Dendritic Cell Subsets are Key Regulators in the Immune System

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Contents

Chapter 1

Introduction I

Exploiting the Innate Immune System to generate Adjuvants: New Tricks in an Old Game

Vaccinations have been given to people for at least 100 years of which 80 years formulated in adjuvants. Despite the massive use of alum adjuvant, its mechanism of action has been only recently addressed. Lately, a lot of focus in improvement on vaccination has resulted in new adjuvants to boost the immune system. Several innate receptors have been proven to be useful targets. Here we introduce the employment of different innate receptor to improve adjuvant capacity.

Vaccination and adjuvants

The first described vaccination was done by Edward Jenner, an English physician, in 1796 [1]. It was performed in a young boy with cowpox (a mild virus related to the deadly smallpox virus). The boy developed fever but no great illness, however more importantly he was protected to smallpox thereafter. Vaccination (Latin: vacca—cow) is so named because the first vaccine was derived from 'cow'-pox. The word 'vaccination' was originally used specifically to describe the injection of smallpox vaccine, but has since been adapted to any immunization that has the purpose to prevent disease, be it microbial or caused by another derangement of the immune system. All present vaccinations work by presenting a foreign antigen (e.g. microbial) to the immune system in order to evoke an immune response, but there are several ways to do this. First, it can be evoked by giving inactivated microbes, for instance heat-inactivated or formaldehyde-inactivated virus particles. Second, it has been administered as an attenuated vaccine, e.g. live virus particles selected to have very low virulence and pathogenicity. Thirdly, a subunit vaccine, an antigen is presented to the immune system without introducing viral particles, whole or otherwise. Vaccines typically contain adjuvants used to boost the immune response. The word 'adjuvant' comes from the Latin verb adjuvare, meaning to help or aid. Tetanus toxoid and hepatitis B subunit vaccines are usually adsorbed onto aluminum hydroxide or aluminum phosphate (alum). In this way the antigen elicits a stronger immune response to the intrinsically harmless protein structure found in a subunit vaccine of a toxoid. Other compounds have also been used as an adjuvant. Oil-based adjuvants are commonly used in some veterinary vaccines and recently MF59, an oil-based adjuvant has been approved for human use [2].

Pattern Recognition Receptors

The innate immune system is the first line of host defense against pathogens, including viruses, bacteria, fungi, and protozoa and is mediated by phagocytes such as macrophages, neutrophils and dendritic cells (DCs). Adaptive immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory [3]. The adaptive immune system with its T cells and B cells has a seemingly endless capacity to recognize foreign antigens, through variations in its T and B cell receptors. The innate immune system lacks these

highly variable receptors and uses a different strategy to recognize a wide variety of pathogens. Every pathogen has certain characteristics that are crucial to its survival and are shared between closely related pathogens. These so-called pathogen-associated molecular patterns (PAMPs) are vital to the survival of the pathogen and consists of e.g. gram-negative cell wall endotoxin, gram-positive peptidoglycan (PGN), or viral double-stranded RNA. Central to the concept of PAMPs is that they are not shared with mammalian cells. PAMPs are recognized by pattern recognition receptors (PPRs) expressed on or in cells of the innate immune system. PPRs including Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are present on the cell surface and in the cytoplasm of macrophages, neutrophils, and DCs [4-7], as well as on some structural cells that line the outside world, like epithelial cells. TLRs and NLRs can also recognize molecular patterns associated with tissue injury or cell death, the so-called damage-associated molecular patterns (DAMPs). These include nuclear and cytosolic proteins, like ATP, uric acid, and HMGB-1 [8]. The evolutionary rationale behind this might be that some pathogens that developed ways to subvert the innate immune system would still induce changes in the tissues which they infect and these changes might be sensed by the immune system.

Other innate sensing receptor, like the C-type lectin-like receptors (CLRs) and retinoic acid induced gene-based (RIG)-I like receptors (RLRs) may also play a role in boosting innate immunity. CLRs like DC-SIGN, DEC-205, and the mannose receptor recognize carbohydrates, which are not only present in pathogens, but also in self-glycoproteins, thereby facilitating cell adhesion, migration, and phagocytosis [9]. RLRs are located in the cytoplasm and can sense ssRNA and dsRNA [10, 11]. Alarmins, like HMGB-1 [12, 13], eosinophil-derived neurotoxin (EDN) [14], and defensins also play an important role in activating the immune system [15]. Another group of endogenous proteins activating the immune system are the endokines, which include heat shock proteins, interleukins (e.g. IL-1α), S100 family proteins, and nucleosomes. In the next paragraphs we will discuss the potency to use TLR or NLR agonist as adjuvants to boost the immune system to improve vaccinations.

Innate immunity shapes adaptive immunity

Triggering the PRRs mentioned above not only activates innate immunity, it also shapes the character of the ensuing adaptive immune response. Dendritic cells (DCs) are the master controllers of adaptive immunity through their capacity to take up and process antigen in antigen exposed areas, and present it in an immunogenic form to naive T cells following migration to the central lymphoid organs. DCs express virtually all PRRs as part of their descent from innate immune phagocytes. Triggering PRRs on the surface of DCs induces their functional maturation in that they become competent to induce T cell proliferation and their differentiation into effector cells. Additionally, PRR triggering leads to the production of polarizing cytokines by DCs that

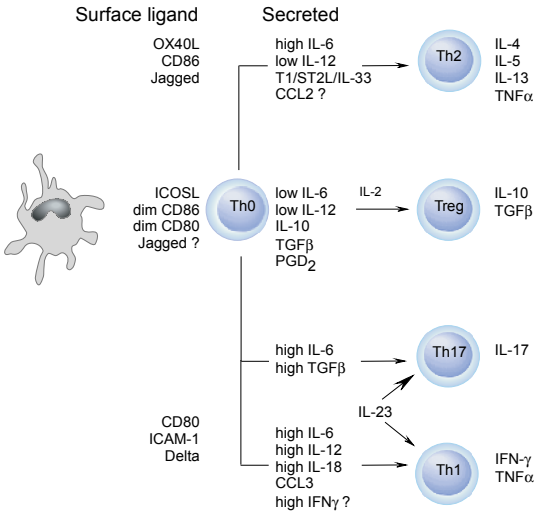

Figure 1
DCs promote different differentiation of T cells
DCs promote Th2 cell differentiation under the influence of OX-40L, CD86, and jagged expression and they differentiate under the influence of high IL-6 levels, low IL-12 levels, IL-33, and possibly CCL2. Th2 cells produce IL-4, IL-5, IL-13, and TNFα. Regulatory T cells (Treg) develop under the influence of ICOSL, low CD86, low CD80 expression on DCs and low IL-6 levels, low IL-12 levels, IL-10, TGFβ, and PGD_2. Tregs produce either IL-10 or TGFβ or suppress via cell-cell contact. Th1 and Th17 cells differentiate when DCs express CD80, ICAM-1 and delta. However, soluble factor determine if Th1 or Th17 cells will develop. Th1 differentiation needs high IL-6, IL-12, IL-18, and possibly IFNγ levels, whereas Th17 differentiation depends on high IL-6 and TGFβ levels. Th17 cells produce mainly IL-17, whereas Th1 cells produce high levels of IFNγ and TNFα.

induce differentiation of naive T cells into either Th1 cells (IFNγ, IL-2 production), Th2 cells (IL-4, IL-5, IL-13 production), or Th17 cells (IL-17, IL-21, IL-22 production) (see figure 1 for molecules necessary to induce differentiation).

Dendritic cell subsets

Dendritic cells (DCs) are the most potent antigen presenting cells present in the immune system. There are different subset of DCs, which each has its own characteristics discriminating them from each other. During steady state, the DCs are divided into conventional (c) DCs and plasmacytoid (p) DCs. In lymphoid organs, like lymph nodes and spleen, the cDCs can be subdivided into $CD8\alpha^+$ and $CD8\alpha^-$ cDCs, with a further subdivision by there CD4 expression [16]. Also, pDCs are found in the lymphoid organs and they migrate between lymph nodes via the lymphatics [17], whereas the cDCs do not migrate between lymphoid organs [18]. Each population of DCs is characterized by their expression of a particular combination of surface markers, on which we will focus on mouse cells. Mouse pDCs express bone marrow stromal antigen-2 (BST2, recognized by the mAbs 120G8 and PDCA-1), the B cell marker B220, intermediate levels of CD11c, MHC II, and Gr-1, and low expression of CD11b [19]. On the other hand, cDCs express high levels of CD11c and MHC II. $CD8\alpha^+$ cDCs furthermore express CD8α, DEC205, DNGR-1, and low CD11b, whereas $CD8\alpha^-$ cDCs express 33D1 and CD11b [20-22]. Furthermore, the cDCs reside in different anatomical locations in the spleen. The $CD8\alpha^+$ cDCs are located in the T cell zone, whereas the $CD8\alpha^-$ cDCs reside in the red pulp and marginal zone of the spleen.
In an activated state of the immune system, so during infection or inflammation, all DC subtypes can be activated. pDCs are activated during virus infections and will release massive amounts

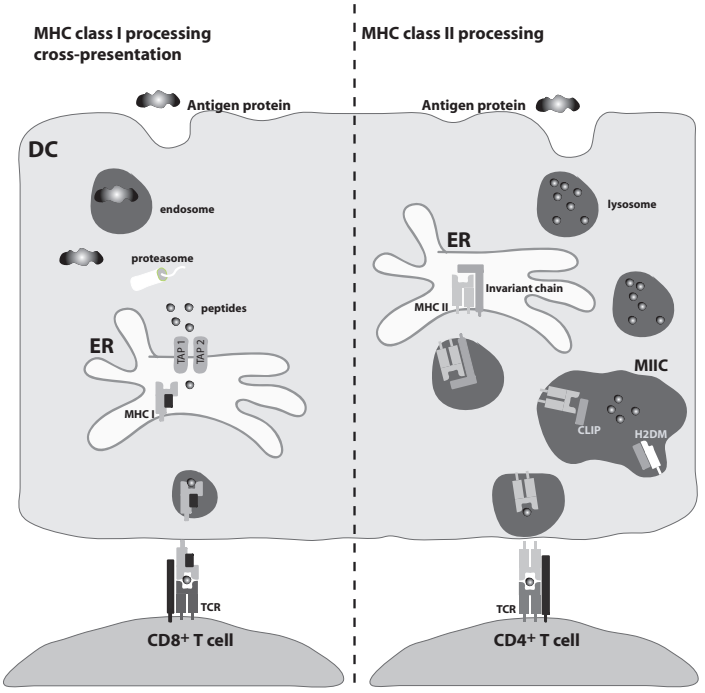

Figure 2
Antigen processing pathways in DCs
DCs can process exogenous antigen for MHC I presentation (called cross-presentation) or for MHC II presentation. Cross-presentation occurs when an antigen becomes cytoplasmatic and is broken down in peptides by the proteosome. These peptides are loaded into MHC I in the ER, thereafter this complex is translocated to the cellsurface for presentation to $CD8^+$ T cells.
For MHC II processing, the antigen in cleaved into peptides in the lysosome. The MHC II molecule is made in the ER and translocated to the MIIC (MHC II compartment). In the MIIC, the MHC II is loaded with antigenic peptides from the lysosome. Thereafter, the complex is exposed on the cell membrane to $CD4^+$ T cells.

of type 1 interferons, which have potent antiviral capacities [23-25]. Upon activation, cDCs will upregulate different co-stimulatory molecules, which are needed to induce a strong T cell response, necessary to clear infection or dampen inflammation. During inflammation or infection a fourth DC subtype emerges, the inflammatory DC. This subtype is characterized by the expression of CD11c, CD11b, MHC II on the cell surface and iNOS expression intracellular [26]. They also produce a lot of the cytokine, tumor necrosis factor (TNF)α. These DCs are excellent antigen presenting cells and take up their antigen on the site of inflammation or infection and migrate to the draining lymph nodes to activate adaptive immunity. A further description of DC subsets in the periphery, e.g. in the lung, is given in chapter 5.

Antigen presentation by DCs

The different subsets of DCs do not only express different markers, but they also process antigens in different ways. There is a distinction for each subset in acquiring antigen and processing in the MHC I or II pathways. For exogenous non-viral antigens, $CD8\alpha^+$ DCs are known to be good at cross-presenting antigens in the MHC I molecule, whereby the antigen in presented to cytotoxic $CD8^+$ T cells (CTLs) (see figure 2 for details). The $CD8\alpha^-$ DCs are primarily focused on presenting exogenous antigens in the MHC II molecule to $CD4^+$ T cells [27, 28]. It has been shown that pDCs are capable of MHC I presentation of endogenous antigens [29]. The role in MHC II antigen presentation for pDCs remains controversial.

There are different routes of acquiring exogenous antigens which can lead to either MHC I or MHC II processing and presentation [30]. For an exogenous antigen to be directed into the MHC I pathway, a process called cross-presentation, it has to be acquired by the DC via the phagocytic pathway (e.g. soluble antigen), preferentially via mannose receptors [30-32] or via Fc receptor mediated endocytosis (e.g. immune-complexes) [33]. When the antigen is present in the phagosome, there are two pathways possible. In the first, the phagosome will release the antigen, possibly by retro-translocation via Sec61 [34, 35], into the cytosol where it will be broken down to antigenic peptides by the proteasome. TAP1 and TAP2, proteins that span the membrane of the endoplasmic reticulum (ER), transport the antigenic peptides into the lumen of the ER. In the ER chaperone proteins, like calnexin, calreticulin, and ERp57 facilitate the proper folding of MHC I. The partially folded MHC I molecule then interacts with TAP1 and TAP2 via tapasin (TAP binding protein). Once the antigenic peptide is transported into the ER, it will bind into the groove of the MHC I molecule, which is then stabilized and will be transported to the cell membrane. In the second possible pathway of cross-presentation, endosomal proteases especially cathepsin S will break down the protein into peptides in the phagosome. Then it can fuse with MHC I containing vesicles leaving the ER. The peptides will then be loaded onto MHC I and the complex is transported to the cell membrane.
For the MHC II pathway, the antigen has to enter the DC either via pinocytosis or via scavenger receptors [30]. Then it will be directed into the lysosome, where acid-dependent proteases, like GILT, cathepsin S, H, and C degrade the antigen into antigenic peptides. MHC II is synthesized in the ER associated with invariant chain (Ii, trimeric form) which prevents binding of endogenous peptides and stabilizes the MHC II molecule. Invariant chain also facilitates transport of MHC II from the ER via vesicles. These vesicles will fuse with a late endosome containing the antigenic peptides, thereafter called the MHC II compartment, MIIC. There the MHC II-Ii complex is the broken down, also done by cathepsin S and L, leaving only a small fragment called CLIP (Class II associated Invariant chain peptides) in the peptide binding groove. An MHC II-like structure, H2-DM, removes CLIP from the MHC II molecule and replaces it with an antigenic peptide from the endosome. The stable MHC II-peptide complex is then presented on the cell membrane to $CD4^+$ T cells and will induce proliferation.

Adaptive immune response

The adaptive immune response is activated by antigen presenting cells and thus by the potent DCs. It consists of a cell-mediated immune response, which is governed by T cells and a humoral immune response in which B cells play a large role. After antigen presentation to T cells by DCs, these T cells will proliferate and differentiate (see figure 1). The effector $CD4^+$ T cells will secrete different cytokines, which will regulate the immune response. Activated $CD8^+$ cytotoxic T cells

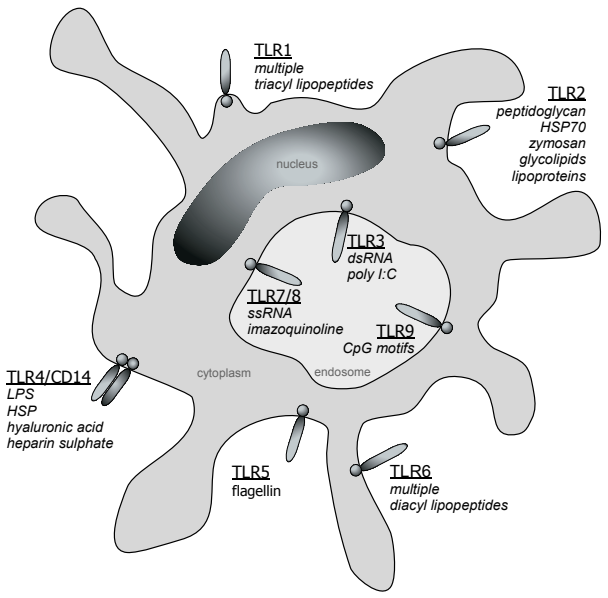

Figure 3
TLR expression on DCs
TLR2 in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl and diacyl lipopeptide, respectively. TLR2 further recognizes peptidoglycan, HSP70, zymosan, glycolipids, and lipoproteins. TLR3 recognizes dsRNA. TLR4 recognizes bacterial LPS. TLR5 recognizes bacterial flagellin. TLR7/8 mediates recognition of imidazoquinolines and ssRNA. TLR9 recognizes CpG DNA of bacteria and viruses. TLR1, 2, 4, 5, and 6 are located on the cell membrane, whereas TLR3, 7/8, and 9 are located inside endosomes

will destroy virally infected cells and/or tumor cells. Furthermore, both $CD4^+$ and $CD8^+$ T cells can become memory T cells. These memory cells are a subset of antigen-specific T cells that persists long-term after an infection has resolved. Their advantage is that they can quickly expand to large numbers of effector T cells upon a secondary exposure to their antigen. Thereby they facilitate the immune system with a 'memory' against past infections. Memory T cells compromises of 2 subsets, the central memory T_{CM} cells and the effector memory T_{EM} cells.
Effector $CD4^+$ T cells can activate B cells. Activated B cells subsequently produce antibodies which assist in inhibiting pathogens until phagocytes or the complement system clears the host of the pathogen. Most antigens which induce B cell activation are T cell-dependent, meaning T cell help is required for maximal antibody production. With a T cell-dependent antigen, the first signal comes from antigen cross-linking of the B cell receptor (BCR) and the second signal comes from co-stimulation provided by Th2 cells. These Th2 cells produce cytokines which will influence B cell proliferation and differentiation. Isotype switching to IgG, IgA, and IgE and memory cell generation also depends on cytokines present in the local environment. As an example, IL-4 produced by Th2 cells will skew B cells to undergo class switching until IgE.

Toll-like receptors and the adaptive immune response

To date, 13 members of the TLR family have been identified in human and mice. TLRs are type I integral membrane glycoproteins characterized by extracellular domains containing varying

numbers of leucine-rich-repeat (LRR) motifs which recognize the PAMPs [4]). TLR activation helps to eliminate invading pathogens, coordinate systemic defenses, and initiate adaptive immune responses [4]. Triggering of the TLR induces maturation of macrophages and DCs, which is essential for the adaptive immune response and thereby memory response (see figure 3 for ligands and location of the different TLRs). Some TLRs have been highlighted as potential candidates for improving vaccines against cancer and infectious disease, including TLR2, TLR4, TLR7/8, and TLR9 [36-38]. The best investigated ligand for TLR4, LPS has been shown to have good stimulatory effects in mice, inducing both innate and adaptive immunity [39, 40]. However, LPS can also induce septic shock, since it so efficiently promotes the release of the innate cytokines IL-1β and IL-6 [41]. Over the last decade, LPS has been modulated, keeping its beneficial effects on immunity and losing its toxicity by modifying the saccharide groups. This resulted in the immuno-active lipid A fraction, monophosphoryl lipid A (MPL), which has been shown to induce good immunity to a secondary viral infection in mice [42]. Also, GlaxoSmithKline Biologicals (Belgium) made a novel hepatitis B vaccine containing MPL (Fendrix ®), which demonstrates that MPL is safe to use. Of note, this vaccine also contains aluminum phosphate. Vaccinations are not only used to induce immunity against viruses and bacteria, but also to boost anti-tumor immunity. In this prospect, the synthetic TLR2/1 agonist [tripalmitoyl-S-(bis(palmitoyloxy)pr opyl)-Cys-Ser-(Lys)3-Lys] was used in a model of melanoma cancer and a reduced tumor load was observed due to directly activating the CTL response [43]. Besides exploiting the adjuvant effects of membrane-bound TLRs, a lot of research has focused on the stimulation of intra-cellular TLRs, like TLR7/8 and TLR9. In human and primates, the expression of TLR9 is limited to plasmacytoid DCs and B cells, whereas in mice also conventional DCs express TLR9 [44]. In different models, like allergy, tumor and viral immunology, administration of CpG motifs as a TLR9 ligand has been investigated intensively [45-48]. CpG immuno-stimulatory motifs reduce the Th2 allergic response (both prophylactic and therapeutic properties) [49-51] and increase the Th1 and CTL responses [52, 53]. TLR7/8 ligands, like imiquimod and R848 have also been shown to be an effective adjuvant in both infection and tumor immunology [37, 54]. Imiquimod works even as a double-edged sword by promoting by itself tumor reduction in vulvar cancer patients without use of an additional tumor antigen [55].

Nod-like receptors and adjuvenicity

The NOD-like receptors (NLRs) are cytoplasmic PRRs that may have a variety of functions in regulation of inflammatory and apoptotic responses. Approximately 20 of these proteins have been found in the mammalian genome and include two major subfamilies called NODs and NALPs, and some other molecules (e.g. MHC Class II transactivator (CIITA), IPAF, and BIRC1) (see figure 4 for ligand of different NLRs) [6]. The NOD proteins include NOD1 and NOD2,

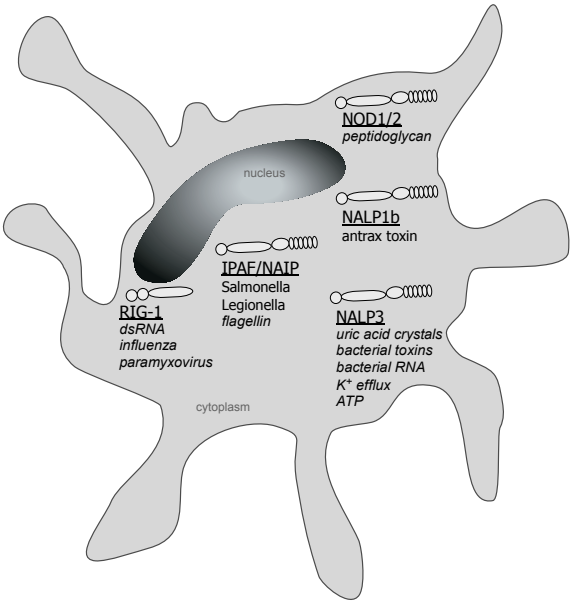

Figure 4
NLR expression on DCs
All NLRs are located in the cytoplasm. NOD1/2 recognize peptidoglycan from bacteria. RIG-1 recognizes dsRNA, influenza, and paramyxoviruses. Inflammasomes like IPAF and NALP1β are activated by different bateria and toxins, like *Salmonella*, *Legionella*, and antrax toxin. The NALP3 inflammasome is activated by uric acid crystals, bacterial toxins, bacterial RNA, K^+ efflux, and ATP.

which are intracellular bacterial sensors and activate NF-κB signaling [5]. The NALP proteins contain 14 members and are cytosolic sensors of pathogens; however whether they directly recognize endogenous PAMPs or DAMPs is not known at the moment. Activated NALPs form oligomers that activate inflammatory caspases (e.g. caspase-1), causing cleavage and activation of inflammatory cytokines such as IL-1β, IL-18 and IL-33, and/or activate the NF-κB signaling pathway to induce production of inflammatory molecules [56]. From this family, the NALP1 and NALP3 inflammasome have been most studied. NALP1 is mainly associated with induction of apoptosis, through activation of caspase-2. It can also promote inflammation by inducing IL-1β through activation of caspase-1 and caspase-5 [57]. The last years, the NALP3 inflammasome has been investigated thoroughly in its role in the pathogenesis of gout [58]. It has been shown be activated by uric acid crystals, which are associated for decades with gout [59]. The use of uric acid as an adjuvant has been shown through its capacity to induce a good CTL response [60]. Furthermore, the use of uric acid is beneficial in autoimmune responses, like diabetes and cancer [61, 62]. Uric acid is not the only factor which can activate the NALP3 inflammasome. ATP has been shown to induce K^+ influx by activating the purinergic P2X7 receptor on which the inactive inflammasome changes confirmation to become active [63]. This process also involves the pannexin-1 hemi-channel that forms a complex with P2X7 receptor, thus potentially shuttling endocytosed particles to the cytoplasm. Recently, ATP has also been shown to induce Th2 responses [64]. The adjuvant aluminum hydroxide has recently been shown to exert its function

partially through the NALP3 inflammasome [65, 66], which specifies that the NALP family is a good target to also improve vaccinations.

Targeting antigen to nature's adjuvant, the dendritic cell

Instead of using adjuvants to boost the immune system via activation of DCs via PRRs, it has been shown that it is possible to improve vaccinations by targeting the antigen to endocytic PRRs of the C-type lectin family directly. Antigens, especially tumor antigens have been coupled to antibodies, recognizing one of the CLRs and thereby facilitating antigen uptake and processing by the DCs [21]. However, an additional maturation stimulus has to be given to induce immunity [67], otherwise the outcome is tolerance [21, 68, 69]. One member of the CLRs is DEC-205 (CD205), which has been targeted as a vaccination strategy. In a melanoma tumor model, DEC-205 targeting promoted anti-tumor immunity [70]. Besides DEC-205, another member of the CLR family, DC-SIGN has been used to target antigen to DCs. In human, DC-SIGN is one of the most specific DC markers, making targeting more specific. Unfortunally, in mice such experiments cannot be done, since they express several forms of DC-SIGN [71]. However, in monkeys this strategy has also been shown successful in inducing proper T cell responses [72]. Recently, targeting of a new member of the CLRs DNGR-1 (DC, NK lectin group receptor-1), more DC-specific than DEC-205 in mice, was shown to be highly effective in a therapeutic approach in a mouse tumor melanoma model [20]. The above described targeted CLRs all initiate strong $CD8^+$ T cell responses as these are expressed on $CD8\alpha^+33D1^-$ cDCs, which are know to be efficient cross-presenters [30, 73]. Other CLRs are expressed on another subset of cDCs, namely the $CD8\alpha^-33D1^+$ cDCs which are good $CD4^+$ T cell stimulators [74]. For instance, DCIR2 (DC inhibitory receptor 2) and dectin-1 targeting on $CD8\alpha^-$ cDCs results in strong $CD4^+$ T cell responses [22, 75]. Although, one should be aware that the outcome of targeting different receptors is not merely dictated by the DC subset, but also other cell types which express the different receptors. Furthermore, antigens can be routed differently intracellular to different compartments (e.g. MHC I or MHC II pathway) due to the specific receptor. Furthermore, receptor internalization can be achieved by other endocytic receptors, like Fc receptors. It has been shown that human plasmacytoid DCs (pDCs) internalize antigen via FcγRII [76]. Other endocytic CLRs are also expressed on pDCs, like Siglec-H and BST2 [77, 78]. Targeting of CLRs on pDCs instead of cDCs will induce a different immune response.

Concluding remarks

All vaccination strategies will come down to essentially one target, the antigen presenting cell, of which the DC is the most potent. Promoting immunity through DCs by stimulating the innate

response via PRRs has been proven effectively, however, the road to human use is long. Secondly, stimulating and targeting other endocytic receptors on DCs has proven to be effective in mice. Studies should be undertaken to translate this to human use.

Aim and scope of this thesis

It is clear from the described above that DCs play a central role in the immune system, in both the innate and the adaptive immune response. Furthermore, different DC subsets provide different signals to the immune system, induce different reactions, and respond to different stimuli. In the first part of this thesis we investigated the different DC subsets in the primary and innate immune response in the context of adjuvantia and induction of Th2 responses, like allergic asthma. In chapter 2, the mechanism of aluminum adjuvant was examined and a role for inflammatory monocytes and DCs was observed. An unexpected finding was the release of uric acid during aluminum adjuvant injection. In chapter 3, the molecular mechanism of aluminum adjuvant was investigated. The activation of DCs by aluminum adjuvant was partially mediated via the innate receptor, the NALP3 inflammasome. In chapter 4, the adjuvanicity of uric acid crystals was studied and a clear induction of Th2 responses was detected. Most likely, this was achieved by activating inflammatory monocytes and DCs.
In the second part of this thesis, we investigated the function of plasmacytoid DCs. In chapter 6, the role of pDCs in the secondary immune response was investigated in a mouse model of experimental asthma. PDCs were shown to down-regulate the eosinophilic airway inflammation in already immunized mice. In chapter 7, the antigen presentation capacity of pDCs was studied. Un-stimulated pDCs could not present exogenous antigens to both $CD4^+$ and $CD8^+$ T cells. However, to induce $CD8^+$ T cell stimulation pDCs had to be activated by CpG motifs. To achieve $CD4^+$ T cell proliferation, pDCs should either be pre-activated with CpG motifs or exposed to antigen-specific immunoglobulins.
Finally, in chapter 8 the main results concerning DC subsets are summarized and discussed.

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Chapter 2

Alum Adjuvant boosts Adaptive Immunity by inducing Uric Acid and activating Inflammatory Dendritic Cells

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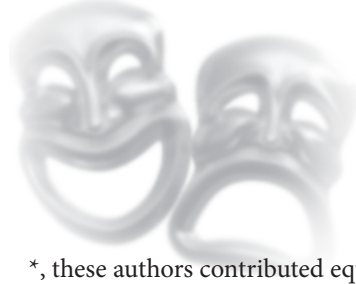

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Alum is the most widely used adjuvant in human vaccines but the mechanism of its adjuvanticity remains unknown. In vitro studies showed no stimulatory effects on dendritic cells (DCs). In the absence of adjuvant, Ag was taken up by LN resident DCs that acquired soluble antigen via afferent lymphatics, while after injection of alum, antigen was taken up, processed and presented by inflammatory monocytes that migrated from the peritoneum, thus becoming inflammatory DCs that induced a persistent Th2 response. The enhancing effects of alum on both cellular and humoral immunity were completely abolished when $CD11c^+$ monocytes and DCs were conditionally depleted during immunization. Mechanistically, DC-driven responses were abolished in MyD88-deficient mice and following uricase treatment, implying the induction of uric acid. These findings suggest that alum adjuvant is immunogenic by exploiting 'nature's adjuvant', the inflammatory dendritic cell through induction of the endogenous danger signal uric acid.

Aluminum-containing adjuvants have historically served as immunopotentiators in vaccines and continue to be the most widely used clinical adjuvants [1]. Despite the fact that millions of doses of aluminum-containing adjuvants have been given to healthy populations, it is surprising that there is no consensus regarding the mechanisms by which they potentiate the immune system [2-7]. Three potential mechanisms are frequently cited to explain how these adjuvants increase humoral immunity, although scarce experimental evidence is publicly available; (I) The formation of a depot by which the antigen is slowly released to enhance the antibody production. (II) The induction of inflammation thus recruiting and activating antigen presenting cells that capture the antigen [8]. (III) The conversion of soluble antigen into a particulate form so that it is phagocytosed by antigen presenting cells such as macrophages, dendritic cells, and B cells. It is common knowledge that aluminum -containing adjuvants (alum) predominantly induce humoral immunity, further supported by the recent discovery that alum induces B cell priming and Ca^{2+} mobilization via a splenic $Gr\text{-}1^+$ myeloid IL-4-producing cell type [5]. Classical cell-mediated immunity measured by DTH responses and induction of $CD8^+$ CTL responses to a range of polypeptide and protein antigens is poorly induced by alum, due to a lack of cross-priming [1, 9, 10]. However, proliferative responses of $CD4^+$ T cells as well as Th2 cytokine production have been found to be enhanced in a number of murine and human studies, suggesting that alum boosts humoral immunity by providing Th2 cell help to follicular B cells [2, 8, 11].

Dendritic cells are seen as nature's adjuvant and have the potential to recognize foreign antigen, process it into small peptides for presentation onto MHC molecules to the TCR, and to provide the essential costimulatory molecules for activation of naive $CD4^+$ and $CD8^+$ T cells [12]. Dendritic cells have an immature phenotype in peripheral tissues, specialized for antigen uptake, but upon recognition of exogenous or endogenous 'danger signals' like uric acid or

extracellular ATP, migrate to the lymph node T cell paracortex where they arrive as mature cells, expressing all costimulatory molecules and having lost the capacity to take up antigens [13, 14]. The response of DCs to exposure to foreign antigens is part of the innate immune response, and by providing a link between antigen recognition and antigen processing for presentation to naive T cells, these cells bridge innate and adaptive immunity [15].

Many agents with adjuvant activity such as bacterial endotoxin, Freund's adjuvant, bacterial CpG motifs, monophosphoryl lipid A, MF59, and β-galactosylceramide boost immunity through induction of dendritic cell maturation [16-19]. It has been less clear if and how aluminum-containing adjuvants can induce dendritic cell mobilization and maturation. At least in vitro, alum did not enhance costimulatory molecule expression and DC maturation, although this finding would not preclude such an effect in vivo as endogenously released danger signals from damaged or inflammatory cells might indirectly activate DCs [13, 20, 21]. The issue is even more complex as Toll-like receptors and TLR signaling through the MyD88 or TRIF adaptor pathway, classical activators of innate immunity and the DC network in vivo, were not always necessary for alum to act as an adjuvant for humoral immunity [6, 21, 22]. In view of the crucial role of DCs in activation of adaptive immunity, we therefore set out to carefully study the effects of alum on DCs and their monocytic precursors in vivo following intraperitoneal and intramuscular injection of antigen (Ag) in alum and studied T cell activation using an adoptive transfer system of traceable Ag-specific T cells. Our experiments revealed a hitherto unappreciated role for monocyte derived inflammatory DCs and uric acid release in boosting adaptive immunity in alum formulated Ag preparations.

Material and Methods

Mice

BALB/c mice (6–8 wk old) were purchased from Harlan (Zeist, The Netherlands). OVA-TCR transgenic mice (DO11.10), CD11c-DTR transgenic mice on a BALB/c background [31], CD45.1 and CD45.2 C57Bl/6 mice were bred at Erasmus University (Rotterdam, The Netherlands). MyD88-/- mice were provided by B. Ryffel (Centre Nationale de la Recherche, UMR6218, Orleans, France) and originally made by S. Akira (Osaka University, Osaka, Japan). All experiments were approved by the animal ethics committee at the Erasmus Medical Centre.

Antigens and adjuvant

Ovalbumin (OVA) was purchased from Worthington Biochemical Corp (Lakewood, NJ). At the dose used in our experiments, the endotoxin level of OVA measured by a limulus-amebocyte lysate assay (Biowhittaker, Verviers, Belgium) was <0.001 μg/ml. Imject-alum (Pierce Biochemicals) is a mixture of aluminum hydroxide and magnesium hydroxide and was mixed at a 1:20 ratio with a solution of OVA antigen in saline followed by stirring for at least 1 h. For immunization 500 μl of Imject-alum suspension (1 mg) containing 10 μg of OVA (OVA-alum) was injected i.p. in the right lower quadrant using a 26 G needle, or alternatively 10 μg of OVA in 500 μl saline was injected.

Detection of the primary T cell response to i.p. injection of OVA

OVA-specific TCR Tg T cells were collected form the lymphoid organs of naïve 4-6 week old DO11.10 mice and stained with CFSE (Carboxyfluorescein diacetate succinimidyl ester from Molecular Probes, Eugene, OR) as described [23]. $10x10^6$ Cells were injected intravenously in the lateral tail vein of BALB/c mice (day -1). On day 0, the

mice received an i.p. injection of 10 µg OVA, OVA-alum, saline, or alum. On day 0, 1, 2, 4, 7, and 14 cervical lymph nodes (CLN), axillary (A)LNs, inguinal (I)LNs, mesenteric (Mes)LNs, mediastinal (M)LNs and spleens were removed and individual cell suspensions prepared as described [23].

In experiments to address the functional role of DCs in peritoneal responses, $CD11c^+$ cells were depleted by injecting 100 ng of diphteria toxin (DT) either intratracheally (i.t.) or i.p. in CD11c-DTR Tg mice [31, 32]. In these mice, $CD4^+$ T cells were purified from OVA-specific TCR DO11.10 cells using magnetic separation ($CD4^+$ T cell isolation kit, Miltenyi), and 2-5x10^6 cells were injected i.v..

In experiments to address if monocytes could restore the phenotype in the DT-treated CD11c-DTR Tg mice, 7,5*10^5 $CD11b^+Ly6C^+CD31^-$ monocytes sorted from bone marrow of BALB/c mice were given i.p. 2 hr after OVA or OVA-alum injection.

In separate experiments the intramuscular (i.m.) route of administration was investigated by giving 10 µg OVA coupled to 1 mg alum in 50 µl in the left hind limb. Four and 7 days after the injection the sacral (S)LNs, popliteal (P)LNs, ILNs, and muscles were removed and prepared for single cell suspensions.

Effector cytokine production

LN and spleen cells (200,000 cells/well in triplicates) were resuspended in culture medium in 96-well plates. Four days later, supernatants were harvested and analyzed for the presence of cytokines by ELISA (IL-4 and IL-5 from eBioscience, IL-10 and IFNγ from BD Biosciences)

Chemokine production

The peritoneal lavage was taken 2 hr after injection of 10 µg OVA, OVA-alum, or saline to determine levels of different chemokines in the supernatant by ELISA (MCP-1 and KC from R&D systems, eotaxine from eBioscience).

Flow cytometry

For detection of OVA-specific T cell responses, cells were gated for living (PI-negative) lymphocytes with CD4-APC and the clonotypic anti-OVA TCR antibody KJ1-26-PE. To acquire clear CFSE division profiles, 2.5–10 x 10^5 events were collected. The term 'CFSE content' gives an estimate of the original number of CFSE-labeled donor cells from which the donor-derived, divided population has arisen and was calculated as described [23]. It can be used to calculate whether the number of cells at analyzed site has been affected by recruitment, migration, or cell death, in addition to division.

For detection and sorting of DCs and monocytes, single cell suspensions of LNs or peritoneal lavage were prepared as described [49] or prepared from bone marrow cultures. Cells were subsequently stained with moAbs directed against CD11c, CD11b, MHCII, CD80, CD86, CD40 (eBiosciences), F4/80, Ly6C, or Ly6G (BD Biosciences) and with pDC specific moAb 120G8 (kindly provided by C. Asselin-Paturel). Sorting of $CD11c^+$ $MHCII^+$ DCs, $F4/80^{int}CD11b^+Ly6G^-Ly6C^+$ inflammatory monocytes and $F4/80^+CD11b^+$ macrophages was performed on a FacsAria high-speed cell sorter (BD).

For antigen uptake and processing studies, 10 µg of OVA-Alexa Fluor 647 (OVA-AF647) or OVA-DQ (Molecular Probes) was coupled or not to Imject-alum, injected i.p., and detected 24 hrs later in the peritoneal cavity or various nodes. Peritoneal lavage and LN cells were stained for seven or nine-colour flow cytometry using combinations of live (DAPI-negative), Ly6C-FITC and Ly6G-PE or CD19-PE, mPDCA1-APC, CD8α-PerCP-Cy5.5 or CD11b-PerCP-Cy5.5, CD11c-PE-Texas Red, Ly6G-PE-Cy7 or CD8α-PE-Cy7, MHCII-biotin or F4/80-biotin followed by streptavidin-APC-Cy7, CD11b-Pacific blue, combined with uptake of OVA-DQ or OVA-AF647.

Acquisition of 4 colour samples was on a FacsCalibur cytometer equipped with Cellquest software (Becton Dickinson) and 7-9 colour samples on a FacsAria cytometer equipped with FacsDIVA software. Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

Generation of BM-DCs

Bone marrow cells were cultured for 9 days in DC culture medium (DC-CM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 50 µg/ml gentamicin (Invitrogen), and 20 ng/ml recombinant mouse GM-CSF (a kind gift from Prof. K. Thielemans, Vrije Universiteit Brussel, Brussels, Belgium). 16 hrs before harvesting, DCs were exposed either to 10 µg/ml of OVA, alum or OVA-alum suspension.

Statistical analysis

For all experiments, the difference between groups was

calculated using the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Differences were considered significant when $p<0.05$.

Results

Distribution of the primary immune response after i.p. injection of OVA

Despite the wide use of i.p. injection of experimental antigens coupled to alum adjuvant, the precise localisation of primary T cell activation after i.p. injection of antigen has not been studied in great detail. By analogy with the rapid resorption of drugs following i.p. injection, it is often assumed that i.p. injection leads to rapid systemic resorption of antigen and therefore i.p. injection is often regarded as systemic immunization. To study primary T cell activation, naive BALB/c mice received a cohort of CFSE-labeled OVA-specific TCR Tg cells obtained from DO11.10 mice, and mice were subsequently immunized one day later with 10 μg of OVA via an i.p. injection in the right lower quadrant. Two days later, primary T cell divisions were readily noticed in the inguinal LN (ILN) on the right side, and in the mediastinal LN (MLN). In Ag-injected mice, only the $CD4^+$ Ag-specific T cells, recognized by the KJ1-26 Ab divided, whereas a co-injected fraction of TCR Tg- population did not. Strikingly, there were no primary divisions in the contralateral left ILN nor in the spleen

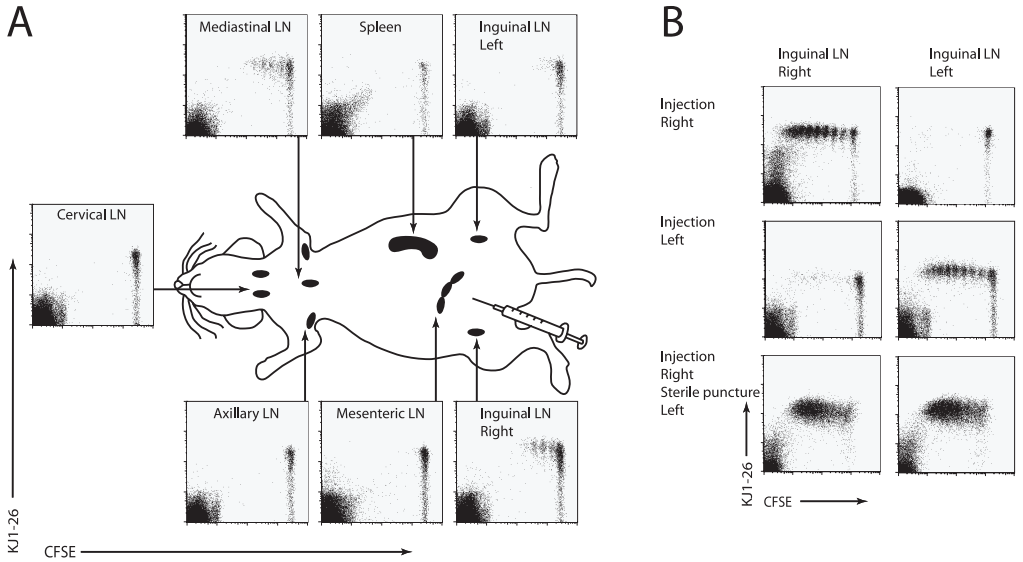

Figure 1

The mediastinal and ipsilateral inguinal lymph node drain the peritoneal cavity following i.p. injection

Mice were injected with CFSE-labelled DO11.10 OVA-TCR Tg cells 1 day before the intraperitoneal injection of OVA in the right lower quadrant. **(A)** Two days after OVA injection, different lymph nodes and spleen were taken and T cell proliferation was assessed with flow cytometry, by following CFSE dilution. Only KJ1-26^+ T cells divide. **(B)** Immediately after the administration of OVA in the right lower quadrant, a sterile puncture was made at the left lower quadrant. Four days later proliferation was measured in the left and right inguinal LN. An example is shown of 4 mice (representative of at least two independent experiments).

(Fig 1A). The fact that antigen presentation occurred only in the ipsilateral ILN was due to the fact that the needle injection caused a break in the peritoneal and skin barrier. This was clear when we injected the Ag in the left lower quadrant, in which case divisions occurred in the left ILN, or when we combined a right Ag injection with a left sterile puncture containing no Ag, in which case both ILNs reacted to the antigen (Fig 1B). When the right ILN was resected prior to Ag injection on the right side, the ipsilateral axillary LN became Ag reactive, illustrating that the antigen reached the LN via the afferent lymphatics (data not shown).

When T cell division was followed over time (Suppl Fig 1), it was evident that Ag-specific T cell responses were restricted to the draining right ILN and MLN for the first 3 days of the

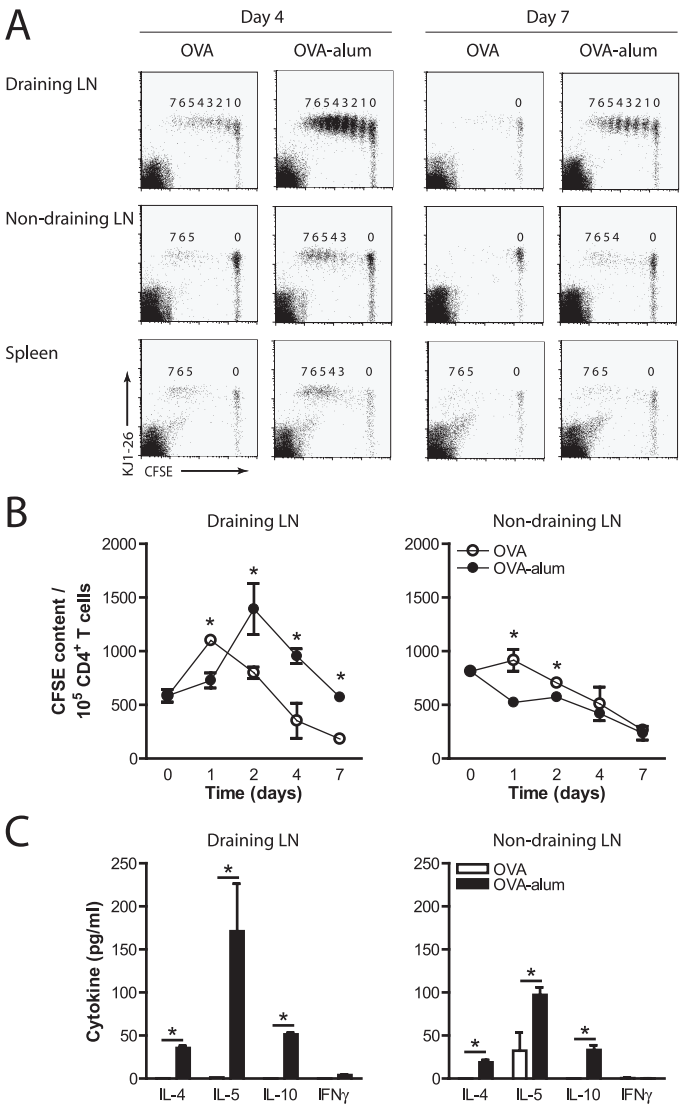

Figure 2
Addition of alum adjuvant to OVA leads to a stronger, more peristent and recirculating Th2 immune response

Mice were injected with CFSE-labelled DO11.10 OVA-TCR Tg cells 1 day before the i.p. injection of OVA or OVA-alum. **(A)** Four and 7 days after the injection the draining LN (MLN), non-draining LN (CLN), and the spleen were analyzed for T cell proliferation with flow cytometry (n=4 mice, experiment performed three times). **(B)** CFSE content was calculated as described in Material and Methods, and is shown for draining LN (MLN) and non-draining LN (ALN). Open symbols represent the OVA-injected mice, the black closed symbols the OVA-alum injected mice. **(C)** Seven days after the i.p. injection, LN cells (draining LN: MLN, non-draining LN: ALN) were taken and re-stimulated in vitro for 4 days with OVA. Cytokines were measured in the supernatants by ELISA. White bars represent OVA-injected mice, black bars the OVA-alum injected mice. Data are shown as mean ± SEM, * $p<0.05$. n=4-6 mice per group.

response. By day 4, cells that had divided at least three times began to appear in the non-draining lymph nodes and importantly also in the spleen. These cells expressed high levels of CD44 and low levels of CD69, consistent with their phenotype of recirculating primed T cells as previously reported [23, 24] (data not shown). However, by day 7 and 14 the majority of divided cells had disappeared from the lymphoid organs and could not be retrieved from the peritoneum.

Effect of alum adjuvant on the immune response induced by an i.p. or i.m. injection of OVA

When OVA was emulsified in alum adjuvant, the localisation of the primary immune response after i.p. injection into the lower right quadrant was again restricted to the ipsilateral ILN and MLN. In some mice, there was also a clear primary proliferation of OVA-specific TCR Tg cells in the mesenteric nodes, as previously reported for $CD8^+$ T cell responses following i.p. injection (data not shown and [25]). By day 4 of the response, the primary T cell response in the draining MLN (and ILN) was more pronounced in mice receiving both OVA and alum compared with OVA alone, with a total percentage of $CFSE^+$ Tg cells of 1.7% vs. 0.6% in the mediastinal nodes (Fig 2A). By day 2-4 of the response there was a clear increase in CFSE content (i.e. the number of $CFSE^+$ KJ1-26^+ cells per 10^5 $CD4^+$ T cells, correcting for the multiplying effect of cell division) in OVA-alum immunized mice compared with mice only immunized with OVA or saline control, signifying that the increase was not only due to division but also recruitment of naïve Ag-specific T to these nodes (Fig 2B). However, such recruitment did not occur in non-draining nodes. Also significantly more divided $CFSE^+$ Tg^+ recirculating effector cells were seen in the non-draining node and spleen. By day 7, a time point when the majority of OVA-specific TCR Tg T cells have disappeared in mice receiving OVA, the Tg T cells persisted in the draining and non-draining nodes and spleen in mice receiving OVA-alum (Fig 2A, right panel and 2B). These persisting cells in the mediastinal nodes had Th2 effector potential in the OVA-alum group as they produced IL-4, IL-5 and IL-10, but little IFNγ (Fig 2C). Bulk cultures of mediastinal node cells from OVA immunized mice did not produce significant levels of cytokines in response to OVA restimulation. In the non-draining nodes, there was similarly a clear increase in Th2 cytokine production in mice receiving OVA-alum, most likely due to recirculating primed T cells.

The i.p. route is most often used for immunization of animals because of its ease of use, but in humans most alum formulated adjuvants are injected intramuscularly or subcutaneously. We therefore also studied the response to OVA-alum and OVA following i.m. injection into the gluteal muscle of mice. In these mice, the primary draining LN site was the sacral LN. Again, OVA injection alone led to transient T cell activation followed by deletion of dividing cells, whereas alum injection left behind a persistent and recirculating T cell response, which was most prominent in the sacral node (Suppl Fig 2A).

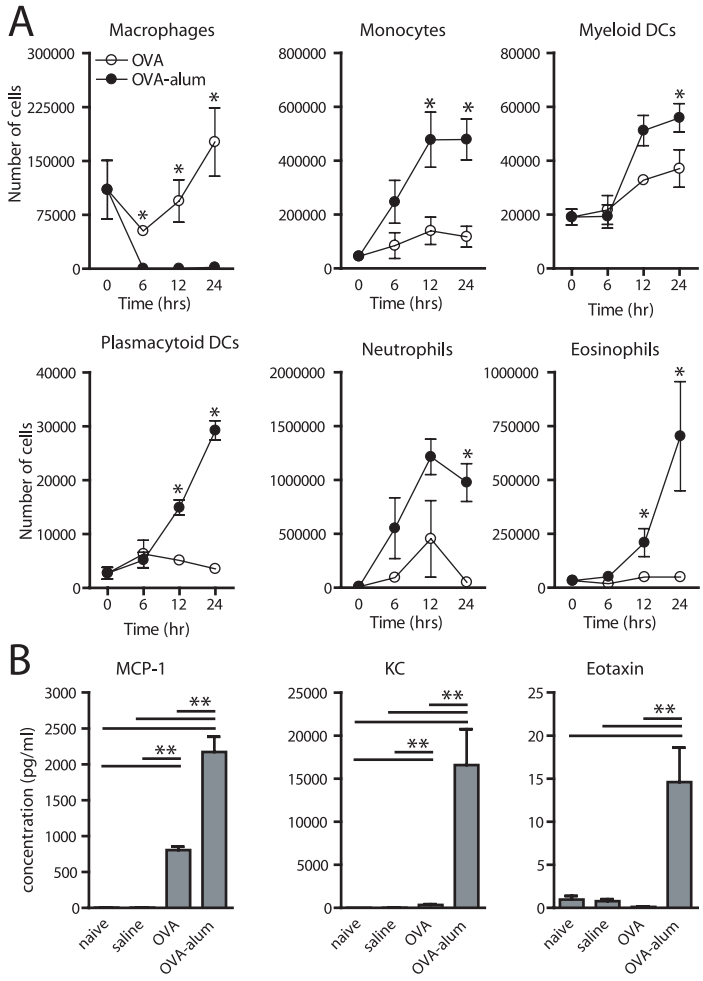

Figure 3
Alum recruits innate immune cells to the peritoneal cavity
Mice were injected i.p. with OVA or OVA alum. **(A)** Six, 12, and 24h after injection, the peritoneal lavage was taken and the number of macrophages (F4/80^{high}CD11b^{+}SSC^{high}), monocytes (CD11b^{+}Ly6C^{high}Ly6G^{-}F4/80^{int}), myeloid dendritic cells (MHCII^{high}CD11c^{+}F4/80^{low}), plasmacytoid dendritic cells (120G8^{+}CD11b^{dim}CD11c^{int}), neutrophils (CD11b^{+}Ly6C^{+}Ly6G^{high}F4/80^{-}), and eosinophils (CD11b^{+}Ly6C^{int}Ly6G^{int}F4/80^{int}) was determined. Open symbols represent the OVA-injected mice, the black symbols the OVA-alum injected mice. **(B)** Two hour after injection, the peritoneal lavage was taken and chemokine levels were determined in the supernatant by ELISA. Data shown are mean ± SEM, * p<0.05, n=4-6 mice per group.

Response of innate immune system cells to i.p. injection of antigen in adjuvant

Having identified the mediastinal LN as the most physiological draining site following i.p. injection of antigen in alum, we next directed our interest to the innate immune response to adjuvant in the peritoneum and how cells would take up and translocate antigen from the peritoneum to this node. By analogy with other adjuvants, it is possible that alum adjuvant is immunogenic due to its induction of inflammation at the site of injection, thus recruiting antigen presenting cells to the site of antigen exposure [7]. One of the most prominent cell types found in the peritoneal cavity of unimmunized mice are the resident F4/80^{high}CD11b^{high} peritoneal macrophages. Within 6 hrs after injection, there was a dramatic reduction in these resident macrophages in mice receiving OVA-alum, but not OVA (Fig 3A). On the contrary, OVA-alum induced a rapid recruitment into the peritoneal cavity of CD11b^{+}F4/80^{int}Ly6G^{-}Ly6C^{high} inflammatory

monocytes, previously shown to be immediate precursors for DCs [26, 27]. Twelve and 24 hrs after injection of OVA-alum a significant increase in the number of myeloid DCs (defined as MHCII^{high}CD11c^{+}F4/80^{low}) and plasmacytoid DCs (120G8^{+}CD11b^{dim}CD11c^{int}) could be found compared to OVA-injected mice. Furthermore, OVA-alum led to a marked increase in the numbers of neutrophils (CD11b^{+} Ly6C^{+}Ly6G^{high}F4/80^{-} cells) and eosinophils (CD11b^{+}Ly6C^{dim}Ly6G^{dim}F4/80^{dim}) recruited into the peritoneal cavity. In attempt to explain the increase in innate immune cells so early after injection of alum, we also measured the levels of chemokines in the peritoneal lavage at 2 hrs following injection of saline, OVA or OVA-alum. There was a marked increase in the levels of the the monocyte chemotactic protein (MCP-1; CCL2), the neutrophil chemotaxin KC (CXCL1), and the eosinophil chemotaxin eotaxin-1 (CCL11) in mice receiving OVA-alum versus OVA or saline (Fig 3B). OVA by itself induced an intermediate level of MCP-1 compared with saline or OVA-alum injected mice.

At 24 hrs after injection, we also studied the presence of a population of IL-4-producing Gr-1^{+} myeloid cells, previously shown to be involved in inducing splenic B cell priming after alum injection [5]. Using IL-4-GFP reporter mice (4-Get mice [28]), we could detect an alum-induced increase in this population in the peritoneum and spleen, but not mediastinal LN. In addition, we found that alum induced two populations of CD11b^{+}Gr1^{+} myeloid cells expressing IL-4 in the peritoneum, the highest Gr1^{+} one most likely being granular F4/80^{+} eosinophils, the intermediate Gr1-expressing one being monocytes (Suppl Fig 3).

Antigen uptake and processing by recruited dendritic cells

The increase in DCs following injection of OVA-alum led us to study the effects of alum on several functional aspects of DCs including antigen uptake, processing and functional maturation. To investigate if alum had an effect on the antigen uptake by DCs, OVA-AF647 was injected i.p. either alone or emulsified in alum and uptake by CD11c^{+} DCs in the peritoneal cavity was measured 6 and 24 hrs later. Even in the absence of alum, DCs captured the antigen, but the mean fluorescence intensity representing the amount of Ag taken up was higher when alum was added (Fig 4A, results at 24 hrs shown). Under the same conditions, also peritoneal B cells took up more fluorescent antigen when alum was added whereas recruited eosinophils and neutrophils did not take up OVA-AF647, even in the presence of alum (data not shown).

To analyze antigen processing of internalized antigen, we utilized OVA-DQ, a form of OVA that is highly conjugated to the BODIPY fluorochrome that fluoresces in the green channel when taken up by cells and in the red channel when it accumulates at high densities inside endosomal antigen processing compartments. CD11c^{+}MHCII^{+} DCs from OVA-DQ-alum treated mice took up and processed more antigen than OVA-DQ treated mice (Fig 4B). When the CD11c^{+}OVA-DQ$^{double\ pos}$ cells were analyzed in the OVA-DQ-alum

treated mice, they expressed more MHC II than the OVA-DQ^{neg} cells, indicating that the DCs that took up and processed antigen, also functionally matured (Fig 4C). After injection of OVA-alum, we observed that the DC maturation marker CD86 (and CD40, data not shown) was induced on peritoneal lavage CD11c^{+}MHCII^{+} DCs within 6 hrs and started to return to baseline from 24 hrs onwards compared to an injection of OVA or saline (Fig 4D). This effect of alum on DC maturation was most likely indirect as exposure of purified BM-derived DCs to alum in vitro did not lead to any direct DC activation (Fig 4D, right panel and [20, 21]). The ultimate definition of DC function is the potential to present antigen to naive T cells. When CD11c^{+}MHCII^{+} DCs were sorted from the peritoneum of immunized mice, only DCs derived from OVA-alum immunized mice induced T cell proliferation of naive DO11.10 OVA-TCR-specific T cells ex vivo (Fig 4E), best explained by induction of DC maturation (Fig 4D) and more efficient antigen processing by these cells (Fig 4B).

One aspect of DC biology that cannot be overestimated is their potential to migrate to the draining lymph node. Uptake studies in mice receiving OVA-DQ-alum revealed that within 24 hrs, 10% of CD11c^{+}MHCII^{+} DCs in the MLN had taken up antigen and processed it into immunogenic fragments, as did 2% of CD19^{+}MHCII^{+} B cells and 10% of 120G8^{+}CD11c^{int} pDCs. The addition of alum to OVA led to a strong increase in cells positive for processed OVA-DQ, from 0.05% to 0.87% of all live draining LN cells, particularly in DCs and B cells (FACS plots not shown). We could not detect significant OVA-DQ processing in any APC population in non-draining nodes.

Injection of alum promotes antigen uptake by recruited monocytes and induces their migration and conversion into CD11c^{+} DCs in the draining nodes

Injection of OVA-alum induced the recruitment of inflammatory CD11b^{+}Ly6G^{-}Ly6C^{+}F4/80^{int} monocytes to the peritoneal cavity (see Fig 3A). When fluorescent OVA-AF647 was mixed with alum, these inflammatory monocytes massively took up more antigen compared with OVA-AF647 injected alone (Fig 5A, upper panels). Particularly in OVA-alum-immunized mice, Ly6C^{high}CD11b^{+}monocytes (identified using the same gating strategy as in the peritoneum) carrying fluorescent OVA-AF647 could also be found in the mediastinal nodes by 24 hrs after immunization (Fig 5A, lower panels). Due to incompatible staining reagents, we could however not measure OVA-DQ processing in this monocyte subset. To prove however that they were processing the internalized antigen, Ly6C^{+} monocytes were sorted from mediastinal LN, and they induced ex vivo proliferation of DO11.10 T cells when obtained from OVA-alum-immunized mice (Fig 5B). The T cell division induced by sorted monocytes was even greater than the one induced by sorted mediastinal LN DCs (sorted based on classical characteristics). Consistent with the induction of T cell proliferation, monocytes arriving in the mediastinal LN and carrying Ag to these nodes (measured by OVA-AF647) acquired costimulatory molecule expression (CD86

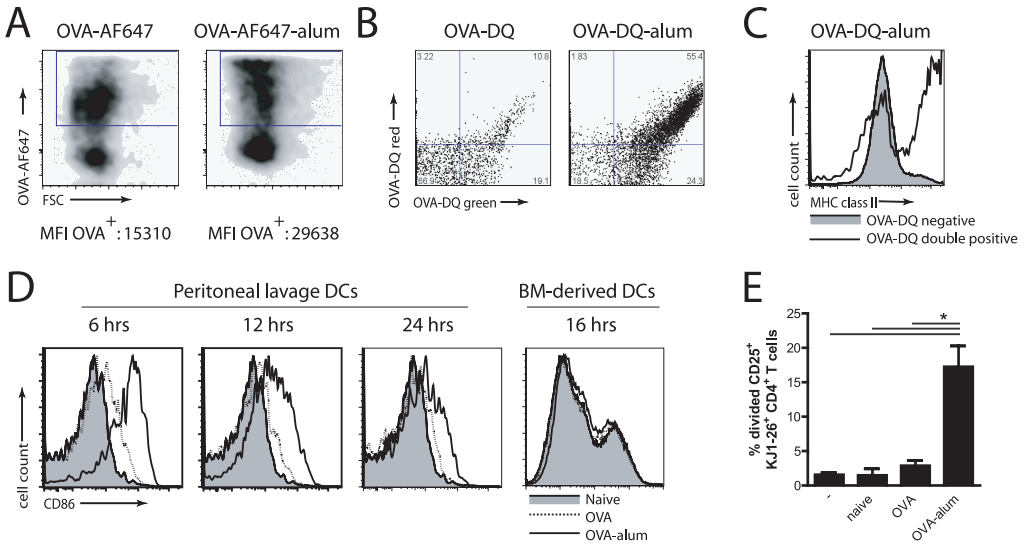

Figure 4

Alum adjuvant stimulates DC function in vivo

(A) Mice were injected i.p. with OVA-AF647 or OVA-AF647-alum. 24 hrs after injection, the peritoneal lavage was taken and the uptake of OVA-AF647 was assessed in F4/80$^-$MHCII$^+$CD11c$^+$ DCs. **(B)** Mice were injected with OVA-DQ or OVA-DQ-alum i.p. and 24 hrs later the mDCs (F4/80$^-$MHCII$^+$CD11c$^+$) in the peritoneal lavage analyzed for the uptake and processing of DQ by flow cytometry. OVA-DQ is fluoresces green when processed in acidified lysosomes. Red fluorescence is caused by accumulation of OVA-DQ in endosomal processing compartments in the cell. **(C)** The CD11c$^+$ cells were also analyzed for the expression of MHCII in the DQ negative or double positive gate. Grey filled histograms represent OVA-DQ negative and black line histograms represent OVA-DQ double positive CD11c$^+$ cells. **(D)** Maturation of cDCs in the peritoneal lavage was assayed 6, 12, and 24 hrs after injection of OVA or OVA-alum by flow cytometry. BM-derived DCs (BM-DCs) were pulsed for 16 hrs with OVA or OVA-alum. Grey filled histograms represent naive mice or unpulsed BM-DCs, black dotted line histograms OVA-injected mice or OVA-pulsed BM-DCs, and black solid line histograms OVA-alum-injected mice or OVA-alum-pulsed BM-DCs. **(E)** Mice were injected with OVA or OVA-alum and 6 hrs later the F4/80$^-$MHCII$^+$CD11c$^+$ DCs were sorted from the peritoneal lavage and placed in co-culture with CFSE-labelled DO11.10 Tg CD4$^+$ T cells. After 4 days, cells were analyzed for proliferation and gated for CD4$^+$, KJ1-26$^+$ and CD25$^+$.

and CD40), upregulated MHC II and most importantly also acquired the CD11c integrin, a classical marker of DCs. These changes were most pronounced in monocytes that had taken up antigen (Fig 5C). As these findings were largely descriptive and not excluding the possibility that LN resident monocytes acquired the antigen and upregulated CD11c in situ, we performed adoptive transfer experiments of flow cytometry sorted Ly6C$^+$CD11b$^+$CD31$^-$ bone marrow monocytes obtained from CD45.2 congenic mice that were injected intraperitoneally into CD45.1 recipients, 2 hrs after injection of OVA or OVA-alum. As shown in Fig 5D, CD45.2 monocytes migrated from the peritoneal cavity to the mediastinal LN, and this migration was strongly amplified by addition of alum. When CD45.2 bone marrow monocytes were phenotyped prior to i.p. injection, they were negative for the DC markers CD11c and MHC II. However, CD45.2 monocytes recovered from the mediastinal LN 36 hrs after i.p. injection now strongly expressed MHC II and CD11c, up to 25% of

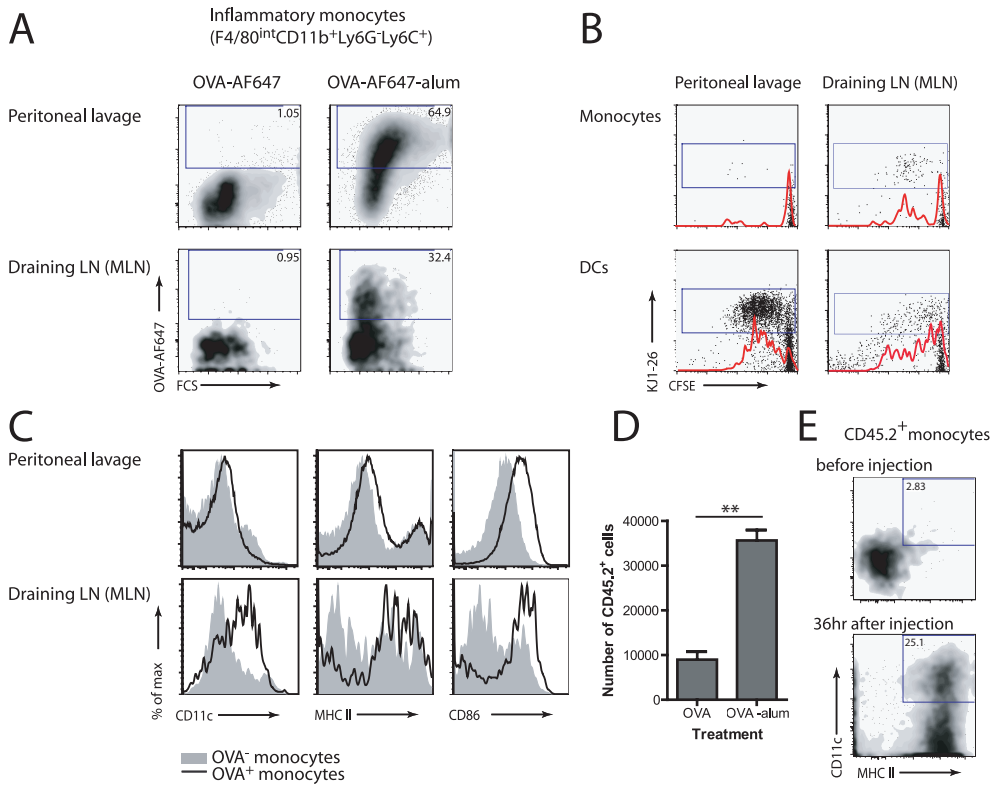

Figure 5

Inflammatory monocytes recruited by alum take up antigen, migrate to draining LN and acquire a DC phenotype

Mice were injected with OVA-AF647 or OVA-AF647-alum and 24h later the peritoneal lavage and draining LN (MLN) were taken. **(A)** Presence of OVA-AF647 in inflammatory monocytes (defined as CD11b$^+$Ly6C^{high}Ly6G$^-$F4/80^{int}) is shown in the peritoneal lavage and MLN. **(B)** Inflammatory monocytes and mDCs (CD11b$^+$MHCII^{high}Ly6C$^-$) were sorted and placed in co-culture with CFSE-labelled DO11.10 Tg CD4$^+$ T cells. T cell proliferation was assayed at day 4 and plots depict PI-negative CD4$^+$ cells. **(C)** Expression of CD11c, MHC II and CD86 on inflammatory monocytes determined by 9-colour flow cytometry. Grey filled histograms represent the OVA-AF647-negative monocytes, whereas the black line histogram represents the OVA-AF647-positive ones. An example is shown of 4 mice. **(D)** CD45.1 mice were injected with OVA or OVA-alum. 2 hr later they received CD45.2$^+$ monocytes sorted from bone marrow (purity >95%). 36 hrs later the number of CD45.2$^+$ cells in the MLN were determined by flowcytometry. Data shown are mean ± SEM, ** p<0.01, n=4-5 mice per group. **(E)** The CD11c and MHC II expression was assessed on the CD45.2$^+$ cells before injection and 36hr later in the MLN.

cells expressing both markers, indicative of conversion to DCs.

When we studied the uptake and transport of fluorescent antigen after i.m. injection into the gluteal muscle, we could similarly detect antigen uptake by DCs (Suppl Fig 2B) and inflammatory monocytes (Suppl Fig 2C) in the muscle. Particularly when OVA-alum was administered did antigen-laden inflammatory monocytes also accumulate in higher numbers in the draining sacral nodes.

Functional effect of depleting resident or recruited DCs on T cell priming and humoral

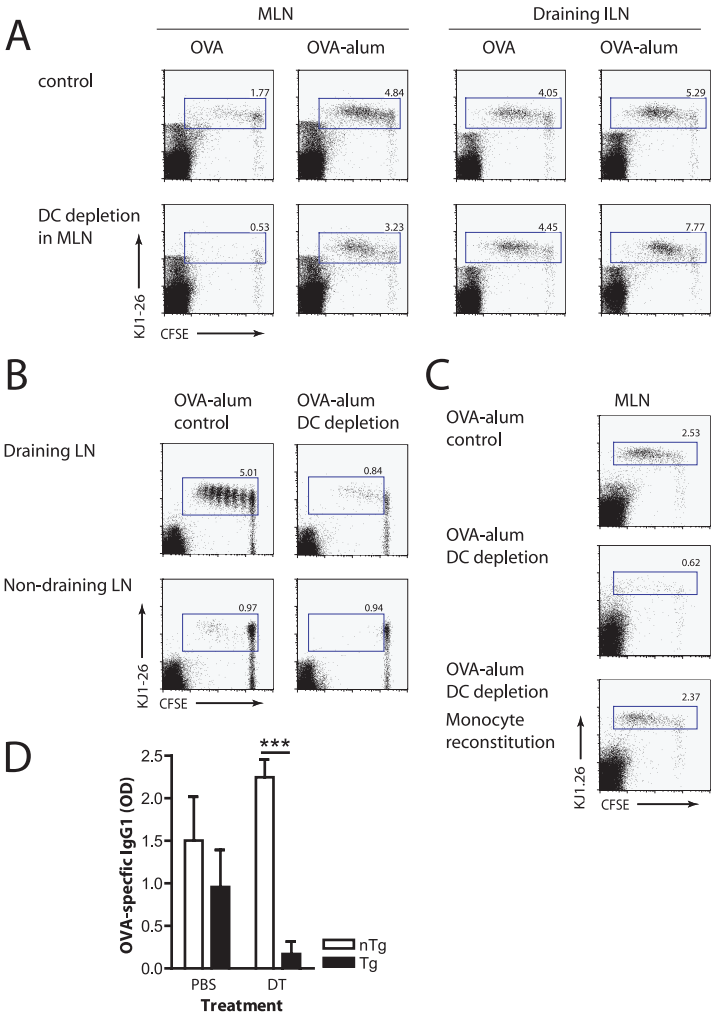

Figure 6
Contribution of resident versus recruited CD11c$^+$ DCs on antigen presentation and immunopotentiating effect of alum
(A) CD11c-DTR Tg mice were depleted of resident MLN DCs by an i.t. injection of 100 ng diphtheria toxin (DT) or PBS as a control. One day before DT, they received a cohort of CFSE-labelled CD4$^+$ DO11.10 T cells i.v.. One day after DT, OVA or OVA-alum was given i.p.. Three days after the last injection, proliferation of Tg T cells were determined in the draining MLN and draining right ILN. Percentages in the plots are the percentage Tg cells from total CD4$^+$ T cells. **(B)** To deplete all CD11c$^+$ cells (resident and recruited) CD11c-DTR Tg mice were injected i.p. instead of i.t.. The rest of the treatment was the same as described in (A). **(C)** CD11c-DTR Tg mice were depleted of DCs by an i.p. injection of 100 ng DT or PBS as a control. One day after DT, OVA or OVA-alum was given i.p. with or without sorted monocytes from BALB/c mice. Thereafter treatment was the same as described in (A). **(D)** CD11c-DTR Tg mice and nTg mice were injected with PBS or DT and received an i.p. injection of OVA-alum and 10 days later serum samples were taken. OVA-specific IgG1 levels were determined by ELISA. Data are shown as mean ± SEM, * $p<0.05$. n=4-5 mice per group.

immune response induced by OVA or OVA-alum

The i.p. injection of OVA by itself did not lead to peritoneal DC activation and antigen presentation (Fig 4), nor recruitment of inflammatory antigen laden monocytes to the mediastinal nodes (Fig 5), yet T cell divisions were induced in these nodes by OVA (Figs 1,2 Suppl Fig 1). One possibility would be that i.p. injected antigen reaches the LN via the flow of afferent lymph from the peritoneum in the absence of cell migration. We indeed observed that 2h after injection of OVA-AF647 with or without alum, the mediastinal LN subcapsular sinus and B cell area became strongly fluorescent when we imaged sections directly without hydration (data not shown), as previously shown by others [29, 30]. We therefore hypothesized that in the absence of

alum, antigen was presented by resident non-migratory lymph node APCs that acquired the antigen via the afferent lymph, whereas in the presence of alum, antigen was presented by recruited inflammatory monocytes and DCs that migrated to the nodes. To address this hypothesis, we used transgenic mice in which DCs can be conditionally depleted by administration of diphtheria toxin (DT). In these mice the human DTR receptor is expressed under the control of the murine CD11c promotor, leading to the rapid killing of CD11c^{hi} cells upon DT administration [31, 32]. Control mice in these experiments were non-transgenic littermates that received a similar treatment with DT. DT was administered locally via the intratracheal route, leading to a depletion of mediastinal resident LN (Suppl

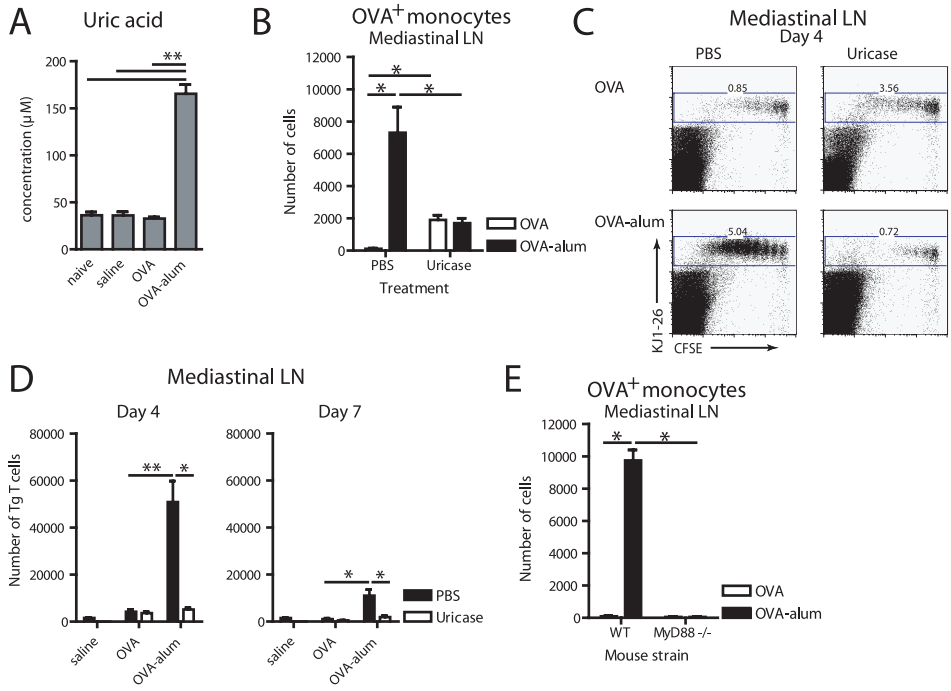

Figure 7

The alum response in mice depends on uric acid and MyD88 signaling

(A) Mice were injected with saline, OVA, or OVA-alum and after 2h uric acid levels were determined in the peritoneal lavage. Data are shown as mean ± SEM, ** p<0.01, n=5-6 mice per group. **(B)** Mice were injected with uricase 1 day and 5 min before OVA-AF647 or OVA-AF647-alum and 24h later the draining LN (MLN) were taken. The number of OVA-AF647^{+} inflammatory monocytes (defined as CD11b^{+}Ly6C^{high}Ly6G^{-}F4/80^{int}) are shown. Data are shown as mean ± SEM, * p<0.05, n=4-5 mice per group. **(C)** At day 0, mice received a cohort of CFSE-labelled DO11.10 T cells i.v. and uricase i.p.. At day 1, mice received another injection with uricase and 5 min thereafter OVA or OVA-alum i.p.. Four days after the last injection, proliferation of Tg T cells were determined in the draining MLN. Percentages in the plots are the percentage Tg cells from total CD4^{+} T cells. An example is shown from 4 mice, experiment is repeated twice. **(D)** Quantification of the number of Tg cells from 4 and 7 days after OVA or OVA-alum plotted in C. Data are shown as mean ± SEM, * p<0.05, **p<0.01, n=4 mice per group. **(E)** MyD88$^{-/-}$ and WT mice were injected with OVA-AF647 or OVA-AF647-alum and 24 hrs later the draining LN (MLN) were examined. The number of OVA-AF647^{+} inflammatory monocytes (defined as CD11b^{+}Ly6C^{high}Ly6G^{-}F4/80^{int}) are shown. Data are shown as mean ± SEM, * p<0.05, n=4 mice per group.

Fig 4) as well as lung-derived migratory DCs, while leaving all other DCs unaffected [32]. By taking advantage of the unique feature that the mediastinal LN drains both the lung and peritoneum, we could deplete LN resident DCs also draining the peritoneum without having to administer the toxin to the peritoneum. When resident DC depleted mice received an i.p. injection of antigen one day later, T cell proliferation (measured three days after injection of OVA) was abolished in the mediastinal LN in mice receiving OVA, but not those receiving OVA-alum (Fig 6A). On the contrary, the ipsilateral draining inguinal LN still demonstrated T cell proliferation in response to OVA even when DT was administered to the lung, illustrating that the toxin did not affect resident DC function outside the mediastinal node.

To finally study the function of migratory DCs and monocyte derived DCs in the priming of the immune system by OVA-alum, we also administered the DT toxin systemically through the peritoneal route, depleting all $CD11c^{hi}$ cells, including the resident ones [31]. When CD11c-DTR Tg mice received an i.p. injection of OVA-alum, adoptively transferred DO11.10 T cells divided strongly when $CD11c^{+}$ cells were present (Fig 6B). In CD11c-depleted mice, there was a very strong reduction in T cell divisions at day 4 of the response, and there was no occurrence of recirculating divided T cells in the non-draining nodes. As the population of recruited inflammatory $CD11b^{+}Ly6G^{-}Ly6C^{+}$ $F4/80^{int}$ monocytes differentiated into DCs in vivo (see Fig 5E) we finally tested whether sorted BM-derived monocytes injected i.p. could restore divisions in mice depleted of $CD11c^{hi}$ cells. As shown in Fig 6C, injection of monocytes 2 hrs after injection of OVA-alum restored T cell divisions in Ag-specific T cells in the mediastinal LN of mice depleted of DCs.

Aluminum adjuvant is widely used for its strong induction of the humoral response, possibly through induction of direct priming of B cells by a myeloid IL-4-producing cell type [5] and by induction of T cell help for class switching. To examine if the humoral response would also be dependent on DCs, we treated CD11c-DTR mice or non Tg littermates with DT and injected them with OVA-alum. Ten days after the injection, serum samples were taken and OVA-specific IgG1 and IgE levels were determined. In DC-depleted mice (CD11c-DTR Tg mice given DT) a significant reduction in the levels of OVA-specific IgG1 was found (Fig 6D). OVA-specific IgE levels in serum also showed this trend, although it did not reach significance (data not shown).

The immuno-potentiating effect of alum depends on induction of uric acid and signalling through the MyD88 pathway

The fact that monocytes are recruited to the peritoneum and differentiate into full blown antigen presenting DCs does not explain how these cells get activated. One striking finding was that alum induced a strong neutrophilic influx, accompanied by the production of CXCL1 (KC) and CCL2 (MCP-1)(Fig 3), as well as IL-1β and IL-18 (data not shown)[21] akin to the response seen when the endogenous danger signal uric acid is injected into the peritoneal

cavity [33, 34]. We therefore measured the level of uric acid in the peritoneal lavage 2 hrs following injection of saline, OVA or OVA-alum and found that only alum induced a strong increase in uric acid levels (Fig 7A). To test the functional significance of this induced uric acid, we neutralized it by treating mice with the uric acid degrading enzyme uricase prior to administration of OVA-AF647 in the presence or absence of alum. The recruitment of OVA-laden $CD11b^{+}Ly6G^{-}Ly6C^{+}F4/80^{int}$ inflammatory monocytes to the mediastinal LN was completely abolished in mice treated with uricase (Fig 7B) and consequently, T cell division that is normally avidly induced in vivo in the presence of alum was abolished back to the control level when uricase was given (Fig 7C and D for quantification). It was previously shown that the peritoneal response to uric acid is heavily dependent on the IL-1 receptor and downstream MyD88 signaling. To test this possibility, we gave OVA-AF647 +/- alum to $MyD88^{-/-}$ C57Bl/6 mice and found that the recuitment of inflammatory monocytes to the mediastinal LN was grossly reduced compared with WT animals (Fig 7E).

Discussion

By carefully studying the kinetics and distribution of the innate and adaptive T cell immune response following i.p. injection of OVA in alum we have uncovered a previously unappreciated role for monocyte derived DCs in mediating the adjuvant effects of alum on cellular and humoral immunity. This is underscored by the fact that inflammatory monocytes and DCs were attracted to the peritoneum following injection of OVA-alum, that they took up and processed the antigen on their way to the mediastinal LNs, that they acquired a functional phenotype of mature DCs once in the LN, and finally that removal of $CD11c^{+}$ DCs abolished T cell proliferation in OVA-alum immunized mice, an effect that was restored by adoptive transfer of $Ly6C^{high}$ monocytes.

One of the aspects in our study that made us uncover this new mechanism of action of alum was the assessment of the precise localisation where antigen presentation occurred following i.p. injection. The peritoneal route is easily accessible and often used as a site for immunization to test the protective effect of novel vaccines against subsequent infection. The good resorption of drugs from the peritoneal cavity has mislead the immunological community, as it is often assumed that intraperitoneal administration of a protein antigen leads to rapid systemic resorption into the bloodstream, leading to the common notion that intraperitoneally administered antigens are presented by antigen presenting cells in the spleen, similar to intravenously injected antigens. Often therefore, i.p. immunization is equalled to 'systemic immunization' and investigators studying the immunogenicity of alum have focussed on the spleen as a site where immune activation might occur [5, 22]. Here we show that i.p. injection of the OVA antigen in the right lower quadrant of the peritoneal cavity leads to Ag presentation and antigen-induced T cell proliferation in the mediastinal LN and

the ipsilateral inguinal LN, but however not in the spleen. By following T cell divisions over time, it was clear that only after 4 days, when T cells had undergone at least 3-4 divisions, could we detect divided cells in non-draining lymph nodes and spleen, very similar to what we and others described for immunization with Ags via the skin or respiratory mucosa [23]. Therefore, the antigen-specific reactivity that can be measured in the spleen ex vivo is the result of recirculating effector and/or memory cells. This finding does not exclude that there is immune activation occurring in the spleen before day 3-4 of the response. Within 24 hrs of injection of OVA-alum, there was induction in the spleen of an IL-4-producing $Gr1^+$ myeloid cell, as described before by others 6 days after injection of alum [5]. The induction of divisions in the ipsilateral ILN was unexpected, but was due to an artefact induced by skin puncture. One important lesson is that ILN nodes should not be taken as 'control non-draining nodes', as is often done because of their easy accessibility, for Ags that are injected intraperitoneally.

It was less surprising that Ag was presented in the MLN as previous studies in rat, mice and sheep have shown that the peritoneal cavity has a lymphatic drainage consisting of stomata that cross the diaphragm and drain into the parathymic and mediastinal LN [26, 35, 36]. The transport of antigen could be either through free flowing lymph, gaining access to the subcapsular sinus and conduit system of mediastinal LNs and thus to resident DCs and to follicular B cells [29, 30, 37] or could be mediated by DCs or other APCs that pick up antigen in the peritoneal cavity and migrate to these nodes [38]. Both scenarios might come into play. By visualizing unhydrated LN slides, we could detect a massive amount of fluorescent Ag in the mediastinal LN within 2 hrs after i.p. injection, irrespective of wether alum was added or not, which would never be caused by cell transport alone. Cell-mediated transport by inflammatory $Ly6C^{high}$ monocytes and DCs occurred particularly when alum was added.

What is the reason for the dramatic difference in T cell outcome when alum adjuvant is added to an antigen? We demonstrated that in the absence of alum, antigen was presented predominantly by non-migratory LN resident DCs that acquired the antigen via afferent lymph, as evidenced in experiments is which these resident DCs were depleted locally in the mediastinal node prior to OVA administration (Fig 6A). Itano et al. have demonstrated that after skin puncture, there is a rapid flux of cell-free Ag from the site of injection to the skin-draining node, leading to T cell divisions in Ag-specific T cells, without generation of T cell effector potential [37]. We speculate that the physiological drainage of the peritoneal cavity through the stomata in the diaphragm also leads to presentation of antigen in a tolerogenic form by immature resident DCs, inducing deletional T cell proliferation [39, 40]. In contrast, when inflammation is induced by alum, there is additional recruitment of inflammatory monocytes and activation of already resident peritoneal DCs that migrate to the LN and arrive as $CD11c^+$ mature cells expressing the necessary costimulatory molecules for naive T cell activation and generation of memory cells

[41]. Several groups have recently shown that $CCR2^{+}Ly6C^{+}$ monocytes are the immediate precursors of inflammatory type DCs, also called 'TIP'-DCs under conditions of Listeria infection [42], with an enhanced potential to induce effector T cells [27, 43, 44]. We believe that our data support the notion that alum boosts immunity by inducing these 'inflammatory' DCs. When the resident LN DCs were depleted in the mediastinal LN using lung application of a selective DC depleting diphteria toxin [32], the induction of T cell division by OVA-alum was not suppressed, whereas when these inflammatory monocytes and DCs were depleted using peritoneal administration the toxin [31], almost all T cell division disappeared (Fig 6B), and there was no longer any priming for humoral immune responses (Fig 6D). The effects of DC depletion on T cell division were however completely restored when we performed an adoptive transfer of bone marrow derived $Ly6C^{+}$ monocytes, cells that acquired a DC phenotype following arrival in the MLN. These data suggest that inflammatory DCs are strongly involved in mediating the enhancing effects of alum on adaptive immunity and also demonstrate that uptake and processing by other APCs is not sufficient for generating immunity in the absence of DCs. This change of function in monocytes could be the result of their phagocytosis of particulate alum particles, as previously shown for phagocytosis of latex beads injected into the peritoneal cavity [26]. One striking feature was that all APCs contained more intracellular antigen when it was emulisfied in alum (Fig 4&5 and data not shown for B cells). Particularly in monocytes, the cells that had internalized antigen demonstrated the shift in CD11c, costimulatory molecules and MHC II, suggesting that indeed antigen uptake was associated with DC differentiation. Inflammatory monocytes in the peritoneum contained fluorescent antigen by 6 hrs, whereas the same cells were found in the MLN only by 24 hrs, suggesting indeed migration to these nodes, a finding also supported by adoptive transfer experiments of CD45.2 congenic donors.

As our own in vitro experiments (Fig 4D) and experiments by others [20] did not reveal a direct activation of monocytes and DCs by alum, we hypothesized that an endogenous danger signal might be released following injection of alum in vivo. We measured very high levels of the endogenous danger signal uric acid when alum was injected and more importantly, recruitment of neutrophils, inflammatory monocytes as well as T cell activation induced by alum in the mediatinal LN was abolished when uric acid was neutralized by uricase treatment. Uric acid is released by necrotic cells and alum has been shown to induce a considerable degree of necrosis. It is well known that alum injection i.p. leads to cell death and when injected into muscle alum leads to myofasciitis. The release of uric acid could explain the high degree of neutrophilic inflammation as well as CXCL1 production, as a very similar response is seen when uric acid is injected intraperitoneally [13, 33]. Moreover, work by others [21] as well as our own unpublished work demonstrated that alum, like uric acid, activates caspase-1 and leads to the release of IL-1β and IL-18 [33]. In

support for a predominant role for this pathway in activating inflammatory DCs, we found that the alum response was abrogated in mice deficient in the signalling molecule MyD88, involved in transducing signaling from the IL-1 and IL-18 receptor. What we cannot explain however at present is the fact that the humoral immune response measured several weeks after injection of alum is variably dependent on MyD88 and/or IL-1 [6, 45, 46]. Although these differences might depend on timing of analysis and contamination or addition of different TLR ligands to alum, it could also be that for induction of humoral responses, IL-1 signaling via MyD88 is redundant, whereas for T cell responses it is crucial [8]. Whether uric acid is the only endogenous innate trigger for DC activation remains to be shown, but the fact that uricase was so effective points towards a predominant role for it. Just like uric acid, alum adjuvant can activate several other aspects of innate immunity including activation of the coagulation and complement cascade, known to influence DC function [7, 47]. As alum does not activate bone-marrow derived DCs in vitro, it is tempting to speculate that non-hematopoeitic structural cells of the peritoneal cavity might undergo necrosis and subsequently release uric acid, although formal proof of this is lacking. The rapid recruitment of neutrophils and eosinophils within 6 hrs along with DCs could subsequently be responsible for the indirect activation of DCs. Indeed neutrophils have been shown to activate DCs through CD11b-DC-SIGN interactions, leading to secretion of chemokines and cytokines [48]. If eosinophils could perform the same task is unclear at present, but they could certainly represent an early source of Th2-polarizing cytokines, necessary for Th2 induction by alum [28]. It has been shown that alum induces a Gr-1$^+$IL-4$^+$ myeloid population (eosinophils and monocytes) in the spleen 10 days after injection [5]. We did see an increase in Gr-1$^+$IL-4$^+$ cells in the peritoneum and spleen, but not mediastinal LN, within 24 hrs after injection of alum, but do not know at present whether this population could be involved in activaton of the monocytes and DCs.

In conclusion, through a series of in vivo experiments, we showed that alum adjuvant promotes adaptive immunity by releasing the endogenous danger signal uric acid thus inducing the differentiation of nature's adjuvant, the inflammatory dendritic cell from recruited monocytes.

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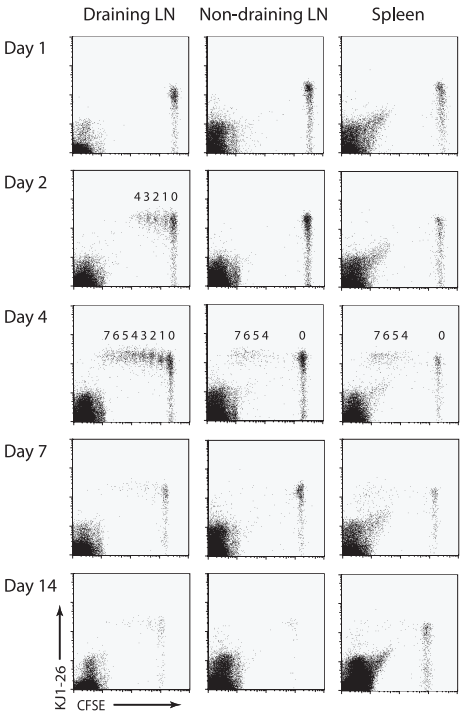
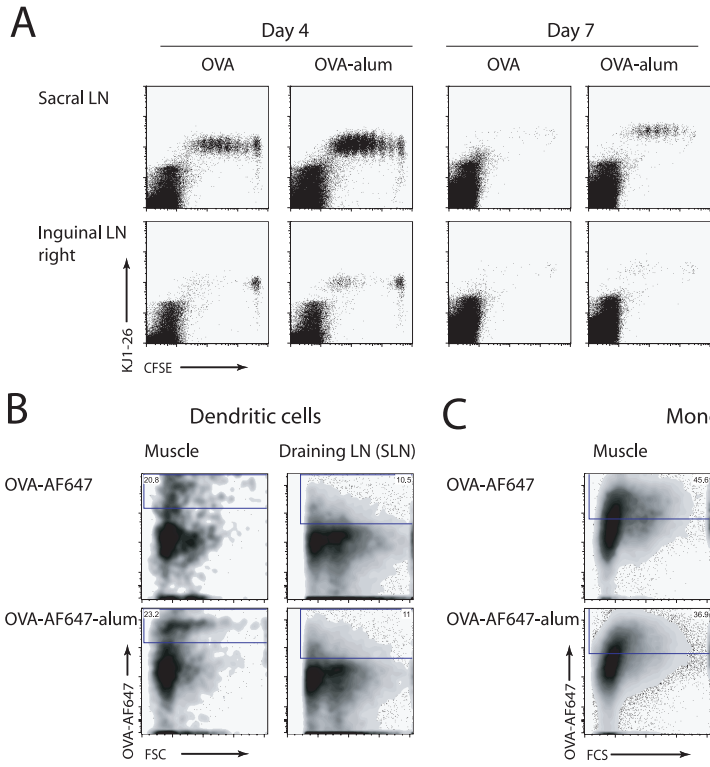
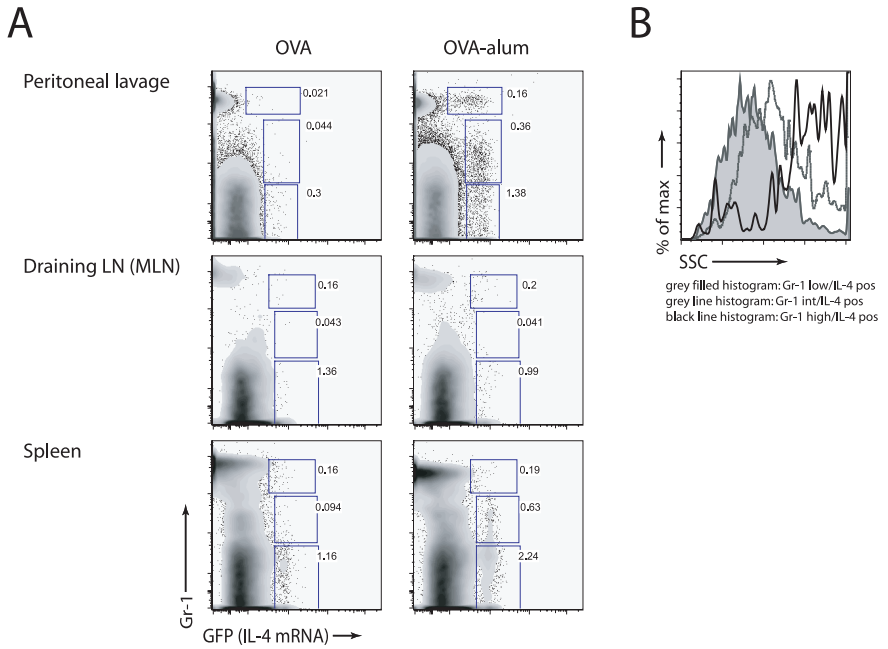


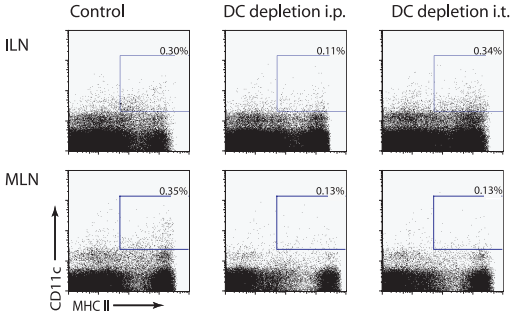
Supplementary figure 1
Time kinetics of the T cell proliferation induced by i.p. injection of OVA
Mice were injected with CFSE-labelled DO11.10 OVA-TCR Tg cells 1 day before the intraperitoneal injection of OVA. One, 2, 4, 7, or 14 days after the OVA injection the draining LN (MLN), non-draining LN (CLN), and the spleen were analyzed with flow cytometry. An example is shown of 4 mice; the experiment was repeated at least two times.



Supplementary figure 2
Intramuscular injection of OVA alum follows the same pattern of T cell activation and inflammatory cell recruitment as i.p. injection.
(A) Mice were injected with CFSE-labelled DO11.10 OVA-TCR Tg cells 1 day before the intramuscular injection of OVA or OVA-alum. The intramuscular injection was given in the left gluteus muscle. After 4 and 7 days, draining LN (sacral LN) and non-draining LN (right ILN) were isolated and proliferation was determined by flow cytometry. Dot plots depict PI-negative $CD4^+$ cells. **(B)** Mice were injected i.m. with OVA-Alexa Fluor 647 (OVA-AF647) or OVA-AF647-alum and 24h later the muscle and draining LN (SLN) were taken. The presence of OVA-AF647 was determined in $F4/80^-$ $MHCII^+CD11c^+$ mDCs. **(C)** Presence of OVA-AF647 in inflammatory monocytes is shown. Examples are shown from 4 mice.



Supplementary figure 3
Alum injection induces IL-4$^+$ Gr-1$^+$ myeloid cells in the spleen, peritoneal cavity, but not the mediastinal LN
(A) 4get mice with a GFP fluorescent reporter under the IL-4 promotor were injected with OVA or OVA-alum. 24 hr after the injection, different organs were analyzed for the presence of IL-4$^-$GFP$^+$Gr1$^+$ cells using flow cytometry on live CD11b$^+$ DAPI$^-$ cells. Particularly in the peritoneum, a population of Gr1^{high} cells was found, and this population was more granular (SSC^{high}) that the Gr1^{int} population **(B)**.



Supplementary figure 4
DCs are depleted following diphteria toxin treatment in CD11c-DTR Tg mice.
CD11c-DTR mice were injected either i.t. or i.p. with 100 ng diphteria toxin. After 24 hr, the mediastinal LN and inguinal LN were examined for the presence of DCs by flow cytometry. The numbers in the plots represent the percentage of DCs from living (PI$^-$) cells. An example is shown out of 4 mice.

Chapter 3

Cutting Edge:
Alum Adjuvant stimulates Inflammatory Dendritic Cells through activation of the NALP3 Inflammasome

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Adjuvants are vaccine additives that stimulate the immune system without having any specific antigenic effect in itself. In this study we show that alum adjuvant induces the release of IL-1β from macrophages and dendritic cells and that this is abrogated in cells lacking various NALP3 inflammasome components. The NALP3 inflammasome is also required in vivo for the innate immune response to ovalbumin (OVA) in alum. The early production of IL-1β and the influx of inflammatory cells into the peritoneal cavity is strongly reduced in NALP3-deficient mice. The activation of adaptive cellular immunity to OVA-alum is initiated by monocytic dendritic cell precursors that induce expansion of antigen-specific T cells in a NALP3-dependent way. We propose that in addition to TLR stimulators, agonists of the NALP3 inflammasome should be considered as vaccine adjuvants.

Activation of dendritic cells (DCs) is a crucial mechanism by which vaccine adjuvants stimulate protective adaptive immunity to cancer or microbial infection [1]. Agonists to several Toll-like receptors (TLRs) are in clinical development. The most promising TLR ligand is unmethylated CpG dinucleotide containing DNA that stimulates TLR9 in a pathway requiring the adaptor MyD88, leading to the activation of DCs [2, 3]. Nonetheless, there is a debate whether TLR stimulation is essential for the Ab-enhancing effect of vaccine adjuvants like alum. Although initial studies showed that MyD88-deficient mice are defective in mounting a Th1 and B cell responses [4], this notion was recently challenged [5]. It has also become clear that an alternative pathogen detection system exists in addition to TLRs, which relies on a family of intracellular receptors called NOD-like receptors (NLRs) [6-8]. In addition to pathogen-associated molecular patterns (PAMPs), some of these NLRs also sense the presence of endogenous danger signals. The most studied NLR member is NALP3, which, together with Cardinal and ASC forms a caspase-1 activating complex, the so-called inflammasome [9]. This complex is activated by multiple agonists including bacterially derived muramyl dipeptide or endogenous ATP and uric acid, leading to the processing and release of IL-1β [10, 11]. We therefore considered the possibility that NLRs, in addition to TLRs, are implicated in the effect of some adjuvants.

Methods

Mice

Wild-type (WT), ASC$^{-/-}$, IPAF$^{-/-}$ (gifts from V. Dixit; IPAF is IL-1β-converting enzyme protease activating factor), MyD88$^{-/-}$, and NALP3$^{-/-}$ mice (all mice on C57Bl/6 background) were bred at University of Lausanne, Switzerland and at the University Hospital Gent, Belgium. Male and female mice were used between the ages of 8 and 14 wks. All in vivo experiments were approved by the animal ethics committee of Ghent University.

Isolation and activation of mouse primary cells

Peritoneal macrophages were isolated 3 days after an i.p. injection with 10% thioglycollate and primed in vitro with 500 ng/ml LPS as previously described [20]. Uric acid, uricase, and cytochalasin D were bought from Sigma-Aldrich and crystals were generated as previously described [12].

Antigens and adjuvant

Ovalbumin (OVA) was purchased from Worthington Biochemical Corp (Lakewood, NJ). In antigen-tracking experiments, OVA-Alexa Fluor 647 (Molecular Probes) was used. Imject-alum (Pierce Biochemicals; hereafter simply called alum) is a mixture of aluminum hydroxide and magnesium hydroxide and was mixed at a 1:20 ratio with a solution of OVA antigen in saline followed by stirring for at least 1 hr. For immunization 500 µl of Imject-alum suspension (1 mg) containing 10 µg of OVA (OVA-alum) was injected i.p., or alternatively 10 µg of OVA in 500 µl saline was injected.

Detection of cellular influx in vivo after OVA-alum injection

In WT and NALP3$^{-/-}$ mice, 2, 6, and 24 hrs after injection, the peritoneal cavity was washed with 3 ml PBS containing EDTA (peritoneal lavage) and the mediastinal lymph nodes ware harvested and used for flow cytometry as previously described [19].

Statistical analysis

For all experiments, the difference between groups was calculated using the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Differences were considered significant when $p<0.05$.

Results

Alum adjuvant activates the NALP3 inflammasome

We first exposed peritoneal macrophages from WT, NALP3$^{-/-}$ and ASC$^{-/-}$ mice to various amounts of alum (see Material and Methods for composition). Even at low doses, a substantial amount of processed IL-1β was detected in the cellular supernatant, which compared well with the quantities of this cytokine observed with crystalline monosodium urate (MSU), one of the most potent activators of the NALP3 inflammasome [12] (Fig 1A). IL-1β was absent completely absent in supernatants from macrophages from NALP3- and ASC-deficient mice, indicating a crucial involvement of NALP3 in caspase-1-mediated pro-IL-1β processing.

Activation of caspase-1 occurs not only upon

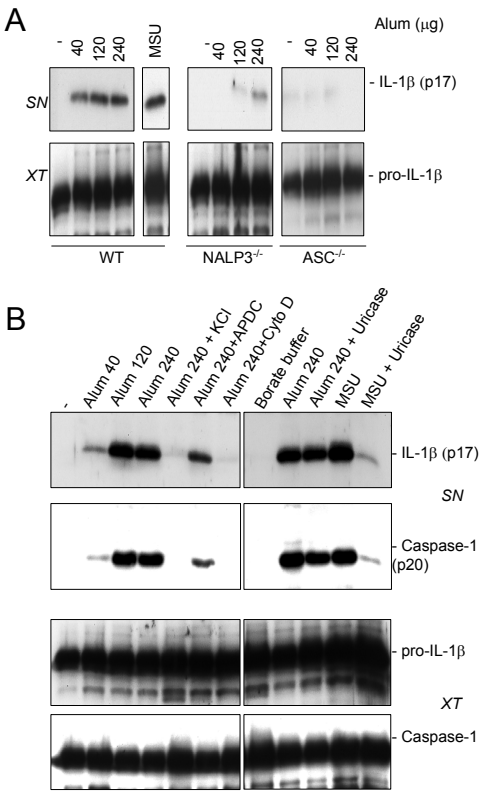

Figure 1
Role of NALP3 and ASC in alum-mediated IL-1β production

(A) Macrophages from WT, NALP3$^{-/-}$, and ASC$^{-/-}$ mice were primed overnight with ultrapure LPS (500 ng/ml) and then stimulated with different amounts of Imject-alum. MSU (150 µg/ml) was used as a control. Production of mature IL-1β was measured by Western blotting in supernatants (SN) and cell extracts (XT). **(B)** Alum-induced IL-1β secretion is dependent on phagocytosis, K^+ efflux and ROS. Peritoneal macrophages were primed as described in (A), treated with alum in the presence and absence of high concentrations of extracellular K^+ (130mM), the ROS inhibitor APDC (100 µM), cytochalysin D (2 µM), and uricase (0.1 U/ml).

assembly of the NALP3, but also the IPAF inflammasome [13]. However, a contribution of this complex in alum-triggered caspase-1 activation is unlikely, because alum-induced IL-1β secretion from macrophages derived from IPAF-deficient mice was still observed (data not shown). Also, alum adjuvant continued to activate caspase-1 in the absence of MyD88, excluding a major role of TLRs (ref [14] and data not shown).

Mechanism of NALP3 inflammasome activation by alum and uric acid is similar

We next investigated by which mechanism alum activates the NALP3 inflammasome. Recent studies have shown that NALP3 inflammasome activation is dependent on K^+ efflux [15, 16] and the generation of reactive oxygen species (ROS) [15]. K^+ efflux can be blocked by the addition of high concentrations of K^+ (130mM) to the extracellular medium. The blockade of K^+ efflux resulted in the inhibition of alum-induced caspase-1 activation and IL-1β secretion (Fig. 1B). Activation of inflammasome was also reduced when the ROS inhibitor (2 R, 4 R)-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) was added (Fig. 1B).

We could not exclude the possibility that activation of the inflammasome by alum occured indirectly through one of the two endogenous danger signals known to activate NALP3, namely ATP and uric acid [17] [12]. ATP, which is thought to be released from necrotic cells, potently activates the inflammasome via binding to the P2X7 receptor [17], while uric acid can form crystals that activate the

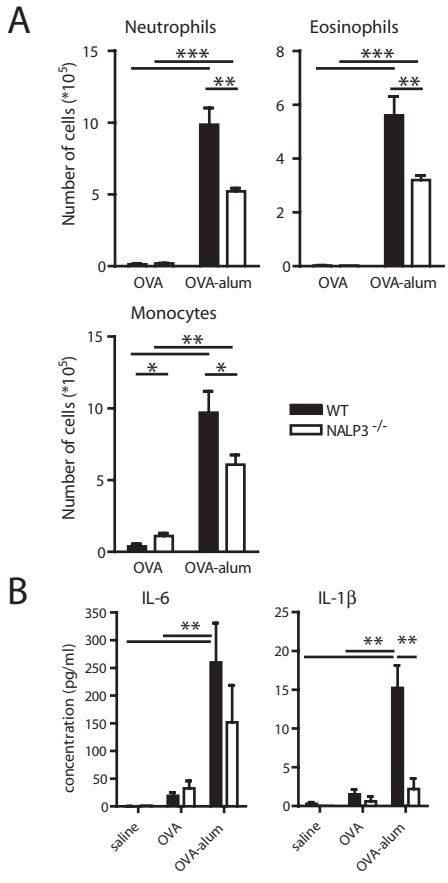

Figure 2
Innate inflammatory response induced by alum is NALP3 dependent
NALP3$^{-/-}$ or WT mice were injected i.p. with OVA or OVA-alum. **(A)** 24 hrs after injection, the peritoneal lavage was taken and the number of neutrophils ($CD11b^+Ly6C^+Ly6G^{high}F4/80^-$), eosinophils ($CD11b^+Ly6C^{int}Ly6G^{int}F4/80^{int}$), and monocytes ($CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$) was determined by flowcytometry. **(B)** 2 hrs after OVA or OVA-alum injection, the peritoneal lavage was taken and cytokine levels were determined in the supernatant by ELISA. Data shown are mean ± SEM, * $p<0.05$,** $p<0.01$, *** $p<0.001$ n=4-6 mice per group.

inflammasome by an as yet poorly understood mechanism. We have recently reported that extracellular ATP triggers inflammation by activating dendritic cells [18] and that in vivo injection of alum into the peritoneal cavity

leads to uric acid (MSU) release [19]. It was therefore possible that MSU or ATP or both, were released from alum-treated cells, leading to the subsequent activation of the NALP3 inflammasome. However, we obtained no evidence for this hypothesis. Uricase, which catalyzes the conversion of uric acid to allantoin [19] had only a minor effect on IL-1β processing in vitro (Fig. 1B). Alum also still activated the inflammasome in macrophages of P2X7-deficient mice (data not shown).

Despite the fact that both ATP and MSU activate the NALP3 inflammasome, the initial pathways leading to inflammasome activation are not identical. MSU is a particulate matter and requires endocytosis [12]. In fact, endocytosis seems to be required for particles in general, including asbestos and silica to activate the inflammasome [20]. It was therefore expected that alum also required endocytosis. The effect of alum, but not ATP (data not shown), was abrogated by the actin-destabilizing drug cytochalasin D (Fig. 1B). Taken together, NALP3 inflammasome activation in vitro by alum requires endocytosis of the particle, K^+ efflux, and generation of ROS, but not release of ATP or uric acid.

Recruitment of inflammatory cells to the site of alum injection is decreased in the absence of NALP3

Intraperitoneal injection of alum is frequently used in mice to induce cellular Th2 immunity and humoral immunity to emulsified protein antigens [21]. To assess the role of the NALP3 inflammasome in alum adjuvanticity in vivo, we examined the innate inflammatory response caused by alum upon i.p. injection of OVA adsorbed to alum. Twenty four hours after injection there was a massive recruitment of neutrophils ($Ly6G^+F4/80^-CD11b^+$ cells), eosinophils ($F4/80^{dim}Ly6G^+CD11b^+$ cells), and inflammatory monocytes ($CD11b^+F4/80^+Ly6C^+$ cells) into the peritoneal cavity that was not seen when OVA was injected without alum (Fig 2A). This innate response was severely reduced in NALP3-deficient animals but nevertheless still present. Compared with injection of saline or OVA alone, injection of OVA-alum induced a rapid increase in the peritoneal cavity of the innate cytokines IL6 and IL-1β (Fig 2B). Strikingly, in $NALP3^{-/-}$ mice the increase in IL-1β was completely reduced to the level seen in mice receiving saline or OVA, whereas the level of IL-6 was only reduced but not abolished (Fig 2B).

Activation of dendritic cells is IL-1β and NALP3 dependent

DCs are seen as nature's adjuvant and have a unique potential to induce adaptive immunity. We have recently reported that induction of T cell adaptive immunity following intraperitoneal injection of alum depends on uptake and transport of antigen from the peritoneal cavity to the mediastinal LN by resident $CD11c^+$ DCs and by inflammatory $CD11b^+Ly6C^+$ monocytes that become $CD11c^+$ DCs upon migration to the draining mediastinal lymph node (MLN) [19]. To address the contribution of inflammasome activation in this process, we measured the uptake of fluorescent OVA

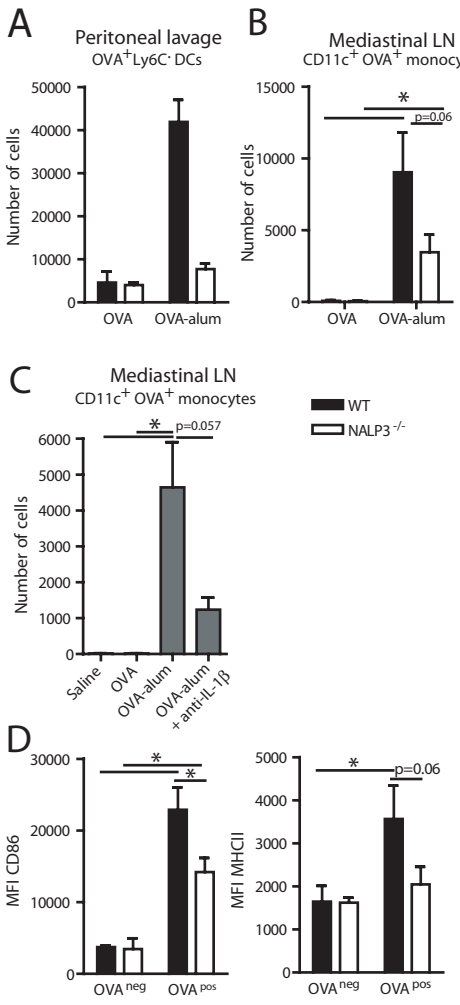

Figure 3

Activation of DCs by alum is NALP3 dependent

NALP3$^{-/-}$ or WT mice were injected i.p. with OVA-Alexa Fluor 647 (AF647) or OVA-AF647-alum. **(A)** 24 hrs after injection the number of OVA^{+} resident Ly6C^{-}CD11c^{+} DCs was determined in the peritoneal lavage by flowcytometry. **(B)** 6 and 24 hrs after injection the number of CD11c^{+}OVA^{+} monocytes in the MLN was measured. **(C)** Mice were treated with anti-IL-1β mAb before OVA-alum injection. The influx of CD11c^{+}OVA^{+} monocytes in the MLN was measured 24 hrs after injection. **(D)** CD86 and MHC II expression were measured on the OVA^{-} and OVA^{+} monocytes in the MLN 24 hrs after injection of OVA-alum in WT or NALP3$^{-/-}$ mice. Data are shown as mean ± SEM, * $p<0.05$; n=4-6 mice per group.

in resident Ly6C^{-}CD11c^{+} DCs following injection of OVA or OVA-alum. Adsorption of OVA-Alexa Fluor 647 in alum led to a strong increase in DCs carrying antigenic cargo in wild type mice, but not in NALP3$^{-/-}$ mice (Fig 3A). We also followed the occurrence of OVA-laden Ly6C^{+} inflammatory monocytes in the MLN (Fig 3B). In WT mice there was a time-dependent increase in the number of inflammatory monocytes in the MLN at 24 hrs, and a majority of these carried OVA. In contrast, in NALP3$^{-/-}$ mice this increase was severely diminished. This deficiency is most likely explained by a defective generation of bioactive IL-1β in NALP3$^{-/-}$ mice, as the alum-induced increase in OVA-laden CD11c^{+} monocytes in the MLN was severely reduced when IL-1β was neutralized in vivo, implying an important functional role for this cytokine in promoting monocyte migration and differentiation (Fig 3C). Provision of costimulation is a necessary requisite for induction of adaptive immunity. Injection of OVA-alum induced the upregulation of the costimulatory molecule CD86 on inflammatory CD11c^{+} Ly6C^{+} monocytes in the MLN of wild type, but to a much lesser extent in NALP3$^{-/-}$ mice (Fig 3D). A similar effect was seen for MHC class II (Fig 3D).

The reduced migration of OVA-laden Ly6C^{+} monocytic cells with DC characteristics to the mediastinal nodes of NALP3$^{-/-}$ mice prompted us to study the activation of naive OVA-specific CD4^{+} T cells. For this, mice first received a cohort of CFSE-labeled T cells obtained from OT-II TCR Tg mice followed by injection of OVA or OVA-alum, and accumulation of

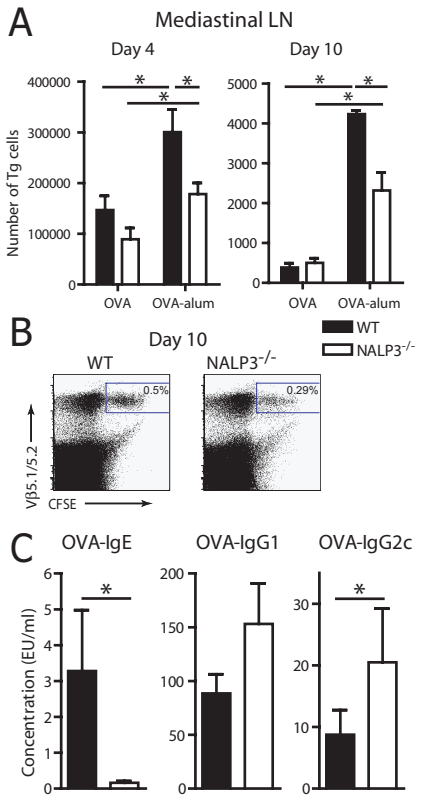

Figure 4
Induction of adaptive immunity by alum is NALP3 dependent
(A) NALP3$^{-/-}$ or WT mice were injected with CFSE-labeled OT-2 OVA-TCR Tg CD4^{+} T cells 1 day before the i.p. injection of OVA or OVA-alum. 4 and 10 days after the injection the MLN was analyzed for T cell proliferation with flow cytometry (n=4 mice, experiment performed twice). **(B)** Representative CFSE profiles of adoptively transferred T cells on day 10 after injection. **(C)** On day 0, NALP3$^{-/-}$ or WT mice were injected i.p. with OVA-alum and on day 7 boosted with OVA i.p.. At day 14 serum samples were taken and OVA-specific IgE, IgG1, and IgG2a levels were determined by ELISA. Data are shown as mean ± SEM, * $p<0.05$. n=5-11 mice per group.

OVA-specific T cells was measured in the lymph nodes. In WT mice OVA-alum caused a marked increase in the number of Ag-reactive T cells in the mediastinal LN at days 4 and day 10 post immunization (Fig 4A) compared with injection of OVA alone. In NALP3$^{-/-}$ mice, the number of T cells induced by OVA-alum was severely reduced but still higher compared with injection of OVA alone. These changes in accumulation of T cells were mainly due to altered degrees of T cell division, as measured using CFSE dilution (Fig 4B).

We finally measured the effect of NALP3 deficiency on the type of immunoglobulins induced by OVA-alum cells (Fig 4C). Mice receiving OVA alone have generally low Ab titers to OVA (data not shown). In WT mice OVA-alum induced the production of IgE, IgG1 and somewhat lower levels of IgG2c. In NALP3$^{-/-}$ mice there was a significant decrease in OVA-specific IgE antibodies and an increase in OVA-specific IgG2c antibodies. Strikingly, IgG1 levels were unaffected.

Discussion

In 1926, the adjuvant effect of aluminium compounds was first described by Glenny et al. [22]. Despite this early discovery, to this day little is known about the mode of action of alum adjuvant. Adjuvants containing a pathogen-associated molecular patterns act as ligands for the Toll-like receptors (TLRs). For example, TLR9 was shown to be essential for the adjuvant effect of CpG oligodeoxynucleotides [2]. However, incubation of DCs with alum adjuvant fails to activate the DCs as indicated by increased expression of MHC II and co-stimulatory molecules [23]; yet, several authors describe an increased production of IL-1β secretion [24, 25]. Mice lacking TLR signaling components still mount

a robust antibody response if alum is given as an adjuvant [5]. Thus, alum adjuvants, in contrast to the bacteria-derived adjuvants, do not directly activate TLRs.

Entirely in agreement with two recent studies by Eisenbarth and Li and colleagues [14, 26], our data show that alum adjuvant triggers activation of the NALP3 inflammasome. The potential of alum to trigger the NALP3 inflammasome leads to early activation of the innate cytokine IL-1β and an innate cellular immune response at the site of injection. Activation of the NALP3 inflammasome and subsequent release of IL-1β leads to recruitment of immature monocytes and DCs. Production of IL-1β also leads to the activation of inflammatory monocytes and their migration to the lymph nodes draining the peritoneum. This situation resembles the migration of skin Langerhans cells to the lymph nodes, which also requires IL-1β for functional migration and maturation to occur [27]. In OVA-alum-immunized mice, OVA-laden DCs and inflammatory monocytes take their cargo to the MLN and up-regulate the expression of critical costimulatory molecules like CD86 with the subsequent expansion of antigen-specific T cells [19]. All these steps are highly impaired in NALP3-deficient mice, indicating that activation of the NALP3 inflammasome contributes not only to the immediate inflammatory innate response at the site of injection of alum but also for the generation of adaptive cellular immunity. Furthermore, the levels of serum immunoglobulins are also to the generation of adaptive cellular immunity.

Levels of serum Igs are also influenced in the NALP3-deficient mice, as a decreased level of OVA-IgE and increased levels of OVA-IgG2c are found. This pattern of Ig synthesis is most likely the result of a shift towards IFNγ-secreting Th1-like helper cells necessary for Ig class switching to IgG2c and equally able to suppress IgE synthesis. Interestingly, we found that NALP3 does not influence IgG1 levels, whereas decreased levels of this subclass were found in the Eisenbarth report [14]. This striking difference may be due to a different experimental setup, as in their study Ab generation was determined after an intranasal challenge with OVA [14] whereas we boosted mice by injecting OVA i.p. The data are most consistent with the notion that NALP3-dependent IL-1β production by DCs skews the adaptive cellular immune response towards a Th2 type of response, as recently proposed [25].

Strikingly, alum adjuvant activates the NALP3 inflammasome directly in vitro, in agreement with two recent reports showing IL-1β secretion from alum-treated DCs [14, 24, 28]. We have recently found that alum also induces high level production of uric acid in vivo and that this increased level of uric acid was required for the infiltration of inflammatory cells [19]. Although how this is done and which cells generate or release uric acid upon alum administration is an open question, it suggests that the increased level of uric acid leads to an amplification of NALP3 inflammasome activation and, thus, IL-1β. Interestingly, uric acid was identified not only as one of the most potent danger signals released from dying cells, but also as an excellent adjuvant [29]. Thus we propose NALP3 as the newest member of a list

of innate receptors that could be exploited for the design of new adjuvants.

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Chapter 4

MSU Crystals induce Th2 Immunization independent of the NALP3 Inflammasome

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Recent studies have shown that uric acid is an endogenous danger signal released from dying cells. Also, during aluminum adjuvant administration, which is used to induce Th2 responses, uric acid is released at the site of injection. Prior work has shown that uric acid crystals (MSU crystals) activate immune effectors of both the innate and adaptive immune system, including neutrophils and CD8+ T cell immunity. However, it was unclear whether MSU crystals were involved in CD4+ Th2 cell immunity. Here we show that when uric acid is degraded using uricase during aluminum adjuvant immunization, all Th2 asthma characteristics are abolished. In addition, MSU crystals injected with antigen could induce Th2 responses. MSU crystals have been shown to activate the NALP3 inflammasome, but surprisingly the induction of Th2 responses by MSU crystals did not depend on the NALP3 inflammasome. In a more relevant asthma model using house dust mite as an antigen, uric acid is released during immunization and when degraded with uricase all Th2 asthma features are diminished. In conclusion, uric acid does not only stimulate the immune system for a proper antitumor response, but can also sensitize for Th2 responses. Thereby, targeting the breakdown of uric acid in asthmatic patients could be a therapeutic strategy in future.

Sensing the presence of pathogens or allergens is the first step in mounting an effective immune response. The wide expression of pattern recognition receptors (PRRs) on innate immune cells and other structural cells, including epithelial cells, is important to allow cells to rapidly respond to antigen exposure. Toll-like receptors (TLRs) were highlighted as the key recognition structures of the innate immune system in the past few years [1]. TLRs are capable of sensing organisms ranging from bacteria to fungi, protozoa, and viruses. Some allergens with a protease activity, such as house dust mite (HDM) extracts [2] can be recognized by protease-activated receptors (PAR1–PAR4) [2, 3], but can also trigger TLR signaling [4]. However, host recognition and response is not restricted to TLRs. A second main group of innate PRRs is the Nod-like receptor (NLR) family. These receptors are located in the cytoplasm and include proteins such as NOD1 (nucleotide-binding oligomerization domain 1), NOD2, NALPs (NACHT-, LRR- and pyrin-domain-containing proteins), IPAF (IL-1β converting enzyme-protease activating factor), and NAIPs (neuronal apoptosis inhibitor proteins) [5]. The intracellular localization of NLRs allows the detection of intracellular pathogens, TLR ligands, such as flagellin and peptidoglycan [6, 7], but also endogenous danger signals released from activated or dying cells. The recognition of ligands by NLRs can lead to the activation of caspase-1 through the assembly of a cytosolic protein complex that is known as the inflammasome. Caspase-1 is required for the processing and the subsequent release of active pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 [8-10]. IL-33, a cytokine that is involved in generating a T helper 2 (Th2)-cell response, is also cleaved by caspase-1 [11]. Therefore, antigen recognition by NLRs might influence the outcome of the immune response. Notably, polymorphisms in

the NLR family have also been linked to Th2 diseases, like asthma and atopic dermatitis [12-14]. Although endogenous danger signals can signal via NLRs, they can also act independently of PRRs. For instance, ATP activates dendritic cells to induce Th2-associated responses [15] most likely through the purinergic receptor P2X7 as already reported for other cell types [16, 17]. Recently, EDN (eosinophil-derived neurotoxin), an endogenous factor released by eosinophils has been shown to promote and/or enhance Th2 responses via the activation of TLR2 [18].

In the present study, we investigated the role of the endogenous danger signal uric acid in the onset of Th2 sensitization. We have previously shown that uric acid is released after injection with aluminum hydroxide adjuvant [19]. Here, we show that the release of uric acid during priming is essential to develop strong Th2 responses. Furthermore, a Th2 sensitization can be achieved by injecting mice uric acid crystals either i.p. or directly into the airways.

Material and Methods

Mice

BALB/c mice and C57Bl/6 mice (6–9 wks old) were purchased from Harlan (Zeist, The Netherlands). OVA-TCR Tg mice (DO11.10, BALB/c background) were bred at Erasmus University Medical Centre. NALP3$^{-/-}$ and WT C57Bl/6 mice (6-10 wks old) were bred at University Hospital Gent (Gent, Belgium). All experiments were approved by the animal ethics committee at Gent University and at Erasmus University Medical Center.

Alum sensitization

Ovalbumin (OVA) was purchased from Worthington Biochemical Corp (Lakewood, NJ). The endotoxin level of OVA measured by a limulus-amebocyte lysate assay (Biowhittaker, Verviers, Belgium) was <0.001 μg/ml at the dosage used in our experiments. Imject-alum (Pierce Biochemicals) was mixed at a 1:20 ratio with a solution of OVA antigen in saline followed by stirring for at least 1 hr. For immunization of BALB/c mice, 500 μl of Imject-alum suspension (1 mg) containing 10 μg of OVA (OVA-alum) was injected i.p. or alternatively 10 μg of OVA in 500 μl saline was injected. Mice were boosted with OVA (10 μg/500 μl) on day 7 and challenged with OVA aerosols (1% for 30 min; Sigma, grade III) on day 17-19. 24 hrs after the last OVA exposure, blood, bronchoalveolar lavage (BAL), mediastinal LN, and lungs were taken. Serum was acquired from the blood by centrifugation and stored until further analysis at -20°C. BAL cells were stained as described previously [20] and BAL supernatant was stored until further analysis. Lungs were frozen and 5-μm sections were prepared and stained with periodic acid to visualize goblet cell hyperplasia.

To assess the role of uric acid during alum sensitization, mice were treated 5 min before injection of OVA-alum with 50U of uricase i.p. (Sigma).

Monosodium urate crystal preparation

MSU crystals were prepared as described [21]. Briefly, saturated uric acid (5 mg/ml, Sigma) in 0.1M borate buffer (pH 8.5) was incubated at room temperature for 48 hrs. After harvesting the crystals, they were washed with alcohol and acetone and air-dried for at least 2 days.

MSU crystal sensitization

For immunization with MSU crystals, different amounts of MSU crystals (1-7.5mg in 500 μl) mixed with 10 μg of OVA or 10 μg of OVA in 500 μl PBS was injected i.p.. 24 hrs after injection, mice were sacrificed and peritoneal lavage and mediastinal LN were isolated.

In experiments where asthma was induced, mice were boosted with OVA on day 7 and challenged with OVA aerosols on day 17-19. 24 hrs after the last OVA exposure, blood, BAL, mediastinal LN, and lungs were taken.

To assess the role of uric acid during sensitization, mice were treated 5 min before injection of OVA/MSU (2.5mg) with 50U of uricase (Sigma).

For mucosal immunization, MSU crystals were instilled intratracheally (500 μg in 80 μl) together with 100 μg of OVA (grade V, Sigma) on day 0. Mice were challenged with OVA aerosols (1% for 30 min) on day 10-12. 24 hrs after the last OVA exposure, blood, BAL, mediastinal LN, and

lungs were harvested.

House dust mite sensitization

C57Bl/6 mice were immunized with 40 µl house dust mite ((HDM) 10 µg, Greer Laboratories) intranasally (i.n.) under isoflurane anesthesia on day 0. 24 hrs later BAL fluid was taken to asses the amount of uric acid (Amplex Red Uric Acid/Uricase assay, Invitrogen).

To investigate the role of uric acid during immunization, mice were treated i.n. with 10U uricase. To induce asthma, mice were subsequently challenged from day 7-9 with 40 µl HDM (10 µg) i.n. under isoflurane anesthesia. On day 11, eosinophilic airway inflammation and bronchial hyper-responsiveness was assessed.

Effector cytokine production

MLN cells (200,000 cells/well) were resuspended in culture medium in 96-well plates with 100 µg/ml OVA (Worthington). Four days later, supernatants were harvested and analyzed for the presence of cytokines by ELISA (IL-4 and IL-5 from eBioscience, IL-10 and IFNγ from BD Biosciences, IL-13 from R&D Systems)

Determination of OVA-specific immunoglobulins

Serum samples of mice exposed to OVA aerosols were measured for OVA-specific IgG1, IgE, and IgG2a with sandwich ELISA.

Determination of broncho-hyperresponsiveness

Twenty-four hrs after the last aerosol (alum sensitization) or 48 hrs after the last HDM instillation, invasive broncho-hyperresponsiveness was determined.

To measure dynamic resistance and compliance, mice were anesthetized with urethane (Sigma), tracheotomized with an 18-gauge catheter, paralyzed using 100 µl of 0.125% D-tubocurarine i.v. (Sigma), followed by mechanical ventilation with a Flexivent apparatus (SCIREQ). Respiratory frequency was set at 120 breaths/min with a tidal volume of 0.2 ml, and a positive end-expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of metacholine (0–600 µg/kg BW, Sigma) were administered via a catheter in the jugular vein. Dynamic resistance (R) and compliance (C) was recorded after a standardized inhalation maneuver given every 10 seconds for 3 minutes.

Flow cytometric analysis

For innate response, antigen uptake, and DC migration studies, 10 µg of OVA-Alexa Fluor 647 (OVA-AF647; Molecular Probes) was mixed with MSU crystals or not, injected i.p. or i.t., and detected 24-36 hrs later in the peritoneal cavity, mediastinal lymph nodes, or lungs. Peritoneal lavage and MLN cells were stained for nine color flow cytometry using living (DAPI-negative), Ly6C-FITC, CD86-PE, CD11c-PE-Texas Red, MHC II-PE-Cy5.5, Ly6G-PE-Cy7, CD11b-AF700, F4/80-APC-AF750 (mAbs from BD Biociences and eBioscience), combined with uptake of fluorescent antigen OVA-AF647.

Phenotyping of cultured BM-derived DCs was done by staining of living (PI-negative) cells with MHC II-FITC, CD11c-APC, and in PE CD40, CD80, CD86, ICAM-1, ICOS-L, PD-L1, PD-L2 or isotype Igs (mAbs from eBioscience, BD Biosciences, and Molecular Probes).

For detection of OVA-specific T cell responses, cells were gated for living (PI-negative) lymphocytes with CD4-APC and the clonotypic anti-OVA TCR antibody KJ1-26-PE.

Acquisition of 4-color samples was done on a FacsCalibur cytometer equipped with Cellquest software (BD Biosciences), and 9-color samples were acquired on a FACS LSR II cytometer equipped with FacsDIVA software. Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

Statistical analysis

For all experiments unless stated otherwise, the difference between groups was calculated using the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Differences were considered significant when $p<0.05$.

Results

Uric acid depletion during OVA-alum sensitization inhibits experimental asthma

We have previously reported that the sensitization of mice with OVA admixed to the adjuvant alum leads to a rapid increase in the levels of uric acid in the peritoneal cavity [19]. We next wanted to address whether uric acid

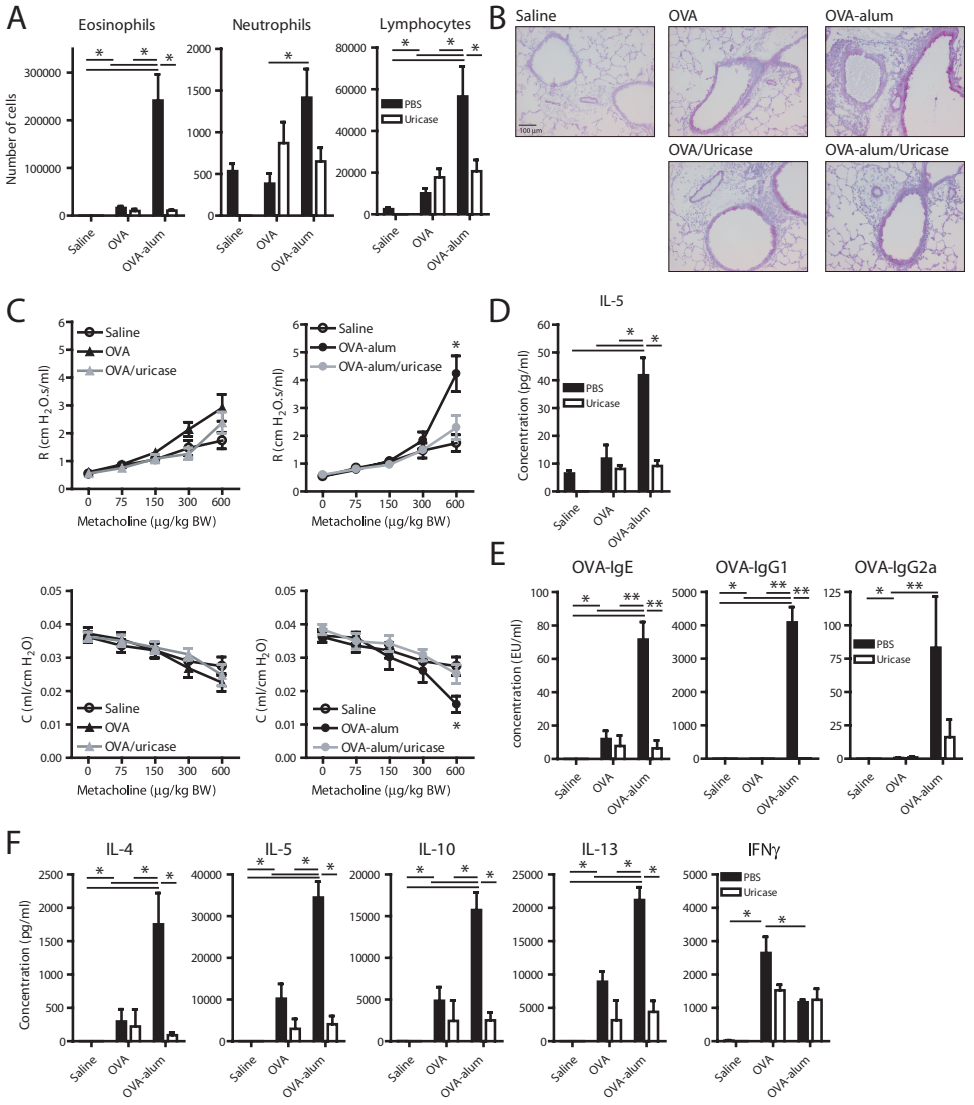

Figure 1

Degradation of uric acid by uricase during alum sensitization completely abrogated Th2 sensitization and allergic asthma induction

Mice were treated with PBS or uricase 5 minutes before sensitization with OVA or OVA-alum i.p. on day 0. On day 7 all mice were boosted with OVA i.p. and on days 17-19 challenged by OVA inhalations. On day 20 mice were sacrificed. **(A)** The cellular composition of the BAL fluid was determined by flowcytometry. **(B)** Lung sections were PAS stained to visualize goblet cell hyperplasia (pink staining). Bar: 100 µm. **(C)** Bronchial hyperresponsiveness (resistance (R) and lung compliance (C)) to metacholine was determined. **(D)** The BAL fluid supernatant was measured for IL-5. **(E)** Serum levels of OVA-specific Igs were determined with ELISA. **(F)** Cytokine levels of in vitro restimulated MLN cells. Data are shown as mean ± SEM, * $p<0.05$, ** $p<0.01$, n=4-8 mice/group. Experiments were done twice.

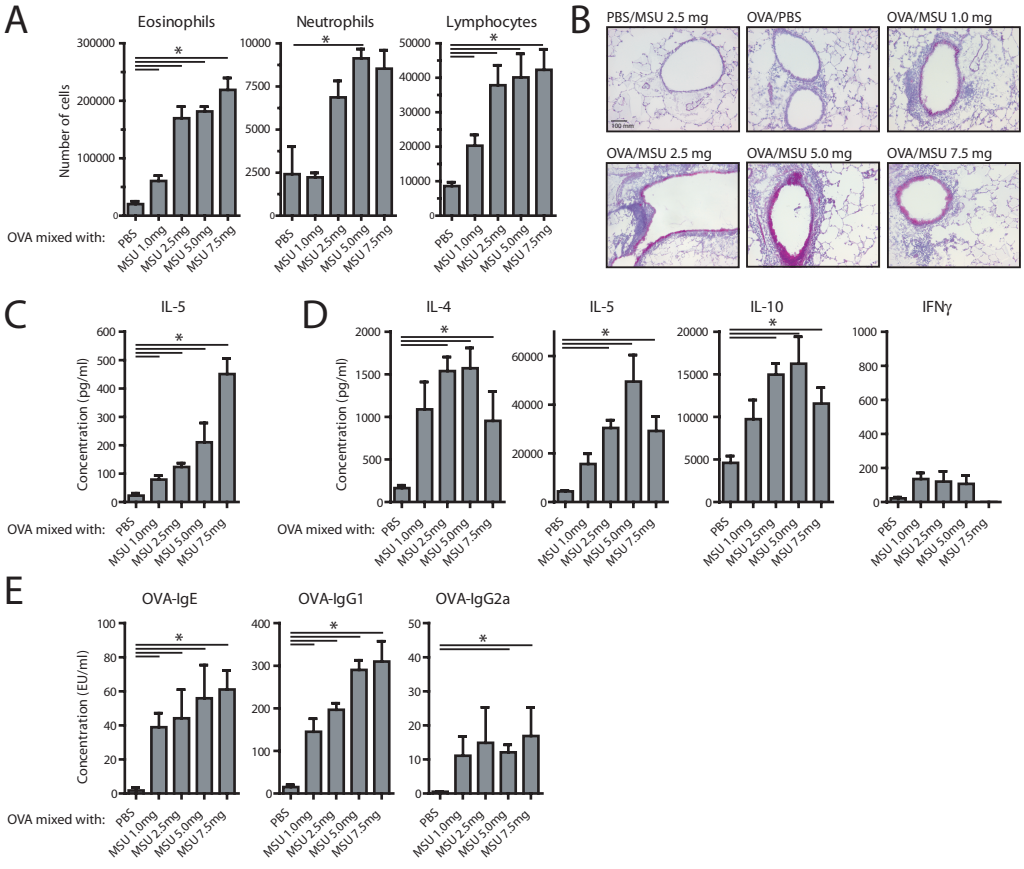

Figure 2
Intraperitoneal injection of MSU crystals mixed with OVA can induce eosinophilic airway inflammation and Th2 immunization
Mice were sensitizated with OVA or OVA mixed with different amounts of MSU crystals i.p. on day 0. On day 7 all mice were boosted with OVA i.p. and on days 17-19 challenged by OVA inhalations. On day 20 mice were sacrificed. **(A)** The cellular composition of the BAL fluid was determined by flowcytometry. **(B)** Lung sections were PAS stained to visualize goblet cell hyperplasia (pink staining). Bar: 100 µm. **(C)** The BAL fluid supernatant was measured for IL-5. **(D)** Cytokine levels of in vitro restimulated MLN cells. **(E)** Serum levels of OVA-specific Igs were determined with ELISA. Data are shown as mean ± SEM, * $p<0.05$, n=4 mice/group. Experiment was repeated twice.

contributes to Th2 sensitization using a model of experimental asthma. In these experiments, Th2 sensitization was achieved following OVA-alum administration on day 0. Mice were pretreated with PBS or uricase before the OVA-alum injection, and were challenged with OVA aerosols 10 days after the OVA boost on day 7. Mice pretreated with PBS before OVA-alum sensitization developed BAL fluid and lung tissue eosinophilia and lymphocytosis (Fig 1A and B), as well as goblet cell hyperplasia (Fig 1B). Moreover, PBS-pretreated OVA-alum sensitized mice showed a substantial Th2 cytokine production in the BAL fluids and in

mediastinal LN (MLN) cell culture (Fig 1D and 1F), associated with high amounts of Th2-associated IgE and IgG1 in the serum (Fig 1E). The pretreatment with uricase before OVA-alum sensitization prevented the development of eosinophilic airway inflammation (Fig 1A and B), strongly reduced the levels of Th2 cytokines in the BAL fluid and in MLN cell culture, as well as the levels of IgE and IgG1 in the serum compared to PBS-pretreated OVA-alum sensitized mice (Fig 1D, F). IFNγ production in MLN cultures was not affected by the uricase treatment (Fig 1F). Notably, the uricase pretreatment of OVA-sensitized mice did not significantly affect any of the features studies compared to PBS-pretreated OVA-sensitized animals.

One of the key features of allergic asthma is the development of bronchial hyperresponsiveness (BHR) in response to aspecific stimuli, like metacholine. BHR was assessed using an invasive method to measure resistance and lung compliance. At the dose of 600 μg/kg BW metacholine, PBS-pretreated OVA-alum-sensitized mice developed a higher resistance and a lower compliance compared to mice sensitized with saline or OVA (Fig 1C). When mice were pretreated with uricase before the sensitization with OVA-alum, the degree of bronchoconstriction was similar to the one observed in mice sensitized with PBS.

These data indicate that the degradation of uric acid at the time of antigen sensitization prevents the development of asthma features.

MSU crystals induce Th2 sensitization and lead to asthma development

We next addressed whether uric acid might be directly involved in the induction of Th2 responses by administering monosodium urate (MSU) crystals to mice at the time of sensitization. OVA was mixed with different amounts of MSU crystals or PBS and injected i.p. on day 0. The mice received a boost with OVA on day 7 and were challenged with OVA aerosols 10 days later. 24 hrs after the last aerosol the mice were sacrificed.

Mice immunized with 2 injections of OVA admixed to PBS developed a very mild inflammation, as indicated by the small numbers of eosinophils and lymphocytes in the BAL fluid (Fig 2A). Mice injected with MSU crystals mixed with OVA showed an increase in the number of eosinophils, neutrophils, and lymphocytes in the BAL compared to mice injected with OVA and PBS. This response was dependent on the dose of MSU crystals injected and reached its maximum with the dose of 2.5 mg of MSU crystals (Fig 2A). Also, when lung histology was examined of mice sensitized with MSU crystals, they developed more goblet cell hyperplasia then mice sensitized only with OVA (Fig 2B). Moreover, the amount of IL-5 in the BAL fluid increased dose-dependent to the amount of MSU crystals (Fig 2C). Likewise, the cytokine response measured in the MLN also shown an induction in the Th2 cytokines, like IL-4, IL-5, IL-10 but almost no induction of the Th1 cytokine IFNγ could be observed (Fig 2D). In addition, the Th2-associated immunoglobulins OVA-specific IgE and IgG1 were also induced when OVA was mixed with different amounts of MSU crystals (Fig 2E).

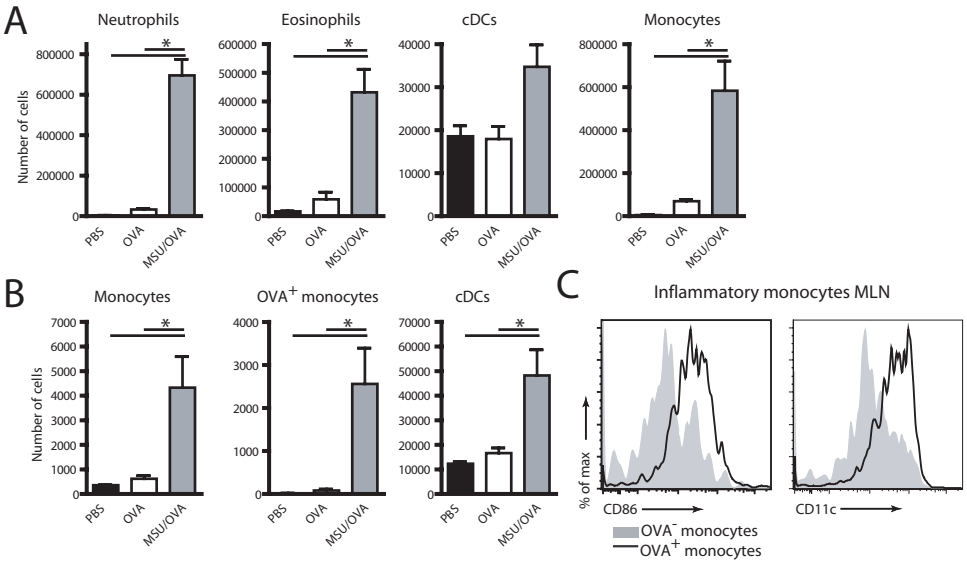

Figure 3

MSU crystals induce the recruitment of eosinophils and inflammatory monocytes

Mice were injected with OVA-Alexa Fluor 647 (OVA-AF647) or OVA-AF647 mixed with 2.5 mg MSU crystals and 24 hrs later the peritoneal lavage and draining LN (MLN) were taken. **(A)** The number of neutrophils ($CD11b^+Ly6C^+Ly6G^{high}F4/80^-$), and eosinophils ($CD11b^+Ly6C^{int}Ly6G^{int}F4/80^{int}$), conventional DCs ($MHCII^{high}CD11c^+F4/80^{low}$), and inflammatory monocytes ($CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$) was determined in the peritoneal lavage. **(B)** The number of inflammatory monocytes ($CD11b^+Ly6C^{high}Ly6G^-F4/80^{int}$), OVA^+ monocytes, and cDCs ($MHCII^{high}CD11c^+F4/80^{low}$) in the MLN was determined by flowcytometry. Data are shown as mean ± SEM, * $p<0.05$, n=4 mice/group. **(C)** Expression of CD11c, MHC II and CD86 on inflammatory monocytes determined by 9-color flow cytometry. Grey filled histograms represent the OVA-AF647-negative monocytes, whereas the black line histogram represents the OVA-AF647-positive ones. An example is shown of 4 mice.

MSU crystals promote Th2 sensitization by inducing influx of inflammatory monocytes

Having shown that MSU crystals promote Th2 responses, we next turned our attention to the peritoneum and looked at the innate immune cells recruited following MSU administration. By analogy with other adjuvants such as alum, it is possible that MSU crystals are immunogenic because of its induction of inflammation at the site of injection, thus recruiting APCs to the site of Ag exposure [19]. As shown in figure 3A, the injection of MSU/OVA i.p. induced a significant influx of neutrophils to the peritoneum compared to mice injected with OVA alone (Fig 3A). Furthermore, the number of eosinophils, DCs, and $Ly6C^{high}$ inflammatory monocytes was significantly increased in MSU/OVA-injected mice compared to OVA-injected mice. In the draining mediastinal LN of mice injected with MSU/OVA, a marked increase in the number inflammatory monocytes loaded with fluorescent OVA was observed (Fig 3B). This increased number of OVA^+ monocytes was absent in mice injected with OVA. Also, the number of $Ly6C^-$ cDCs was increased following the injection of MSU/OVA crystals compared to OVA alone. The OVA^+ monocytes that arrived in the MLN after MSU/OVA injection were more mature than OVA^- monocytes,

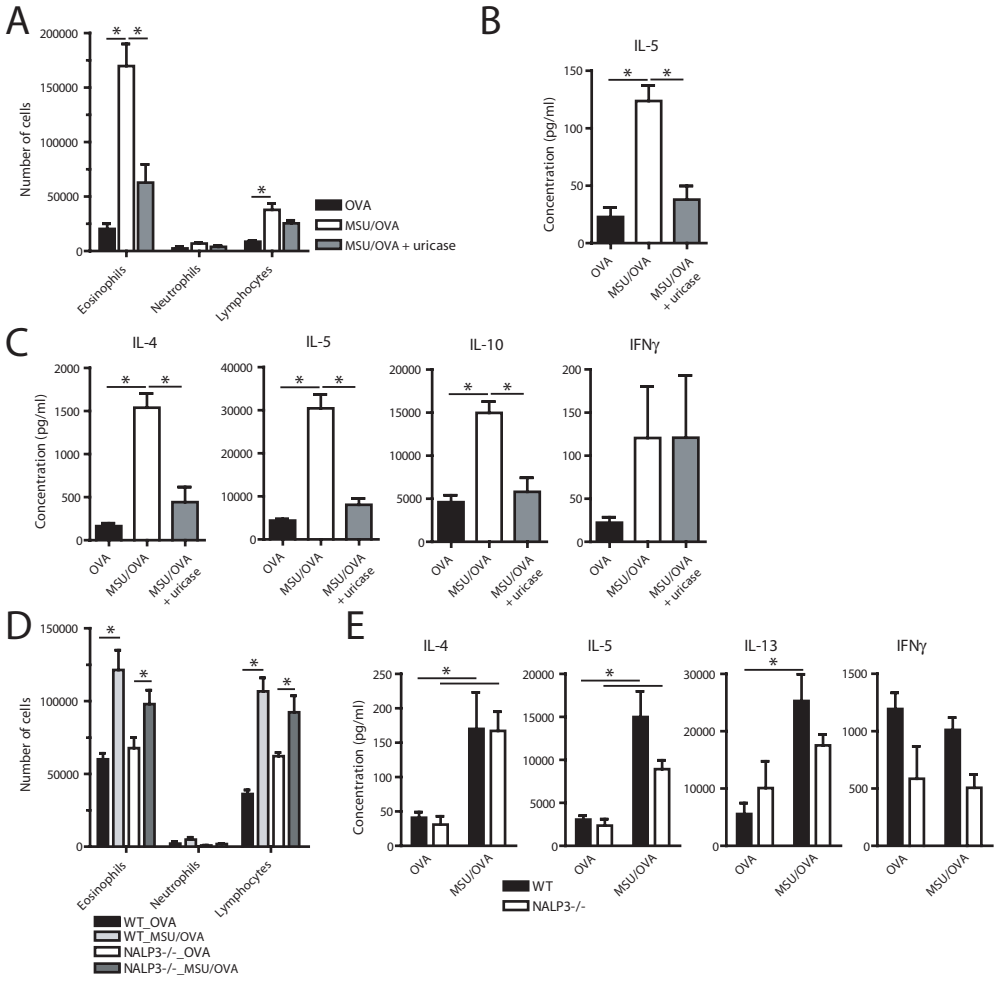

Figure 4

Th2 immunization by MSU crystals can be blocked by uricase treatment, but is NOT absent in NALP3$^{-/-}$ mice

BALB/c mice were treated with PBS or uricase 5 minutes before sensitization with OVA or OVA mixed with 2.5 mg MSU crystals i.p. on day 0. On day 7, all mice were boosted with OVA i.p. and on days 17-19 challenged by OVA aerosols. On day 20 mice were sacrificed. **(A)** The cellular composition of the BAL fluid was determined by flowcytometry. **(B)** The BAL fluid supernatant was measured for IL-5. **(C)** Cytokine levels of in vitro restimulated MLN cells. **(D)** WT (C57Bl/6) or NALP3$^{-/-}$ mice were sensitized with OVA or with 2.5 mg MSU crystals mixed with OVA mixed i.p. on day 0. On day 7, all mice were boosted with OVA i.p. and on days 17-19 challenged by OVA inhalations. On day 20 mice were sacrificed. The cellular composition of the BAL fluid was determined by flowcytometry. **(E)** Cytokine levels of in vitro restimulated MLN cells. Data are shown as mean ± SEM, * $p<0.05$, n=4 mice/group.

as they expressed higher levels of CD86 and CD11c (Fig 3C).

Sensitization induced by MSU crystals is blocked with uricase pretreatment

We have shown that MSU crystals, injected i.p. into mice, promoted Th2 responses in the airways (Fig 1). We next investigated whether MSU-induced Th2 sensitization was due to a direct effect of the crystals. To address this,

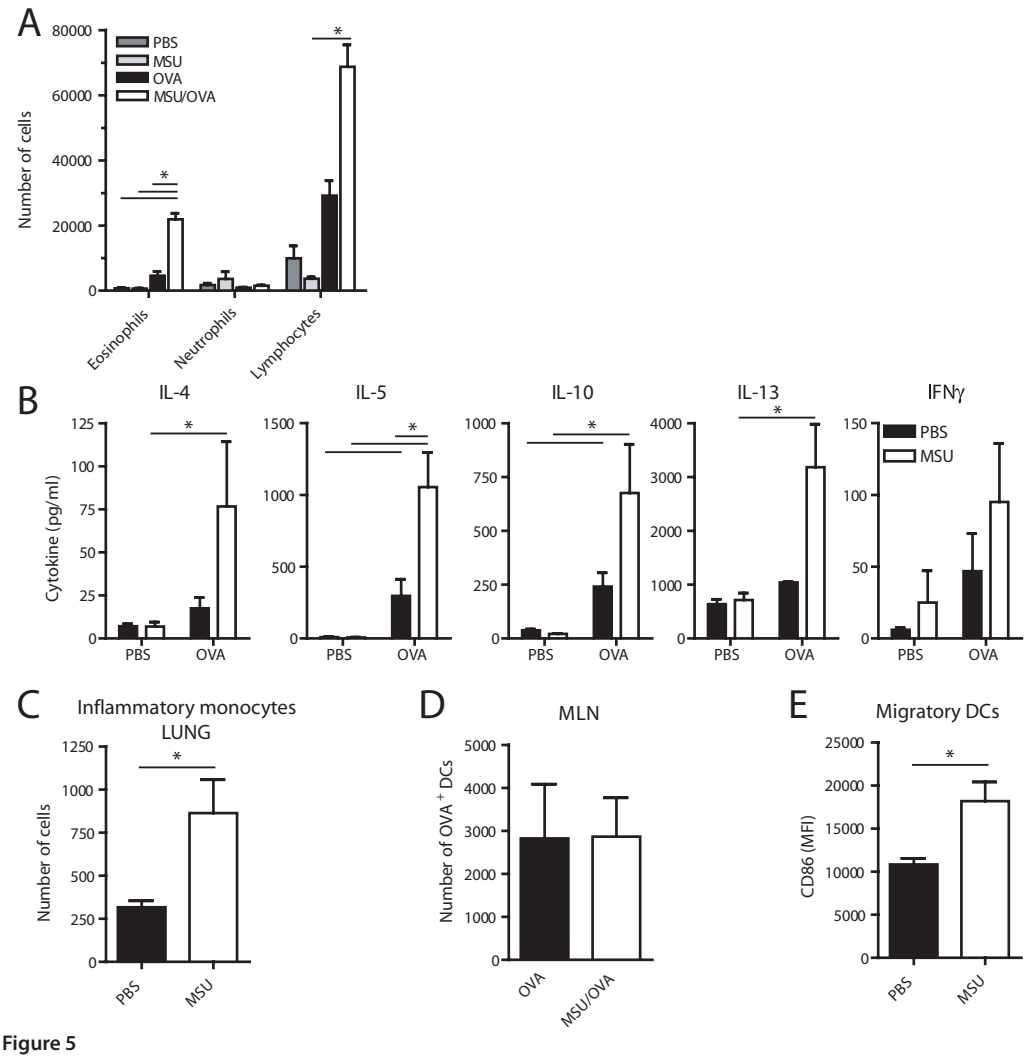

Figure 5

MSU crystals in the lung break tolerance to OVA

Mice were sensitized with OVA or OVA mixed with 500 μg MSU crystals i.t. on day 0. On days 10-12, all mice were challenged by OVA inhalations. Mice were sacrificed on 24 hrs later. **(A)** The cellular composition of the BAL fluid was determined by flowcytometry. **(B)** Cytokine levels of in vitro restimulated MLN cells. **(C)** Number of inflammatory monocytes present in the lung 36 hrs after PBS or MSU instillation. **(D)** OVA^+ DCs in the MLN 36 hrs after instillation of OVA-AF647 or MSU/OVA-AF647. **(E)** CD86 expression on migratory DCs (MHC II high) in the MLN 36 hrs after PBS or MSU (500 μg) instillation. Data are shown as mean ± SEM, * $p<0.05$, n=4 mice/group. Experiment was done twice.

mice were given uricase i.p. 5 minutes prior to the injection of MSU crystals (2.5 mg) admixed with OVA. Mice receiving MSU/OVA showed an increase in the number of eosinophils and in the levels of IL-5 in the BAL compared to the control mice injected with PBS (Fig 4A and B). The pretreatment of MSU/OVA-injected mice with uricase dramatically reduced the number of eosinophils and the levels of IL-5 (Fig 4A and B). Notably, the reduced Th2

response in the airways of uricase-pretreated animals was supported by a substantially lower Th2 cytokine production in MLN cell cultures compared to MSU/OVA-injected mice (Fig 4C). To address whether the effect of uricase was specific, we pretreated mice with uricase i.p. 5 minutes before inducing sensitization by the i.p. administration of OVA-pulsed DCs. In this experiment, the OVA-DC-induced eosinophilic airway inflammation and the induction of Th2 cytokines was not affected by the pretreatment with uricase (data not shown).

Sensitization induced by MSU crystals is not dependent on NALP3 inflammasome

MSU crystals have been shown to recruit innate immune cells such as neutrophils through the activation of the NALP3 inflammasome. We next sensitized NALP3 deficient mice with MSU/OVA and examined the degree of inflammation. NALP3 deficient mice sensitized with MSU/OVA showed an increased eosinophilia and lymphocytosis in the BAL fluid that was comparable to MSU/OVA-injected WT mice (Fig 4D). Notably, the levels of IL-4 and IL-5 in the MLN cultures were increased in NALP3$^{-/-}$ and WT mice subjected to MSU/OVA compared to animals injected with PBS/OVA (Fig 4E).

MSU crystals promote Th2 sensitization when directly administered in the lungs

As uric acid/MSU crystals can act as danger signal [22, 23] and can induce Th2 responses upon i.p injection (Fig 1), we next investigated whether a local exposure (e.g. in the airways) to MSU crystals would be sufficient to induce sensitization. To investigate this, mice were injected with OVA intratracheally and challenged 10 days later with OVA aerosols. We have previously shown that this protocol does not lead to the development of airway inflammation but rather induces tolerance [24]. Mice received MSU crystals or PBS, as a control, at the time of the i.t administration of OVA. Mice sensitized with PBS/OVA showed a low number of eosinophils and lymphocytes in the BAL fluid (Fig 5A). However, when MSU crystals were co-administered with OVA, a significant increase in the number of eosinophils and lymphocytes was observed. The levels of IL-4, IL-5, IL-10, and IL-13 were substantially increased in the mediastinal LN cell cultures of mice sensitized with MSU/OVA compared to mice injected with PBS/OVA (Fig 5C).

To better understand how MSU crystals can break inhalation tolerance and promote Th2 sensitization when given locally in the airways, we investigated the changes induced early (i.e. 36 hrs) after the administration of MSU crystals, and looked at the presence of inflammatory cells from the innate immune system in the lungs. In the lung, MSU crystals induced an increase in the number of inflammatory monocytes, direct precursors of DCs under inflammatory conditions (Fig 5D). Increased Th2 sensitization by MSU/OVA did not depend on increased DC migration (Fig 5E), as equal amount of OVA^{+} DCs arrived in the MLN after i.t administration. However, the

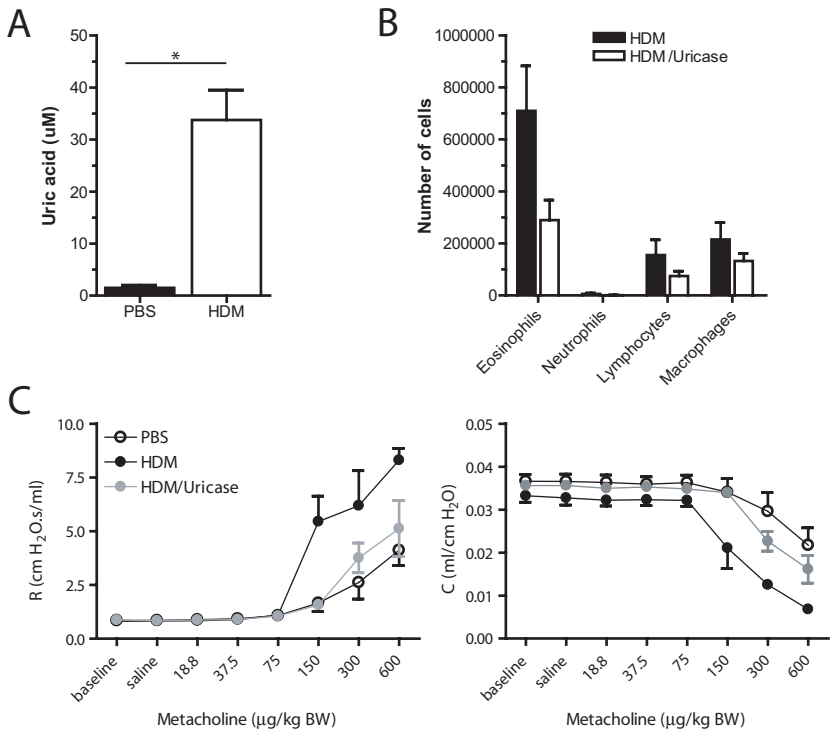

Figure 6

Uric acid plays a key role in the sensitization with house dust mite

(A) Mice were sensitized with HDM or PBS and uric acid levels were determined 24 hrs later in the BAL fluid. **(B)** Mice were sensitized on day 0 with PBS, HDM, or HDM/Uricase and challenged with HDM from day 7-9. On day 11, the BAL fluid was examined with flowcytometry. **(C)** On day 11, BHR of the mice (resistance (R) and lung compliance (C)) to metacholine was determined.

DCs that did arrive expressed more CD86 (Fig 5F).

House dust mite sensitization depends on the release of uric acid

To investigate if uric acid played a role in a more relevant allergic asthma model, we examined the release of uric acid in the BAL fluid of house dust mite (HDM) sensitized mice. 24 hrs after HDM instillation, an increase in the levels of uric acid could be observed in HDM-treated mice when compared to PBS-treated mice (Fig 6A). To examine if uric acid was necessary to mount a proper Th2 response in response to HDM, mice were treated with the uric acid degrading enzyme, uricase, at the time of sensitization. When HDM-sensitized mice were subsequently challenged with HDM, an important influx of eosinophils in the BAL fluid and an increased BHR were observed (Fig 6B and C). When mice were treated with uricase at the time of sensitization, this increased eosinophilia and BHR were strongly reduced.

Discussion

For decades, alum adjuvant has been used to induce Th2 responses in experimental mice. In a previous study, we have shown that alum adjuvant induced the release of uric acid in vivo [19]. However, the direct involvement of uric acid as the potential inducer of alum-driven Th2 responses had not been shown. Here, we report that uric acid also accumulates in the BAL fluids of mice injected with allergens, and that uric acid is necessary and sufficient to promote Th2 responses in vivo when administered i.p or locally in the airways. This allergen-induced increase in uric acid levels was functionally relevant since asthma features were clearly suppressed when uric acid was degraded using uricase.

The exact cellular source of allergen-induced uric acid in vivo is still unknown but it has been reported that uric acid can be released from stressed and injured cells [22]. Although it is known that adjuvants such as alum, or environmental triggers such as ozone, air pollutants and respiratory infections induce high levels of uric acid likely by inducing high levels of stress in cells, we still do not know how allergens mediate this production [19, 25-28]. The excessive levels of uric acid have been associated with the development of several inflammatory diseases including gout and contact hypersensitivity [29, 30]. The levels of uric acid are controlled by uricase, an enzyme that degrades uric acid in most mammals [31]. In humans, however, uricase is absent and thus humans might have a low capacity to control increases in uric acid, thus leading to inflammatory disorders. Notably, we observed that exogenously added MSU promotes sensitization to inhaled antigen. Inhalation of a harmless protein antigen such as ovalbumin normally is a tolerogenic event [24], which can be turned into robust Th2 priming by adding as little as 100 ng of endotoxin, thus triggering cDC maturation [32]. Intrapulmonary application of MSU together with OVA in naive mice similarly potentiated the Th2 response to subsequent OVA challenge, suggesting that uric acid might work as an adjuvant to enhance the immune response, like endotoxin. The adjuvant properties of uric acid are in keeping with the hypothesis that it can function as danger signals that alert the immune system to tissue damage [22, 23].

Since uric acid promotes Th2 responses in vitro, trying to reduce the allergen-induced levels of uric acid might be a good approach to limit Th2 responses. Here, we show that the pretreatment of mice with uricase before sensitization with OVA-alum or HDM strongly reduced the number of innate immune cells recruited to the peritoneum and the subsequent development of asthma features. The exact mechanism by which uric acid neutralization reduces recruitment of inflammatory cells requires further study, but uricase might likely prevent the production of several pro-inflammatory chemokines. Indeed, MSU promote the early recruitment of neutrophils, eosinophils and inflammatory monocytes (Fig 2, [33]). In addition, it has been reported that uric acid can directly activate monocytes and neutrophils to produce chemotactic factors that recruit other inflammatory cells, and in

this way contribute to the amplification of the inflammatory response induced by MSU crystals [34, 35]. Although we did not formally show it, it is tempting to speculate that uricase would control the reactivity of inflammatory cells to the relevant chemokines, and in this way limit the extent of Th2 sensitization. There are also other possible scenarios to explain why inflammation is reduced when uric acid is blocked, one of them being alteration of DC function. DCs are crucial for asthmatic inflammation because they recruit Th2 lymphocytes to the airway wall and trigger local Th2 effector cytokine production [36, 37]. MSU crystals have been reported to directly activate DCs in vitro and in vivo by increasing the expression of co-stimulatory molecules, and to promote strong DC-induced T cell response [30, 38]. Some of these molecules (CD80 and CD86) are necessary to drive Th2 differentiation [39]. Alternatively, it has been suggested that MSU crystals activate caspase-1 and lead to the release of IL-1β and IL-18 through the activation of the NALP3 inflammasome [11, 21, 40]. However, although NALP3 deficient mice showed a small reduction in the degree of eosinophilia, some features of Th2 responses such as the antibody production remained intact [38], suggesting a NALP3 independent pathway used by uric acid. Our data are in line with this possibility since Th2 sensitization by MSU crystals only partially depended on NALP3 inflammasome activation (Fig 4). Other pathways by which MSU crystals can activate the immune system have also been shown. Liu-Bryan et al. showed that the uptake of MSU crystals could be mediated by the pattern recognition receptors CD14, TLR2, and TLR4 [41, 42]. Notably, we previously showed that some effects induced by alum adjuvant administration were abolished in MyD88$^{-/-}$ mice [19]. Although we did not formally address this point, we would like to envision the activation of this pathway by alum-induced uric acid.

In summary, we have identified uric acid as an endogenous danger signals that can promote and contribute to the maintenance of Th2 responses in the lung. The neutralization of uric acid might be of interest to suppress asthmatic inflammation.

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Chapter 5

Introduction II

Dendritic Cells in Asthma and COPD: Opportunities for Drug Development

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The lung contains many subsets of dendritic cells that are distributed in various anatomical compartments. In homeostatic conditions, a fine-tuned balance exists between plasmacytoid and myeloid dendritic cells necessary for maintaining tolerance to inhaled antigen and avoiding overt inflammation. These subsets of DCs also play important roles in establishment of airway inflammation seen in asthma and chronic obstructive pulmonary disease. Based on these new insights on airway DC biology, several approaches that interfere with DC function show potential as new intervention strategies for these ever increasing diseases.

Obstructive airway disease, broadly divided clinically into asthma or chronic obstructive pulmonary disease (COPD) are a significant cause of morbidity and mortality. In allergic asthma, allergen-specific T helper type 2 (Th2) cells produce key cytokines like IL-4, IL-5 and IL-13 that regulate the synthesis of allergen-specific IgE and control tissue eosinophilic airway inflammation and remodeling of the airways. In the lungs of COPD patients, predominantly CD8 lymphocytes and neutrophilic airway inflammation are seen, concomittant with remodeling of small airways and destruction of distal air spaces (called emphysema). It is increasingly clear that dendritic cells (DCs) are essential for inducing activation and differentiation of not only naive but also effector $CD4^+$ T and $CD8^+$ T cells in response to inhaled antigen, and it has been well established that these cells play a pivotal role in the initiation and maintenance phase of airway inflammation [1]. In this review, we will highlight the recent discoveries in airway DC biology with special emphasis on mouse models of asthma and COPD. Where possible, the applicability to the human situation and the therapeutic potential of novel findings will be discussed.

Lung dendritic cell subsets

It has long been established that the various lung compartments (conducting airways, lung parenchyma, alveolar compartment, pleura) contain numerous DCs, of which the precise lineage or origin have been poorly defined. Recently however, many groups have refined the ways in which lung DCs should be studied, both in mouse [2-5] and man [6, 7]. It is clear that different DC subsets can be found in the lung, each with functional specialization. In the mouse, all of these express the integrin CD11c and subsets are further defined based on the expression of the myeloid marker CD11b, as well as anatomical location in the lung. The trachea and large conducting airways have a well developed network of intraepithelial DCs, even in steady state conditions. These cells in some way resemble skin Langerhans' cells, and have been shown to express langerin and CD103 while lacking expression of CD11b [4, 8]. In the submucosa of the conducting airways, $CD103^-CD11b^+CD11c^+$ myeloid DCs can be found particularly under conditions of inflammation, and these cells are particularly suited for priming and restimulating effector $CD4^+$ T cells in the lung [8, 9]. The lung interstitium that is accessible by enzymatic digestion also contains $CD11b^+$ and $CD11b^-$ DCs that access the alveolar lumen and migrate to

the MLN [2, 5, 10]. It should be noted that this population is however contaminated with DCs lining the small intrapulmonary bronchioles as well as those lining the vessel walls. In the nearby alveolar lumen, $CD11c^{high}$ alveolar macrophages control the function of these interstitial DCs. Plasmacytoid DCs are $CD11b^{-}CD11c^{int}$ cells expressing SiglecH and bone marrow stromal cell Ag-2 (recognized by the moAbs mPDCA-1 or 120G8). In the lungs, pDCs are predominantly found in the lung interstitium and produce large amounts of IFNα in response to triggering by CpG motifs or viral infection ex vivo [11]. How, where, and by which DC subset inhaled antigen is sampled from the airway lumen has been a matter of debate. Jahnsen demonstrated that, analogous to that reported in the gut, a subset of rat airway intraepithelial DCs extend their processes into the airway lumen. This 'periscope up' function is constitutively expressed within the airway mucosal DC population, providing a mechanism for continuous immune surveillance of the airway luminal surface in the absence of 'danger' signals [12]. In the mouse, $CD103^{+}CD11b^{-}$ intraepithelial DCs express the tight junction proteins claudin-1, claudin-7, and zonula-2, allowing the sampling of airway luminal contents while keeping the epithelial barrier function intact [4]. This subset is also found in the alveolar septa and DCs lining the alveolar wall can take up inhaled harmless ovalbumin or bacterial Anthrax spores by forming intra-alveolar extensions and migrate to the mediastinal LN in a CCR7-dependent way [5, 8, 10]. It is still a matter of debate however whether the uptake and transport of inhaled antigen occurs exclusively by alveolar wall DCs, by intraepithelial DCs lining the large conducting airways, or by both [2, 5]. Another controversial issue is the location and extent by which plasmacytoid DCs take up inhaled antigen. Two reports describe that within 24-48 hrs following exposure of inhaled fluorescently labeled Ag almost 50-60% of pDCs are antigen positive [11, 13], whereas another report saw only a minor percentage of Ag uptake in this subset [5]. It remains to be demonstrated if pDCs take up antigen in the periphery of the lung and subsequently migrate, whether they get their antigen from another migratory DC [14], or whether they take up free afferent lymph while resident in the LN. How much antigen crosses the epithelial barrier passively in the absence of DC uptake is unknown, but heavily depends on the molecular weight of the Ag, its dose, as well as the potential to disrupt epithelial tight junctions. Control of epithelial barrier function could be under important genetic control as well, as many of the gene polymorphisms associated with atopy in humans control epithelial integrity (e.g. Spink5, S100 family). It is similarly possible that Ag uptake by lung pDCs would be facilitated by the presence of Ag specific immunoglobulins acting on Fc receptors, thus enhancing endocytosis [15].

Outcome of antigen inhalation depends on the functional state of myeloid and plasmacytoid DCs

The usual outcome of inhalation of harmless protein antigen in the lungs is immunological

tolerance (see figure 1 for a model depicting cellular interactions). In true sense this means that when the antigen is subsequently given to mice in an adjuvant setting (e.g. in combination with the Th2 adjuvant alum) it no longers induces an immunological response that leads to effector cells causing inflammation [11, 16]. Inhalational tolerance is mediated in part by deletion of Ag -reactive T cells as well as induction and/or expansion of regulatory T cells in the mediastinal nodes [14, 16-18]. The latter type of tolerance is dominant and can be transferred to other mice by adoptive transfer. Induction of tolerance to inhaled antigen is a function of lung DC subsets that migrate from the lung in a CCR7-dependent way [14]. It is often claimed that induction of tolerance is a function of 'immature' DCs, meaning that these cells lack the expression of high levels of MHC, adhesion and co-stimulatory molecules, However, Reis e Sousa recently argued that the term 'mature DC' should be reserved for those DCs that have the potential to generate

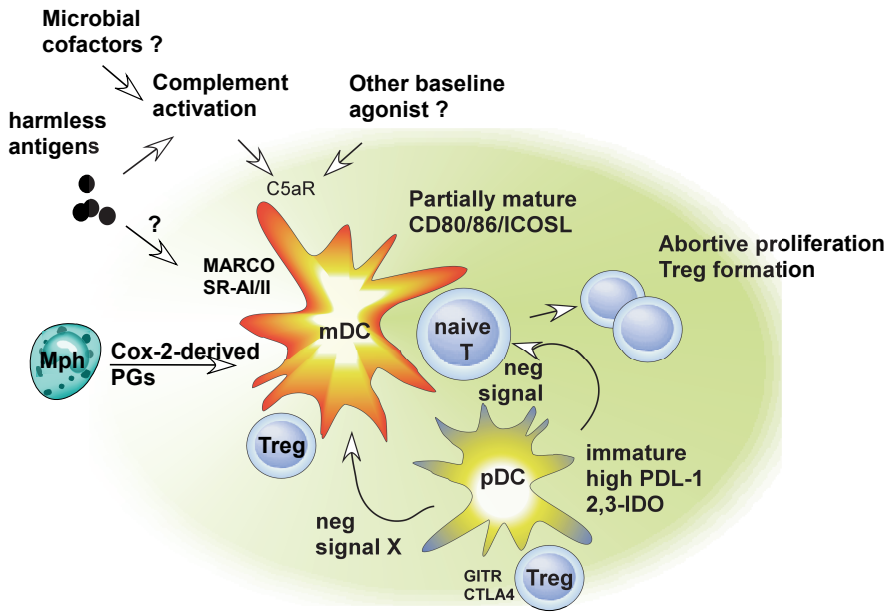

Figure 1

Dendritic cell function following inhalation of harmless antigen in homeostatic conditions

When an inhaled antigen reaches the deeper airways and it is not cleared by mucociliary transport or by macrophages, it will be taken up by airway dendritic cells. Both mDCs and pDCs take up antigen. mDCs seem to do this in the periphery, whereas pDCs only do this in the mediastinal node. In baseline conditions, the mDCs that reach the nodes are only partly "mature" and the T cell response that they induce is characterized mainly by division, but not by differentiation to effector cells. Eventually many dividing cells die. Additionally, mDCs induce Treg cells that suppress inflammation. At the same time, the pDCs control the level of activity of the mDCs so that these cells are kept in a quiescent state. This function of pDCs is controlled by Treg control occurring via GITR-GITRL ligand and CLTA4-CD80/86 interactions. The signals involved are not precisely known but could involve indoleamine 2,3-dioxygenase (2,3-IDO), or some surface expressed ligand on pDCs (programmed death ligand-1, PDL1). The function of mDCs is constantly kept in check, amongst others by tonic inhibition by cyclooxygenase-2 (Cox-2) derived prostaglandins (PGs), as well as by complement activation acting on the C5a receptor (C5a). The precise ligand for the class A scavenger receptors (MARCO and SR-AI/II) are not known.

effector T cells, and that expression of costimulatory molecules by DCs does not exclude the possibility that tolerance would be induced [19]. In the lungs, inhaled tolerance is dependent on signals delivered by CD86 and/or ICOSL on DCs, supporting this view [17].

Conventional lung DCs (either $CD11b^+$ or $CD11b^-$) are necessary for tolerance induction [14], but are also responsible for inducing Th2 sensitization providing there is some form of activation (either LPS or TNFα)[20] leading to functional DC differentiation and their capacity to prime Th2 effector cells (see figure 2 for a model depicting cellular interactions leading to Th2 sensitization in the airways). In further support, Th2 sensitization can be induced by adoptive intratracheal transfer of GM-CSF-cultured bone marrow DCs, most closely resembling mature monocyte-derived $CD11b^+$ DCs, but not by Flt3L-cultured bone marrow derived DCs that more resemble the immature steady state DCs resident in the lymph nodes and spleen [11]. As activation of lung DCs is the common event leading to Th2 sensitization, it is likely that under homeostatic conditions, the degree of DC maturation is therefore constantly kept in check. One such pathway of tonic DC suppression seems to involve COX-2 derived prostaglandins or their metabolites, most likely derived from nearby alveolar macrophages [21]. Chimeric mice in which the PGD_2 receptor DP1 was selectively deleted on hematopoietic cells demonstrated spontaneous maturation of lung cDCs and subsequently, response to harmless antigen were greatly enhanced, suggesting tonic inhibition of DC function by PGD_2 in the lung [22]. When exposed to selective PGD2 agonists, myeloid DCs induced the formation of $Foxp3^+$ Ag specific Tregs that subsequently suppressed airway inflammation. A similar mechanism on DCs was found for stable PGI_2 analogues [23, 24].

When pDCs are depleted from the lungs, inhaled tolerance is abolished, and consequently pDC/mDC balance is the lung is tightly regulated, amongst others by the cytokine osteopontin as well as complement C5a [11, 13, 25, 26]. How exactly pDC depletion leads to sensitization is still unsolved, but in vitro and in vivo data suggest that pDCs directly suppress the potential of mDCs to generate effector T cells [11, 26]. Plasmacytoid DCs can also stimulate the formation of Treg cells, possibly in an ICOS-L dependent way [11, 27]. Tregs expressing GITR could also induce the production of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase through reverse signaling in pDCs [28]. In mice depleted of pDCs, there was endogenous release of extracellular ATP responsible for inducing mDC maturation and Th2 skewing potential. Th2 sensitization to inhaled OVA was abolished when ATP signalling was blocked using the broad spectrum P2X and P2Y receptor antagonist suramin. On the contrary, a non-degradable form of ATP was able to break inhalation tolerance to OVA [29]. How exactly purinergic receptor triggering on DCs promotes Th2 development is unclear at present, but could involve the formation of the inflammasome, a multi-protein complex that leads to activation of caspase-1 and processing and release of IL-1β, IL-18 and possibly IL-33. The conditions regulating ATP release in the lungs will have to be studied more carefully before we can conclude how important the pathway of

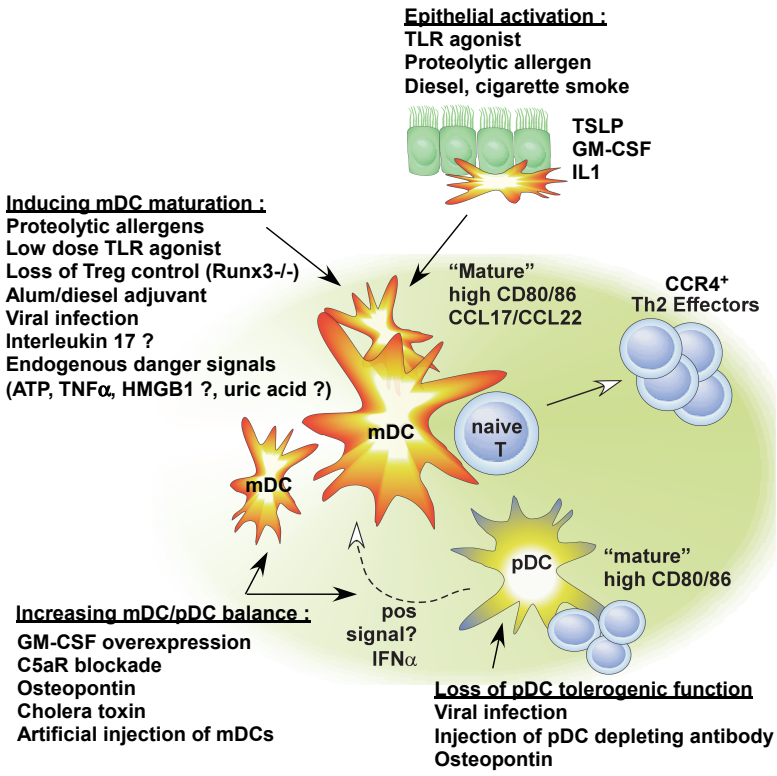

Figure 2
Model of dendritic cell function during Th2 sensitization
Several known risk factors for atopy have been shown to interfere with DC function in the airways. Also, several experimental models have been developed that have seen sensitization to occur even after inhalation of harmless antigens to the lung, providing there is some form of DC activation. In these models, respiratory tolerance is broken. Some models have induced a shift in the pDC/mDC balance, and consequently mDCs induce priming because they are no longer suppressed by adequate numbers of pDCs. Activated mDCs also produce chemokines like CCL17 or CCL22 to further attrack Th2 cells into the response. Some adjuvants induce proper activation of mDCs (yet not sufficient to induce IL-12) so that they now induce effector Th2 cells rather than regulatory T cells. Some stimuli, like concomittant viral infection, might have an additional effect by inducing maturation of pDCs and their production of IFNα. This is a know maturation stimulus for mDCs and in this way, these cells might even contribute to sensitization upon viral infections. Activation of epithelial cells by proteolytic allergens, virus infection, toll like receptor (TLR) ligands or air pollutants is an indirect way of activating and polarizing the DC network, through release of thymic stromal lymphopoeitin (TSLP) or granulocyte-macrophage colony stimulating factor (GM-CSF) or interleukin-1 (IL-1). The precise source and role of endogenous danger signals like ATP, tumor necrosis factor α (TNFα), high mobility group box 1 (HMGB1) or uric acid is currently being investigated.

purinergic signaling is in sensitization to more common allergens, like house dust mite.

Direct or indirect mechanisms of Th2 sensitization to inhaled antigen

Induction of tolerance or immunity to inhaled antigen by DCs is tightly controlled by signals from

alveolar macrophages, Tregs, NKT cells, complement activation, nervous system interactions, and epithelial activation. When studying the literature on particular substances that can break inhalation tolerance and induce Th2 priming, one needs to wonder therefore whether a stimulus acts directly on mDCs or pDCs or whether its effects are mediated indirectly though modification of any of the above interactions. For systemically administered TLR agonists, like endotoxin, the activation of DCs occurs mainly through direct recognition by TLR4 expressed on the DC, but in epithelia, the response could be clearly different [30]. As an example, low dose endotoxin was able to break inhalation tolerance to inhaled OVA by inducing mDC/cDC maturation [20]. These effects could be mediated directly via TLR and MyD88-depedent pathways in DCs, but could also be mediated via TLRs on bronchial epithelial cells [31]. Bronchial epithelial cells could produce chemokines as well as crucial growth and differentiation factors that subsequently attract, activate and polarize lung DCs to prime Th2 responses. In this regard, the epithelial cytokines TSLP and GM-CSF might be crucial, as their overexpression in the lungs breaks inhalational tolerance [32, 33]. On the contrary, neutralization of these cytokines during priming regimens, eliminates much of the adjuvant effects of diesel exhaust particles (DEP) [34, 35] or pro-allergic effects of house dust mite [36]. Importantly, the production of these cytokines by bronchial epithelial cells in response to these triggers might be genetically regulated and this could be the explanation why some individuals become primed to inhaled antigen under the right environmental exposure [37]. Under some conditions, predictions about sensitizers or adjuvants can be made form in vitro experiments. Ambient particulate matter (APM) is ubiquitous in the environment and is associated with allergic diseases in inner cities. In vitro, inhaled APM can act directly on human DCs as a danger signal to direct a pro-allergic pattern of innate immune activation thus explaining why it acts as an adjuvant [38]. Likewise, DEP induce maturation of human DCs indirectly, via promoting GM-CSF production in bronchial epithelial cells in vitro [35]. In mice, DEP and APM induce altered DC maturation directly, via nuclear factor-erythroid 2 (NF-E2)-related factor 2-mediated signaling, implicating oxidative stress in the activation of DCs [39]. Whether enhancement of inflammation in Nrf-2 deficient mice, who are hypersensitive to oxidative stress, is also the result of overzealous DC activation remains to be shown [40]. Another known sensitizer is cigarette smoke. When given concomitantly with harmless OVA, it induces Th2 responses, and this response is associated with enhanced DC maturation and migration [41]. DCs developed in a nicotinic environment (nicDCs) fail to support the terminal development of effector memory Th1 cells due to their differential expression of costimulatory molecules and lack of IL-12 production. In both man and mouse, nicDCs promoted the development of Th2 responses [42]. As maternal cigarette smoking is a solid risk factor for becoming sensitized in early life, it will be important to elucidate how it leads to DC activation (e.g. whether any indirect mechanisms acting via epithelial TLR4 contribute) as this might provide novel intervention strategies. Another unsolved question is how the nearby nervous endings react to viruses or air

pollution and how this could affect the way DCs react to inhaled allergen. In this regard, the remodeling that occurs in the airway unmyelinated nerves following RSV virus could partly explain the subsequent enhanced risk of Th2 sensitization in mice.

Function of dendritic cells in allergic inflammation and tissue remodeling

Not only do DCs play a role in the primary immune response to inhaled allergens, they are also crucial during the effector phase of asthma. The number of $CD11b^+$ DCs is increased in the conducting airways and lung interstitium of sensitized and challenged mice during the acute phase of the response [9, 43, 44]. However, during the chronic phase of the pulmonary response, induced by prolonged exposure to a large number of aerosols, respiratory tolerance develops through unclear mechanisms. During this regulatory phase, the number of mDCs as well as their costimulatory molecules in the lungs steadily decreased, and this was associated with a reduction of BHR, possibly mediated by the action of Treg cells [16, 45, 46]. Inflammation however reappeared when mature inflammatory $CD11b^+$ DCs were given [45]. Increased levels of class A scavenger receptors (MARCO and SR-AI/II) were found in the lungs of asthmatic mice, possibly suppressing DC-driven inflammation. These receptors are expressed on lung macrophages, DCs and basophils. Receptor deficient mice had more eosinophilic airway inflammation, BHR, and increased migration of DCs to the MLN [47].

The role of mDCs in the secondary immune response was further supported by the fact that their depletion at the time of allergen challenge abrogated all the features of asthma, including airway inflammation, goblet cell hyperplasia and bronchial hyperresponsiveness [9]. Again the defect was restored by intratracheal injection of GM-CSF cultured $CD11b^+CD11c^+$ mDCs, most closely resembling monocyte derived 'inflammatory DCs'. The same effects were observed when DCs were depleted in the nose in an animal model for allergic rhinitis [48]. It therefore seems that inflammatory DCs are both necessary and sufficient for secondary immune responses to allergen. Upon allergen challenge, lung DCs upregulate the expression of CD40, CD80, CD86, ICOS-L, PD-L1 and PD-L2, particularly upon contact with Th2 cells [9, 11, 43, 44, 49]. Costimulatory molecules might be involved in activation of effector T cells in the tissues, or in regulation of Treg activity. In allergen-challenged mice, DCs might also be a prominent source of the inflammatory chemokines CCL17 and CCL22, involved in attracting $CCR4^+$ Th2 cells to the airways, and in producing eosinophil-selective chemokines [26, 44]. In helminth infections, recruitment of Th2 cells and eosinophils depends on a IL-4/IL-13 responsive bone marrow derived cell, most likely a DC or alternatively activated macrophage population [50]. A number of cytokines and innate immune response elements control the production of these chemokines. The pro-allergic cytokine TSLP induces the production of large amounts of CCL17 by mDCs, thus contributing to the recruitment of Th2 cells to the airways, explaining how it may act to enhance inflammation [32].

The complement factor C5a suppresses the production of CCL17 and CCL22 [26]. A similar effect was seen with the cytokine IL-17, explaining how it may suppress allergic inflammation when given during allergen challenge. In vitro, IL-17 reduced CCL17 production and antigen uptake by DCs and IL-5 and IL-13 production in regional lymph nodes in vivo. Furthermore, IL-17 is regulated in an IL-4-dependent manner since mice deficient for IL-4Rα signaling showed a marked increase in IL-17 concentration with inhibited eosinophil recruitment [51]. Emerging evidence suggests that IL-4Rα expression on lung DCs is an important feedback mechanism through which IL-4 producing cells (effector Th2 cells, eosinophils, basophils) might promote further Th2 polarization in ongoing responses [52].

As the number and activation status of lung $CD11b^+$ DCs during secondary challenge seems critical for controlling allergic inflammation, studying the factors that control recruitment, survival or egress from the lung during allergic inflammation will be important, as this might reveal therapeutic targets. In an elegant study using mixed bone marrow chimeras in which half the hematopoietic cells were $CCR2^{-/-}$ and half were $CCR2^{+/+}$, it was shown by Robays et al. that CCR2 (and not CCR5 or CCR6) is crucial for releasing DC precursors from the bone marrow and attracting them into allergically inflamed lung. This was unexpected, as CCR6 is generally seen as the chemokine receptor attracting immature DCs into peripheral tissues [53]. Lung mDCs use CCR7 ligands and CCR8 for emigration to the draining lymph node, but not the leukotriene C4 transporter multidrug-related protein-1 as they do in the skin [54]. Unexpectedly, disruption of CCR7 selective chemokines in paucity of lymphocyte T cell (plt) mutant mice, deficient in CCL21 and CCL19, airway inflammation and Th2 activity were enhanced [55]. Still, increased numbers of mDCs could be found in the draining lymph node of these mice. So, other factors than CCR7 ligands are involved in the migration of DCs to the draining LN, including other chemokine receptors [54]. Eicosanoid lipid mediators, like prostaglandins and leukotrienes can also influence the migration of lung DCs [22]. Leukotriene LTB4 promoted the migration of immature and mature skin DCs, but these effects seem to be indirect [56]. It will be important to study if well known inducers of LTB4 in the lungs, such as the environmental biopolymer chitin, derived from fungi, helminths and insects, also induces DC migration [57]. Additional 'druggable' factors promoting the migration of DCs to the draining mediastinal nodes during inflammatory responses could be sphingosine-1-P and extracellular ATP [29, 58].

In humans, allergen challenge leads to an accumulation of myeloid, but not plasmacytoid DCs to the airways of asthmatics, concomitantly with a reduction in circulating $CD11c^+$ cells, showing that these cells are recruited from the bloodstream in response to allergen challenge [59, 60]. A recent report suggests that pDCs are also recruited into the BAL fluid, but are poor APCs [60]. The exact role of pDCs in ongoing allergen specific responses in asthma is currently unknown. It was shown that pDCs accumulate in the nose, but not lungs, of allergen challenged atopics [61]. When pDCs were pulsed with pollen allergens, they were as efficient as mDCs in inducing Th2

proliferation and effector function [62]. Others have suggested, as in the mouse, that pDCs might also confer protection against allergic responses [13]. In children at high risk of developing atopic disease, the number of circulating pDCs was reduced.

Dendritic cells as drug targets in allergic diseases

If DCs are so crucial in mounting immune responses during ongoing inflammation in the lung, nose and skin, then interfering with their function could constitute a novel form of treatment for allergic diseases. Additionally, pharmacological modification of DCs might fundamentally reset the balance of the allergic immune response in favor of regulatory T cells and thus lead to a more long lasting effect on the natural course of allergic disease. Steroids are currently the cornerstone of anti-inflammatory treatment in allergic disease. Inhaled steroids reduce the number of lung and nose DCs in patients with allergic asthma and allergic rhinitis [63]. Steroids might also interfere with a GITRL driven induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in plasmacytoid DCs, thus broadly suppressing inflammation [28]. Recently several other new molecules have surfaced that may alter DC function in allergic inflammation and thus treat disease. Many of these compounds were first discovered by their potential to interfere with DC driven Th2 sensitization. The sphingosine-1-P analogue FTY720 is currently used in clinical trials for multiple sclerosis and transplant rejection. When given to the lungs of mice with established inflammation, it strongly reduced inflammation by suppressing the T cell stimulatory capacity and migratory behavior of lung DCs [58]. Also selective agonists of particular prostaglandin series receptors might suppress DC function. The DP1 agonist BW245C strongly suppressed airway inflammation and bronchial hyperreactivity when given to allergic mice by inhibiting the maturation of lung DCs [22]. A very similar mechanism was described for inhaled iloprost, a prostacyclin analogue acting on the IP receptor expressed by lung DCs [24]. Extracellular ATP might be released by platelets upon allergen challenge. Neutralization of ATP via administration of the enzyme apyrase or the broad spectrum P2 receptor antagonist suramin reduced all the cardinal features of asthma by interfering with DC function [29]. A specific small molecule compound (VAF347) that blocks the function of B cells and DCs was also shown to be effective in suppressing allergic airway inflammation in a mouse model of asthma [64]. Finally, specific inhibitors of syk kinase were shown to suppress DC function and cure established inflammation [65].

A role for dendritic cells in COPD

Several investigators have initiated studies to investigate the role of DCs in COPD. In this disorder, inflammation is predominantly rich in neutrophils and $CD8^+$ lymphocytes. In a model

of cigarette smoke exposure, increased numbers of $CD11c^+$ DCs were seen upon sub-acute (5 weeks) and chronic (6 months) exposure, the increase being explained by increased levels of MCP-1 and MIP3α. Increase numbers of DCs as well as the occurrence of emphysema was also seen in SCID mice lacking adaptive immune cells [66]. Lung DCs exposed to cigarette smoke produced increased levels of the matrix metalloproteinase MMP12, possibly contributing to tissue damage [67]. In support, mice deficient in CCR6, the chemokine receptor expressed on pulmonary DCs as well as B cells, had reduced emphysema formation [68]. Studying occurrence of emphysema in mice depleted of DCs is less convenient as no models currently exist that allow a prolonged depletion of lung DCs in vivo.

It is possible that this pathway is also operative in COPD patients, as increased numbers of DCs were found in the epithelium and adventitia of small airways, correlating with increased levels of MIP3α in induced sputum [69]. One report did not find an increase in mucosal DCs in smoking asthma patients, suggesting that the increase in DCs might be specific for COPD [70]. One striking finding that awaits further study is the discovery that airway smooth muscle cells of COPD patients produce more TSLP, a critical DC maturation factor [71]. How exactly DCs contribute to initiating or perpetuating airway inflammation and emphysema is still largely unknown, further complicated by the fact that the antigen specificity of $CD8^+$ T cells in COPD is not known. Whether DCs also contribute to the occurrence of organized lymphoid tissues seen in end stage COPD remains to be studied.

Conclusion

Our understanding of DC biology in the airways has grown considerably. The concept that different subtypes of DCs perform different functions not only during sensitization but also during established inflammation is a theme that will persist in the coming years. Slowly, therapeutic strategies are emerging from these basic studies in animal models of asthma and COPD, which could one day reach clinical application. However, detailed knowledge of DC biology in human airways is still lacking.

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Chapter 6

An Anti-Inflammatory Role for Plasmacytoid Dendritic Cells in Allergic Airway Inflammation

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It was previously shown that administration of recombinant human Fms-like tyrosine kinase receptor-3 ligand (Flt3-L) prior to allergen challenge of sensitized mice suppresses the cardinal features of asthma through unclear mechanisms. Here, we show that Flt3-L dramatically alters the balance of conventional to plasmacytoid dendritic cells (cDC/pDC) in the lung favoring the accumulation of pDCs. Selective removal of pDCs abolished the anti-inflammatory effect of rhFlt3-L, suggesting a regulatory role for these cells in ongoing asthmatic inflammation. In support, we found that immature pDCs are recruited to the lungs of allergen-challenged mice irrespective of rhFlt3-L treatment. Selective removal of pDCs during allergen challenge enhanced airway inflammation, whereas adoptive transfer of cultured pDCs prior to allergen challenge suppressed inflammation. Experiments in which TLR9 agonist CpG motifs were administered in vitro or in vivo demonstrated that pDCs were anti-inflammatory irrespective of their maturation state. These findings suggest a specialized immunoregulatory role for pDCs in airway inflammation. Enhancing the anti-inflammatory properties of pDCs could be employed as a novel strategy in asthma treatment.

Allergic asthma is a chronic disease of the airways characterized by bronchial hyper reactivity (BHR) to non-specific stimuli, chronic eosinophilic airway inflammation, goblet cell hyperplasia, and airway structural changes in response to a chronic Th2 immune response to inhaled antigen, also reflected by Th2-dependent increased serum levels of allergen-specific immunoglobulin E (IgE) and immunoglobulin G1 (IgG1)[1, 2].

Various forms of (anti-) cytokine therapy have been employed in an attempt to suppress allergic inflammation, with variable success. One such recently described strategy was to administer the hematopoietic growth factor Fms-like tyrosine kinase receptor-3 ligand (Flt3-L) to allergic mice prior to allergen challenge. In these mice, all the cardinal features of asthma were abolished [3-5]. The precise mechanism of this anti-inflammatory effect has not been addressed. Flt3-L has broad actions in the hematopoietic and immune system and was shown to selectively enhance the number of NK cells and dendritic cells (DCs) in vivo [6, 7]. A specific subset of DCs induced by this cytokine in vivo and in vitro are the so-called plasmacytoid (p) DCs, mainly known for their function in anti-viral immunity and rapid production of type I interferons [8]. It was also shown that systemic administration of Flt3-L to mice led to an expansion of semi-mature lung conventional (c)DCs [9].

The effects of Flt3-L on DCs could be important to understand the anti-inflammatory effects of this cytokine in asthma. There is evidence for distinct roles of cDCs and pDCs in regulating T cell mediated adaptive immunity in the lung [10, 11]. Inflammatory DCs promote Th2 sensitization to inhaled antigen after reaching the mediastinal lymph nodes [12-14], whereas pDCs mediate tolerance to inhaled antigen through induction of regulatory T cells [15-18]. Increasing evidence supports the notion that DCs also contribute to ongoing airway inflammation beyond the sensitization phase of asthma as CD11c-diphteria toxin receptor

transgenic mice no longer developed signs of asthma when airway DCs were depleted during secondary and tertiary challenge with antigen [14].

In this paper, we show that rhFlt3-L greatly enhanced the number of pDCs in the lungs of allergen-challenged mice, while reducing Th2 associated eosinophilic inflammation. Removing pDCs from rhFlt3-L-treated mice prior to allergen challenge abolished the anti-inflammatory actions of this cytokine. These studies also led us to investigate the contribution of pDCs in ongoing allergic responses to aerosolized antigen. Upon allergen challenge of sensitized mice, pDCs were recruited to the lungs. Selective removal of pDCs using a depleting antibody 120G8 enhanced inflammation whereas adoptive transfer of cultured pDCs suppressed it. These effects occurred irrespective of pDC maturation state. Our data therefore demonstrate a previously unrecognized function of pDCs in controlling ongoing allergic inflammation that could be exploited for the better design of anti-inflammatory compounds.

Material and Methods

Animals

Female BALB/c mice were purchased from Harlan (Zeist, The Netherlands). All mice were housed under specific pathogen-free conditions and were between 6-8 weeks of age at the start of an experiment. All experiments were conducted with the approval of the Animal Care Committee of Erasmus Medical Center, Rotterdam, The Netherlands.

Induction of experimental asthma

Mice were sensitized by two intraperitoneal (i.p.) injections of 10 μg ovalbumin (OVA: Worthington) adsorbed onto 1 mg aluminum hydroxide (alum; Imject, Pierce) in 500 μl saline (OVA-alum) on days 0 and 7. Ten days after the last injection, the mice were challenged by inhalation of OVA aerosols (grade III, 1% wt/vol in PBS (Sigma)) generated by a jet nebulizer for 30 min. Depending on the experiment, the aerosols were administered between 1 to 7 times on consecutive days.

Treatment of mice with recombinant Flt3-L

Mice were injected with 10 μg recombinant human Flt3-L (rhFlt3-L; kindly provided by Amgen) i.p. for 8 days before the secondary challenge phase.

Treatment of mice with pDC-depleting antibodies

Mice were treated i.p. with 200 μg monoclonal 120G8 antibodies (kindly provided by C. Asselin-Paturel, Schering-Plough, Dardilly, France; [20]) or as a control with 200 μg rat IgG (Sigma). Mice were either injected for 3 days on consecutive days starting 1 day before OVA challenge or treated also after the OVA challenge on alternate days for 3 times (see Fig 3A and C).

Treatment of mice with CpG motifs

Unmethylated CpG motifs (10 μg; ISS-ODN 1680; CpG (5'-TGACTGTG-AACGTTCCGAGATGA-3'); Sigma-Genosys) [53] were administered intratracheally (i.t.) in a volume of 80 μl PBS in OVA-alum sensitized mice. One day after the i.t. treatment mice were exposed to three OVA aerosols (see Fig 5F).

Purification of plasmacytoid dendritic cells (pDCs)

Bone marrow was cultured for 4 days with culture medium (CM) supplemented with 200 ng/ml rhFlt3-L and 50 ng/ml murine rmSCF (Peprotech) in a 6-well plate, at a concentration of $1*10^6$ cells/ml. At day 4 of the culture, all non-adherent cells were collected, centrifuged and placed back into culture with 200 ng/ml rhFlt3-L. On day 10 of the culture, the cells were pulsed with LPS-low OVA (Worthington; LPS contamination of 2.9 ng/mg protein) and/or 1 μM unmethylated CpG motifs. On day 11, the cells were sorted based on their expression of 120G8, B220, CD11c, and CD11b using a FACSAria flow cytometer (BD). A purity of ≥96% was obtained. Three days before the start of OVA aerosols, $1*10^6$ pDCs were injected intravenously via the tail vein (see Fig 4A).

Isolation of Broncho-alveolar lavage, lungs, and MLN

Mice were sacrificed by an overdose of 2.5% avertin, followed by bleeding. Broncho-alveolar lavage (BAL) was performed by inserting a canula in the trachea of the mouse. By lavage of 3 x 1 ml PBS containing 0.1 mM EDTA, cells were extracted from the lung alveolar space. Lungs were either inflated with PBS/OCT for histology or perfused with PBS through the pulmonary artery prior to mincing them to obtain a single cell suspension as described before [54]. To determine the cytokine levels, MLN cells were plated in 96-wells round bottom plates at a density of $2*10^5$ cells per well and re-stimulated with 10 or 100 μg/ml OVA for 4 days. After 4 days, supernatants were harvested and stored at –20°C until further examination. Using ELISA, the amount of IL-4, IL-5, IFNγ (all eBioscience), IL-10 (BD Bioscience), and IL-13 (R&D Systems) was measured in the supernatants.

Flow cytometry of BAL, lung, and MLN

All staining reactions were performed at 4°C. In all staining reactions, 2.4G2 Fc receptor Ab (CD16/CD32) was added to reduce non-specific binding. Dead cells were excluded using propidium iodide and the cellular composition of the BAL fluid was determined as described before [55]. The BAL, lung, and MLN were furthermore stained for DC subsets using 120G8-FITC, B220-PE (RA3-6B2) or MHC class II-PE (M5/114; BD Biosciences), and CD11c-APC (HL3; eBioscience) or CD11b-APC (MAC-1; BD Biosciences). In experiments in which the expression of the costimulatory molecules was investigated, 120G8-FITC and CD11c-APC, and in FL-2 channel CD80-PE (16-10A1; BD Biosciences), ICOSL-PE (B7rp1; BD Biosciences), or isotype-PE were used.

Histology

Frozen sections (6 μm) of the lung were stained with Periodic Acid Schiff's reagent (Sigma-Aldrich) to address the amount of inflammation and goblet cell hyperplasia. To identify pDCs, immuno-histochemical staining was performed on formaldehyde-fixed sections. Endogenous peroxidase was blocked with 0.1% NaN_3/0.01% H_2O_2 in PBS for 30 min, non-specific binding was blocked with 10% NGS, and sections were subsequently incubated with 120G8, goat-anti-rat Ig-AP, rat-APAAP, hamster anti-CD11c, and goat anti–Armenian hamster PO. PO was visualized with AEC substrate and AP with Fast blue substrate.

Statistical analysis

For all experiments, the difference between groups was calculated using the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Differences were considered significant when $p<0.05$.

Results

Effect of systemic rhFlt3-L administration on allergic airway inflammation in OVA-sensitized mice

It has been shown that administration of Flt3-L for 10 days prior to a secondary OVA allergen challenge in OVA-sensitized mice suppresses the cardinal features of asthma [4]. Using a modified protocol, we administered rhFlt3-L intraperitoneally for 8 days prior to giving a series of three OVA aerosols to OVA-sensitized mice (Fig 1A) and focused on dendritic cells (DCs). To allow later on comparisons with pDC depleting Abs, mice received isotype control rat IgG Abs during secondary challenge (see below). As expected in OVA-sensitized and -challenged mice, PBS-treated mice developed a strong eosinophilia in the BAL fluid (Fig 1B) and showed inflammatory lesions around the bronchi and the blood vessels in the lung (Fig 1C). Challenge with PBS aerosols did not induce any changes of airway inflammation (data not shown and [13, 19]). The eosinophilic inflammation was associated with substantial Th2 cytokine production by MLN cells in vitro (Fig 1D). Compared with administration of PBS, Flt3-L treatment strongly suppressed eosinophilic and lymphocytic airway

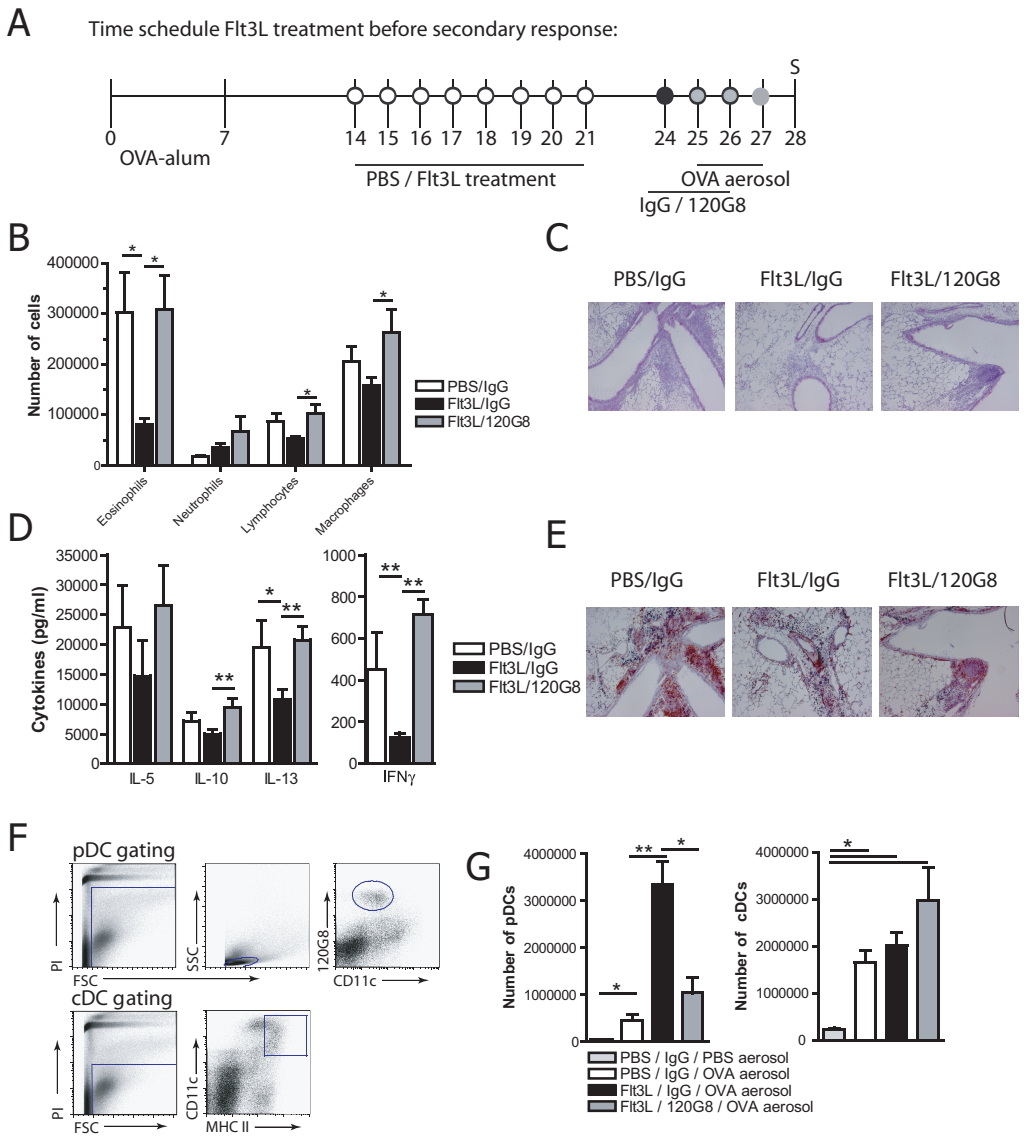


Figure 1

Effect of Flt3-L treatment is dependent on pDCs

(A) Mice were sensitized by 2 i.p. injections of OVA-alum on days 0 and 7. Seven days after the last injection, the mice were treated with 8 injections of rhFlt3-L (10 µg i.p.) or with PBS as a control. 3 days after the last rhFlt3-L injection, mice were treated with mAb 120G8 or rat IgG (200 µg i.p.). Thereafter, mice were subjected to 3 OVA aerosols. **(B)** Inflammation was analyzed in the BAL with flow cytometry. **(C)** A PAS staining was done on lung slides (50x magnification) to visualize goblet cell hyperplasia. **(D)** Cytokine production in the supernatants of MLN cells 4 days after restimulation with OVA in vitro. **(E)** Lung slides were stained for DCs (in blue 120G8 and red CD11c). pDCs were double positive for 120G8 and CD11c, whereas cDCs are positive for CD11c. **(F)** Gating strategy for pDCs and cDCs on lung cells. pDCs are depicted as small cells, $120G8^{+}$, and $CD11c^{int}$. cDCs are shown low autofluorescence, MHC II^{+}, $CD11c^{+}$. An example is shown of a rhFlt3-L-treated mouse. **(G)** Number of pDCs and cDCs were determined lung by flow cytometry. Results are expressed as mean ± SEM for n=5-6 mice/group. Experiment was repeated twice.

inflammation as assessed by BAL fluid analysis (Fig 1B), tissue infiltration of eosinophils and goblet cell hyperplasia (Fig 1C). Upon rhFlt3-L treatment, the levels of IL-13 and IFNγ, but not IL-5 or IL-10 were reduced. In view of the known alteration of DC numbers by Flt3-L, we determined the number and assessed the phenotype of lung DC subsets in OVA-sensitized and -challenged mice, treated with rhFlt3-L or not. On lung histology, we identified a clear increase in the number of $CD11c^+$ cells in the lung interstitium of mice receiving Flt3-L, and a significant proportion of these cells also expressed the marker bone marrow stromal antigen-2, recognized by the monoclonal 120G8 Ab, consistent with a pDC phenotype [20] (Fig 1E). To more carefully address the precise subset of DC induced, we confirmed the presence of pDCs and cDCs in lung tissue using flow cytometry. Plasmacytoid DCs in the lung were identified based on live/dead discrimination, small size, intermediate expression of CD11c and expression of 120G8 marker, as previously described (Fig 1F) [21] [15], whereas conventional DCs were identified based on lack of auto-fluorescence and strong expression of CD11c and MHC II [22] (Fig 1F). OVA challenges in OVA-sensitized mice increased the number of pDCs and cDCs in the lungs compared to PBS challenges (Fig 1G). In OVA-challenged mice, rhFlt3-L treatment led to a further substantial and selective increase in the number of pDCs compared to PBS-treated OVA-challenged mice. (Fig 1G).

To study whether the selective increase in pDC number in rhFlt3-L-treated mice was responsible for the associated reduction in airway inflammation, we injected the pDC depleting 120G8 Ab or control rat IgG Abs into Flt3-L-treated mice [15, 20] at the time of OVA challenge (Fig 1A). Through this treatment, the increase in number of pDCs in the lungs of Flt3-L-treated mice could be prevented (Fig 1G), whereas it had no effect on the number of cDCs. Furthermore, airway inflammation was completely restored, as seen in eosinophilic and lymphocytic recruitment to the lung interstitium (Fig 1B) and goblet cell hyperplasia (Fig 1C). Also, cytokine levels produced by MLN cells were restored to the levels produced by PBS-treated OVA-challenged mice (Fig 1D). These data clearly demonstrate that the anti-inflammatory effects of Flt3-L are in a large part mediated by pDCs.

Effect of OVA aerosol challenge on the number of pDCs in OVA-sensitized mice

We next addressed if the number of pDCs in the lung would also be affected during airway inflammation in the absence of Flt3-L treatment. In these experiments, OVA-sensitized mice were subjected to different numbers of OVA aerosols (Fig 2A). As expected the degree of eosinophilic inflammation increased with increasing numbers of OVA aerosols (Fig 2B). Previous work by van Rijt et al [19] reported that different number of PBS aerosols did not change the composition of the BAL fluid. Therefore, as a control, mice were only exposed to the maximum number of PBS aerosols. The number of pDCs in the BAL fluid of allergic mice was increased up to 7 times with increasing numbers of aerosols (Fig 2C). The

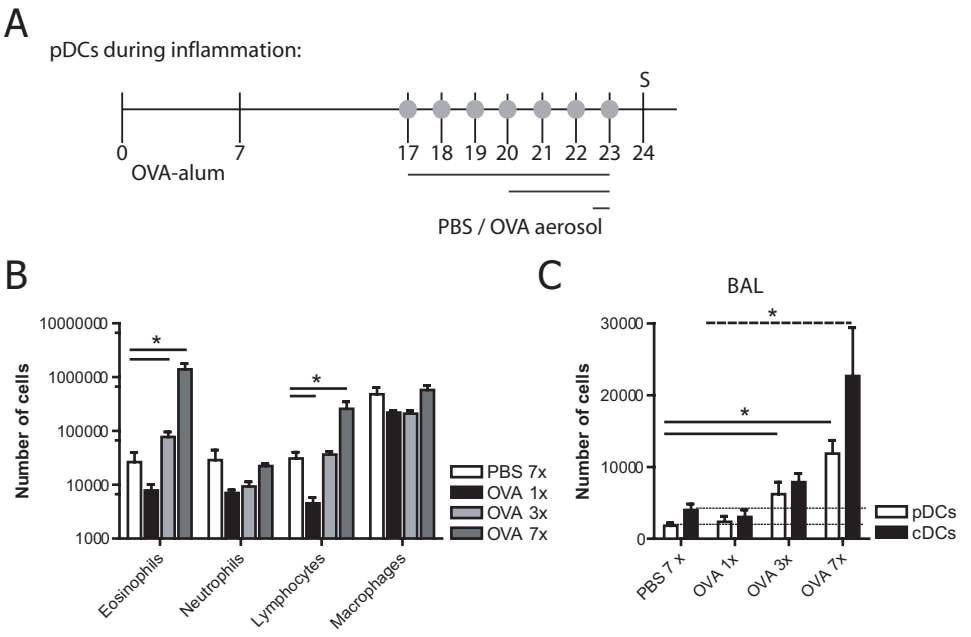


Figure 2
Effect of OVA aerosol challenge on pDC numbers in the BAL
(A) Mice were sensitized by 2 i.p. injections of OVA-alum on days 0 and 7. Ten days after the last injection, they were subjected to either 7x PBS, 1x, 3x, or 7x OVA aerosols. **(B)** Inflammation in the BAL fluid of PBS aerosolized mice (white bars) or mice subjected to 1 (black bars), 3 (light shaded bars) or 7 OVA aerosols (dark shaded bars). **(C)** The number of pDCs (white bars) and cDCs (black bars) in the BAL fluid. Results are expressed as mean ± SEM for n=4 mice/group, *p<0.05. Similar results were obtained in two different experiments.

increase in cDC numbers in the BAL followed the same trend as for pDCs. We next looked whether the level of maturation of pDCs and cDCs would be affected by increasing numbers of OVA aerosols. In the BAL fluid, lungs, and MLN of OVA-challenged mice, pDCs showed no increase in the expression CD40, CD80, CD86, MHC II, PD-L1, PD-L2 or ICOS-L. However, we could observe an upregulation of costimulatory molecules (CD40, PD-L2) on cDCs in the BAL as previously reported ([14] and data not shown).

Effect of selective removal of pDCs on OVA-induced airway inflammation in OVA-sensitized mice

As OVA-induced airway inflammation caused an increase in the number of pDCs, we wondered what effect depletion of pDCs would have on the severity of eosinophilic inflammation. To address this point, we depleted pDCs using 120G8, administered at the time of challenge (Fig 3A). Injection of 120G8 Abs decreased the number of pDCs in most organs by 80-90%, and the effect of 120G8 administrations was observed for at least 4 days after the last injection (data not

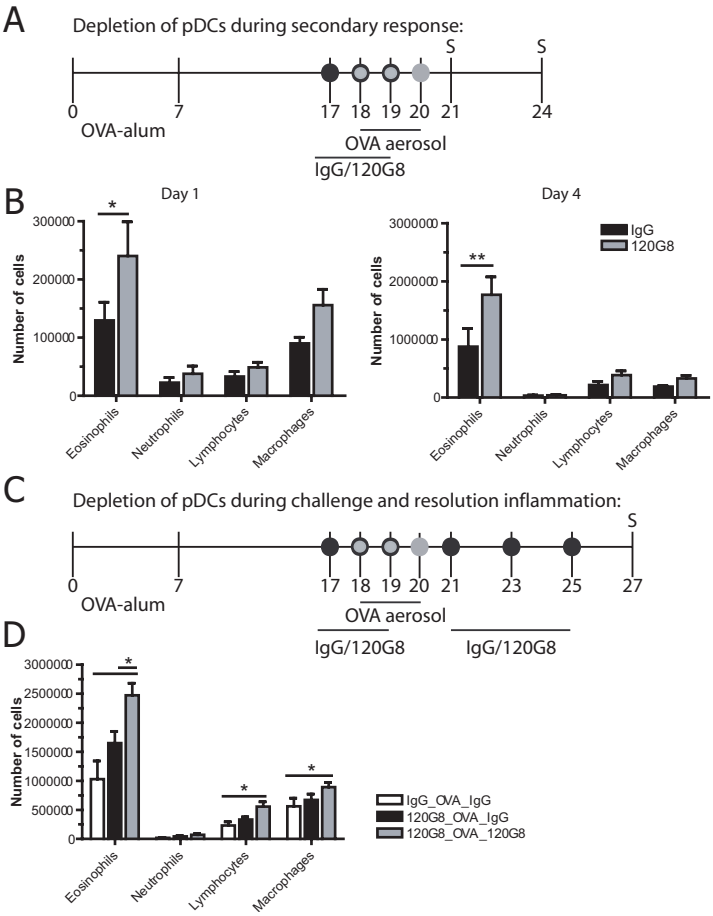


Figure 3
pDCs are important in regulating inflammation in the secondary response
(A) Mice were sensitized by 2 i.p. injections of OVA-alum on days 0 and 7. Ten days after the last injection, they were subjected to 3 OVA aerosols. During or after the challenge, mice were treated with the 120G8 Ab or rat IgG (200 µg i.p.) as a control. **(B)** Inflammation in the BAL fluid was determined by flow cytometry. **(C)** Mice were treated during challenge and during the resolution phase with 120G8 or rat IgG (200 µg i.p.). **(D)** BAL inflammation was determined by flow cytometry. White bars represent the rat IgG-treated OVA challenged mice, the black bars the OVA challenged mice treated with 120G8 during challenge, and the grey bars OVA challenged mice treated with 120G8 during challenge and resolution. Results are expressed as mean ± SEM for n=4-6 mice/group, *p<0.05, **p<0.01. Similar results were obtained in two different experiments.

shown). As expected in OVA-sensitized and –challenged groups, mice injected with rat IgG control Ab developed eosinophilic airway inflammation (Fig 3B, D) and showed a Th2 cytokine profile in the draining MLN (data not shown). In mice depleted of pDCs by injection of 120G8 Ab, the number of eosinophils in the BAL was increased 24 hrs and 4 days after the last aerosol (Fig 3B). Seven days after the last challenge, the eosinophilia in the BAL fluid was no longer different between the groups, and the inflammation was completely resolved by day 14 after the last challenge (data not shown).

The fact that at day 7 after the last aerosol, airway inflammation was no longer different between 120G8 Ab- and rat IgG-treated mice could suggest that pDC depletion was no longer complete during the resolution phase of airway inflammation. Therefore, mice were additionally given 120G8 Ab during the resolution phase (Fig 3C). Mice treated with rat IgG throughout challenge and resolution still demonstrated a strong BAL lymphocytosis and eosinophilia 7 days after the last aerosol exposure (Fig 3D, white bars), accompanied by Th2 cytokine production by MLN cells

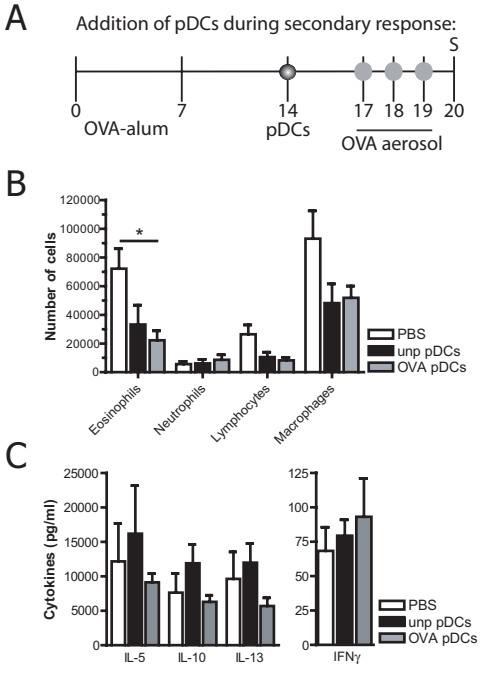


Figure 4
Effect of exogenous pDCs in the secondary response of lung inflammation
(A) Mice were sensitized by 2 i.p. injections of OVA-alum on days 0 and 7. Three days before the first aerosol, mice received $1*10^6$ pDCs i.v.. **(B)** Inflammation of the BAL was analyzed. White bars represent mice receiving PBS, black bars mice receiving unpulsed pDCs, and shaded bars OVA-pulsed pDCs before challenge. **(C)** Cytokine levels in the supernatants of MLN cell cultures re-stimulated in vitro with OVA for 4 days. Results are expressed as mean ± SEM for n=4-6 mice/group, *p<0.05. Similar results were obtained in two different experiments.

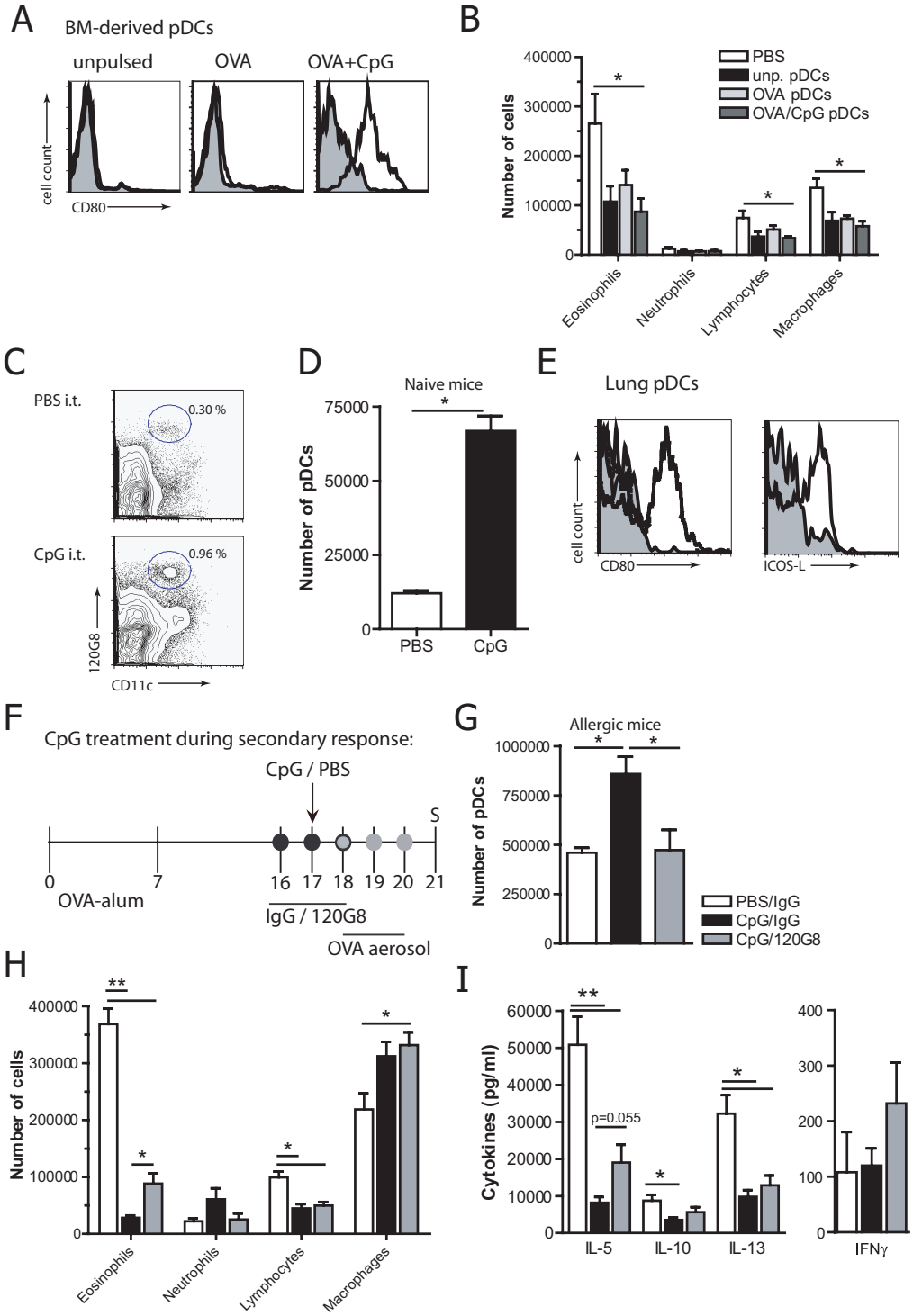
(data not shown). In mice depleted of pDCs only during challenge (black bars), the number of inflammatory cells in the BAL fluid was not different from this response. However, in mice depleted of pDCs during the challenge and resolution phase, the degree of airway inflammation was again dramatically increased (Fig 3D, grey bars).

Effect of exogenous pDC administration on OVA-induced airway inflammation in OVA-sensitized mice

Since depletion of pDCs during the secondary response altered the inflammatory response (Fig 3), and the anti-inflammatory effect of Flt3-L was pDC-dependent (Fig 1), we examined if ex-vivo cultured pDCs would also have an effect on airway inflammation. Mice were injected with bone marrow (BM)-derived pDCs 3 days before submitting them to OVA aerosols (Fig 4A). As expected in OVA-sensitized and -challenged mice not injected with pDCs, lymphocytosis and eosinophilia was observed in the BAL (Fig 4B) and Th2 cytokine production by MLN cells (Fig 4C) was induced. A strong decrease in the number of BAL fluid eosinophils and lymphocytes was observed in pDC-injected mice (Fig 4B). This decrease occurred irrespective of whether pDCs were pulsed in vitro with OVA or not. The administration of pDC had no effect on the levels of Th2 cytokines produced by MLN cells (Fig 4C).

Effect of pDC maturation state on their capacity to control airway inflammation

Since endogenous and exogenous pDCs can suppress allergen-induced eosinophilic airway inflammation (Fig 1, 3 and 4), we next studied if this effect was dependent on their maturation status. BM-derived pDCs were exposed to unmethylated CpG motifs (ISS ODN 1680) in


A
BM-derived pDCs
unpulsed
OVA
OVA+CpG
cell count
CD80
B
PBS
unp. pDCs
OVA pDCs
OVA/CpG pDCs
Number of cells
400000
300000
200000
100000
0
Eosinophils
Neutrophils
Lymphocytes
Macrophages
C
PBS i.t.
0.30 %
CpG i.t.
0.96 %
120G8
CD11c
D
Naive mice
Number of pDCs
75000
50000
25000
0
PBS
CpG
E
Lung pDCs
cell count
CD80
ICOS-L
F
CpG treatment during secondary response:
CpG / PBS
S
0
7
16
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20
21
OVA-alum
IgG / 120G8
OVA aerosol
G
Allergic mice
Number of pDCs
1000000
750000
500000
250000
0
PBS/IgG
CpG/IgG
CpG/120G8
H
Number of cells
400000
300000
200000
100000
0
Eosinophils
Neutrophils
Lymphocytes
Macrophages
I
Cytokines (pg/ml)
60000
50000
40000
30000
20000
10000
0
p=0.055
IL-5
IL-10
IL-13
400
300
200
100
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IFNγ

vitro, before injecting them into mice. pDCs exposed to CpG showed an up regulation of CD80 (Fig 5A), CD40, CD86, ICOS-L, and MHC II (data not shown) [23, 24]. When injected, fully mature pDCs pulsed with OVA and CpG were as efficient as immature OVA-pulsed pDCs or unpulsed pDCs in reducing the number of inflammatory cells in BAL fluids (Fig 5B). These data show that cultured pDCs are able to dampen airway inflammation irrespective of their degree of maturation. We next addressed whether endogenous maturing pDCs exposed to CpG motifs in vivo would have the same capacity. Direct instillation of unmethylated CpG motifs in naive mice induced a rapid and massive increase in the number of pDC in the lungs compared to PBS instillation (Fig 5C/D). In mice injected with CpG motifs, lung pDCs showed a mature phenotype as assessed by the upregulation of CD80 and ICOS-L compared to pDCs of mice injected with PBS (Fig 5E). It has been shown by others that instillation of CpG motifs in sensitized animals before subjecting them to OVA aerosols led to a marked decrease in eosinophilic inflammation [25-28]. In support, we also observed a decrease in airway inflammation and Th2 cytokine profile following local CpG motif treatment (Fig 5H). Similar to the effects of CpG motifs in naive mice, the lungs of CpG motif-treated OVA-challenged mice (CpG/IgG) showed an increase in the number of pDCs compared to PBS-treated OVA-challenged mice (Fig 5G). To address a role for pDCs in the CpG motif-induced reduction in airway inflammation, mice were concomitantly treated with CpG motifs and 120G8 Abs (Fig 5F). The increase in pDC number by CpG motif instillation was neutralized by 120G8 treatment (Fig 5G). A partial restoration of the airway eosinophilia could be observed when pDCs were depleted in CpG motif-treated mice (Fig 5H). This could also be seen in the cytokines, like IL-5 produced by MLN cells (Fig 5I).

Discussion

Several key findings in this paper point to an intrinsic anti-inflammatory role for pDCs in eosinophilic airway inflammation in allergically

Figure 5
Mature pDCs are still able to dampen eosinophilic inflammation
(A) CD80 expression of BM-derived pDCs after 20 hrs pulse with OVA (100 μg/ml) with or without CpG (1 μM). **(B)** Inflammation was analyzed in the BAL of mice treated i.v with PBS (white bars), $1*10^6$ unpulsed pDCs (black bars), OVA-pulsed pDCs (light shaded bars), or OVA and CpG motif-pulsed pDCs (dark shaded bars). **(C)** Mice were injected i.t. with 10 μg CpG motifs or PBS, lungs were analyzed 48 hrs later for pDCs and cDCs. A typical example is shown of the lungs stained with 120G8 and CD11c. **(D)** Number of pDCs in the lung of CpG-motif-treated mice (black bar) or PBS-treated mice (white bar), analyzed with flow cytometry. **(E)** CD80 and ICOS-L expression of lung pDCs in vivo 48 hrs after i.t. instillation of CpG (10 μg) (open histogram) or PBS (shaded histogram). **(F)** Mice were sensitized by 2 i.p. injections of OVA-alum on days 0 and 7. Nine days after the last injection, the mice were treated i.t. with CpG (10 μg) or PBS. Thereafter, they were subjected to 3 OVA aerosols. During i.t. instillation, mice were also treated with either moAb 120G8 or rat IgG (200 μg i.p.). **(G)** The number of pDCs was examined in the lungs by flow cytometry, 1 day after the last challenge. The white bar represents the PBS-treated OVA-challenged mice, the black bar CpG-motif/IgG- treated OVA-challenged mice, and the grey bar the CpG motif/120G8-treated OVA-challenged mice. **(H)** Inflammation in the BAL analyzed with flow cytometry. **(I)** Cytokine levels in the supernatants of MLN cell cultures re-stimulated in vitro with OVA for 4 days. Results are expressed as mean ± SEM for n=4-6 mice/group, *$p<0.05$. Similar results were obtained in two different experiments.

sensitized mice. First, pDCs are recruited to the airways of allergen challenged mice with kinetics similar to other inflammatory cells. Second, their removal using a specific pDC depleting antibody enhances inflammation and prevents the resolution of inflammation once allergen challenge is stopped. Finally, increasing the numbers of pDCs through adoptive transfer of cultured pDCs, or expansion of endogenous pDCs by administration of Flt3-L dampens inflammation.

In previous studies, Flt3-L was shown to suppress all the cardinal features of asthma in models of experimental airway inflammation [3, 4]. In this study, we specifically set out to study the mechanism of this anti-inflammatory effect. Systemic administration of Flt3-L is known to expand the number of NK cells, conventional DCs (cDCs), and plasmacytoid DCs (pDCs) in vivo [6, 7]. All of these cell types have been implicated in the pathogenesis of asthma. Although we did not directly test the effect of Flt3-L on lung NK cell distribution or function in asthma, it is unlikely that the anti-inflammatory effects of Flt3-L were caused by NK cell alterations. Korsgren et al. have previously shown that depletion of NK cells in a very similar model of asthma decreased disease severity, suggesting that it is a pro-inflammatory player in this disease [29]. We therefore favor the hypothesis that the effects of Flt3-L were due to alterations in DC homeostasis. DCs are thought to be the most important antigen presenting cells that determine the outcome of allergen inhalation in the lung [11]. Inflammatory dendritic cells (iDCs) are held responsible for inducing Th2 sensitization to inhaled antigen and are similarly crucial for the establishment of secondary immune responses to inhaled allergen in allergen-sensitized mice [12, 14, 19]. As administration of cDCs to mice exacerbates the severity of the disease, and their depletion ameliorates disease [14], it is unlikely that Flt3-L would have its anti-inflammatory effects via increasing the number and/or function of this subset. We hypothesized that it is predominantly through the recruitment of pDCs that Flt3-L suppresses inflammation. In support of this hypothesis, we have previously reported that pDCs play an essential role in maintaining tolerance in the lung when naive mice were exposed to harmless inhaled antigens [15]. The critical involvement of DCs in promoting or dampening inflammation has been reported in several other models, in which also viral antigens were used [30-35]. However, these experiments were done in the context of a primary immune response as pDCs were given or depleted at the time of sensitization. In the present paper, we show that pDCs, like cDCs, play a role beyond the sensitization phase. Flt3-L treatment given before the secondary challenge induced a massive recruitment of pDCs, accompanied by a suppression of eosinophilic airway inflammation. Importantly, this pDC recruitment was necessary for the anti-inflammatory effect, as removal of pDCs using the specific pDC depleting Ab 120G8 restored airway eosinophilia and Th2 cytokine levels in draining nodes.

The exposure of OVA-sensitized mice to OVA aerosols resulted in a Th2-dependent airway eosinophilic inflammation and in the recruitment of cDCs and iDCs to the lung

and the broncho-alveolar lavage, as already reported [14]. Interestingly, the number of pDCs was also increased following OVA challenges, and correlated with the degree of airway inflammation. Again, the function of these cells was to dampen inflammation as 120G8 treatment exacerbated the degree of immuno-pathology. How pDCs are recruited to inflamed airways is still unclear but could involve several inflammatory mediators [36]. Indeed, inflammatory chemokines such as CCL2 (MCP-1), CCL3 (MIP1α), CCL5 (RANTES), CCL12 (MCP-5), CXCL10 (IP10), CXCL12 (SDF-1) and are upregulated in allergic lungs [37] and pDCs express the receptors for most of these chemokines (CCR1, CCR2, CCR5, CCR7, CXCR3, and CXCR4 [38-40]). A second possibility would be the involvement of ChemR23, a receptor involved in DC migration and expressed on pDCs [41]. Its ligand, chemerin, is found in inflammatory fluids [42], but more importantly directs pDC migration to inflamed tissues [41, 43]. Whether chemerin is expressed in the lungs after repeated exposures to OVA aerosols remains to be established. Alternatively, DC subsets might be recruited to the airways by osteopontin, a cytokine involved in autoimmune and allergic diseases. Osteopontin is increased in BAL fluids of OVA-sensitized and challenged mice, but has the potential to dampen airway inflammation and BHR, and interestingly contributes to pDC recruitment during allergic responses [33].

Increasing pDC numbers at the site of allergic airway inflammation may be part of an inherent protective mechanism, as suggested by the fact that the disease was exacerbated following removal of these cells at the time of challenge, and by the fact that administration of pDCs prior to secondary challenge diminished the severity of inflammation. One striking observation was the fact that prior to adoptive transfer, pDCs did not have to be pulsed with OVA antigen for the suppressive effect to occur. This is in contrast to the situation in naive mice, where pDCs had to be pulsed with OVA antigen for prevention of alum-induced sensitization, as previously reported by our group [15]. The most likely explanation is that in sensitized mice, unpulsed pDCs acquired and processed inhaled antigen delivered via aerosol exposure. In already sensitized mice, the uptake of OVA could be facilitated by the presence of OVA-specific immunoglobulins, as previously reported for human pDCs [44] and our unpublished data suggest.

How pDCs exert their suppressive functions in vivo still remains to be elucidated. There is evidence showing that pDCs can down-modulate immune responses by directly interfering with effector T cell function, by inhibiting cDC function or by inducing regulatory T cells [17]. Increasing regulatory T cells numbers and functions during the secondary phase is a strategy to inhibit airway inflammation [45-47]. We have previously reported that immature lung OVA-pulsed pDCs could induce regulatory T cells when co-cultured with naive $CD4^+$ T cells [15]. One way by which pDCs might promote the formation of regulatory T cells, is their poor expression of co-stimulatory molecules. In our experiments, the instillation of CpG motifs strongly reduced airway inflammation, similar

to previous reports [25-27]. The explanation for this effect has been the inhibition of both Th2 cell activation and IgE-mediated cytokine induction [28]. In our paper, we show not only that the effect induced by CpG motifs was partially mediated by pDCs but also that the degree of maturation of these pDCs did not seem to influence their suppressive effect. Indeed, CpG motif-exposed pDCs, although very mature, were still very potent at suppressing eosinophilic airway inflammation in vivo. Recently, Gilliet's group demonstrated that mature CpG motif-pulsed pDCs were able to induce the generation of IL-10-producing regulatory T cells via the ICOS/ICOS-L pathway [16, 48]. Interestingly, in mice instilled with CpG motifs, pDCs in the lung upregulated ICOS-L. It is tempting to speculate that CpG motif-exposed pDCs exert their function in the lung by increasing regulatory T cell numbers in an ICOS-L dependent way. Certainly, when CpG motif-matured pDCs were co-cultured in vitro with $CD4^+$ T cells, we could observe an increased number of $Foxp3^+$ T cells, consistent with the phenotype of regulatory T cells (data not shown). However, ICOS-$L^{-/-}$ pDCs in our asthma model could still reduce eosinophilic airway inflammation (data not shown), so their suppressive capacity does rely on ICOS-ICOSL interactions. It is however noteworthy that pDCs are still able to induce regulatory T cells when mature, whereas cDCs drive the generation of IL-10–producing regulatory T cells only when they are immature and lose this ability upon maturation [49, 50]. This difference may suggest a specialized role for pDCs in immuno-regulation during allergic airway inflammation.

Another mechanism that might be used by pDCs to suppress eosinophilic airway inflammation would be to down-regulate the function of cDCs [17]. cDCs are known to be critical in the maintenance of airway inflammation as their removal at the time of challenge reduced the features of asthma [14]. Moreover, cDCs of allergen-sensitized and -challenged animals selectively produce CCR4 ligands CCL17 (TARC) and CCL22 (MDC) as a means to recruit Th2 cells to sites of inflammation [17, 51]. Kohl et al elegantly showed that in conditions where the cDC/pDC ratio is shifted to pDCs, the levels of MDC and TARC were decreased [17]. This might offer a likely explanation as to why Th2-dependent airway inflammation is decreased when the number of pDCs is increased in the airways. Alternatively, pDCs stimulated with unmethylated CpG motifs are a major source of IFNα. There is evidence that IFNα can block the recruitment of eosinophils and $CD4^+$ T cells to the site of inflammation [52]. In an attempt to pinpoint a role for this cytokine in the suppressive effect induced by pDCs, the type I IFN receptor was blocked using specific antibodies at the time of pDC administration i.e. before the secondary challenge. No differences in terms of degree of airway inflammation were found between anti-IFN receptor mAb and isotype treated allergic mice (unpublished data), ruling out a role for this cytokine in the pDC-induced suppression.

In conclusion, our paper provides definite proof that pDCs serve an anti-inflammatory role during eosinophilic airway inflammation

irrespective of their maturation state. The therapeutic effectiveness of Flt3-L on airway inflammation validates the concept that enhancing pDC functions during established airway inflammation could be a potential new strategy for the treatment of asthma.

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Chapter 7

Exposure to Immune Serum and TLR agonists determines the Capacity of Murine Plasmacytoid Dendritic Cells to present Soluble Antigen to $CD4^+$ and $CD8^+$ T cells

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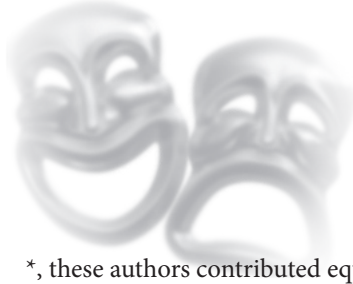

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Dendritic cells (DCs) are the most potent antigen presenting cells. There are different subsets of DCs, that each has a different capacity to present exogenous and endogenous antigens to either $CD4^+$ or CD8+ T cells. However, how and when murine plasmacytoid (p) DCs present exogenous antigen to either $CD4^+$ or $CD8^+$ T cells is currently not clear. Is this study we investigated the capacity of pDCs to induce $CD4^+$ or $CD8^+$ T cell responses in vitro. Plasmacytoid DCs can present exogenous antigens to $CD4^+$ and $CD8^+$ T cells, providing they have been stimulated properly or took up the antigen in the proper context. Simultaneous or sequential activation with the TLR9 agonist CpG motifs and exogenous antigen to pDCs made them capable of inducing $CD8^+$ or $CD4^+$ T cell proliferation. $CD4^+$ T cell proliferation could also be induced when pDCs were exposed to exogenous antigen in the presence of antigen-specific immunoglobulins. In conclusion, pDCs can act as proper antigen presenting cells as long as they are in the appropriate milieu.

Adaptive immunity requires activation of T lymphocytes by dendritic cells (DCs) which present antigen bound by major histocompatibility complex (MHC) molecules. 'Endogenous' antigens (Ag) that are synthesized intracellularly (for example those of viral or tumor origin) are presented by MHC I molecules and activate $CD8^+$ cytotoxic T cells. In contrast, 'exogenous' (extracellular) Ag that gain access to the cell via an endosomal route are presented by MHC II molecules to activate $CD4^+$ T cells. However, the separation of these distinct pools of source proteins is less stringent than originally believed. A mechanism termed cross-presentation permits some forms of exogenous antigen to also stimulate $CD8^+$ T cells via the MHC I pathway [1]. On the other hand, intracellular endogenous proteins can be presented by MHC II to CD4 cells, in a process termed autophagy whereby cytoplasmic content is engulfed into the endosomal pathway [2].

There are various subtypes of antigen presenting DCs and a commonly emerging theme is that there is division of labor between various subsets in various anatomical locations. Splenic $CD8\alpha^+$ conventional (c)DCs are specialized in cross-presenting exogenous antigens to $CD8^+$ T cells, whereas $CD8\alpha^-33D1^+$ cDCs are specialized in presenting exogenous Ag to $CD4^+$ T cells [1, 3]. The reason for these specializations are determined by differential expression of antigen uptake receptors [4, 5] or by differential expression levels of the various enzymes and chaperone molecules associated with antigen processing [6]. Although this division of labor has been clearly described in baseline conditions, it is possible that upon prolonged microbial stimulation, through ligation of pattern recognition receptors like Toll-like receptors (TLRs), the different cDC subtypes can perform multiple functions.

The most recent member of the DC family is the plasmacytoid (p)DC. They are a specialized cell type that produce high levels of type I interferon upon viral stimulation and play a role in the clearance of some viral infections [7-10]. Plasmacytoid DCs also play a critical role in immunoregulation by preventing

overzealous inflammation in the course of a cellular immune response, by inducing the formation of regulatory T cells [11-15].

Although the potential of pDCs to present peptide Ag or to stimulate allo-specific T cells during the mixed leukocyte reaction has been shown on numerous occasions [16, 17], the relative strength of soluble antigen uptake and processing of pDCs versus cDCs for mounting $CD4^+$ and $CD8^+$ T cell responses is still a controversial issue [11, 18, 19]. For presentation to $CD8^+$ T cells, some authors conclude that pDCs only present endogenous Ag, but fail to cross-present exogenous Ag [5, 20, 21]. It could be that cross-presentation only occurs in special conditions, such like TLR triggering by bacterial CpG motifs or viral infection [22, 23], or following targeting of soluble Ag to specific endocytic receptors expressed by pDCs [23, 24]. For presentation to $CD4^+$ T cells, pDCs were initially thought to present exogenous protein Ag only to already primed T cells [11, 25]. Contrasting these early reports, it was shown recently that lymph node pDCs were able to present soluble Ag to naive $CD4^+$ T cells when the antigen was targeted to the bone marrow stromal Ag-2 receptor (BST2) via mPDCA-1, but strangely enough this effect was not seen in splenic pDCs. [21]. Additionally, it was demonstrated that the presence of Ag-specific immunoglobulins stimulate the uptake of Ag by human pDCs, thus promoting the presentation to primed $CD4^+$ Th cells [26]. In many of these studies the possibility that ex vivo purified pDCs or pDC specific targeting studies were contaminated by Ag presentation by other APC subsets was not rigorously excluded.

In view of these emerging, yet conflicting and somewhat confusing studies we thought it would be of interest to study in greater detail under which conditions highly purified pDCs acquire and process exogenous and endogenous antigen and present it to $CD4^+$ and $CD8^+$ T cells. Our results demonstrate that pDCs express the necessary enzymes for processing Ag for MHC I loading and are good at presenting endogenous and cross-presenting exogenous Ag to $CD8^+$ T cells, providing there is simultaneous TLR stimulation. We found that pDCs present exogenous antigen to naive $CD4^+$ T cells only after prolonged TLR stimulation, leading to upregulation of critical MHC II-loading molecules, and when immune serum immunoglobulins target the Ag to acidic MHC II antigen processing organelles via the intermediate affinity Fcγ receptor IIB for immunoglobulin G. Presentation of endogenous Ag on MHC II to $CD4^+$ T cells was not constitutive, and required the presence of a specific Ii80 sorting signal, directing the cytosolic antigen to the endosomal compartment.

Material and Methods

Mice

Female C57BL/6 mice were purchased from Harlan (Zeist, The Netherlands). OT-I (C57BL/6 background) Tg mice containing OVA-specific TCR $CD8^+$ T cells [27] and OT-II (C57BL/6 background) Tg mice containing OVA-specific TCR $CD4^+$ T cells [28] were bred at Erasmus University. At the start of an experiment, mice were between 6-11 wks of age. All experiments were approved by the animal ethics committee at the Erasmus Medical Centre.

Generation of BM DCs in hFlt3-L

Bone marrow (BM) cells were seeded in petridishes (10^6 cells/ml) in DC culture medium (DC-CM, RPMI 1640 containing GlutaMAX-I (Invitrogen) supplemented with 5% FCS (HyClone), 50 µM 2-mercapthoethanol (Sigma) and 50 µg/ml gentamycin (Invitrogen)) with 200 ng/ml human FMS-like tyrosine kinase 3 ligand (hFlt3-L, Amgen) and 50 ng/ml recombinant murine stem cell factor (rmSCF, PeproTech). At day 4, cells were harvested and re-plated ($1*10^6$ cells/ml) in DC-CM supplemented with 200 ng/ml hFlt3-L. At day 7 fresh medium was added. The DCs were harvested and sorted at day 10. BM cells were cultured with hFlt3-L alone to obtain pDCs, $CD24^+$ and $CD24^-$ cDCs to evaluate the mRNA levels of MHC I and MHC II associated proteins.

Flow cytometry

For sorting of pDCs and cDCs, the BM Flt3-L/SCF culture was stained with mAbs against 120G8-FITC (provided by C. Asselin-Paturel), CD11c-APC, CD11b-PercP-Cy5.5 and B220-PE-Cy7 (eBioscience). Sorting of $120G8^+$ $CD11b^-$ $B220^+$ $CD11c^+$ pDCs and $120G8^-$ $CD11b^+$ $B220^-$ $CD11c^+$ cDCs was performed on a FACS Aria (BD Biosciences). BM Flt3-L culture was additionally stained with anti-CD24-PE (BD Bioscience) to be able to discriminate between $CD24^-$ and $CD24^+$ cDCs.

For analysis of antigen uptake, BM Flt3-L/SCF culture was incubated o/n with 10 µg/ml OVA-Alexa Fluor 647 (Molecular Probes). Cells were harvested and stained with 120G8, CD11c, CD11b and B220 to determine the antigen uptake by pDCs and cDCs. To analyze the antigen uptake and processing capacity inside acidic endosomes, OVA-DQ (Molecular Probes) was added to sorted pDCs and cDCs. After o/n incubation fluorescence emitted by OVA-DQ was measured.

To determine the expression of Fc receptors on pDCs, the BM Flt3-L/SCF culture was stained with mAbs directed against CD11c, CD11b, B220, 120G8, FcεRI alpha (eBioscience), FcγRI (BD), FcγRIII and FcγRII/III (R&D). Normal human serum was used to prevent nonspecific antibody binding.

For sorting experiments and T cell stainings, 2.4G2 (anti FcγRII/III) was used to reduce non-specific antibody binding. Acquisition of 6 color samples was done on a FACS ARIA (BD Biosciences). Acquisition of 4 color samples was done on a FACS Calibur (BD Biosciences) and FlowJo software (Tree Star, Inc.) was used for analysis. In all flow cytometry experiments either propidium iodide (PI), TOPRO-3 (Molecular Probes), or DAPI (Molecular Probes) was used to discriminate between live and dead cells.

Antigen uptake and processing by pDCs

To determine the antigen uptake and processing sorted pDCs were plated at a concentration of $1*10^6$ cells/ml with 10 µg/ml OVA-DQ with or without CpG motifs (ISS-ODN 1668, 10 µg/ml). Simultaneously heat-inactivated normal mouse serum (NMS), OVA-immune serum (serum from mice sensitized twice with OVA-alum and challenged with OVA aerosols [29]), or OVA-immune serum pre-treated with protein G-sepharose beads was added to the pDCs.

To investigate the role of FcγRIIB, pDCs were pre-incubated with 10 µg/ml K9361 (anti FcγRIIB, a kind gift from J.N. Samsom, ErasmusMC, The Netherlands, [30]) for 1 hr at RT, thereafter the antibody was present during o/n incubation.

In some experiments, part of the pDCs was pulsed with CpG motifs (10 µg/ml) o/n before exposure to OVA-DQ. Uptake and processing of OVA-DQ was measured 8 hrs after exposure to OVA-DQ.

Antigen presentation to T cells in vitro

OVA-specific TCR Tg $CD8^+$ T cells (OT-I) or $CD4^+$ T cells (OT-II) were isolated from spleen and lymph nodes by depletion of non-$CD8^+$ or non-$CD4^+$ T cells respectively, using MACS (Miltenyi Biotec) and labeled with CFSE (Invitrogen) as previously described [31]. $2*10^4$ pDCs or cDCs were cultured with $2*10^5$ $CD8^+$ or $CD4^+$ T cells in the presence of OVA-peptide (2 ng/ml SIINFEKL (MHC I peptide) or 0.1 µg/ml ISQAVHAAHAEINEAGR (MH II peptide, $OVA_{323-336}$) or 10 µg/ml OVA protein (Worthington). After 4 days, cells were harvested and OVA-specific T cell proliferation was determined by staining CFSE labeled OT-I or OT-II cells with anti-Vβ5-PE (BD Bioscience) and anti-Vα2-APC (eBioscience) [32]. Data are shown as percentage of T cells recruited into cell division, calculated as previously described [33].

Retroviral transduction of BM cells

For the production of retrovirus the retroviral vector MFG, derived from moloney murine leukemia virus was used. MFG-tOVA contains cDNA encoding a truncated form of

the OVA protein (amino acids 40-386) and MFG-Ii80-OVA contains amino acids 242-353 of the ovalbumin protein fused to invariant chain of which the CLIP sequence was skipped. Retroviral particles were produced by transient transfection of phoenixECO as described [34].

BM DCs were generated as described above with minor modifications. At the start of the culture lineage positive cells are removed from the BM using anti-CD4, anti-CD8, anti-CD45R, anti-Ly6G and anti-I-A/I-E antibodies. The lineage negative cells are plated in 24-well culture plates (10^6 cells/ml, 1 ml/well) with DC-TC supplemented with 200 ng/ml hFlt3-L and 50 ng/ml rmSCF. Transduction with MFG-tOVA and MFG-Ii80-OVA was performed as previously described [35].

Real-time quantitative RT-PCR

Frozen cell pellets were homogenized, RNA was isolated using RNeasy miniprep (Qiagen) with on column DNase I treatment according to manufacturer's protocol. 100 ng RNA was reverse transcribed using random hexamers (Amersham Biosciences, Roosendaal) and Superscript II (Invitrogen) for 50 min at 42°C. Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA) and pre-designed 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 secs at 95°C and 60°C for 1 min using an ABI PRISM 7000 (Applied Biosystems). PCR amplification of the housekeeping gene ubiquitin C was performed during each run for each sample to allow normalization between samples.

Results

Plasmacytoid DCs cross-present exogenous protein antigen to CD8$^+$ T cells only when triggered simultaneously with TLR9 agonist, but fail to present it to CD4$^+$ T cells

There a several papers describing pDCs to be good APCs for CD4$^+$ and CD8$^+$ T cell responses, although some only show antigen presentation of antigenic peptides and not whole protein. Also, the purity of the pDCs used for stimulation could confuse the interpretation of data, since we found that contamination of only 4-5% cDCs contributes significantly to the antigen presentation capacity of pDCs (data not shown). We therefore used highly purified pDCs, derived from bone marrow cultures in Flt3-L and SCF, and purified using high speed cell sorting to over 98% purity based on expression of CD11c, BST2 (recognized by the clone 120G8), B220, and lack of expression of CD11b. Conversely, cDCs were identified based on expression of CD11c, CD11b and lack of 120G8 and B220, as previously described [31]. To exclude any possible bystander effects of cDCs on the potential of pDCs to take up and process antigen, pDCs were separated from cDCs before exposure to ovalbumin (OVA) antigen overnight. BM-derived pDCs were viable after overnight culture in the presence of exogenous OVA, but were incapable of inducing cell division in purified OVA-specific CD8$^+$ or CD4$^+$ T cells taken from naive OT-I or OT-II mice respectively (Fig 1A). However, sorted pDCs loaded with OVA MHC I or MHC II peptide induced CD8$^+$ and CD4$^+$ T cell proliferation. The induction of naive T cell division by APCs is a function of provision of signal 1 (MHC-peptide) and signal 2 also known as costimulation, that is often induced by triggering of innate pattern recognition receptors such as TLRs. In the presence of the well known endosomal TLR9 agonist bacterial CpG motif, OVA-pulsed pDCs upregulated the expression of numerous costimulatory molecules (including CD80, CD86 and CD40, data not shown) and at this

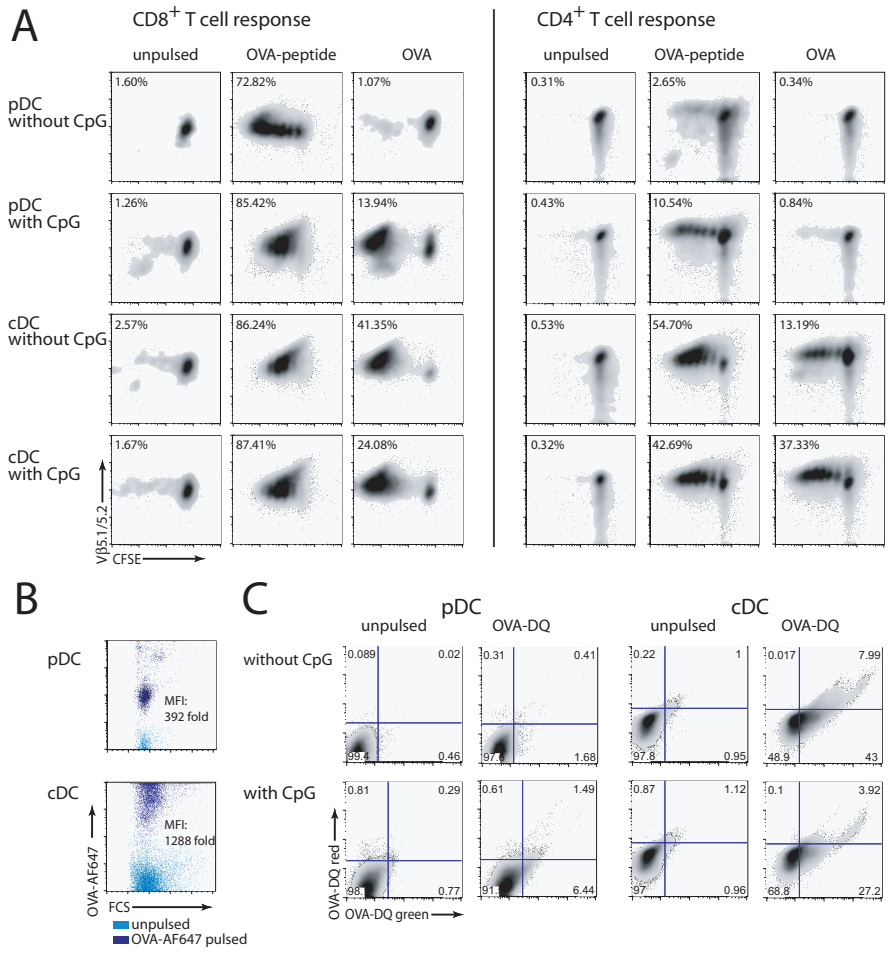

Figure 1

Mouse plasmacytoid DCs are incapable of presenting exogenous antigen to CD4⁺ T cells

Plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L and SCF. On day 9, they were purified by FACS sorting. **(A)** Purified cells were pulsed o/n with OVA or OVA-peptide with or without CpG motifs. On day 10, the cells were washed and placed in co-culture with either CFSE-labeled CD8⁺ T cells or CD4⁺ T cells. On day 14, T cell proliferation was measured by flowcytometry. Percentages in plots indicate percentage of cells recruited into cell division (see M&M for calculation). **(B)** Purified cells were pulsed o/n with OVA-Alexa Fluor 647 (AF647). On day 10 the antigen uptake was measured by flowcytometry. **(C)** Purified cells were pulsed o/n with OVA-DQ with or without CpG motifs. On day 10 the antigen processing was measured by flowcytometry. All plots represent experiments repeated 2-5 times.

time induced CD8⁺ T cell proliferation, as shown by an increase in the percentage of OT-I cells recruited into cell division (from 1.07% without CpG to 13.94% with CpG). However, a concomitant CD4⁺ T cell response was not induced under these conditions. As a control, cDCs could induce both a CD8⁺ and CD4⁺ T cell response after exposure to the OVA protein and MHC I and MHC II peptide, and additional maturation with CpG motifs showed

a stronger $CD4^+$ T cell proliferative response and a reduction in $CD8^+$ T cell proliferation (Fig 1A, bottom panels).

The absence of T cell responses to exogenous OVA protein induced by immature pDCs could be due to a lack of uptake of the protein. However, the capability of freshly sorted pDCs to internalize protein antigen was shown by uptake of OVA-Alexa Fluor 647 (Fig 1B), although on a per cell basis, pDCs acquired less antigen than cDCs (392 fold increase of the fluorescent signal over unpulsed cells compared to 1288 fold increase in cDCs). Loading of MHC II molecules occurs in an acidic endosomal compartment, rich in MHC II molecules, the so-called MIIC compartment. To follow if OVA would be targeted to these acidic organelles, OVA-DQ was used as a tracer. OVA-DQ is a quenched non-fluorescent protein that becomes fluorescent green when present in acidic endosomes and also fluorescent red when it accumulates at high concentrations in these compartments. Unstimulated pDCs did not show any positive signal for either OVA-DQ green or red, showing an incapability of targeting the antigen to acidic endosomes or an inefficiency in acidifying the endosomes (Fig 1C), whereas cDCs showed clear accumulation of OVA-DQ in these endosomes (50% of cDCs showing evidence of accumulation). The addition of CpG motifs only marginally increased the processing of OVA-DQ in pDCs from 2.09% to 7.93%.

pDCs and cDCs differentially express enzymes and chaperones involved in MHC I and MHC II processing

Many of the proteins that regulate MHC I and MHC II processing pathways have been described and their expression documented in DCs [6]. To evaluate if pDCs had intrinsic differences compared with cDCs in the expression of any of these proteins, the mRNA levels were compared by q-RT-PCR. In view of the recent findings that different subtypes of cDCs have differential intrinsic expression of these factors [6], we additionally separated $CD24^+$ cDCs (in vitro equivalent of splenic $CD8\alpha^+$ cDCs [31]) from $CD24^-$ cDCs (equivalent to $CD8\alpha^-$ splenic cDCs) from the Flt3-L BM culture. As expected, and lending further proof to the idea that CD24 expression reliably separates the different subsets of cDCs, we found that $CD24^-$ cDCs and $CD24^+$ cDCs differentially expressed components of the MHC I and MHC II pathway. mRNA levels of proteins which regulate the MHC I processing (such as the chaperone molecule calnexin, the peptide loading complex consisting of TAP, thiol-oxidoreductase ERp57, calreticulin and tapasin; and the ER retrotranslocation machinery Sec61a1, Sec61b and Sec61g) were expressed in both subsets of cDCs (Fig 2A). Notably however, expression of the MHC II degradative enzymes (Cathepsins S, H and C; asparagine endopeptidase; GILT and the cathepsin regulator Cystatin C), the chaperone molecule Invariant chain (Ii), and the CLIP exchanger H2-DMb1 were highest in the $CD24^-$ cDC subset. In comparison with cDCs, the pDCs had lower expression of TAP1 and TAP2, although the rest of the MHC I processing machinery resembled the $CD24^+$ cDCs. pDCs had very little MHC II processing machinery

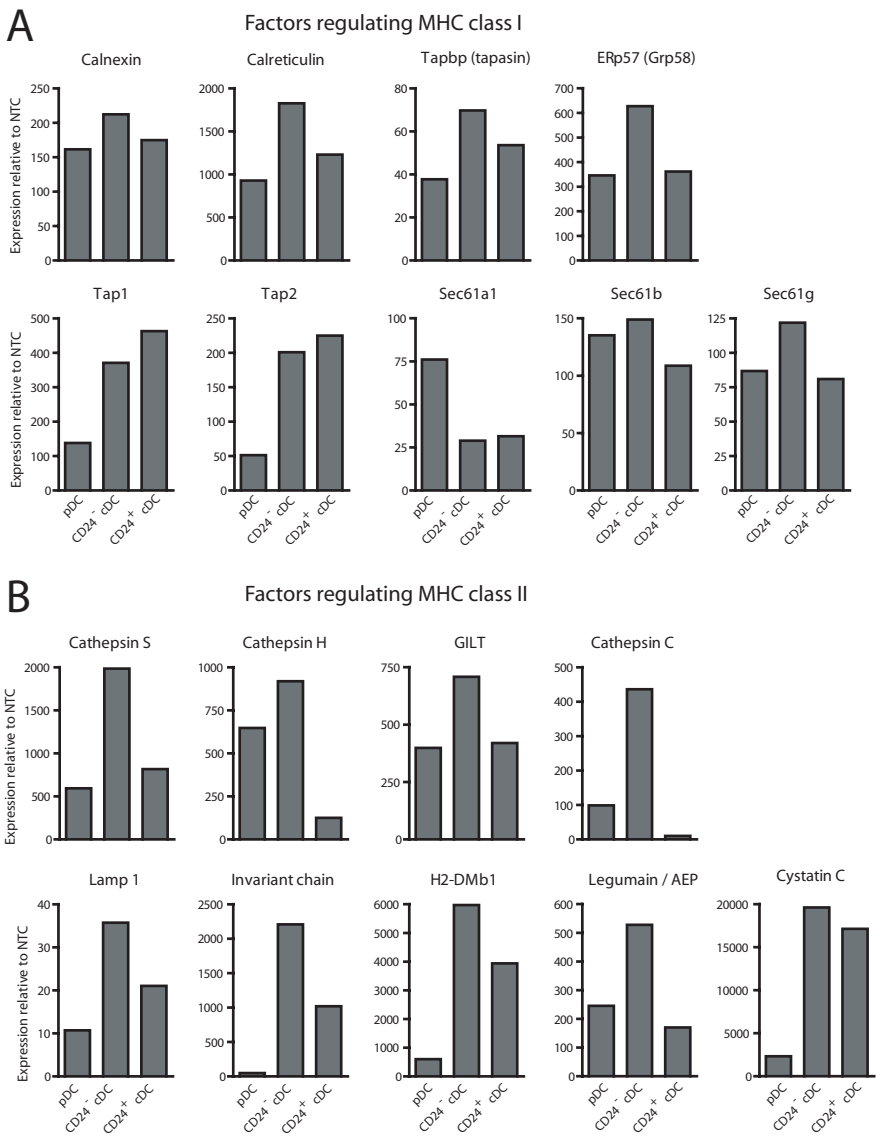

Figure 2

Mouse plasmacytoid DCs express low RNA levels of proteins associated with the MHC II pathway

Plasmacytoid and conventional CD24$^+$ and CD24$^-$ DCs were cultured from bone marrow with Flt3-L. On day 9, all 3 cell populations were purified by FACS sorting. **(A)** RNA levels of proteins associated with the MHC I pathway. **(B)** RNA levels of proteins associated with the MHC II pathway.

compared with CD24$^-$ cDCs, and notably the expression of the chaperone molecule Ii and the CLIP exchanger H2-DMb1 was almost absent in freshly isolated pDCs (Fig 2B).

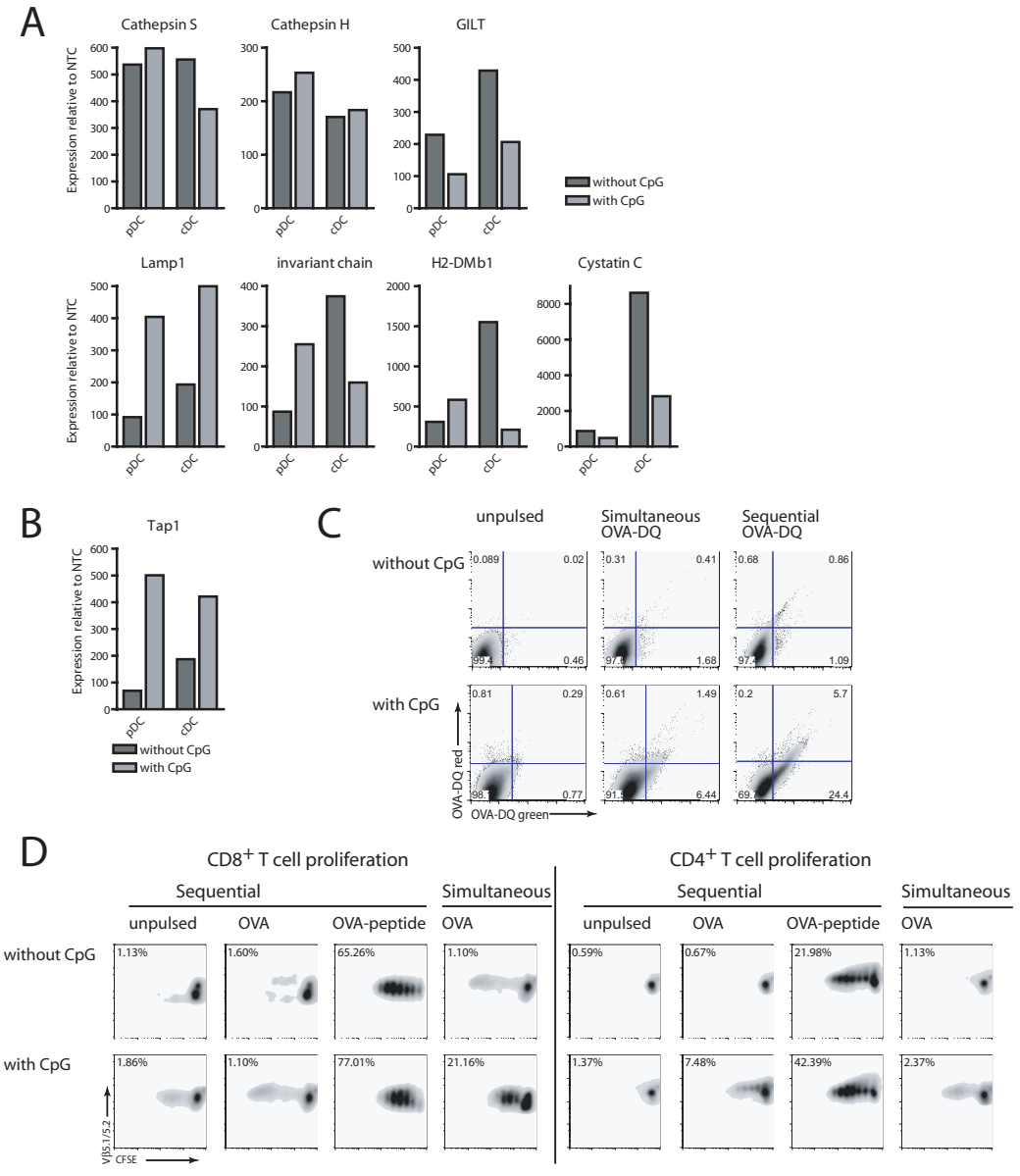

Figure 3

Activation of mouse plasmacytoid DCs with CpG motifs increases MHC II antigen processing

Plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L and SCF. **(A)** On day 9, cells were purified by FACS sorting and pulsed o/n with or without CpG motifs. RNA levels of proteins associated with the MHC II pathway. **(B)** RNA levels of TAP1 associated with the MHC I pathway. **(C)** On day 9, purified pDCs were either simultaneous or sequentially pulsed with or without CpG motifs and OVA-DQ. **(D)** On day 9, purified pDCs were either simultaneous or sequentially pulsed with or without CpG motifs and OVA. On day 10 they were placed in co-culture with CFSE-labeled $CD8^+$ or $CD4^+$ T cells. On day 14, T cell proliferation was measured by flowcytometry. Percentages in plots indicate percentage of cells recruited into cell division. All experiments are repeated at least 2 times.

TLR9 ligation by CpG motifs influences the MHC class I and II processing machinery in pDCs and cDCs differentially

Since pDCs could induce $CD8^+$ T cell proliferation after exposure to OVA antigen mixed with CpG motifs (Fig 1A), we wondered whether the expression profile of the critical MHC I and II pathway molecules would be influenced by CpG motif exposure. Indeed, factors regulating the MHC II pathway, like H2-DMb1, cathepsin H, cathepsin S, Lamp1, and invariant chain were increased when pDCs were exposed to CpG motifs (Fig. 3A). Especially the increased expression H2-DMb1, cathepsin S and invariant chain was striking, since the expression level of these mRNA levels in cDCs were regulated in a differential manner, showing a decrease upon exposure. Furthermore, TAP1, associated with MHC I pathway was increased in both pDCs and cDCs when compared to non-pulsed cells (Fig 3B). Alternatively, in both pDCs and cDCs the expression of cystatin C was decreased.

Since the expression of cathepsin S increased and its negative regulator cystatin C decreased whereas that of Ii invariant chain was strongly increased upon CpG exposure, we wondered if pDCs that were pretreated with CpG motifs before exposure to the exogenous protein antigen would increase their capacity to process the antigen for MHC II presentation. For this we exposed the purified pDCs for 18 hrs with CpG motifs and thereafter exposed cells for 8 hrs with OVA-DQ (sequential administration) or exposed them for 18 hrs with CpG motifs and OVA-DQ simultaneously (Fig 3C). pDCs exposed simultaneously to CpG and OVA-DQ showed only a minor fraction of cells (6.4%) that had processed the antigen in acid organelles (as shown by green fluorescence of OVA-DQ), whereas 24.4% exposed sequentially to CpG and OVA-DQ showed signs of processing, and a small fraction of cells accumulated OVA at high density inside these acidic organelles, as shown by red OVA-DQ fluorescence. To address if pre-activation of pDCs with CpG induced a better $CD4^+$ T cell response, the sequentially treated pDCs were placed in co-culture with T cells and proliferation was measured. pDCs exposed to OVA and CpG simultaneously stimulated only 2.4% $CD4^+$ T cell to go into division, whereas pDCs sequentially exposed to CpG and OVA 7.5% T cell went into division (Fig 3D, right panel). Strikingly, pDC pre-activation reduced their capacity to induce $CD8^+$ T cell proliferation (Fig 3D, left panel), as only 1.1% of T cells divided compared to 21.2% when simultaneously pulsed.

The presence of immune serum dramatically alters the potential of pDCs to process Ag for presentation to $CD4^+$ T cells

It has been shown that human pDCs could internalize antigen only in the presence of antigen-specific serum [26]. As these findings are at odds to our findings of constitutive antigen uptake by BM-derived pDCs in vitro (Fig 1B), we investigated the influence of OVA-immune serum on OVA uptake and processing by pDCs, and focused specifically on the capacity of serum to boost $CD4^+$ T cell responses. We first examined the effect of serum

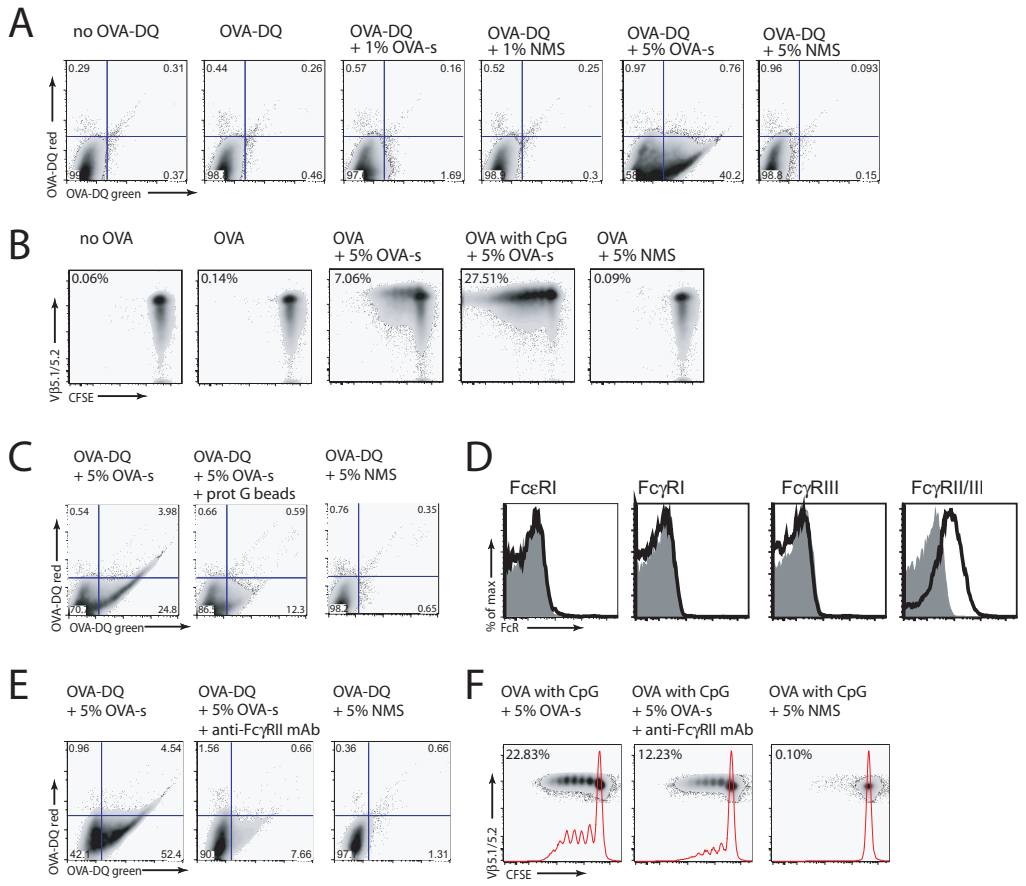

Figure 4

Exposure of mouse plasmacytoid DCs with antigen and antigen-specific serum induces MHC II processing dependent on FcγRII expression

Plasmacytoid DCs were cultured from bone marrow with Flt3-L and SCF. **(A)** On day 9, cells were purified by FACS sorting were pulsed o/n with OVA-DQ in the presence of 1 or 5% NMS or OVA-immune serum. On day 10, antigen processing was measured by flowcytometry. **(B)** On day 10, purified pulsed pDCs were placed in co-culture with CFSE-labeled CD4+ T cells. On day 14, T cell proliferation was measured. Percentages indicated in the plots represent the percentage of cell recruited into cell division. **(C)** OVA-immune serum was pretreated with protein-G beads before exposure to purified pDCs and OVA-DQ o/n. **(D)** Expression levels of different FcR on mouse pDCs. Grey histograms represent isotype control, black line the depicted FcR. **(E)** Purified pDCs were treated with a blocking FcγRII mAb before and during exposure to OVA-immune serum and OVA-DQ o/n. After 20 hrs antigen processing was measured by flowcytometry. **(F)** Purified pDCs were treated with a blocking FcγRII mAb before and during exposure to OVA-immune serum and OVA o/n. After 20 hrs, treated pDCs were placed in co-culture with CFSE-labeled $CD4^+$ T cells. Percentages in plots represent percentage of T cells recruited into cell division. All expts were repeated 2-4 times.

on the capacity to direct the antigen in acidic endosomes for MHC II loading by exposing pDCs to OVA-DQ. As expected, pDCs exposed to OVA-DQ without serum did not show any signs of OVA-DQ processing. However, 40% of pDCs exposed to OVA-DQ in the presence of 5% OVA-immunized serum showed signs of accumulation inside acidic endosomes, an effect

not seen when NMS was added (Fig 4A). To address the effect of serum on Ag presentation, pDCs exposed to OVA-immunized serum and OVA were cultured with antigen-specific CD4$^+$ T cells. Again, pDCs pulsed with OVA did not induce CD4$^+$ T cell proliferation, but pDCs exposed to OVA in the presence of OVA-immune serum could induce CD4$^+$ T cell proliferation, an effect that was further enhanced when CpG motifs were added (Fig 4B). The effect of serum could be any serum factor, like complement or immunoglobulins, or the presence of a cytokine factor. Heat-inactivated OVA-immunized serum induced a comparable processing of OVA-DQ as non-heat-inactivated OVA-immunized serum (data not shown), arguing against a role for a cytokine or complement activation. However, when OVA-immunized serum was depleted of IgGs by protein G-sepharose beads, OVA-DQ processing was decreased by at least 50% (Fig 4C).

Immunoglobulins bind to a variety of Fc receptors (FcγRI-IV) on the surface of phagocytic cells. Examination of FcγRs and FcεRs on pDCs showed that only FcγRII was expressed as the mAb detecting FcγRIII did not show specific staining whereas the 2.4G2 antibody recognizing FcγRII/III did (Fig 4D). Blocking the FcγRII using a specific Ab before and during exposure to OVA-immune serum and OVA-DQ reduced the processing of OVA-DQ significantly (from 52.4% OVA-DQ green to 7.66%) (Fig 4E) and consequently, T cell proliferation was also reduced, implying that the presence of serum immunoglobulins enhanced Ag presentation by routing Ag to the acidic endosomal compartment where MHC II is loaded with peptide.

Presentation of endogenous Ag by pDCs to CD4$^+$ and CD8$^+$ cells depends on its localization inside the cell

It has been suggested that pDCs only present endogenous, but not exogenous protein and

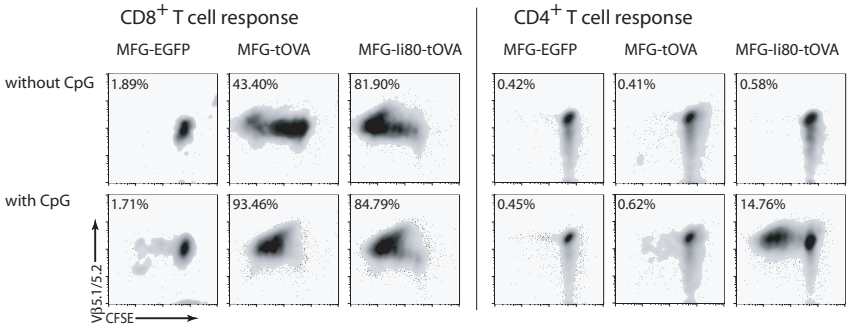

Figure 5
Only when OVA is targeted to MIIC mouse plasmacytoid DCs can induce CD4$^+$ T cell proliferation
Plasmacytoid DCs were cultured from bone marrow with Flt3-L and SCF and transduced with MFG-EGFP, MFG-tOVA or MFG-li89-tOVA as described in the M&M. On day 10, pDCs were purified using a FACS sorter and placed in co-culture with CFSE-labeled CD8$^+$ or CD4$^+$ T cells. On day 14, T cell proliferation was measured. Percentages indicated in the plots represent the percentage of cell recruited into cell division. Transduction efficiency was 46%. Experiment was repeated twice.

viral antigen to $CD8^+$ and $CD4^+$ T cells [20]. To address this question, we made use of retroviral over-expression of a cytoplasmic truncated form of OVA (tOVA), coding for amino acids 40-386, from which the ER translocation sequence is removed and thus is not secreted by transduced cDCs in the supernatant, nor presented to $CD4^+$ T cells [34]. pDCs transduced with this form of OVA did present Ag to $CD8^+$ T cells, an effect that was strongly enhanced when also exposed to CpG bacterial motifs overnight prior to T cell stimulation (Fig 5, left panel). Some endogenous Ag can be presented by MHC II molecules, in a process relying on autophagy. However, tOVA-transduced DCs did not present to $CD4^+$ T cells, not even after CpG exposure overnight (Fig 5, right panel). One of the possible reasons for this deficiency to present to $CD4^+$ T cells might be the expression of tOVA in the cytoplasm, thus effectively shielding the antigen from endosomal routing. To redirect the tOVA to the MHC II rich endosomal compartment (MIIC), we additionally transduced pDCs with a retrovirus carrying a fusion protein of tOVA and the Ii sorting sequence (Ii80) that is involved in routing of Ii to the MIIC compartment [36]. When pDCs were transduced with Ii80-tOVA (Fig 5, right panel), they were still able to induce $CD8^+$ T cell proliferation, but strikingly also capable of inducing strong $CD4^+$ T cell proliferation, but only when cells were pulsed with CpG motifs.

Discussion

One of the most predominant tasks of a dendritic cell is its potential to take up, process and present antigen to $CD4^+$ and $CD8^+$ T cells. Antigen uptake and processing for MHC I or MHC II restricted Ag presentation might depend on intrinsic properties of particular DC subsets [4, 37]. As such it was suggested that the way antigen is taken up by DC subsets differs fundamentally between cDC subsets, endocytosis via the mannose receptor and early endosomes favoring cross-presentation to $CD8^+$ cells and uptake via pinocytosis or scavenger receptors leading to routing to acidic late endosomes and lysosomes and thus favoring $CD4^+$ T cell responses. Additionally, cDC subsets differ fundamentally in the level of expression of enzymes and chaperones implicated in MHC I and MHC II processing [6].

Here, we have addressed the controversial issue whether pDCs are good antigen presenting cells and perform specialized tasks differentially from cDCs in presenting exogenous or endogenous Ag to $CD4^+$ or $CD8^+$ T cells. For this, we have used highly purified pDCs that we obtained from the bone marrow of mice grown in Flt3-L, and sorted to purity by high speed cell sorting prior to exposure to antigen, to avoid any bystander effects of other APCs on the potential of pDCs to process and present antigen. Indeed, a contamination of even a few percents of cDCs to a pDC preparation might dramatically mislead the conclusion whether a pDC can present or not.

Plasmacytoid DCs were first considered to have a minor role in antigen uptake [19] compared with cDCs [11]. It was only very recently shown again that human blood-derived pDCs did not

take up soluble antigen, unless antigen specific immune serum was added in vitro, acting to enhance Ag uptake [26]. This is at odds with a report by Sapovnikov et al showing uptake of subcutaneously injected Ag by mouse lymph node pDCs and our own previous studies in which inhaled fluorescent OVA was found inside vacuoles of mediastinal LN pDCs of naive mice [11, 21]. The acquisition of soluble Ag could be indirect in the latter studies, and obtained after transfer from other APCs. We now show in this paper that highly purified bone marrow-derived pDCs do take up soluble antigen in the absence of serum components. Conventional DCs were compared with pDCs obtained from the same Flt3-L culture, and were also shown to take up Ag constitutively, albeit at higher levels than pDCs. The addition of serum immunoglobulins did affect the cellular routing of antigen by pDCs, as assessed by the use of the pH-sensitive OVA-DQ probe, allowing us to see a routing of Ag to acidic endosomal organelles, where antigen is loaded onto MHC II molecules, in the presence of serum. The cDCs did not require the presence of serum to route OVA-DQ into these acidic endosomes. The serum-mediated routing of pDCs was due to a heat stable component in serum, was only seen when OVA-immune serum was used, was neutralized by prior treatment and extraction of serum by protein G affinity magnetic beads and was neutralized to a large extent when the FcγRII receptor was blocked. This strongly implies the influence of serum Ag-specific immunoglobulins in influencing the routing of Ag to acidic organelles. The internalization of immune complexes via FcγRII might have a similar effect as entry via the scavenger receptor found on CD8α$^-$ splenic cDCs and macrophages, which also route their Ag to the acidic endosomes [4]. The same FcγRII receptor was also implied in a prior human study to mediate Ag uptake by human pDCs [26]. We cannot explain at present however why human pDCs would also rely on this receptor for uptake in addition to routing of internalized antigen.

We also addressed the functional capacities to present Ag to CD4$^+$ and CD8$^+$ T cells. For presentation to CD4$^+$ T cells, pDCs were initially thought to present exogenous protein Ag only to already primed but not to naive T cells [11, 25]. Contrasting these early reports, it was shown recently that lymph node, but not splenic, pDCs were able to present soluble Ag to naive CD4$^+$ T cells in vivo when the antigen was targeted to the BST2 receptor via mPDCA-1. [21]. In the latter study, residual Ag presentation in mice depleted of cDCs using CD11c-mediated expression of diphteria toxin receptor was taken as evidence that pDCs presented Ag to CD4$^+$ T cells. These studies are not without technical difficulties and in our hands cDC depletion is never total in CD11c-DTR mice given DT [10, 38, 39]. We found, using BM-derived pDCs that fresh BM-pDCs, in contrast to cDCs sorted from the same cultures, did not present exogenous Ag to CD4$^+$ T cells, whereas they readily did present processed peptide to CD4$^+$ T cells, particularly when exposed to CpG motifs to induce phenotypical maturation. The reason for the failure to present Ag to CD4$^+$ T cells might be the lack of expression of invariant chain

(Ii), critical for sorting of MHC II molecules to the acidic MIIC endosomal compartment and of the other enzymes involved in MHC II processing such as cathepsin S, Cathepsin C, GILT, LAMP1, legumain and the peptide exchanger H2-DMb1. In this regard, pDCs resembled the expression pattern of the $CD24^+$ cDCs from the same Flt3-L culture. It is exactly this subset that is the in vitro equivalent of the $CD8\alpha^+$ cDC subset from the spleen that is also known to be poor at presenting exogenous Ag to $CD4^+$ T cells [6, 31]. Another explanation for the lack of $CD4^+$ T cell responses to exogenous Ag induced by pDCs might be the routing of internalized Ag away from acidic endosomes (see discussion above) where MHC II complexes are loaded.

An overnight exposure to CpG motifs followed in sequence by Ag exposure led to an increase in Ii expression and H2-DMb1 expression and consequently, more $CD4^+$ T cell proliferation was induced. Strikingly, the inverse was seen in cDCs exposed to CpG motifs. There was also increased routing of internalized OVA-DQ into acidic organelles by sequential CpG motifs and OVA exposure (see Fig 3C). Antigen uptake could have changed due to endocytic receptor expression changes, thereby directing cargo to different cellular compartments. This seems unlikely as we could not detect any increase in c-type lectin receptor (CD206, Ly49Q) expression on pDCs after CpG activation ([40] and data not shown). Even more strikingly, the combination of CpG motifs and OVA-immune serum on pDCs induced T cell proliferation in 27% of $CD4^+$ T cells (Fig 4B), an effect of almost identical strength as that induced by cDCs exposed to CpG motifs (37% Fig 1A).

We also addressed whether endogenous Ag, expressed from a retroviral vector as a truncated form that is not secreted into the supernatant, would be presented to $CD4^+$ T cells by pDCs. This process has been shown to occur in cells undergoing macro-autophagy or chaperone mediated autophagy (MCA), a process by which cytosolic proteins can gain access to the acidic endosomal organelles [41]. We did not find evidence for this pathway in pDCs (Fig 5). However, when the endogenous Ag was artificially routed to these acidic endosomes using the Ii80 sorting sequence [34], strong $CD4^+$ T cell responses were seen, particularly following CpG motifs exposure, illustrating that there is an intrinsic capacity of pDCs to present to $CD4^+$ T cells, providing the antigen is in the right intracellular compartment. We therefore favor the view that pDCs can prime $CD4^+$ T cell responses predominantly to exogenous Ag, but only when previously stimulated adequately by microbial stimuli and in the presence of specific immune globulins.

For presentation to $CD8^+$ T cells, some authors have suggested that pDCs only present endogenous Ag, but fail to cross-present exogenous Ag [5, 20, 21]. Our findings now clearly show that purified pDCs can indeed present an endogenously expressed antigen to naive $CD8^+$ T cells almost constitutively (Fig 5), and the process is enhanced by exposure to CpG motifs, the latter acting most likely by increasing the expression of co-stimulatory molecules that promote T cell expansion. In support, pDCs expressed all the machinery to load cytosolic peptides into the ER for loading

onto MHC I (Fig 2).

As for cross-presentation of exogenous Ag (Fig 1), we found that exogenous OVA was presented only under particular conditions, and pDCs were weaker at presentation compared with cDCs. Concurrent exposure of OVA antigen with CpG motifs was necessary to promote cross-presentation by pDCs (Fig 1). The CpG motifs induced the expression of TAP1 to levels seen in cDCs. Recently, it has been shown that cross-presentation is biased to antigens which contain microbial molecular patterns [37], which could also explain why pDCs pulsed with CpG and antigen simultaneously induce stronger $CD8^+$ T cell responses. Additionally, the antigens which are cross-presented are better protected from proteases when high NADPH oxidase (NOX2) levels are present [42]. It has been shown that pDCs release more O_2^- radicals (NADPH oxidase is the source of O_2^- radicals) after TLR ligation [43]. Strikingly, a sequential exposure of CpG motifs followed by exogenous Ag abolished the potential of pDCs to cross-present OVA (Fig 3) whereas it boosted $CD4^+$ T cell responses under these conditions. This could be due to the routing of Ag into more acidic organelles induced by prior stimulation by CpG motifs (see Fig 3C), or an acidification of endosomal organelles in response to prolonged CpG, thus exposing endocytosed material to a more hostile proteolytic environment not conductive to cross-presentation [37, 44].

We believe therefore that the conclusions drawn by Salio on the inefficiency of CpG-matured pDCs to cross-present soluble Ag to $CD8^+$ T cells can be explained by effects of timing of CpG exposure in relation to Ag exposure [20]. On the contrary, the findings of Sapoznikov that pDCs fail to cross-present might be explained by the fact that no maturation stimulus in the form of CpG or viral infection was used at all to boost pDC maturation [21]. As supported by our data, cross-presentation by pDCs has indeed been shown to occur only after TLR triggering by bacterial CpG motifs [22], viral infection [23], or targeting of soluble Ag to the SiglecH endocytic receptor expressed by mouse pDCs [24] or BDCA2 on human pDCs [23] in combination with a proper pDC maturation stimulus.

In conclusion, we have demonstrated the conditions under which pDCs might better present endogenous or exogenous antigen to $CD4^+$ and $CD8^+$ T cells. Although our study was performed purely in vitro, our findings might help to understand immunoregulation by pDCs in vivo. As the function of constitutive presentation of endogenous self antigen to $CD8^+$ T cells in the absence of pDC maturation might be the maintenance of peripheral tolerance in the immune system [45], this might have implications for understanding autoimmunity. Cross-presentation occurs only upon simultaneous exposure of antigen and CpG motifs, as one would expect during acute infection, and might contribute to the generation of $CD8^+$ T cells that are necessary to kill infected cells [8, 22]. Conversely, pDCs present antigen to $CD4^+$ T cells only after prolonged TLR stimulation and in the presence of Ag specific immunoglobulins, a conditions under which they no longer cross-present to $CD8^+$ T cells. This situation could occur

late during viral or bacterial infections when serum immunoglobulins start to be produced. We have some preliminary evidence that pDCs exposed to Ag specific serum and CpG motifs promote the generation of $CD4^+Foxp3^+$ Treg cells, which might serve to terminate the inflammatory reaction towards the end of a viral or bacterial infection. Alternatively, pDCs might work by promoting $CD4^+$ T cell responses that might help immunoglobulin class switching, thus acting as a positive feedback loop on immunoglobulin synthesis [10]. Further in vivo work will have to be performed to address these possibilities.

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Chapter 8

General Discussion and Summary

Dendritic cells (DCs) are the masters of command of innate and adaptive immunity. First, through their capacity to release cytokines and chemokines necessary to kill an invading microbe and attract/stimulate other cells involved in innate immunity, like neutrophils and macrophages. Secondly, through their capacity to take up and process antigen in antigen exposed areas, and present it in an immunogenic form to naive T cells after migration to the central lymphoid organs. However, in steady state DCs also play a crucial role in regulating the immune response to self-antigens.

Steady state

DCs are heterogeneous. Recently, the discrimination has been made accordingly to difference between steady state and during inflammation and/or infection [1]. In steady state, the different DC subsets can be divided into plasmacytoid DC (pDC), precursor DC (monocytes), and conventional DC (cDC) (see figure 1). The latter can be further divided into migratory and lymphoid tissue-resident cDC. The migratory cDCs serve as sentinels in the periphery, e.g. lungs or skin. After antigen capture in the peripheral tissue, these cDCs migrate to the draining lymph nodes, and present antigen to naive T cells in the lymph node.
The lymphoid tissue-resident DC can be divided into $CD8\alpha^+$ cDC and $CD8\alpha^-$ cDC, with a further subdivision classified by CD4 expression. These cDCs do not migrate through lymph vessels, but they collect their antigen from the surrounding lymph. This can either be self-antigens or soluble antigens which have reached the lymph node via the lymphatics. Yet, the $CD8\alpha^+$ cDCs process the antigen different than the $CD8\alpha^-$ cDCs. It has been shown that $CD8\alpha^+$ cDCs are good at cross-presentation (presenting exogenous antigen in MHC I) and $CD8\alpha^-$ cDCs at MCH II presentation [2]. We could confirm by q-RT-PCR that indeed the $CD24^+$ cDCs (in vitro equivalent of $CD8\alpha^+$ cDCs in Flt3-L culture) expressed more TAP1 and TAP 2 (associated with MHC I processing [3]) than $CD24^-$ cDCs (**Chapter 7**). On the other hand, $CD24^-$ cDCs (in vitro equivalent $CD8\alpha^-$ cDCs in Flt3-L culture) expressed more MHC II proteins than $CD24^+$ cDCs. This confirmed that the $CD24^+$ and $CD24^-$ cDCs in culture resemble the $CD8\alpha^+$ cDC and $CD8\alpha^-$ cDCs in vivo and have the same antigen processing capacities. The pDCs found in steady state are thought to have a regulatory role, facilitating either the formation of regulatory T cells [4], T cell anergy [5], or keeping the cDCs immature (unpublished data). **Chapter 7** shows that pDCs do not present exogenous antigen to either $CD4^+$ or $CD8^+$ T cells in an immature state. However, when the antigen is endogenously present (in the case of viral transduction of pDCs with OVA), the antigen is presented only to naive $CD8^+$ T cells. The phenomenon that pDCs can present endogenous antigens has been described before for viral antigens [6, 7].

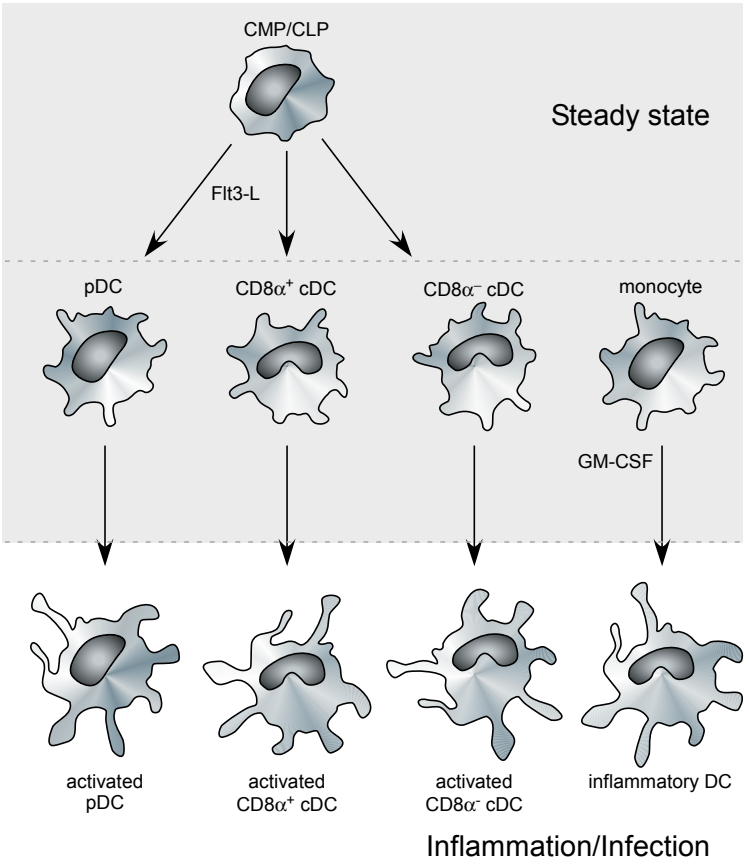

Figure 1
Different dendritic cell subsets in steady-state and inflammation or infection
Steady state DCs (pDC, CD8α$^+$ cDC, and CD8α$^-$ cDC) arise from common myeloid progenitors (CMP) and/or common lymphoid progenitors (CLP) and differentiate under the influence of Flt3-L. In inflammation or infection these steady state DCs all become activated and inflammatory DCs arise from inflammatory monocytes under the influence of GM-CSF.

Inflammation or infection

During inflammation or infection, DCs behave in a different way. First, they are activated through recognition of microbes via their pattern recognition receptors (PRRs), after which they release different anti-viral or anti-inflammatory cytokines [8, 9]. pDCs are well known for their anti-viral function [10-13]. Upon encounter with a virus, the pDC is activated via TLR9, TLR7, or TLR8, which recognizes either unmethylated CpG motifs (DNA) or ssRNA [14]. The activated pDCs release massive amounts of type 1 interferon, mainly IFNα, which has strong anti-viral properties [15]. After activation, pDCs can acquire antigen and present it to either memory CD8$^+$ or memory CD4$^+$ T cells [7, 13, 16-18]. **Mainly endogenous antigens (e.g. viral antigens) have been described**

for pDC antigen presentation and not exogenous antigens [6, 19, 20]. In **Chapter 7**, we discuss the antigen presentation capacity of pDCs and found that they can present exogenous antigen to naive $CD8^+$ T cells when matured by CpG motifs. Furthermore, they can acquire antigen, process it and present it on MHC II to naive $CD4^+$ T cells when either pre-activated to CpG motifs or exposed to exogenous antigen in the context of antigen-specific immunoglobulins. 20 hrs after activation with CpG motifs, mRNA levels of the proteins associated with MCH II processing are upregulated, indicating that the processing machinery is turned on after activation.

pDCs have been shown to be important in several viral infections [10, 21], however their other immunological settings, like allergy or transplantation [22] pDCs have also been shown to have effects. De Heer et al have shown that pDCs are crucial to keep the lungs in a tolerant state [23]; moreover in tolerant mice pDCs outnumber cDCs [24]. Normally when an antigen is presented via the lung mucosa without an activation stimulus the outcome will be tolerance. As a consequence, when pDCs are depleted during exposure of a harmless antigen, the outcome is immunity [23]. In **Chapter 6** we studied the capacity of pDCs to dampen an immune response in immunized mice, so if they are able to regulate a secondary encounter with antigens. Exogenous generated pDCs could dampen the eosinophilic inflammation induced by OVA aerosols, when administered before the challenge. Moreover, by increasing the endogenous pDCs with growth factor Flt3-L before the challenge phase the inflammation was also dampened, an effect already described by others [25-27]. Administration of Flt3-L increases both pDCs and cDCs. We show that the anti-inflammatory effect of Flt3-L was completely dependent on the increase of pDCs, as depleting pDCs with the mAb 120G8 completely abolished the Flt3-L effect. Surprisingly, pDCs can reduce eosinophilic airway inflammation irrespective of their maturation state. However, it has been described that only mature pDCs could induce regulatory T cells [4, 17, 28-32]. Liu et al showed that pDCs can induce regulatory T cells, which are known to dampen inflammation, probably via the interaction ICOS-ICOS-L on T cells and pDCs [33]. Only activated pDCs have ICOS-L expression on their cell surface.

In inflammation, also the cDCs become more activated and will then prime T cells. This can either lead to tolerance, protective immunity, or autoimmunity [34-36]. In **Chapter 2**, it was shown that an antigen (in this case OVA coupled to a fluorescent dye for tracking) injected intraperitoneally can passively flux to the draining mediastinal lymph node. There it will be taken up by the lymphoid-resident cDCs, processed and presented to $CD4^+$ T cells. However, without an additional activation stimulus, after 7 days these proliferating $CD4^+$ T cells have died. It was also clear that the lymphoid-resident cDCs were responsible for antigen presentation when only OVA (so no danger signal) was injected, since depletion of the lymphoid-resident cDCs (by intratracheal administration of diphteria toxin in CD11c-DTR mice) blocked the T cell

response.

Under inflammatory conditions, circulating monocytes can differentiate into inflammatory DCs [37-41], and not when injected into non-inflamed or non-infected mice [42, 43]. These inflammatory monocytes ($Ly6C^+$ $CCR2^+$) are attracted to site of inflammation as they respond to the chemokine MCP-1 [44-46] produced by several cells after activation. In mice injected with alum adjuvant (**Chapter 2**) or mono-sodium urate (MSU) crystals (**Chapter 4**) an increase in MCP-1 and in the number of $Ly6C^+$ monocytes can be detected already 2-6 hrs after i.p. injection into the peritoneal cavity. These monocytes efficiently take up the antigen, migrate to the draining mediastinal LN and differentiate into inflammatory DCs. There, these inflammatory DCs efficiently prime $CD4^+$ T cells and induce effector T cells [47, 48]. In vitro the equivalent of these inflammatory DCs can be generated by culturing bone marrow cells with GM-CSF. These DCs resemble the TIP (TNFα-iNOS producing)-DCs [49], which efficiently prime for Th2 responses, when injected intratracheally [50]. However, the Flt3-L generated cDCs and pDCs can not induce immunity when injected intratracheally (unpublished data).

Therapeutic implications to target the different DC subsets

The use of adjuvants has been around for at least 80 years, when people started to vaccinate with a booster (adjuvant) in the formula. Aluminum hydroxide and/or aluminum phosphate (alum) had been found to have these adjuvant activities. However, nowadays alum adjuvant is not only given to vaccinate against viral pathogens (e.g. hepatitis), but also to promote desensitization in the case of severe allergy. In this way, people try to overcome the aberrant Th2 response. The choice to use alum as an adjuvant is quit striking, since in mice alum adjuvant is used to induce strong Th2 responses [51]. So, in this way one should think if this approach is clever or not. However, in humans it has been shown that alum induces the formation of IgG4 (an isotype of not much knowledge in the mouse) [52]. IgG4 in humans is believed to act by neutralizing allergen and blocking both IgE-dependent activation of mast cells and basophils [53, 54]. **B cells** switch to the production of IgG4 by IL-10 and IL-4 [55], cytokines also produced in mice after alum sensitization **(Chapter 2)**. It will need further investigations if the production of IgG4 is the main difference between human and mice in the context of alum immunotherapy.

In mice, alum injection leads to the release of uric acid (**Chapter 2**). **Chapter 4** shows that uric acid crystals (monosodium urate (MSU) crystals) can mimic Th2 responses induced in mice comparable to the responses induces by alum sensitization. It can either be delivered intraperitoneally (as alum is) or via the airways. The latter would suggest that there maybe a role for uric acid in ongoing allergic disease, as during some environmental triggers induce the release

of uric acid in the epithelial ling fluid. Uric acid can be released as an anti-oxidant reaction, but when it is locally present in high concentrations, it can form MSU crystals. Higher uric acid levels can also be found in mice exposed to influenza [56]or house dust mite (**Chapter 4**), maybe implicating a role for uric acid in exacerbating allergic disease, e.g. asthma.

MSU crystals have also been shown effective in promoting $CD8^+$ T cytotoxic cell responses [57-59], which is a beneficial property for an adjuvant. MSU crystals bind to a member of innate PPRs, namely the NALP3 inflammasome [60]. Different bacterial compounds, like muramyl dipeptide (MDP, product of bacterial cell wall, bacterial DNA, or even whole bacteria (e.g. Staphylococcus aureus) can activate the NALP3 inflammasome [61]. We (**Chapter 3**) and others have shown that the effect of alum adjuvant partially works via the NALP3 inflammasome. As ligands for another group of PPRs, namely the Toll-like receptors (TLRs) are subjugated to generate new adjuvants, exploitations of ligands of Nod-like receptors (NLRs) of which NALP3 is a member should also be proven a successful strategy.

Recently, strategies to target antigen in vivo to inflammatory or steady-state cDCs have been used in tumor immunology [62, 63]. In addition, in vitro generated DCs pulsed with antigen could reduced tumor load in different cancer models [64-68]. The use of DCs or targeting to specific DC subsets has been proven to be very efficient in promoting CTL responses and thereby reducing tumor load. Recently, DNGR-1, a specific receptor on $CD8\alpha^+$ cDCs, was used target with mAbs to the $CD8\alpha^+$ cDCs, thereby induced strong $CD8^+$ T cell responses, and facilitating a reduced tumor load [62]. However, in the concept of using Abs to target DC subsets should be important that targeting to specific DCs should be done carefully. The Ab of use should be extremely specific, as expression on other celltypes can be unfavorable.

Tricks to target inflammatory DCs or cDCs to reduce inflammation can be exploited as well. Targeting the cDCs with antigen in vivo without any maturation stimulus promotes tolerance (e.g. T cell anergy or regulatory T cells) instead of immunity [62, 69, 70]. This strategy could be proven useful in models of autoimmunity or allergy. Furthermore, targeting antigen to plasmacytoid DCs could be exploited to generate responses to dampen inflammation, as pDCs induce regulatory T cells. pDCs express markers specific for this population in naive mice, however in inflammation some markers are also upregulated on other cells, like BST2 (bone marrow stromal antigen-2 recognized by PDCA-1 and 120G8 mAb) [71]. On the other hand, increasing pDCs by Flt3-L administration completely abolishes the eosinophilic airway responses. So, this pathway should also need further investigation to be proven useful as an adjuvant strategy. Indeed, it has been shown that Flt3-L administration increases T and B cell responses, obligatory properties of an adjuvant [72]. However, in this context they describe the effects found to the activation and number of cDCs found in the lymphoid organs [72].

Triggering adjuvanicity via TLRs, like CpG motifs is an approach used in anti-tumor and anti-viral vaccinations [73, 74]. Furthermore, CpG motifs have been used to dampen eosinophilic airway inflammation [75-78], an effect partially dependent on the recruitment of pDCs to the lung compartment (**Chapter 6**). The dampening effect of pDCs on eosinophilic inflammation did not depend on there maturation status (**Chapter 6**), but rather improved the reduction.
Concluding, DC subsets can be used to promote either immunity or tolerance and thereby are key to regulating the immune system.

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Nederlandse Samenvatting

Het immuunsysteem beschermt het lichaam tegen gevaarlijke microben, zoals bacteriën en virussen, maar ook tegen het ontstaan van tumoren. De Latijnse term *immunis* betekent vrijgesteld, verwijzend naar bescherming tegen indringers van buiten. Dit wordt gedaan door verschillende cellen die in het immuunsysteem voorkomen. De reactie wordt in gang gezet door eiwitten die door het immuunsysteem herkend worden, zogenaamde antigenen. Deze antigenen kunnen worden gepresenteerd door speciale antigeen-presenterende cellen, waarvan de dendritisch cellen (DC's) enorm effectief zijn. Deze antigenen worden gepresenteerd via bepaalde structuren die op het celoppervlak tot expressie worden gebracht, zogenaamde MHC moleculen. Het MHC II/antigeen complex wordt herkend door T cellen, die effectief kunnen reageren om een anti-virale of anti-bacteriele response op gang te zetten. De zogenaamde $CD8^+$ T cellen, cytotoxische T cellen, zullen geinfecteerde cellen aanvallen en doden. De $CD4^+$ helper T cellen worden geactiveerd en maken mediatoren die helpen bij het klaren van de ontsteking. Wat voor soort T cel er ontwikkelt uit de naive T cel, komt door verschillende signalen die ze van de dendritische cellen krijgen en dit wordt een zogenaamde 'verworven reactie. Dit wordt gedaan in de zogenaamde lymfoide weefsels, zoals de lymfeklieren en de milt. Het aanpassings (verworven) immuunsysteem kan dagen tot weken de tijd nemen na een eerste besmetting om een effect te hebben.

Naast de bovenstaande functies van DC's hebben zij ook functies tijdens de primaire reactie van een infectie (zogenaamde aangeboren reactie). Hierdoor worden ze ook wel 'bevelhebbers' van de aangeboren en verworven immuniteit genoemd. De aangeboren immuniteit bestrijdt ziekteverwekkers gebruikend defensie die snel wordt gemobiliseerd en door receptoren teweeg te brengen die een breed spectrum van ziekteverwekkers herkennen. Tijdens de aangeboren reactie maken DC's cytokines en chemokines, stoffen die nodig zijn om de geinfecteerde cellen te doden en andere cellen die de infectie tegengaan aan te trekken. Dit wordt gedaan op de plaats van ontsteking of infectie.

Tijdens de 'rust' staat van het lichaam, dus als er geen infectie of tumor aanwezig is, vervullen DC's ook een cruciale rol. Zij zorgen voor tolerantie tegen 'zelf'-antigenen. Dit is cruciaal, aangezien het lichaam zichzelf niet moet aanvallen, maar alleen alles wat 'niet'-zelf is.

Rust status

Dendritische cellen zijn heterogeen. Er wordt een onderscheid gemaakt tussen stabiliteit en tijdens infectie of ontsteking. In ruststaat, het onderscheidt in DC subsets kan gemaakt worden in plasmacytoide DC (pDC), voorloper DC (ook wel monocyten genoemd) en conventionele DC (cDC). cDC's kunnen nog verder opgedeeld worden in migrerende ('transporterende') and lymfeklier-residente cDC. De migrerende cDC's zijn 'schildwachten' in de periferie van het lichaam, zoals in de longen of de huid. Na opname van antigenen in de periferie,

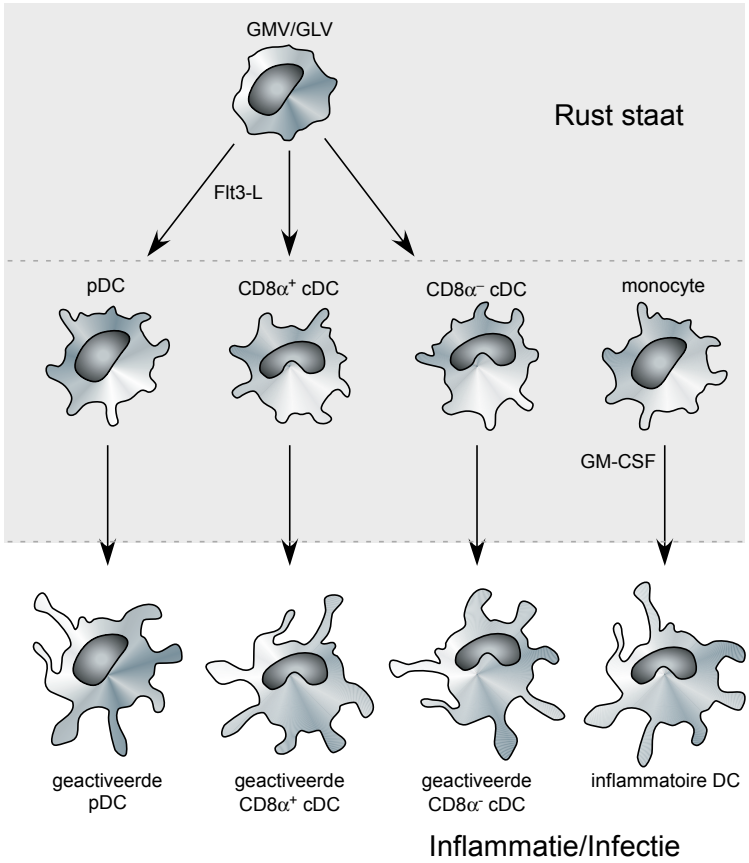


Figure 1
Verschillende dendritische cel subsets in rust en ontsteking/infectie
Tijdens rust ontwikkelen DC's (pDC, CD8α+ cDC, and CD8α- cDC) zich van gemeenschappelijke myeloide voorlopers (GMV) or gemeenschappelijke lymfoide voorlopers (GLV) onder de invloed van de groeifactor Flt3-L. Tijdens inflammation of infectie worden deze 'in rust zijnde' DC's geactiveerd. Verder ontstaat er een subset, de inflammatoire DC vanuit monocyten onder invloed van de groeifactor GM-CSF. Tijdens rust komen deze DC's niet voor.

transporteren deze cDCs het antigeen naar de drainerende lymfeklieren, waar ze het presenteren aan naive T cellen. De lymfeklier-residente cDC's worden verder opgedeelt in CD8α+ cDC's en CD8α- cDC's. Deze cDC's migreren niet door lymfevaten, maar nemen hun antigeen op uit de lymfevloeistof. Dit kunnen 'zelf'-antigenen zijn of oplosbare antigenen die in de lymfevloeistof aanwezig zijn en daardoor de lymfeklier bereikt hebben. In hoofdstuk 2 laten we zien dat als antigeen in de buikholte wordt gespoten het naar de drainerende lymfeklier kan gaan via de lymfevloeistof. Op deze wijze kunnen de lymfeklier-residente cDC's het antigeen opnemen en presenteren aan CD4+ T cellen. Echter, wanneer dit gebeurd zonder activatie van de DC's zullen 7 dagen na injectie van het antigeen zijn de CD4+ T cellen dood gegaan.
Er is een verschil in antigeen opname en verwerking tussen de CD8α+ cDC en CD8α- cDC. De

CD8α$^+$ cDC's zijn goede 'cross-presentators', dit betekend dat zij antigenen uit de omgeving presenteren aan CD8$^+$ T cellen via MHC klasse I moleculen. Daarintegen zijn de CD8α$^-$ cDC's goed in MHC klasse II presentatie, wat betekend dat zij goed CD4$^+$ T cellen kunnen activeren. Dit hebben wij ook kunnen aantonen in hoofdstuk 7, waar de CD24$^+$ cDCs (in vitro gelijkwaardig aan de CD8α$^+$ cDCs van de beenmerg Flt3-L kweek) hogere expressie hadden van MHC klasse I geassocieerde eiwitten, en de CD24$^-$ cDC's (in vitro gelijkwaardig aan de CD8α$^-$ cDC's) hogere expressie van MHC klasse II geassocieerde eiwitten. Dit bevestigde ook dat deze gekweekte cDC's lijken op de in het lichaam-aanwezige cDC's en dezelfde antigeen processing capaciteiten hebben. Verder worden er in de rust status ook pDCs gevonden. Van deze DC's wordt gedacht dat zij een regulerende werking hebben door de vorming van een suppressieve T cel, de zogenaamde regulatoire T cel te induceren. In hoofdstuk 7 wordt de antigeen presenterende capaciteit van pDCs besproken en duidelijk is dat zij exogeen (in de omgeving aanwezige, extern) eiwitten niet aan naive CD4$^+$ of CD8 T cellen kunnen presenteren als ze onrijp zijn. Daarintegen presenteren ze als ze onrijp zijn wel endogene (in de cel aanwezige, intern) eiwitten aan naive CD8 T cellen. Dit laatste is ook bekend van virale eiwitten.

Ontsteking of infectie staat

Tijdens ontstekingen of infecties handelen de DC's anders. Eerst worden zij geactiveerd/gerijpt door herkenning van microben door speciale receptoren, de 'Patroon Herkenning Receptoren' (PRRs). Deze receptoren vallen onder het aangeboren immuunsysteem. Na deze activatie zullen de DC's verschillende anti-virale en anti-inflammatoire cytokines uitscheiden. De pDC's staan bekend om hun goede anti-virale werking. Als pDC's virussen tegenkomen, worden zij geactiveerd via de PRRs door herkenning van stukjes DNA, zogenaamde CpG motieven. De geactiveerde pDC's zetten enorme hoeveelheden type 1 interferonen vrij, welke een sterke virale werking hebben. Echter, na activatie kunnen pDC's ook antigeen presenteren aan memory (geheugen) CD4$^+$ of memory CD8$^+$ T cellen. In hoofdstuk 7 bleek dat pDC's antigeen kunnen presenteren in de context van infectie of ontsteking. Als pDC's geactiveerd worden met CpG motieven (context infectie) of blootgesteld worden aan antigeen met antigeen immune serum (context herhaalde ontsteking) dan kunnen zij aan naive CD4$^+$ en aan naive CD8$^+$ T cellen antigeen presenteren. De anti-virale werking van pDC's is hierboven al genoemd, echter de werking van pDCs in immunologische omstandigheden, zoals allergy of transplantie is nog maar weinig van bekend. Tijdens een eerste kontakt met een antigeen via de luchtwegen, zonder dat daarbij een activatie aanwezig is, zal het immuunsysteem zo reageren dat er tolerantie optreedt tegen dat antigeen. Nu is al eerder bewezen dat wanneer de pDC's niet in de longen aanwezig zijn tijdens zo'n eerste kontakt, immuniteit (dus allergie) optreedt in plaats van tolerantie. In hoofdstuk 6 is de remmende werking van pDC's onderzocht tijdens een herhaalde blootstelling met het antigeen,

dus in een al geimmuniseerde muis. Gekweekte pDC's gegeven voor de herhaalde blootstelling konden de eosinofiele ontsteking, welke karateristiek is voor allergie, onderdrukken. Verrassend genoeg was de remming van pDC's niet afhankelijk van hun rijpe status, zelfs hele rijpe pDC's (met CpG motieven behandeld) konden de ontsteking remmen. Het is beschreven dat juist rijpe pDC's regulatoire T cellen kan induceren.

Tijdens ontsteking of infectie zullen de cDC's ook meer geactiveerd worden en beter T cellen kunnen activeren. Dit kan leiden tot tolerantie, beschermende immuniteit of auto-immuniteit. In hoofdstuk 2 en 4 laten we zien dat als een antigeen in een adjuvant (=hulpstof), zoals alum gegeven wordt, of met urinezuur kristallen worden er DC's aangetrokken die de T cellen wel een goede stimulatie geven. Dit zijn de zogenaamde inflammatoire DC's, welke uit de monocyten rijpen. Deze inflammatoire monocyten zijn zeer efficient in de opname van antigenen en zullen migreren naar de drainerende lymfeklier waar ze uitrijpen naar inflammatoire DC's. Hier zullen zij een sterke $CD4^+$ en/of $CD8^+$ T cel response aanzetten.

Concluderend, de verschillende subsets van dendritische cellen hebben elk specifieke functies. Ook hun reactie is verschillend als het immuunsysteem in rust is of als er een infectie is. Echter, de DC subsets kunnen zeer goed fungeren als doelwitten voor toekomstige therapien, afhankelijk van de reactie die nodig is op dat moment in het lichaam.

List of Abbreviations

1-9

7-AAD	7-aminoactinomycin

A

alum	aluminum hydroxide and magnesium hydroxide (Imject, Pierce)
ALN	axillar lymph nodes
APC	antigen-presenting cell
APC	allophycocyanin
APC-AF750	allophycocyanin-alexa fluor 750
APC-Cy7	allophycocyanin-indotricarbocyanine 7
Ag	antigen
AF647	alexa fluor 647
ASC	apoptosis-associated speck-like protein containing a CARD domain

B

BAL	broncho alveolar lavage
BCR	B cell receptor
BHR	bronchial hyperreactivity
BM	bone marrow
BSA	bovine serum albumin
BST2	bone marrow stromal antigen 2

C

CCR	chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
CFSE	carboxy fluorescein diacetate succinimidylester
CLIP	class II-associated invariant peptide
CLR	C-type lectin receptor
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CM	culture medium
COPD	chronic obstructive pulmonary disease
CTL	cytotoxic T lymphocyte

D

DAPI	4'-6-diamidinc-2-phenylindole
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCIR2	DC inhibitory receptor 2
DNGR-1	DC, NK lectin group receptor-1
DLN	draining lymph node
DTH	delayed type hypersensitivity
DT	diphteria toxin
DTR	diphteria toxin receptor

E

EDTA	ethylene-diamine-tetra acetic acid
EDN	eosinophil-derived neurotoxin
ELISA	enzyme-linked immuno sorbent assay
ER	endoplasmatic reticulum

F

FACS	fluorescence activated cell sorter
FSC	forward scatter
FITC	fluorescein isothiocyanate
Flt3-L	FMS-like tyrosine kinase 3 ligand
FcR	Fc receptor
FCS	fetal calf serum
FoxP	forkhead protein

G

GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor

H

h	human
HDM	house dust mite
hrs	hours

I

ICAM	intracellular adhesion molecule
ICOS-L	inducible co-stimulator ligand
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
ILN	inguinal lymph node
i.n.	intranasal
iNOS	inducible nitric oxide synthase
IPAF	IL-1β-converting enzyme protease activating factor
i.p.	intraperitoneal
IP-10	interferon inducible protein-10
i.t.	intratracheal
i.v.	intravenous

L

LN	lymph node
LPS	lipopolysaccharide
LRR	leucin-rich repeats

M

MACS	magnet activated cell sorting
mAb	monoclonal antibody
MCP	monocytes chemoattractant protein
mDC	myeloid dendritic cell
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MLN	mediastinal lymph nodes
MSU	monosodium urate
MPL	monophosphoryl lipid A
MyD88	myeloid differentiation factor 88

N

NALP	NACHT-associated domain, leucine-rich repeat and pyrin region
NAIP	neuronal apoptosis inhibitor protein
NF-κB	nuclear factor κB
NLR	NOD-like receptor
NK	natural killer cell
NKT	natural killer T cell
NOD	nucleotide-binding oligomerization domain

O

OVA	ovalbumin

P

PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PAR	protease activated receptor
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
PD-L	programmed cell death ligand
PE	phycoerythrin
PE-Cy5	phycoerythrin-indotricarbocyanine 5
PE-Cy7	phycoerythrin-indotricarbocyanine 7
PGN	peptidoglycan
PGD	prostaglandin
PI	propidium iodide
PRR	pattern recognition receptor

R

r	recombinant
RLR	Rig-I like receptor
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcription-polymerase chain reaction

Q

q-RT-PCR	quantitative reverse transcription-polymerase chain reaction

S

SCF	stem cell factor
SEM	standard error of the mean

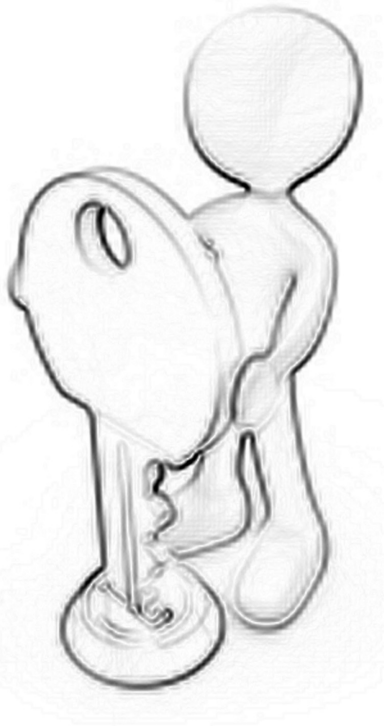
T

TAP	transporter associated with antigen processing
TCR	T cell receptor
Th	T helper cell
TIP-DC	TNF-producing, iNOS-expressing dendritic cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TRIF	TIR (Toll/IL-1R)-domain containing adaptor protein inducing IFNβ

W

WT	wild type

Dankwoord



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Dan natuurlijk het lab Longziekten. Ik twijfelde de eerste dag waar ik nu terecht was gekomen, een bureautje op de gang….. gelukkig was Hamida zo aardig om me te zeggen dat er een 'gewoon' bureau was. De les die je hier uit moet leren is dat je niet op 1 april een nieuwe baan moet

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Het enige wat ik nu nog kan zeggen; je bent mijn schat!!!

Mirjam

List of Publications

Kool M, Soullié T, van Nimwegen M, Willart MA, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN: Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 2008, 205:869-882.

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De auteur van dit proefschrift werd geboren op 2 november 1975 te Utrecht. Na het behalen van het HAVO diploma aan het Cals College in Nieuwegein in 1993, begon zij aan de studie Laboratoriumonderzoek aan de Hogeschool van Utrecht. Vanaf 1998 was zij werkzaam bij de afdeling Farmacologie en Pathofysiologie aan de faculteit Farmaceutische Wetenschappen van de Universiteit Utrecht. Onder leiding van Prof.dr. Frans Nijkamp, Dr. Aletta Kraneveld en Dr. Frank Redegeld werd gewerkt aan de rol van mestcellen in niet-allergische asthma. In 2004 is zij haar promotie onderzoek bij de afdeling Longziekten aan de faculteit Geneeskunde van het Erasmus Medisch Centrum / Erasmus Univesiteit begonnen. Hier heeft zij onderzoek gedaan aan de rol van verschillende dendritische cel subsets in de primaire en secundaire allergische response onder leiding van Prof.dr. Bart Lambrecht, Prof.dr. Henk C. Hoogsteden en Dr. Hamida Hammad. Tijdens dit onderzoek is zij bij Prof.dr. Stephen Galli op Stanford University in Amerika geweest om met een bepaalde stam muizen te werken. De resultaten van het promotie-onderzoek staan beschreven in dit proefschrift.