassas, VA) was included in all cell surface stainings. To study T cell priming in vitro or the primary immune response in adoptive transfer experiments, T cells were labeled with CFSE and with the anti-clonotypic DO11.10 TCR Ab KJ1-26 [28]. For the in vivo experiments anti-CD4-APC (RM4-5) was also included. Dead cells were excluded by labeling with TOPRO-3 (Molecular Probes, Leiden, The Netherlands) or propidium iodide (PI) prior to acquisition. Cell divisions were quantified as described [8]. Briefly, the CFSE data set was fitted with algorithms provided by the analysis program FlowJo (Treestar, San Carlos, CA), resulting in two parameters that describe the proliferation. The proliferation index (PRI) has been defined as the average number of divisions of the cell fraction that divided and the responder frequency (%D) as the percentage of input cells that responded to stimulation by dividing.

Anti-CCR3-PE was used to detect eosinophils in the lung [32], together with anti-CD8-PECy5 (53-6.7) and anti-CD4-APC to determine the cellular composition in BAL. All fluorochrome-conjugated antibodies were purchased from BD Biosciences, except anti-CCR3-PE, which was from R&D Systems (Minneapolis, MN) and anti-clonotypic-TCR-PE (KJ1-26), which was from Caltag Laboratories (Burlingame, CA). Events were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Cytokine measurements

Levels of IL-12p70, IL-4, IL-5, IL-10 and IFN-γ in culture supernatants or BAL were measured using OptEIA kits (BD Biosciences) according to manufacturer's instructions. IL-13 levels were measured using a commercially available kit from R&D Systems.

Real-time quantitative RT-PCR

Frozen lung tissue was homogenized, RNA isolated with RNeasy midi-prep columns (Qiagen, Hilden, Germany) and treated on-column with DNaseI, according to the manufacturer's protocol. 1 µg RNA was reverse transcribed using SuperscriptII (Invit-

rogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 120 min at 42 °C. Primer sequences are listed in table 1. PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min using an ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA) and SYBR Green mastermix (Stratagene, La Jolla, CA). Water controls were included to ensure specificity and primer pairs were evaluated for integrity by analysis of the amplification plot, dissociation curves and efficiency of PCR amplification. PCR amplification of the housekeeping gene ubiquitin C was performed during each run for each sample to allow normalization between samples.

Statistical analysis

Reported values are expressed as mean ± standard error of the mean (SEM), unless indicated otherwise. Statistical analyses were performed with SPSS (SPPS Inc, Chicago, IL) using a Mann-Whitney U-test. p-values less than 0.05 were considered significant.

Results

Genetically engineered DCs expressing high levels of IL-12 polarize naïve CD4+ T cells towards Th1 in vitro

Following transduction of DCs with a retroviral vector encoding for IL-12p70 (IL12-DC), we detected significantly higher levels of IL-12 p70 in the supernatant at day 11 (median 183 ng/ml, range 73-919 ng/ml, n=8 transductions) compared with DCs transduced with the control constructs (control-DC; median 0 ng/ml, range 0-2.2 ng/ml, n=8 transductions). Phenotypic analysis at day 11 by FACS revealed that the cells exhibited cell surface expression of markers characteristic for DCs (CD11c, MHCII, CD40, CD80, CD86; data not shown), which has been previously shown by us and others [30, 33]. It should be noted that no differences

Table 1: Primer sequences used for real-time quantitative RT-PCR

gene	forward primer	reverse primer	Amplicon (bp)
UbiquitinC[a]	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACA	80
IL-4[b]	CTCATGGAGCTGCAGAGACTCTT	CATTCATGGTGCAGCTTATCGA	70
IL-5[b]	CTCACCGAGCTCTGTTGACAAG	CCAATGCATAGCTGGTGATTTTTAT	79
IL-6[b]	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA	84
IL-10[a]	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	191
IL-13[b]	TGACCAACATCTCCAATTGCA	TTGTTATAAAGTGGGCTACTTCGATTT	132
IFN-γ[a]	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	92
CCL2[a]	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA	127
CCL7[b]	GGGAAGCTGTTATCTTCAAGACAAA	CTCCTCGACCCACTTCTGATG	74
CCL11[b]	CCAGGCTCCATCCCAACTT	TGGTGATTCTTTTGTAGCTCTTCAGT	88
CCL17[b]	GGATGCCATCGTGTTTCTGA	GCCTTCTTCACATGTTTGTCTTTG	75
CXCL5[c]	TCACACTATCGGAAATTGTGATACTTTA	AATGCAATAGTCACCCTCAGTTCA	96
CXCL9[b]	TGCACGATGCTCCTGCA	AGGTCTTTGAGGGATTTGTAGTGG	63
CXCL10[b]	GACGGTCCGCTGCAACTG	GCTTCCCTATGGCCCTCATT	65

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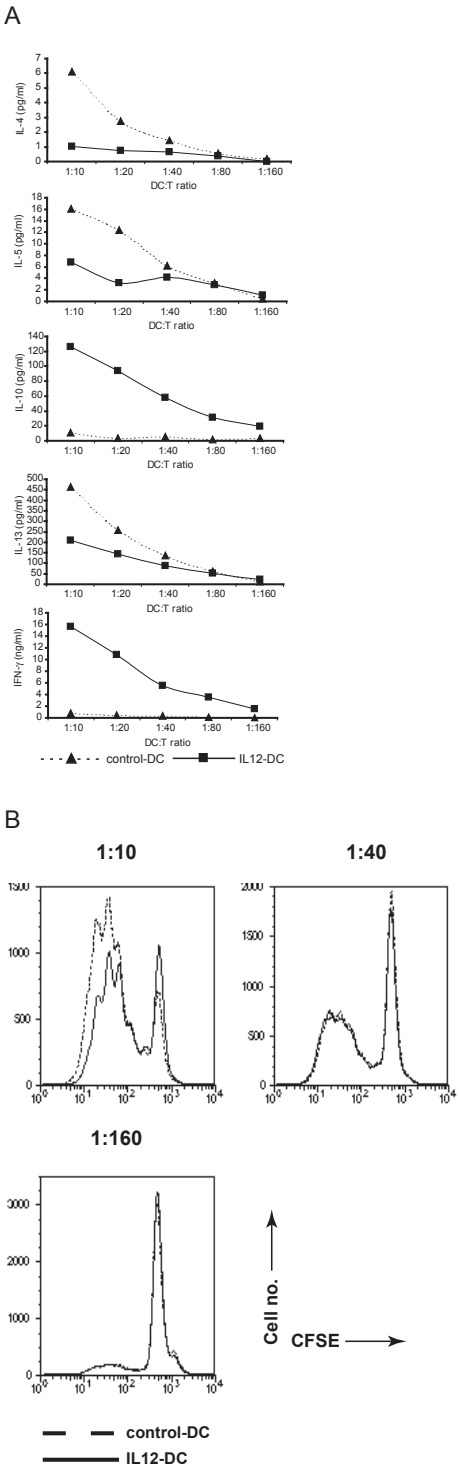

Figure 1 IL-12 producing DCs have equal stimulatory capacity but skew naïve $CD4^+$ T cells towards Th1 in vitro. BM derived DCs were retrovirally transduced with a vector encoding IL-12p70 (IL12-DC) or a control construct (control-DC) and pulsed with OVA (100 µg/ml). After 24 h, cells were harvested, washed and put into culture with purified, CFSE labeled, OVA-specific (DO11.10) $CD4^+$ T cells (5×10^5) at various ratios. Four days later, cells and supernatant were harvested, the division profile of CFSE labeled cells as well as cytokine levels in the supernatant were determined. A, Levels of cytokines in the supernatant at various DC to T cell ratios. Results are representative for two independent experiments. Note the different scale of the graph depicting IFN-γ levels. B, Division profile of living ($TOPRO\text{-}3^-$), OVA-specific ($KJ1\text{-}26^+$) $CD4^+$ cells, stimulated with either control-DC (dashed line) or IL12-DC (solid line). The various ratios of DC to T cells are indicated above each histogram. Of note, there was no cell division when unpulsed DCs were used as APC (data not shown).

could be observed in the levels of maturation markers between control and IL-12 transduced DCs as determined by FACS analysis (data not shown and [30]).

As DC-derived IL-12 is a potent inducer of Th1 responses in vitro as well as in vivo [18], we first established the T cell polarizing capacity of IL12-DC in vitro. OVA-specific, CFSE labeled CD4+ T cells were cultured with OVA-pulsed IL12-DC or control-DC at various ratios of DC and T cells. After four days the proliferation of OVA-specific T cells and the levels of cytokines in the culture supernatants were assayed. There was a major difference in cytokine secretion pattern, with increased levels of the Th1 prototype cytokine IFN- γ as well as IL-10 when IL12-DC were used as APC at a high DC-T cell ratio, while priming with control-DC resulted in higher levels of the Th2-associated cytokines IL-4, IL-5 and IL-13 compared with IL12-DC (Fig. 1A). Because cytokine production was not quantified on the single-cell level but on the whole population and it has been reported that cytokine produc-

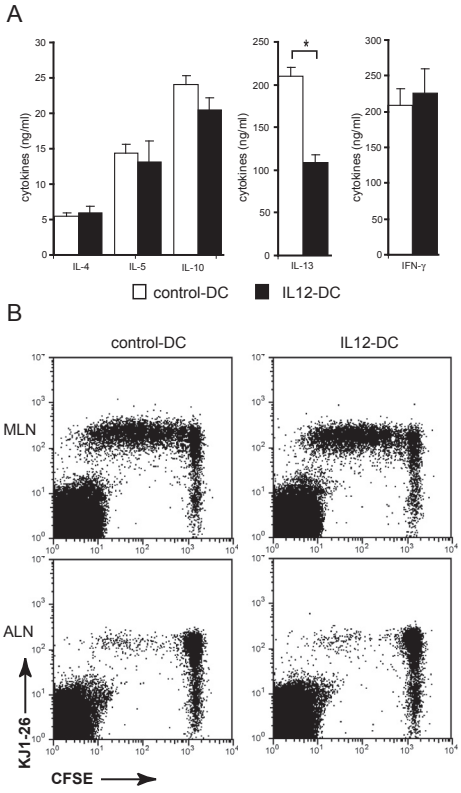

Figure 2 IL12-DC have equal stimulatory capacity but a distinct T helper cell differentiation capacity in vivo. Naïve Balb/c mice received a cohort of DO11.10 T cells and were subsequently immunized with either OVA pulsed IL12-DC (n=8-10) or OVA pulsed control-DC (n=8-9). Four days later, mediastinal lymph nodes (MLN) and axillar lymph nodes (ALN) were resected and analyzed for the presence of OVA-specific, $CFSE^+$, $CD4^+$ T cells directly or stimulated with OVA in vitro for four days. A, Cytokine levels after in vitro restimulation with OVA. No cytokines could be detected in cultures of ALN (data not shown). Results are expressed as mean ± SEM from 7-8 mice per group. *: $p< 0.05$. B, Cell division profile of OVA transgenic T cells ($KJ1\text{-}26^+$, PI^-, $CD4^+$) in the MLN and ALN was assessed by flow cytometry. Dot plots shown are from representative mice of each group. Results are representative for one (cytokines) or two (division profile) independent experiments.

tion may be dependent on the number of T cell divisions [34], we also verified whether the observed differences in cytokine production were attributable to differences in division profiles of divided T cells. The division profiles of T cells stimulated with either IL12-DC or control-DC were identical, except for the highest DC to T cell ratio, when IL12-DC driven T cell proliferation was slightly lower compared to the control-DC group (Fig. 1B). Thus, the observed differences in cytokine expression patterns are likely to be caused by differences in polarization of primed T cells and not in differences in total CD4+ T cell number or division history. Moreover, the efficient proliferation of naïve CD4+ T cell demonstrated that retrovirally transduced DCs are functional APC.

Genetically engineered DCs expressing high levels of IL-12 migrate efficiently to lung draining lymph nodes but do not strongly polarize naïve $CD4^+$ T cells in vivo

Next, we investigated the polarizing capacity of IL12-DC in the draining LN of the lung. OVA-specific T cells were adoptively transferred into syngeneic recipient mice which were subsequently immunized with OVA-pulsed IL12-DC or control-DC. Four days later the MLN and ALN were removed, lymphocytes cultured ex-vivo and cytokines in the supernatant determined (Fig. 2A). In contrast to the in vitro studies, the Th2-type cytokines IL-4 and IL-5 were similar when IL12-DC were used as APC, only the IL-13 levels were statistically significantly decreased compared to control-DC. High levels of IFN-γ were produced in mice immunized with both control-DC and IL12-DC. Also, IL-10 levels were decreased in the IL12-DC immunized group, contradictorily to the in vitro studies.

To rule out the possibility that the priming of T cell was weaker or altered due to decreased migration or increased cell death of i.t. injected DCs, we inject-

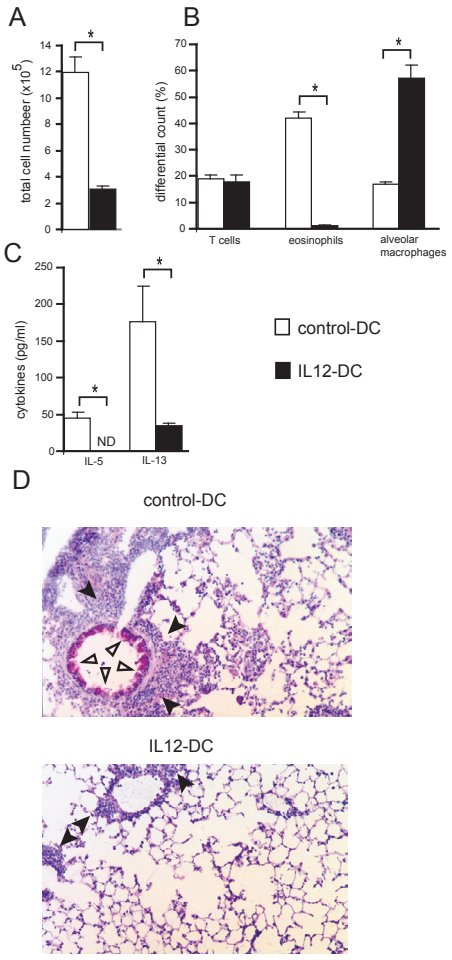

Figure 3 IL12-DC fail to induce eosinophilic airway inflammation. On day 0, groups of mice were immunized by i.t. administration of 1 x 10^6 IL12-DC or control-DC. On days 10 to13, mice were exposed to OVA aerosols for 30 min daily. At 24 h after the last exposure mice were sacrificed, BAL and lung histology performed, and lymph nodes isolated as described in *Materials and methods.* A, total cell number in BAL fluid. B, Cellular composition of BAL fluid. Alveolar macrophages were characterized by their light scatter and autofluorescence properties. Eosinophils are defined by their $CCR3^+$, $CD4^-$, $CD8^-$ staining pattern. The T cell fraction consists of $CD4^+$ and $CD8^+$ cells within the appropriate light scatter gate. C, Cytokine levels in BAL fluid. D, Leukocyte infiltration and PAS staining in lungs of control-DC or IL12-DC immunized mice. Solid arrowheads indicate cellular infiltrate, open arrowheads indicate mucus accumulation (PAS^+). Results are expressed as means ± SEM. *: $p < 0.05$. Data shown is representative of 3 independent experiments with 4-10 mice per group. ND, none detected

ed mice with a cohort of CFSE labeled, OVA-TCR $CD4^+$ T cells which we subsequently immunized with OVA-pulsed IL12-DC or control-DC. Four days later, MLN and ALN were dissected and the division profile of OVA-specific T cells was analyzed. The division profile in the MLN was identical in control-DC and IL12-DC immunized animals (Fig. 2B, upper panels). This was confirmed by quantification of the cell proliferation, which revealed a slight increase in both the average number of divisions of divided T cells (PRI) and the responder frequency, defined as the cell population that participated in clonal expansion (Table 2). In the ALN, representing the non-draining lymph nodes, some OVA-specific T cells that underwent multiple divisions (>5) were present (Fig. 2B, lower panels). As no cells that had divided 1-2 times were present at this non-draining site these cells represent recirculating effector cells, as previously shown [27]. Thus, these data show that IL-12 transduced DCs have a similar stimulatory capacity in vivo compared with control-DC and induce a T cell response characterized by a decreased IL-13 production.

Table 2: Quantification of OVA-specific $CD4^+$ T cell proliferation in the MLN

Immunization	Proliferation index (PRI) [a]	Responder frequency[b]
IL12-DC	2.34 ± 0.05[c]	24.4 ± 3.5
control-DC	2.19 ± 0.04	19.9 ± 2.5

[a] defined as the average number of divisions of the $CD4^+$, $KJ1\text{-}26^+$, PI^- cell population that divided

[b] defined as the percentage of input $CD4^+$, $KJ1\text{-}26^+$, PI^- cells that responded to stimulation by dividing

[c] values represent the mean ± SEM of the data (n=8 animals per group)

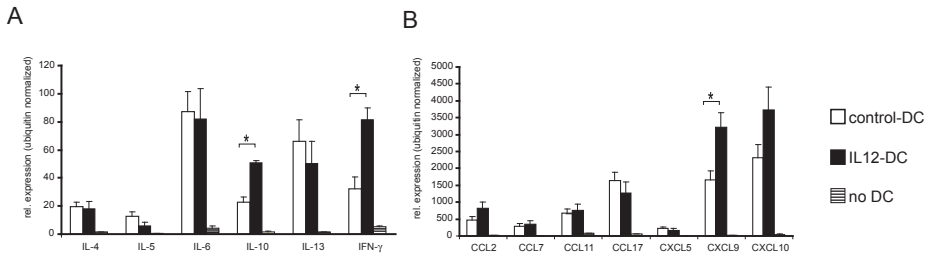

Figure 4 Gene expression pattern in the lungs of IL12-DC immunized mice suggests a Th1 response. Mice were immunized (4-5 per group) as described for figure 3. To account for OVA aerosol induced effects, a control group of non-immunized mice receiving only OVA aerosols was included. At day 14, lungs were excised, snap-frozen, RNA isolated and gene expression of selected cytokines and chemokines was analyzed with real-time quantitative RT-PCR. A, expression levels of cytokine transcripts. B, expression levels of chemokine transcripts. Data shown are the ubiquitin-normalized values of transcript RNAs. Results are expressed as mean ± SEM. *: $p < 0.05$.

Genetically engineered DCs expressing high levels of IL-12 fail to induce eosinophilic airway inflammation

We have previously shown that OVA-pulsed BM-DCs injected into the trachea of naïve Balb/c mice induce sensitization to inhaled OVA and to Th2 dependent eosinophilic airway inflammation upon OVA aerosol challenge [4]. This model was therefore well suited to study the effect of IL-12 overexpression on the potential of these cells to induce Th2 sensitization. As a measure of airway inflammation, we measured the total number of BAL fluid cells 24 h after a series of OVA aerosol challenges and observed that cellularity was lower when mice were immunized with OVA-pulsed IL12-DC compared with control DCs (Fig. 3A). Unpulsed DCs did not induce airway inflammation, as previously reported (data not shown and [4]). Further characterization revealed that the remaining cell population in IL-12 DC immunized group was almost devoid of eosinophils, in contrast to control-DC immunized mice. However, both groups contained identical frequencies of T lymphocytes, well above the level seen in mice immunized with unpulsed DCs (3-5%, ref [4]) (Fig. 3B). The levels of the Th2-associated cytokines IL-5 and IL-13 in the BAL fluid were also significantly decreased in the IL-12 DC group compared with the control-DC group (Fig. 3C). As reported, histological examination of the lungs demonstrated large peribronchial infiltrates rich in eosinophils, as well as PAS-positive goblet cell hyperplasia in the OVA-pulsed control-DC immunized group [4]. Animals immunized with IL12-DC however, showed only small peribronchial infiltrates devoid of eosinophils and almost no mucus production (Fig. 3D), which is consistent with the BAL fluid data.

Altogether, these data demonstrated that immunization with IL-12 producing DCs did not lead to eosinophilic airway inflammation, although the presence of peribronchial infiltrates in the lung and increased numbers of T cells in the BAL fluid indicated that mild inflammation was present. To further characterize the type of inflammation induced, we measured an array of cytokines and chemokines which have been shown to be highly informative of the type of induced Th response in the lung [35]. Mice were immunized with OVA-pulsed IL12-DC or control-

DC and subsequently challenged via the airways. As a control, for the effect of OVA exposure per se, mice that did not receive DCs were challenged as well. 24 h after the final aerosolization, lungs were harvested, snap-frozen and processed for RNA. Gene expression levels were determined with real-time quantitative RT-PCR. Compared to S-DC and IL12-DC immunized animals, almost no cytokine and chemokine expression was observed in the lungs of mice that were solely challenged with OVA aerosol ('no DC' group, Fig. 4A). The cytokine expression pattern observed in the lung when animals were immunized with IL12-DC consisted of a Th1-like response, with significantly elevated levels of IFN-γ mRNA and a trend towards a lower expression of the Th2-type cytokines IL-5 and IL-13 (Fig. 4A). Of note is the concurrent increased expression of IL-10 in IL-12 DC immunized mice, also observed during in vitro $CD4^+$ T cell priming by these DCs (Fig. 1A). The chemokine expression pattern in the lung of these mice correlated highly with the Th1-type cytokine expression pattern, as the IFN-γ-inducible chemokines IP10/CXCL10 and MIG/CXCL9 were strongly increased compared to control-DC immunized mice. Also, the levels of MCP-1/CCL2 mRNA were increased. However, gene expression of the Th2-associated chemokines eotaxin/CCL3, TARC/CCL17 and LIX/CCL5 was similar or slightly decreased when compared to control-DC immunized mice (Fig. 4B). Taken together, it can be concluded that the overall gene expression pattern of cytokines and chemokines of IL12-DC-immunized mice suggested a Th1-type of response in the lung, which is in agreement with the cellular composition and cytokine levels in the BAL fluid (Fig. 3B&C).

Genetically engineered DCs expressing IL-12 fail to revert Th2 sensitization and exacerbate Th2-dependent lung inflammation in sensitized mice.

In view of the potential of Th1 cells to suppress the development of Th2 responses, it has been suggested that Th1 cells might be of potential therapeutic benefit for Th2 mediated diseases. Therefore, we next investigated if IL12-DC could suppress or revert a developing Th2 response (secondary prevention). Mice were first sensitized to OVA in the Th2 adjuvant alum and subsequently treated with OVA-pulsed IL-12 DC or OVA-pulsed control-DC ten days later. Another 10 days later, mice were challenged with 3 OVA aerosols. As a control, mice received unpulsed IL12-DC, control DC, or no DCs. As shown in figure 5, administration of unpulsed IL12-DC or control DC after priming did not affect eosinophilic airway inflammation and Th2 cytokine in the BAL fluid. Exposure of mice to OVA-pulsed DCs, irrespective of their IL-12 production capacity, led to severely enhanced eosinophilic airway inflammation, with enhanced total cell recovery, increase in the frequency of eosinophils (Fig. 5A) and increase of IL-5 and IL-13 in the BAL fluid (Fig. 5B). Thus, IL12-DC are not capable of suppressing a developing Th2-dependent airway response, but rather enhance it when presenting OVA to previously sensitized mice.

Discussion

In this paper, we have directly addressed whether high level expression and secretion of IL-12 in DCs is sufficient to reduce sensitization to inhaled antigens. For this, we first overexpressed the IL-12 p35 and p40 subunit using a retroviral vector that minimally

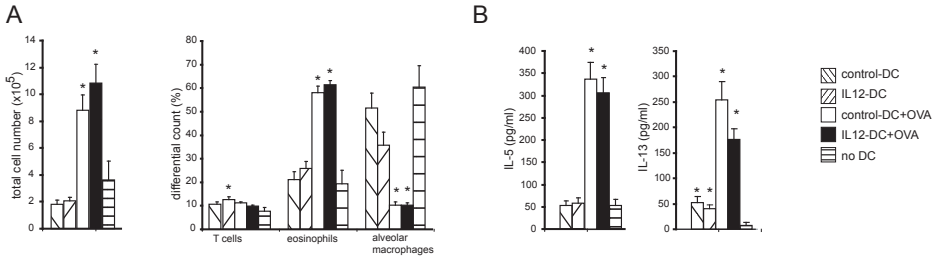

Figure 5 IL12-DC are unable to suppress a developing eosinophilic airway inflammation. Mice were immunized by i.p. injection of OVA/alum. Ten days later, mice were injected i.t. with IL12-DC and control-DC, either or not pulsed with OVA. To control for the effect of i.p. immunization alone, one group did not receive DCs ('no DC'). From day 20 onwards, mice were challenged with OVA aerosol for three consecutive days. Airway inflammation was analyzed at day 24, as described for figure 3. Results are expressed as mean ± SEM from 9-10 mice per group. *: $p < 0.05$ vs. 'no DC' group.

affected the expression of costimulatory molecules on DCs and studied the effect on Th differentiation in vitro and in vivo by injecting DCs into the Th2 prone lung compartment [10]. In vitro, we observed a profound polarization of the cytokine production of naïve $CD4^+$ T cells towards a Th1 phenotype when IL-12 producing DCs were used as APC. This was reflected by decreased levels of IL-4, IL-5, IL-13 and increased levels of IFN-γ compared to control-DC. A striking observation was the concurrent increase of IL-10 with IFN-ã when IL12-DC were used to prime $CD4^+$ T cells. This was shown before for human T cells derived from cell cultures primed in the presence of IL-12 [36, 37]. As we examined IL-10 gene expression at the population level but not at the single-cell level, we cannot conclude that IL-10 and IFN-γ are produced by the same cell. An appropriate candidate would be a subset of regulatory T cells, which produce IL-10 and IFN-γ and have been shown to be generated in the lungs of mice upon challenge with microbial pathogens that induce IL-12 [38]. At a high DC to T cell ratio (1:10), T cell proliferation was slightly lower when IL12-DC were used as APC. Grogan et al., reported a similar phenomenon, when they stimulated naïve $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies under strong Th1 polarizing conditions, implicating that lower proliferation is intrinsic to strongly polarized Th1 cells [39]. At lower DC to T cell ratios, proliferation was identical between both groups, indicating that Th1 polarization was a direct effect of IL-12 overexpression and was not due to the fact that IL12-DC were more mature APCs and thus provided a stronger strength of stimulus to T cells, known to influence Th polarization [40-42]. Ex-vivo lymph node recall responses to OVA antigen from mice immunized with IL12-DC were less clearly polarized. As only a small percentage of i.t. injected DCs migrate to the MLN [8], a possible explanation might be that naïve T cells became less polarized in vivo due to a lower DC to T cell ratio, as supported by the in vitro data.

The Th2 hypothesis of asthma proposes that airway inflammation arises from an imbalance between Th1 and Th2 $CD4^+$ T lymphocyte subsets. Th2 cells release many cytokines that have been shown to regulate the inflammatory response, while it has been postulated that Th1 cytokines may counteract this response [1]. Not surprisingly, systemic or lung administration of the Th1-polarizing cytokines IFN-γ or IL-12 during sensitization to inhaled allergen

was able to inhibit all the cardinal features of asthma through induction of a counterregulatory Th1 subset [43-46]. It was therefore of interest to study whether IL-12 overexpression in DCs also abolished the potential of these cells to prime for eosinophilic airway inflammation, a defining characteristic of asthma. DCs overexpressing IL-12 p70 no longer induced eosinophilic airway inflammation or goblet cell hyperplasia, and concomitantly a reduction was seen in IL-5 and IL-13, known to cause eosinophilic airway inflammation and goblet cell hyperplasia [47, 48]. In contrast, untransfected DCs producing low levels of bioactive IL-12 caused all the salient features of asthma, as previously reported [4, 8]. These observations have important implications for understanding the mechanisms underlying Th2 sensitization and development of allergic diseases. Several authors have suggested that low level production of bioactive IL-12 in APCs underlies the propensity of atopic individuals to develop Th2 responses to commonly inhaled allergens [19, 20, 24]. Conversely, the level of exposure to microbial stimuli in early life, known to induce high level production of IL-12 in APCs, is inversely correlated with the development of atopic sensitization [49]. Several animal models have shown that bacterial stimuli such as heat killed *Listeria monocytogenes*, bacterial LPS and mycobacterium Bacille Calmette Guérin (BCG) reduce the onset of Th2 sensitization and eosinophilic airway inflammation typical of asthma, while at the same time strongly enhancing the production of IL-12 in vivo [7, 8, 50-53]. Our data suggest that the induction of IL-12 by these microbial patterns is more than just a marker of the Th1 bias induced by microbes but is causally related to the inhibition of sensitization.

Although airway inflammation was strongly reduced in IL12-DC immunized mice, compared with control DC immunized mice, there was still induction of BAL fluid lymphocytosis (18%, Fig. 3A) and mild lymphocytic infiltration around blood vessels (Fig. 3D), well above the levels of 1-5% normally seen in naïve or sham sensitized mice [4]. Although this could be the reflection of a Th1 response to OVA aerosol, it was striking to see no associated neutrophilic inflammation, classically associated with Th1 responses to inhaled OVA [54-56]. It was therefore of interest to study the cytokine and chemokine expression pattern in the lungs of IL12-DC immunized mice [35, 57]. By quantitating the mRNA expression level of a panel of cytokines and chemokines, we obtained evidence that immunization with IL12-DC resulted in a Th1 dominated lung pathology, with significantly increased levels of IFN-ã mRNA in the lung. The chemokine expression pattern was in accordance with a predominant Th1 response as increased expression of CXCL9/MIG and CXCL10/IP-10 was seen [39, 57, 58]. CXCL9 and CXCL10 are ligands for CXCR3, a chemokine receptor highly expressed on Th1 cells and believed to be important for migration of these cells to the lung [58-60]. The mRNA levels of the macrophage derived CCL2/MCP-1 chemokine, were also slightly increased as a reflection of Th1 mediated pathology, the most likely source being the increased numbers of alveolar macrophages [35]. Strikingly, BAL fluid lymphocytosis and monocytosis is characteristic of many Th1 mediated lung diseases such as sarcoidosis, hypersensitivity pneumonitis and early transplant rejection. In these diseases, there is an increased production of IL-12, eventually leading to expression of CXCL10/IP-10 chemokine within the

lung [61, 62]. Our data suggest that IL-12 overproduction in DCs in response to recognition of the known or unknown antigens might be the cause of the deranged Th1 response leading to lymphocytary and monocytary alveolitis.

Although these findings suggest a predominant Th1 response, we did not see a decrease in the expression of Th2 associated chemokine CCL11/eotaxin mRNA nor in levels ofIL-5 and IL-13 transcripts between the IL12-DC and control-DC group. As these chemokines and cytokines are known to attract eosinophils to the lung [63], it was striking that there was a dramatic reduction in airway eosinophilia in IL12-DC immunized mice. This might reflect a discrepancy between mRNA levels and protein levels, as for IL-5 and IL-13 it is known that protein levels in the BAL are significantly decreased (Fig. 3C). Another explanation could be the induction of IL-10 and IFN-γ production by IL12-DC. These cytokines have been shown to directly inhibit airway eosinophilia by reducing the recruitment of eosinophils or by inducing their apoptosis [64, 65]. Moreover, it was very recently shown that CXCL9/MIG can act as a direct antagonist of eotaxin-mediated eosinophil recruitment to the lungs [66]. Therefore the high levels of CXCL9/MIG in IL12-DC mice might explain the absence of airway eosinophilia despite high levels of eotaxin production in the lung.

In view of the induction of a polarized Th1 response by IL12-DC in the lung, we questioned whether IL-12 DC were also able to revert or suppress a developing Th2 response to OVA antigen. In contrast to the strong inhibition of Th2 responses seen in the primary immune response to OVA, IL12-DC were unable to suppress eosinophilic airway inflammation in OVA/alum Th2 sensitized mice. Rather, DCs given after Th2 sensitization strongly enhanced airway inflammation and Th2 cytokine production, irrespective of IL-12 production. It is known that polarization of primed Th2 cells is hard to revert, due to loss of IL-12R expression on these cells [67]. It has also been shown that antigen-specific Th1 lymphocytes can enhance the potential of Th2 lymphocytes to cause all the cardinal features of asthma, and therefore IL-12 DCs might exacerbate disease via Th1 induction [68, 69]. Despite this, exogenous administration of recombinant IL-12 can inhibit the salient features of asthma, even when given during the challenge phase in Th2 sensitized mice [43, 44, 70]. It is likely that these treatments lead to effects of IL-12 that are not mediated directly on T cell polarization. It has been shown that high systemic levels of IL-12 suppress the bone marrow output of eosinophil precursors and can directly induce the apoptosis of eosinophils in the lungs [71]. Moreover, IL-12 directly suppresses the formation of eotaxin by lung epithelial cells, independently of IFN-γ [72]. As overexpression of IL-12 was limited to injected DCs in our system, high local levels of IL-12 are only thought to occur in the draining mediastinal lymph nodes during interaction with T cells, not leading to high systemic or lung concentrations, which might explain the differences between recombinant IL-12 administration and IL-12 overexpression in DCs.

In summary, we show in this report that high level expression of IL-12 in DCs renders these cells incapable of inducing Th2 sensitization to inhaled antigen. Finding strategies that enhance IL-12 production in endogenous lung DCs might lead to novel forms of prevention of allergic sensitization.

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5

Constitutive overexpression of programmed death ligand -1 or -2 in dendritic cells inhibits IL-2 production in responding T cells but does not inhibit immune response induction

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Abstract

T cell activation is a complex, multi-step complex involving many molecules. One subset of molecules of particular importance is the family of costimulatory molecules expressed by antigen presenting cells. New members of this family have been discovered in recent years including molecules with inhibitory properties such as programmed death ligand-1 (PD-L1) and PD-L2. PD-L1 (also known as B7-H1) and PD-L2 (B7-DC) are members of the B7 family of costimulatory molecules which are expressed on a variety of cells, including professional APCs such as dendritic cells (DC), but also non-lymphoid organs such as heart, lung and placenta. The receptor for these ligands is PD-1, expressed by T and B cells, which contains inhibitory ITIM/ ITSM domains. PD-1 deficient mice demonstrate generalized T cell hyperreactivity and mice develop signs of severe auto-immunity. Interestingly, PD-L1 expression by human tumors has been implicated in immune evasion. These data have raised the interesting hypothesis that overexpression of PDL-1 and/or PDL-2 might be used to engineer APCs to induce tolerance to self or foreign antigens. We engineered bone marrow-derived DCs to overexpress murine PD-L1 or murine PD-L2 and used these engineered DCs to stimulate naïve OVA-specific (DO11.10) $CD4^+$ T cells with increasing concentrations of antigen. Our results demonstrate that despite profound effects of PD1 ligation on IL-2 production, there were no defects in primary immune response induction in vitro or in vivo when DCs overexpressed PDL-1 or PDL-2, not even when the CD80 and CD86 pathway were eliminated by genetic targeting. These data suggest that strategies aimed at overexpressing negative costimulatory ligands on APCs to induce tolerance to self or transplantation antigens or to allergens are unlikely to be successful. PD-L1 and PD-L2 expression by non-APCs on peripheral tissues might be a more effective means of silencing T cell effector responses.

Introduction

According to the two signal hypothesis of T cell activation, triggering of the TCR by peptide-MHC molecules (signal 1) needs to be accompanied by a second signal that cooperates with TCR signaling to induce optimal T cell activation and avoidance of anergy. Costimulatory molecules were originally defined as signals that are necessary for activation of T cells but have no function in the absence of TCR signaling [1]. However, numerous subsequent studies have shown that elimination of even crucial costimulatory molecules such as CD80 and CD86 or of the receptor CD28 on T cells does not eliminate T cell responses in vivo, illustrating redundancy of co-stimulatory molecules [2-4]. The two-signal hypothesis was further refined with the discovery of co-signaling molecules that deliver inhibitory signals, such as programmed death ligand-1 (PD-L1) and PD-L2 [5, 6]. These findings, together with new insights in the role of antigen affinity in T cell activation as well as the contribution of the duration of T cell-APC interactions to this process, has culminated in the definition of a progressive model of T cell activation. This model proposes that total signal strength delivered to T cells regulates the progression of T cells through hierarchical thresholds of proliferation, differentiation an cell death [7]. As a consequence, costimulation can be viewed as a component of the

total signal strength, capable of either stimulating or inhibiting TCR mediated signaling. Under physiological conditions, T cell stimulation occurs at the dendritic cell-T cell synapse. Dendritic cells (DCs) are unsurpassed in their capability to activate naïve $CD4^+$ T cells and express many costimulatory signals besides CD80 (B7-1), CD86 (B7-2), including PDL-1 and PDL-2 [8, 9]. They also produce cytokines such as IL-2, IL-6 and IL-15 that have important roles in T cell stimulation and differentiation into effector cells [10-13].

Among the new inhibitory co-signaling molecules, PD-L1 (also known as B7-H1) and PD-L2 (B7-DC) are members of the B7 family of costimulatory molecules which are expressed on a variety of cells, including professional APCs such as dendritic cells (DC), but also non-lymphoid organs such as heart, lung and placenta [5, 6, 14-17]. Interestingly, PD-L1 expression by human tumors has been implicated in immune evasion [18, 19]. The receptor for these ligands is PD-1 [20], which contains an ITIM motif and an ITSM motif in its cytoplasmic tail [21, 22]. PD-1 deficient mice demonstrate generalized T cell hyperreactivity and mice develop signs of severe auto-immunity [23, 24]. Upon PD-1 ligation in the presence of CD3/CD28 signaling, these domains recruit the phosphatases SHP-1 and SHP-2, leading to attenuation of TCR-mediated signaling [25, 26]. Consequently, stimulation of human and mouse $CD4^+$ T cells in the presence of PD-1 signaling inhibits proliferation and cytokine production [14, 15, 27, 28]. These data have raised the interesting hypothesis that overexpression of PDL-1 and/or PDL-2 might be used to engineer APCs to induce tolerance to self or foreign antigens, by analogy to what has been performed with overexpression of FasL on DCs [29].

As DCs are the most relevant APCs that express costimulatory signals that contribute to the total signal strength, we decided to overexpress PDL-1 and PDL-2 during interaction with naïve $CD4^+$ T cells. We engineered bone marrow-derived DCs to overexpress murine PD-L1 or murine PD-L2 and used these engineered DCs to stimulate naïve OVA-specific (DO11.10) $CD4^+$ T cells with increasing concentrations of antigen, thereby providing a more physiological stimulus. Our results demonstrate that despite profound effects of PD1 ligation on IL-2 production, there were no defects in primary immune response induction *in vitro* or *in vivo* when DCs overexpressed PDL-1 or PDL-2, not even when the CD80 and CD86 pathway were eliminated by genetic targeting. These data suggest that strategies aimed at overexpressing negative costimulatory ligands on APCs to induce tolerance to self or transplantation antigens or to allergens are unlikely to be successful. PD-L1 and PD-L2 expression by non-APCs on peripheral tissues might be a more effective means of silencing T cell effector responses.

Material & Methods

Mice

Female Balb/c mice (6-10 wks old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHCII restricted, TCR transgenic (DO11.10) mice [30] were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Mice deficient in B7-1 and B7-2 on the Balb/c background [31] were a kind gift from Dr M. Oosterwegel (Utrecht University, Utrecht, The Netherlands). Mice were housed in microisolators under specified pathogen-free conditions and experiments were performed under approval of the Erasmus MC committee for animal ethics.

Vector construction

The retroviral vector expressing murine PD-L1 was constructed by PCR amplification of the murine PD-L1 cDNA using pMET7-mPD-L1 as a template (a gift from Dr. A. Coyle, Millenium Pharmaceuticals, Boston, MA) with the forward primer 5'-AGATCTTCTCCTCGCCTGCAGATAGT-3', containing a BglII restriction site, and the reverse primer 5'-CTCGAGAAGCTGCCAATCGACGATCA-3', containing a XhoI restriction site. The PCR product was cloned into pGEMTeasy (Promega, Madison, USA) and sequenced. The BglII/XhoI PD-L1 cDNA fragment was ligated into the BglII/XhoI digested retroviral vector pRES-GFP RV ([32]; provided by K.M. Murphy), resulting in pmPD-L1-IRES-GFP RV.

The retroviral vector expressing murine PD-L2 was constructed by PCR amplification of the murine PD-L2 cDNA using pBluescriptSK$^+$-mPD-L2 as a template (a gift from Dr. A. Coyle, Millenium Pharmaceuticals, Boston, MA) with the forward primer 5'- GAAGATCTCACCATGCTGCTCCTGCT-3', containing a BglII restriction site, and the reverse primer 5'- CCTCGAGCCCTGCTCTAGATTAGATCCT-3', containing an AvaI restriction site. The BglII/AvaI digested PCR product was cloned into BglII/XhoI digested pIRES-GFP-RV, resulting in pmPD-L2-IRES-GFP RV. For verification, the cloned mPD-L2 cDNA fragment was fully sequenced.

DC transduction

Retroviral particles were produced by transient transfection as described [33]. Bone marrow-derived DCs were transduced as described [33]. At day 11, DCs were harvested by gentle pipetting, resuspended in PBS and GFP-positive DCs were sorted with a FACSDiva or FACSAria flow cytometer (BDBiosciences, Erembodegem, Belgium). DCs transduced with IRES-GFP-RV, mPD-L1-IRES-GFP RV or mPD-L2-IRES-GFP RV are hereafter designated control-DC, PDL1-DC and PDL2-DC, respectively.

DC-T cell coculture

Spleen and lymph node (LN) cells were obtained from DO11.10 mice and untouched CD4$^+$ T cells isolated by negative selection using the CD4 T cell isolation kit and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) The resulting population was typically > 90% CD4$^+$. Cells were labeled with CFSE as described previously [34].

5 x 10^5 CFSE-labeled CD4$^+$ T cells were cultured with either 2.5 x 10^4 control-DC, PDL1-DC or PDL2-DC in 48-wells plates and indicated concentrations of $OVA_{323-339}$ peptide (Ansynth, Roosendaal, The Netherlands) After four days, cells and supernatant were harvested and OVA-specific T cells proliferation and IL-2 levels determined.

Cytokine measurements

Levels of cytokines in culture supernatants or BAL were measured using OptEIA kits (BD Biosciences) according to manufacturer's instructions except for IL-13 levels, which were measured using a commercially available kit from R&D Systems (Minneapolis, MN).

In vivo studies

To induce eosinophilic airway inflammation, groups of mice (n=3-4 per group) were immunized and challenged as described [34], with the modification that 0.5*10^6 EGFP$^+$ sorted control-DC, PDL-1 DC or PDL2-DC, pulsed overnight with OVA, were used for immunization. Twenty-four hours after the last aerosol exposure, mice were sacrificed and bronchoalveolar lavage (BAL) performed.

Flow cytometry

Functional expression of murine PD-L1 and PD-L2 after transfection was confirmed by staining with biotinylated anti-PD-L1 (MIH5) and biotinylated anti-PD-L2 (TY25) (both purchased from ebioscience, San Diego, USA) against these molecules, followed by streptavadin-APC (BD Biosciences). For the DC-T cell coculture experiments and BAL stainings, anti-FcγRIII/II antibody (2.4G2, ATCC, Manassas, VA) was included in all cell surface stainings to reduce non-specific antibody binding,. Dead cells were excluded by labeling with TOPRO-3 (Molecular Probes, Leiden, The Netherlands) prior to acquisition. To study DC mediated T cell division in vitro, cells were labeled with CFSE and with the anti-clonotypic DO11.10 TCR mAb KJ1-26 (Caltag Laboratories, Burlingame, USA) [30]. Anti-CCR3-PE (R&D Systems) was used to detect eosinophils in

the lung [35], together with anti-CD8-PECy5 (53-6.7; BD Biosciences) and anti-CD4-APC (RM4-5; BD Biosciences) to determine the cellular composition in BAL fluid. Events were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR).

Results

Isolation of PD-L1 or PD-L2 overexpressing DCs

PD-L1 and PD-L2 function in a cell-cell contact dependent manner which necessitates purification of transduced DC from a bulk population consisting of both transduced and nontransduced DCs. To accomplish this, we transduced bonemarrow cultures supplemented with GM-CSF with a bicistronic retroviral vector expressing GFP in combination with either PD-L1 or PD-L2. This permits sorting of DCs based on GFP expression (Fig 1). Purity of GFP-positive DCs after sorting was on average 88% (85-92%, 95% confidence interval).

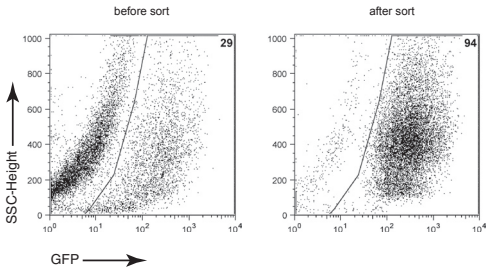

Figure 1 Sorting of transduced DCs. Bonemarrow cells, depleted of lineage positive cells, were transduced at day 2, 3 and 4 with either IRES-GFP-RV, mPD-L1-IRES-GFP RV or mPD-L2-IRES-GFP RV in the presence of GM-CSF. At day 11, cells were harvested, washed with PBS and sorted based on GFP expression. Shown are plots of a representative sort of IRES-GFP-RV transduced DCs. Numbers shown in dotplots are percentages of GFP-positive cells of total cells.

Effect of overexpression of PD-L1 or PD-L2 on DC-driven IL-2 production and T cell proliferation

The majority of data concerning the suppressive function of PD-L1 and PD-L2 have been obtained using highly artificial in vitro systems, including overexpression of PD-L1 and PD-L2 in cell lines co-transfected with MHCII molecules [14, 28]. To obtain insight in the role of PD-L1 and PD-L2 in a more physiological system, we transduced DCs with retroviral vectors expressing either murine PD-L1 or PD-L2. Although mature bone marrow-derived DCs already express PD-L1 and PD-L2 at high levels (data not shown and [16]), we wondered whether constitutive overexpression of PD-L1 and PD-L2 would result in an additional inhibitory effect on T cell proliferation and IL-2 production, as previously observed with artificial APCs. To address this issue, we cultured bonemarrow-derived DC transduced with either mPD-L1 (PDL1-DC), mPD-L2 (PDL2-DC) or control vector (control-DC) with CFSE labeled DO11.10 $CD4^+$ T cells, which have an OVA-specific T cell receptor, at various OVA peptide concentrations. After four days, analysis of the CFSE dilution pattern between control-DC stimulated and PDL1-DC or PDL2-DC stimulated T cells revealed no differences in T cell proliferation at any of the OVA peptide concentrations tested (Fig. 2A). IL-2 levels however, were decreased in the cultures containing PDL1-DC or PDL2-DC, in particular at OVA peptide concentrations of 10 and 1 µg/ml (Fig. 2B). Thus, although DCs constitutively expressing PD-L1 or PD-L2 were not capable of decreasing $CD4^+$ T cell proliferation, production of the autocrine growth factor IL-2 was impaired, demonstrating that even in a strong stimulatory setting as provided by mature DCs constitutive overexpression of inhibitory ligands could convey an inhibitory signal to T cells.

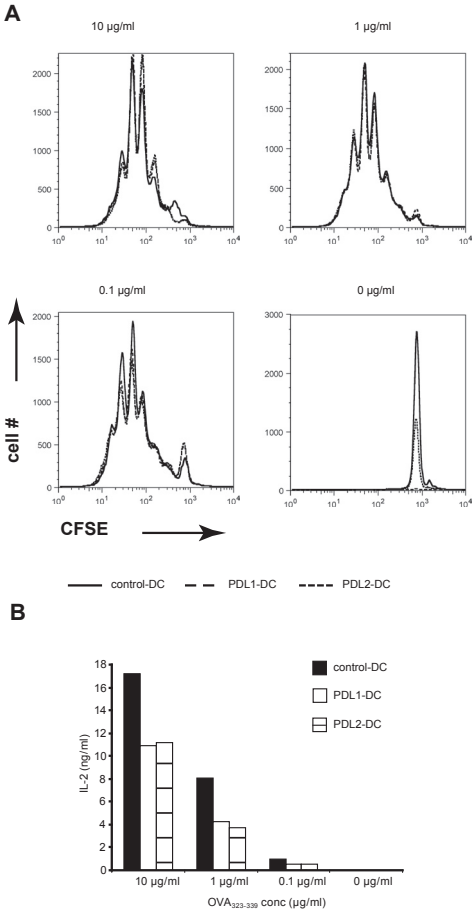

Figure 2 Overexpression of PD-1 ligands by DCs has a partial inhibitory effect on CD4+ T cell responses. CFSE-labeled CD4+ T cells were co-cultured with DC transduced with either IRES-GFP-RV (control-DC), mPD-L1-IRES-GFP RV (PDL1-DC) or mPD-L2-IRES-GFP RV (PDL2-DC) in the presence of indicated concentrations $OVA_{323-339}$ peptide. At day four T cell division was analyzed and levels of IL-2 in the supernatant determined. A, CFSE profile of gated TOPRO-3+, KJ1-26+ cells. B, IL-2 levels in supernatants of DC-T cell cultures. Data representative for one out of three experiments are shown.

Inhibitory effect of PD-L1 or PD-L2 overexpression in the absence of B7-1 and B7-2 costimulation on DCs

With the discovery of inhibitory co-signaling molecules such as PD-L1 and PD-L2, the original two-signal theory of lymphocyte activation has been further refined. Typically, the current view on T cell activation is that signals delivered via co-signaling molecules, either stimulatory or inhibitory, are integrated with TCR-mediated signaling to determine a net effect on T cell activation [5]. Considering the small inhibitory effect seen on T cell activation when DCs constitutively expressing PD-L1 or PD-L2 were used as APCs, we next wondered whether this inhibitory effect would be more pronounced if strong costimulatory molecules for naïve T cells were absent. To address this question, we transduced DCs obtained from CD80/CD86 deficient bonemarrow cells (B7KO DCs) with PD-L1, PD-L2 or control vector and used these DCs to stimulate naïve CD4+ T cells at varying antigen concentrations. After four days, we analyzed the CD4+ T cell division profile and IL-2 production. Not surprisingly, T cell proliferation was decreased at any indicated concentration of antigen when B7KO DCs were used to stimulate T cells (Fig. 3A). However, in the absence of B7 costimulation, we did not observe any inhibition in cell proliferation when PD-L1 or PD-L2 transduced DCs were used compared to control-DC. Indeed, at an antigen concentration of 10 µg/ml, we even observed a slightly increased proliferation of PDL1-DC and PDL2-DC stimulated T cells compared to control-DC, suggestive of a costimulatory role of PD-L1 or PD-L2 (Fig. 2A, right panels).

IL-2 concentrations in T cell cultures stimulated with B7-1 and B7-2 deficient APCs were lower compared to wildtype DC cultures, irrespective of the construct transduced into DCs, except for an OVA peptide concentration of 0.001 µg/ml, where PD-L1 and PD-L2 transduced DCs result in higher IL-2 levels (Fig. 3B). Within the B7KO DC group,

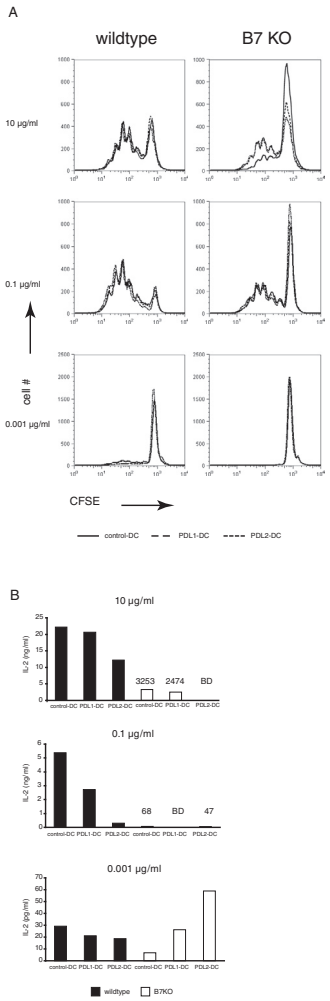

Figure 3 Stimulation of CD4+ T cells by B7 deficient DCs constitutively overexpressing PD-1 ligands does not inhibit T cell proliferation. CFSE-labeled CD4+ T cells were co-cultured with bonemarrow-derived DCs from wildtype (wildtype DCs) or B7 deficient mice (B7KO DCs), transduced with either IRES-GFP-RV (control-DC), mPD-L1-IRES-GFP RV (PDL1-DC) or mPD-L2-IRES-GFP RV (PDL2-DC) in the presence of indicated concentrations $OVA_{323-339}$ peptide. At day four T cell division was analyzed and levels of IL-2 in the supernatant determined. A, CFSE profile of gated TOPRO-3+, KJ1-26+ cells. B, IL-2 levels in supernatants of DC-T cell cultures. Numbers above B7KO DCs cultures indicate levels of IL-2 in pg/ml. No CD4+ T cell division occurred in the absence of antigen (data not shown). BD; below detection.

constitutive expression of PD-L1 and PD-L2 resulted in a strong decrease in IL-2 production at a high antigen dose, which is in contrast with the observed increased proliferation. Surprisingly, at a low OVA peptide concentration of 0.001 µg/ml, the IL-2 production is increased when PD-L1 or PD-L2 DCs were used as stimulators (Fig. 3B; open bars), but again no correlation was observed with T cell division. To summarize, DCs deficient in B7-1 and B7-2 are poor T cell stimulators compared to wildtype DC, as defined by T cell division and IL-2 production. However, transduction of the inhibitory costimulatory molecules PD-L1 and PD-L2 still did not result in decreased T cell divisions. It should also be noted that at high OVA peptide concentrations, there was no correlation between T cell division and IL-2 production in B7KO DC.

Effect of PD-L1 and PD-L2 overexpression on DCs to generate effector T cell responses in vivo

Despite reduced levels of IL-2 in our in vitro culture systems when APCs constitutively expressed the inhibitory 'signal-2' molecules PD-L1 or PD-L2, we could not observe any decrease in proliferation. Gett and co-workers recently showed that priming of naïve T cells in vitro under suboptimal conditions resulted in similar T cell division compared to stimulation under optimal conditions. However, survival of T cells that received a low overall stimulus-strength was much lower, in vitro as well as in vivo, than survival of fully stimulated T cells, a phenomenon termed 'T cell fitness' [36]. To test whether CD4+ T cells stimulated with PDL1-DC or PDL2-DC in vivo have a reduced T cell fitness, we analyzed the immune response in a well-established mouse model of effector T cell dependent eosinophilic air-

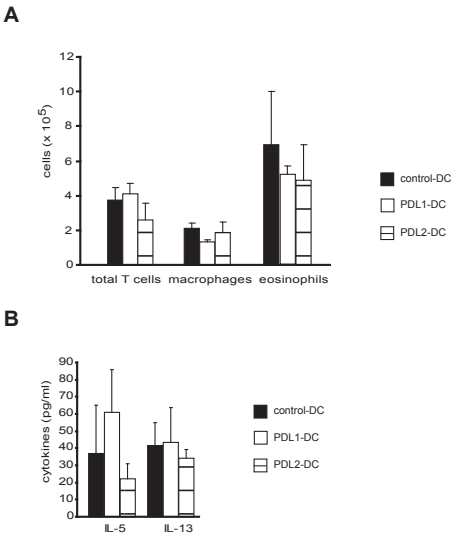

Figure 4 PD-L1 and PD-L2 overexpression by DCs can not suppres eosinophilic airway inflammation development. On day 0, groups of mice were immunized by intratracheal administration of $0.5*10^6$ control-DC, PDL1-DC or PDL2-DC. On days 10-13, mice were exposed to OVA aerosols for 30 min. daily. At 24 h after the last exposure mice were sacrificed, BAL performed and lymph nodes isolated. A, Cellular composition of BAL fluid. Alveolar macrophages were characterized by their light scatter and autofluorescence properties. Eosinophils are defined by their $CCR3^+$, $CD4^-$, $CD8^-$ staining pattern. The T cell fraction consists of $CD4^+$ and $CD8^+$ cells within the appropriate light scatter gate. B, Cytokine levels in BAL fluid. Results are expressed as mean ± SEM from 3-4 mice per group. Data are representative for 2 independent experiments.

way inflammation [37], after immunization with either OVA-pulsed control-DC, PDL1-DC or PDL2-DC. In this model, sensitization to OVA is induced by intratracheal injection of OVA-pulsed DC, followed by OVA aerosol challenge 10 days later. This results in peribronchial and perivascular eosinophilic airway inflammation, which is analyzed 24 hours after the last aerosol exposure through analysis of the cellular composition of BAL fluid, BAL cytokine levels and cytokine production profile in the draining lymph nodes of the lung to determine the type and magnitude of immune response. No statistical significant decrease in the number of eosinophils could be detected in the groups immunized with PDL1-DC or PDL2-DC compared to control-DC (Fig. 4A). Likewise, the levels of the Th2 associated cytokines IL-5 and IL-13 in BAL fluid did not differ significantly between groups (Fig. 4B). In conclusion, based on these in vivo data, it can not be concluded that constitutive, high-level expression of PD-L1 and PD-L2 inhibits the development of eosinophilic airway inflammation, although a trend towards lower inflammation can be observed when PDL2-DC were used for sensitization.

Discussion

Most of the studies that have addressed the role of PD-1 ligation on T cell activation have used either antibody-mediated delivery of TCR-and CD28 costimulatory signals, or cell lines artificially expressing peptide-MHC complexes and CD80/86 to stimulate T cells. This approach carries the risk of over-interpreting the results due to the often high signal strength delivered and the limited number of co-signaling pathways used. Moreover, use of artificial APCs or direct ligation of purified T cells by antibodies largely ignores the role that cytokines released by antigen presenting cells (IL-2, IL-15, IL-6) play in T cell activation [10-13].

In this study, we investigated the contribution of constitutively expressed inhibitory 'signal 2' molecules PD-L1 and PD-L2 to the total signal strength provided by mature, bonemarrow-derived DCs. DCs differentiated in vitro from bonemarrow cells in the presence of GM-CSF express high levels of PD-L1 and PD-L2 (data not shown and [16]), questioning the relevance of this

method. However, constitutive overexpression of molecules already endogenously expressed by DCs such as IL-12 or OX40L have shown beneficial effects in $CD4^+$ T cell polarization and antitumor respectively [33, 38]. Despite the high expression of PD-L1 and PD-L2 by mature bonemarrow-derived DCs, constitutive overexpression of these inhibitory molecules resulted in decreased IL-2 production by T cells, in agreement with other reports [14, 28]. A mechanistic explanation was provided by analysis of the signal transduction pathways affected by PD-1 signaling. Ligation of PD-1 in combination with TCR signaling inhibits phosphorylation of ZAP70 and its association with TCR ζ [25]. In addition, phosphorylation of PKC θ, a novel PKC family member required for IL-2 production [39], is strongly inhibited in the presence of PD-1 signaling [25]. However, at none of the antigen concentrations used in this study did we observe an inhibition of T cell proliferation when PDL1-DC or PDL2-DC were used as APC. Latchman and colleagues, using a similar OVA-restricted TCR T cell assay, reported similar findings, with no inhibition of T cell proliferation at higher antigen concentrations [14]. Another study, using a bead-based T cell activation system with agonistic anti-CD3 antibodies, showed profound inhibition of T cell proliferation in the presence of PD-L1 protein. However, when costimulation was provided by anti-CD28 antibodies, the inhibitory effect of PD-L1 was absent or reduced. Interestingly, exogenous IL-2 could overcome inhibition of proliferation at all time points examined [28]. Although the different experimental setups used make direct comparison between IL-2 levels as reported in this work and the studies mentioned above difficult, we observe much higher levels of IL-2 in our culture systems. As CD28-mediated costimulation acts to a large extent by promoting IL-2 production [40], it appears that mature bonemarrow DCs as used in our experimental setup provide sufficient costimulation for T cell proliferation, even at low antigen concentrations. These findings were supported by an in vitro study blocking PD-L1/2-PD-1 pathway in an allogeneic monocyte-derived human DC-T cell assay. The effects of PD-L1/2 inhibition were more pronounced when immature DC were used as APC compared to mature DC, indicating that the expression of costimulatory molecules such as CD40, CD80 and CD86, which highly correlates with the maturation level of DCs, can balance the negative signals provided by PD-L1 and PD-L2 [41]. One explanation for the fact that overexpressio of PDL-1 and PDL-2 in DCs did not result in T cell suppression might be the production of IL-2 and IL-15 by dendritic cells. It was indeed shown that PD-1 mediated inhibition of human cells was overcome by IL2, IL-7 and IL-15, acting to increase the phosphorylation of STAT-5 and thus upregulating the high affinity IL-2 receptor rendering cells more sensitive to low amounts of IL-2 [42]. It is known that mouse and human DCs produce both IL-2 and IL-15 and this could be the explanation why we still see proliferation despite low IL-2 levels

T cells stimulated with B7-1 and B7-2 deficient DCs (B7KO DCs) exhibited decreased proliferation and IL-2 production, as expected [43]. However, even in the absence of costimulation provided by B7-1 and B7-2, we could not detect any differences in proliferation when B7 KO DC were transduced with PD-L1 or PD-L2. On the contrary, at an antigen concentration of 10 µg/ml OVA protein we consistently observed increased proliferation compared to DC

transduced with control vector. We can not explain these data in light of the total signal strength hypothesis. It has been proposed that a second, unidentified, receptor expressed by T cells exists for PD-L1 and PD-L2 that has costimulatory properties [5], which might be dominant over PD-1 expression or signal transduction in the absence of B7-2/B7-2. In line with this speculation is the observation that PD-1 expression of T cell is lower at higher antigen concentrations [14]. Alternatively, there might be some redundancy in costimulatory molecules, as various other costimulators of the B7 family have been recently described, such as B7-RP1 [44], which could counter-balance inhibitory PD-1 signaling even in the absence of B7-1 and B-2.

Despite the fact that no differences in T cell proliferation could be detected, IL-2 production was consistently lower when T cells were primed with PD-L1 or PD-L2 transduced DCs. Models of T cell proliferation *in vitro* incorporating the effects of IL-2 reveal that small differences in IL-2 concentrations below a threshold concentration have large differences in the final total cell number [45]. Furthermore, analysis of T cell proliferation *in vivo* indicated that T cell exposure to IL-2 is more restricted than *in vitro* [46]. Taken together, based on these studies it can be predicted that inhibition of IL-2 production *in vivo* by PD-L1 or PD-L2 transduced DC could have large outcome on 'T cell fitness' [36] and on the subsequent effector response generated. Therefore, we analyzed the immune response *in vivo* after priming with PD-L1 and PD-L2 transduced DC. We did not observe any statistically significant inhibition of the Th2-dependent eosinophilic airway inflammation, suggesting that the $CD4^+$ T cell response was independent of PD-L1 and PD-L2 overexpression. It could be argued that antigen transfer from adoptively transferred DCs to endogenous DCs dissociate antigen presentation from PD-L1/2 overexpression. However, from experiments involving adoptive transfer of antigen loaded class II deficient DCs it can be concluded that antigen transfer is limited in this particular model of eosinophilic airway inflammation (HK, data not shown)

Based on the data derived from this study it can be concluded that the costimulatory function of PD-L1 and PD-L2 expressed by DCs is relatively minor compared to other pathways such as CD28 and CTLA-4 signaling. Unlike CTLA-4, PD-1 lacks the cysteine residue that allow CTLA-4 to homodimerize. This CTLA-4 conformation allows binding of B7 molecules in a zipper-like oligomerization, forming the basis of the unusually stable signaling complexes at the T cell surface [47]. Therefore, it is now postulated that the principal role of PD-1 signaling is to downregulate immune responses in peripheral tissues through expression of PD-L1 and PD-L2 by 'non-professional' APC [5]. This hypothesis supported by the tissue distribution of these proteins and the abundant PD-L1 expression by many types of tumors [48]. Our data disprove the hypothesis that DCs overexpressing the inhibitory cosignaling molecules PDL-1 or PDL-2 can be used to suppress T cell responses.

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6

Soluble PD-1 inhibits dendritic cell driven T cell activation and eosinophilic airway inflammation

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Abstract

PD-L1 and PD-L2 are members of the B7-family of costimulating molecules, highly expressed by dendritic cells. Although signaling via their receptor PD-1 is thought to inhibit T cell activation, recent studies have suggested a co-stimulatory role for these molecules as well. In this study we used soluble PD-1 to block both PD-L1 and PD-L2 in a more physiologically relevant dendritic cell driven T cell activation system, using OVA-specific TCR transgenic T cells. The addition of soluble PD-1 to DC-T cell cultures decreased IL-2 production and T cell proliferation, particularly when the amount of antigen was limiting, while at the same time increasing the production of IL-10. These inhibitory effects of soluble PD-1 were mainly affecting DCs which acquired a suppressive phenotype, expressing less CD80 and CD86 but producing more IL-10 upon exposure. The high expression of PD-L1 and PD-L2 on pulmonary DCs during T cell driven eosinophilic airway inflammation prompted us to investigate the inhibitory effects of soluble PD-1 in vivo. Administration of soluble PD-1 during sensitization and challenge to OVA in a DC-driven mouse model of asthma resulted in less eosinophilia, IL-5 and IL-13 cytokine production in the BAL fluid of inflamed lungs. Our findings using soluble PD-1 suggest either a costimulatory role for PD-L1/PD-L2 via signaling through a yet unidentified receptor, or similar to soluble CTLA-4, soluble PD-1 exerts its inhibitory effects through acting on DCs. Soluble PD-1 might have therapeutic potential in T cell mediated diseases such as asthma.

Introduction

Dendritic cells (DCs) are extremely proficient inducers of T cell activation due to their high expression level of peptide-MHC and an array of accessory molecules involved in cell migration, adhesion and co-signaling molecules. In particular the B7-1/2-CD28/CTLA-4 axis of co-signaling molecules has been shown to play an important role in this respect. A new layer of complexity has been added with the discovery of new B7 family members, both with stimulatory as well as inhibitory properties. Two of these newly described molecules are PD-L1 (also known as B7-H1) [1, 2] and PD-L2 (also known as B7-DC) [3, 4]. PD-L1 is expressed on a broad variety of murine tissues and cells, including DCs, and can be upregulated by inflammatory stimuli. PD-L2 expression is restricted to macrophages and DCs [5]. Both co-stimulatory molecules share the same receptor PD-1 which is expressed on activated T cells. The majority of experimental data suggests that ligation of PD-1 inhibits T cell activation or induces cell death [6]. PD-1 contains an immunoreceptor tyrosine-based inhibitory motif and mice deficient in PD-1 develop autoimmune disorders suggesting a defect in peripheral tolerance [7, 8]. Whereas most investigators agree that PD-1 delivers an inhibitory signal to T cell, various studies have shown that PD-L1 and PD-L2 can act as co-simulators [2, 4, 9, 10] as well as co-inhibitors [1, 11, 12] of T cell activation in vitro. A possible explanation for the observed discrepancies could be the fact that most studies used different highly artificial T cell stimuli, consisting of CD3-or TCR-specific agonistic antibodies or artificial antigen presenting cell lines expressing MHC-peptide complexes and transfected with PD-L1

or PD-L2.

Due to the absence of physiologically relevant APCs (DCs, macrophages, B cells) a possible caveat in these studies could be the lack of the recently described phenomenon of 'reverse signaling'. This feedback mechanism consists of B7 family members acquiring receptor functions and transmitting signals into the APC, upon triggering of their receptor (CD28 related immunoglublin superfamily members). Studies by Grohmann and colleagues showed that stimulation of CD80/CD86 on DCs with soluble CTLA-4 (CTLA4-Fc fusion protein) resulted in upregulation of the enzyme indoleamine 2,3-dioxygenase (IDO) activity, which subsequently inhibited T cell activation by tryptophan depletion, while stimulation of the same pathway with soluble CD28 results in DC activation [13, 14]. Along similar lines, stimulation of DCs with a PD-L2 cross-linking IgM antibody resulted in activation of DCs [15]. Another possibility explaining the contrasting results between studies might be the existence of a second receptor for PD-L1 and/or PD-L2 that has co-stimulatory properties. In support, engineered PD-L1 and PD-L2 mutants that lost the capacity to bind to PD-1 still possessed co-stimulatory properties [16], and cytokine production of PD-1-deficient T cells stimulated with PD-L2 is similar to wild-type T cells [10].

In this study, we have used bone marrow (BM)-derived DCs to stimulate OVA-specific $CD4^+$ T cells in the presence of soluble PD-1, consisting of the extracellular domain of PD-1 fused to the constant domain of human IgG1. In addition to providing a more physiologically relevant T cell stimulus, the use of DCs as APCs also enabled us to study possible occurrence of PD-L1/L2-mediated reverse signaling in DCs. We also addressed the potential of soluble PD-1 to block T cell activation in vivo, in a T cell mediated model of asthma. Together our data demonstrate that soluble PD-1 inhibits DC-driven T cell activation in vitro and in vivo, and that this effect may be mediated in part via reverse signaling into DCs.

Materials & Methods

Mice

Female Balb/c mice (6-10 wks old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHCII restricted, TCR transgenic (DO11.10) mice [17] were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Mice were housed in microisolators under specified pathogen-free conditions and experiments were performed under approval of the Erasmus MC committee for animal ethics.

Generation of dendritic cells

For the DC-T cell co-culture and DC stimulation experiments, DCs were generated from bone marrow (BM) progenitors as described [18], with some minor modifications. At day 1, cells were reseeded at 2.5 x 10^5 cells per ml in DC culture medium (DC-CM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Biocell, Rancho Dominguez, CA), 50 µM β-ME (Sigma, St Louis, MO) and 50 µg/ml gentamycin (Invitrogen) supplemented with 20 ng/ml recombinant mouse GM-CSF and 10 ng/ml recombinant human Flt3-L. Medium was refreshed on day 4, day 7 and day 9 (Flt3-L was omitted at day 7 and 9). On day 10 or day 11, mature DCs were harvested by gentle pipetting.

BM-derived DCs used as APCs in the mouse model of eosinophilic airway inflammation were generated as described [19].

Soluble PD-1 in vitro studies

Soluble PD-1 consists of the extracellular domain of murine PD-1 fused to the Fc fragment of human IgG1 (Chimerigen Laboratories, Allston, MA). As a control, human IgG1 (hIgG1, Sigma-Aldrich) was

used.

For the DC-T cell co-culture and T cell stimulation experiments, spleen and lymph node (LN) cells were obtained from DO11.10 or Balb/c mice and untouched $CD4^+$ T cells isolated by negative depletion with a commercially available panel of biotin-conjugated antibodies, followed by labeling with anti-biotin MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) The resulting population was typically > 95% $CD4^+$.

To measure proliferation through [^{3}H]thymidine incorporation, $1x10^5$ $CD4^+$ T cells were cultured together with $5x10^3$ DCs and various concentrations of $OVA_{323-339}$ peptide (Ansynth, Roosendaal, The Netherlands) in 96-wells U-bottom plates for four days. soluble PD-1 or hIgG1 was present at a concentration of 30 µg/ml. [^{3}H]thymidine (0.5 µCi/well) was added for the last 8 h.

For the CFSE labeling studies, cells were labeled with CFSE as described previously [19]. 5 x 10^5 CFSE-labeled $CD4^+$ T cells were cultured with 2.5 x 10^4 BM-derived DCs in 48-wells plates and various concentrations of $OVA_{323-339}$ peptide (Ansynth, Roosendaal, The Netherlands) in the presence of 30 µg/ml soluble PD-1 or 30 µg/ml hIgG1 as a control. After 96 h, cells and supernatant were harvested and OVA-specific T cells proliferation and cytokine levels determined.

To stimulate $CD4^+$ T cells in the absence of DCs, 96-wells U-bottom plates were coated wit anti-CD3 mAb (145-2C11; BD Biosciences) at various concentrations. Next, 2 x 10^5 purified $CD4^+$ T cells from Balb/c mice were stimulated in the presence of 2.5 µg/ml anti-CD28 mAb (37.51; BD Biosciences) and soluble PD-1 or hIgG1 (30 µg/ml). Proliferation was measured by [3H]thymidine incorporation (0.5 µCi/well) for the last 8 h of a 72 h culture.

For DC stimulation experiments, DCs were harvested at day 10, washed, replated at 2 x 10^5 DCs in 96-wells U-bottom plates and stimulated for 24h with soluble PD-1 or hIgG1 (30 µg/ml). In some experiments, DCs were cultured in the presence of recombinant murine IFN-γ (200 U/ml; Peprotech, Rocky Hill, NY) to upregulate IDO expression. After 24h, cells and supernatants were harvested to determine cell surface marker expression and cytokine levels, respectively. For quantitative RT-PCR, cell pellets were snapfrozen in liquid nitrogen and stored at –80 °C until RNA isolation.

Real-time quantitative RT-PCR

Frozen cell pellets were homogenized, RNA isolated with RNeasy mini-prep columns (Qiagen, Hilden, Germany) and treated on-column with DnaseI, according to the manufacturer's protocol. 1 µg RNA was reverse transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 120 min at 42 °C. Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) and preformulated primers and probe mixes ('Assay on Demand', Applied Biosystems). PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min using an ABI PRISM 7900 HT (Applied Biosystems). PCR amplification of the housekeeping gene ubiquitin C was performed during each run for each sample to allow normalization between samples.

Eosinophilic airway inflammation model

Eosinophilic inflammation in the lower airways was induced essentially as described [19]. In brief, mice were sensitized to OVA by intratracheal injection of 1 x 10^6 OVA-pulsed DCs on day 0, followed by OVA aerosol challenge (1% OVA in PBS using a jet nebulizer) on day 10-12. 100 µg soluble PD-1 or hIgG1 as a control was injected i.p. daily during the sensitization period (day -1, 0, 1, 2) and during the challenge period (day 9, 10, 11, 12). One group of mice did not receive any i.p. injection ('no treatment' group). Twenty-four hours after the last exposure mice were sacrificed and broncho alveolar lavage (BAL) was performed. To determine PD-L1 and PD-L2 expression on DCs in the lung tissue compartment, the right middle lobe was excised and enzymatically digested as described [20].

Flow cytometry

To reduce non-specific antibody binding, anti-FcγRIII/II antibody (2.4G2, ATCC, Manassas, VA) was included in all cell surface stainings. In the T cell proliferation experiments, dead cells were

excluded by labeling with TO-PRO-3 (Invitrogen) prior to acquisition. To study DC mediated T cell division in vitro, cells were labeled with CFSE and with the anti-clonotypic DO11.10 TCR mAb. KJ1-26 [17]. For the DC stimulation experiments, DCs were stained with anti-I-A/I-E-FITC (2G9) in combination with anti-CD11c-APC (HL3) and anti-CD80-PE (16-10A1), anti-CD86-PE (GL1) or anti-CD40-PE (3/23).

Anti-CCR3-PE was used to detect eosinophils in the lung [21], together with anti-CD8-PECy5 (53-6.7) and anti-CD4-APC (RM4-5) to determine the cellular composition in BAL. To determine PD-L1 and PD-L2 expression on DCs in BAL and lung digests, cell populations were stained with anti-I-A/I-E-FITC in combination with anti-CD11c-APC and either biotinylated anti-PD-L1 (MIH5) followed by streptavadin-PE or anti-PD-L2-PE (TY25).

All fluorochrome-conjugated antibodies were purchased from BD Biosciences, except anti-CCR3-PE, which was from R&D Systems (Minneapolis, MN), anti-clonotypic-TCR-PE (KJ1-26), which was from Caltag Laboratories (Burlingame, CA) and biotinylated anti-PD-L1 and anti-PD-L2-PE, which were from eBioscience (San Diego, CA). Events were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR).

Cytokine measurements

Levels of cytokines in culture supernatants or BAL were measured using OptEIA kits (BD Biosciences) according to manufacturer's instructions except for IL-13 levels, which were measured using a commercially available kit from R&D Systems (Minneapolis, MN).

Statistical analysis

Reported values are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed with SPSS (SPPS Inc, Chicago, IL) using a Mann-Whitney U-test. p-values less than 0.05 were considered significant.

Results

Inhibition of T cell proliferation in the presence of soluble PD-1 is consistent with the observed cytokine production pattern

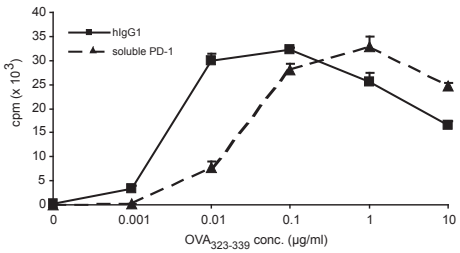

Figure 1 Soluble PD-1 blocks T cell proliferation in vitro at low antigen concentrations. 1 x 10^5 $OVA_{323-339}$-specific $CD4^+$ T cells were plated with 5 x 10^3 BM-derived DCs in the presence of indicated concentrations of $OVA_{323-339}$ peptide and soluble PD-1 (dashed line; triangles) or hIgG1 (solid line; squares) at 30 µg/ml. Proliferation was measured after 4 days by ^{3}[H]thymidine incorporation (0.5 µCi/well) for the last 8 h of culture. Results are expressed as mean ± SEM of three wells

In an attempt to further clarify whether PD-L1 and PD-L2 expressed by DCs have a costimulatory or inhibitory role in $CD4^+$ T cell stimulation, we cultured $OVA_{323-339}$-specific T cells with $OVA_{323-339}$-presenting DCs in the presence or absence of soluble PD-1 and measured the proliferation of T cells. To take into account any aspecific effects of the immunoglobulin heavy chain, T cells were exposed to human IgG1 as a control protein. As it has been shown that contribution of the PD-L1 and PD-L2 pathways to T cell stimulation is dependent on the TCR stimulus [1, 3], we titrated the antigen concentration. When hIgG1 was present during the T cell stimulation, proliferation was maximal at 0.1 µg/ml $OVA_{323-339}$ and decreasing at higher concentrations, presumably to activation induced cell death. However, when soluble PD-1 was added to the culture, $CD4^+$ T cell proliferation was decreased

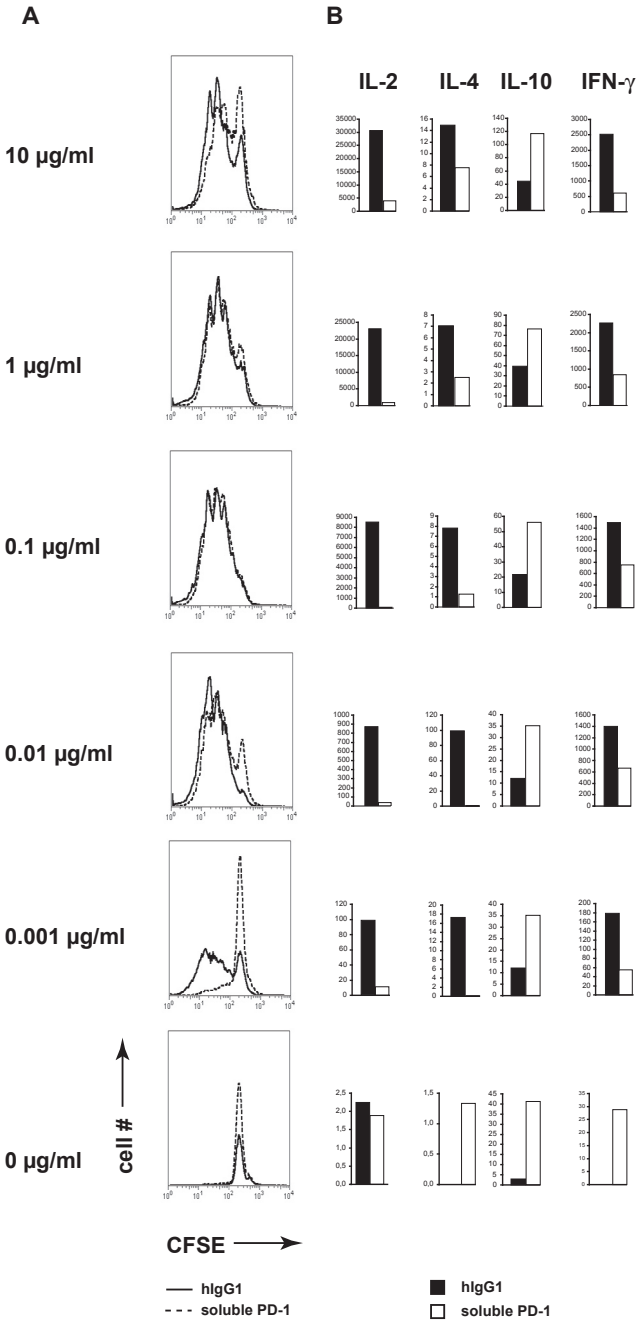

Figure 2 T cell stimulation in the presence of soluble PD-1 decreases IL-2 production but augments IL-10 levels. 5 x 10^5, CFSE labeled, $OVA_{323-339}$-specific ($KJ1\text{-}26^+$), $CD4^+$ T cells were plated with 2.5 x 10^4 BM-derived DCs in the presence of indicated concentrations of $OVA_{323-339}$ peptide and soluble PD-1 or hIgG1 (30 µg/ml). Four days later, cells and supernatant were harvested. A, Division profile of TO-$PRO\text{-}3^-$, $KJ1\text{-}26^+$ cells. B, cytokine levels in the supernatant. All cytokine concentrations are expressed in pg/ml. Shown results are representative for 5 independent experiments.

at lower antigen concentrations but increased at higher concentrations (1 and 10 μg/ml; Figure 1). This suggests that soluble PD-1 inhibits $CD4^+$ T cell activation by increasing the threshold of T cell activation.

Because measurement of T cell proliferation through ^{3}[H]thymidine incorporation only captures the proliferation during the last 8 h of the assay, we repeated the assay with CFSE-labeled $CD4^+$ T cells which permits visualization of the entire division history. The division profile of T cells obtained through CFSE labeling correlated with the proliferation data of radioactive thymidine incorporation, with decreased proliferation at lower antigen concentrations when soluble PD-1 was present during the T cell stimulation (Fig. 2A). Thus, these proliferation data support a costimulatory role for the PD-L1 and/or PD-L2 pathway rather than an inhibitory function.

Next, we analyzed whether soluble PD-1 also influences the cytokine profile of stimulated $CD4^+$ T cells. After day four of the T cell stimulation, supernatants were harvested and assayed for cytokines known to be involved in T cell proliferation or differentiation. Levels of the autocrine growth factor IL-2 were significantly decreased when soluble PD-1 was present during the T cell stimulation, suggesting a mechanism for the observed inhibition of T cell proliferation (Fig. 2B). Analysis of the levels of the Th1 prototypic cytokine IFN-γ and the key Th2 cytokine IL-4 revealed a reduction of both cytokines in the soluble PD-1-containing DC-T cell cultures (Fig. 2B), indicating that soluble PD-1 does not influence $CD4^+$ T cell polarization. Another cytokine with known T cell suppressive properties is IL-10. In our in vitro $CD4^+$ T cell culture system, levels of IL-10 were increased in the presence of soluble PD-1 compared to hIgG1, irrespective of antigen dose (Fig. 2B). Thus, to summarize, soluble PD-1 blocks T cell proliferation when the TCR signal is limited, possibly due to inhibition of IL-2 production or an increase in IL-10 production.

Soluble PD-1 cannot inhibit T cell proliferation in the absence of DCs

It has been reported that the known ligands for PD-1, PD-L1 and PD-L2, are expressed on murine $CD4^+$ T cells [5], and that reverse signaling via these ligands can occur in human $CD4^+$ T cells [22]. Therefore, we examined the effect of soluble PD-1 on pure $CD4^+$ T cells, without any other cell population present. Purified $CD4^+$ T cells were activated with anti-CD3 mAb and anti-CD28 mAb in the presence of soluble PD-1 or hIgG1 and after 72 h the proliferation was measured.

As shown in figure 3, proliferation in response to increasing CD3 stimulation in the presence of soluble PD-1 was nearly identical to hIgG1 exposed T cells, although a slightly lower but consistent inhibition of proliferation was observed when T cells were exposed to soluble PD-1. Taken together, these results suggest that although signaling via PD-1 ligands on T cells might have a minor contribution to suppression of $CD4^+$ T cell activity, this pathway probably does not solely account for the strong inhibition of T cell proliferation and cytokine expression pattern as observed in the Ag-specific assays.

DCs exposed to soluble PD-1 acquire a suppressive phenotype

Because direct PD-1 stimulation of $CD4^+$ T cells did not result in a significant inhibition of proliferation, we next focused our attention on the direct effects of soluble PD-1 on DCs. BM-derived DCs

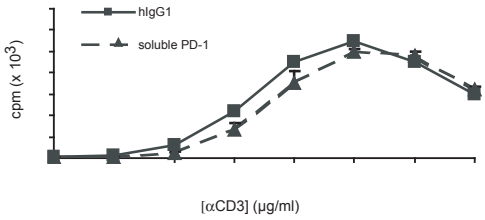

Figure 3 soluble PD-1 does not exert its effect directly on T cells. 2 x 10^5 purified Balb/c $CD4^+$ T cells were stimulated with indicated concentrations of plate-bound anti-CD3, soluble anti-CD28 (2.5 μg/ml) in the presence of soluble PD-1 or hIgG1 (30 μg/ml) for 72 h. Proliferation was measured after 4 days by ^{3}[H]thymidine incorporation (0.5 μCi/well) for the last 8 h of culture. Results are expressed as mean ± SEM of three wells. Results are representative for two independent experiments.

were harvested at day 10 and cultured for 24 h in the presence of soluble PD-1 or hIgG1, followed by analysis of maturation-associated cell surface markers and cytokine production. The percentage of DCs, as defined by CD11c and MHCII expression, was similar in the soluble PD-1 culture compared to hIgG1 exposed DCs (data not shown). However, when soluble PD-1 was added, the levels of the cell surface expression of the maturation markers CD80 and CD86 on DCs was lower compared to the hIgG1 exposed DC culture, while CD40 expression was similar (Fig. 4A). It has been shown that 'reverse signaling' via either B7-1 or B7-2 leads to alteration of DC phenotype and function, which manifests either as upregulation of IDO activity [13] or IL-6 production [14], depending on the stimulus used. Furthermore cross-linking of PD-L2 on DCs results in IL-12 production [15]. Therefore, we analyzed cytokine production and ido expression in soluble PD-1 stimulated DCs. The production of IL-6 and IL-12 of soluble PD-1 stimulated DCs were similar to hIgG1 exposed DCs, but levels of the immunosuppressive cytokine IL-10 were increased in the supernatants of soluble PD-1 stimulated DC cultures (Fig. 4B). As shown in Figure 4C, soluble PD-1 stimulation, in contrast to IFN-γ, did not lead to upregulation of IDO gene expression as assessed by quantitative RT-PCR. We conclude that DCs stimulated with soluble PD-1 acquire a suppressive phenotype as defined by increased IL-10 production and decreased expression of maturation markers, which may explain the observed inhibition of $CD4^+$ T cell proliferation.

PD-L1 and PD-L2 are upregulated during eosinophilic airway inflammation

Having shown the inhibitory effects of soluble PD-1 on DC driven T cell activation, we next questioned whether this molecule would also inhibit T cell activation in vivo making use of a T cell driven model of allergic asthma that uses the same OVA antigen as in our in vitro studies. As it has been reported that Th2 cytokines upregulate PD-L1 and PD-L2 on DCs [5, 23], we decided to determine the expression of PD-L1 and PD-L2 on DCs isolated from BAL fluid and lung tissue from mice with Th2-dependent eosinophilic airway inflammation. Mice were sensitized to OVA via intratracheal injection of OVA-pulsed DC, followed by OVA aerosol challenge 10 days later (OVA-group). This results in peribronchial and perivascular eosinophilic airway inflammation, which is analyzed 24 h after the last aerosol exposure [24].As a control, mice were immunized with unpulsed DC followed by PBS aerosols (control-group). PD-L1 and PD-L2 expression on DCs ($CD11c^+$, $MHCII^{hi}$) was assessed by flowcytometric analysis of BAL fluid and digested whole lungs. PD-L1 was constitutively expressed on DCs isolated from BAL fluid or lung tissue under non-inflammatory conditions (Fig. 5A, left panels),

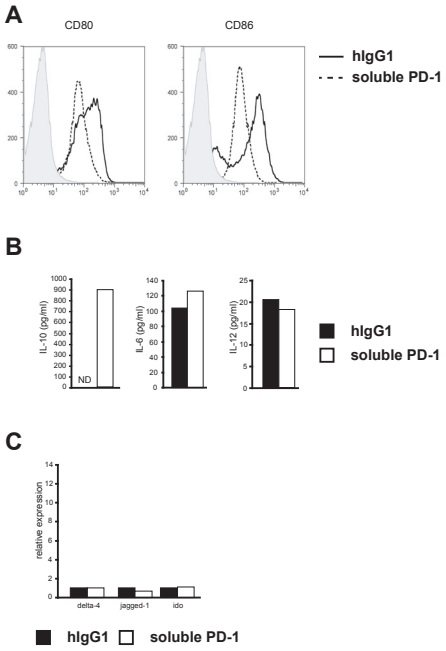

Figure 4 DCs exposed to soluble PD-1 acquire a suppressive phenotype. BM-derived DCs were harvested at day 10 and reseeded at 1 x 10^6 DCs/ml in 96-wells U-bottom plates in the presence of soluble PD-1 or hIgG1 (30 µg/ml). Twenty-four hours later, cells and supernatants were harvested. A, Cell-surface expression of CD40, CD80 and CD86 on mature ($CD11c^+$, $MHCII^+$) DCs. Shaded histograms indicate isotype control stainings and mean fluorescence intensity is shown for CD80 and CD86 stainings. B, cytokine levels in culture supernatant. ND; none detected. C, IDO gene expression.

and only slightly upregulated when eosinophilic airway inflammation was induced (Fig. 5A, right panels). In contrast, PD-L2 expression was absent in lungs and BAL fluid of naïve mice (Fig. 5B, left panels), but strongly upregulated during pulmonary inflammation (Fig. 5B, right panels).

Soluble PD-1 suppresses eosinophilic airway inflammation

Based on the observation that PD-L1 and PD-L2 are highly expressed on pulmonary DCs during eosinophilic airway inflammation, we were interested in the effects of soluble PD-1 on a pulmonary inflammation response. When mice received soluble PD-1 fusion construct during the sensitization and challenge phase, inflammation was slightly suppressed, as judged by total cell number in the BAL fluid (Fig. 6A). Concomitantly, analysis of the cellular composition of the BAL fluid revealed a decrease in the frequency of eosinophils retrieved (Fig. 6B) and levels of the prototypic eosinophilic airway inflammation associated cytokines IL-5 and IL-13 were significantly decreased (Fig. 6C). These data implicate that even in a more complex in vivo environment, where PD-L1 is likely also expressed by a variety of other tissues during lung inflammation, soluble PD-1 is still capable of blocking $CD4^+$ T cell proliferation.

Discussion

The data obtained in this study with OVA-specific $CD4^+$ T cell cultures stimulated with OVA-pulsed DCs indicate that, in this experimental set up, PD-L1 and PD-L2 have a co-stimulatory function. This confirms the findings of other studies stimulating T cells in vitro in the presence of PD-L1 and PD-L2 blocking agents [2, 4, 9], but contradict with other studies [1, 11]. Interpretation of the data is complicated by the variety of experimental conditions used, and all these studies have in common that they use agonistic anti-CD3 antibody to mimic antigen signaling in combination with PD-L1-immunoglobulin or PD-L2-immunoglobulin. As a consequence, only T cell mediated signaling events, either via PD-1 or a putative co-stimulatory receptor, can be investigated.
Two recent reports however, did use a more physiological approach to dissect the role of the PD-1 ligands in DC-T cell interaction, using human $CD4^+$ T cells cultured with allogenic DC in the pres-

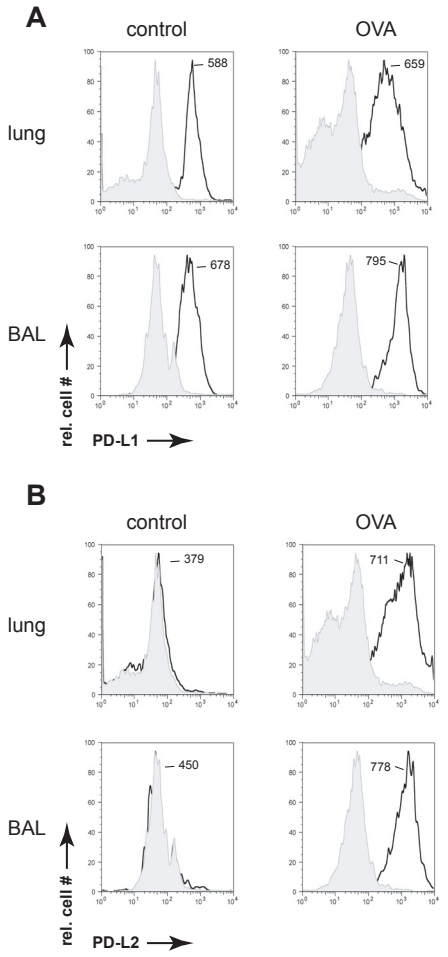

Figure 5 PD-L1 and PD-L2 expression pattern on pulmonary DCs. On day 0, groups of mice were immunized by intratracheal administration of 1 x 10^6 OVA-pulsed BM-DC (OVA group) or unpulsed BM-DC (control group). On days 10-12, mice were challenged with OVA aerosols (OVA group) or PBS (control group) for 30 min. daily. At 24 h after the last aerosol exposure mice were sacrificed, BAL performed, right-middle lobe excised and single-cell suspensions were prepared from enzymatically digested lungs. To acquire sufficient events for analysis, 15 BAL samples of control mice, 10 BAL samples of the OVA group, and 5 lung cell suspensions of each group were pooled. Cells were stained for CD11c and MHCII to characterize DCs, in combination with PD-L1 or PD-L2. A, PD-L1 expression of DCs in BAL and lungs of unsensitized mice (control) or mice with eosinophilic airway inflammation (OVA). B, PD-L2 expression of pulmonary DCs. All shown populations are gated on $CD11c^+$, $MHCII^{hi}$. Numbers in histograms denote mean fluorescence intensity. Shaded histograms indicate isotype control stainings.

ence of antibodies to PD-L1, PD-L2 or both [12, 25]. Contrarily to our observations, they found an increase of T cell proliferation when antibodies against PD-L1 or PD-L2 were present, favoring an inhibitory role for PD-L1 and PD-L2 during T cell stimulation. IFN-γ production was increased in both studies, but IL-10 levels were decreased in one study [25], while Brown et al., similar to our findings, observed an increase in IL-10 levels in the mixed DC-T cell culture [12]. A possible explanation for this discrepancy between studies might be the use of different blocking reagents, the extracellular domain of PD-1 in this study versus antibodies against PD-L1 and PD-L2 in the other two studies. Furthermore, although not published before for this pathway, there might be a difference in co-signaling effects of PD-L1 or PD-L2 between species, as previously noted for the B7 family member B7-H3 [26, 27].

Several reports have shown the existence of reverse signaling of B7 family ligands into DCs, as shown in studies using soluble CTLA-4 or CD28, and has been illustrated for the HVEM-BLTA pathway as well [13, 14, 28]. More specifically, a recent study demonstrated that reverse signaling via PD-L2 expressed by DCs lead to activation of DCs, as measured by enhanced T cell activation ability, migration capacity, IL-12 production and survival. Interestingly, maturation markers CD80 and CD86 remained similar between the treatment and control group [15]. The antibody directed against PD-L1 isolated by Selenko-Gebauer and co-workers did not activate DCs, as judged by CD80 expression, CD86 expression and cytokine production [25]. In our experi-

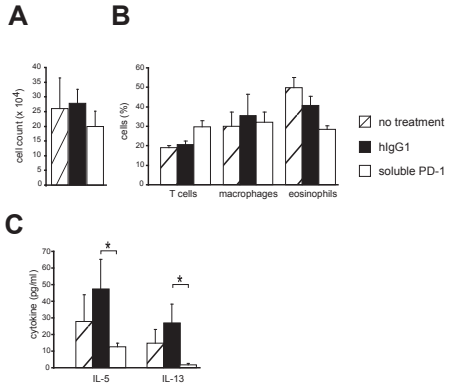

Figure 6 soluble PD-1 suppresses eosinophilic airway inflammation. On day 0, groups of mice (n=3-4) were immunized by intratracheal administration of 1 x 10^6 OVA-pulsed BM-DC. On days 10-12, mice were challenged with OVA aerosols for 30 min. daily. During sensitization and challenge mice were treated with 100 µg soluble PD-1 or hIgG1 i.p. daily for four consecutive days. One group was not administered protein ('no treatment' group). At 24 h after the last OVA aerosol exposure mice were sacrificed, BAL performed and lungs isolated. A, total cell number in the BAL. B, cellular composition of BAL fluid. Alveolar macrophages were characterized by their light scatter and autofluorescence properties. Eosinophils are defined by their $CCR3^+$, $CD4^-$, $CD8^-$ staining pattern. The T cell fraction consists of $CD4^+$ and $CD8^+$ cells within the appropriate light scatter gate. C, IL-5 and IL-13 levels in BAL fluid. Results are expressed as means ± SEM. Data are representative for 2 independent experiments. *: $p < 0.05$

ments, DCs stimulated with soluble PD-1 acquired a suppressive phenotype, secreting IL-10 and decreased expression of CD80 and CD86, while we did not detect increased IL-12 production. Whether these contrasting effects of PD-1 ligand-mediated reverse signaling are dependent on the type of stimulus used (PD-1- fusion protein versus a patient-isolated anti-PD-L2 IgM) remains to be seen. Moreover, we have not formally established whether signaling occurs via PD-L1, PD-L2 or perhaps another ligand for PD-1. DC stimulation experiments using BM-derived DC from PD-L1 and PD-L2 deficient animals are necessary to verify this.

As PD-L1 is also expressed on activated $CD4^+$ T cells, we also analyzed the effect of soluble PD-1 on $CD4^+$ T cell proliferation directly. Proliferation was similar to hIgG1 control, indicating no reverse signaling occurred via PD-L1 expressed by $CD4^+$ T cells. Such signaling is possible, as antibodies have been isolated from serum of rheumatoid arthritis patient that stimulated $CD4^+$ T cell proliferation and IL-10 production [22]. Intriguingly, in this study soluble PD-1 was also able to deliver these costimulatory signals. This increased proliferation was accompanied by increased apoptosis, which could be partially blocked by anti-IL-10 antibody. Although we have not directly assessed apoptosis of soluble PD-1 stimulated T cells and we also found increased IL-10 levels in DC-T cell cultures, we do not observe increased proliferation in response to PD-1 ligand signaling. Moreover, both anti-IL-10 mAb and anti-IL-10R could not restore the soluble PD-1-mediated inhibition of T cell proliferation in DC-T cell cultures (data not shown), suggesting that IL-10-mediated $CD4^+$ T cell apoptosis is not responsible for the inhibition in cell cycling. This is in agreement with findings of Latchman *et al.* who observed cell cycle arrest but not apoptosis upon stimulation of $CD4^+$ T cell with PD-L2-Ig [3].

We also addressed the potential of soluble PD-1 to block DC-driven T cell activation *in vivo*, taking advantage of a DC driven model of asthma, where T cells play a key effector role by inducing airway eosinophilia through the release of IL-5 and IL-13. Interpretation of the effects of soluble PD-1 *in vivo* is bound to be more difficult than interpretation of the more defined *in vitro* culture system, especially because PD-L1 and PD-L2 are not only expressed on pulmonary DCs

and macrophages (this study and [23, 29], but PD-L1 is also expressed on a variety of tissues of non-hematopoietic origin, particularly under inflammatory conditions [6]. Nonetheless, the results of treatment with soluble PD-1 in this model of Th2-dependent asthma are in line with our in vitro data as we observe a decrease in eosinophils in the BAL fluid and, even more importantly, a concomitant decrease in the Th2-associated cytokines IL-4 and IL-13. This suggests that also in vivo soluble PD-1 is capable of blocking T cell activation. As we have previously shown that airway DCs are crucial for inducing Th2 effector function after allergen challenge to the airway [30], it is very likely that soluble PD-1 had its effect through altering DC function. We are currently investigating the phenotype of pulmonary DCs in inflamed airways during treatment with soluble PD-1.

The majority of studies interfering with PD-L1 or PD-L2 signaling in vivo supported a inhibitory role for these molecules [23, 31-33]. One such study involved eosinophilic airway inflammation. Matsumoto and colleagues showed that treatment with anti-PD-L2 mAb at the time of allergen challenge in a mouse model of eosinophilic airway inflammation lead to increased airway inflammation, consistent with an inhibitory role for PD-L2 [29]. A possible explanation for the contrasting results between our work and this study might be the way in which PD-L2 blocking is achieved; the monoclonal antibody used by Matsumoto might only have blocked the signaling via the inhibitory PD-1 receptor on T cells, while soluble PD-1 used by us could have exerted its effect additionally through reverse signaling via PD-L1 or PD-L2 expressed on DCs and macrophages, thus reducing immunostimulatory potential. This latter pathway leading to less efficient T cell stimulation might be dominant over the direct effect of inhibiting the crosslinking of PD-1, very similar to what has been shown for CTLA-4-Ig.

Despite these studies supporting an inhibitory role for PD-L1/PD-L2, some studies have clearly shown the opposite. A costimulatory role for PD-L1 in vivo has been noted in a murine model of colitis [29, 34]. Likewise, transplantation of PD-L1 expressing Langerhans islets resulted in increased incidence of diabetes [29, 34]. Another very recent study by Oflazoglu and colleagues using an agonistic mPDL-2-hFc fusion protein demonstrated that PDL2 induced Th2 effector cytokines and enhanced eosinophilic airway inflammation, consistent with a costimulatory role for this molecule and consistent with our findings [23]. The feasibility of an immunotherapy against eosinophilic airway inflammation based on modulation of PD-1 ligand signaling has already been shown by Radhakrishnan and colleagues, who demonstrated that administration of an IgM antibody crosslinking PD-L2 could block eosinophilic airway inflammation, even when it was fully established [35]. It remains to be determined however whether soluble PD-1 functions similar to this crosslinking IgM antibody, which suppresses the Th2-associated airway inflammation via a STAT4-dependent polarization towards a suppressive Th1 response. A plausible mechanism for this Th1 polarization is IL-12 production by DCs upon PD-L2 crosslinking [15], which is capable of preventing the development of eosinophilic airway inflammation [36]. However, it is also of interest to note the increase in IL-10 production by restimulated splenocytes isolated of anti-PD-L2 IgM antibody treated mice, which resembles the observed production of IL-10 in our in vi-

tro system. As it has been reported that IL-10 producing DCs have the capacity to induce IL-10 producing regulatory T cells [37], we are currently investigating whether soluble PD-1 stimulation leads to the development of regulatory T cells in vitro and in vivo.

In conclusion, the data described in this paper implicate that soluble PD-1 blocks $CD4^+$ T cell stimulation in vitro and in vivo, presumably via reverse signaling into DCs as has been described for B7-1, B7-2 and BLTA [13, 14, 28]. Further studies are required however, to delineate the signal transduction pathways involved in reverse signaling and the effector molecules responsible for T cell inhibition. Nevertheless, the observed suppression of eosinophilic airway inflammation after treatment with soluble PD-1 offers a new perspective for immunotherapy against T cell mediated allergic disorders.

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7

Modification of dendritic cell function as a tool to prevent and treat allergic asthma (discussion)

Vaccine, 2005. in press

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Abstract

Atopic asthma is a chronic inflammatory disease of the airways, characterized by airway hyperreactivity and mucus hypersecretion that result in intermittent airway obstruction. This chronic inflammation is the result of an aberrant Th2-mediated response to innocuous environmental proteins. The prevalence of this disease has increased dramatically in the industrialized world in the last decades. Current treatment is mainly based on pharmacological interventions which control the disease but are not curative. Although the etiology is not completely understood, it becomes increasingly clear that dendritic cells play an important role in both the sensitization phase and maintenance of the disease. In this review we explore the different possibilities to exploit dendritic cell vaccines in order to prevent the development of (or inhibit established) atopic asthma.

Introduction : the natural history of asthma

Atopic asthma is a chronic inflammatory disease of the airways, characterized by airway hyperreactivity and mucus hyperssecretion that result in intermittent airway obstruction [1]. Histologically, eosinophilic airway inflammation, goblet cell hyperplasia and epithelial fragility are consistent findings [2]. The natural history of allergic asthma begins by an early and persistent Th2 skewed immune response to commonly ingested (cow milk) and inhaled allergens (house dust mite, pollen allergens, animal dander), as reflected by the occurrence of Th2 dependent allergen-specific IgE antibodies in the serum. The immunological basis of asthma is supported by some genetic susceptibility studies that have seen an association between polymorphisms at the Th2 cytokine gene cluster at locus 5q23-31 (containing the IL-4, IL-9 and IL-13 gene locus) and risk of developing atopy and asthma [3, 4]. At young age, some sensitized children develop atopic dermatitis and/or intermittent periods of wheezing, coughing and shortness of breath upon contact with allergens or upon non-specific stimuli such as cold air or exercise. In adolescents, asthma subsequently disappears clinically (although subclinically bronchial hyperreactivity and/or inflammation can often persist [5]), whereas in others the disease progresses into a chronic phase into adulthood.

It is currently believed that the symptoms associated with chronic asthma are a result of structural changes in the lung in response to chronic inflammation and remodeling of the airway wall, leading to fixed airway obstruction. The chronic nature is exemplified by the observation that airway biopsies of asthmatic children show already signs of remodeling and inflammation [6]. Second, an indirect finding indicating the relative importance of airway remodeling is the identification of the asthma susceptibility gene *Adam33* [7]. ADAM33 is a member of the dysintegrin and metalloproteinase family and expressed in smooth muscle cells, fibroblasts and myofibroblasts but not in T lymphocytes or other cells of the immune system, suggesting it is involved in remodeling rather than the immunological aspects of disease.

Risk factors for developing asthma

The best known and validated risk factor for developing asthma is Th2 sensitization to commonly inhaled allergens. The contribution of allergen sensitization to the risk of subsequently developing asthma seems inversely correlated with the age at which allergy initially manifests [8], reinforcing the idea that the respiratory system is particularly vulnerable to inflammatory damage during early life [9]. Asthma is inherited as a polygenic trait, but clearly, environmental factors greatly influence the manifestation of asthma, including family size, early day-care attendance and maternal smoking during pregnancy [10, 11]. Another important early life risk factor for developing asthma is the occurrence of childhood respiratory infections [8, 12]. The incidence of atopic diseases has risen dramatically in westernalized countries since the 1950's, concomitantly with a reduced incidence of infectious diseases such as measles, tuberculosis, hepatitis A virus, streptococcal infection as well as helminth infections [13]. This reduction in infectious diseases has been the result of increased hygiene, large scale vaccination campaigns and early use of antibiotics. The immunological explanation of the hygiene hypothesis is surrounded by controversy. Most likely, the reduced infectious pressure from the environment leads to aberrant or persistent Th2 responses because of a lack of counterregulatory anti-infectious Th1 responses or anti-inflammatory regulatory T cells (Tregs). As a nice illustration to the hygiene hypothesis, it was shown that children raised at a farm, with frequent contact with cows and drinking unpasteurized milk in the first year of life were protected from developing atopic sensitization and atopic diseases possibly due to high level exposure to LPS [14]. Subsequently, the same group showed that even in non-farmers, the level of LPS exposure in mattress covers in the bedroom are negatively associated with the risk of developing atopic symptoms [15].

Understanding the origin of the sensitization process : a critical role for dendritic cells.

From the above it is clear that the initiation of a Th2 response to inhaled allergens is a critical checkpoint in the development of the 'atopic march' towards persistent asthma. If we are to intervene at this point, it is imperative that we understand the process of sensitization much better and also study how this process is influenced by genetic and environmental factors. In the last decades, antigen presenting dendritic cells (DCs) have emerged as the principal cell that orchestrates adaptive immunity [16]. By their expression of a vast array of antigen receptors and Toll like receptors shared with the cells of the innate immune system, these cells react to most harmless (self) antigens as well as dangerous pathogens. Besides activation of T and B cells, DCs also influence the differentiation of $CD4^+$ T cells, depending on the type of immune response required to eradicate the pathogen [17]. In particular the role of DCs in polarization towards Th1 effector T cells has been well studied, with DC-derived IL-12 being of paramount importance for this differentiation route [18, 19]. We and others have also shown that airway DCs are at the very heart of the sensitization process that leads to Th2 immunity in the lung [20, 21]. In a series of experiments, we

were able to demonstrate that myeloid dendritic cells (mDCs) are sufficient to induce Th2 sensitization in naïve mice [20, 22]. In these experiments, animals were sensitized to a model allergen (mostly OVA) via intratracheal injection of allergen-pulsed, bone marrow derived, DCs. Ten days later, mice were challenged with OVA aerosols on three consecutive days. The allergen challenge led to allergen-specific Th2 cytokine synthesis, eosinophilic airway inflammation, goblet cell hyperplasia and bronchial hyperreactivity, all salient features of asthma (Figure 1). Not only adoptively transferred DCs but also endogenous (myeloid) DCs induce proper Th2 sensitization in response to inhaled antigen, providing that the antigen is capable of inducing the maturation of lung DCs [23, 24].
The subset of lung DCs presenting the inhaled antigen is also likely to influence the immunization process, either directly or indirectly. De Heer et al, showed a suppressive effect of plasmacytoid DCs (pDCs) on the outcome of airway immunization, most likely via inhibiting myeloid DC function [25]. In the absence of pDCs, inhalation of harmless antigen led to stable Th2 immunity and features of asthma, while adoptive transfer of pDCs rendered mice tolerant to the antigen. These studies are supported by recent findings that the balance of particular subsets of DCs presenting antigen in the draining lymph nodes of mice airways was dependent on the immunization protocol: predominantly pDCs were recovered under tolerizing conditions (OVA in the absence of adjuvant), while mDCs were isolated after an airway inflammation inducing regimen (OVA combined with cholera toxin) [26]. Thus, the final outcome of an immune response is depending on the type of DC and/or activation status of the DC presenting the antigen.
In humans, less is known about the precise role of DCs in the Th2 sensitization process at the basis of atopic asthma. Indirect evidence that points towards a role for DC-derived IL-12 in atopic sensitization is based on epidemiological studies, reporting an inverse correlation between endotoxin exposure at young age, a well-known IL-12 inducer, and atopic sensitization [15, 27]. More direct evidence was derived from studies examining the IL-12 production of PBMCs (including DCs) in atopic patients, which was shown to be subsequently lower compared to healthy individuals [28, 29]. Moreover, polymorphisms in the IL-12B gene promoter have been correlated with an enhanced severity of atopic asthma in children, presumably due to lower levels of IL-12 production by DCs [30-32]. Some of the most common allergens such as house dust mite and pollen allergens contain substances that clearly polarize DC function towards Th2 induction. In house dust mite, the proteolytic activity of the major allergen Der p 1 induces IL-10 production and CD86 expression, while suppressing IL-12 production in DCs obtained from house dust mite-allergic patients [33]. In pollen allergens, the plant phytoprostanes, closely related to mammalian prostaglandins, strongly suppress IL-12 secretion in DCs, leading to Th2 induction [34].

Altering DC function to prevent sensitization

The predominant influence of DCs on the critical decision between tolerance or Th1/Th2 adaptive immunity might be exploited to the benefit of preventing atopic Th2 sensitization in high risk individuals. Over the last years we have performed some proof of concept studies that this is a feasible strategy. In particular, we

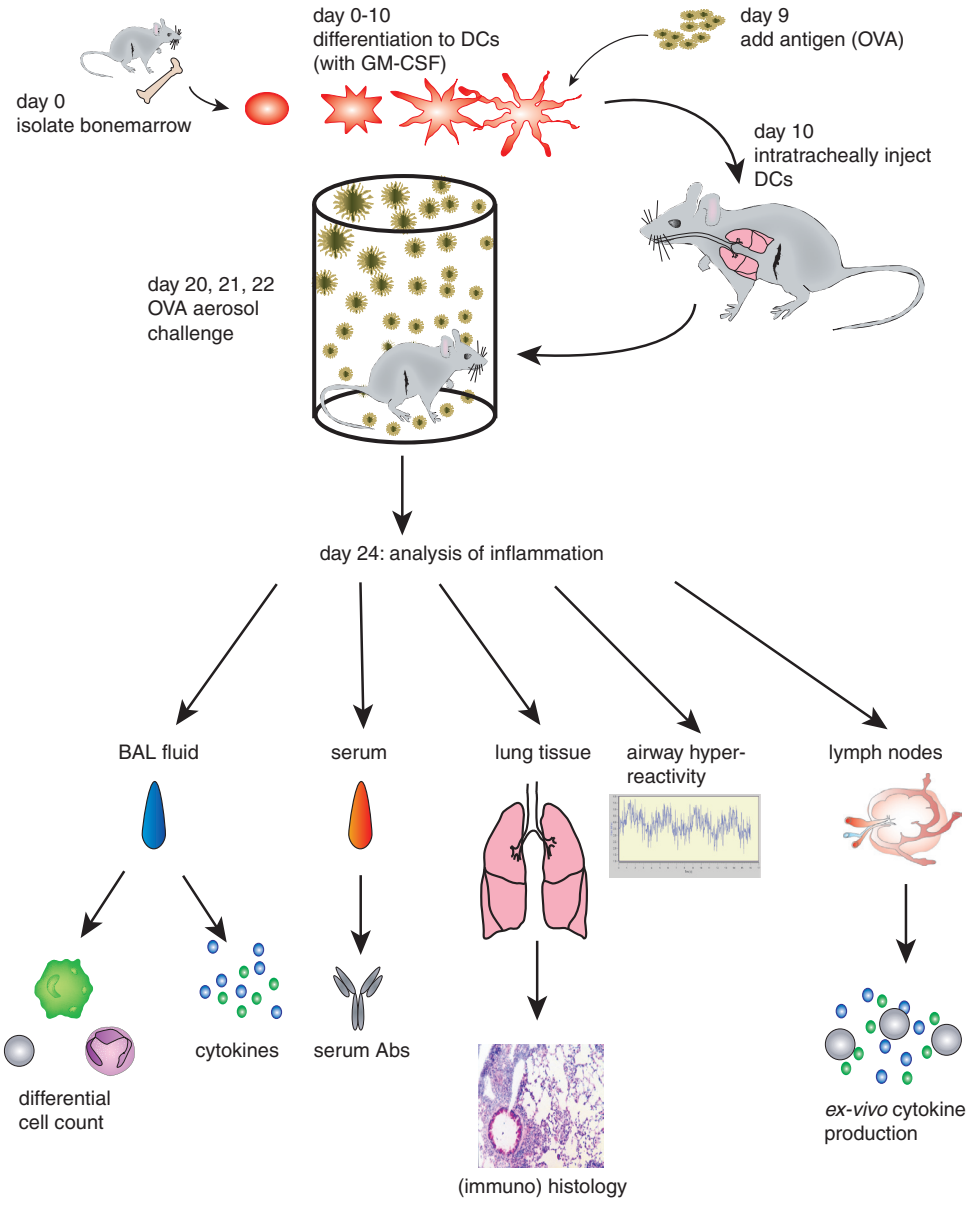

Figure 1 *DC-mediated murine model of eosinophilic airway inflammation*

have used an animal model of asthma where intratracheal injection of bone-marrow derived DCs is used to prime for Th2 dependent eosinophilic airway inflammation (figure 1 and [22]. In such a system, we can selectively modify DCs in vitro and subsequently observe what the effects of such modulation are on the process of DC-driven sensitization. As atopic asthma is associated with Th2-dependent disease, deviation towards other pathways of $CD4^+$ T cell development or suppressing of the Th2 response might be beneficial.

Altering the functional behavior of dendritic cells through bacterial products

As stated before, infectious pressure and associated levels of environmental endotoxin at a young age profoundly influence the incidence of atopic asthma in later life. As DCs are the predominant cell type that steers adaptive immunity to allergens and also reacts to microbial stimuli we speculated that direct exposure to common microbial factors such as LPS might alter the potential of DCs to promote Th2 responses. When we exposed myeloid DCs to endotoxin and the model allergen OVA, the Th cell polarization process in vitro and in vivo was profoundly changed from a default Th2 pathway to a strong Th1 response. Not surprisingly, when mice were subsequently challenged with OVA aerosol, airway eosinophilia no longer developed [35]. These direct inhibitory effects of LPS exposure on Th2 sensitization are in agreement with various other studies investigating the relationship between LPS exposure during intraperitoneal priming and eosinophilic airway inflammation [23, 36, 37]. They are new in the way that we now offer an explanation as to how LPS might work to induce suppression of asthma, i.e. by modeling the phenotype and function of DCs. Although in our system, we modified DCs with LPS in vitro, we believe that this is a reflection of what might happen in vivo. Indeed, recently our findings were corroborated by studies revealing that LPS modulation of atopic sensitization via the airways in murine models of eosinophilic airway inflammation were strongly dependent on TLR4 and MyD88 signaling, implicating that modulation of endogenous lung DC function is the mechanism by which high dose LPS suppresses Th2 development [23, 24]. An intriguing aspect of our study and other studies was the finding that the suppressive effect of LPS exposure on Th2 development and induction of Th1 development was independent of IL-12 production by DCs altogether [38]. One of the most plausible explanations for IL-12 independent Th1 development is rescue of Th1 differentiation by IL-27, which is produced by DCs upon LPS stimulation [39]. However, subsequent studies have revealed that IL-27 has also anti-inflammatory properties [40]. IL-23 might be another candidate, as it is produced by DCs and has been reported to drive IL-12 independent development of a highly pathogenic $CD4^+$ T cell subset [41]. In addition to their fundamental role in T cell development, the Notch ligand families Delta and Jagged are also implicated in DC-mediated $CD4^+$ T cell polarization [42], with LPS increasing the cell-surface expression of the Th1 favoring Delta-4 molecule on DCs. Future studies will address whether other microbial compounds such as β-glucans, CpG motifs or peptidoglycans, many of which also act upon members of the TLR family, also suppress sensitization by altering DC function. The ultimate goal of this strategy will be to find those compounds that act on endogenous DCs can be safely and reliably be administered and can exert their function in a non-toxic way.

Altering the functional behavior of dendritic cells through genetic modification

It remains a matter of debate whether IL-12 production capacity of APCs is fundamentally lower in atopics compared to healthy controls. IL-12 production is probably dependent upon the age of the individual, making comparisons between studies more difficult [28, 29, 43-45]. Despite the fact that LPS suppresses Th2 development in an IL-12-independent way, it is still possible that overexpres-

sion of IL-12 is sufficient to prevent Th2 development. To prove this point, we have retrovirally overexpressed bioactive IL-12p70 in BM-DCs and see whether IL-12 overexpression would abolish the potential of these cells to induce Th2 sensitization in the lungs [46]. To our surprise, we observed indeed that IL-12 overexpression turned DCs into Th1 priming cells, even in the Th2 prone milieu of the lungs. These results were confirmed by another recent study that examined the feasibility of IL-12 transduced DC in prevention of eosinophilic airway inflammation [47].

As less is known about DC signals directing naïve $CD4^+$ T cell differentiation towards the type 2 subset compared to Th1 signals (e.g. IL-12), the strategies to directly inhibit Th2 development by DCs are limited. The cytokine IL-4 is critical for Th2 development, but is not produced by DCs. Recently, administration of plasmid DNA or a viral vector encoding for an IL-4R antagonist (A mutated form of IL-4 with binding capacity but lacking signaling capacity, $IL\text{-}4^{Q/Y}$) could prevent eosinophilic airway inflammation in a murine asthma model [48, 49]. However, some preliminary experiments using DCs retrovirally expressing $IL\text{-}4^{Q/Y}$ could not prevent antigen sensitization in our eosinophilic airway inflammation model (unpublished observations).

Significant ethical and practical problems will have to be solved before we can ever envisage a genetic strategy to prevent asthma. However, based upon our data in this mouse model we believe that genetic targeting of genes to DCs might be an efficient way of fundamentally altering Th priming in vivo. A potential caveat that should be taken into account is Th1-mediated breakdown of tolerance, as it was shown that mucosal tolerance to harmless antigen could be broken by administration of IL-12 [50, 51]. These findings suggest that, translated to a clinical setting, patients receiving Th1 inducing immunotherapy should be carefully monitored for breakdown of self-tolerance, which could eventually lead to autoimmunity.

Using DCs to revert existing Th2 responses : a novel form of immunotherapy ?

After the process of sensitization has occurred, it is hard to convert existing polarized pathogenic Th2 responses into protective Th1 or Tregs responses in an attempt to prevent the clinical manifestations of Th2 mediated diseases such as asthma, allergic rhinitis or atopic dermatitis (secondary prevention strategies). This is however clinically the most relevant question, as it would allow the secondary prevention of high risk sensitized children before any diseases have developed. In recent years, immunotherapy based on the unparalleled ability of DCs to activate and control the adaptive immune response has been pioneered, mainly in the field of cancer therapy and aimed at eliciting a tumor-specific immune response [52]. The techniques for generating large amounts of clinical grade DCs have become widely available, as are the techniques for (genetically) modifying the phenotype and degree of maturation of dendritic cells. In the field of cancer, the primary emphasis has been to generate DCs with a strong potential to induce $CD4^+$ Th1 responses and cytotoxic $CD8^+$ responses directed against the tumor. We have explored the ability of gene-modified DCs to modulate an

allergen-specific immune response, either by changing $CD4^+$ T cell differentiation or suppressing Th2 development.

Counterbalancing Th2 responses by Th1 inducing DCs

The controversy about the relationship between IL-12 and allergic asthma (see before) does not exclude the possibility to employ Th1-inducing DCs for therapeutic purposes. Unfortunately, when we adoptively transferred IL-12 overexpressing DCs to already Th2 sensitized mice, to induce a counterregulatory antigen-specific Th1 population, we could not prevent the occurrence of eosinophilic airway inflammation upon rechallenge of the mice with relevant allergen [46]. In other words, our secondary prevention strategy failed. In line with these results, it has been shown that adoptive transfer of antigen-specific Th1 cells does not suppress, or even enhances, Th2-mediated airway eosinophilia [16, 53, 54]. One study that did report a beneficial effect of Th1 cell transfer on eosinophilic airway inflammation saw only a decrease in the number of eosinophils in the BAL fluid compartment, not in tissue eosinophilia [55]. A mechanistic explanation was provided by Cohn *et al*, as it was shown that IFN-γ inhibits eosinophil migration to the airway lumen [56]. It should be emphasized though that all these studies transferred *in vitro* polarized Th1 cells, while in our DC-immunotherapy protocol naïve $CD4^+$ T cell polarization took place *in vivo*. Recent evidence suggests however, that in vivo differentiated antigen-specific Th1 cells have limited ability to inhibit Th2 cells development in vivo [57]. To summarize, despite the initial promising reports of IL-12 administration in murine models of asthma to prevent or treat asthma [58], the subsequent adverse side-effects noted in clinical trials makes this approach not realistic [59]. Although we and the study of Ye [60] show that eosinophilic airway inflammation can be prevented through Th1-directed antigen sensitization, the subsequent lymphocytosis we and others observed [61] still represents a deviation of the normal state (no inflammation) and is likely to have also detrimental effects for the patient.

Direct suppression of Th2 responses

We have recently shown that conditional depletion of $CD11c^+$ DCs from the airways of mice was able to completely suppress Th2 mediated effector responses and cardinal features of asthma [62]. Therefore, altering the function of DCs might also directly suppress Th2 activation in an already sensitized host. In fact, the current standard therapy of asthma consists of inhaled steroids. A number of studies have already shown that inhaled and systemic corticosteroids reduce the number and function of airway DCs *in vivo* or *ex vivo* [63-65]. However, based on our knowledge of Th2 activation by DCs in asthmatic airways, we have attempted a more direct way of inhibiting Th2 activation by DCs.

It is increasingly clear that the process of T cell activation involves not only costimulatory molecules being expressed on DCs, but that there are also inhibitory molecules that have the potential to suppress or switch off naïve and/or activated T cells in an antigen specific manner [66]. Overexpression of these negative signals on DCs could be exploited as a strategy to suppress only antigen-specific T cell responses. The strategy of targeting activated T cells selectively has the clear advantage that the immune response to allergens or self antigens is abolished

instead of deviated, theoretically decreasing the risk of severe side-effects. As a nice illustration it was shown that DCs overexpressing the death receptor ligand FasL (CD178) and a specific antigen were able to kill selectively the activated antigen specific T cells expressing Fas [67]. This led to a reduction in auto-immune disease [68]. With respect to asthma, it has been shown that intratracheal delivery of adenovirus encoding FasL could significantly suppress pulmonary immune responses [69]. However, this strategy has been disputed a lot as some recent reports have shown that DCs overexpressing FasL induce massive pulmonary inflammation, pleuritis and acute skin allograft rejection [70, 71].

In an attempt to devise a similar, less toxic way of eliminating antigen-specific Th2 responses, we have chosen to overexpress the ligands for the inhibitory receptor PD-1 on DCs. PD-1 is expressed on activated T and B cells and its inhibitory function is illustrated by the fact mice deficient for PD-1 develop autoimmune diseases [72]. The ligands for PD-1 identified so far are PD-L1 (B7-H1) and PD-L2 (B7-DC), which show some homology to other B7 family members such as B7-1 and B7-2. Expression of PD-L2 is limited to activated macrophages and DCs, while PD-L1 is also expressed on non-hematopoietic cells [66, 72]. Unfortunately, the efficacy of the inhibitory molecules PD-L1 and PD-L2 was limited in our DC-mediated model of eosinophilic airway inflammation, and these gene modified DCs were not able to abolish established Th2 priming (unpublished observations). Accumulating evidence suggests that the PD-L1/2-PD-1 'signal 2' pathway is primarily important in peripheral tolerance, mediated by non-professional APCs, possibly to limit the extent of immunopathology. The expression pattern of PD-L1 is in agreement with this hypothesis, as it is not only expressed on cells of hematopoietic origin but also in other organs such as heart, lungs and placenta [47, 66]. Furthermore, it might be that this pathway is not as efficient as other similar costimulatory pathways with an inhibitory role such as the CTLA-4 cascade, as mice deficient for CTLA-4 exhibit massive lymphoproliverative disorders and die at 3-4 weeks of age [73]. PD-1 deficient mice also spontaneously develop autoimmune diseases, but more slowly and succumb after 5-6 months [72].

Another strategy one can exploit to suppress Th2 immune responses is based on a phenomenon termed 'reverse signaling', where molecules classically defined as ligands turn into receptors, transmitting signals into the cell from which they are expressed Reverse signaling has recently been described for DCs via CD80, CD86 as well as BTLA. [74-76]. We have obtained evidence that PD-L1 and/or PD-L2 can fulfill a similar role, as soluble PD-1 (the extracellular domain of PD-1 fused to the constant domain of human IgG1) could inhibit $CD4^+$ T cell activation in vitro. This inhibition was accompanied by an increased IL-10 production. Subsequent analysis of the mode of action has revealed that soluble PD-1 acts via PD-L1 and/or PD-L2 on DCs, which acquire a suppressive phenotype consisting of downregulation of CD80 and CD86, in combination with enhanced IL-10 production (manuscript submitted). It is currently not clear whether these immunosuppressive DCs are directly responsible for suppressing T cell activation or generate inducible regulatory T cells (Tregs; discussed below). Irrespective of the mode of action, this phenomenon could be exploited for therapeutic purposes, as pulmonary DCs express high levels of PD-L1 and PD-L2 during eosinophilic

airway inflammation [62, 77, 78]. Indeed, we observed that soluble PD-1 had the capacity to suppress Th2 dependent eosinophilic airway inflammation.
The enzyme indoleamine 2,3- dioxygenase (IDO) is capable of inhibiting T cell responses, either by depletion of tryptophan or the production of inhibitory metabolites such as kynurenine, and thought to be involved in DC-mediated tolerance [79]. The therapeutic effects of CpG motifs, also seem to be mediated by induction of IDO enzyme activity in the lung [80]. We found that tolerogenic pDCs isolated from the lungs of tolerized mice produce high levels of IDO (unpublised observations) and we reasoned that this enzyme could endow these cells with their tolerogenic properties [25] Based on these findings, we have undertaken several attempts to generate DCs overexpressing IDO. Unfortunately, despite IDO gene transcription and IDO protein production in transduced DCs, we did not succeed in inhibiting $CD4^+$ T cell responses *in vitro* (unpublished observations).

Indirect suppression via induction of regulatory T cells

A promising new strategy to develop immunotherapy against disorders ranging from cancer to allergies currently under intense scrutiny of immunologists is based on regulatory T cells (Tregs) As a thorough review of this subset of $CD4^+$ T cells falls outside the scope of this review, we will provide a brief summary here (see [81] for a review). Two major populations have been described so far, the natural occurring regulatory T cells and inducible Tregs. The natural occurring Tregs cells represent 5–10% of the $CD4^+$ T lymphocytes in healthy adult mice and humans and are characterized by expression of CD25 in a naïve state, hence the designation $CD4^+CD25^+$, and express the transcription factor Foxp3. Inducible Tregs appear *in vivo* under regimens of antigen administration known to generate anergy/tolerance. The exact mechanisms of action of these regulatory T cell subsets are not understood in detail. Several mediators have been reported for $CD4^+CD25^+$ Tregs, including IL-10, TGF-ß, CTLA-4 and GITR, but this list is not believed to be complete. Inducible Tregs mainly exert their effect via secreted IL-10, although in some studies it has also been found that cell-cell contact is required, suggesting other effector mechanisms.
The specific interest to employ these cells for immunotherapy against atopic disorders was fuelled by recent discoveries that naturally occurring $CD4^+CD25^+$ regulatory T cells from allergic patients have limited ability to suppress activation of allergen-specific effector T cells compared to healthy controls [82]. In addition, tolerance induction to cow's milk allergens was associated with an increased frequency of circulating $CD4^+CD25^+$ regulatory T cells and a decreased *in vitro* response to a major allergen [83]. Another piece of evidence underscoring the importance of these naturally occurring regulatory T cells is the provided by the observation that humans suffering from the IPEX syndrome, caused by a mutation in the *foxp3* gene, is not only associated with autoimmune disease but with allergy as well [84].
Furthermore, the proportion of allergen-specific induced regulatory T cells was reduced in atopic individuals compared to healthy controls [85]. These findings suggest that function and/or frequency of regulatory T cells are different in atopics compared to healthy individuals.
Animal models of eosinophilic airway inflammation suggest a role for pulmonary

DCs in the induction of regulatory T cells [86, 87]. Similarly, we could also show that a specific subset of plasmacytoid DCs has the potential to capture airborne antigen and induce the formation of Tregs ex vivo [25].
Thus, engineering DCs in such a manner that they induce subsets of regulatory T cells might have great therapeutic potential, not only for allergic disorders but also for other pathologies characterized by unwanted immune responses.
The precise signals governing the induction of Tregs are currently unknown. It has been shown that antigen-specific tolerance as a result of a respiratory exposure regime is mediated by pulmonary DCs that produce IL-10. These DCs subsequently induce IL-10 producing T cells in a B7RP-1-ICOS dependent manner [87]. Interestingly, in our hands, IL-12 producing DCs lead to increased production of IL-10 and IFN-γ by $CD4^+$ T cells [46]. Although we did not formally show that in our assays these cytokines were simultaneously produced by the same cell, it was previously shown that IL-12 and strong polarization stimuli lead to IL-10/IFN-γ secreting $CD4^+$ T cells [88]. These particular subset was also identified in vivo after infection with pathogens causing chronic inflammation and believed to protect against severe inflammatory pathology [89]. More recently, a $CD4^+$T cell subset with a similar cytokine production phenotype but also expressing T-bet and Foxp3 was found to protect against airway hyperreactivity [90]. Moreover, this T cell subset with regulatory properties could only be induced by a particular DC subset expressing IL-12. It will be of interest to know whether IL-12 transduced DC can drive $CD4^+$ T cell differentiation into the same regulatory subset. Surface molecules expressed by DCs might also induce the generation of Tregs. This was elegantly shown in a model of respiratory allergy where DCs transfected with the Notch ligand Jagged 1 was able to specifically induce antigen specific Tregs [91].

Using persistently immature DCs for tolerance induction

Induction of DC maturation by ligation of TLR receptors on DCs is a critical event in the decision between immunity or tolerance [92]. With elegant experiments using DCs that could conditionally express a viral antigen it was shown that antigen expression under inflammatory conditions resulted in the generation of an effective immune response towards this virus, while under steady-state conditions, antigen presentation leads to T cell tolerance, which could not be broken by subsequent infection with the virus [93]. Along similar lines, Brimnes and colleagues showed that inhalation of OVA protein alone leads to tolerance, while simultaneous exposure to OVA and influenza virus results in a immune response towards OVA as well as influenza [94]. Further evidence underscoring the importance of the maturation status of DCs was obtained by silencing SOCS1 expression in DCs, leading to increased antigen presentation and enhanced anti-tumor immunity [95]. Therefore, keeping DCs in a persistent immature state and using these for immunotherapeutic purposes can be seen as a strategy to induce T cell tolerance. Two tactics can be employed. First, antigens could be targeted to endogenous immature DCs, providing we know the receptors for specifically targeting antigens to these cells. The feasibility of this approach was illustrated by specific targeting of antigen to murine DCs via conjugation of antigen to a mono-

clonal directed against DEC-205, a C-type lectin. Targeting OVA antigen to DCs under non-inflammatory conditions led to tolerance, and mice were unresponsive to subsequent OVA challenges in the presence of strong adjuvants [96]. This principle of achieving tolerance via DC targeting is currently pursued for therapeutic purposes through identification of the natural ligands for this (and other) lectins, followed by antigen conjugation. The different approaches described above are summarized in figure 2. A second strategy would be to culture immature DCs ex vivo, pulse them with antigen and reinject those in a persistently immature form to mediate tolerance to the antigen. There have been several attempts at this strategy. Martin et al [97] used CD40 deficient DCs, resisting T cell mediated DC maturation, to induce Tregs in vivo and these cells could suppress T cell mediated pathologies. Others have used a dominant negative inhibitor of NF-Kb signaling to keep DCs in an immature state to generate Tregs [98].

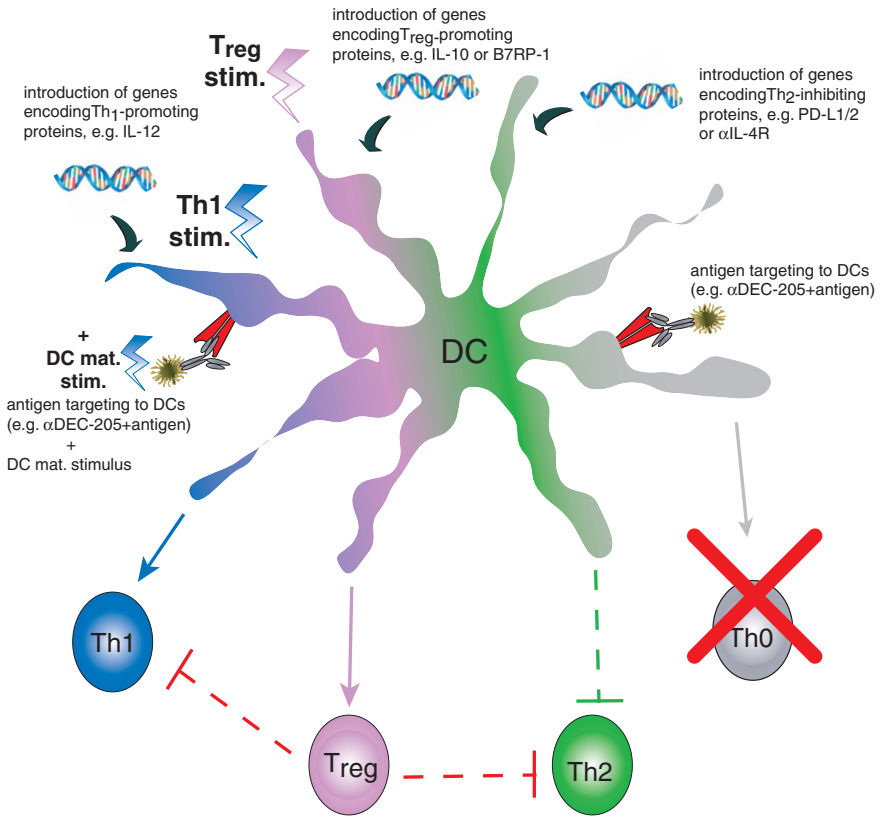

Figure 2 *Strategies for DC-mediated ($CD4^+$) immunotherapy*
See text for explanation

Conditions for successful DC-mediated immunotherapy

Despite these exciting possibilities for DC based immunotherapy for a range of human diseases looming at the horizon, some basic questions need to be elucidated before routine clinical use becomes a reality.

How can we control DC activation?

The context in which antigen is presented to naïve T cells by DCs is of paramount importance for the class of immune response generated [17]. The discovery of pattern recognition receptors (PRRs), such as TLRs, provided a molecular mechanism for the transition of immature, tolerizing, DCs into effective APCs. To tailor a DC vaccine in such a way that it generates the desired immune response requires more detailed knowledge about these secondary signals. For example, which stimuli (or absence of stimuli) are required to induce tolerance against a particular allergen without inducing an immune response against self-antigens, resulting in autoimmunity? Conversely, for tumor eradication a robust immune response needs to be generated and inadequate innate signals may make an individual even more susceptible to further tumor growth by actively tolerizing the antigen-specific lymphocytes.

What is the optimal route and dose of administration of DC?

The optimal route of administration for immunotherapy for allergic disease has not been settled. In current practice, standard desensitization immunotherapy using allergen extracts is administered subcutaneously or sublingually. Based on the findings in DC-immunotherapy in cancer patients, it might similarly be practical to inject DCs subcutaneously or directly into the skin draining node using an echography-guided approach. However, interfering with a mucosal disease such as asthma might also require the administration of tolerogenic DCs to the nasal or lung mucosa. Preclinical experiments will have to be performed in the future in mouse models to address these points in greater detail.

What is the antigen-specificity and are the targets of regulatory T cells?

Although the presence of regulatory T cells has been confirmed in many disease models [99], less is known about the antigen specificity and targets of these cells. The naturally occurring $CD4^+CD25^+$ T cell population mainly seems to recognize an antigen repertoire of peripheral self-antigens [100, 101], but have also been implicated to control pathogen infections [102]. Inducible regulatory T cells, on the other hand, arise during antigen administration regimes known to induce tolerance/anergy [81], and antigen-specific clones have also been generated from lymphocytes isolated from *B. pertussis* infected lungs [103]. Besides the antigen specificity of the cell populations, its is also important to unravel their mechanism of action. The effector molecules of induced Tregs identified so far, IL-10 and TGF-ß, suggest that this subset is activated in an antigen-dependent manner, but suppresses immune responses in an antigen-independent fashion. Therefore, careful examination of the regulation of regulatory T cell activation and termination needs to be undertaken to avoid general immunosuppresion due to Treg-inducing immunotherapy.

Concluding remarks : early immunomodulatory intervention is the key to altering the natural history of asthma

Current asthma therapy is largely based on the use of inhaled corticosteroids [104], which are adequate and safe but concerns remain about their long-term use, particularly in children [105]. Although inhaled corticosteroids reduce eosinophilic airway inflammation and provide symptomatic relief, it is unclear if they also modify airway remodeling [106-108]. Upon withdrawal of treatment, the symptoms recur, suggesting that they do not alter the natural history of asthma and that the various structural changes in airway tissue, are of increasing importance in established disease. The only way to intervene with the natural history of asthma is to alter the aberrant Th2 response to inhaled allergens, and in this way halt 'the atopic march'. Various strategies of immunotherapy have been employed, some of them involving regular subcutaneous injections of allergen extracts or recombinant allergen, others using sublingual administration of allergens, in the hope of downregulating the allergen-specific Th2 response [109]. Many of these studies have been performed in adults with chronic, therapy resistant disease. Based on our current understanding of the pathology of established asthma, and on our experience with DC driven immunotherapy, it can be concluded that immunotherapy will likely be of limited use for treatment at the chronic phase of the disease. Instead, immunotherapeutic interventions should be of greater benefit at an earlier stage, before onset of symptoms [110]. It is known that the lung undergoes profound changes during infancy driven by rapid growth and differentiation, establishing structure-function relationships that are central to respiratory health throughout the rest of life [111]. Accumulating evidence suggests that lung injury during this critical period may initiate developmental changes that are amplified during subsequent growth and eventually culminate in asthma. So, (immunotherapeutic) intervention at this early stage of disease development might have great impact on the final disease outcome. The key element of this strategy will be to predict which infants are most likely to progress towards adult asthma. Advances in susceptibly gene discovery will most certainly aid in identification of children that have a high risk to develop chronic allergic airway inflammation, together with large-scale prospective cohort studies, involving tracking of individual children over a period of years, from first manifestation of infant wheezing through to the development of persistent asthma in adult life [112]. Together with increased understanding of DC biology, in particular in the area of $CD4^+$ T cell polarization, this knowledge may ultimately result in DC-based vaccines to prevent atopic asthma.

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Summary

Asthma is a chronic disease of the lower airways, characterized by intermittent episodes of airway narrowing, causing symptoms of chest tightness, wheezing and shortness of breath. Although rarely life-threatening, the disease significantly affects the quality of life and places a large economic burden on the healthcare system. Moreover, for yet unknown reasons, the disease is reaching epidemic proportions in the industrialized world.

Although the cause of asthma is complex and multifactorial, is it certain that the immune system provides a large contribution. The symptoms of asthma are correlated with inflammation of the lung tissue, as evidenced by the accumulation of cells of the immunesystem, such as eosinophils, mast cells, lymphocytes and dendritic cells. Mediators produced by these cells are also increased in the lungs of asthmatic patients. Several phases can be distinguished during the development of this disease Upon encounter with an allergen (any compound that can trigger a so-called allergic reaction), sentinel cells that line all tissues exposed to the outside world, termed dendritic cells (DCs) , take up the allergen and transport it to specialized organs of the body, lymph nodes. Here, DCs interact with $CD4^+$ T lymphocytes that recognize the allergen, resulting in T cell activation. These T cells subsequently differentiate into a specialized subset of T cells, called Th2 cells. These Th2 cells force another type of lymphocytes, B cells, to secrete allergen-specific IgE that binds to mast cells. People susceptible to develop asthma are now said to be sensitized. Upon subsequent encounter with allergen during the so called challenge phase, this allergen binds to the IgE bound to mast cells, which triggers the release of all sorts of products responsible for immediate effect such as bronchoconstriction leading to airway narrowing and mucus production, which plugs the airways. This immediate response is called the 'early asthmatic reaction' to allergen and is mainly mediated by mast cells. Moreover, local DCs can stimulate previously activated T cells which migrated to the site of inflammation. These stimulated Th2 cells in turn also secrete mediators responsible for characteristics ranging form mucus production to attracting and activating eosinophils. This phase occurs 3-6 hours after allergen challenge and is called the 'late asthmatic reaction'. Repeated exposure to allergen leads to chronic inflammation and eventually leads to permanent alterations of the lung tissue. What probably was already deduced from the above mentioned sequence of events is the pivotal role of DCs and Th2 cells during the developmental as well established phases of the disease. Indeed, depletion of these cells in animal models of asthma completely abrogated the symptoms of this disease.

Despite the wealth of information acquired in the last decades about the role of the immunologic component of the disease, current treatment is still rather crude and mainly consists of inhalation of corticosteroids, which non-specifically suppress the entire immune system, in combination with bronchodilators. Corticosteroids only suppress symptoms though, and do not cure the disease. Furthermore, although current therapy is considered safe, some concerns remain about potential long-term use of corticosteriods in particular in the light of the chronic nature of the disease and the trend towards an earlier onset of therapy in children.

In this thesis, we investigated whether we could use dendritic cells to prevent development of asthma or suppress established asthma, in an effort to more specifically target the immune pathways involved in airway inflammation. To that end, we used a previously established mouse model of asthma in which animals were sensitized to a model allergen by presentation of this allergen by DCs applied to the airways. In this way, we were able to deliver signals simultaneously with the allergen that modify the response of the allergen-specific T cells. In this thesis we have followed several strategies to modify the T cell response.

In the context of asthma, T cells differentiate towards the so-called Th2 subset, as discussed above. However, another subset of T cells is the Th1 subset. This subset is involved in helping other cells of the immune system to fight off intracellular pathogens, e.g. viruses. Moreover, it was postulated that these subsets suppress each others' development and function. Thus, Th1 cells suppress the formation and function of Th2 cells. Depending on the type of stimulus, DCs are potent inducers of Th1 cells. If DCs are stimulated with Th1-inducing stimuli, DCs secrete soluble messengers molecules (cytokines) that act on T cells and drive Th1 differentiation. A very important molecule in this regard is IL-12. To examine whether deviation towards a Th1 immune response protected against the development of asthma in mice, we incubated DCs with a compound derived from the cell wall of bacteria (LPS) know for its potent IL-12 production capacity by DCs. Indeed, as described in **chapter 3**, LPS stimulated DCs induce Th1 formation in a cell-culture system (in vitro) as well as in mice (in vivo), as judged by cytokine production patterns specific for Th1 cells. LPS stimulated DCs were also capable to reduce Th2-specific airway inflammation, with a reduction in Th2 cytokines, eosinophils and mucus production. Interestingly, production of IL-12 by LPS stimulated DCs was not necessary to reduce eosinophilic airway inflammation. It was suggested that endogenous cells could rescue IL-12 production, but repetition of the experiments in mice deficient for IL-12 showed similar results. Thus, it can be concluded that stimulating the formation of Th1 cells can suppress the development of Th2-dependent eosinophilic airway inflammation, but that IL-12 is not per se necessary for these suppressive effects.
However, as shown in **chapter 4**, IL-12 is sufficient to drive the Th1 differentiation of naïve T cells. In this chapter, we transduced DCs with a retroviral vector harboring the genes encoding IL-12. In this way, DCs continuously produce high levels of IL-12. Analogous to LPS stimulated DCs, IL-12 transduced DCs have potent Th1 skewing capabilities in vitro and efficiently inhibit the development of eosinophilic airway inflammation. The Th1 response in the lung was confirmed by quantitative analysis of expression levels in the lungs of a whole array of messenger molecules (cytokines and chemokines), which revealed an expression pattern consistent with previously published reported Th1 expression patterns. Unfortunately, injecting IL-12 producing DCs into pre-sensitized animals did not result in suppression of asthma symptoms but rather enhanced the disease.
To summarize, deviation of the immune response towards Th1was beneficial to suppress the development of asthma. However, inducing the formation of aller-

gen-specific Th1 cells in the presence allergen-specific Th2 cells worsened disease symptoms considerably, making this approach not suitable for treatment of established disease, which is the present situation in human patients. In addition, it can be questioned whether substituting one type of immune response (Th2) for another type (Th1) is the most optimal treatment strategy.
To circumvent this problem, we also investigated an alternative strategy consisting of direct suppression of allergen-specific Th2 lymphocytes. This strategy was based on a group of molecules collectively called 'co-stimulators'. These molecules are expressed by DCs and together with antigen required to optimally activate T cells. However, recently new members of this family, called PD-L1 and PD-L2, have been discovered that possess inhibitory properties that inhibit T cell function instead of stimulating it. In this thesis, we examined whether overexpressing these molecules by DCs presenting a model allergen could inhibit T cell function in cell cultures as well as in our mouse model of asthma (**chapter 5**). The cell culture experiments showed no differences in T cell division, a marker for activation, when PD-L1 or PD-L2 overexpressing DCs were compared to mock-infected DCs. However, levels of an important activation cytokine, IL-2, were lower. We next reasoned that expression levels of stimulatory co-stimulator molecules, notably CD80 and CD86, were too high to permit any inhibitory effect of PD-L1 or PD-L2. Therefore, we repeated the cell-culture experiments with DCs lacking CD80 and CD86. However, even in the absence of these potent stimulatory molecules, PD-L1 or PD-L2 were not capable of inhibiting T cell division. Still, the consistent observation that PD-L1 and PD-L2 decrease IL-2 production by T cells suggested a suboptimal activation of T cells. To examine whether this would result in a suboptimal immune response in vivo, we sensitized mice in our asthma model with DCs overexpressing PD-L1 or PD-L2 and measured lung inflammation. We did not detect a significantly lower inflammation, indicating that despite the decreased IL-2 production in vitro, T cells were sufficiently activated in vivo to cause airway inflammation.
Finally, we attempted to exploit for therapeutic purposes a phenomenon we observed when we added a soluble form of the receptor for PD-L1 and PD-L2, PD-1, to DC-T cell cultures. When soluble PD-1 was present, T cells divided less and produced less IL-2, but the amount of the suppressive cytokine IL-10 in the culture was increased.. Subsequent dissection of the cell type involved demonstrated that soluble PD-1 acted upon DCs, which acquired a suppressive phenotype, secreting IL-10 and downregulating costimulatory molecules. This indicates the occurrence of a phenomenon known as reverse signaling, where ligands (PD-L1 and/or PD-L2) turn into receptors, and has also been described for homologous costimulatory ligands and their receptors such as CTLA-4 and CD28. Analysis of PD-L1 and PD-L2 expression on DCs isolated from asthmatic mice showed high expression of these ligands, prompting us to investigate the therapeutic effect of soluble PD-1 in our mouse model of asthma. We observed a slight decrease in the number of eosinophils obtained from lung fluid, and decreased levels of prototypic Th2 messenger molecules, but this decrease is not sufficient to warrant therapeutic purposes (**chapter 6**).
In conclusion, the concept of DC mediated immunotherapy directed against asthma still holds potential, as judged from chapters 3 to 6. However, some re-

quirements need to be fulfilled before asthma immunotherapy becomes reality. First and foremost, the results presented in chapter 4 indicate that the timing of immunological intervention is an important parameter, i.e. the earlier the better. In this regard, it is currently not possibly to accurately predict which atopic children progress from episodes of wheezing into full blown asthma and thus may qualify for immunotherapy. Longitudinal cohort studies following up children from birth up into their teens are now underway and may yield prognostic tools that could help to better identify children at risk. Secondly, from chapters 3 and 4 it is evident that although substituting a Th2 response with a Th1 response inhibits the development of allergic airway inflammation, it still represents a deviation from the normal, non-inflamed, situation in the lung. The recent discovery of the involvement of regulatory T cells (Tregs) in allergic diseases might therefore offer new therapeutic strategies. Tregs are a subset of $CD4^+$ T cells that suppress other cells of the immunesystem. It has been reported that lung DCs are able to deliver signals that support the formation and activation of these cells that subsequently inhibit lung inflammation. However, before this approach can be applied in a clinical setting, more data is required about the DC-mediated signals that result in Treg formation/activation. Furthermore, the precise mechanism of Treg activation and target cells need to be elucidated, to avoid potentially dangerous general immunesuppression. Providing that we can fill these specific gaps in our current knowledge, DCs based immunotherapy ultimately may become reality for an array of immunological disorders, including asthma.

Samenvatting

Astma is een chronische ontsteking van de luchtwegen die wordt gekarakteriseerd door periodieke luchtwegvernauwing resulterend in een drukkend gevoel op de borst, piepende ademhaling en benauwdheid. Ondanks dat deze ziekte zelden levensbedreigend is, kan de kwaliteit van leven ernstig worden aangetast en zijn de directe en indirecte kosten groot. Bovendien neemt de ziekte epidemische vormen aan in de westerse wereld.
De precieze oorzaak van astma is complex en niet precies bekend. Wel is het zeker dat het immuunsysteem een grote bijdrage levert. De symptomen van astma correleren met ontstekingen in de longen, zoals kan worden afgeleid aan de ophoping van ontstekingscellen zoals eosinofielen, mestcellen, lymfocyten en dendritische cellen. Stoffen geproduceerd door deze cellen komen ook in verhoogde mate in de longen van astma patiënten voor. Verscheidende fasen kunnen worden onderscheiden in het verloop van de ziekte. Als de longen in contact komen met een allergeen (elke stof die een zogenoemde allergische reactie kan veroorzaken) wordt dit opgenomen door 'schildwacht' cellen die zich bevinden in alle weefsels die in contact staan met de buitenwereld. Deze cellen worden dendritische cellen (DCs) genoemd. Vervolgens transporteren DCs het allergeen naar gespecialiseerde organen van het lichaam, de lymfeklieren. Hier komen deze DCs in contact met $CD4^+$ T lymfocyten die het allergeen herkennen wat leidt tot T cel activatie. Deze geactiveerde T cellen differentiëren vervolgens in een speciale subset van T cellen, de Th2 cellen. Stoffen geproduceerd door deze T cellen zetten een ander type lymfocyten, B cellen, aan tot de productie van allergeen-specifiek IgE dat bindt aan mest cellen. Vanaf dit moment spreekt men van sensibilisatie voor een specifiek allergeen. Bij ieder volgend contact met allergeen bindt dit aan het IgE op mestcellen. Hierdoor scheiden de mestcellen allerhande stoffen uit die verantwoordelijk zijn voor directe effecten in de longen zoals luchtwegvernauwing en slijm productie dat de luchtwegen verstopt. Deze eerste reactie op allergeen wordt de 'vroege astmatische reactie' genoemd en wordt voornamelijk veroorzaakt door mest cellen. Ook kunnen lokaal aanwezige DCs geactiveerde Th2 cellen stimuleren die naar de plek van de ontsteking zijn gemigreerd. Deze gestimuleerde T cellen produceren op hun beurt allerlei mediatoren verantwoordelijk voor allerhande ontstekingsprocessen, variërend van slijm productie tot het aantrekken en activeren van eosinofielen. Deze fase vindt 3-6 uur na allergeen blootstelling plaats en wordt de 'late astmatische reactie' genoemd. Herhaalde blootstelling aan allergenen leidt tot chronische ontsteking en uiteindelijk tot permanente veranderingen van het longweefsel. Wat kan worden afgeleid uit het bovenstaande is dat DCs en Th2 cellen een essentiële rol spelen in zowel ziekte ontwikkeling als het in stand houden van de symptomen. Dit wordt bevestigd in diermodellen van astma, waarbij deletie van deze cellen leidt tot het compleet verdwijnen van de ziekte symptomen.
Ondanks alle kennis die gedurende de laatste jaren is vergaard over de rol van het immuunsysteem in deze ziekte, is de huidige behandeling niet echt verfijnd en bestaat voornamelijk uit inhalatie van corticosteroïden, die op een niet-specifieke wijze het gehele immuun systeem onderdrukken, in combinatie met luchtwegverwijderaars. Door deze behandelwijze worden echter alleen de symptomen best-

reden en niet de onderliggende oorzaken. Verder bestaan er, alhoewel de huidige therapie als veilig wordt beschouwd, zorgen over de lange-termijn effecten van langdurig steroïden gebruik. Dit vooral in het licht van de chronische aard van de ziekte en de trend om behandeling op steeds jongere leeftijd te beginnen.

In dit proefschrift is onderzochte of dendritische cellen gebruikt konden worden om het ontstaan van astma te voorkomen of bestaand astma te onderdrukken en daardoor op meer specifieke wijze te interveniëren in de immunologische processen betrokken bij de ontsteking in de longen. Om dit te realiseren is er gebruik gemaakt van een bestaand muizenmodel van astma waarin dieren worden gesensibiliseerd tegen een model allergeen dat wordt gepresenteerd door DCs geïnjecteerd in de luchtwegen. Op deze wijze waren we in staat om de DCs, tegelijkertijd met het allergeen, specifieke signalen te laten afgeven die de respons van allergeen-specifieke T cellen beïnvloeden. In dit proefschrift werden verschillende strategieën gevolgd teneinde de T cel respons te beïnvloeden.

In context van astma differentiëren T cellen naar de bovengenoemde Th2 subset. Echter, er bestaat nog een andere subset van T lymfocyten, de Th1 cellen. Deze subset is betrokken bij de afweer tegen intracellulaire ziekteverwekkers, b.v. virussen. Er zijn bewijzen dat deze subsets elkaars formatie en functie afremmen. Dus, Th1 cellen onderdrukken de vorming en functie van Th2 cellen. DCs zijn, afhankelijk van het type stimulus, zeer goed in staat om T cellen richting Th1 te laten differentiëren. Blootstelling van DCs aan zogenaamde. Th1 stimuli leidt tot de secretie van boodschapper molekulen (cytokines) welke T cel differentiatie sturen richting de Th1 subset. Een belangrijke molecuul in dit verband is IL-12. Om te onderzoeken of een Th1 respons beschermt tegen de ontwikkeling van astma in muizen stelden we DCs bloot aan een celwand component van bacteriën (LPS) waarvan bekend is dat deze IL-12 produktie van DCs stimuleert. Inderdaad, zoals beschreven in **hoofdstuk 3**, kon uit het cytokine profiel worden afgeleid dat LPS-gestimuleerde DCs Th1 cellen induceerden in zowel een laboratorium celkweek systeem (in vitro) alsmede in muizen (in vivo). LPS gestimuleerde DCs waren ook in staat om Th2-specifieke luchtwegontsteking te verminderen, met een vermindering van Th2 cytokinen, het aantal eosinofielen en slijmproductie. Interessant genoeg bleek dat IL-12 hiervoor niet nodig was. Hierop werd gesuggereerd dat endogene cellen (van de muis zelf) de benodigde IL-12 produceerden, maar herhaling van de experimenten in muizen die geen IL-12 produceerden gaf dezelfde resultaten. Samenvattend kan hieruit geconcludeerd worden dat het stimuleren van de vorming van Th1 cellen de ontwikkeling van Th2-afhankelijke luchtwegontsteking kan voorkomen maar dat IL-12 hiervoor niet per se nodig is.
Echter, uit de resultaten beschreven in **hoofdstuk 4** bleek dat IL-12 alleen voldoende is om de T cel ontwikkeling richting Th1 te sturen. Daartoe maakten we gebruik van een proces dat transductie wordt genoemd; het inbrengen van een gen in een cel door middel van een (kreupel) virus. In dit hoofdstuk transduceerden we DCs met een retrovirale vector die genen coderend voor IL-12 bev-

atte. Op deze manier produceerden DCs continu grote hoeveelheden IL-12. IL-12 getransduceerde DCs waren, net als LPS gestimuleerde DCs, sterke Th1 inducers in vitro en remden de ontwikkeling van eosinofiele luchtwegontsteking. De Th1-gemedieerde respons in de longen werd bevestigd door kwantitatieve analyse van de expressie niveaus van een reeks boodschapper moleculen (cytokinen en chemokinen) die typisch zijn voor een Th1 respons. Helaas bleken IL-12 producerende DCs niet in staat astma af te remmen als de muizen al gesensibiliseerd waren, maar werden de symptomen hierdoor alleen maar verergerd.
Samenvattend kan gezegd worden dat sturing van de immuunrespons richting Th1 in staat was om de vorming van astma te voorkomen, maar dat de vorming van allergeen-specifieke Th1 cellen in de aanwezigheid van allergeen-specifieke Th2 cellen de ziekte verergerde. Hierdoor is deze strategie niet bruikbaar om bestaand astma te behandelen. Daarnaast kan men zich afvragen of het vervangen van het ene type immuun respons (Th2) door het andere (Th1) de meest ideale strategie is.
Om dit probleem te omzeilen is ook onderzocht of direkte afremming van allergeen-specifieke Th2 lymfocyten een mogelijkheid was om astma te voorkomen of te verminderen. Deze benadering was gebaseerd op een groep moleculen die 'co-stimulators'genoemd worden. Deze moleculen worden (onder andere) tot expressie gebracht door DCs en zijn samen met het allergeen nodig voor een optimale activatie van T cellen. Echter, recentelijk zijn nieuwe leden van deze familie, PD-L1 en PD-L2, ontdekt die T cel activering afremmen in plaats van stimuleren. In dit proefschrift is onderzocht of overexpressie van deze moleculen door DCs die een model allergeen presenteerden aan T cellen leidde tot verminderde T cel activatie, zowel in vitro alsmede in ons muizemodel van astma (**hoofdstuk 5**). Vergelijking van deze DCs met controle DCs liet geen verschillen zien in T cel deling (karakterisitiek voor T cel activatie). Echter, de hoeveelheden van een belangrijk activatie cytokine, IL-2, waren lager. Vervolgens redeneerden we dat expressie niveaus van positieve co-stimulators, met name CD80 en CD86, zodanig waren dat een eventueel remmend effect van PD-L1 en/of PD-L2 niet waarneembaar was.Hierop werden de celkweek experimenten herhaald met DCs die CD80 en CD86 misten. Maar zelfs in de afwezigheid van deze potente T cel stimulatoren waren PD-L1 en PD-L2 niet in staat om T cel deling te remmen. Echter, de consistent lagere IL-2 productie duidde op een suboptimale T cel activatie. Om te onderzoeken of dit ook leidde tot een suboptimale immuun respons in vivo werden muizen in ons astmamodel gesensibiliseerd met DCs die PD-L1 of PD-L2 tot overexpressie brachten en vervolgens werd de mate van luchtwegontsteking geanalyseerd. Er was geen sprake van verminderderde ontsteking, waaruit geconcludeerd kan worden dat ondanks de lagere IL-2 productie in vitro T cellen in vivo voldoende waren geactiveerd om luchtweg inflammatie te veroorzaken.
Tenslotte werd er een poging ondernomen om astma af te remmen met behulp van de niet-membraan gebonden vorm ('vrij') van PD-1. Wanneer vrij PD1, de receptor voor PD-L1 en PD-L2 werd toegevoegd aan DC-T cel kweken deelden T cellen minder en produceerden minder IL-2, maar was er meer van het remmende cytokine IL-10 aanwezig in de kweek. Uit vervolgonderzoek bleek dat vrij PD-1 deze effecten veroorzaakte via DCs, welke na incubatie met vrij PD-1 een remmend karakter kregen bestaande uit verminderde expressie van co-

stimulator moleculen en productie van IL-10. Dit duidt op het optreden van een fenomeen dat bekend staat als 'reverse signaling', waarbij liganden (PD-L1 en/of PD-L2) veranderen in receptoren en dat ook beschreven is voor homologe receptor-ligand paren zoals CTLA-4 en CD28. Uit analyse van DCs geïsoleerd uit de longen van muizen met eosinofiele (astma) luchtwegontsteking bleek dat PD-L1 en PD-L2 hoog tot expressie kwamen,waarop het effect van vrij PD-1 op astma in ons muizemodel werd onderzocht. Een lichte afname in het aantal eosinofielen in long spoelvloeistof werd gemeten, evenals verminderde hoeveelheden karakteristieke Th2 cytokines, maar deze vermindering lijkt echter onvoldoende voor therapeutische doeleinden (**hoofdstuk 6**).

Concluderend bezit het concept van immunotherapie tegen astma met behulp van DCs potentieel, zoals kan worden afgeleid van hoofdstuk 3 t/m 6. Echter, er zal aan enkele voorwaarden voldaan moeten worden voordat astma immunotherapie realiteit wordt. Allereerst blijkt uit de resultaten van hoofdstuk 4 dat de timing van interventie een belangrijke parameter is; hoe vroeger hoe beter. In dit opzicht is het een probleem dat op het moment niet accuraat voorspeld kan worden welke allergische kinderen uiteindelijk astma ontwikkelen en daardoor in aanmerking komen voor immunotherapie. Er lopen momenteel longitudinale cohort studies die kinderen volgen vanaf de geboorte tot in de tienerjaren en die mogelijk leiden tot hulpmiddelen waarmee beter voorspeld kan worden welke kinderen uiteindelijk astma ontwikkelen. Ten tweede wordt uit hoofdstuk 3 en 4 duidelijk dat ondanks dat vervanging van een Th2 respons door een Th1 respons de ontwikkeling van astma kan afremmen er nog steeds sprake is van een immuunrespons, wat een afwijking van de normale situatie (geen immuunrespons) inhoudt. De recent ontdekking dat regulatoire T cellen (Tregs) ook een rol spelen bij allergieën kan en aanknopingspunt zijn voor nieuwe behandelings mogelijkheden. Tregs zijn een subset van $CD4^+$ T cellen die andere cellen van het immuunsysteem onderdrukken. Er zijn aanwijzingen dat DCs uit de longen in staat zijn om het ontstaan en activatie van deze Tregs te bevorderen en dat deze cellen vervolgens luchtwegontsteking afremmen. Voordat deze strategie echter toegepast kan worden in de kliniek moet er eerst meer bekend zijn over de precieze DC signalen die betrokken zijn bij Treg formatie/activatie. Verder moet er ook meer bekend zijn over de omstandigheden waarin deze cellen geactiveerd worden en welke het doelwit zijn van deze Treg cellen, om potentieel gevaarlijk niet-specifieke onderdrukking van het immuunsysteem te voorkomen. Vooropgesteld dat we deze hiaten in onze kennis kunnen opvullen zou immunotherapie met behulp van DCs uiteindelijk realiteit kunnen worden voor een reeks van ziekten waarbij het immuunsysteem verstoord is, waaronder astma.

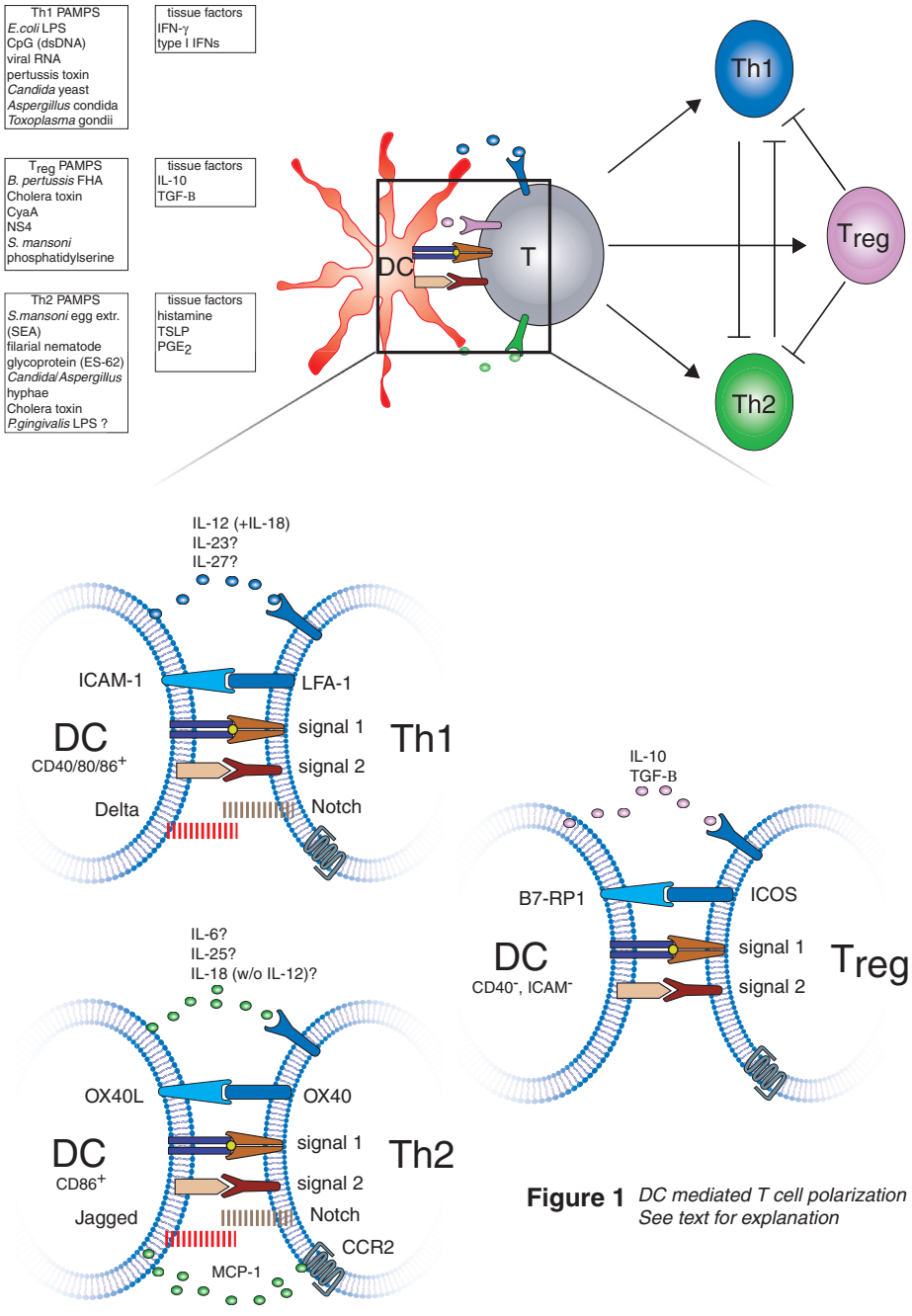


Figure 1 *DC mediated T cell polarization*
See text for explanation

Chapter 1, figure 1

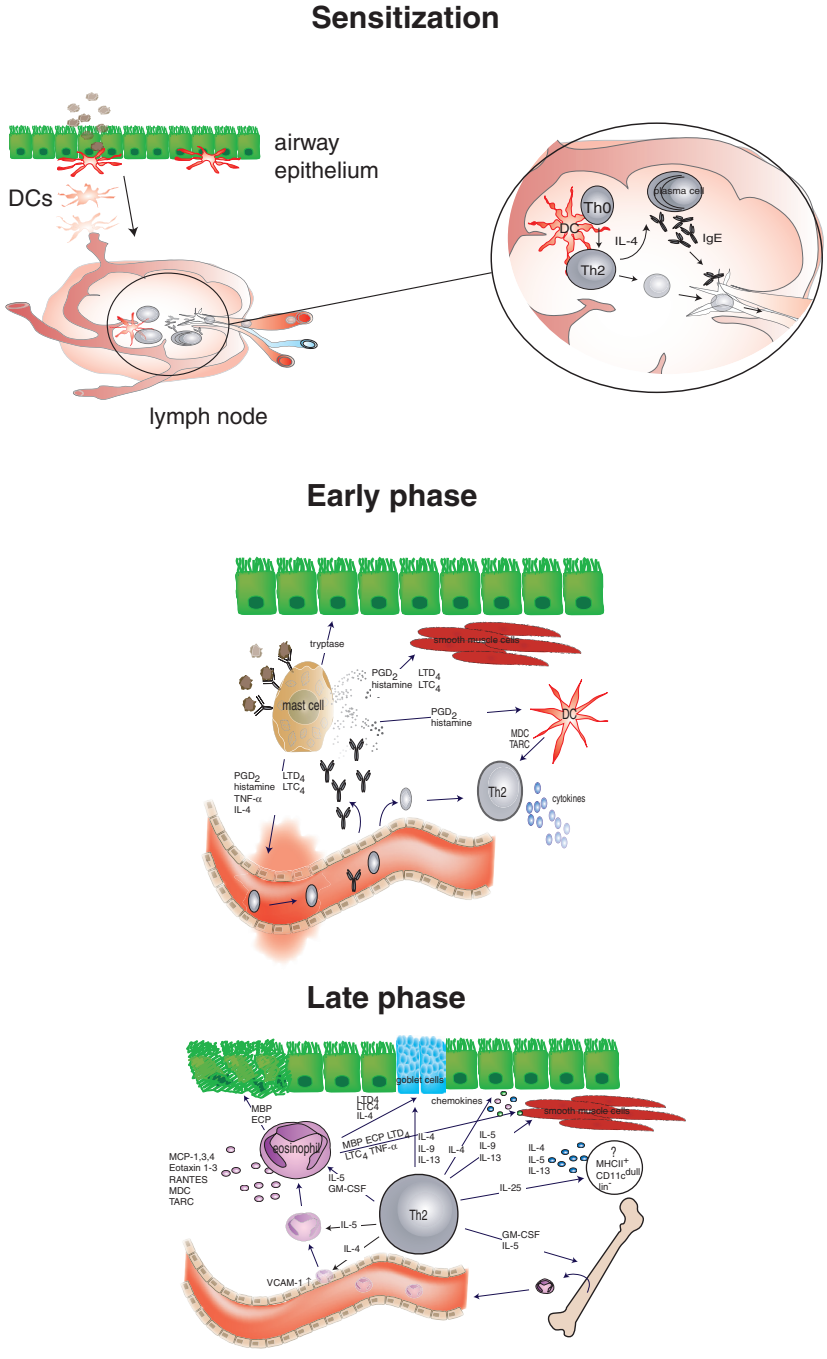
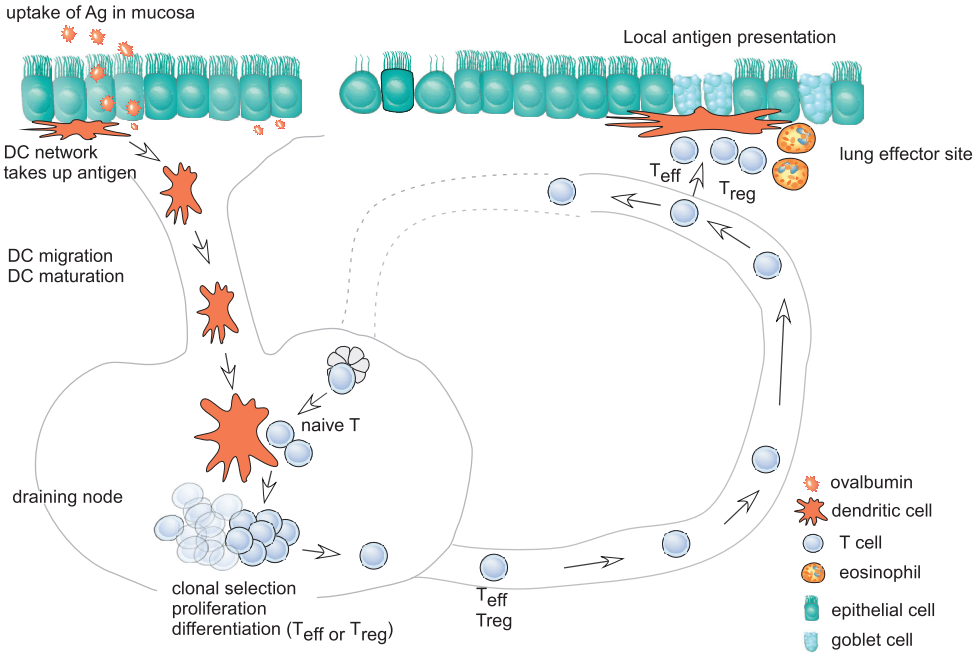
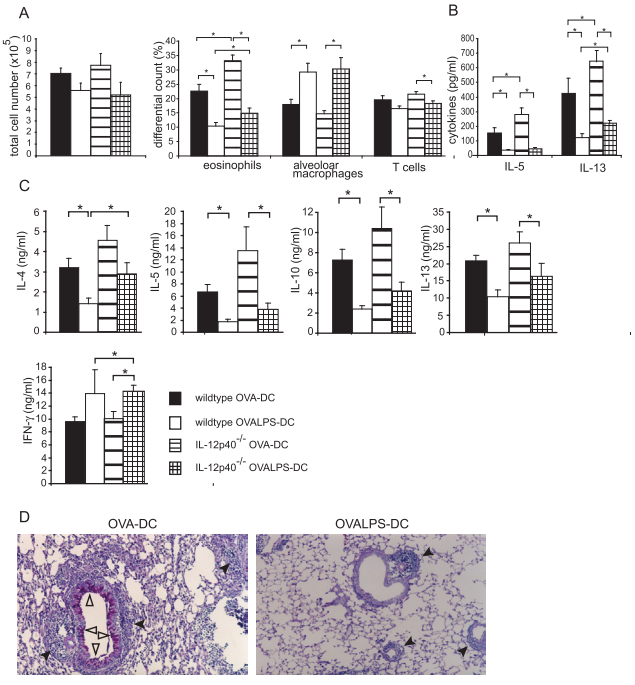


Figure 2 *Immunology of eosinophilic airway inflammation. See text for explanation*

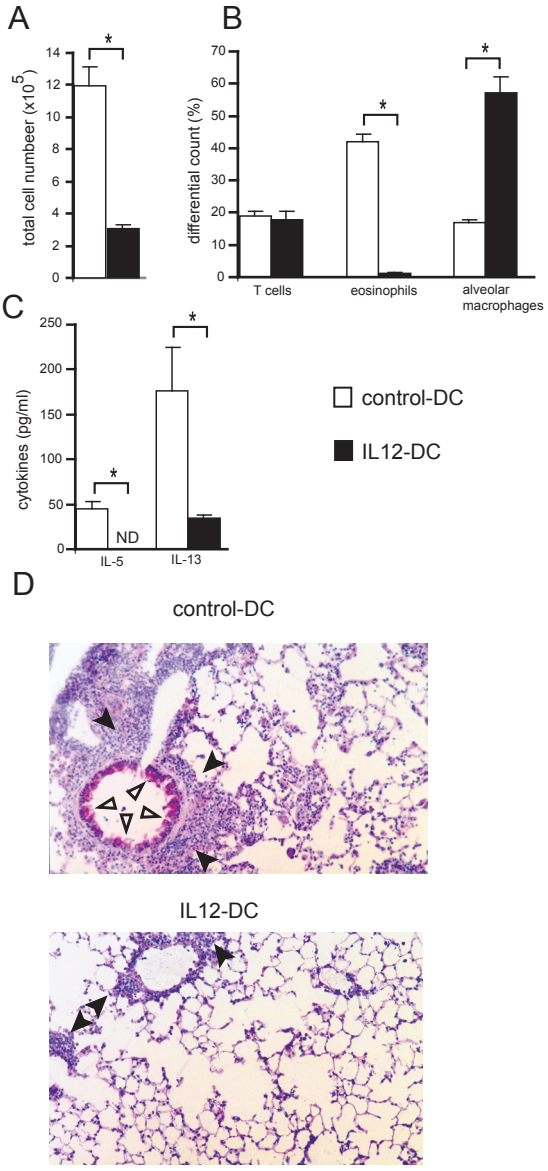
Chapter 1, figure 2



Chapter 2, figure 1



Chapter 3, figure 6



Chapter 4, figure 3

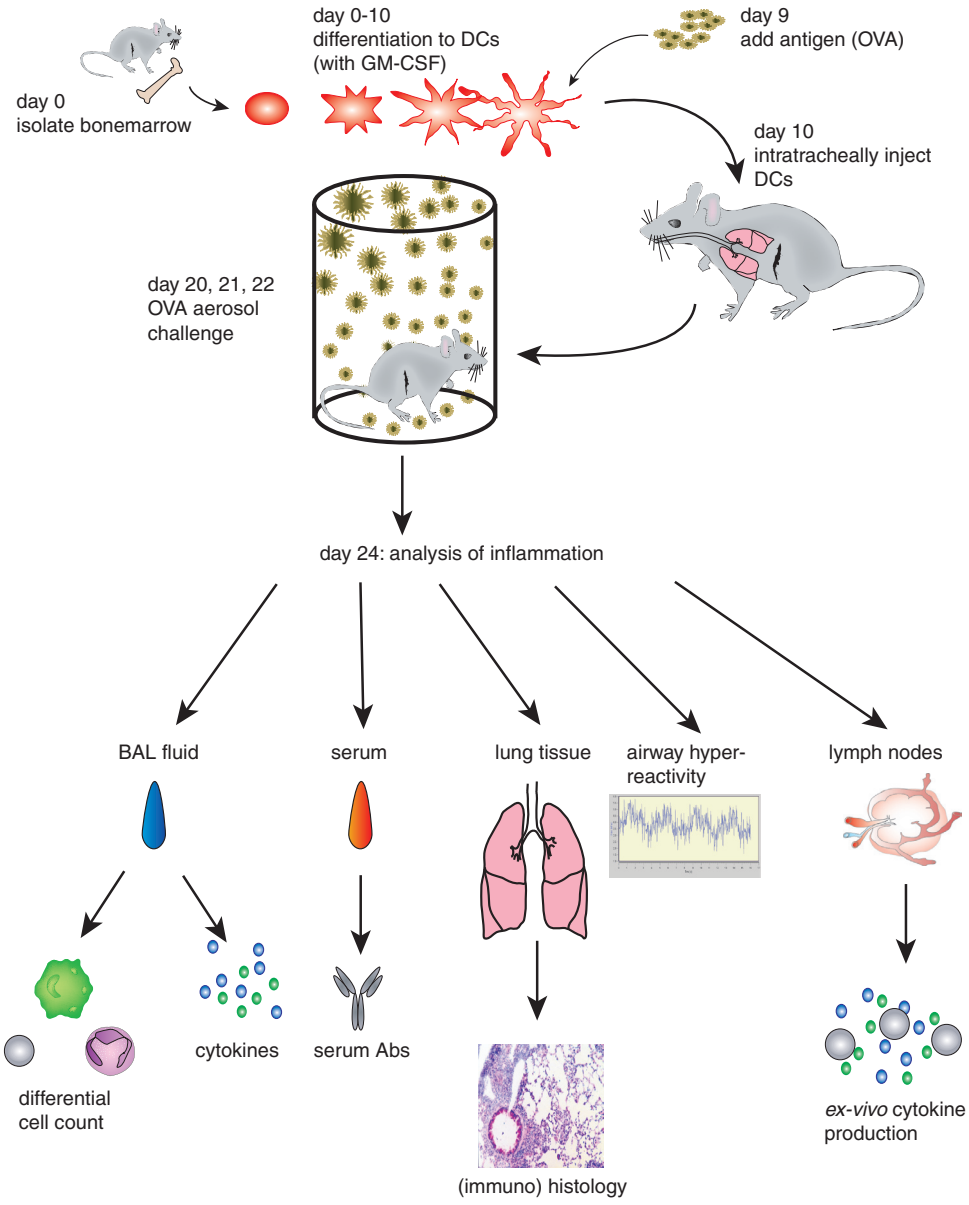


Figure 1 *DC-mediated murine model of eosinophilic airway inflammation*

Chapter 7, figure 1

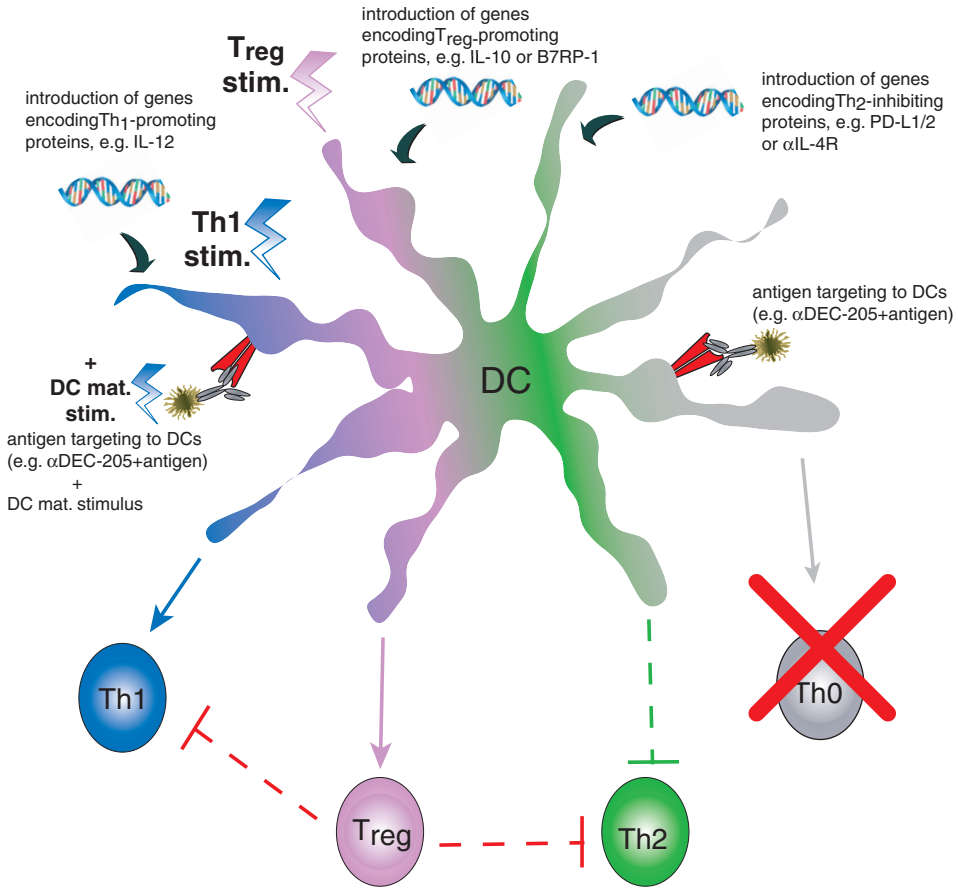


Figure 2 *Strategies for DC-mediated ($CD4^+$) immunotherapy*
See text for explanation

Chapter 7, figure 2

List of abbreviations

7-AAD	7 aminoactinomycin
Ag	antigen
ALN	axillar lymph nodes
APC	antigen-presenting cell
APC	allophycocyanin
Ag	antigen
AICD	activation induced cell death
BAL	broncho alveolar lavage
BHR	bronchial hyperreactivity
BSA	bovine serum albumin
BM	bone marrow
CD	cluster of differentiation
CFSE	carboxy fluorescein diacetate succinimidylester
CM	culture medium
d	day
DC	dendritic cell
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
FACS	fluorescence activated cell sorter
FSC	forward scatter
FITC	fluorescein isothiocyanate
Flt-3L	Flt-3 ligand
FCS	fetal calf serum
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
ICAM	intracellular adhesion molecule
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
IP-10	interferon inducible protein-10
i.t.	intratracheal
i.v.	intravenous
LN	lymph node
LPS	lipopolysaccharide

MACS	magnet activated cell sorting
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLN	mediastinal lymph nodes
NK	natural killer cell
NKT	natural killer T cell
NF-kB	nuclear factor kB
OVA	Ovalbumin
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
pDC	plasmacytoid DC
PE	phycoerythrin
PECy5	phycoerythrin-cychrome 5
PI	propidium iodide
PRI	proliferation index
PRR	pattern-recognition receptor
RPMI	Roswell Park Memorial Institute medium
Sav	streptavidin
sCD40L	soluble CD40 ligand
SEM	standard error of the mean
STAT	Signal Transducers and Activators of Transcription
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
WT	wild type

Het 'boekje' is af. Wat rest is het dankwoord. Niet het moeilijkste gedeelte zou je denken. Echter, de wetenschap dat dit het meest gelezen stukje is èn dat je niemand wil vergeten maken het schrijven van een dankwoord niet zo makkelijk als het lijkt. Laat ik een poging wagen…

Allereerst professor Hoogsteden, die mij in de gelegenheid heeft gesteld om onderzoek te doen op de afdeling Longziekten waarvan hier het resultaat.

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wat betreft
onderzoeksonderwerp
al snel gedag zei, heb ik heel veel van je
geleerd wat betreft de fijne kneepjes van het
labwerk en was je daarnaast gewoon een super
kamergenoot. Succes met je eigen laatste loodjes.

Not all my PhD time was spent in Rotterdam. I performed the initial virus experiments at Millenium Pharmaceuticals in Boston. First of all, thanks to Tony Coyle for giving me the opportunity to work in his department for 3 months. I would also like to thank all the people in his department who took time to help me out, in particular Jane Tian and Steve Manning.

At the end of my PhD I had another chance to 'escape' Rotterdam, this time to master the art of Flexiventing in (not inconvenient) Perth in the lab of Peter Sly. I learned a lot about this technique, but I would also like to mention the effort you all at ICHR did to make us feel home, not only me but also Marieke. Thanks.

Gelukkig is het niet alleen werk wat de klok slaat. Zo was daar ook het 'kliekje' oud-Wageningers waar regelmatig een biertje mee gedronken werd of een goeie film bezocht (de '13th warrior' is voor iedereen een aanrader). Met name Josien en Marieke M. bedankt, met wie het als (nu oud-) collega's altijd goed klagen was hoe zwaar het leven van een AIO wel niet was (ach en wee). Bedankt allemaal.

Arne, Bram, Sander, Floris, Friso, Rutger, Wouter en Ad, alias Quark, een constante factor sinds Wageningen. Bedankt voor alle interesse, adviezen maar vooral de afleiding van het werk. Three down, one to go..

Ruud en Hetty, bedankt voor de belangstelling voor mijn onderzoek en niet te vergeten die overheerlijke Indische rijsttafels in Rhoon.

Lieve Kristien, ondanks dat we in sommige opzichten elkaars tegenpolen zijn kan ik me geen betere zus wensen. Jij èn Marc bedankt voor de feestjes, skivakanties, KvK-avonden, kortom al die niet-curriculaire activiteiten die het leven zo leuk maken.

Lieve papa en mama, bedankt voor alle liefde, steun en vertrouwen (vooral in 4 VWO), anders hadden jullie nu geen exemplaar van dit 'boekje' ontvangen. Ik weet 't, het is een cliché, maar clichés zijn waar!

Liefde is...
... begrip als ik weer eens zat te mokken over een mislukt experiment of met mijn gedachten er niet bij was
... zeggen dat promoveren wèl een knappe prestatie is
... niet boos worden als het een uurtje (of 2) later wordt door uitgelopen experimenten.

Dankjewel lief, voor je begrip, geloof en liefde. Beiden het AIO-schap 'overleefd', een mooie basis voor de toekomst. ...ik ook van jou.

Hanmjan

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Curriculum Vitae

Harmjan Kuipers was born on October 8, 1973 in Emmen. From 1986 to 1992 he attended grammar school in Emmen and subsequently enrolled for the study bioprocestechnology at Wageningen University, specialization animal cell technology. As part of this study, he performed several graduation projects. First at the Department of Virology, Wageningen University, under supervision of Just Vlak, followed by training period at the Netherlands Cancer Institute, Department of Immunology under supervision of Grada van Bleek. Finally, he went to the Australian Red Cross Bloodbank (ARCBS) in Sydney, Australia and performed a research project under supervision of John Sullivan. He graduated in 1997 and subsequently stayed at the ARCBS as a research assistant. In 1999, he started his PhD training at the department of Pulmonary Medicine of the Erasmus MC in Rotterdam under supervision of dr. Bart Lambrecht. The subject of his thesis research was the use of dendritic cells (DCs) as tools for immunotherapy against asthma. During his PhD training he stayed at Millenium Pharmaceuticals in Boston to acquire knowledge about retro/lentiviral transductions of DCs and visited the lab of prof. Sly in Perth, Australia to get instructed in forced oscillation lung function measurements in mice. He received his PhD in 2005 and is currently employed as a post-doc in the same department, responsible for implementing lentiviral mediated RNA interference in several projects currently running at the department.