Division of Labor between Dendritic Cell Subsets in the Lung during Influenza Virus Infection

De rolverdeling van dendritische cel subtypes in de long tijdens influenza virus infectie

Proefschrift

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"Science sans conscience n'est que ruine de l'âme …"
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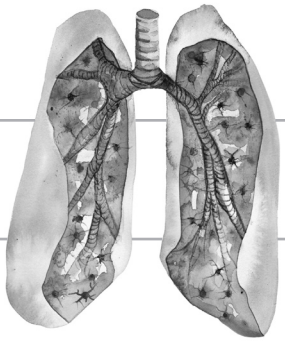

Chapter 1

Introduction and outline of the thesis

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Influenza virus infection

Influenza disease, often referred to as "flu", is caused by the influenza viruses. These viruses are most only responsible for epidemics of variable severity almost every winter (1-3) but occasionally cause major pandemic outbreaks. The term "influenza" has been derived from the Italian astrologers in the mid-1300s, who thought the flu was due to the "influence" of the heavenly bodies. Yet, the aetiology of the disease and the explanation for it's peculiar behavior remained elusive. At the turn of the 19th century, influenza was thought to be due to a bacterial infection with Haemophilus influenzae. It was not until 1931 that Richard Shope showed that the causative agent was a virus (4). A few years later, Smith and co-workers for the first time isolated an influenza virus from humans with respiratory illness (5).

The burden of influenza virus for the society, not only from a clinical but also from an economic perspective, is often underestimated (6). This relates particularly to the recurring annual winter epidemics. Fortunately, since the virus was first discovered, efficient means to contain the infection have been developed. Vaccination is the primary method for the prevention of influenza. However, due to the continuous genetic and antigenic variation that influenza viruses undergo, a constant global surveillance is required to identify and select new variants with epidemic or pandemic potential. Therefore, improvement of vaccination strategies against epidemic influenza and development of effective vaccines against potential pandemic viruses are a public health priority. New strategies for influenza vaccines include altering the dose, site or method of delivery of inactivated vaccines, the use of adjuvants or immunomodulators to enhance immune responses, or targeting of viral proteins that may promote broader, cross protective responses (7).

In order to develop novel intervention strategies, profound knowledge of both the influenza virus and the immunological response of the host to the virus are essential. Influenza viruses belong to the family of Orthomyxoviridae, and are enveloped, single stranded negative-sense RNA viruses with a segmented genome. There are three types of influenza viruses (A, B and C), which can be distinguished on the basis of antigenic characteristics of the internal proteins. The 8 RNA segments of influenza viruses A and B and seven segments of influenza C are independently encapsidated by the viral nucleoprotein (NP) and associated with polymerase proteins to form a ribonucleoprotein complex (RNP). The RNP complexes are located inside a shell of matrix protein (M1), which lines the viral

envelope and provides rigidity to the viral structure. The envelope of influenza viruses is derived from the plasma membrane of the host cell in which the virus has replicated. Influenza A and B viruses have two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), which form a typical layer of spikes radiating outward from the lipid envelope and that respectively mediate cell entry and release of virus particles. Influenza C virus exhibits a single multi-functional glycoprotein. The matrix protein M2 functions as an ion channel and together with HA and NA interacts with the internal M1 protein (figure 1).

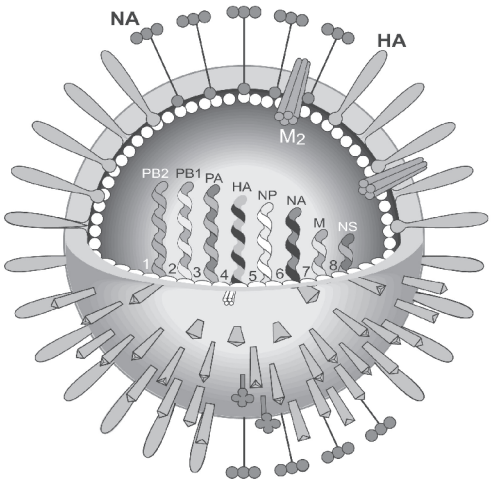

Figure 1: Schematic representation of an influenza A virus particle.
Adapted from Smolinski *et al.,* Microbial Threats to Health: The Threat of Pandemic Influenza (ISBN:0-0309-09717-7)

In the initial phase of influenza virus infection, the HA on the influenza virus envelope binds to sialic acid-containing receptors on the host cells (figure 2 and ref 8,9). Upon binding, the virus particle becomes endocytosed by the cell and internalized. Human influenza A viruses preferentially bind sialic acid residues attached to galactose by an α2,6 linkage, whereas avian influenza viruses prefer sialic acid attached by an α2,3 linkage (10, 11).The low pH in the endosome causes an acidification of the interior of the virus resulting in a dissociation of the M1 from the RNP, allowing the release of RNP. The acid pH also triggers a structural change in the HA, which induces fusion of the viral envelope with the membrane of the endosome, resulting in uncoating and entry of the viral RNP into the cytoplasm and

subsequent transport of the RNPs into the nucleus. In the nucleus, the viral RNA is transcribed into messenger RNA for the translation of viral proteins and copy RNA that serves as a template for viral RNA synthesis. The newly synthesized viral RNA forms RNPs with the newly translated polymerase proteins and NP, which are then transported to the cell membrane. Together with the other structural proteins that have assembled there, the RNPs are packaged into new virions, which bud from the cell surface. The efficient release of the viral particles is facilitated by the NA surface glycoprotein that cleaves sialic acid residues from the cell surface thereby allowing virion detachment from the cell and preventing virion aggregation. The enzym responsible for cleavage of the HA precursor molecule is a trypsin-like protease, which is released from cells in the respiratory epithelium. Due to the limited distribution of this enzyme, influenza virus infection is normally restricted to the respiratory tract. Progeny virus can infect other cells or can be transmitted to new individuals. The entire process of viral infection seriously disrupts the normal cell physiology, eventually leading to cell death. However, this cytopathic effect does not occur until the cell has produced many thousands of new virus particles.

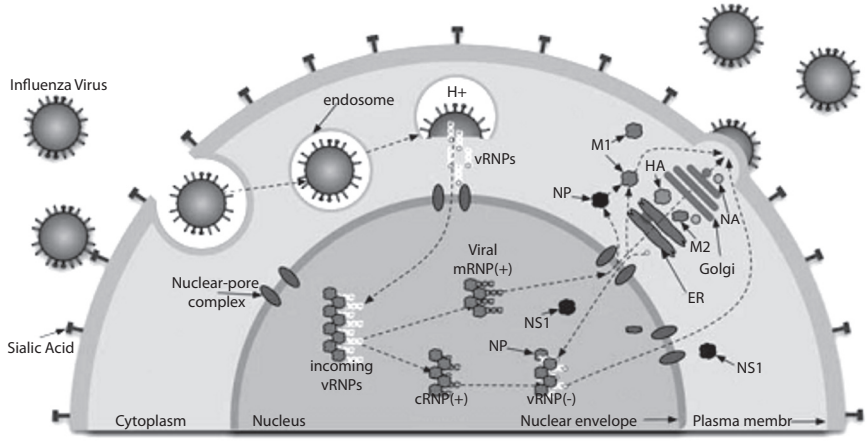

Figure 2: Schematic representation of the replication cycle of influenza A virus.
Adapted from http://www.reactome.org/figures/influenza_life_cycle_overview

Since influenza A viruses are responsible for all pandemic and most epidemic outbreaks, studies described in this these are restricted to influenza A virus. The clinical outcome of influenza A virus infection causing upper and lower respiratory tract infection, is mainly determined by the way in which the innate and adaptive immune system initially recognizes and deals with the replicating virus (12-16). The innate immune response is a rapid response, generally regarded as predetermined and directed towards a broad range of pathogens. This rapid innate response leads to high-level production of type I interferons by infected lung epithelial cells, alveolar macrophages and natural interferon producing cells (also known as pre-pDCs). Innate chemokines promote the recruitment of neutrophils, natural killer (NK) cells and dendritic cells (DCs) (17, 18). Following this innate response, the adaptive immune response will be activated. This is an acquired response, relying on specific viral recognition and reactivity. The adaptive response can be divided into a humoral antibody-mediated response and a cellular T-lymphocyte-mediated response. Neutralizing anibodies are secreted by B-lymphocytes, and bind to virus particles and infected cells that present virus proteins on their membranes, thereby blocking virus-entry into host cells and promoting phagocytosis by macrophages (19, 20). The T-lymphocyte mediated response consists of two distinct populations of $CD3^+$ T cells, the $CD8^+$ cytotoxic T-lymphocytes (CTL) and the $CD4^+$ T helper lymphocytes. Upon recognition of antigen presented by DCs, naïve $CD8^+$ T lymphocytes differentiate and expand clonally into mature activated effector cells, which subsequently migrate from the lymphe nodes or spleen into blood circulation and intercellular fluids. These CTL act via secretory and cell contact-dependent pathways by T cell receptor (TCR) contact. The TCR recognizes infected cells via foreign peptides presented by MHC class-I molecules. Although virus-specific CTL cannot prevent infection of host cell, like virus neutralizing antibodies, they promote viral clearance and accelerate recovery from infection by eliminating virus infected cells, thereby limiting the production of progeny virus (21-23). The $CD4^+$ T helper lymphocytes have an immune response promoting and regulating function. By secreting cytokines and interferons they facilitate proliferation and differentiation of CTL and B-lymphocytes.

In both innate and adaptive immune responses DCs have been described to either induce or mediate the response and thereby have a function beyond just CTL activation. With the studies described in this thesis we have focussed on the role of the different lung DC subsets during influenza virus infection.

Introducing DC heterogeneity

Dendritic cells have been described by Steinman 25 years ago, as a population of cells with antigen presenting capacity found in the spleen and lymph nodes of mice. It was initially thought that these cells represented a unique population of cells that could be isolated based on physical characteristics (low density in comparison with other cells, formation of long dendrites), expression of cell surface molecules like the integrin CD11c, and the functional characteristic of being able to stimulate naïve T cell proliferation(18). In the decades following initial discovery, there has been an explosion of papers describing the precise surface phenotype of DCs in various tissues, the ways in which these cells can be generated *in vitro*, as well as the various ways in which these cells contribute to disease pathogenesis. This has lead to a seemingly endless list of papers describing the surface marker expression of DCs. Only recently there have been attempts to try to streamline this information into a general paradigm on how we can subdivide DC subsets that is comprehensive to a novice to the field of DC biology (24, 25). At least in murine central lymphoid organs, there is a broad division into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). The interested reader is referred to specialized literature on this topic, but broadly speaking, there are at least 3 subsets of $CD11c^{hi}$ cDCs ($CD8\alpha^+$, $CD4^+$ and $CD8\alpha^-CD4^-$) and 1 population of $CD11c^{int}$ pDC in the spleen, whereas in the skin draining lymph nodes, there are at least 5 subsets of cells, the extra one representing skin derived Langerhans cells (26). In contrast to cDCs, mouse pDCs uniquely express the endocytic receptor Siglec-H (recognized by the moAb 440c) (27), the bone-marrow stromal antigen-1 (the latter is recognized by the monoclonal antibodies mPDCA-1 or 120G8) and the B cell marker B220. Plasmacytoid DCs also express high levels of L-selectin and Ly6C, recognized by the Gr1 antibody (anti-Ly6C/Ly6G) which also marks granulocytes and subsets of monocytes (28). In mice both cDC and pDC subsets can express MHCII, CD80, CD86, CD40 and OX40L and should therefore both be capable of antigen presentation to T cells(29). It is however clear now that different subsets of DCs perform different tasks, some subsets of cDCs being better at crosspresentation of antigen to CD8 cells on MHCI molecules and others better at presenting endocytosed antigen to CD4 T cells on MHCII molecules (30) (31) (32) (33). These differences might relate to intrinsic differences between DC subsets in expression of endocytic receptors that target their cargo to well defined intracellular processing compartments (30),

or to intrinsic differences in the expression levels of MHC processing machinery proteins in various subsets(32).

Heterogeneity of mouse lung DC subsets

The subject of lung DC heterogeneity has been appreciated from the very starting point when lung DCs were being unravelled in the mouse and rat, and it has since become clear that different DC subsets exert different functions (34-36). Here, we will mainly discuss the DC subsets of murine lung (see figure 3), as this species is the most studied in various animal models of disease.

In mouse lungs, DCs are found in most tissue compartments, including the large extrathoracic and intrathoracic conducting airways, the lung parenchyma accessible by lung tissue digestion, the alveolar compartment accessible by bronchoalveolar lavage, the pleura, the perivascular space, as well marginating inside the pulmonary lung vessels (37, 38). Although the lung is not a central

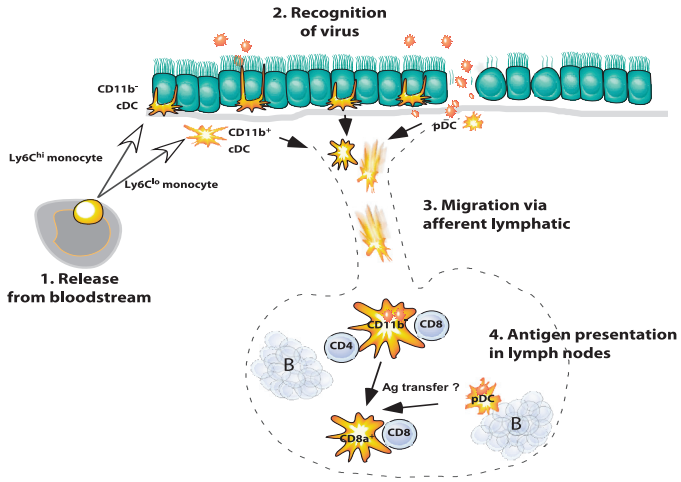

Figure 3. Dendritic cell subsets in the airways.
The conducting airways are composed of airway epithelial cells, which act as a molecular sieve excluding inhaled antigens and pathogens. Two populations of resident DCs are located underneath the epithelial layer. Mucosal CD11b- DCs are situated in the basolateral space, and can extend their processes between epithelial cells directly into the airway lumen. This "periscope" function provides a mechanism for continuous immune surveillance of the airway luminal surface. CD11b+ resident DCs and pDCs are located underneath the basal membrane. In the alveolar space alveolar DCs and macrophages are located. Upon inflammatory conditions like respiratory viral infections, an activated population of inflammatory DCs expressing CD11b and Ly6C can be found in the lung tissue as well as the interferon producing killer DCs (IKDC).

lymphoid organ, most recent papers now also broadly subdivide lung DCs into cDCs and pDCs (13, 14, 39), while the denominators 'lymphoid' and 'myeloid' are best abandoned. All cDCs in the mouse express high levels of the integrin CD11c, and these cells can be further subdivided into $CD11b^+$ or $CD11b^-$ (40-42). The trachea and large conducting airways have a well-developed network of intraepithelial DCs, even in steady-state conditions (43). These cells resemble skin Langerhans cells, and have been shown to express langerin and CD103 but not CD11b (14,40). In the submucosa of the conducting airways, $CD103^-CD11b^+CD11c^+$ cDCs can be found, and these cells are particularly suited for priming and restimulating effector $CD4^+$ T cells in the lung (44, 45).

The lung interstitium, which is accessible by enzymatic digestion, also contains $CD11b^+$ and $CD11b^-$ cDCs that access the alveolar lumen (41, 42,46). It should be noted that this population of cells also comprises DCs that line the small intrapulmonary bronchioles, as well as those that line the vessel walls. Intravascular DCs are also highly enriched in the lung capillaries and most authors studying lung DCs try to eliminate this subset prior to enzymatic digestion of the lung by rinsing the right heart circulation via the pulmonary artery (37). In the nearby alveolar lumen, autofluorescent $CD11b^{lo}CD11c^{hi}$ alveolar macrophages and alveolar $CD11b^{hi}$ $CD11c^{hi}$ DCs can be found. One needs to be particularly carefull not to confuse $CD11b^{lo}CD11c^{hi}$ alveolar macrophages with $CD11b^{lo}$ cDCs, as macrophages can profoundly suppress functional characteristics of lung DCs (47). Therefore, protocols that solely rely on purification of lung DCs by CD11c magnetic beads are confounded. We and others commonly use the characteristic autofluorescence of alveolar macrophages to discriminate the two when performing flow-cytometry based sorting experiments on lung DCs (48-50). Lung plasmacytoid DCs in steady state only represent a minor population of $CD11c^{int}$ cells that also express bone marrow stromal antigen-1 (recognized by moAb 120G8 and mPDCA-1), Siglec-H, Ly6C, and B220 (51-53) and can be found in large conducting airways and lung interstitium (41).

Under inflammatory conditions, things become even more complicated. Inflammatory stimuli like TLR ligands, virus or bacterial infection, or environmental exposure to pollutants like cigarette smoke or ozone trigger the production of chemokines that attrack monocytes and other inflammatory cells to the lungs in a CCR2 and/or CCR5 dependent manner (13, 14, 54-56). These $CCR2^+$ monocytes can be the immediate precursors to so-called inflammatory $CD11b^{hi}$ $CD11c^{hi}$ DCs,

that still express high levels of Ly6C as a remnant of their monocytic descent (56). Under some conditions, these DCs have been called TIP-DCs for TNF-producing iNOS-producing DCs (57), and in some conditions lung iDCs have been shown to produce iNOS(56). These inflammatory DCs closely resemble the steady state CD11b^{+} cDCs and therefore some authors have suggested that cDCs 'mature' or alter phenotype or even proliferate under inflammatory conditions (39,58). A second confounder when studying DC subsets under inflammatory conditions, particularly when high levels of IFNα are being produced during viral infection, is the fact that the bone marrow stromal Ag-1 and B220 is also induced on inflammatory type CD11b^{+} DCs, considerably confounding the discrimination beween pDCs and cDCs if the characteristics of size and expression of Siglec-H is not taken into account (GeurtsvanKessel, unpublished). This has led to the misinterpretation that pDCs can differentiate to cDCs and vice versa, or that pDCs become myeloid-like (13, 59, 60). Finally, a recently described population of NK cells with high MHCII and intermediate CD11c was discovered and named IKDC (61). We have recently observed that this subset is also found inside the lungs following inflammatory stimuli (see chapter 3). As it has a CD11c^{dim}, B220^{dim}, MHCII^{dim} CD11b^{-}, CD19^{-}, CD3^{-} phenotype, it is easy to confuse this subset with pDCs if one does not use Siglec-H or a specific NK marker like NK1.1 or NKp46 to discriminate the two.

Origin of lung DC subsets

Studies performed by Holt's group in the rat and mouse have suggested that the turnover rate of tracheal DCs is in the order of 2-3 days, whereas interstitial lung DCs are much more long lived (42, 62). Both studies suggested repopulation of DC subsets by a circulating precursor cell derived from the bone-marrow. Recent studies performed to elaborate on the origin of the two main cDC populations, CD11b^{-}CD103^{+} DCs and CD11b^{+}CD103^{-} DCs, have demonstrated an origin from distinct circulating monocyte precursors, even in steady state conditions(63). Two distinct monocyte populations have been described in the mouse. A population of Ly-6C^{high}CCR2^{high} (Gr-1high) monocytes are characterized as the more classical monocytes that readily emigrate to sites of ongoing inflammation (64, 65), whereas Ly-6C^{low}CCR2^{low} (Gr-1^{low}) monocytes expressing high levels of CX3CR1 do not robustly emigrate to many tissues, but do migrate well to lung even in absence of inflammation(64). Randolph and colleagues revealed that CD103^{+}CD11b^{-}

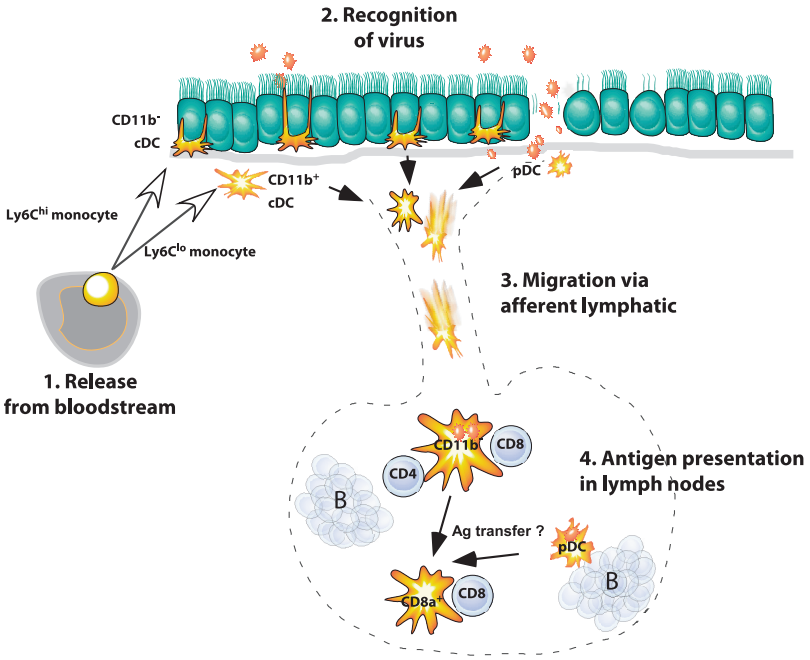

Figure 4. Antigen uptake and presentation by dendritic cells in the airways.
Dendritic cells are released from the bloodstream and give rise to CD11b- cDCs (Ly6Chi) or CD11b+ cDCs (Ly6Clo). CD11b- DCs can directly recognize viral particles in the airway lumen by sensing with their processes. In contrast, CD11b+ DCs and pDCs are located underneath the basal membrane and do not pass the epithelial barrier with their processes but pickup antigen which has passed the basal membrane. The different DC populations migrate to the lymphe nodes by afferent lymphatics and exert their specific functions. Whereas the migrated CD11b- DCs can present their antigen to both CD4 and CD8 T cells, the resident lymphe node population of CD8α+ DCs only presents to CD8 T cells. These resident DCs might obtain their antigen from the migrated CD11b+ DCs or pDCs which present antigen less efficiently themselves.

DCs preferentially derived from CCR2^{hi} monocytes, whereas CD11b^{high} cDC preferentially arise from CCR2^{lo} monocytes, thus lending proof to the concept that subsets of monocytes recruited to the same tissue undergo differential differentiation pathways within the DC lineage. Others working on lung DC ontogeny have however refuted this idea (56, 66). Studies have been performed in which a spleen-resident precursor of steady state cDCs was identified. These cells were not monocytes but could nevertheless generate DCs with relatively high efficiency (67, 68) and therefore were named pre-DC. The equivalent of these pre-DCs were also found in BM and other lymphoid organs (67) (69), implicating that DCs can arise from a precursor that is resident within the lymphoid organ itself and was preprogrammed in the bone marrow as a macrophage-DC precursor (MDP)

(24). It remains to be determined whether a MDP or pre-DC population can also be found in the lungs.

Function of lung DC subsets

The general paradigm of DC function states that DCs reside in an immature state in the periphery of the lung, where they sample the inhaled air for incoming antigen (see figure 4). Upon triggering of pattern recognition receptors like Toll like receptors, the cells migrate via the afferent lymphatics to the T cell area where they select and activate naïve T cells and differentiate them into either tolerizing Treg cells or cytokine secreting T effector cells. Considerable progress has recently been made to suggest that different lung DC subsets perform various aspects of this paradigm differentially. The conducting airways are lined with a mucociliary blanket and composed of airway epithelial cells that are connected by tight junctions and zonula adherens. The epithelial-cell layer acts as a molecular sieve that excludes inhaled antigens and pathogens based on their molecular weight. Intraepithelial $CD11b^-$ DCs are situated in the basolateral space, only separated from the inhaled air by the epithelium tight-junction barrier. In the airways, as well as in the gut, DCs can extend their processes between epithelial cells directly into the airway lumen. This 'periscope' function is constitutively active in the airway mucosal DC population, providing a mechanism for continuous immune surveillance of the airway luminal surface (70-72). At least in mouse lungs, intraepithelial $CD103^+$ DCs express the tight junction proteins claudin-1, claudin-7 and zonula-2, which form tight junctions with airway epithelial cells, thereby explaining how DCs can sample the content of the airway lumen while keeping the epithelium barrier function intact (40). Following sampling of airway luminal contents, there is transport of antigen to the mediastinal nodes. When large fluorescently labelled antigens are injected into the lungs of mice, different subsets of DCs become antigen positive in the draining mediastinal lymph nodes as early as 12 hours after antigen administration (41, 50).

There are several mechanisms by which the inhaled antigen could reach the lymph nodes (for more detailed discussion on this topic see (41). Most of the experimental evidence suggest that antigen is taken up by DCs in the lung that then migrate in a CC-chemokine receptor 7 (CCR7)- and CCR8-dependent manner to the draining mediastinal lymph nodes, which is analogous to the directed migration

of skin DCs (41, 73). It is still unclear exactly where and by which DC subset the inhaled antigen is sampled from the airways, as both mucosal cDCs that line the conducting airways and cDCs that are situated in the alveolar wall are exposed to inhaled antigens, and have the potential to migrate to the lymph nodes (53,41,46, 74). Similarly, only one paper briefly showed that also pDCs might migrate from the periphery of the lung to the mediastinal node during viral infection (39).

In addition to cell-mediated transport, the tight junction barrier might act as a molecular sieve that allows the passive leakage of smaller antigens into the afferent lymph vessels. Antigen sampled in such a passive manner might gain access to resident DCs in the draining lymph node, such as $CD8\alpha^+$ DCs or plasmacytoid DCs (pDCs)(53). Although there is indeed evidence for the passive transfer of fluorescently labelled molecules to the mediastinal lymph nodes, no studies to date have demonstrated that antigen that reaches the resident DCs in this way can result in the induction of T-cell division (41, 50, 53). The final and most important aspect of DC function is the capacity of these cells to program T cell responses. Regarding functional differences in DC populations from airway mucosa versus parenchymal tissue von Garnier et al (41, 42) demonstrated that airway mucosal cDC were more endocytic and presented peptide to naive $CD4^+$ T cells more efficiently than their lung counterparts. However, only DCs matured *in vitro* or after migration to lymphe nodes *in vivo* could present whole protein to T cells and induce T cell activation. This finding indicates a mechanism in which DC function is regulated at the level of protein processing, rather than peptide loading.

It has been shown that inhalation of harmless antigen leads to T cell unresponsiveness in process that is accompanied by DC-driven T cell proliferation and formation of Tregs (75, 76). Whether this is a function of particular lung DC subsets still remains a matter of debate. We and others have shown that inhalation tolerance clearly depends on the presence of pDCs that take up inhaled harmless antigen and lead to formation of Treg cells (51-53). Others have shown that this process also required cDCs that depend on CCR7 for migration to the mediastinal nodes (45, 73). In this regard, del Rio suggested that $CD103^+CD11b^-$ cDCs were specialized in crosstolerizing CD8 responses, whereas $CD11b^{hi}$ cDCs were specialized in tolerizing CD4 T cell responses to inhaled harmless antigen.

Antigen encountered in the lung by DCs as part of a respiratory pathogen, or model protein antigens administered together with different TLR ligands can induce different types of effector T helper responses (77). A detailed description on

how T cell effector responses are programmed is beyond the scope of this article, but suffice it to say that cytokines and costimulatory molecules are the most important factors that will determine the outcome of a pulmonary immune response driven by lung DCs. Stumbles (78) and Dodge (79) have demonstrated that lung DCs are biased towards inducing Th2 responses, through production of IL-6, but it is not yet clear whether this property could be linked to the distinct properties of a particular cDC subset in a particular location. In any case, depending on the type of inflammatory stimulus, lung DCs can induce CTL responses, and Th1, Th2 or Th17 Th effector responses. In the coming chapters we will illustrate how a better understanding of DC subsets of the lungs has provided novel insights into how effector T cell responses are induced in the lung.

Outline of the thesis

As this introduction has pointed out, the emerging field of DC research has recently focused on seemingly antagonistic ideas on the role of DC subsets. Functional specialization of DC subsets, plasticity (multitasking) and crosstalk between functionally distinct DC subsets has been described, but the situation in inflammatory conditions remained to be unraveled. With the studies described in this thesis we aimed to unravel the complexity of the DC system during influenza virus infection, and provide a conceptual framework to understand how cooperation between functionally distinct DC subsets can shape immunity and immunological memory to pathogens. To start, **chapter 2** gives an extensive description of the various DC subsets in the airways of influenza-infected mice by using 10-color flow cytometry. Migration patterns of these subsets and their antigen presenting capacity were demonstrated. To study distinct functions during infection, we depleted specific DC subsets by using either transgenic mouse models or antibody depletion. In **chapter 3** we demonstrate a recently described DC subset in the lungs of influenza virus infected mice, called interferon-killer DC (IKDC). Both antigen presenting capacity and killer potential was demonstrated in this cell population and they thereby resembled conventional NK cells. **Chapter 4** shows a previously unappreciated role of inflammatory DCs (iDC) after influenza virus has been cleared from the lungs. iDCs controlled the maintenance of local lymphoid structures in the lung following influenza virus infection, the so called inducible bronchus associated lymphoid tissue (iBALT). IBALT following influenza virus

infection did not only contribute to local immunity, but was also a source of virus specific plasma cells migrating to the bone marrow. In **chapter 5** we elaborate on antigen presenting capacities of bone-marrow cultured DC subsets during *in vitro* influenza virus infection and demonstrated differences in antigen processing. Finally, the findings presented in this thesis are evaluated in a summarizing discussion (**chapter 6**).

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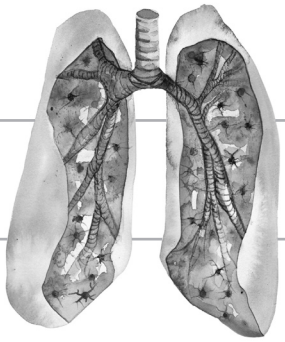

Chapter 2

Clearance of influenza virus from the lung depends on langerin^{+}CD11b^{-} but not plasmacytoid dendritic cells

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ABSTRACT

Although dendritic cells (DCs) play an important role in mediating protection against influenza virus, the precise role of lung DC subsets such as $CD11b^-$ and $CD11b^+$ conventional DCs or plasmacytoid DCs in different lung compartments is currently unknown. Early after intranasal infection, tracheal $CD11b^-CD11c^{hi}$ DCs migrated to the mediastinal LN, acquiring costimulatory molecules in the process. This emigration from the lung was followed by an accumulation of $CD11b^+CD11c^{hi}$ DCs into the trachea and lung interstitium. In the mediastinal nodes, the $CD11b^+$ DCs contained viral nucleoprotein abundantly, but these cells failed to present antigen to CD4 or CD8 T cells, whereas resident $CD11b^-CD8a^+$ DCs presented to CD8 cells and migratory $CD11b^-CD8a^-$ DCs presented to CD4 and CD8 T cells. When lung $CD11c^{hi}$ DCs and macrophages or $langerin^+CD11b^-CD11c^{hi}$ DCs were depleted using either CD11c-DTR or langerin-DTR mice, the development of virus specific $CD8^+$ T cells was severely delayed, which correlated with increased clinical severity and a delayed viral clearance. Plasmacytoid $120G8^+$ $CD11c^{int}$ DCs also accumulated in the lung and lymph nodes carrying viral nucleoprotein but in their induced absence, there was no effect on viral clearance or clinical severity. Rather, in pDC-depleted mice, there was a reduction in antiviral antibody production following lung clearance of the virus. This suggests that multiple DCs are endowed with different tasks in mediating protection against influenza virus.

INTRODUCTION

Influenza type A is a cytolytic virus that causes acute respiratory infection of which the clinical outcome can vary greatly. The way in which the innate and adaptive immune system initially recognizes and deals with replicating virus could be decisive in determining outcome of infection, as this might heavily influence the kinetics of viral clearance (1-3). Little is known about the initial recognition event of Influenza virus by the lung immune system *in vivo*.

In immediate response to viral infection, innate defence mechanisms consist of high level production of type I interferons by infected epithelial cells, alveolar macrophages and natural interferon producing cells (also known as pre-plasmacytoid DCs), as well as recruitment of conventional (c)DCs, neutrophils and NK cells (4, 5). By expressing a wide array of microbial pattern recognition receptors shared with cells of the innate immune response and at the same time displaying the potential to process and present antigen to naive T cells, DCs bridge innate and adaptive immunity. After recognizing foreign antigen in the periphery of the body, DCs migrate via afferent lymphatics into the draining LN, where they can induce antigen-specific protective CD8 cytotoxic T lymphocyte (CTL) responses as well as CD4 T helper cells that enforce cellular and humoral immunity (6).

In the lung, DCs are situated in immediate proximity to the respiratory epithelial cells where they form an elaborate network that rapidly reacts to all kinds of foreign antigens and inflammatory stimuli, including respiratory viruses (7-10). Different DC subsets can be found in the lung, each with functional specialization (11-15). In the mouse, all DC subsets express the integrin CD11c and subsets are further defined based on the expression of the marker CD11b, as well as anatomical location (11, 13, 16). The trachea and large conducting airways have a well developed network of intraepithelial DCs, even in steady state conditions (17, 18). These cells have been shown to express langerin and CD103 while lacking expression of CD11b (10, 13, 15). In the submucosa of the conducting airways, $CD103^{-}CD11b^{+}CD11c^{+}$ conventional DCs can be found, particularly under conditions of inflammation, and these cells might be particularly suited for priming and restimulating effector CD4 cells in the lung in response to protein antigens (15, 19, 20). 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 (11, 14). In the nearby alveolar lumen, $CD11c^{hi}$ alveolar macrophages control the function of these interstitial DCs

(21). Plasmacytoid DCs are $CD11b^{-}CD11c^{int}$ cells expressing SiglecH and bone marrow stromal Ag-2 (recognized by the moAbs mPDCA-1 or 120G8) (22, 23). In the lungs, pDCs are predominantly found in the lung interstitium and produce large amounts of IFN-α in response to triggering by CpG motifs or viral infection *ex vivo* (5, 24). Under particular conditions, lung pDCs can prevent immunopathology in response to inhalation of harmless antigens or in response to respiratory syncitial virus (RSV) (24-26)

Several investigators have studied the involvement of antigen presenting cells such as DCs, macrophages and B cells in mediating protective immunity to Influenza virus (27). It was shown that a mouse adapted strain of influenza virus induced the *in vivo* maturation of $CD11c^{+}$ DCs in the lung (28) and their migration to the mediastinal LNs (2, 8, 29). Dendritic cells isolated *ex vivo* from the mediastinal LN of influenza infected mice presented viral nucleoprotein derived peptides to virus specific CD8 T cells *in vitro* for at least 10 days, a peak occuring around 72 h post infection (27, 29-32). In humans, both monocyte derived DCs and plasmacytoid DCs were shown to be capable of activating already primed influenza specific T cells *in vitro* (33, 34) and upon adoptive transfer *in vivo* (35).

Although these studies certainly suggest the strong involvement of DCs in mediating protective immunity to influenza A virus, direct description of the functional *in vivo* involvement of the now well described DC subsets in the different compartments of the lung during early infection is lacking. Here, using 8 colour flow cytometry, we have carefully studied the kinetics of reaction of different DC subsets and alveolar macrophages in different lung compartments, to intranasal infection with influenza virus X-31 (H3N2), focusing particularly on $CD11b^{+}$ and $CD11b^{-}$ subsets as well as on pDCs, describing which subsets carry viral nucleoprotein to the nodes, and carefully dissecting which subsets present antigen to specific CD8 and CD4 T cells. Using genetic cell specific targeting techniques, $CD11c^{hi}$ DCs and macrophages or $langerin^{+}CD11c^{hi}$ DCs could be depleted in CD11c-DTR or langerin-DTR mice carrying a diphteria toxin receptor (36, 37) and pDCs could be depleted using moAbs. Together, our results demonstrate that different DC subsets perform specialized tasks during primary encounter with influenza virus in the lung.

RESULTS

Lung dendritic cell subtypes and alveolar macrophages respond to influenza virus infection differentially

As expected following intranasal inoculation with $5x10^5$ $TCID_{50}$ influenza A virus X-31 (H3N2), there was induction of a vigorous innate and adaptive immune response in the lung. The innate cellular immune response was already present 1 dpi and peaked at 4 dpi, exemplified by a strong increase in neutrophils. B lymphocytes ($CD19^+$) cells were present in the lung by day 4 and peaked around day 10. Total $CD8^+$ cells in the lung peaked around day 10 (see suppl. figure 1). To investigate the number of DC subsets and macrophages after influenza virus infection, enzymatically digested lungs (from which the large conducting airways were dissected) were analysed at different time points after infection by 8 color flow-cytometry. Digested lung cells were first gated for live leukocytes and expression of the pan-leukocyte marker CD45. Conventional DCs were characterised as low fluorescent cells, with a $CD45^{hi,}$ $MHCII^{hi}CD11c^{hi}$ phenotype (suppl. figure 2A), and further subdivided into $CD11b^+$ or $CD11b^-$ (11, 13, 15, 30). As previously described, pDCs were small, low autofluorescent cells expressing a $CD45^{hi}$, $CD11b^{lo}$, MHC^{int} $CD11c^{int}$ $mPDCA\text{-}1^+$ phenotype (Suppl. figure 2B). Alveolar macrophages were identified in the BAL fluid as highly fluorescent cells with a $CD11c^{hi}$, $CD11b^{lo}$, $MHCII^{lo}$, $F4/80^{hi}$ phenotype (Suppl. figure 2C). The use of CD11c and CD11b expression in combination with autofluorescence typically seen in alveolar macrophages adequately discriminated alveolar DCs from macrophages, as has been described (11, 38, 39).

In the mock situation, 65% of $CD11c^{hi}$ DCs were $CD11b^-$ and 35% were $CD11b^+$. Following infection with influenza virus X-31, a significant increase in both $CD11b^+$ (figure 1A) and $CD11b^-$ (figure 1B) lung DCs was seen from 2 dpi and these DC subsets remained increased up to at least 10 dpi. The DCs expressed high levels of MHCII and upregulated maturation markers such as CD86 (figure 1A,B), CD40, CD80 and ICAM-1 (data not shown). The increase in the expression level of these markers peaked shortly after inoculation (2 days) and gradually returned back to the levels seen in mock infected mice (see bar diagrams). In contrast, the number of pDCs peaked shortly after infection (2 dpi) and then returned back to base-line condition (figure 1C). This increased recruitment of pDCs was accompanied by a

distinct but very transient upregulation of maturation markers (CD86 is shown). Alveolar macrophages were studied in the BAL fluid accessible by broncho alveolar lavage. This compartment contains a large amount of resident alveolar macrophages in mock situation. Following influenza virus infection there was a non-significant trend of increase in alveolar macrophage number at 4 dpi (figure 1D), accompanied by a temporary increase in CD86 and more continue increase in MHCII expression (average MFI of 820 in mock to 2769 at 10 dpi).

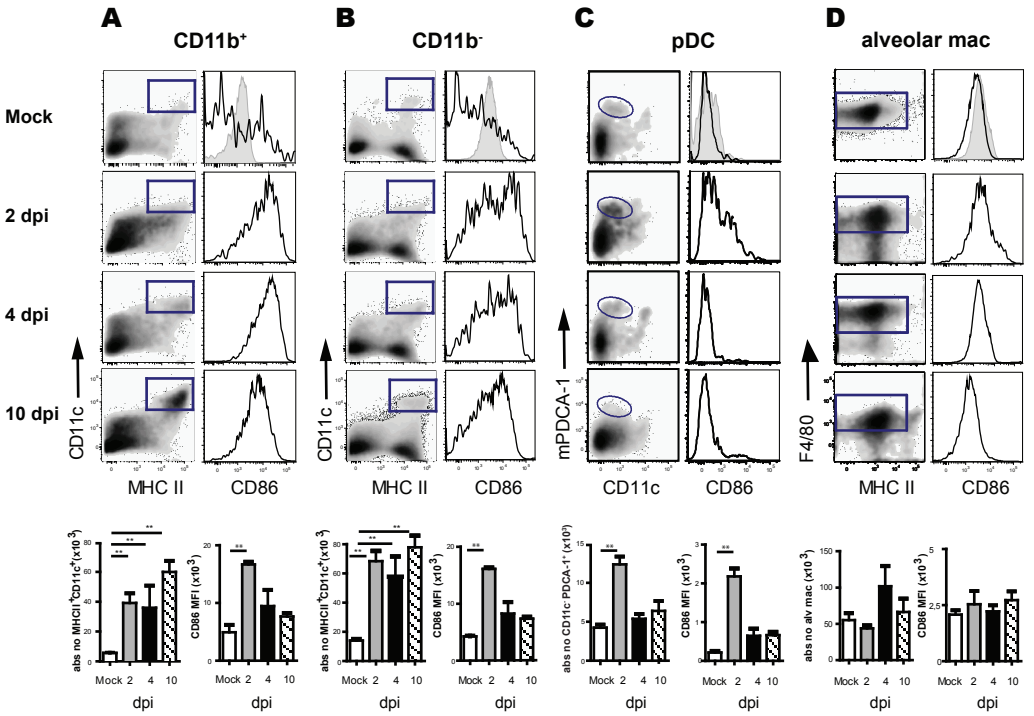


Figure 1: Number and surface phenotype of mouse lung CD11b$^+$ and CD11b$^-$ DCs, pDCs and alveolar macrophages following influenza infection.

Populations were gated as shown in supplementary figure 2, and indicated by gate in left column of each panel. (A) CD11b$^+$ DCs significantly increased after infection and remained increased up to 10 dpi (left panel); CD86 expression was plotted in histograms in the right panel with an average MFI in the lower graph. (B) The increase in CD11b$^-$ DCs demonstrated in flowcytometric plots in left panel, with absolute numbers in the lower graph. CD86 expression was upregulated and plotted as average MFI (mean fluorescence intensity) in the lower graph (C) pDCs increased significantly at 2 dpi and then returned back to base-line. Recruitment of pDCs was accompanied by upregulation of CD86 (right panel). (D) Alveolar macrophages slightly increased in number, but CD86 expression was not increased. Grey histograms represent isotype controls and were measured on 4 dpi. The values are representative of 5 mice/group and expressed as mean ± SEM. Similar results were obtained from at least 3 separate experiments. * p<0,05 **p<0,01

Response of DC subsets in the large conducting airways to influenza virus infection

To examine the DC network in the large conducting airways, we stained *in vivo* fixed and permeabilized tracheal whole mounts with a monoclonal antibody to MHCII revealing the presence of a highly developed network of DCs demonstrating delicate dendritic processes in between bronchial epithelial cells (figure 2A). Following infection, there was an increase in $MHCII^+$ cells at 2 dpi and cells had a more rounded appearance, making it difficult to quantify DC density. At 4 dpi there was a strong decrease in density of cells staining for MHCII, followed by a restoration to baseline density and morphology by 10 dpi. To quantify these changes better, 8-colour flow cytometry was performed on tracheal digests, demonstrating considerable heterogeneity in DCs at various time points post infection (figure 2B).In the mock situation, all $CD45^+$ $MHCII^+$ cells expressed CD11c, identifying them as DCs. The majority of these cells (appr. 75%) were CD11b negative and expressed the mucosal aE-integrin marker CD103 (figure 2C). At 2 dpi, this subset was depleted from the tracheal digest and a majority of cells now expressed CD11b but lacked CD103, most consistent with the phenotype of a freshly recruited monocyte derived DC (figure 2B&C). A temporary increase in the expression level of CD86 was found on these $CD11b^+$ DCs at day 2 (figure 2B). At 10 dpi, the subset balance between $CD11b^+$ and $CD11b^-$ DCs was almost restored to baseline, and cells again showed a highly dendritic morphology and CD86 expression level returned to baseline levels. As reported before by others, we detected a minor percentage (0.35% of live tracheal $CD45^+$ leukocytes) of mPDCA-1^+ pDCs in tracheal digests of mock infected mice (figure 2D) (11). Again, pDCs increased following influenza virus infection and temporarily upregulated their expression of costimulatory molecules (figure 2E).

Influenza virus infection increases DC subsets in MLN

Upon recognition of antigen, lung DCs are known to migrate to the mediastinal LN. We followed the kinetics of increase and expression of maturation markers on various DC subsets within this node. Conventional DC subsets were $CD11c^+MHCII^+$ cells (figure 3A) that were further discriminated based on expression of the myeloid

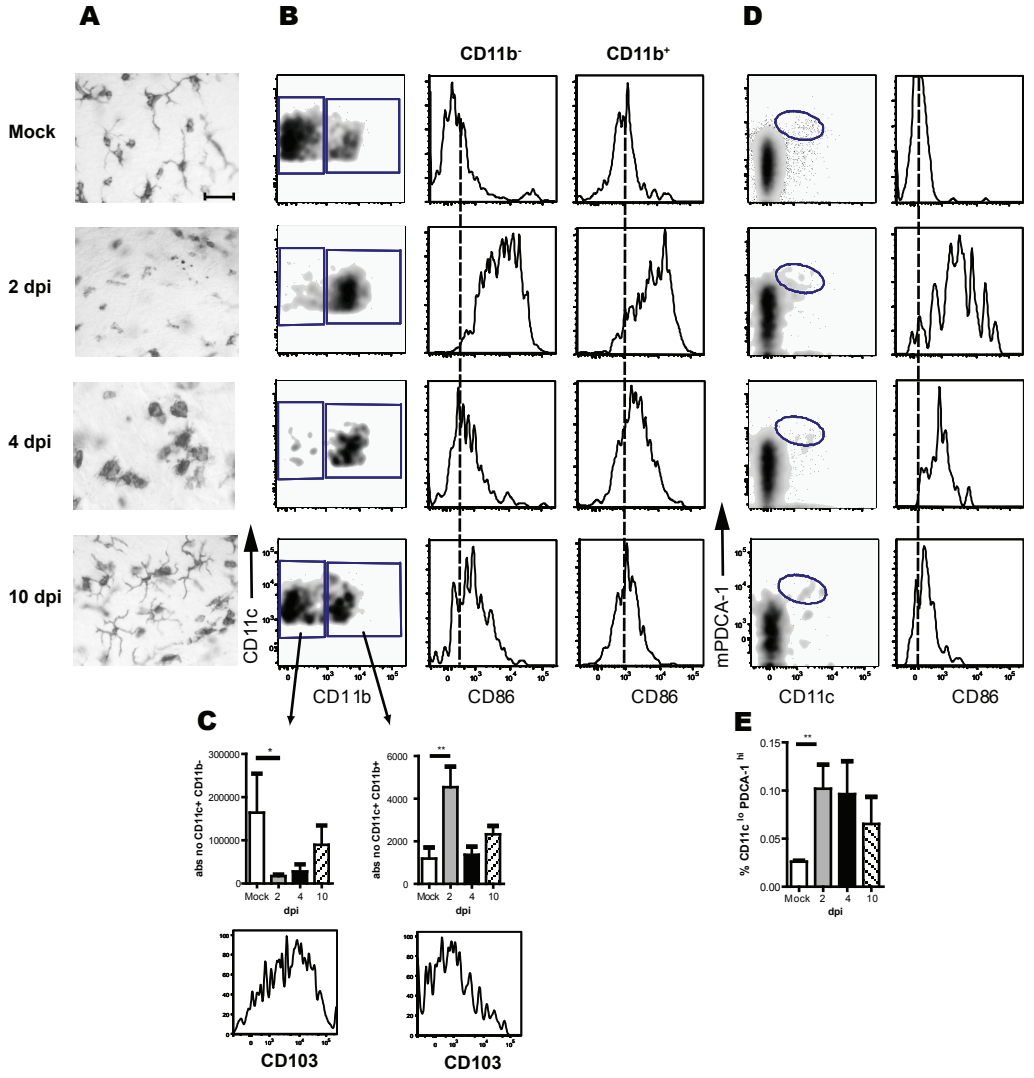


Figure 2: Dendritic cell subtypes in tracheal tissue following influenza infection.
(A) Tracheal whole mount sections stained for MHCII expression were performed at various days pi. Scale bar represents 35 μm. (B) Flow cytometric analysis of DCs in tracheal cell suspensions stained for CD45, CD11c, CD11b, F4/80 and CD103. CD11c$^+$ cells contained two subsets, one CD11b$^-$, the other CD11b$^+$. Histograms represent CD86 expression on both subsets. The dotted line indicates the MFI at mock situation. (C) Absolute numbers of the two subsets following influenza infection. Histograms represent CD103 expression on both subsets. (D) Plasmacytoid DCs were identified as CD11c^{int}PDCA-1$^+$ cells representing a minor percentage of CD45$^+$ leukocytes in tracheal cell suspensions, and only temporarily expressing CD86 (histogram). (E) Absolute number of pDCs at various days following infection. * p<0,05 ** p<0,01

marker CD11b (figure 3B).

Following infection, there was an increase in the absolute amount of $CD11c^+MHCII^+CD11b^-$ as well as $CD11c^+MHCII^+CD11b^+$ DCs, starting at 2dpi and the increase was more pronounced in the $CD11b^+$ subset. Within the $CD11b^-CD11c^{hi}$ cells, there was a clear increase in cells co-expressing langerin and CD103 (30×10^4at 4 dpi vs 2×10^4 in mock infected mice). Belz et al. previously suggested that a resident subset of $CD11b^-CD8a^+$ DCs was responsible for generating virus specific CD8 CTLs, while the population of $CD11b^-CD11c^+$ cells transported antigen from the periphery (30). Therefore, cells were further discriminated based on CD8a (figure 3C). Compared with mock, at 4 dpi there was a generalized increase in all $CD8a^-$ subsets ($CD11b^-$ and $CD11b^+$) while the resident $CD8a^+CD11b^-$ DCs were not increased. Following infection, $CD11c^+MHCII^+$ cells had higher levels of the CD86 maturation marker compared with mock infected mice, consistent with their potential to prime CD8 T cell responses (figure 3D), and this was found in all subsets of DCs.

To test the antigen-presenting potential of the various DC subsets in the MLN, mice were infected with influenza virus encoding either the immunodominant $OVA_{257-264}$ Kb restricted MHCI epitope recognized by the OT-I TCR-transgenic strain (40) or carrying the $OVA_{323-339}$ MHCII epitope recognized by the OT-II TCR transgenic strain (41), allowing us to probe presentation of sorted lung DC subsets to naive OVA-specific CD8 and CD4 T cells directly *ex vivo*. To have an indication about the uptake of viral antigen or virally infected apoptotic cells, preparations of sorted DC subsets from the MLN were also stained for the presence of viral nucleoprotein using a specific Ab (figure 3E), and confirmed using 8 color flow cytometric staining on permeabilized cells (figure 3F). Viral nucleoprotein was found particularly in the $CD11b^+CD8a^-$ subset as well as abundantly in the pDC subset, and was practically absent from the $CD11b^-$ subsets (figure 3F). When DC subsets were sorted and cocultured with OVA-specific OTI cells (figure 3G, top panels) or OTII cells (figure 3G, lower panels), the $CD11b^-CD8a^-$ subset presented antigen to both CD8 and CD4 T cells, whereas the $CD11b^-CD8a^+$ resident DCs presented exclusively to CD8 cells. Despite the fact that these cells had seen viral antigens (see nucleoprotein staining, figure 3E&F), the $CD11b^+$ DCs and the pDCs did not present antigen to naive CD4 or CD8 T cells. As a control, OT-I or OT-II T cells incubated with total MLN DCs obtained from mice infected with the virus containing the OVA-MHCII or –MHCI epitope respectively, failed to proliferate.

Conditional depletion of lung CD11c^{hi} cells aggravates features of infection

DCs are extremely potent APCs that are uniquely suited to prime naïve T cells. To study the immune response against Influenza virus infection with and without CD11c^{hi} DCs, CD11c-DTR mice were treated with DT intratracheally 1 day prior to infection with influenza virus X-31. By this localized treatment, lung CD11c^{hi} DCs (both CD11b^{+} and CD11b^{-}) and alveolar macrophages were efficiently depleted from the lungs (figure 4A). In the trachea, the localized treatment with DT led to a reduction of CD11b^{+} DCs, but not to depletion of CD11b^{-} DCs (suppl. figure 3A) or pDCs (data not shown). In the mediastinal LN all DC subsets were partially depleted after DT treatment with the biggest depletion occurring in the resident CD11b^{-}CD8a^{+} DCs (suppl. figure 3B and (19, 37)). On the contrary, CD11c^{hi} cells in the spleen or non-draining LNs were not affected (data not shown) (19, 37). At different time-points post-infection the clinical, immune response and viral replication were analyzed. First, clinical severity of infection was determined by measuring body weight. In general, mice show a maximal 10 % weight loss after a mild X-31 infection, and rapidly regain weight once virus has been cleared from the lungs. When CD11c-DTR Tg mice were treated with DT prior to infection the clinical severity of infection dramatically increased and mice lost up to 20% weight, representing a sublethal infection. This weight loss could not be contributed to DT treatment, as the control group, treated with DT prior to Mock infection, did not loose weight (figure 4B). As weight loss might be related to initial severity of infection or failure of the immune system to clear infectious particles from the lung, we next studied the generation of an efficient immune response in CD11c^{hi} depleted mice. For efficient lung clearance of a primary influenza virus infection, CD8^{+} CTLs play an important role. We therefore measured the number of nucleoprotein $(NP)_{366-374}$ peptide/H-2Db specific CTL cells using tetramer reagents and correlated their numbers to viral clearance. In DT treated and infected animals, the number of virus specific CTL cells was significantly reduced in lung, spleen (figure 4C) and MLN (data not shown). In addition to the measurement of CTL, CD3-stimulated IFNγ production by CD4 and CD8 T cells from MLN was determined and dramatically suppressed in the absence of DCs (figure 4D). As induction of virus-specific CTL responses is necessary for viral clearance from the lung, we measured viral titers in the lungs. In this mild infection model, virus is normally cleared completely at 8 dpi

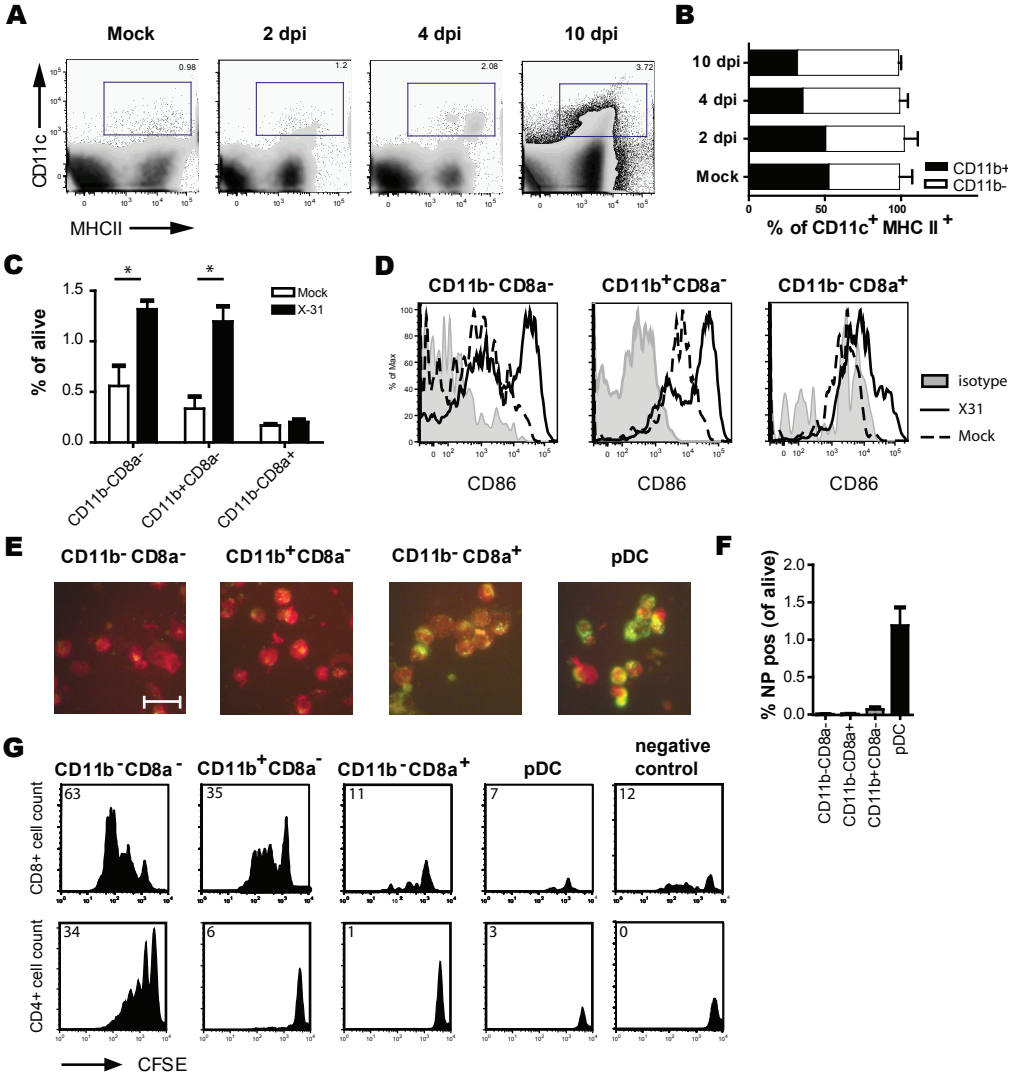
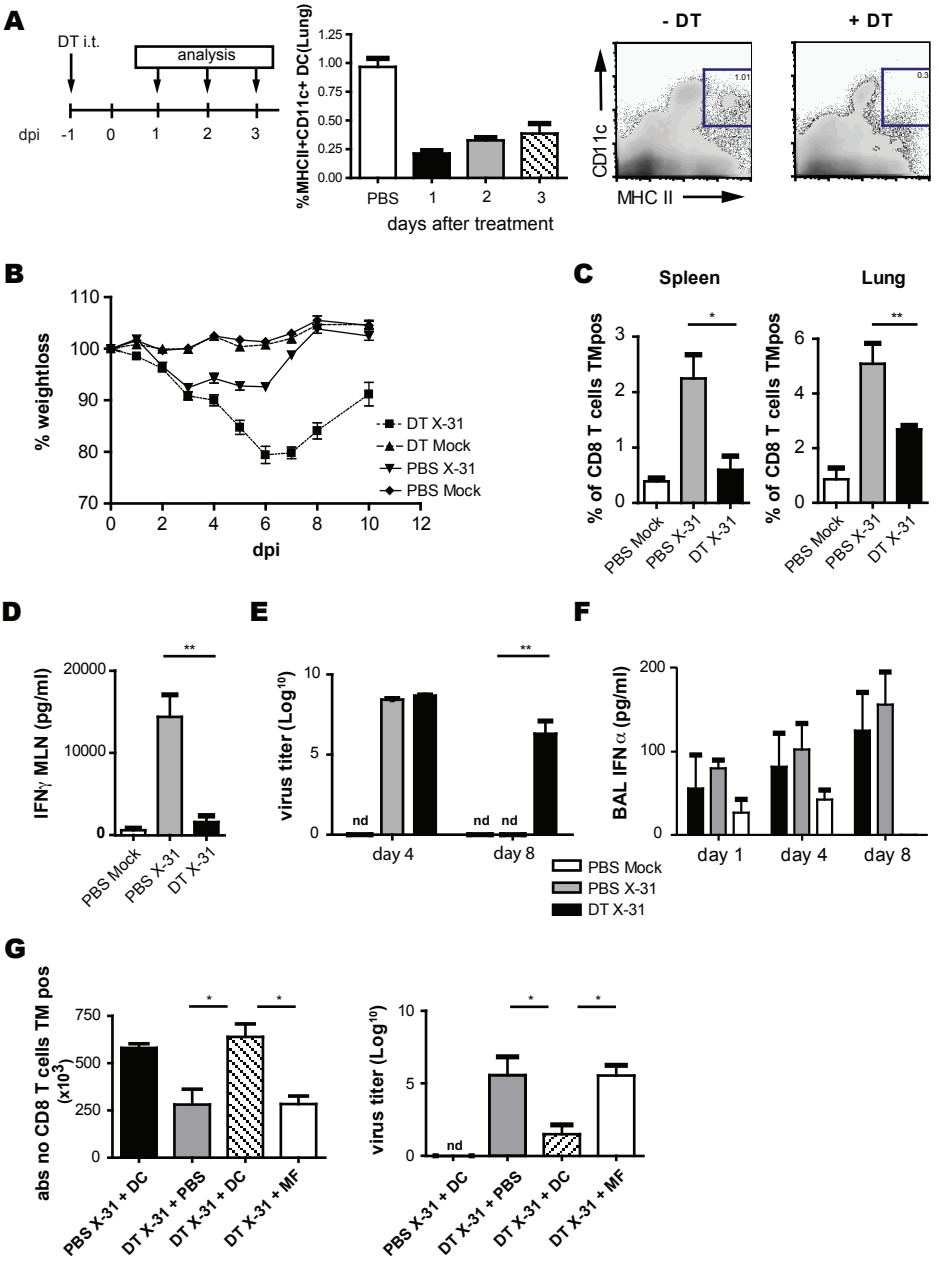


Figure 3: DC subsets in MLN following influenza virus infection.
(A) kinetics of CD11c$^+$MHCII$^+$ DCs demonstrating almost 4-fold increase following infection (B) expression of CD11b by CD11c$^+$ DCs gated in A. Black bar represents CD11b$^+$ population and the white bar CD11b$^-$. (C) CD8a expression on different DC subsets demonstrated in combination with CD11b, with percentages of the different populations plotted in the graph. (D) CD86 expression on different MLN subsets. (E) Sorted DC subsets were stained for intracellular NP. Uninfected cells stain dull red due to evans blue in the solution, the green fluorescence indicates NP. Scale bar represents 20 μm. (F) Flowcytometric analysis of total detectable amount of intracellular NP in DC subsets, as a percentage of total live LN cells per DC subset. (G) Plots represent CFSE labeled T cell proliferation 4 days after co-culture with different sorted DC populations obtained from pooled LNs of 20 influenza-infected mice. Top row shows CD8$^+$ T cells, lower graphs show CD4$^+$ T cell proliferation. Numbers in left corner of the plot represent the % of cells recruited into cell division. All figures are representative of at least 5 mice/group and expressed as mean ± SEM. Similar results were obtained from at least two separate experiments. * $p<0,05$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day 4
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abs no CD8 T cells TM pos (x10³)
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Figure 4: Infection parameters after conditional depletion of CD11chi cells in a CD11c-DTR transgenic mouse model.CD11c-DTR transgenic mice received an i.t. injection of DT on day -1, followed by X-31 intranasal infection. (A) Efficient depletion of lung CD11c^{hi} cells by DT treatment compared to PBS treatment. Right plots demonstrate flowcytometry data, numbers indicate % of live cells within the gate (B) Body weight following influenza infection. (C) Virus specific CTL response in spleen and lung measured by $Flu_{peptide}$/H-2Db tetramer (TM) staining. (D) IFN-α levels in supernatants of MLN cell cultures re-stimulated with anti-CD3 Ab. (E) Viral titers measured in lung tissue following influenza X-31 infection. N.d. signifies non-detectable (F) IFNIFα levels in BAL fluid. (G) Adoptive transfer of wild type DCs and alveolar macs into DT treated CD11c-DTR Tg mice prior to viral infection. CD11c-DTR Tg mice were either treated with PBS or diphteria toxin (DT) and received either DCs or macrophages prior to influenza infection. Numbers of $Flu_{peptide}$/H-2Db specific CTL in spleen suspensions (left graph) and viral titer in lung tissue (right graph). The values are representative of 5 mice/group and expressed as mean ± SEM. Similar results were obtained from at least 2 separate experiments. * p<0,05 **p<0,01.

(figure 4E and (42)). However, in the absence of lung CD11c^{hi} cells and mediastinal CD8a^{+}CD11c^{hi} cells, the virus had not been cleared at this time. A delay in viral clearance could be due to a direct defect in CTL priming, but could also result from a less efficient innate response consisting mainly of type I IFN production. IFN-α has been described as an important anti-viral cytokine, mainly produced by epithelial cells and alveolar macrophages, but also by pDCs and even cDCs following influenza infection *in vitro* (3, 5). We therefore determined the IFN-α production in the BAL fluid at different time points after infection in CD11c^{hi} depleted mice. *In vivo*, IFN-α was produced in the lungs following X-31 infection, but levels were not affected by CD11c^{hi} cell depletion (figure 4F).

The above data show that lung CD11c^{hi} cells are required for an efficient immune response against influenza virus infection. However, as CD11c is also highly expressed by alveolar macrophages and these cells are also depleted by local i.t. administration of DT we performed adoptive transfer reconstitution experiments (19, 37). Tg mice depleted of CD11c^{hi} cells therefore received i.t. unpulsed wild type CD11c^{hi} DCs or alveolar macrophages at the moment of infection. DCs were capable of restoring the CTL response (figure 4G) and as a consequence viral clearance from the lung was complete by day 8 (figure 4G, right panel). Alveolar macrophages were not capable of restoring anti-viral immunity or viral clearance. These experiments demonstrated that CD11c^{hi} DCs are sufficient for inducing an adequate immune response against influenza virus infection.

Depletion of lung langerin^{+} DCs aggravates infection parameters

The conditional depletion of CD11c^{hi} cells in CD11c-DTR mice did not allow us to address the contribution of tracheal CD11b^{-}CD11c^{hi} DCs as tracheal CD11b^{-}

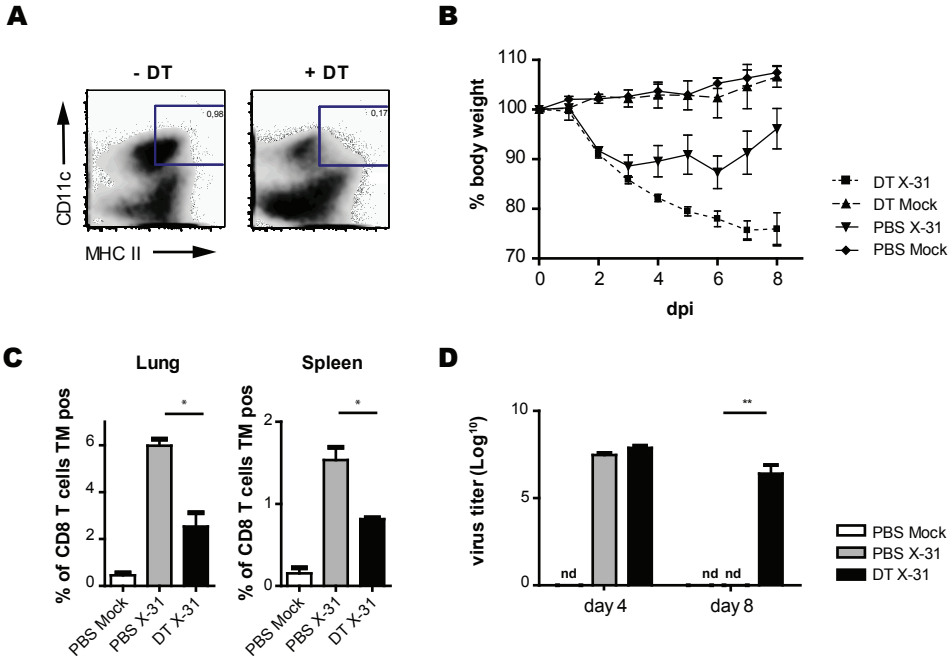

Figure 5: Effect of conditional depletion of Langerin+ DCs cells during influenza infection.
Langerin-DTR transgenic mice received an i.t. injection of DT on day -1, followed by X-31 intranasal infection. (A) Efficient depletion of MHCII^{+}CD11c^{+} DCs in Lung after DT treatment. Stated number indicates % of live cells within the gate (B) Body weight following influenza infection. More than twenty % weight loss represents a sublethal infection. (C) Virus specific CTL response in spleen and lung measured by $Flu_{peptide}$/H-2Db tetramer (TM) staining. (D) Viral titers measured in lung tissue following influenza X-31 infection. Virus is normally cleared completely at 8 dpi. N.d. signifies non-detectable. The values are representative of 5 mice/group and expressed as mean ± SEM. Similar results were obtained from at least 2 separate experiments. * p<0,05 **p<0,01.

CD11c^{hi} DCs nor their CD11b^{-} progeny in the MLN were depleted in CD11c-DTR mice. Therefore we performed experiments in langerin-DTR mice. When DT was intratracheally administered to these mice, there was a strong reduction in CD11b^{-} CD11c^{hi} DCs in the trachea, whereas tracheal CD11b^{+} DCs were unaffected. In the lungs, CD11c^{hi} DCs were depleted whereas alveolar macrophages were unaffected. The CD8a^{+}CD11b^{-} resident MLN cDC subset do not express langerin, and consequently, lung administration of DT to these mice only led to reduction in the lung-derived CD11b^{-}CD8a^{-} migratory DC (see suppl figure 3). Depletion of langerin^{+} DCs resulted in a severe weight loss in the mice until 8 dpi (figure 5B) and this was correlated with a significant decrease in CTL response (figure 5C) and a deficient viral clearance at 8 dpi (figure 5D).

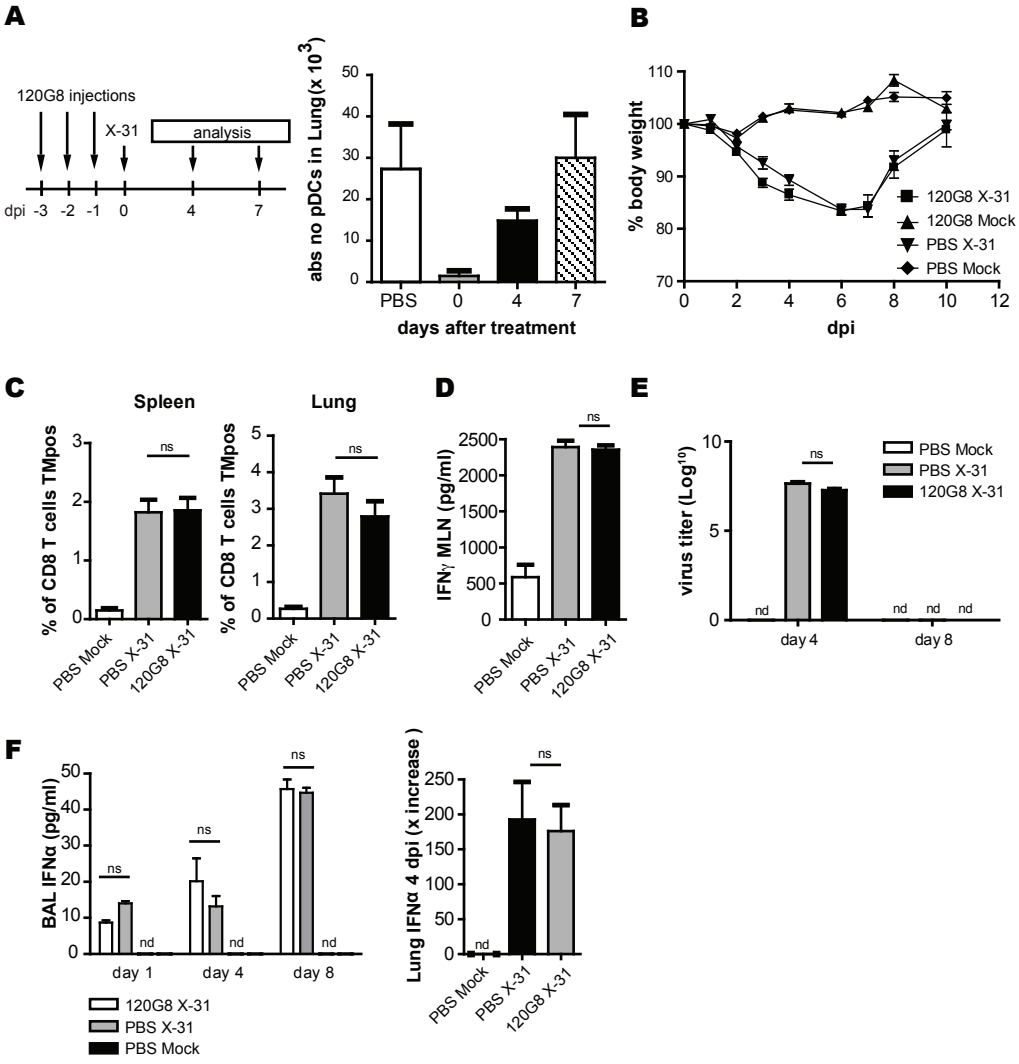

Figure 6: Infection parameters following influenza in mice depleted of pDC.
Mice received three i.p injections of depleting moAb 120G8 prior to infection with influenza on day 0. (A) Efficient depletion of lung pDCs after 3 days of 120G8 i.p. treatment compared with PBS treatment. (B) Body weight following infection (C) Virus specific CTL response in spleen and lung cell suspensions measured by $Flu_{peptide}$/H-2Db tetramer staining (D) IFNγ levels in supernatants of MLN cell cultures re-stimulated with anti-CD3 Ab (E) Viral titers in lung following influenza infection. (F) IFN-α levels in BAL fluid. The values are representative of 5 mice/group and expressed as mean ± SEM. Similar results were obtained from at least 2 separate experiments. *p<0,05 **p<0,01.

Depletion of pDCs did not alter the course of infection

The experiments using CD11cDTR and langerin-DTR mice mainly depleted CD11c^{hi} cells, whereas CD11c^{int} pDCs are globally not affected by this targeting strategy (43). To additionally address the role of pDCs, we performed experiments in which pDCs were depleted by injection of the 120G8 monoclonal Ab (22). Using this antibody, an effective depletion of pDCs to less than 10% of baseline numbers was achieved in the lung (figure 6A), as measured using Abs directed against both bone marrow stromal Ag and Siglec-H (24). Surprisingly, this efficient depletion of pDCs did not affect any of the infection parameters and virus was cleared efficiently by 8 dpi (figure 6B-E). As pDCs have been described as main IFN-α producing cells in response to a viral infection (44) it was similarly striking that there was no reduction in the protein level of IFN-α in the BAL fluid or in the mRNA level for IFN-α in lung tissue following depletion of pDCs (figure 6F).

The production of hemagglutinin specific antibodies depends on pDCs and not on CD11c^{hi} DCs.

The induction of antiviral CD8 T cell responses is only one aspect of adaptive immunity to influenza. We also measured the production of serum hemaglutinin

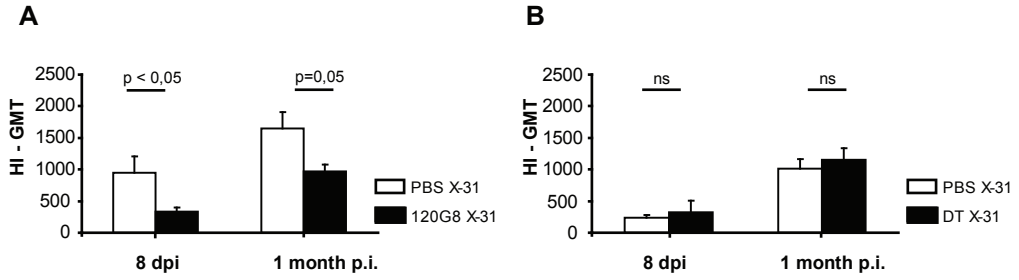

Figure 7: Virus specific serum antibodies of influenza virus infected mice after DC subset depletion.
(A) Virus specific antibodies in serum of C57BL/6 mice after i.p. treatment with 120G8 depleting Ab or PBS. (B) or in serum of CD11c-DTR mice after i.t. treatment with DT or PBS, measured at 8 dpi and 1 month p.i. The values are representative of at least 5 mice/group and expressed as geometric mean titre (GMT) of hemagglutinin inhibition (HI) ± SEM. The differences in HI titer can be explained by the background of the mice. CD11c-DTR mice were F1 (Balb/c x C57Bl/6) animals and developed less high viral titers and HI titers then the pure C57BL/6 mice that were used in 120G8 depletion experiments. Similar results were obtained from at least 2 separate experiments. * p<0,05 **p<0,01.

specific antibodies at 8 dpi and 28 dpi, by measuring hemaglutinin inhibition titers. Whereas there was no effect on induction of CD8 responses following 120G8-mediated pDC depletion, virus specific antibody titers measured at both time points were significantly reduced (figure 7A). We found no evidence for 120G8 staining on lung B-cells, making it unlikely that this would be a depleting effect of 120G8 on B cells directly (data not shown). Antibody responses were maintained in mice depleted of $CD11c^{hi}$ DCs by DT treatment of CD11cDTR mice one day prior to infection in CD11c-DTR mice (figure 7B).

DISCUSSION

Belz et al. have elegantly described that following influenza infection, both $CD11b^{-}CD8a^{+}$ resident mediastinal LN DCs as well as $CD11b^{-}CD8a^{-}$ lung derived migratory DC subset presented Ag to naïve $CD8^{+}$ T cells *ex vivo* (30, 32). However, these authors have not described in detail where exactly in the lung these $CD11b^{-}$ migratory DCs originated from, whether any of the DCs would also present to $CD4^{+}$ T cells nor have they studied in detail the precise contribution of inflammatory $CD11b^{+}$ or pDCs that are recruited to inflamed lungs.

Our studies on the various anatomical lung compartments suggest that a predominant source of $CD11b^{-}$ DCs arriving in the mediastinal nodes are from the network of highly dendritic shaped $CD103^{+}$ intraepithelial DCs that line the large conducting airways (see figure 1 and (13, 18). There was a marked decrease in this subset of highly dendriform tracheal $CD11b^{-}CD103^{+}$ DCs at 2dpi, at a time that the $langerin^{+}CD103^{+}CD11b^{-}CD8a^{-}$ DCs started to accumulate in the MLN. The disappearance of $CD11b^{-}CD11c^{+}$ tracheal DCs was accompanied by a new influx of $CD11b^{+}CD11c^{+}$ DCs into the trachea (present as more rounded cells at day 2 pi on whole mounts) and into the lung interstitium. These cells most likely differentiate from recruited $Ly6C^{+}$ monocytes that give rise to inflammatory-type DCs (16, 45), or arrose from local proliferation and differentiation of a myeloid precursor population that also generates alveolar macrophages (46). An identical subset of $CD11b^{+}CD11c^{+}$ DCs was found to be increased in the MLN from day 2-4 onwards, suggesting again migration from the lungs to the MLN. It was striking that there were several subsets of DCs found to be recruited and/or activated in the mediastinal LN, following influenza infection, strongly suggesting the division of labor between various APCs. Recently, several papers have shown that the

cross presentation of exogenous harmless or viral antigen to $CD8^+$ lymphocytes or presentation of exogenous antigen to CD4 T cells is a mutually exclusive function of $CD8a^+$ or $CD8a^-$ DC subsets respectively (47-49). We therefore performed a head-to-head comparison of the potential to present viral antigen to naïve CD4 or CD8 T cells, taking advantage of influenza virus encoding either the MHCI or MHCII ovalbumin immunodominant epitope.

As previously described by Belz et al, $CD8a^+CD11b^-$ resident DCs presented viral antigen to naïve CD8 cells, supported by the fact that these cells had upregulated costimulatory molecules. Strikingly, the population of airway derived $CD11b^-CD8a^-$ DCs also upregulated costimulatory molecules and presented not only to CD8 T cells but as well to CD4 T cells. This suggests that processing for and presentation on both MHCI and MHCII molecules can occur in a single cell population *in vivo* in the lung, contrary to what was shown for the spleen or lymph nodes (30). Vermaelen et al. also previously demonstrated that Ag presentation of harmless antigen to naive CD4 T cells in the MLN was an exclusive function of a migratory DC population (50). Despite the fact that both $CD8a^+$ and $CD8a^-CD11b^-$ DCs presented antigen to naïve T cells, the strength of viral NP staining was not abundant. This could be explained by the fact that viral NP was digested in these subsets as part of an antigen processing step, leading to a loss of immunoreactivity towards the NP-specific antibody. Alternatively, Belz previously suggested that resident $CD8a^+$ cells acquired the antigen from another migratory APC, proposedly the $CD11b^-CD8a^-$ lung derived DCs, although this was never directly demonstrated (30). Our data on viral NP staining suggest that the $CD11b^+$ DCs as well as the pDCs were abundantly positive for viral antigen in the mediastinal LN, and therefore could be the most important source for providing viral antigens to resident $CD8a^+$ DCs.

Another striking observation was the fact that the $CD11b^+CD11c^+$ subset found to be increased in the trachea, lung and mediastinal LN following influenza infection hardly presented any viral antigen to CD4 or CD8 T cells. Clearly, the $CD11b^+$ DCs had seen viral antigens (either directly or through phagocytosis of virally infected apoptotic epithelial cells) as they carried an abundant amount of viral nucleoprotein in their cytoplasm. The absence of APC function by this subset is in striking contrast to the situation when harmless non-inflammatory antigen is inhaled (15). What could be the purpose of recruitment of a different DC subset, in addition to $CD11b^-$DCs, if these cells do not present antigen to naïve T cells? First,

as these cells have been shown to massively produce inflammatory chemokines, $CD11b^+$ DCs might be crucial in attracking effector CD4 and CD8 cells that have been generated in the LN back to the lung and trachea, where they would mediate effector function (51). Secondly, $CD11b^+$ DCs might have direct innate antiviral activity by producing TNFα and iNOS dependent NO, analogous to the situation seen with Listera monocytogenes infection (52, 53). Thirdly, recruited $CD11b^+$ DCs might also stimulate the innate antiviral activity of NK cells. The important function of inflammatory DCs in initiating the innate response is supported by the fact that influenza produces the non-structural protein-1 (NS-1), with a specific aim to subvert the innate immune function of the $CD11b^+$ DC subset (3, 54). Future studies in our laboratory will have to address the direct or indirect innate and adaptive functions of inflammatory $CD11b^+$ DCs in influenza infection.

Another way to study the function of lung DC subsets is to deplete them using various cell specific genetic targeting strategies employing the expression of the diphteria toxin receptor under control of a specific promotor and administration of the diphteria toxin via the airways (19). In CD11c-DTR mice, there is a predominant depletion of lung $CD11c^{hi}$ DCs, of tracheal $CD11b^+CD11c^{hi}$ DCs and of resident mediastinal LN $CD8a^+CD11c^{hi}$ DCs. As $CD11b^-CD11c^{hi}$ migratory tracheal DCs are not depleted in these mice, we consequently observed no decrease in MLN $CD11b^-$ $CD8a^-$ DCs. In CD11c-DTR mice given DT, we noticed that generation of virus specific $CD8^+$ CTLs as well as production of effector cytokines (IFNγ) by MLN cells was severely diminished. Based on our antigen presentation studies and based on previous work of Belz et al, we propose that this is due to depletion of the resident $CD8a^+CD11c^{hi}$ DCs or to the depletion of chemokine producing lung $CD11b^+$ DCs. In CD11c-DTR mice, $CD11c^{hi}$ alveolar macrophages are also depleted, although it is unlikely that this contributed to a decrease in antiviral immunity as adoptive transfer of wild type DCs but not macrophages restored immunity.

To address the specific role of migratory tracheal $CD11b^-$DCs, we performed experiments in langerin-DTR mice (36). Langerin was found to be present particularly on mucosal $CD11b^-CD103^+$ DCs in the lung (13), a population of cells that strictly relies on CCR7 to migrate to the mediastinal LN (15). In accordance, we also found increased numbers of $langerin^+CD103^+CD11b^-CD11c^+$ DCs in the mediastinal LN following influenza infection. Following intratracheal administration of DT, there was a selective depletion of $CD11b^-CD8a^-$ migratory DCs in the trachea and MLN, while leaving the $CD8a^+$ resident LN population or

alveolar macrophages unaffected. In the absence of the migratory langerin$^+$ CD11b- DC population, influenza ran a particularly severe course. Strikingly, these data also suggest that the langerin$^+$ DCs of the lung are much more immunogenic than their langerin$^+$ Langerhans' cell counterparts in the skin (36, 55, 56).

Finally, we also addressed the *in vivo* function of lung plasmacytoid DCs, previously known as natural IFN-α producing cells (44). In humans, these cells produce copious amounts of IFN-α when exposed to influenza virus *in vitro* (57) and have been shown to stimulate already primed influenza-specifc CD4 and CD8 T cells (34). It has therefore been suggested that upon proper stimulation, plasmacytoid DCs develop into bona fide APCs that stimulate primarily antiviral immune response (58). To address the antigen presenting function of pDCs, we sorted pDCs from the mediastinal nodes of infected mice, and found no evidence for presentation to either CD4 or CD8 T cells *ex vivo*, despite the fact that the cells contained copious amounts of viral NP intracelullarly. Previous studies with other respiratory viruses have suggested that pDCs have a more immunoregulatory role, crucial for preventing excessive immune activation and immunopathology (12, 24, 25). Although pDCs were attracted to the lungs and tracheal wall during influenza infection, their confirmed depletion using 120G8 Ab did not affect viral titers, generation of virus specific T cells, or severity of infection, arguing against a predominant role for pDCs as APCs, immunoregulatory cells or innate immune cells in this mild infection model. It was similarly striking to see that there was no effect on IFN-α production when pDCs were effectively depleted. Infections in epithelial surfaces that bathe in IFN-α (produced by epthelial cells) might be less dependent on IFN-α production by plasmacytoid DCs. Akira's group demonstrated recently that during lung infection with RNA viruses, alveolar macrophages are a predominant source of type I interferon, and that pDCs only start producing IFN-α when macrophages are depleted from the lungs (5). The only effect of treatment with the 120G8 antibody was a significant reduction of the titer of virus-specific HI antibodies at 8 dpi and 28 dpi, signifying a possible role for pDCs in stimulating humoral antiviral immunity, as previously suggested (59). Although we found no evidence for 120G8 staining on lung B cells following influenza infection, treatment with 120G8 Ab could also deplete plasma cells directly and in this way reduce HI Ab titers. Therefore we are awaiting more specific (e.g. genetic) pDC targeting strategies to address this point further.

In conclusion, our paper demonstrates a division of labor between

different DC subsets during pulmonary influenza infection, knowledge that might be employed for the design of better influenza vaccines and could increase understanding why particular strains of influenza are more pathogenic than others.

MATERIALS AND METHODS

Mice

C57BL/6 mice (6-8 weeks) were purchased from Harlan (Zeist, The Netherlands). The generation and screening of CD11c-DTR Tg mice has been reported previously (37). Male Balb/c background CD11c-DTR Tg (H2-Dd) were crossed to C57BL/6 (H2-Db) to obtain F1 progeny, to allow detection of H2-Db tetramer. $CD11c^{hi}$ cells were depleted in CD11c-DTR x C57BL/6 Tg mice by i.t. injection of 50 ng DT, a dose previously determined by titration (15) . PDCs were depleted by i.p. injection of pDC-selective depleting 120G8 antibody (22). All experiments were approved by an independent animal ethics committee of Erasmus MC Rotterdam, the Netherlands.

Influenza virus infection

Influenza virus X-31 (MRC, Cambridge, England) were inoculated in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested after 2 days. Infectious virus titers were determined in Madin-Darby Canine Kidney (MDCK) cells (ATCC: Product CCL-34 (NBL-2) as described previously (60). Virus titers were obtained at day 4 and 8 post infection. Lungs were stored at -70 C. Lungs were homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection medium (Eagles Minimal Essential Medium (EMEM), Bovine serum albumin (fraction V 0,3 %), 4 μg/ml trypsin, 2mM l-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, 0,15% NaHCO3, 20mM Hepes, non essential amino acids 0,1 mM). Ten-fold serial dilutions of these samples were used in eight-fold to determine the virus titers in MDCK cells as described previously (60). For antigen presentation assays of lymph node DCs, mice were infected with WSN influenza virus encoding $OVA_{257-264}$ Kb restricted MHC I epitope in the neuraminidase (40) and X-31 influenza virus encoding $OVA_{323-339}$ MHC II epitope in hemagglutinin of the virus (41) The OVA viruses were kindly provided by Dr. R. Webby (St. Jude Children's Hospital, Memphis,USA).

Flow cytometry

For detection and phenotyping of DCs at day 2, 4 and 10 post infection, single cell suspensions of MLN and lung samples were prepared as described previously (24). Trachea was digested in collagenase solution for 1 hour at 37˚C to promote release of DCs. Cells were subsequently stained with moAbs directed against MHCII FITC, intracellular NP FITC, CD86 PE, CD11c PE Texas Red, CD45 PECy5, CD103 PECy7, intracellular Langerin APC, mPDCA-1 APC, F4/80 APC Cy7, CD11b Pacific Blue and a fixable live/dead marker in Aqua (Molecular Probes). Acquisition of 8-9 colour samples was on a FacsAria cytometer equipped with FacsDIVA software. Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA). Cell sorting of DC subsets from MLN was performed on a FacsAria cytometer. Purity of sorted populations was > 95%.

Immunofluorescence on sorted DC subsets

Sorted cell populations were spotted on microscope slides, dried and fixed in acetone. Subsequently, the slides were incubated with FITC labelled influenza A nucleoprotein specific antibody (IMAGENTM Influenza virus, DakoCytomation Diagnostics) at 37 ˚C for 15 minutes. The slides were washed with PBS twice and once with distilled water, dried and embedded into a glycerol–PBS solution (Citifluor, UKC Chemlab, Canterbury). Uninfected cells stain dull red due to the Evan's blue in the solution. Green and yellow signal indicates the presence of NP. Fluorescence was scored using a fluorescence microscope (Carl Zeiss Jena, Jena, Germany).

Analysis of T cell proliferation

OT-1 and OT-2 transgenic T cells were isolated from spleens and LNs of respective mice, enriched by MACS cell sorting with anti-CD8 or -CD4 antibodies according to manufacturer's protocol (Miltenyi Biotec GmbH) and labelled with CFSE (61). Sorted DC subsets were co-cultured with T cells in v-bottom plate at 1/10 ratio for 4 days. T cell divisions were measured by flow cytometry. The percentage of cells recruited into cell division was calculated by dividing the number of individed cells by CFSE content as described previously using the formula 100 x {1 - [n0/(n0 + n1/2 + n2/4 + n3/8 + n4/16 + n5/32 + n6/64 + n7/128)]} to correct for the multiplying effect of division (61)

Tracheal whole mount staining

Animals were anesthetized with a lethal dose of Nembutal and perfused *in vivo* with 1 % paraformaldehyde fixative in PBS (pH7.4) for 2 minutes through the ascending aorta (18). Tracheas were removed, opened by a midline incision and pinned flat on silicone-coated petri dishes. After permeabilization in PBS containing 0,3 % Triton X-100(Sigma), tissues were preblocked with rabbit serum, incubated with M5/114 mAb (rat IgG2, anti-I-A, and I-Eb,d,k, Boehringer Mannheim, Indianapolis, IN), for 36 hours at room temperature, washed, incubated with peroxidase conjugated rabbit-anti-rat IgG, followed by incubation for 20 minutes in 0,05 % diaminobenzidine (DAB)in Tris-buffered saline (pH7,6). Tissues were dehydrated in serial alcohol steps and cleared in toluene. The entire trachea was mounted in D.P.X. mounting medium.

Detection of virus-specific CTL by tetramer-staining

Single cell suspensions of lung and spleen samples were prepared as described previously (24). Red blood cells were removed using erythrocyte lysis buffer (Roche, Almere, the Netherlands). The cells were washed with 0.5% BSA in PBS and stained 20 minutes at room temperature with antibodies: CD3e-PerCP, CD8b.2-FITC (PharMingen, San Diego, United States), ToPro 3-APC (Molecular Probes,Eugene, United States) and PE labeled H-2Db tetramer with the $NP_{366–374}$ epitope ASNENMETM (62) (Sanquin Research, Amsterdam, The Netherlands)

Effector cytokine production

On 4 dpi, single cell suspensions of lung-draining lymph nodes were prepared. Cells were cultured in RPMI 1640 medium (RPMI 1640 with 5% FCS) at a concentration of 2 x 10^6 cells/ml in the presence of either 1 μg/ml plate-bound anti-CD3 (BD Biosciences) or medium alone and incubated at 37°C. After 4 days, supernatants were collected and stored at –20°C until ELISA for IFNγ (BD Biosciences) was performed. IFN-α ELISA (PBL Biomedical laboratories) was performed on BAL fluid on several dpi. QRT-PCR for IFN-α was performed on homogenised lung on 4 dpi (Applied Biosystems, Assay-On-Demand).

Generation of BM-DCs

Bone marrow cells were cultured for 9 days in DC medium (DC-CM; RPMI 1640 containing glutamax-I

(Invitrogen, Carlbad, 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, as described (15).

Adoptive transfer

Adoptive transfer reconstitution experiments were performed in DT treated CD11c-DTR x C57BL/6 mice. At the moment of infection they were treated with 2 x 10^6 i.t. bone-marrow –derived DCs and 2 x 10^5 alveolar macrophages, as previously described (19).

Detection of virus specific antibodies in serum

After treatment with cholera filtrate and heat inactivation at 56°C, the serum samples were tested for the presence of anti-HA antibodies. For this purpose, a hemagglutination inhibition (HI) assay was used following a standard protocol of 1% turkey erythrocytes and 4 HA U of H3N2 influenza virus (63).

Statistical analysis

All experiments were performed using 5-10 animals per group. 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 $p<0,05$.

Online Supplemental Materials

Figure S1 shows time kinetics of neutrophils, B cells and CD8+ T cells in the lung following influenza infection. Figure S2 shows the gating strategy for cDCs (A), pDCs (B) and alveolar macrophages (C). Figure S3 shows depletion of DC subsets in trachea (A) and MLN (B) in CD11c-DTR (left column) and Langerin-DTR (right column). The online version of this article is available at http://www.jem.org/cgi/content/full/jem20071365.

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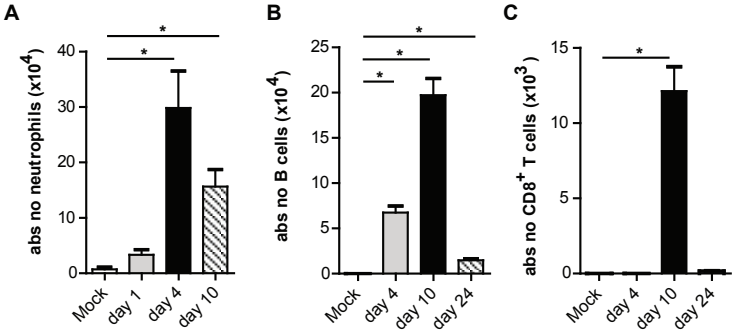
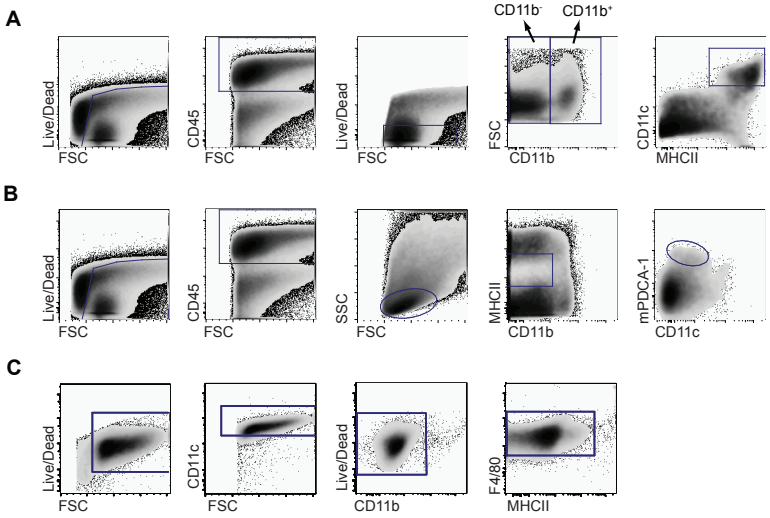
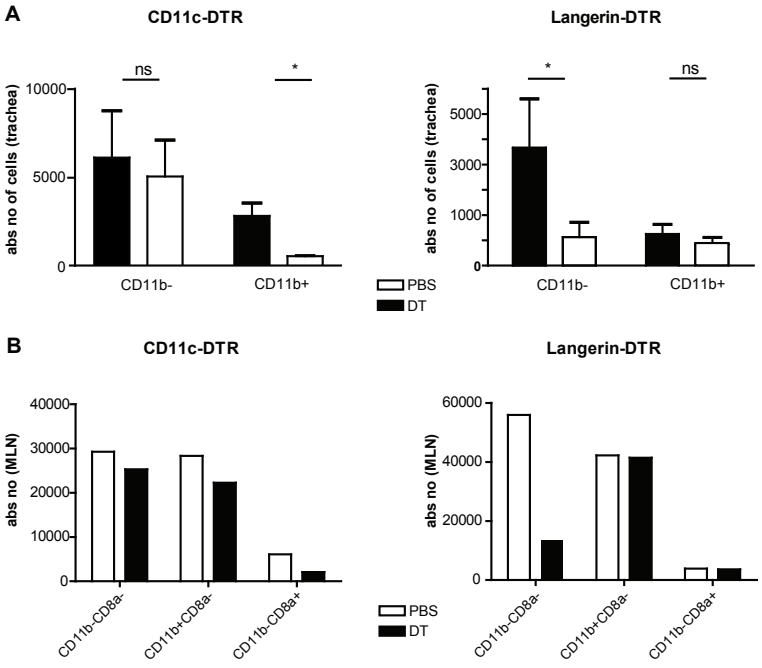
Supplementary figures



Supplementary figure 1: Cellular composition of BAL fluid.
(A) Number of CD11b$^+$ Gr1$^+$ neutrophils (B) Number of CD19$^+$ B cells (C) Number of CD8$^+$ T cells. The values are representative of 5 mice/group and expressed as mean ± SEM. Similar results were obtained from several separate experiments. *p<0,05 **p<0,01

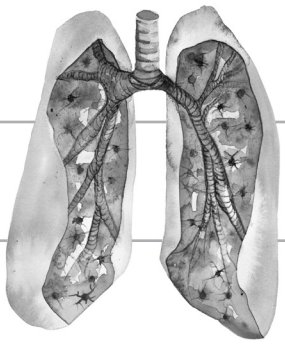


Supplementary figure 2: Gating strategies in 8 color flowcytometric analysis of lung samples.
(A) cDCs were gated as live cells, CD45$^+$ and low fluorescent. Next, CD11b$^+$ and CD11b$^-$ fractions were determined and within these populations the MHCII$^+$CD11c$^+$ DCs were gated. (B) pDCs were gated as live cells, CD45$^+$ in the lymphocyte gate. Next they were gated as CD11b$^-$MHCII^{int} cells expressing CD11c^{int} and high mPDCA-1. (C) Alveolar macrophages were determined in BAL fluid as highly autofluorescent cells, expressing high levels of CD11c. Next, the CD11b$^-$ fraction was gated for F4/80 pos cells.



Supplementary figure 3: Depletion of DC subsets in the trachea and MLN of CD11c-DTR versus Langerin-DTR mice.

CD11c-DTR (left) or langerin-DTR mice (right) received an i.t. injection of DT. One day after treatment tracheal digests (A) and pooled MLN samples (B) were obtained for a subset analysis. Open graphs represent untreated animals whereas black bars represent mice treated with DT. Similar results were obtained from several separate experiments. *p<0,05



Chapter 3

Both conventional and 'IKDC' natural killer cells have antigen presenting capacity during *in vivo* influenza virus infection

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ABSTRACT

Natural killer cells are innate effector cells known for their potential to produce interferon-γ and kill tumour and virus-infected cells. Recently, $B220^{+}CD11c^{int}NK1.1^{+}$ NK cells were found to also have antigen presenting capacity like dendritic cells (DC), hence their name interferon producing killer DC (IKDC). Shortly after discovery, it has already been questioned if IKDC really represent a separate subset of NK cells, or merely represent a state of activation. Despite similarities with DCs, *in vivo* evidence that they behave as bona fide APCs is lacking. Here, using a model of influenza infection, we found recruitment of both conventional B220- NK cells and IKDCs to the lung. To study antigen presenting capacity of NK cell subsets and compare it to cDCs, all cell subsets were sorted from lungs of infected mice and co-cultured *ex vivo* with antigen specific T cells. Both IKDCs and conventional NK cells as well as cDCs presented virus-encoded antigen to CD8 T cells, whereas only cDCs presented to CD4 T cells. The absence of CD4 responses was predominantly due to a deficiency in MHCII processing, as preprocessed peptide antigen was presented equally well by cDCs and IKDCs. *In vivo*, the depletion of NK1.1-positive NK cells and IKDCs reduced the expansion of viral nucleoprotein-specific CD8 T cells in the lung and spleen, but did not affect viral clearance from the lung. In conclusion, we found evidence for APC function of lung NK cells during influenza infection, but this is a feature not exclusive to the IKDC subset.

INTRODUCTION

Influenza type A is a cytolytic virus that causes acute respiratory infection of which the clinical outcome can vary greatly. The way in which the innate and adaptive immune system initially recognizes and deals with replicating virus could be decisive in determining outcome of infection, as this might heavily influence the kinetics of viral clearance (1-3). In immediate response to viral infection, innate defence mechanisms consist of high level production of type I interferons by infected epithelial cells, alveolar macrophages and natural interferon producing cells, as well as recruitment of neutrophils and NK cells (4),(5, 6). NK cells can kill virus-infected cells without prior antigen stimulation (7-9) in a process that is controlled by inhibitory and activating receptors, of which the activating natural killer cell receptor (Ncr1) gene product is most crucial during influenza infection (9-11). How exactly this innate immune response influences or enhances initiation of adaptive immunity is poorly understood.

By expressing a wide array of microbial pattern recognition receptors shared with cells of the innate immune response and at the same time displaying the potential to process and present antigen to naive T cells, lung DCs bridge innate and adaptive immunity (12). During influenza infection, both conventional (cDC) and plasmacytoid (p)DCs exert different functions, but both are necessary to induce an immune response that clears the virus from the lungs and prevents re-infection (13). Recently it has been proposed that new member in the mouse DC family might display both innate immune functions of NK cells, as well as the potential to process and present antigen to naïve T cells, the so-called interferon producing killer DC, or IKDC (14, 15). Phenotypically IKDCs are defined as non-T ($CD3^-$), non-B ($CD19^-$) cells, with expression of intermediate levels of CD11c, B220, and MHC class II, and high-level expression of NK-specific markers NK1.1 and NKp46 controlled by the Ncr1-locus (14-16). Functionally, IKDCs have the capacity to kill NK-sensitive target cells without prior activation, and induce proliferation of naïve T cells when pulsed with antigenic peptides. By contrast, conventional NK cells that lack expression of B220 and MHCII have not been identified as antigen presenting cells in the mouse(17). Although the existence of the IKDC population would make evolutionary sense, it has been controversial whether IKDCs really represent a separate DC lineage or nothing else but activated NK cells, endowed with antigen presenting capacity (18). The latter view is supported by the fact that

human NK cells have long been known to have APC-like activity (19, 20). The developmental pathways between NK cells and IKDCs also overlap as both rely on the IL-2Rβ, IL-5Rβ and common γ chain (and thus a functional IL-15R complex) and IL-15 for development, whereas cDCs and pDCs develop in the absence of a functional IL-15R (15, 16). Furthermore there was some doubt whether resting or CpG activated splenic IKDCs could present protein antigen or relevant pathogens (as opposed to pre-processed peptide) to CD8 and CD4 T cells *ex vivo* (21). Therefore, some authors suggest that IKDCs are functionally and developmentally closer to NK cells than to the two best-known DC family members.

Here, we have studied the antigen presenting capacities of conventional NK cells, IKDCs and cDCs during influenza infection. Subsets of conventional NK and IKDCs were sorted from the lungs of infected mice to study their APC potential, in direct comparison with conventional DCs. We found clear evidence for recruitment of NK subsets to lungs of infected mice and both conventional and IKDC subsets were able to present virus-encoded antigen to CD8 cells, but not CD4 T cells. In support, NK1.1 depletion led to a reduced expansion of virus specific CD8 T cells in the spleen and lung. However, viral clearance was unaffected. These data support an APC function for NK cells, which however is not unique to the IKDC subset.

RESULTS

Surface phenotype analysis of lung DC subsets and NK cells following influenza virus infection

There is considerable overlap in the level of expression of phenotypical markers that have been used to discriminate putative IKDCs from pDCs, cDCs and NK cells. In an attempt to make a head to head comparison of these various subsets in lungs in steady state conditions (mock infected mice) or during influenza infection, we have employed 10-colour flow cytometry to rigorously define phenotypes and activation status. We first gated on live cells in the lung and subsequently gated out the $CD19^+$ B and $CD3^+$ T cells (figure 1A, left plot), while discriminating between $B220^+$ and $B220^-$ cells. Conventional DCs (cDC) are described as being low for B220, while expressing high levels of CD11c and MHCII. As previously described by others, and us, in steady state conditions (mock) these cells can be further

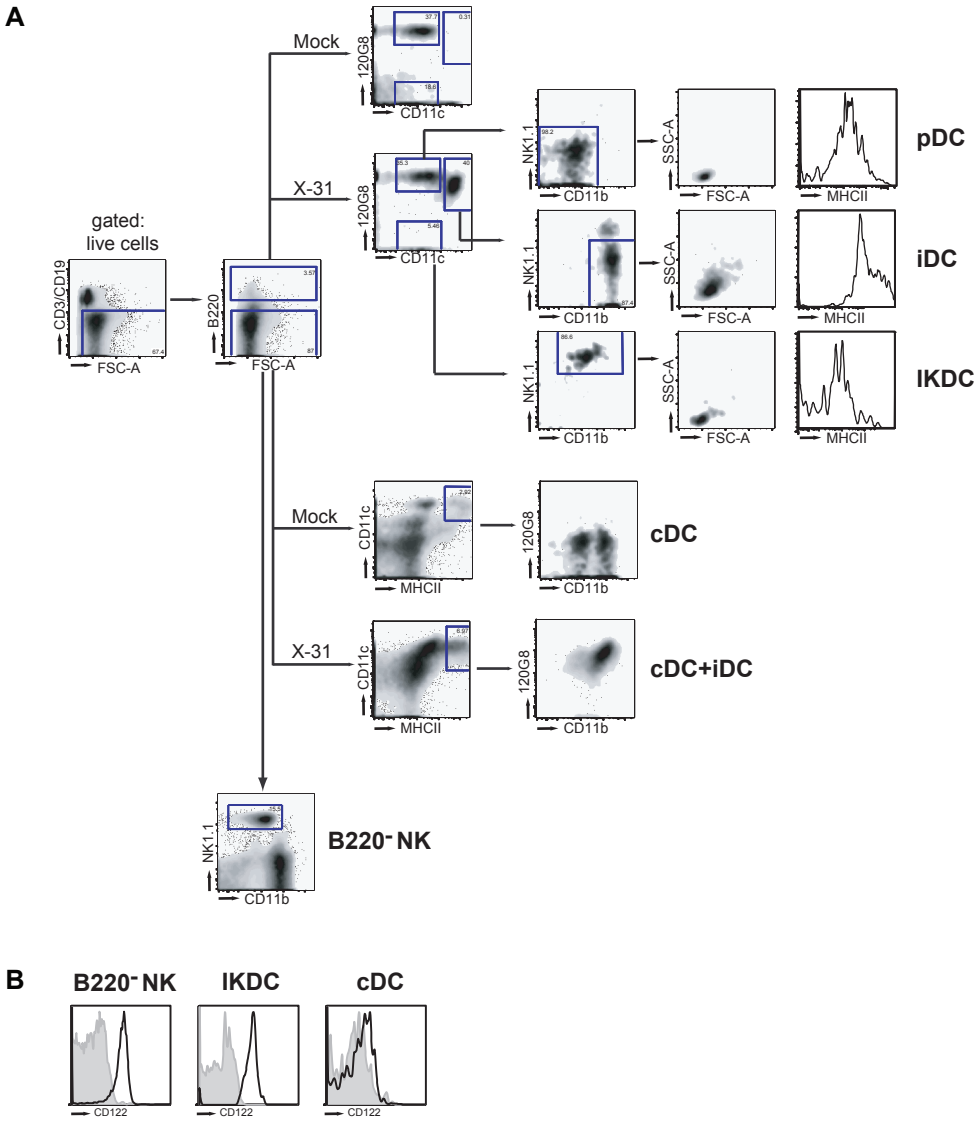


Figure 1: Identification of NK and DC subsets cell in lung tissue
(A) 10-color flowcytometric analysis of lung tissue. A CD3/CD19 negative population was gated from live, low autofluorescent cells in the lung. The B220 high population was further subdivided based on CD11c, 120G8, CD11b and NK1.1. After influenza virus infection, three populations could be clearly demarkated.CD11c^{interm}120G8^{low}NK1.1^{hi} IKDCs, CD11c^{interm}120G8^{hi}NK1.1^{lo}CD11b^{lo} pDCs and CD11c^{hi}120G8^{hi}CD11b^{hi}MHCII^{hi} iDCs. The iDC population was completely absent in steady state condition. In the B220 low population cDCs were gated as MHII^{hi}CD11c^{hi} and NK cells as a NK1.1^{hi} population expressing intermediate levels of CD11b. (B) Histograms demonstrating CD122 expression on NK cells, IKDCs and cDCs. The grey histogram represents isotype control, black histogram represents CD122 expression. Representative of 3 experiments.

discriminated into a $CD11b^+$ and a $CD11b^-$ subpopulation lacking expression of 120G8 as depicted in the lower panels of figure 1A (13, 28). The $CD3^-CD19^-B220^-$ population also contained the conventional $NK1.1^+$ NK cells. When selecting for $CD3^-CD19^-B220^+$ cells, we found two populations of $CD11c^{int}$ cells in steady state conditions, which can be further discriminated into pDCs by expression of the pDC marker bone marrow stromal antigen-2 recognized by the moAb 120G8. The $120G8^+CD11c^{int}$ population represents $NK1.1^-CD11b^-$ pDCs with low forward and side scatter, whereas the $120G8^-CD11c^{int}$ population represents $NK1.1^{hi}CD11b^{int}$ IKDCs, also of small size and scatter (14). In influenza X-31 infected lungs, there was a striking difference in the characteristics of isolated populations found at 4 days post infection. Firstly, within the $B220^-MHCII^{hi}CD11c^{hi}$ cDC, there was a loss of the $CD11b^-$ subset, as recently reported, and the remaining $CD11b^{hi}$ DCs co-expressed 120G8 (a pDC marker shown to be induced by interferon production) (29) (13). Even more strikingly, the $B220^+$ cells now also contained a subset of cells that highly expressed CD11c (like cDCs) as well as 120G8 (like pDCs). Further analysis of this population revealed that it expressed very high levels of CD11b and intermediate levels of NK1.1, although a subset of these cells was as high in expression of NK1.1 as IKDCs and NK cells. These cells were however larger and expressed very high levels of MHCII, most likely resembling inflammatory DCs, recently surged from monocytes (13, 30). Figure 1B demonstrates that the expression of the IL-2-IL-15 receptor β chain (CD122) was only expressed on NK cells and IKDCs, but not on pDCs or cDCs, as previously described (15). In addition, IKDCs in the lungs expressed NKG2D and PDL1 (also known as B7H-1) (data not shown).

During influenza infection, both lung IKDCs and $B220^-$ NK cells lyse NK-sensitive targets

The main function of NK cells is known to be cell lysis without prior activation in a process requiring granzyme A and B. We compared expression of these molecules by qPCR on lung derived NK cell subsets and cDCs, and found higher expression levels of both Granzym A and B on IKDCs than on regular $B220^-$ NK cells. On cDCs no expression was detected (figure 2A). Next, the functional capacity to lyse NK-sensitive YAC target cells was studied by using a CFSE-labeled YAC assay as described previously (25). In line with granzyme expression and expression of

activating NK receptors, both IKDCs and B220$^-$ NK cells sorted from infected lung tissue efficiently lysed YAC-1 target cells (figure 2B), to a degree that is seen when using splenic NK cells as effector cells, supporting the idea that lung CD3$^-$CD19$^-$ B220$^+$CD11c^{int}NK1.1$^+$ cells also functionally behave like IKDCs (15, 21).

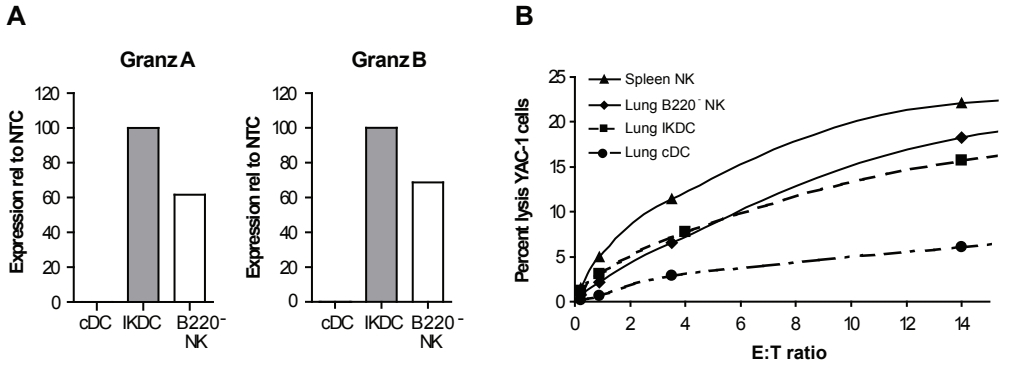


Figure 2: IKDCs and B220- NK cells induce cell lysis
(A) Expression of Granzyme A and B on sorted cell subsets at 4 dpi. Bars represent the values of expression relative to NTC. (B) Percentage YAC-1 cell target lysis after co-culture with different effector DC and NK cell subsets. IKDCs, B220$^-$ NK cells and DCs were sorted from lung tissue at 4 dpi following gating strategies depicted in figure 2 and compared with enriched NK cells from spleen tissue. Cells were incubated with CFSE-labeled NK-sensitive YAC-1 target cells. Lysis at each effector:target (E:T) ratio was determined as described in materials and methods. Representative of 2 experiments.

Kinetics and maturation state of IKDCs, B220$^-$ NK cells and cDCs in lung following influenza virus infection

As influenza X-31 infection is a mild infection localized in the respiratory tract, we studied the effect of infection on the number and maturation status of IKDCs in the respiratory tract (lung, bronchoalveolar compartment (BALf), mediastinal LN (MLN) and the spleen), using the multi-parameter gating strategy as above. A kinetic analysis at various days post infection (dpi) revealed that IKDCs, B220$^-$NK cells and cDCs accumulated in the lungs with different kinetics and to different extents; IKDCs and cDCs peaking at 7dpi whereas NK cells were increased from day 1 to 7 post infection compared with mock infected mice (data shown for digested lung tissue). For clarity reasons we lumped CD11b$^+$ and CD11b$^-$ cDCs together, but we have recently provided detail on this (13). We next defined the co-

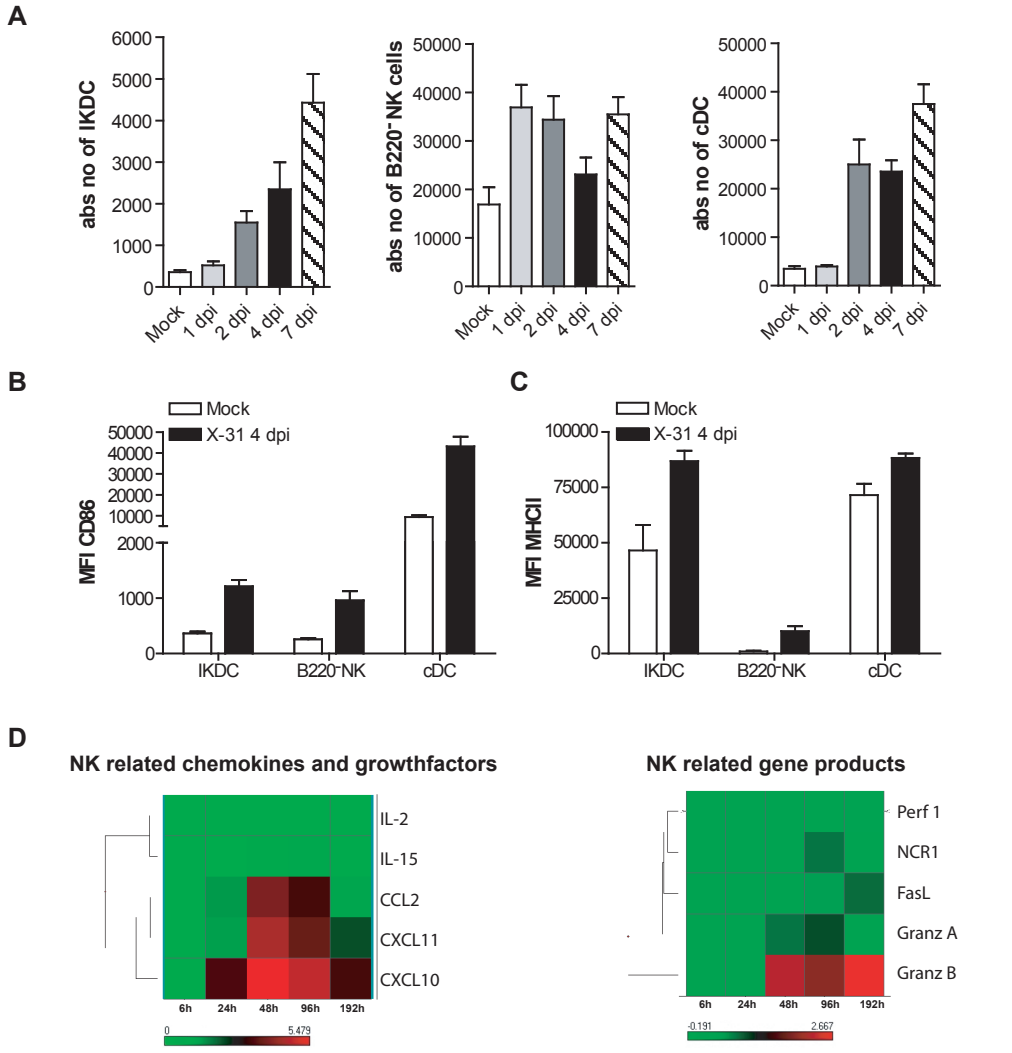


Figure 3: Kinetics of increase in IKDCs, B220-NK cells and cDCs in lung tissue after influenza virus infection
(A) Cell populations were gated as demonstrated in figure 1. Histograms demonstrate absolute numbers of IKDCs, B220$^-$NK cells and cDCs at different time points after infection. Bars represent mean values +/- SEM of at least 5 mice per group. CD86 (B) and MHCII (C) expression on cell populations at 4 dpi expressed as mean MFI +/- SEM, derived from at least five mice per group. Similar results were obtained from three separate experiments (D) Gene expression analysis using Affymetrix GeneChips. Top heat map shows NK related chemokines and growth factors. Lower map shows NK related gene products.

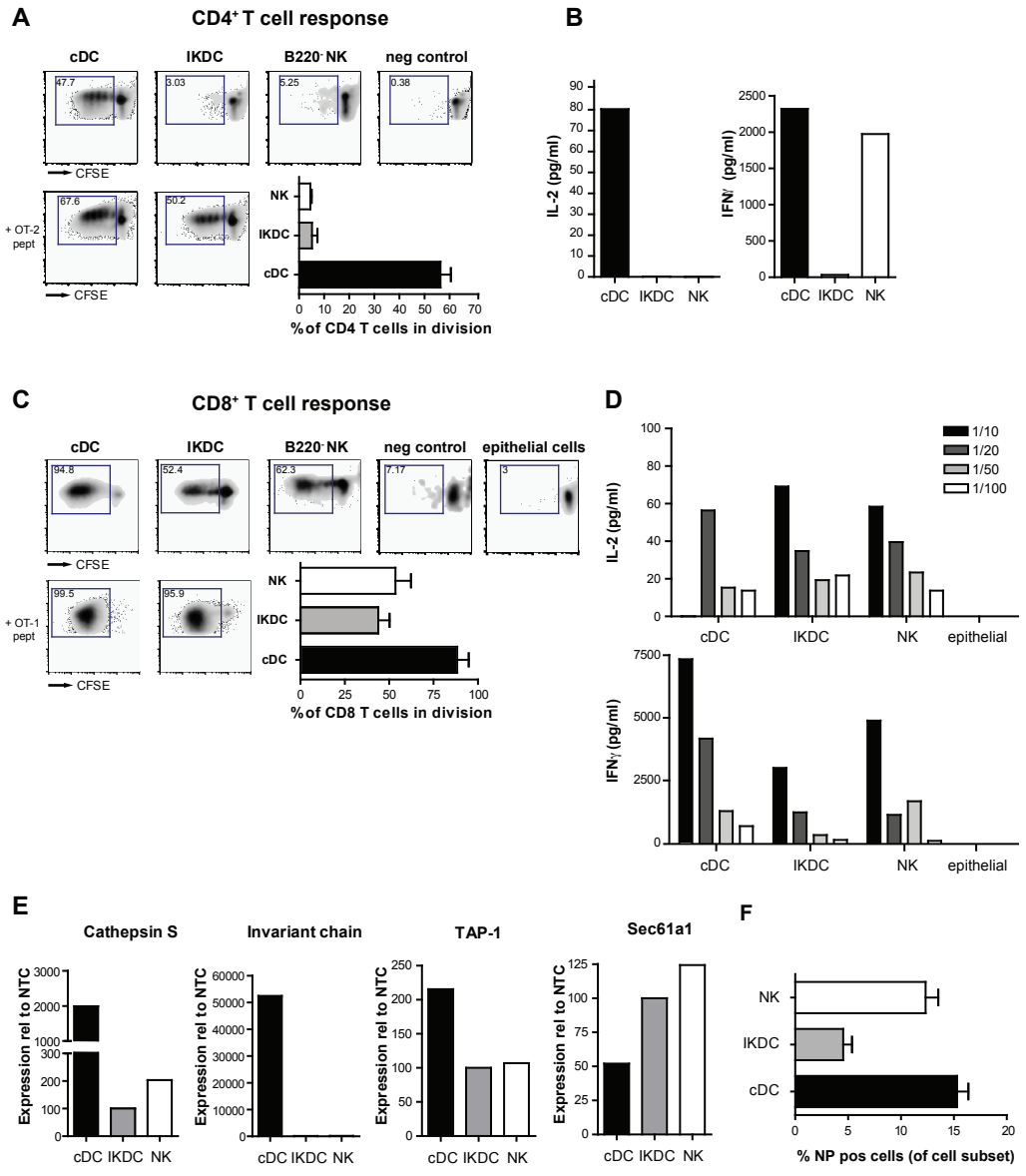
stimulatory molecule expression on these various populations at 4 dpi. Whereas we could find an increased number of IKDCs in all compartments (figure 3A

depicts only the digested lung samples), the increase in expression of the maturation marker CD86 (Fig 3B) and expression of MHC class II (figure 3C) molecules was restricted to the site of primary infection being lung and BALf (figures depict lung tissue) and occurred on both IKDCs, B220$^-$NK cells and cDCs, albeit to different degrees. Notably there were no signs of cDC, NK or IKDC activation in spleen or MLN (data not shown).

An increase in IKDCs could be due to increased chemoattraction of IKDCs to the lung, in a process that might be controlled by CXCR3 and/or CCR2 (31). We therefore examined expression levels of the CXCR3 ligands CXCL10 (IP-10), CXCL11 (ITAC), as well as the CCR2 ligands CCL2 (MCP-1) on a genome-wide expression micro array performed on whole lung homogenate from mock-infected or X-31 infected mice at various hours post infection (hpi) (Fig 3D, full microarray data to be published elsewhere). Analysis revealed that these chemokines were clearly upregulated with similar kinetics as gene products associated with NK and/or IKDC function such as Ncr1, granzyme A and B, klrk1, Nkg7. Alternatively, IKDC accumulation could be due to increased proliferation or survival inside the lung, possibly due to the presence of homeostatic cytokines of the IL-2 family that share the γc chain for signaling (IL-2, IL-15, IL-21).

Antigen presenting capacity of sorted IKDCs, B220-NK cells and cDCs from infected lungs

After observing a clear increase in absolute number and maturation state (MHCII and CD86) of IKDCs in lung tissue at 4 dpi, we decided to sort IKDCs from infected mice and perform a head to head comparison with their two closest family members, cDCs and B220$^-$ NK cells for the capacity to present to CD8 or CD4 T cells. To study the antigen presenting capacity of the different cell subsets we infected mice either with an influenza virus containing the OVA MHCI (SIINFEKL) or MHCII $OVA_{323-339}$ epitope. At 4 dpi DC and NK cell subsets were sorted with a purity exceeding 98% from the lung tissue and put in co-culture with either CFSE-labeled OVA specific $CD8^+$ T cells (OT-1) or $CD4^+$ T cells (OT-2), taken from the respective TCR transgenic animals. As a negative control, co-cultures were set up with influenza virus infected cDCs in which the opposite OVA epitope was expressed (for example: a virus containing the MHCII OVA epitope for presentation to OT-1 cells). To test if any lung cell exposed to the innate cytokine response would be able to induce



proliferation of OT-1 T cells, additional cocultures were set up with CD45- lung epitelial cells as APCs. Four days later, CFSE dilution of T cells was measured by flowcytometry, and expressed as the % of cells that had divided at least once. As shown in Figure 4A, cDCs induced strong CD4 T cell proliferation *ex vivo* following influenza infection (48% recruited into cell division), whereas IKDCs

Figure 4: Antigen presenting capacity of lung cDCs and NK cell subsets
Lung populations were sorted following the gating strategies in figure 1. (A) CFSE-labelled OT-2 ($CD4^+$) - T cell proliferation 4 days after co-culture with cDCs, IKDCs and $B220^-$ NK cells sorted from lung tissue of mice infected with PR-8 containing the OVA-$CD4^+$ epitope. Negative control sample indicates co-culture of OTII cells with cDCs derived from a mouse infected with a MHCI-OVA epitope containing virus. Histogram represents average % of cells divided, from 3 seperately performed experiments. Lower left plots indicate T cell proliferation after addition of OT-2 peptide to the co-cultures. (B) Histogram represent IL-2 and IFN-γ levels in supernatant of OTII co-cultures (C) Panel shows the same data for CFSE-labelled OT-1 ($CD8^+$) - T cell proliferation 4 days after co-culture with cDCs, IKDCs, $B220^-$ NK cells and CD45-negative epithelial cells sorted from lung tissue of mice infected with WSN containing the OVA-$CD8^+$ epitope. Negative controls in this setting are co-culture of OTI cells with cDCs derived from a mouse infected with a MHCII-OVA epitope containing virus. Numbers in top left corners of all plots represent the percentage of cells recruited into cell division and the histogram shows average % cell divisions of 3 separate experiments. (D) Histograms represent IL-2 and IFN-γ levels in supernatant of OTI co-cultures with different ratios of DCs versus OTI T cells. (E) Expression of TAP-1, Sec61a1, Cathepsin S and invariant chain on sorted cell subsets at 4 dpi. Bars represent the values of expression relative to NTC. (D) Percentage of cells that have nucleoprotein (NP) positivity, measured by flowcytometry, and indicating replicative infection. Bars represent an average of at least 5 mice/group +/- SEM.

only induced the proliferation in 3% and $B220^-$ NK cells in 5% of co-cultured CD4 T cells. In the supernatant of the co-culture we determined levels of IL-2 and IFN-γ (figure 4B) and according to the T cell division we found high levels of IL-2 in the co-culture with cDCs. High levels of IFN-γ were induced in the co-culture with cDC and $B220^-$ NK cells but not with IKDCs. Next, we studied the capacity to induce CD8 T cell proliferation (figure 4C). Conventional DCs induced the proliferation of up to 95% of co-cultured $CD8^+$ Ag specific T cells. In contrast to the lack of induction of CD4 responses, IKDCs presented influenza derived OVA epitopes to $CD8^+$ T cells (53% recuited into cell division), however to a degree similar to $B220^-$ NK cells (62 % recuited into cell division). Lung epithelial cells did not induce any T cell divisions. Cytokine profiles in figure 4D supported these data; high levels of both IL-2 and IFN-γ were measured in the cDC co-culture, whereas similar (yet lower) levels were found in cultures with IKDCs and NK cells, and epithelial cells failed to induce IL-2 or IFN-γ.The cytokine production showed a clear dose dependency in all APC populations, which were put into co-culture with several concentrations of OT-1 T cells. The absence of CD4 T cell response induction in the face of clear CD8 activation could signify a defect in processing of viral antigen for MHCII pathway or an absence of co-stimulatory molecules on NK subsets. To address if the MHCII complex and co-stimulation on IKDCs is at least functional, we added pre-processed MHCII-restricted $OVA_{323-332}$ peptide to the co-culture of IKDCs and OT-II cells, which induced efficient proliferation in up to 50% of CD4 cells, similar to the degree of division seen in T cells stimulated with cDCs (67%), indicating that IKDCs do posses a functional MHCII complex. Likewise, addition of MHCI-restricted OVA peptide led to identical T cell proliferation induced

by IKDCs and cDCs. It has been proposed that specializations of DCs to either cross-present to CD8 cells or to CD4 cells is a function of particular DC subsets caused by the expression of specific enzymes involved in antigen processing in particular DC subsets, or differential expression of antigen-uptake receptors (32, 33). To study antigen-processing capacity of the various isolated NK-like cell populations, quantitative PCRs were performed on the sorted cell subsets (figure 4E). Class II processing and loading molecules (cathepsin S and invariant chain) were expressed in high levels by cDCs and to a much lesser extent in IKDCs or $B220^{-}$NK cells. MHCI pathway molecules Sec61a1 (an endoplasmatic reticulum membrane protein translocator involved in crosspresentation to CD8 T cells) and TAP-1 (ATP-binding cassette transporter associated with MHCI loading) were expressed on cDCs and on both IKDC and $B220^{-}$NK cells. Differences in APC capacity might also reflect different degrees by which these cells were infected by influenza virus. To address this point, we studied the degree of infection by staining for nucleoprotein (NP) in the separate cell subsets at 4 dpi (13). Whereas 15% of cDCs and $B220^{-}$ NK cell isolated from the lung were infected, we found that only 5% of IKDCs were NP positive (figure 4F).

Depletion of NK1.1 positive cells during influenza virus infection reduces the innate and adaptive immune response to influenza

The described experiments suggested that IKDCs and B220- NK cells had APC potential for CD8 cells *ex vivo*. To study the role of $NK1.1^{+}$ cells during infection *in vivo* we injected the NK1.1-specific depleting antibody PK136 before and throughout influenza infection or mock infection (34). Both in mock infected and infected mice, intra-peritoneal (i.p.) injection of the antibody efficiently depleted $NKG2D^{+}$ $B220^{-}$NK cells and IKDCs from the lung tissue (figure 5A) when compared to injection with isotype Ig diluted in PBS, although depletion was more absolute in mock infected mice. On the contrast, cDC numbers were unaffected by treatment with NK1.1. Previously an enhancing effect of NK cells on the state of maturation and antigen-presenting capacity of cDCs has been suggested (35-37). To exclude any confounding effects of NK1.1 depletion on cDC functions, we additionally compared the antigen presenting capacity of sorted cDCs after infection with influenza virus containing the OVA MHCI epitope, in mice treated

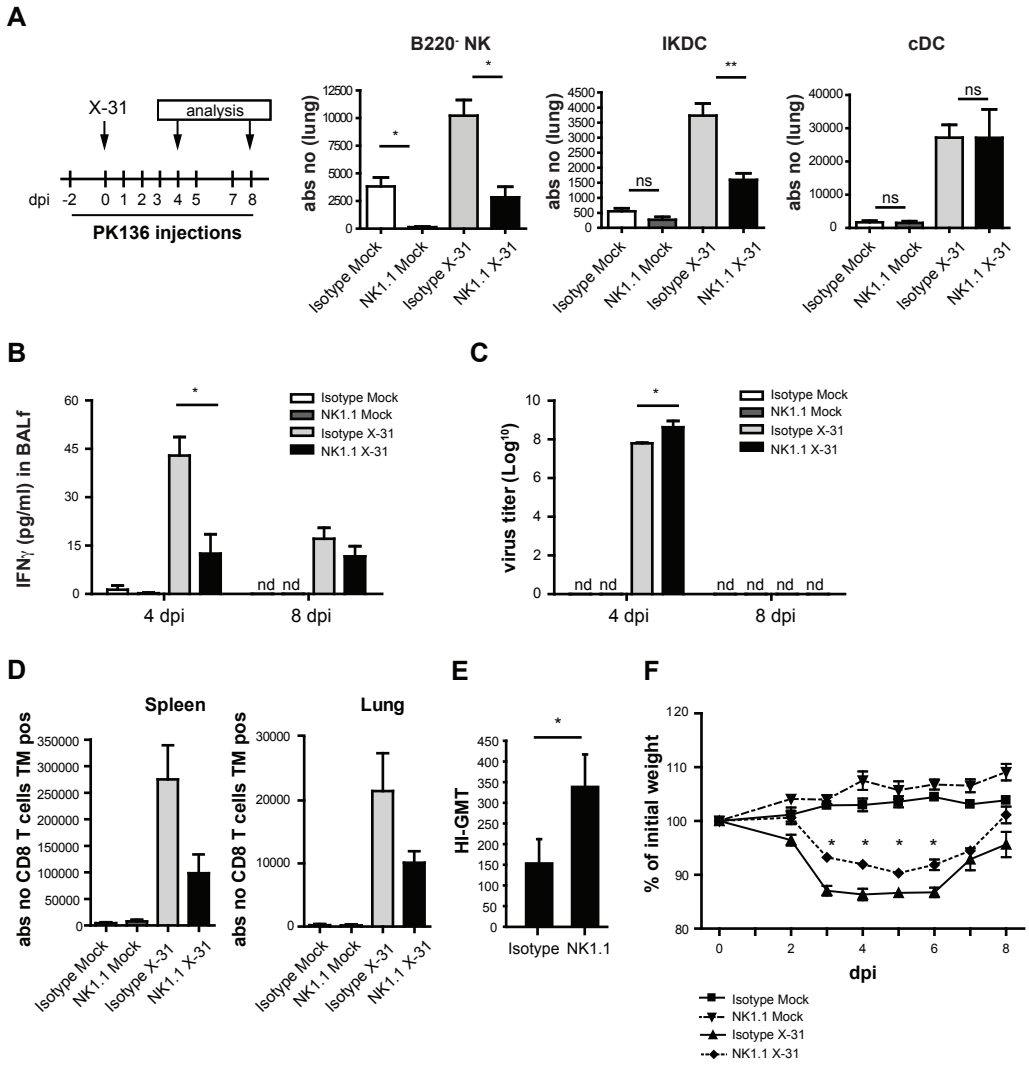


Figure 5: Depletion of NK1.1$^+$ cells during *in vivo* influenza virus infection affects infection parameters
(A) 2 days after injection of PK136 (NK1.1 depleting antibody), mice were infected with X31 virus. Two days later, depletion of NK subsets and cDCs was checked in lung tissue by using the gating strategy in Fig 1. (B) IFN-γ was measured in BALf of infected mice versus mock-infected mice with or without NK1.1+ cell depletion. Bars represent mean values of IFN-γ +/- SEM. At least 5 mice per group were used, * p<0,05. (C) Viral titers were measured in lung tissue of at least 5 mice per group and expressed as mean $TCID_{50}$ +/- SEM. * p< 0,05. (D) Absolute numbers of TM specific CD8$^+$ T cells were measured in spleen and lung tissue at 8 dpi and represented as mean values +/- SEM, of at least 5 mice/group. (E) Hemagluttinin specific antibodies in serum were measured at 8 dpi and depicted as geometric mean titer +/- SEM. * p<0,05. (F) Weight loss of mice during infection depicted as % of initial weight. Values indicate mean of at least 5 mice per group +/- SEM.*p<0,05.

or not with PK136. At 4 dpi cDCs were sorted from both groups and expressed similar levels of co-stimulatory molecules (CD86) and MHCII and induced the same amount of T cell proliferation (supplementary Fig 1). We next addressed the impact of NK1.1 depletion on induction of antiviral innate and adaptive immunity to a mild X-31 pulmonary influenza infection. During the early innate response to influenza, IFN-γ is found in high levels at the site of infection (lung tissue and BAL fluid) (13) and NK cells are the most prominent source of this cytokine (6, 11). Not surprisingly, we found a significant decrease of IFN-γ at 4 dpi in mice depleted of NK1.1 cells. At 8 dpi this mild influenza X-31 infection has been cleared from the lungs and therefore IFN-γ levels start decreasing at this time point (Figure 5B). Viral titers in mice lacking NK1.1 cells were increased 5 fold at 4 dpi compared with mice treated with isotype control antibody. Nevertheless virus was efficiently cleared at 8 dpi (figure 5C). Most importantly, to address the contribution of NK1.1 positive cells on induction of adaptive CTL immunity, we measured the percentage of virus-specific CD8 T cells at day 8 post infection by using a PE labeled H-2Db tetramer with the NP366–374 epitope ASNENMETM (13). At day 8 we found decreased numbers of tetramer (TM) positive cells in both spleen and lung tissue (figure 5D). In addition to this we determined the hemagluttinin specific antibodies in serum at 8 dpi (figure 5E) and found increased levels following NK1.1 depletion, supporting the higher viral titers. Both at earlier and later time points, similar conclusions on tetramer positive cell induction and humoral immune responses were reached (Supplementary figure 2). Surprisingly, NK1.1 depleted mice did not appear to have much more severe systemic morbidity as their weight loss did not exceed 10% of initial weight, in contrast with isotype treated mice, which lost significantly more weight during the peak of infection (Figure 5F). As a final support for an antigen presenting capacity of NK cells *in vivo*, we also found colocalization of naïve OVA-specific CD8 T cells with NK cells in the draining nodes 48 h after infection with influenza-OVA virus but to a much lower extent with wild type influenza virus (supplementary figure 3).

DISCUSSION

NK cells are part of the innate immune system and among the first immune effector cells to arrive at inflammation sites (5, 11). Here, we found rapid accumulation of cells with an IKDC phenotype in infected lungs. The reason for this could be

enhanced recruitment of IKDCs to the lungs, or enhanced proliferation and/or survival of IKDCs in the infected lung. Although little is known about chemokines and their receptors that govern directed migration of IKDCs, CCR2 and CXCR3 are likely candidates based on a recent study (31) and similarities with NK cell migration(38). Accordingly, we found an early yet persistent upregulation of the ligands CXCL10 and CXCL11 as well as CCR2 and CCL2 in virus-infected lungs. We also found increased expression of IL-15 in the lungs, a critical cytokine involved in IKDC development, expansion and survival in steady state (29) (16) (21) and inflammatory conditions (31). Another likely explanation for the increased number of NK-like cells in the lungs following infection could be that cells are interconverting by differentiation, thereby upregulating markers on their surface. We do not exclude that IKDCs could be a highly differentiated NK cell but decided to study both NK cell subsets separately and compare them with cDCs in lung tissue.

Although the predominant effector function of NK cells is the direct attack of virus-infected cells, as illustrated here by an increase in early viral titer in the lungs of NK1.1 depleted mice, increasing evidence suggests that they shape the quality and quantity of the ensuing adaptive immune response by either stimulating or suppressing the function of bona fide APCs like DCs (35-40). NK cells have extensive trafficking capabilities and are found within non-lymphoid and lymphoid tissues, sometimes in close proximity to naïve T cells, as also shown in this study (38, 41). *In vitro* studies revealed that activated human NK cells and NK cell clones can process soluble antigen, up-regulate MHCII and co-stimulatory molecules, and thus stimulate naïve CD4 and CD8 T cell responses (19, 20). The functional relevance of this has been questioned *in vivo*, as mouse NK cells were classically not found to have direct APC function (17, 18). Recently, it was proposed that IKDCs found in central lymphoid organs and tumours are the *in vivo* mouse correlates of human NK cells that display DC-like properties, such as expression of CD86, MHCII and potential to present pre-processed peptides to T cells (14, 15, 17). Although in the original description, these cells were proposed to be a separate and new subset of NK-like DCs, subsequently three different groups refuted this idea and proposed that IKDCs are nothing else but DC-like NK cells (16, 21, 29).

Regardless of these 'family affairs' between NK cells and DCs, the argument that NK cells might be endowed with APC potential made us embark on a detailed study in the mouse in which we evaluated the precise contribution of NK cells to

induction of adaptive immunity during influenza infection of the lung. We repeated an experiment previously reported by Kos and Engleman in which depletion of NK1.1$^+$ cells was shown to hamper the induction of virus-specific CD8 CTLs (34). This experiment had been performed in an era when it was impossible to identify virus-specific CD8 T cells with MHCI-peptide tetramers and multi-colour flow cytometry was not so advanced as to discriminate and sort multiple subsets of NK cells and DCs simultaneously. In our hands, injection of an NK1.1 depleting antibody effectively depleted the NKG2D$^+$ conventional B220-NK cells, as well as IKDCs, which led to a reduction of tetramer-positive NP-specific CD8 T cells upon viral infection, as measured at several time points post infection. Although our data on reduced CTL frequency support the early work of Kos et al, the interpretation has been that NK cells promote the accessory function of DCs via secretion of IFN-γ or direct cell-cell contact (34, 35). However several other explanations are plausible. By studying the expression of MHCII, CD86 and the potential of cDCs to present viral derived antigen to CD8 T cells, we found little evidence for altered DC accessory function when NK1.1-positive cells were depleted during influenza, lending little support to the former theory. Alternatively, NK1.1 Ab treatment might have eliminated CD8 CTLs directly, as NK1.1 is expressed by some 10% of virus specific CD8 CTLs ((42) and our own unpublished data). We do not believe that direct killing of CTLs by NK1.1 antibody was responsible for the observed effect because as many as 50% of tetramer-positive CD8 cells were depleted. CD3-positive NK T cells also express the NK1.1 marker, yet these cells were recently shown not to play a predominant role for antiviral immunity during influenza infection (43). Our data on the antigen presenting capacities of NK cell subsets *ex vivo*, as well as the colocalization of NK cells and naïve CD8 T cells in the central lymphoid organs at early time points post infection favour the theory that CD8 T cell responses were partially reduced because of the depletion of B220$^-$NK cells and IKDCs, as direct actors in the process of antigen presentation to naive antigen-specific CD8 T cells.

As recently suggested by others, we also found that during influenza infection the APC functions of NK cells are present within a subset of lung CD3$^-$ CD19$^-$NK1.1$^+$ B220$^+$CD11c$^+$ cells that also express killing activity for classical NK targets (14, 15). However, this APC feature was not unique to IKDCs and similarly present in B220$^-$ conventional NK cells. Both subsets upregulated the expression of CD86 upon influenza infection and presented to CD8 T cells. This could be

a reflection of direct Ag presentation in case of direct infection of NK subsets by influenza virus or cross-presentation of virally infected lung epithelial cells. At least for IKDCs we favour the latter explanation as we did not find real evidence of direct infection of IKDCs by influenza (low levels of viral nucleoprotein staining compared with cDCs (13, 21)), and as IKDCs highly expressed members of the ER retro translocation machinery Sec61a1 (involved in cross-presentation) and cathepsin S, also recently implicated to play a role in crosspriming (32, 33). How exactly IKDCs acquire antigen for cross-presentation however is unclear at present as we did not address their capacity to phagocytose virus infected cells, nor the causal relationship between killing of virus infected cells and subsequent processing of antigen derived from killed target cells. The division that was induced *ex vivo* in CD8 T cells by IKDCs was not accompanied by vigorous IFN-γ production, in contrast to T cells stimulated with cDCs under identical conditions.

One factor that discriminates lung NK cell subsets is the higher level of expression of MHCII on IKDCs compared with B220$^-$ NK cells in infected lung samples. It was therefore striking to see that IKDCs hardly presented influenza derived antigen to specific CD4 T cells, whereas cDCs readily did under these conditions. The most obvious explanation would be the much higher level of co-stimulatory molecules and MHC molecules on cDCs, and expression of the full enzymatic machinery to process antigen for the MHCII pathway in cDCs. Supporting this idea, we did find that addition of pre-processed MHCII peptide on IKDCs led to T cell divisions, albeit at reduced vigour compared with similarly pulsed cDCs. The apparent lack of mRNA expression of invariant chain by B220$^-$ NK cells and IKDCs, compared with cDCs explains the incapacity to process for MHCII, as invariant chain is crucial for proper intracellular routing and loading of MHCII molecules by endosomal cargo (32, 33, 44). Strikingly, the coculture of B220$^-$ NK cells and naïve CD4 T cells, did lead to a good production of IFN-γ. The fact that this was not accompanied by CD4 T cell division, makes it likely that this IFN-γ response was NK-derived. The idea that IKDCs have MHC processing capacity has been questioned by other studies showing a very weak potential of these cells to present antigen to CD4 T cells *ex vivo* following expansion in Gleevec/IL-2 and maturation by IL-15 (31), or following exposure *in vitro* to soluble protein antigens or pathogens (21). It is possible that IL-15 present in the lungs of influenza infected mice (as detected from our whole lung mRNA array data) shuts down part of the APC function of IKDCs for CD4 T cells, as recently demonstrated *in vitro*

(31).

At first sight our data on the APC capabilities of IKDCs are at odds with recent papers studying this subject. *In vitro* work in the human immune system illustrated how activated NK cells expressing MHCII and co-stimulatory molecules could process influenza hemaglutinin (HA) protein and virus infected targets for presentation to HA-specific $CD8^+$ and $CD4^+$ T cell clones (20). There could be major differences between *in vitro* activated human NK cells and *ex vivo* lung derived murine NK cells that have been part of an innate immune response to live influenza infection. In one of the papers refuting the idea that IKDCs represent a subset of DCs it was shown that splenic IKDCs were unable to present viral antigens to influenza specific T cells following *in vitro* influenza infection, even after CpG stimulation (21). This could reflect the need for a cell population or cytokine response *in vivo* that licences NK cell subsets to become APCs at the site of infection. Clearly, finding such a stimulus could be very instructive in finding ways to improve mucosal vaccines.

In conclusion we have demonstrated that influenza virus infecting the lung tissue seriously affects the phenotypical appearance of cells, which makes multi-color flowcytometry necessary to distinguish multiple cell subsets. We found *in vivo* and *ex vivo* evidence for antigen presenting capacities within IKDCs and conventional NK cells during influenza infection. All together, clearance of influenza virus from the lungs is therefore yet another example of the delicate crosstalk between innate and adaptive immune response that emerged through evolution to safeguard the host from infectious threats while avoiding immune pathology to the host.

MATERIALS AND METHODS

Influenza virus infection

C57BL/6 mice (6-8 weeks) were purchased from Harlan (Zeist, The Netherlands). Influenza virus X-31 (MRC, Cambridge, England) was inoculated in the allantoic cavity of embryonated chicken eggs. Infectious virus titers were determined in Madin-Darby Canine Kidney (MDCK) cells. Lungs were homogenized and ten-fold serial dilutions of these samples were used in eight-fold to determine the virus titers in MDCK cells as described previously(22). All experiments were approved by an independent animal ethics committee at Erasmus MC Rotterdam, the Netherlands.

NK1.1 cell depletion

Naive C57BL/6 mice were treated i.p. with 200mg of anti-NK1.1 mAb (PK136) on day -2 followed by

infection with influenza X-31 at day 0. Subsequent i.p. injections of 100mg anti-NK1.1. were given at day 0, 1, 2, 3, 4, 5 and 7 post infection. As a control mice were treated with isotype IgG from mouse serum (Sigma-Aldrich)

Flow cytometry and cell sorting

For detection and phenotyping of DC subsets in organs, cell suspensions of lung, MLN and spleen were prepared as described previously(23). Cells were subsequently stained with moAbs directed against 120G8 FITC (kindly provided by C.Asselin-Paturel), CD11b PE, MHCII PECy5, B220 PECy7, NKG2D PECy7, CD122 Pacific Orange (eBioscience), CD11c PETxR(Caltag), NK1.1 APC, CD3 APC Cy7, CD19 APC Cy7 (BD Biosciences), and a live/dead marker (DAPI) in Violet. Acquisition of 9-10 color samples was done on a LSRII cytometer. For measurement or viral nucleoprotein (NP) in cells FITC labeld anti-NP Ab (DakoCytomation) was used. Virus-specific CTL were detected by tetramer-staining with following antibodies: CD3e-PerCP, CD8b.2-FITC (PharMingen, San Diego, United States), ToPro 3-APC (Molecular Probes,Eugene, United States) and PE labeled H-2Db tetramer with the $NP_{366–374}$ epitope ASNENMETM (Sanquin Research, Amsterdam, The Netherlands). Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

Flow cytometry-based cytotoxicity assay

Effector cells from infected lungs were sorted on a FacsARIA cytometer and as a positive control spleen NK cells (isolated from infected spleens of the same mice of which lungs had been obtained) were enriched by MACS cell sorting with DX-5 antibodies according to manufacturer's protocol (Miltenyi Biotec GmbH). All effector cells were labeled with CFSE (24) and plated in serial dilution. Target cells (YAC-1) were harvested in exponential growth phase, washed and plated 2000 target cells/well. As a positive control 2% triton was added to a number of wells containing only YAC-1 cells. Cells were centrifuged for 1 minute at 335g to facilitate cell-cell contact, and incubated for 4 hours at 37˚C. At the end of the incubation time samples were put in an ice water bath and Micro Beads were added to allow a constant number of measuring on the LSRII Flowcytometer (BD Bioscience, USA). Prior to measuring, DAPI was added for labeling of dead cells. All samples were analysed using FlowJo software (Treestar, Costa Mesa, CA) as described previously (25).

Antigen presentation assays

For antigen presentation assays of lung DC subsets, mice were infected with WSN influenza virus encoding $OVA_{257-264}$ Kb restricted MHC I epitope in the neuraminidase (26) and PR-8 influenza virus encoding $OVA_{323-339}$ MHC II epitope in hemagglutinin of the virus (27). The OVA viruses were kindly provided by Dr. R.Webby (St. Jude Children's Hospital, Memphis, Tennessee, USA). OT-1 and OT-2 transgenic T cells were isolated from spleens and LN of respective mice, enriched by MACS cell sorting with anti-CD8 or –CD4 antibodies according to manufacturer's protocol (Miltenyi Biotec GmbH) and labeled with CFSE(24). Sorted DC subsets were co-cultured with T cells at 1/10 ratio for 4 days. T cell divisions were measured by flow cytometry and supernatants were collected and stored at -20˚C until ELISA for IFN-γ and IL-2 (BD Biosciences) was performed in 96 wells ELISA plates (Greiner Bio-One).

Affymetrix GeneChip hybridization and analysis

Mice were infected with 10^5 influenza virus X-31 at day 0. At several time points after infection (6, 24, 48, 96, 192 hours) mice were sacrificed, lungs were collected in RNAeasy and stored at -80˚C. Total lung RNA from influenza virus infected lungs was isolated using an RNeasy kit (Qiagen). RNA was biotin labeled and hybridized to mouse micro arrays (Affymetrix Mouse 430.2).

Real-time quantitative RT-PCR

Quantative RT-PCR for TAP-1, Sec61a1, Cathepsin S, invariant chain, Granzym A and Granzym B were performed on RNA from sorted DC subsets and NK cells. Frozen cell pellets were homogenized, RNA was isolated with RNAqueous micro kit (Ambion) and treated with DNaseI, according to the manufacturer's protocol. RNA (100 ng) was reverse transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences) for 50 min at 42 ˚C. Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems) and preformulated primers and probe mixes ('Assay on Demand', Applied Biosystems). PCR conditions were 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 15 s at 95˚C and 60˚C for 1 min using an ABI PRISM 7300 (Applied Biosystems). PCR amplification of the housekeeping gene encoding ubiquitin C was performed during each run for each sample to allow normalization between samples.

Statistical analysis

All experiments were performed using 5-10 animals per group. 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 $p<0,05$.

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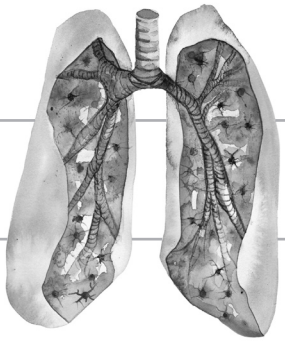

Chapter 4

Dendritic cells control tertiary lymphoid tissue formation in the lung of influenza virus infected mice
Brief Definitive Report

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ABSTRACT

Tertiary lymphoid organs (TLO) are organized aggregates of B and T cells formed in post-embryonic life in response to chronic immune responses to infectious agents or self-antigens. Although CD11c^{+} dendritic cells are consistently found in regions of TLO, their contribution to TLO organization has not been studied in detail. Here, we found that CD11c^{hi} DCs are essential for the organisation of inducible bronchus-associated lymphoid tissue (iBALT), a form of TLO induced in the lungs following influenza virus infection. Elimination of DCs after the virus had been cleared from the lung resulted in iBALT disintegration, and led to significantly reduced numbers of class switched plasma cells in the lung and bone marrow. DC depletion demonstrated a major contribution of iBALT to protective serum immune globulins. Dendritic cells isolated from the lungs of mice with iBALT no longer presented viral antigens to T cells, but rather were a source of homeostatic chemokines and cytokines known to organize TLO structures. Finally, repetitive administration of DCs to the lungs of naïve mice was sufficient to induce BALT like structures. Together our data reveal a previously unappreciated function of DCs in TLO homeostasis and humoral immunity.

INTRODUCTION

The organized accumulation of lymphocytes in lymphoid organs is a biological phenomenon used to optimize both homeostatic immune surveillance, as well as chronic responses to pathogenic stimuli (1). During embryonic development, circulating hemopoietic cells gather at predestined sites throughout the body, where they are subsequently arranged in T and B cell specific areas, characteristic of secondary lymphoid organs (SLO). In contrast, the body seems to harbour a limited second set of selected sites that support neo-formation of organised lymphoid aggregates in adult life. However, these are only revealed at times of local, chronic inflammation when so-called tertiary lymphoid organs (TLO) appear. As such, TLO was found in the liver of mice with chronic hepatitis (2), in the pancreas of mice with autoimmune diabetes (3), around blood vessels in chronic allograft rejection (4) and atherosclerosis (5), and in the brain in experimental allergic encephalitis (6). In humans, TLO has been observed in the joint and lung of rheumatoid arthritis (7), around the airways of COPD patients (8, 9), and in the thyroid (9). Certain infectious diseases are also accompanied by formation of TLO. As an example, influenza virus infection of the respiratory tract leads to formation of inducible bronchus associated lymphoid tissue (iBALT) that supports T and B cell proliferation and productive immunoglobulin class switching in germinal centres (GC) (10, 11).

Although the development of secondary lymphoid organs (SLO) during embryogenesis requires the function of $CD3^{-}CD4^{+}$ lymphoid tissue-inducer cells (LTi), these are not a prerequisite for TLO induction (12). Like SLO, TLO are formed in a highly regulated manner controlled by production of homeostatic chemokines such as CXCL13 and CCL19/CCL21, partially in response to signalling from the heterotrimer lymphotoxin-$\alpha 1\beta 2$ (LT-$\alpha 1\beta 2$) acting on the LTβ-receptor on stromal lymphoid tissue organizer cells (5, 7, 10, 12-14). The instruction of stromal cells leads to formation of specialized high endothelial venules and the organized production of chemokines leads to cellular organization of T cells and B cells in discrete area (14). In all instances where TLO have been described, antigen presenting DCs have been found interspersed with T and B cell area, just as they do in SLO (10, 12, 15-17). So far, the precise role of DCs in the functional organization of TLO has not been studied in great detail. Although DCs are mainly known for their function as antigen presenting cells to T and B cells (18), they are also a prominent

source of homeostatic and inflammatory chemokines that can attract T and B cells and thus may contribute to TLO homeostasis (19, 20). Here, we have studied the precise contribution of DCs in the functional organization of inducible bronchus associated lymphoid tissue (iBALT) a specific form of TLO found in the lung after influenza virus infection (10, 11, 21).

RESULTS

Lung CD11c$^+$ DCs localize to zones of iBALT after clearance of influenza virus

Mice were infected intranasally with a non-lethal strain of influenza A/HK-X-31 (H3N2) that is cleared from the lungs at 8 days post infection (dpi) (22) and is accompanied by formation of iBALT as soon as 10 dpi (11). At various dpi, the presence of CD11c$^+$ DC subsets (CD11b$^+$ and CD11b$^-$)(23) was determined in dispersed lung cells. In mock-infected mice, a majority of DCs were CD11b$^-$. However, up to at least 24 dpi the number and percentage of CD11b$^+$CD11c$^+$ DCs remained increased in influenza infected over mock infected mice (figure 1A and (22)). To precisely localize these CD11c$^+$ cells in the lung, immunohistochemistry was performed 17 dpi (figure 1B). CD11c$^+$ DCs were found within areas of B220$^+$ B cell aggregates, as well as in regions containing more CD4 and CD8 T cells. In accordance to previous reports, these cell aggregates resembled iBALT as the B cell area contained follicular dendritic cells (FDC, recognized by the moAb FDC-M2) and PNA-positive germinal center (GC) B cells (see below). Although iBALT was mainly present in close proximity to bronchi, these structures were also seen in the lung interstitium, as reported(21).

Lung DCs are necessary for maintenance of iBALT after viral clearance

Having observed the presence of DCs inside iBALT structures at 17 dpi, we next addressed whether CD11c^{hi} cells were necessary for preserving iBALT structures. A CD11c-diphteria toxin receptor (DTR) transgenic mouse model was used to deplete CD11c$^+$ DCs from the lungs, as described previously (22, 24). By treating mice at 17 dpi with diphtheria toxin (DT) intra-tracheally, CD11c$^+$ DCs were depleted from the lung (figure 2A) and draining mediastinal lymph nodes (ref (22) and

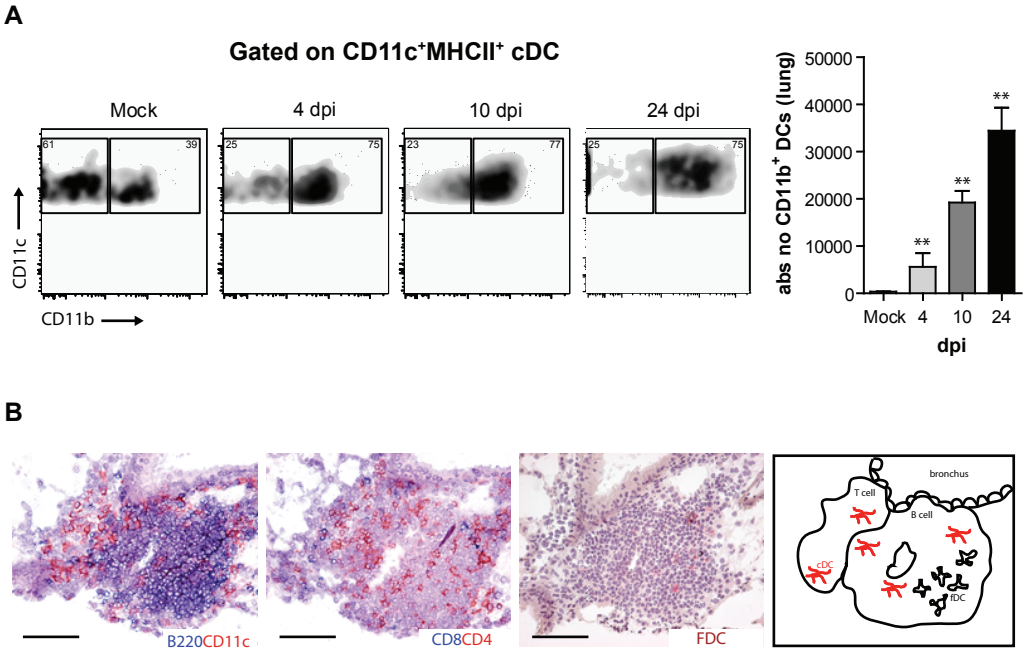

Figure 1: The development of iBALT structures following influenza virus infection.
(A) Flowcytometric analysis of CD11b expression on DCs following infection. Numbers in the corners of the plots indicate % of MHCII$^+$CD11c$^+$ DCs. Histogram indicates absolute numbers of CD11b$^+$ DCs, bars represent mean values of at least 5 mice/group and arrow bars indicate SEM, ** p<0,01 (B) Consecutive histological slides from snap frozen lung tissue at 17 days post infection (dpi). B220$^+$ B cell structures could be identified, with CD11c$^+$ DCs within the aggregates. Mainly CD4 T cells and some CD8 T cells were present in iBALT and follicular DCs (FDC) could be detected. Right panel shows schematic view of the depicted area. Bar represents 100μm.

suppl figure 2), but not from the spleen or other SLO (data not shown). CD11c^{interm} plasmacytoid DCs were minimally affected by diphtheria toxin treatment (data not shown and (25)). One week after depletion of DCs (i.e. at 24 dpi), lungs were examined by immunohistochemistry. Whereas infected CD11c-DTR Tg mice treated with PBS (figure 2B, left upper photographs) or non-transgenic mice treated with DT (data not shown) still had extensive iBALT structures containing B220$^+$ B cells and CD11c$^+$ cells throughout the lung, these structures disintegrated after DT treatment of CD11c-DTR Tg mice (figure 2B lower photographs). Some B220$^+$ B cells were still detected in the lung tissue, clustered into discrete regions, but density of B cells was strongly reduced and these regions no longer contained CD11c$^+$ cells.

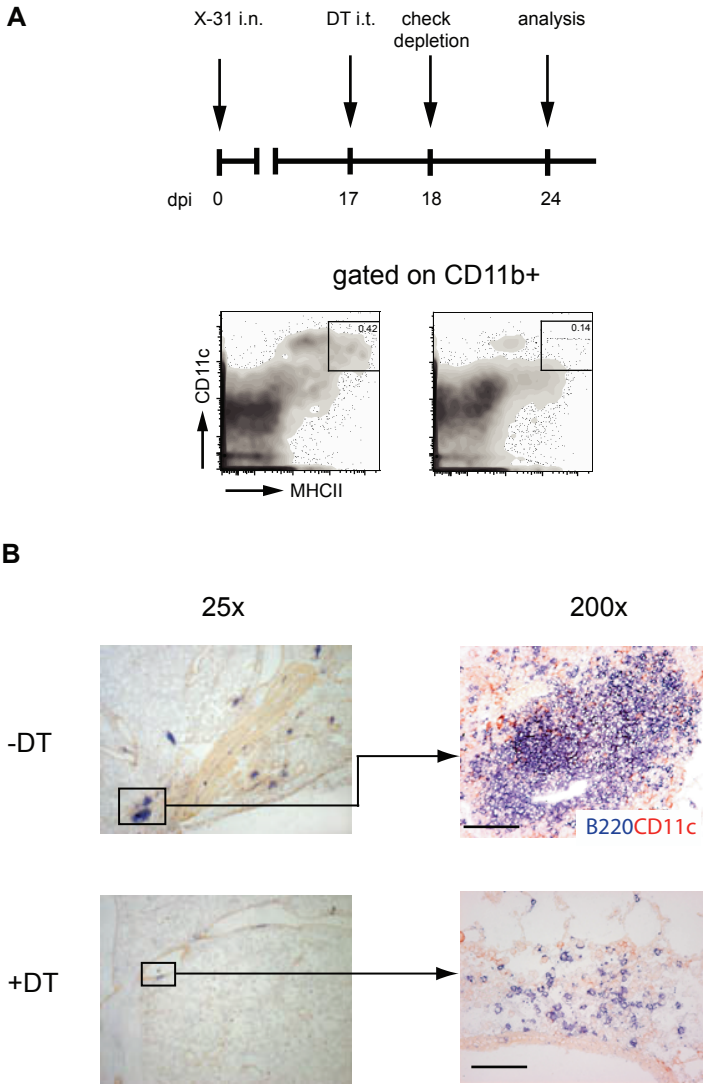

Figure 2: Depletion of CD11c$^+$ DCs in a CD11c-DTR mouse model abrogates iBALT structures from the lung
At 17 dpi mice were treated with DT i.t. and depletion was checked at 18 dpi and 24 dpi. At both time-points the majority of cDCs expressed CD11b. Boxes outline the DC populations, the numbers indicating mean values of % DCs (of cells alive) (B) one week after depletion of CD11c$^+$ DCs dense B220-CD11c aggregates could not be detected in the lungs. Bar represents 100μm.

DC depletion following viral clearance affects local humoral immune responses

The precise contribution of TLO to cellular and humoral immunity is currently unclear but it is thought to serve local immune responses to microbial derived antigens or to autoantigens (3, 9). Although a role for iBALT in mediating humoral immunity to influenza has been proposed, these studies were performed in mice lacking SLO through genetic manipulation (11). The breakdown of iBALT in DC-depleted mice, allowed us to study the functional contribution of these local structures to humoral immunity in mice with normal SLO. GC B cells undergoing active immunoglobulin (Ig) class switching, are typically found within TLO (3, 10). Presence of these GCs was demonstrated by staining consecutive slides for peanut agglutinin (PNA) and Ig isotypes (figure 3A). Next to IgM^+ B cells, IgM, IgG and IgA secreting plasma cells could be detected at the periphery of the iBALT. We next addressed if plasma cells were specific for influenza. For this purpose we developed a novel staining technique, employing recombinant nuclear protein (NP) as a straining reagent to detect NP-specific B cells (see supplementary figure 1). Double staining for NP-specificity with the plasma cell marker CD138 confirmed that the majority of NP-specific cells within iBALT were plasma cells (supplementary figure 1A). Strikingly, after local depletion of DCs by DT treatment at 17 dpi, these NP-specific plasma cells could no longer be detected on histology 24 dpi (figure 3B). This indicated that besides an abrogation of iBALT aggregates, depletion of DCs seriously affected local immunity. To quantify these changes, multi-color flowcytometry was performed on dispersed lung cells taken 1 week after DC depletion. The numbers of total $B220^+CD19^+$ B cells (data not shown), and the number of $B220^+CD19^+$ $IgM^-IgD^-CD95^+$ PNA-positive GC B lymphocytes were significantly decreased (figure 3C). Furthermore, local Ig levels in broncho-alveolar lavage fluid (BALf) were measured. Whereas the levels of class-switch independent IgM remained unaffected by DT treatment, IgA levels were readily decreased (figure 3D). This points out the importance of iBALT structures and intact DC function for the local production of antibodies that rely on germinal centers for Ig class-switching.

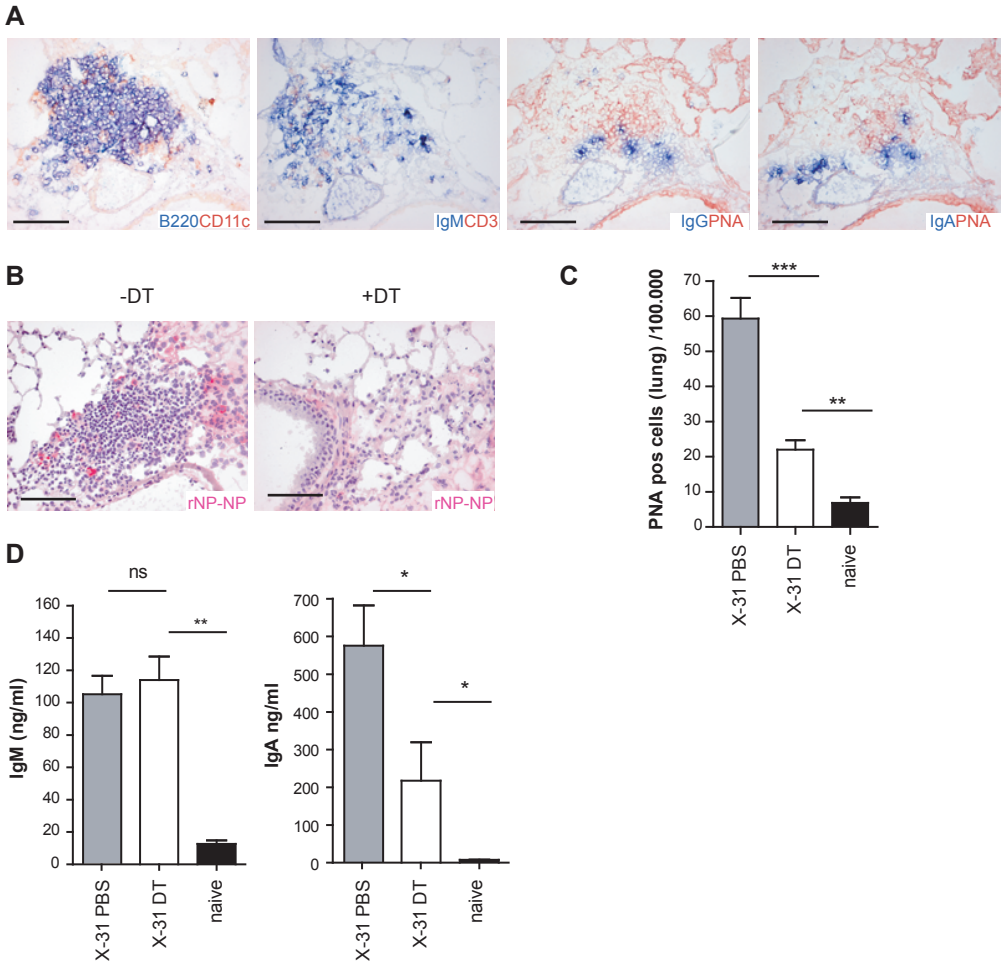

Figure 3: Local humoral immunity is affected by DC depletion
(A) Local class switching in the lung is demonstrated on consecutive slides of lung tissue at 24 dpi which show IgM, IgG and IgA in proximity of PNA positive germinal centers. (B) Depletion of DCs affected presence of NP specific plasma cells. Bars represent 100µm. (C) Histogram represents the decreased number of $B220^{+}CD19^{+}IgM^{-}IgD^{-}PNA^{+}$ cells per 100.000 lung cells one week after DC depletion (DT). (D) Levels of IgM and IgA measured in BALf of mice one week after DC depletion. Bars represent average values with an arrow bar indicating SEM. All results in this figure represent results of at least 5 mice/group and similar results were obtained from at least three separate experiments.
* $p<0,05$ *** $p<0,001$

Major contribution of iBALT to systemic humoral immunity

The contribution of iBALT plasma cells to systemic humoral immunity has not been studied in great detail. Serum hemagluttinin-inhibiting (HAI) antibodies, the gold standard correlate of protective systemic immunity to influenza, were determined at 24 dpi, one week after DCs and iBALT had been abolished by DT treatment. Surprisingly, the local depletion of DCs and iBALT led to significantly decreased HA specific antibodies in serum (figure 4A). Although serum virus-specific antibodies may derive from plasma cell production locally in the lung or draining lymph nodes, an important source of serum antibodies are long-lived bone marrow plasma cells (26). To study this matter, $CD19^{+}CD138^{+}$ NP specific plasma cell numbers in bone marrow were determined by multi color flow cytometry, and found significantly decreased one week after lung DC depletion (24 dpi, figure 4B). These data suggest that even 17 days post infection, the formation of a pool of long lived plasma cells in the bone marrow still depends on continued input of B cells that have undergone class switching in the local TLO of the lung. As we found that DC depletion leaves the differentiation of NP specific plasma cells in lung draining lymph nodes intact (see supplementary figure 2), we conclude that iBALT contributes significantly to systemic humoral immunity.

To study the biological significance of the decrease in bone marrow plasma cells, we reinfected mice with the homologous virus, causing no systemic illness (data not shown). Mice were infected at day 0 and a second time at day 31. Although reinfection did not cause clinical illness, it strongly boosted serum HAI titers and local IgA responses in the BAL fluid, when compared with primo-infected mice. However, when iBALT structures were affected by DC depletion intermittently at 17 dpi, reinfection led to lower titers of these antibodies. However, reinfection in DC-depleted mice still elicited stronger humoral immunity when compared with primary infected mice. This was most likely due to the fact that DC depletion did not fully reduce the pool of long-lived plasma cells in the bone marrow down to the level of non-infected mice (Fig 4 B), nor did it fully abolish local IgA responses (figure 3D).

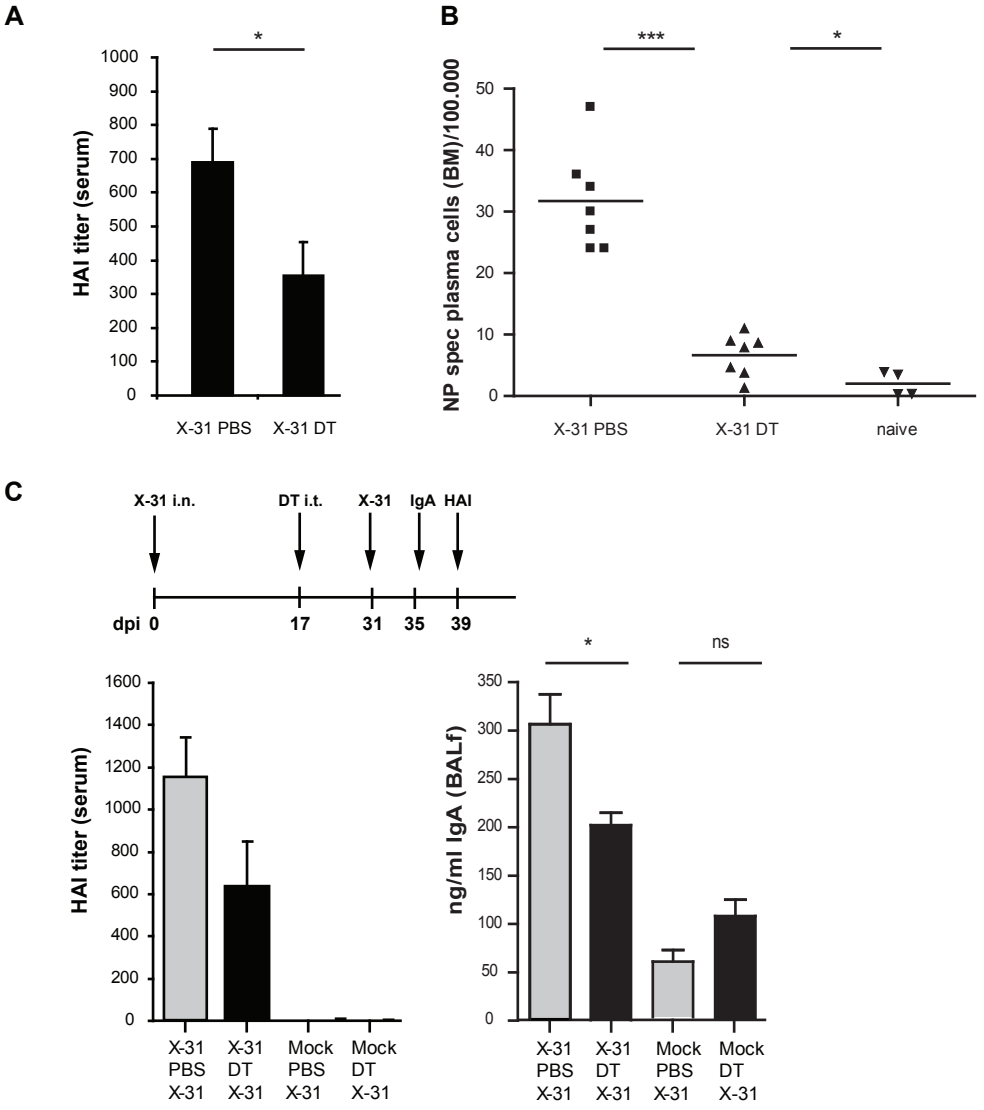

Figure 4: Abrogation of iBALT affected systemic humoral responses
(A) Hemagluttinin (HA) agglutination inhibition titers measured in serum, indicating the titer of HA specific antibodies. Mice were treated with DT at 17 dpi, serum was obtained at 24 dpi. Bars indicate average values with SEM. (B) Number of $CD11b^{-}CD138^{+}CD95^{+}NP^{+}$ plasma cells in bone marrow per 100.000 bone marrow cells. All results in this figure represent results of at least 4 mice/group and similar results were obtained from at least three separate experiments. (C) Secondary homologous influenza virus infection 2 weeks after DC depletion (DT) or PBS. 4 days after secondary challenge IgA in BALf was measured (right plot) and 8 days after secondary challenge HA inhibiting serum titers were measured (left plot). Bars represent average values with an arrow bar indicating SEM. All results in this figure represent results of at least 5 mice/group and similar results were obtained from at least two separate experiments. * $p<0,05$, *** $p<0,001$, ns not significant.

After viral clearance, lung CD11b$^+$ DCs no longer present viral antigen yet express homeostatic chemokines

Taken together, our data demonstrated a crucial role of DCs in maintenance of iBALT structures long after virus had been cleared from the lungs. This could be due to ongoing presentation of viral antigen by DCs to nearby CD4$^+$ or CD8$^+$ T cells (see figure 1), which subsequently help in organizing the iBALT structure and local Ig class switching (12). Previous reports have indeed suggested a possibility of prolonged persistence of viral or parasitic airborne antigen in pulmonary DCs (27, 28). However, this scenario is less likely, as we studied antigen presentation of viral proteins by CD11b$^+$ DCs at various dpi (figure 5A). To this end, we infected mice with a recombinant influenza X-31 strain in which the MHCII $OVA_{323-339}$ epitope was inserted in the hemagglutinin gene of the virus. At 4 dpi and 17 dpi CD11b$^+$ DCs (defined as B220$^-$CD11c$^+$CD11b$^+$MHCII$^+$120G8^{low}) were purified from lung tissue, and co-cultured with CFSE-labeled OVA-specific CD4 T cells (OT-II), taken from OT-II transgenic animals. Four days later, CFSE dilution in T cells was measured by flow cytometry, and expressed as the % of cells that had divided at least once. Figure 5A demonstrates that at 4 dpi lung CD11b$^+$ DCs were capable of efficient OVA antigen presentation to CD4 T cells *ex vivo*. At 17 dpi however, sorted CD11b$^+$ DCs could no longer induce CD4 T cell division, whereas addition of preprocessed OVA peptide showed that they still possessed antigen-presenting capacity for naïve T cells. In the negative control experiment, in which the co-culture was set up with OVA specific CD8 T cells (OT-1) at both time-points, DCs did not induce any cell division (data not shown). These data indicate that the function of DCs within iBALT at 17 dpi is not the presentation of viral antigen to surrounding T cells.

If DCs do not exert their antigen presenting capacity at the moment they are located in iBALT, what then is their function? Previous studies in influenza models indicated that chemokines are mainly produced by stromal cells during iBALT formation (7). However, CD11c$^+$Ly6C^{lo} monocytic precursors to DCs have also been suggested to be a source of these chemokines (5) (23). To study the possible role of DCs as chemokine producers, we sorted CD11c$^+$CD11b$^+$ DCs from digested lung tissue at 17 dpi and compared these with populations sorted at 4 and 10 dpi. Total RNA was isolated and chemokine expression levels were determined by quantitative PCR. Importantly, at 17 dpi expression levels of CXCL12 (stromal cell derived factor 1), CXCL13 (B lymphocyte chemoattractant) and LTβ were increased,

when compared with those at 4 and 10 dpi (figure 5B). These chemokines as well as LTβ, for which recently a major role in aorta TLO formation was demonstrated (5), have been strongly implicated in controling both SLO and TLO development, by recruiting B cells and retaining plasma cells (7, 14, 29, 30). However, formation of iBALT seems to be only minimally affected by the loss of CXCL13 (7) suggesting that several chemokines might have redundant effects during iBALT formation.

Adoptive transfer of DCs induces iBALT

So far, our studies demonstrated that endogenous lung DCs were located within iBALT and were necessary for maintenance of these structures, possibly by producing chemokines and cytokines controlling TLO homeostasis. As a final experiment, we also questioned whether adoptive transfer of $CD11b^+$DCs would be sufficient to induce iBALT. Therefore, GM-CSF cultured bone marrow DCs, which are an *in vitro* equivalent of *in vivo* inflammatory type $CD11b^+$ DCs (31), were administered 5 times intra-tracheally (i.t.) to naïve mice, each with 2 week intervals. 2 days following the last DC injection lungs were immunohistochemically stained and examined for the presence of iBALT. Besides $B220^+$ B cell aggregates containing $CD11c^+$ DCs (figure 5C), germinal centers containing PNA-positive B cells were found, with some IgM or IgG antibody secreting plasma cells. These findings indicate that DCs may well exert their effects on TLO neogenesis directly. However, as we have previously shown that adoptive transfer of DCs to the lungs causes airway inflammation (32), it is also conceivable these effects on TLO neogenesis are induced via chronic inflammation. All together, our data for the first time support a crucial role for DCs in the control of iBALT formation in the lungs following influenza virus infection. First, $CD11b^+CD11c^+$ DCs are found inside iBALT structures, and produce chemokines and cytokines that can retain B cells and organize TLO. Secondly, depletion of $CD11c^+$ DCs abolishes pre-exisiting iBALT structures and induces a concomitant reduction in systemic and local humoral immunity. Finally, administration of just DCs to the lungs of naïve mice induces iBALT structures. It will be interesting to study if $CD11c^+$ DCs would also have a critical role in SLO development and/or homeostasis following infections (33). One study supports this notion, as $c\text{-}Kit^+$ $CD11c^+$ cells were found to be crucial for development of Peyer's patches during embryogenesis (34). A big

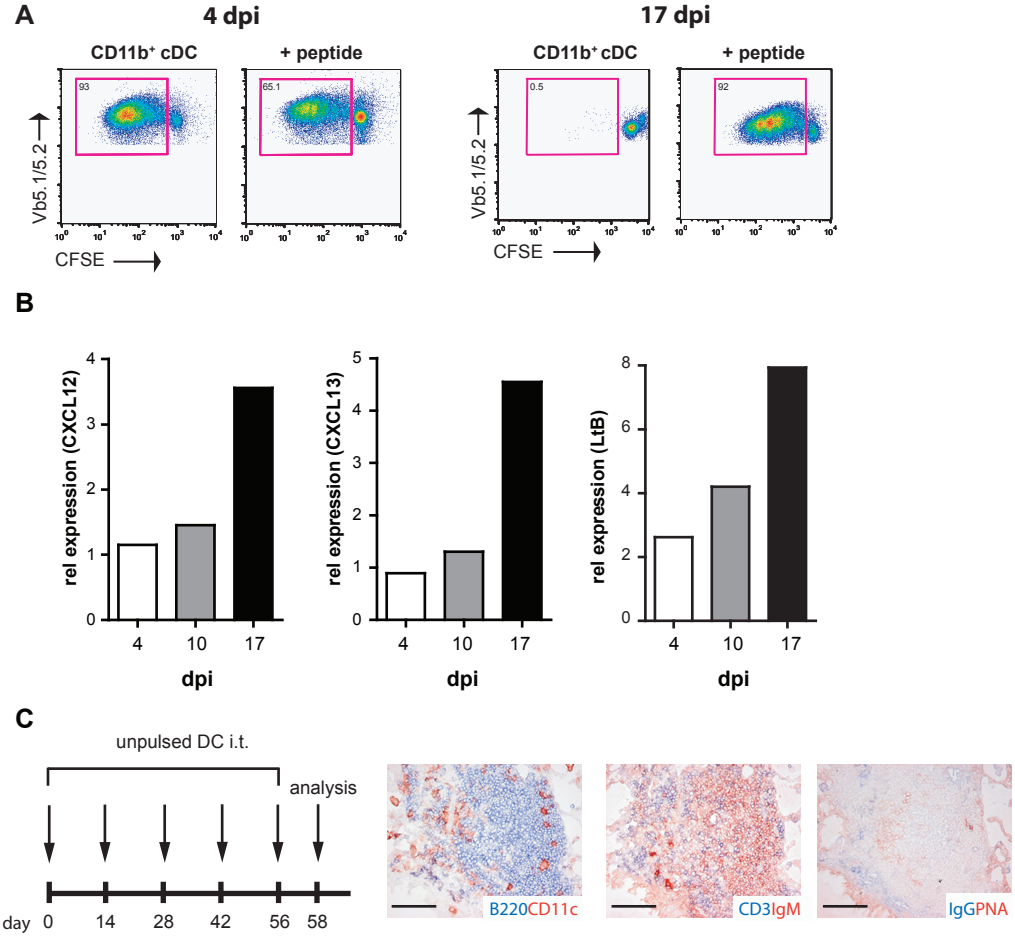

Figure 5 DCs are capable of inducing iBALT due to chemokine expression
(A) Mice were infected with influenza virus containing OVA-class II epitope. DC subsets were sorted from pooled infected lung tissue at 4 dpi and 17 dpi, and co-cultured with CFSE-labeled OVA specific CD4 T cells (OT-II) during 4 days. Left panel demonstrates that at 4 dpi cDCs (B220-CD11c^{+}MHCII^{+}CD11b^{+}120G8^{-}) efficiently presented the OVA antigen to OT-II T cells, but at 17 dpi (right panel) both subsets had lost this capacity. However when peptide was added DC still showed to have APC function at 17 dpi. Boxes line the cells in division and the % of cells in division is indicated in the left corner. The experiments have been performed at least 2 times. (B) Relative expression of CXCL12, CXCL13 and Lymphotoxin-β on sorted CD11b^{+} DCs obtained from pooled lung tissues of 10 infected mice at various days post infection. Bars indicate relative expression. (C) Repetitive adoptive transfer of cultured GM-CSF DCs induces iBALT. DCs were gorown from the bone marow and injected five times with two week intervals. Consecutive histological slides show presence of iBALT aggregates. Bars represent 100μm.

question that remains in the field of ectopic lymphoid tissue development is the precise character of the lymphoid tissue inducer cells that provide the initial trigger for TLO development to stromal organizer cells (12). Although our paper does not answer this question, we provide clear evidence that DCs should be incorporated into any conceptual framework as to how these structures develop.

MATERIALS AND METHODS

Mice

C57BL/6 mice (6-8 weeks) were purchased from Harlan (Zeist, The Netherlands). The generation and screening of CD11c-DTR Tg mice has been reported previously (35). Male Balb/c background CD11c-DTR Tg (H2-Dd) were crossed to C57BL/6 (H2-Db) to obtain F1 progeny. $CD11c^{hi}$ cells were depleted in CD11c-DTR x C57BL/6 Tg mice by i.t. injection of 50 ng DT, a dose previously determined by titration. All experiments were approved by an independent animal ethics committee of Erasmus MC Medical Center, Rotterdam, the Netherlands.

Influenza virus infection

Mice were i.n. infected with 10^5 $TCID_{50}$ H3N2 influenza virus X-31 (MRC, Cambridge, England), diluted in 50 μl PBS. For antigen presentation assays of lung DCs, mice were infected with 10^5 X-31 influenza virus encoding $OVA_{323-339}$ MHC II epitope in hemagglutinin of the virus (36). The OVA virus was kindly provided by Dr. R. Webby (St. Jude Children's Hospital, Memphis, USA).

Histology

Lungs were inflated with OCT, snap-frozen in liquid nitrogen and stored at -80˚C. Frozen sections were fixed in acetone and endogenic peroxidase was blocked prior to immuno-histochemical staining. Immunohistochemical double staining was performed for B220 (BD, RA3-6B2) and CD11c (eBioscience,N418), CD8 (Serotec, KT-15) and CD4 (Ebioscience,RM4-5), IgG (BD, A85-1) and PNA (Sigma, L-6135), IgA (BD, 02262D) and PNA, IgM (BD,II-41) and KT3 (supernatant). FDC (BD, M2) was stained single. For detection of NP positive cells slides were incubated with recombinant NP (37) prior to FITC labelled anti-NP staining.

Detection of antibodies in serum and BALF

For detection of HA-specific antibodies in serum, samples were treated with cholera filtrate and heat inactivated at 56°C. The serum samples were tested for the presence of anti-HA antibodies. For this purpose, a hemagglutination inhibition (HI) assay was used following a standard protocol of 1% turkey erythrocytes and 4 HAU of H3N2 influenza virus (37) in 96-wells plates (Greiner Bio-One). ELISA was performed on BALf for detection of IgA (BD Pharmingen) and IgM (Southern Biotec).

Adoptive transfer of GM-CSF cultured DCs

Bone marrow cells were cultured for 9 days in DC culture medium (DC-TCM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Sigma-Aldrich), 50μM 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). Mice received

5 i.t. injections of 10^6 DCs, each with 2 weeks interval.

Flowcytometry and cell sorting

To check the depletion of cell subsets after treatment with DT and analyze tissues after depletion, single cell suspensions of lung and bone marrow were prepared as described previously (38). Extra-cellular staining for DCs was done by staining with 120G8 FITC (kindly provided by C.Asselin-Paturel), CD11b PE, MHCII PECy5, B220 PECy7(eBioscience), CD11c PETxR(Caltag), CD3 APC Cy7, CD19 APC Cy7 (BD Biosciences), and a live/dead marker (DAPI) in Violet. PNA positive germinal centers in lung were detected with moAbs against IgD FITC, CD95 PE, IgM PECy7,B220 AF700 (BD Biosciences), CD19 PerCp Cy5.5 (eBioscience), biotinylated PNA (Sigma) and DAPI. For detection of NP specific plasma cells in bone marrow, cells were first stained extra-cellular with CD138 PE (BD Biosciences), CD19 PerCp Cy5.5, CD11b AF700 (eBioscience) and a Aqua Live/Dead marker (Invitrogen). Next Cytofix/Cytoperm (BD Bioscience) was added to fixate cells. Recombinant NP was diluted in perm buffer and added to each well; cells were incubated for 30 minutes and then washed with perm buffer. Intra cellular cells were stained with aNP FITC (Dakocytomation), IgM APC, biotinylated IgG2a/2b (BD Bioscience), and secondary streptavidin APC Cy7 (eBioscience). Acquisition of ten color samples was performed on a cytomter (LSR) equipped with FACSDiva software (both BDBiosciences). Final analysis and graphical output were performed using FlowJo software (Tree Star, Inc.). For sorting of DCs, mice were sacrificed at different time points after infection with X-31 influenza virus. Lungs of 10 animals were pooled and digested in a collagenase-DNAse solution for 1 hour at 37 ˚C to promote release of DCs. DCs were stained as above, cell sorting was performed on a FACSAria. The purity of sorted populations was > 98%.

Antigen presentation assay

For the co-culture of sorted DC subsets with OVA specific $CD4^+$ T cells, OT-2 transgenic T cells were isolated from spleen and MLN of a OT-2 mouse, enriched by MACS cell sorting with anti-CD4 antibodies according to the manufacturer's protocol (Milteny Biotec), and labelled with CFSE (39). Sorted DC subsets were co-cultured with T cells in a v-bottom plate at a 1/10 ratio for 4 days. T cell divisions were measured by flow cytometry on a LSR cytometer equipped with FACSDiva software (both from BD Biosciences). Supernatant was stored at –20 ˚C.

Real-time quantitative RT-PCR

Quantative RT-PCR for CXCL12, CXCL13 and LT-β was performed on RNA obtained from sorted lung DC subsets. RNA was isolated with RNAqueous micro kit (Ambion) and treated with DNaseI, according to the manufacturer's protocol. RNA (100 ng) was reverse transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences) for 50 min at 42 ˚C. Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems) and preformulated primers and probe mixes ('Assay on Demand', Applied Biosystems). PCR conditions were 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 15 s at 95˚C and 60˚C for 1 min using an ABI PRISM 7300 (Applied Biosystems). PCR amplification of the housekeeping gene encoding ubiquitin C was performed during each run for each sample to allow normalization between samples.

Statistical analysis

All experiments were performed using 5-10 animals per group. 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 $p<0,05$.

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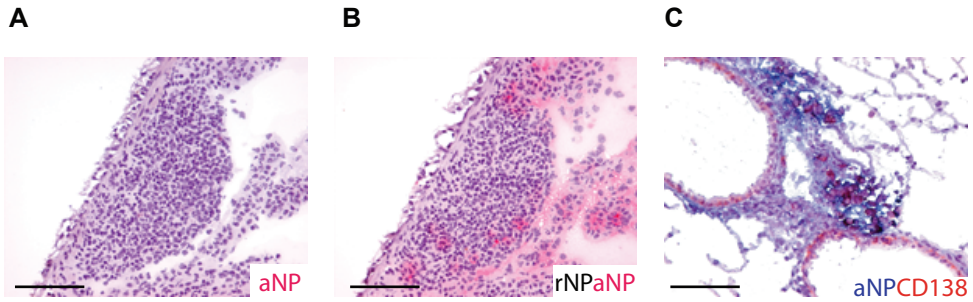
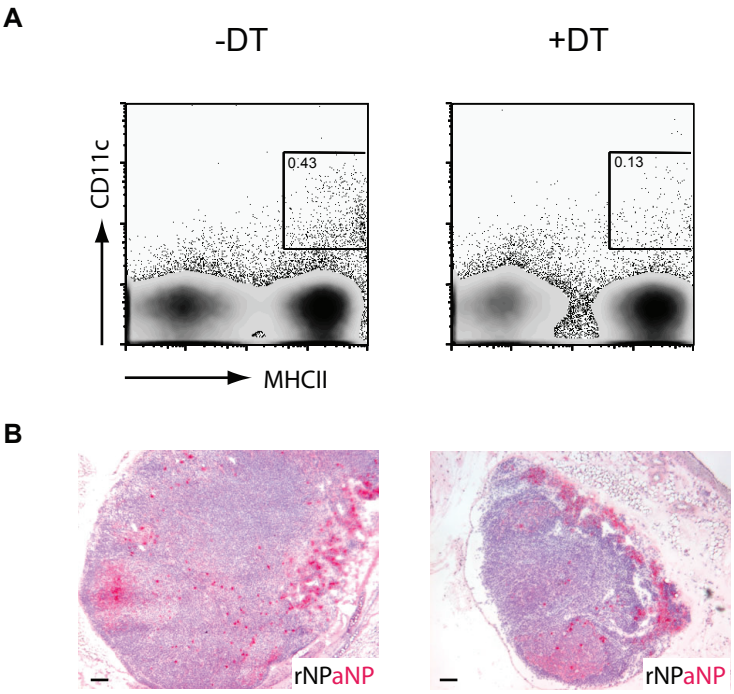
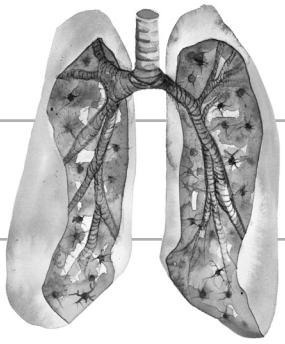
Supplementary figures



Supplementary figure 1: Detection of NP specific plasma cells in iBALT
Use of an antibody to NP detected infected cells of the lungs shortly after infection (data not shown and 22). (A) At 24 dpi staining with this antibody no longer detected NP-positive cells within iBALT, demonstrating that virus had been completely cleared from the lung and that NP antigen was not retained for prolonged periods in the FDC network. (B) Recombinant NP protein was used to reveal NP specific B cells; binding was demonstrated by using NP-specific antibody. (C) Double staining in which slides were coated with rNP, stained for NP positivity and then double stained with CD138 (plasma cell marker). Bars represent 100μm.



Supplementary figure 2: Depletion of DCs from MLN does not affect local NP specific plasma cells
I.t. treatment of CD11-DTR Tg mice with DT depleted CD11c^{+} cells from lung and MLN. (A) FACS plots demonstrate depletion of CD11c^{+}MHCII^{+} DCs in MLN 1 day after DT treatment at day 17 (B)Histology demonstrates intact NP specific plasma cell differentiation in MLN, 1 week after DT treatment (24 dpi). Bar represents 100 μm.



Chapter 5

The antigen presenting capacity of steady state dendritic cell subsets is differentially affected by influenza virus infection

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ABSTRACT

Dendritic cells (DCs) are the most potent antigen presenting cells during influenza virus infection. Two main subsets of DCs are plasmacytoid DCs (pDC) and conventional DCs (cDC). Whereas *in vivo* studies have demonstrated that upon influenza virus infection CD8a$^+$ cDCs efficiently present antigen to both CD4$^+$ and CD8$^+$ T cells, pDCs did not seem to contribute. As this antigen presenting capacity of pDCs in inflammatory conditions remains a controversial issue, we have performed a study to further investigate this point. We used a controlled *in vitro* system in which infection, antigen processing and antigen presentation of DC subsets was studied. Therefore both pDCs and the equivalent of *in vivo* cDCs were cultured in a Flt3-L/SCF bone-marrow culture. Infection patterns following influenza virus infection differed for both subsets and in accordance to our *in vivo* studies, cDCs were most efficient in presenting antigen to T cells. Nevertheless, pDCs did present endogenously processed antigen to naïve CD8$^+$ T cells, especially after maturation by influenza virus infection. Antigen presentation of pDCs to CD4$^+$ T cells remained problematic, probably due to a defective MHCII signaling pathway. In conclusion, this study demonstrates that pDCs in an appropriate (infectious) setting do have antigen presenting capacity. However, due to defective antigen processing they hardly present exogenous antigen to CD4$^+$ T cells. All together their contribution to antigen presentation *in vivo* will be inferior to cDCs.

INTRODUCTION

Influenza virus is a major pathogen causing acute respiratory illness, which can produce severe injury to the respiratory tract. Besides direct morbidity, respiratory viral infection can cause modulation of the airway immune response, leading to increased susceptibility to atopic sensitization and asthmatic disease. During induction of an adaptive immune response against influenza virus infection, activation of T lymphocytes by dendritic cells (DCs) is required. Costimulatory molecule expression on DCs is upregulated and they are recruited to the site of infection where an inflammatory environment is induced. These events lead to enhanced antigen presentation but possibly can also change the normal tolerogenic response to inhaled antigen into an immunogenic response.

Generally, DCs are divided into plasmacytoid (p)DCs and conventional (c)DCs. Within the population of cDCs recent studies have described various subpopulations with a distinct division of labor, depending on anatomical location (1, 2). In the spleen a population of $CD8a^+$ cDCs are specialized in cross-presenting exogenous antigen to $CD8^+$ T cells, whereas a population of $CD8a^-$ cDCs are specialized in $CD4^+$ T cell presentation (3, 4). We and others (5, 6) have demonstrated that upon *in vivo* influenza virus infection, resident $CD8a^+$ DCs are most efficient antigen presenting cells to both $CD4^+$ and $CD8^+$ T cells. Most authors who have previously studied the *in vitro* effect of viral infection on DCs have used GM-CSF cultured DCs. More recent studies have demonstrated that these GM-CSF DCs are an *in vitro* equivalent of *in vivo* inflammatory type DCs (7). In order to compare antigen-presenting capacities of our *in vitro* cultured DCs to the *in vivo* $CD8a^+$ DCs we sorted $MHCII^+CD11c^+$ DCs from a Flt3L/SCF culture, which are the equivalent of *in vivo* $CD8a^+$ cDCs (8).

PDCs are a more recent described member of the DC family and are specialized in producing high levels of type 1 interferon upon viral stimulation (9-14). It was previously assumed that pDCs play an important role in clearance of viral infection, but more recent studies have demonstrated that this is dependent on the type of viral infection (5, 9, 15). Yet, another role of pDCs has been described in immune regulation, by inducing the formation of regulatory T cells (10, 16-19). In our study pDCs were sorted from the same Flt3L/SCF culture to make a comparison with cDCs. Although the potential of pDCs to present peptide antigen or to stimulate allo-specific T cells during a mixed leukocyte reaction has

been shown (13, 20), the relative strength of the antigen uptake and processing of pDCs versus cDCs for mounting effective T cell responses remains controversial, especially under inflammatory conditions. In our recently published *in vivo* studies (5) we were not able to show antigen presentation by pDCs but to confirm this finding we decided to use optimal *in vitro* infection conditions to study infection, antigen processing and antigen presentation of pDCs in comparison to cDCs. We now demonstrate that influenza virus differentially affect pDCs and cDCs *in vitro*. Differences in antigen presenting capacity between DC subsets following influenza virus infection might be related to deficient MHCII processing in pDCs.

RESULTS

The effect of influenza virus infection *in vitro* on conventional DCs and plasmacytoid DCs

To obtain highly purified populations of cDCs and pDCs, murine bone marrow cells were cultured in Flt3-L and SCF (8). After 9 days of culture, DCs were purified using high-speed cell sorting to over 98% purity. PDCs were sorted by expression of intermediate levels 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 (21). Following cell sorting, DCs were infected with influenza X-31 (H3N2) virus, using a MOI of 3, for 1 hour at 37˚C after which virus was washed away and cells were left overnight for infection at 37˚C. The next day DCs and supernatant were harvested and DCs were partly spotted on histological slides to perform a fluorescent nucleoprotein (NP) staining. This fluorescent staining nicely demonstrated infected cDCs by concentrated NP in the nucleus. In contrast, pDCs were not infected at all by this MOI and time-point (figure 1A). The grade of infection was confirmed by flow cytometry. Therefore cells were first stained extra-cellularly using DC markers, then fixed and permeabilized and subsequently NP was stained intra-cellularly. Flowcytometric data confirmed our fluorescent staining on histology and demonstrated an infected percentage of cDCs of 19%, whereas pDCs failed to get infected (figure 1A, histograms)

Next, levels of IFN-α on cell culture supernatant after overnight infection were determined. Previous studies described that pDCs produce high levels of

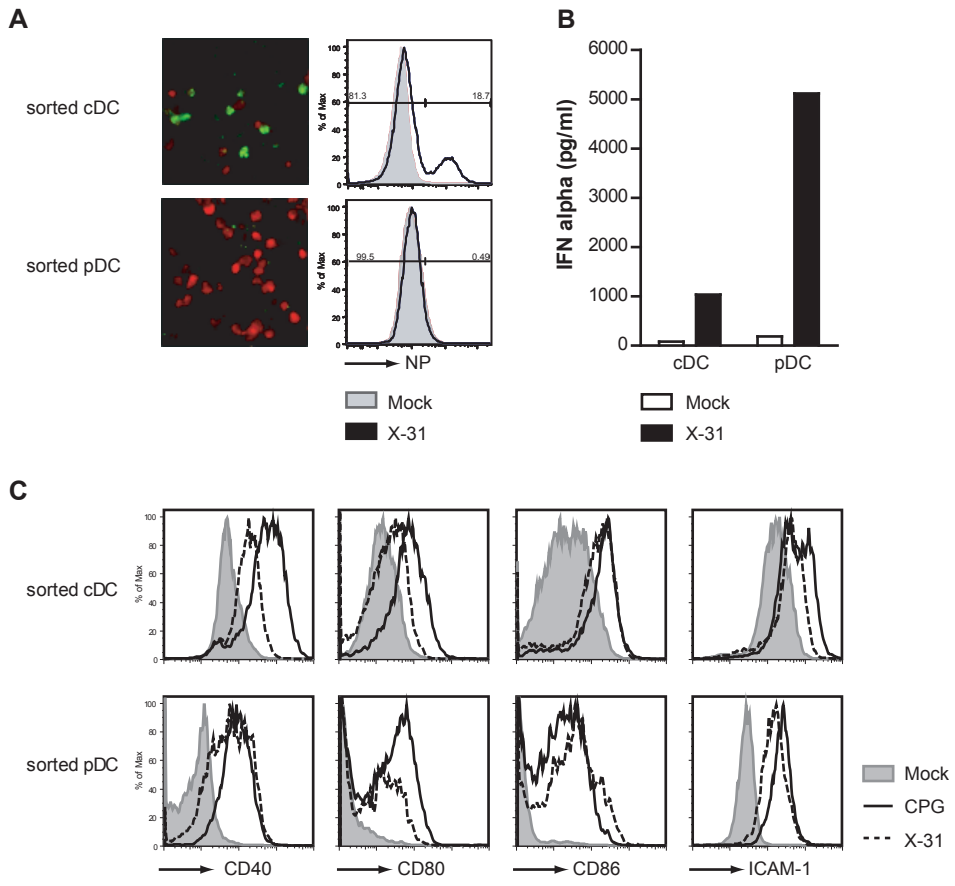


Figure 1: Infection and maturation of DC subsets by influenza virus infection *in vitro*.
Mice plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L. On day 9, cell populations were purified by FACS sorting. (A) Rate of infection of DC subsets after influenza X-31 virus infection (MOI=3) versus mock infection determined by aNP staining. Numbers stated in histograms indicate % of sorted cells. (B) IFN-α measured in supernatant of cultured DC populations after overnight infection with influenza virus or mock. (C) Upregulation of maturation markers on DC subsets following overnight infection with influenza virus or mock. A comparison was made to overnight CPG exposure. Demonstrated data are representative of at least 3 experiments.

IFN-α following viral infection (11, 13, 14, 22-24). Our data confirmed these studies; whereas both cDCs and pDCs increased IFN-α production following infection, levels produced by pDCs were five times higher than cDCs (figure 1B). This illustrates that at least pDCs were viable in our assays.

Finally, we studied the effect of overnight infection on the phenotype of cDCs and pDCs. A comparison was made between mock and influenza virus

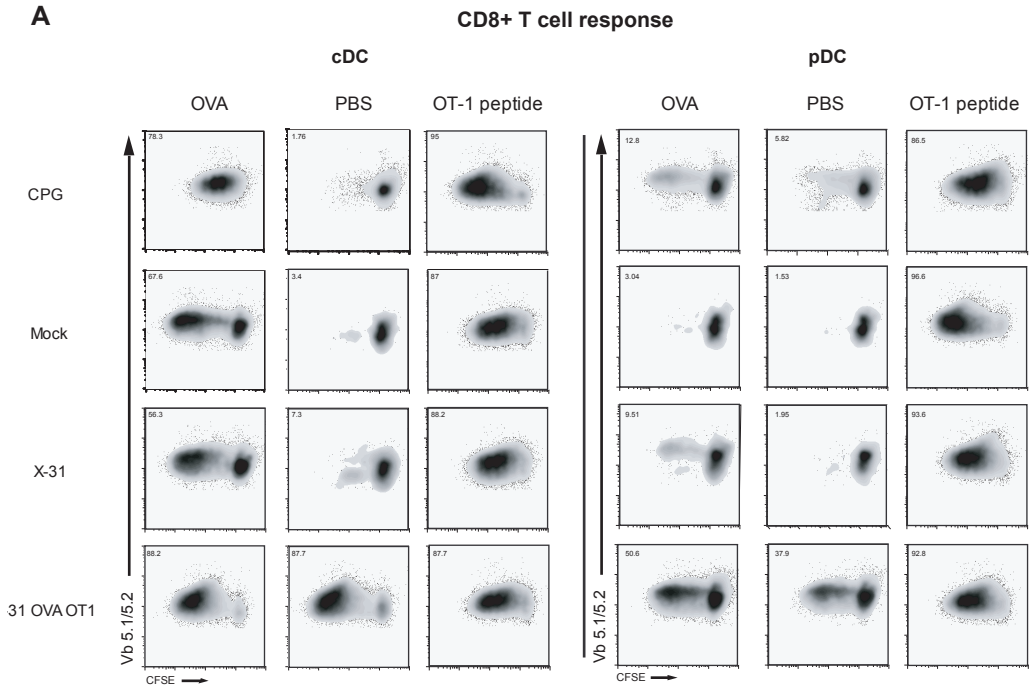
infected DCs. Addition of bacterial CPG motifs (an endosomal TLR9 agonist) to DCs is known for it's effect on cell maturation, and was therefore used as a positive control. Both cDCs and pDCs upregulated the expression of surface molecules as CD40, CD80, CD86 and ICAM-1, in the presence of CPG and influenza virus when compared to mock infection (figure 1C).

Both cDCs and pDCs more efficiently cross-present exogenous protein antigen to CD8$^+$ T cells following influenza infection, but pDCs only induce minor CD4$^+$ T cell proliferation

Whereas cDCs are generally known to be efficient antigen presenting cells (APCs), several papers also describe pDCs as good APCs for CD4 and CD8 T cell responses (12, 25, 26). However, some of these studies just show presentation of antigenic peptides and not whole protein, bypassing the need for antigen presentation. Besides the antigenic stimulus, the purity of used pDCs for stimulation can also be a confusing factor. We have found that a slight contamination with cDCs (of 4–5%) can already significantly contribute to antigen presentation (data not shown), therefore highly purified pDC and cDC populations were used in our study.

Being interested in the antigen presentation capacity of "bystander antigen" during infection, we used ovalbumin (OVA) as a model antigen. Presentation of exogenous antigen was compared to an antigen incorporated in the virus. For this we used recombinant influenza viruses with either an OVA MHI or MHCII construct built into respectively the neuraminidase or hemagluttinin of the viruses. As effector cells we used OVA specific CD4 (OTII) or CD8 (OTI) T cells. Sorted DCs were infected with influenza virus, an OVA-containing influenza virus, mock or exposed to CPG. One hour later, cells were washed, put into fresh DC culture medium and OVA, PBS or pre-processed OVA-peptide was added overnight. The next day cells were harvested and put into co-culture with either CFSE labeled

Figure 2: Antigen presentation of DC subsets to CD8 and CD4 T cells.
Mice plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L. On day 9, cell populations were purified by FACS sorting. (A) Sorted cDC and pDC were infected overnight with influenza virus X-31, mock, influenza virus containing OVA-class I epitope or CPG was added. To study the antigen presentation of bystander antigen to CD8 T cells, OVA, PBS or OVA class I peptide was added to all conditions. The next day they were co-cultured with CFSE-labeled OVA specific CD8 T cells (OT-I) during 4 days. Left panel demonstrates T cell division induced by cDCs, right panel by pDCs. Numbers in the left corners indicate % of divided cells. (B) Experiment performed as described in A, but CD4 T cell response was studied by using OVA specific CD4 T cells (OT-II). The experiments have been performed at least 3 times.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OVA
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OT-1 peptide
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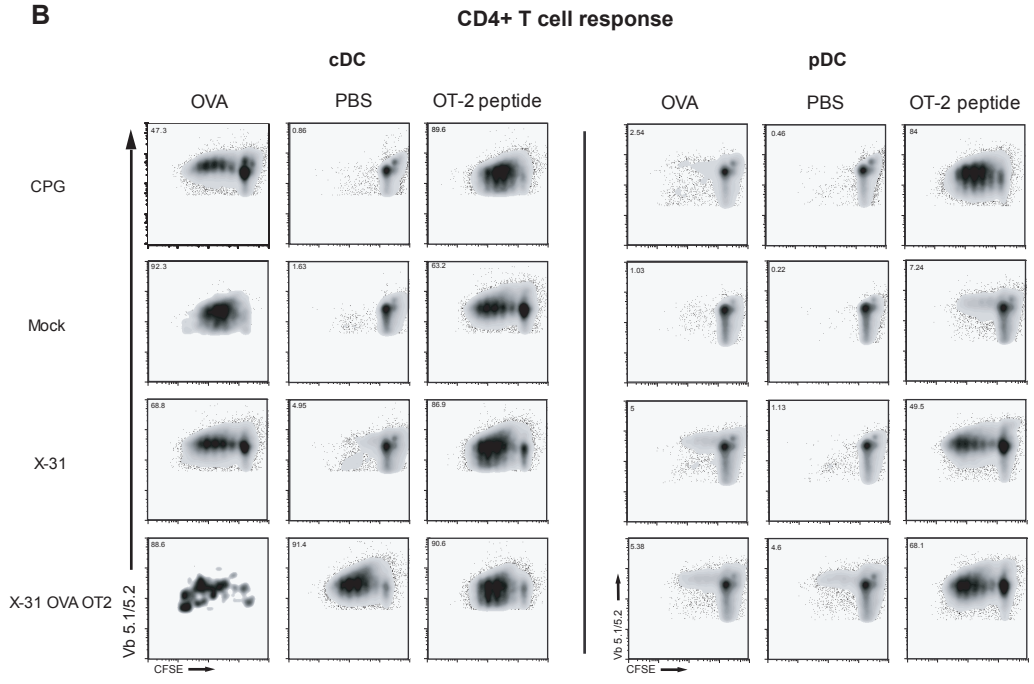
CFSE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CD8 (OTI) or CD4 (OTII) OVA specific T cells. Co-cultures were left for 4 days and proliferation of T cells was measured by CFSE dilution on flowcytometry. As expected, cDCs efficiently presented antigen to $CD8^+$ T cells (figure 2A left panel). Addition of either exogenous OVA antigen (OVA), OVA incorporated into the virus (X-31 OVA OT1) and OVA peptide to cDCs induced clear $CD8^+$ T cell proliferation. PDCs were much less capable of inducing $CD8^+$ T cell proliferation (figure 2A right panel). Whole OVA could be presented, but only after prior DC maturation by CPG or influenza virus infection. In case OVA was either incorporated into the virus or pre-processed (thereby circumventing antigen processing), pDCs became more efficient in presentation to $CD8^+$ T cells.

Figure 2B displays the induction of $CD4^+$ T cell proliferation. CDCs again efficiently presented all forms of antigen to $CD4^+$ T cells, nevertheless it was remarkably to see that exogenous OVA presentation to $CD4^+$ T cells decreased after influenza virus infection or CPG treatment. As the presentation of (pre-processed) OVA class II peptide was not affected this might indicate that maturation of cDCs by these stimuli altered the processing of OVA. Regarding pDCs (figure 2B right panel); in contrast to their minor antigen presenting capacity of pDCs to $CD8^+$ T cells, they presented very weakly to $CD4^+$ T cells in all instances. Only pre-processed OVA class II peptide was efficiently presented after pDCs were either infected or stimulated with CPG motifs (figure 2B).

Antigen processing for MHCII presentation

Following the antigen presentation studies described above, we hypothesized that perhaps pDCs had a problem in processing the OVA within the MHCII machinery. Loading of MHCII molecules occurs in an acidic endosomal compartment, rich in MHCII molecules, the so-called MHCII compartment. To follow if OVA would be targeted to these acidic compartments, 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 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 (figure 3 lower plots), whereas cDCs showed clear accumulation of OVA-DQ in these endosomes (over 50% of cDCs showing evidence of accumulation). The addition of CPG motifs

or influenza infection slightly increased processing of OVA-DQ in pDCs to about 14%.

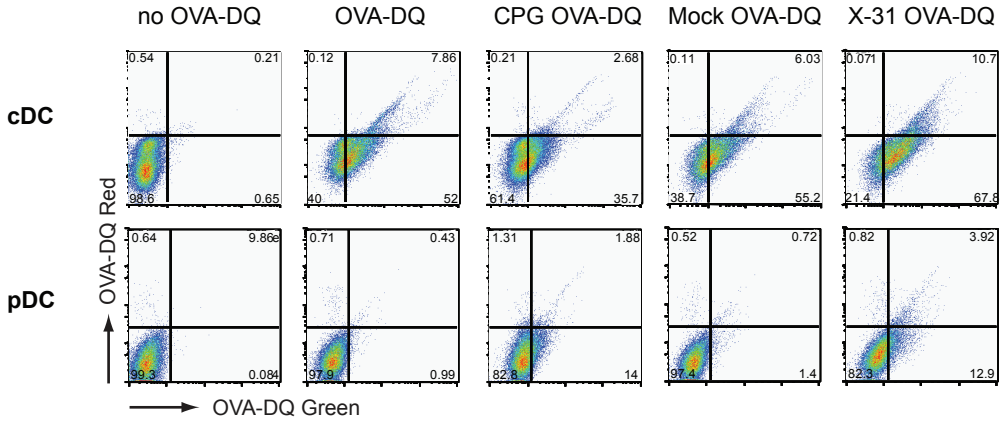

Figure 3: Antigen processing for MHCII presentation
Mice plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L. On day 9, cell populations were purified by FACS sorting and infected with influenza X-31, mock or CPG was added. OVA-DQ was added to cDC and pDC after one hour of infection. Eight hours later uptake and processing of OVA-DQ was measured by flowcytometry. Numbers in the corners indicate % of cells.

PDC and cDCs differentially espress enzymes involved in MHI and MHCII processing

Many of the proteins that regulate MHCI and MHCII processing pathways have been described and their expression has been documented in DCs (27). To evaluate if pDCs had intrinsic differences compared with cDCs in the expression of any of these proteins following infection, the mRNA levels were compared by q-RT-PCR. As an example of a protein regulating the MHCI processing we here show expression of the peptide loading complex consisting of TAP (Tap-1) and for MHCII processing the chaperone molecule H2-DMb1. After infection, the expression of Tap-1 on cDCs generally remained at the same level, but in contrast was dramatically increased on pDCs (figure 4A). This increase might contribute to the improved antigen presentation op pDCs to CD8 T cells after infection. Figure 4B shows H2-DMb1 expression on cDCs, most evident is the decrease following

CPG treatment, which again correlates to depicted CD4 proliferation plots. Expression of H2-DMb1 on pDCs was clearly lower than on cDCs and only very slightly increased following infection or CPG treatment.

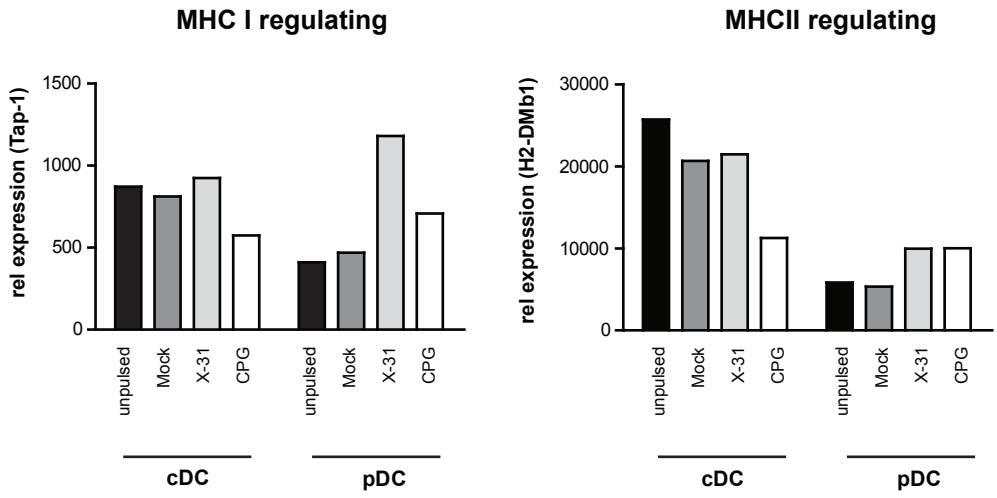

Figure 4: Enzymes involved in MHI and MHCII processing
Mice plasmacytoid and conventional DCs were cultured from bone marrow with Flt3-L. On day 9, cell populations were purifed by FACS sorting. DCs were infected overnight, mock infected or CPG was added. (A) RNA levels of Tap-1 and (B) RNA levels of H2-DMb1.

DISCUSSION

Infection of bone-marrow-derived DCs by influenza virus with triggering of the cytokine production (e.g., IL-6 and IL-12) and type 1 interferon production has been previously shown. Furthermore, efficient induction of $CD8^+$ T cell responses *in vitro* appears to require direct infection of DC by live virus (28-32) although presentation of antigen from noninfectious sources through cross-presentation by DCs has been reported (33). Within the population of cDCs recent studies described differences in function between $CD8a^+$ and $CD8a^-$ populations. These specialized functions are determined by a differential expression of antigen uptake receptors (34, 35) or by differential expression levels of the various enzymes and chaperone molecules associated with the antigen processing (27). Viral infection of the DCs can disturb these base-line conditions, by causing for example ligation

of pattern recognition receptors (like Toll like recepors), leading to upregulation of endocytic receptors, and for alteration of antigen processing signalling.
In this report, we have studied the responses of bone marrow cultured cDCs and pDCs to *in vitro* influenza virus infection. We have demonstrated that the pattern of infection between these subsets was not uniform and could thereby confirm previous studies on this point (36). Whereas a fraction of cDCs was infected by influenza virus and expressed high levels of influenza virus nucleocapsid protein at a MOI of 3, pDCs infected with a same MOI did not show any level of infection. Hao et al have shown a dose dependency of influenza virus infection (36), in purified respiratory tract DCs. It might be that the difficulty of infection of pDCs is due to the very high levels of interferon-a in the culture.

PDCs were first considered to have a minor role in in antigen uptake (37) when compared to cDCs (16). However, more recent reports showed uptake of subcutaneously injected Ag by mouse lymph node pDCs and inhaled fluorescent OVA inside vacuoles of mediastinal lymphe node pDCs of naïve mice (16, 25). In this paper we show that highly purified bone-marrow derived pDCs do take up soluble (OVA-DQ) antigen following influenza virus infection. However, when comparing pDCs with cDCs obtained from the same Flt3L culture, cDCs were much more efficient in uptake of this soluble Ag.

Next, we have addressed the controversial issue on the antigen presenting capacity of pDCs versus cDCs and especially focused on their capacities following *in vitro* infection with a high dosis of influenza virus. For presentation to CD4 T cells, pDCs were initially thought to present exogenous protein Ag only to already primed, but not naïve T cells (16, 38). Contrasting these early reports, more recently it was shown that lymphe node, but not splenic pDCs were able to present soluble Ag to naïve CD4 T cells *in vivo*, but only when the Ag was targeted to the BST2 receptor via mPDCA-1 (25). In our study we found that when using BM-derived pDCs, they did not present exogenous Ag to CD4 T cells, whereas they readily did present pre-processed peptide, particularly when exposed to CPG motifs or influenza virus infection. The reason for the failure to present Ag to CD4 T cells might be the lack of expression of enzymes involved in MHCII processing (as the chaperone molecule H2-DMb1).

Some authors have suggested that pDCs only present endogenous Ag to CD8 T cells, but fail to cross-present exogenous Ag (25, 39, 40). Our findings clearly confirm that purified pDCs can present an endogenously expressed Ag to

naïve CD8 T cells, and the process is enhanced by maturation of pDCs by CPG or influenza virus. Regarding cross-presentation of exogenous Ag, we found that exogenous Ag was only presented under particular conditionsæafter pDCs had been matured. In all cases pDCs were weaker at presentation when compared with cDCs. In conclusion, we have demonstrated that the major DC populations *in vitro* react differentially to influenza virus infection. As cDCs are already very efficient antigen presenters, influenza virus does not enhance this significantly. PDCs however, increased their antigen presenting capacity following infection, but only to CD8 T cells. A defective MHCII signaling pathway might be the cause of their deficient CD4 T cell presentation. Although the studies in this paper were performed purely *in vitro*, our findings might help to understand the role of DC subsets in the response to *in vivo* influenza virus infection. To further unravel the differential responses of DC subsets to influenza virus infection genomic tools will be used. Pathway analyses on *in vitro* infected DCs might provide novel insights in to functionality of distinct DC subsets.

MATERIALS AND METHODS

Mice

C57BL/6 mice (6-8 weeks) were purchased from Harlan (Zeist, The Netherlands). OT-1 (C57BL/6 background) Tg mice containing OVA-specific TCR $CD8^+$ T cells (Hogquist K et al. Cell 1994) and OT-II (C57BL/6 background) Tg mice containing OVA-specific TCR $CD4^+$ T cells (Barnden MJ Immunol Cell Biol 1998) were bred at Erasmus MC. *Mice were used when aged between* 6-12 weeks. All experiments were approved by an independent animal ethics committee of Erasmus MC Medical Center, Rotterdam, the Netherlands.

Generation of BM DCs in hFlt3-L/SCF

Bone marrow (BM) cells were seeded in petridishes (106 cells/ml) in DC culture medium (DC-CM, RPMI 1640 containing GlutaMAX-I (Invitrogen) supplemented with 5% FCS (HyClone), 50 mM 2-mercapthoethanol (Sigma) and 50 mg/ml gentamycin (Invitrogen) with 200 ng/ml human FMS-like trosin 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 (1x 106 cells/ml) in DC-CM supplemented with 200ng/ml hFlt3-L alone to obtain pDCs and cDCs.

Influenza virus infection

DCs were infected *in vitro* with influenza virus X-31(MRC, Cambridge, England), WSN influenza virus encoding $OVA_{257-264}$ Kb restricted MHC I epitope in the neuraminidase (41) or X-31 influenza virus encoding $OVA_{323-339}$ MHC II epitope in hemagglutinin of the virus (42). The OVA viruses were kindly provided by Dr. R. Webby (St. Jude Children's Hospital, Memphis, USA). DCs were infected with an MOI=3 at 37 ˚C and

after 1 hour washed with PBS. DC-TCM was added for overnight infection at 37 ˚C with eventually OVA and/or CPG .

Flow Cytometry and cell sorting

Acquisition of 4 color samples for identification of maturation markers on DCs was done on a FACS Calibur (BD Biosciences) after staining with MHCII FITC, CD11c APC and CD40PE, CD80 PE, CD86 PE, ICAM-I PE. Proponium Iodide (PI) was used as a live/dead markere. FlowJo software (Tree Star Inc.) was used for analysis. For sorting of pDCs and cDCs, the BM Flt3-L/SCF culture was stained with moAbs against 120G8-FITC (kindly provided by C.Asselin-Paturel), CD11c-APC, CD11b-PercP-Cy5.5 and B220-PECy7 (eBioscience). Sorting of $120G8^{+}CD11b^{-}B220^{+}CD11c^{+}$ pDCs and $120G8^{-}CD11b^{+}B220^{-}CD11c^{+}$ cDCs was performed on a FACS Aria (BD Biosciences). 2.4G2 (anti Fc_RII/III) was used to reduce non-specific antibody binding in all stainings. In all flow cytometry experiments either propidium iodide (PI), TOPRO- (Molecular-Probes) or DAPI (Molecular Probes) was used to discriminate between live and dead cells.

Analysis of T cell proliferation

OT-1 and OT-2 transgenic T cells were isolated from spleens and LNs of respective mice, enriched by MACS cell sorting with anti-CD8 or -CD4 antibodies according to manufacturer's protocol (Miltenyi Biotec GmbH) and labelled with CFSE(43). Sorted DC subsets were co-cultured with T cells in v-bottom plates (Greiner Bio-One) at 1/10 ratio for 4 days. T cell divisions were measured by flow cytometry.

Antigen uptake and processing

To determine the antigen uptake and processing, sorted DC subsets after one hour of infection were plated at a concentration of $1*10^{6}$ cells/ml with 10 mg/ml OVA-DQ. Uptake and processing of OVA-DQ was measured 8 hours after exposure.

Real-time quantitative RT-PCR

Quantative RT-PCR for H2DMb1, Cathepsin S and Tap1 was performed on RNA obtained sorted DC populations. RNA was isolated with RNAqueous micro kit (Ambion) and treated with DNaseI, according to the manufacturer's protocol. RNA (100 ng) was reverse transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences) for 50 min at 42 ˚C. Quantitative PCR was performed with Taqman Universal PCR Mastermix (Applied Biosystems) and preformulated primers and probe mixes ('Assay on Demand', Applied Biosystems). PCR conditions were 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 15 s at 95˚C and 60˚C for 1 min using an ABI PRISM 7300 (Applied Biosystems). PCR amplification of the housekeeping gene encoding ubiquitin C was performed during each run for each sample to allow normalization between samples.

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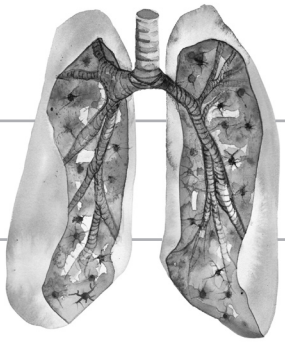

Chapter 6

Summarizing discussion

The studies described in this thesis focus on dendritic cell (DC) subsets present in the lung and elaborate on their distinct functions in the immune response to influenza virus infection. The DC subsets discussed in this final chapter are summarized in figure 1.

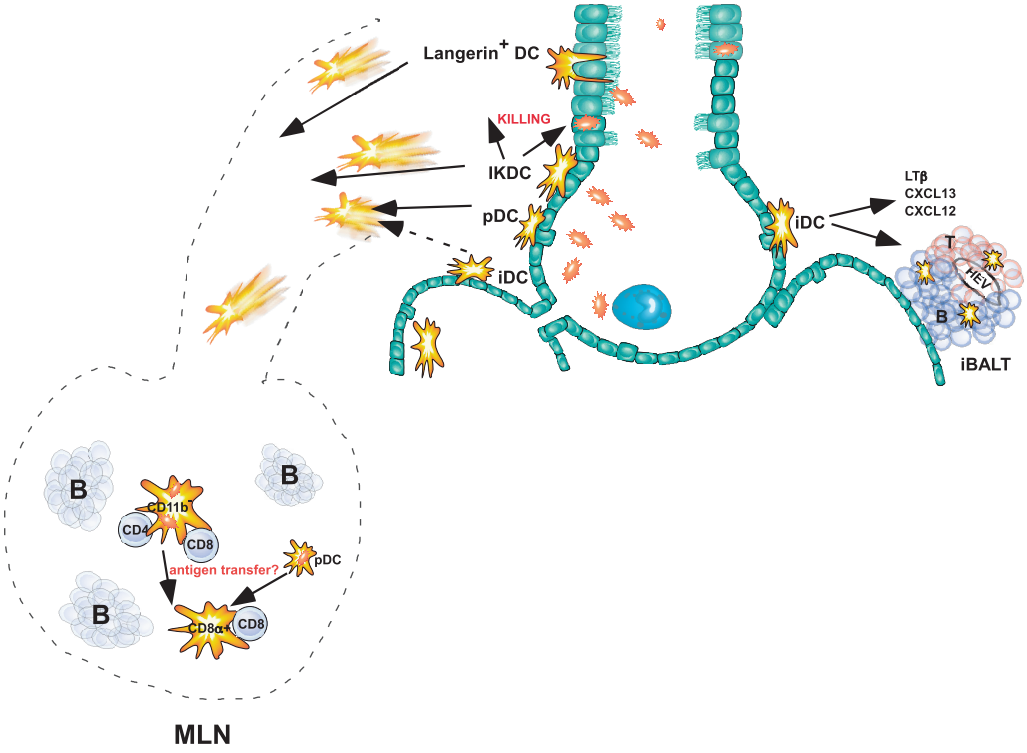

Figure 1: Division of labor between dendritic cell subsets in the airways following influenza virus infection
The conducting airways are composed of airway epithelial cells, which act as a molecular sieve excluding inhaled antigens and pathogens. Underneath the epithelial layer several DC subsets are located. Langerin^{+}CD11b^{-} DCs are situated in the basolateral space, and can extend their processes between epithelial cells directly into the airway lumen. These langerin^{+} DCs can migrate towards the draining MLN and directly present viral derived antigen to CD4^{+} and CD8^{+} T cells. IKDCs can directly kill virus-infected cells in the lung tissue and some migration to the MLN has been observed. Antigen is presented only to CD8^{+} T cells. PDCs migrate towards the MLN but are not capable of presenting antigen. Together with migrating langerin^{+}CD11b^{-} DCs they might be a source of viral antigen for resident CD8α^{+} DCs in the MLN, which can in their turn efficiently present it to CD8^{+} T cells. IDCs slightly migrate towards the MLN, but do not efficiently present antigen. Most importantly they are involved in the control of iBALT following the clearance of infection.

Migration and antigen presentation by DC subsets in the lung

Several investigators have studied the involvement of antigen presenting cells in mediating protective immunity to influenza virus, such as DCs, macrophages and B cells (1-6). It was shown that a mouse-adapted strain of influenza virus induced the *in vivo* maturation of $CD11c^+$ DCs in the lung of mice (7) and their migration to the mediastinal LNs (8-10). In **chapter 2** we describe that during influenza virus infection, the predominant source of $CD11b^-$ DCs arriving in the MLN originated from the network of highly dendritic-shaped $langerin^+CD103^+$ intra-epithelial DCs that lines the large conducting airways (11-13). The disappearance of this $CD11b^-$ population from the trachea was accompanied by a new influx of $CD11b^+$ DCs into the trachea and interstitium. These freshly recruited $CD11b^+$ DCs most likely represent inflammatory-type DCs (14, 15) a highly activated subset of cDCs found in the lungs upon respiratory infection (16). Data in **chapter 4** have demonstrated that during influenza virus infection, these inflammatory DCs expressed B220 and 120G8 in addition to high levels of CD11c and CD11b, considerably confusing the separation from pDC subsets if 10 colour multi-parameter flow cytometry is not used. Some of these $CD11b^+$ inflammatory DCs also migrated towards the draining MLN.

Once arrived in the MLN, both $CD11b^-CD8\alpha^+$ resident (i.e. non-migratory) DCs, and $CD11b^-$ and $CD11b^+$ lung-derived migratory DCs upregulated co-stimulatory molecules, and thus acquired the potential to present antigen. To test the antigen-presenting potential of the various DC subsets in the MLN we took advantage of influenza viruses encoding either the MHCI or MHCII immunodominant OVA epitope (17, 18). 4 days after infection with these viruses, DC subsets were sorted from MLN by flowcytometry and co-cultured ex-vivo with respectively OT-I or OT-II T cells. This allowed us to probe presentation of sorted lung DC subsets to naive OVA-specific CD8 and CD4 T cells directly. There was a clear division of labor between the DC subsets (**chapter 2**). The $CD11b^-$ DCs stimulated CD4 and CD8 responses, whereas $CD11b^+$ DCs were very poor at antigen presentation at all. This suggests that upon influenza virus infection, processing for and presentation on both MHCI and MHCII molecules can occur in a single cell $CD11b^-$ population *in vivo* in the lung, in contrast to what previously has been shown for spleen or lymph node (19-22). In accordance to findings by Belz et al. the resident $CD8\alpha^+$ DCs in the MLN efficiently presented viral antigen,

but exclusively to CD8 T cells (3), whereas pDCs did not present antigen to naïve T cells in the MLN at this time point (11). Despite the fact that both $CD8\alpha^+$ and $CD8\alpha^-CD11b^-$ DCs presented antigen to naïve T cells, the strength of viral NP staining was not abundant. This could be explained by the fact that the viral NP was digested in these subsets as part of an antigen-processing step, leading to a loss of immunoreactivity towards the NP-specific antibody. Alternatively, Belz et al. previously suggested that resident $CD8\alpha^+$ DCs acquired the antigen from other migratory APCs, proposed as the $CD11b^-CD8\alpha^-$ lung derived DCs, but this was not yet demonstrated (3). Our data on viral NP staining of DC subsets in MLN suggest that the $CD11b^+$ DCs as well as the pDCs were abundantly positive for viral antigen in the MLN and therefore, could be the most important source for providing viral antigen to resident $CD8\alpha^+$ DCs.

Finally, our data in **chapter 3** have demonstrated presence of a novel subtype of antigen presenting cell during influenza virus infection. This $B220^+CD11c^{interm}NK1.1^+$ interferon killer dendritic cell (IKDC) will be discussed further on in this discussion, but it should be mentioned that upon influenza virus infection this population was capable of presenting viral derived antigen to naïve CD8 T cells in the lung. However, this APC feature was not unique to IKDCs and similarly present in $B220^+$ conventional NK cells. This antigen presenting function of NK cells supports earlier findings in human situations (23, 24).

Transgenic mouse models

Besides studying the antigen presenting capacities of isolated DCs *ex vivo*, there is another way to study the function of lung DC subsets *in vivo*. In **chapter 2** we depleted DCs by using cell specific genetic targeting strategies employing the expression of the diphteria toxin receptor (DTR) under control of a specific promotor. In CD11c-DTR transgenic mice, the CD11c promotor element drives the expression of the DTR. Murine cells are normally insensitive to DT treatment as they lack the DTR, leading to a conditional depletion of $CD11c^+$ DCs in the airways of CD11c-DTR mice treated with DT intra-tracheally (25, 26). Another DTR transgenice mouse model we used was the langerin-DTR mouse, in which the expression of DTR is driven by the langerin promotor element and therefore langerin-expressing cells could be conditionally depleted (27). We have first carefully studied the depletion of DC subsets in the transgenic mice expressing DTR under DC-specific promoters. In

CD11c-DTR mice, there is a predominant depletion of lung $CD11c^+$ DCs, of tracheal $CD11b^+CD11c^+$ DCs and of resident mediastinal LN $CD8\alpha^+CD11c^+$ DCs after diptheria toxin (DT) treatment. Importantly, $langerin^+CD11b^-CD11c^+$ migratory tracheal DCs are not depleted in these mice, and we consequently observed no decrease in mediastinal LN $CD11b^-CD8\alpha^-$ DCs. In CD11c-DTR mice given DT, we noticed that generation of virus specific $CD8^+$ CTLs as well as production of effector cytokines (IFN-α) by MLN cells was severely diminished. Based on our antigen presentation studies and on previous work of Belz et al, we propose that this is due to depletion of the resident $CD8\alpha^+CD11c^+$ DCs or perhaps to the depletion of chemokine producing lung $CD11b^+$ DCs. In CD11c-DTR mice, $CD11c^+$ alveolar macrophages are also depleted, but it is unlikely that this contributed to a decrease in antiviral immunity as adoptive transfer of wild type DCs but not macrophages restored immunity.

To more specifically address the role of migratory tracheal $CD11b^-$ DCs, we performed experiments in langerin-DTR mice (27). Langerin was found to be present particularly on mucosal $CD11b^-CD103^+$ DCs in the lung (13), a population of cells that strictly relies on CCR7 to migrate to the mediastinal LN (28). In accordance, we also found increased numbers of $langerin^+CD103^+CD11b^-CD11c^+$ DCs in the mediastinal LN following influenza infection. Following intratracheal administration of DT, there was a selective depletion of $CD11b^-CD8\alpha^-$ migratory DCs in the trachea and MLN, while leaving the $CD8\alpha^+$ resident LN population or alveolar macrophages unaffected. In the absence of the migratory $langerin^+CD11b^-$ DC population, influenza ran a particularly severe course. Strikingly, these data also suggest that the $langerin^+$ DCs of the lung are much more immunogenic than their $langerin^+$ Langerhans' cell counterparts in the skin (27, 29, 30).

Inflammatory dendritic cells

We found a clear increase in $CD11b^+$ inflammatory DCs (iDC) during influenza virus infection, but these DCs did not seem to present antigen in the MLN. During our studies, the question therefore arose what might be their contribution to the immune response? Several possibilities were hypothesized; first, lung $CD11b^+$ DCs have been shown to massively produce inflammatory chemokines and might therefore be crucial in attracking effector CD4 and CD8 cells that have been generated in the LN back to the lung and trachea, where they would mediate

effector function (31). Recently, Legge et al also suggested that these inflammatory type DCs might indeed be crucial for boosting effector CD8 responses in the lung, rather than priming of CD8 responses in naïve cells (32). Secondly, $CD11b^+$ DCs might have direct innate antiviral activity by producing TNF and iNOS dependent NO, analogous to the situation seen with Listera monocytogenes infection (33, 34), and thus might also contribute to the features of severe influenza such as lung damage and weight loss (16). Thirdly, recruited $CD11b^+$ DCs might also stimulate the innate antiviral activity through crosstalk with NK cells.

In **chapter 5** we precisely localized these inflammatory $CD11c^+CD11b^+$ DCs in the lung by immunohistochemistry. $CD11c^+$ DCs were found within areas of $B220^+$ B cell aggregates, as well as in regions containing more CD4 and CD8 T cells. In accordance to previous reports, these cell aggregates resembled inducible bronchus associated lymphoid tissue (iBALT) as the B cell area contained follicular dendritic cells and PNA-positive germinal center (GC) B cells. IBALT is a form of so-called tertiary lymphoid organs (TLO), which are revealed at times of infectious diseases or during local, chronic inflammation (35-38). In all instances where TLO have been described, antigen presenting DCs have been found interspersed with T and B cell area, just as they do in secondary lymphoid organs (SLO) (39-43). However, until now, the precise role of DCs in the functional organization of TLO was not studied in great detail. Having observed the presence of DCs inside iBALT structures at 17 dpi, we addressed whether $CD11c^+$ cells were necessary for preserving iBALT structures. Again, the CD11c-DTR transgenic mouse model was used to deplete $CD11c^+$ DCs from the lungs, as described above. $CD11c^+$ DCs were depleted from the airways at day 17 dpi, which led to disintegration of iBALT one week after depletion of DCs (i.e. at 24 dpi). This disintegration resulted in effects on both local and systemic immunity. The local production of antibodies (relying on germinal centres for immune globulin class-switching) diminished, but also virus specific immune globulin levels in serum and even virus specific plasma cells in bone marrow were affected. Long lived bone marrow plasma cells are an important source of serum antibodies (44). Therefore these latter data suggest that even 17 days post infection, the formation of a pool of long lived plasma cells in the bone marrow depends on continued input of B cells that have undergone class switching in the local TLO of the lung. When studying the way DCs controlled iBALT, this was not by presenting long-lived antigen but they rather seemed to be efficient producers of chemokines and cytokines that can retain B cells and organize TLO.

Whereas previous reports have suggested $CD11c^{+}Ly6C^{lo}$ moncytic precursors to be a source of these chemokines (31, 35), we for the first time demonstrated production by sorted iDCs in **chapter 4**. Finally, administrating DCs to the lungs of naïve mice could induce iBALT structures. A big question which remains in the field of ectopic lymphoid tissue development is the precise character of the lymphoid tissue inducer cells, that provide the initial trigger for TLO development to stromal organizer cells (42). Although our study does not answer this question, we provide clear evidence that DCs should be incorporated into any conceptual framework as to how these structures develop.

Plasmacytoid dendritic cells

The precise role of plasmacytoid dendritic cells (pDC) in influenza virus infection remains undetermined from carefully studying the literature. Whereas they are main producers of type 1 interferon during both *in vivo* and *in vitro* influenza virus infection (45, 46) this role seems to be taken over by other (epithelial and alveolar macrophages) cells in case pDCs are depleted (11, 47, 48). Migration of pDCs to MLNs upon infection or inflammation has been demonstrated (11, 49, 50) but their contribution to antigen presentation at this site seems to be negligible *in vivo*, as depletion of pDCs through specific antibodies prior to infection did not affect viral clearance or induction of virus specific CD8 T cells (**chapter 2**, (48)). Recently however, it was shown that human pDCs did have the potential to cross-present viral influenza antigen to CD8 T cells *in vitro* (5). This concept is worked out in **chapter 6** of this thesis, where we compared *in vitro* antigen presenting capacities of pDCs and compared it to cDCs following influenza virus infection. Whereas pDCs *in vitro* had a minor antigen presenting capacity to CD8 T cells following influenza virus infection, cDCs became much more efficient antigen presenting cells (APC). In contrast to earlier reports (49, 51) which demonstrated presentation of exogenous antigen to already primed T cells, we demonstrated that apparently due to a defective MHCII signaling pathway pDCs did not present exogenous antigen to naïve CD4 T cells. *In vivo* infection, it could still be that we failed to detect APC function in lymph node pDCs because we studied the wrong compartment at a wrong point in time. However, it seems that upon influenza virus infection lung pDCs (besides the cDCs) mainly accumulate at the site of infection and do not direct their interest to the MLN (32). It remains to be shown whether *in vivo* lung

(as opposed to MLN) pDCs present viral derived antigen or not to CD4 or CD8 T cells. Also, it could be that pDCs play an important role in contributing to antibody production by B cells (11, 52).

Interferon-producing dendritic cells

There has recently been proposed a new member in the mouse DC family, the so-called interferon producing killer DC (IKDC) (53-55). This DC subset was described in **chapter 4** and displays both innate immune functions of NK cells, as well as the potential to process and present antigen to naïve T cells (54). Functionally, IKDCs have the capacity to kill NK-sensitive target cells without prior activation, and induce proliferation of naïve T cells when pulsed with antigenic peptides. By contrast, conventional NK cells that lack expression of B220 and MHCII have not been identified as antigen presenting cells in the mouse (56). Although the existence of the IKDC population would make evolutionary sense, it has been controversial whether IKDCs really represent a separate DC lineage or nothing else but activated NK cells, endowed with antigen presenting capacity (57-59). The latter view is supported by the fact that human NK cells have long been known to have APC-like activity (23, 24). In our studies we used 10-color flowcytometry to clearly demonstrate the presence of IKDCs following influenza virus infection and to distinguish the population from other inflammatory cell types during influenza virus infection. These data demonstrated the complexity of defining cell populations during an inflammatory state. Phenotypical markers were easily upregulated during inflammation, making the use of multi-color flowcytometry indispensable. As described above we found evidence for antigen presenting capacities within both IKDCs and conventional NK cells during influenza infection.

General conclusion

All together the studies described in this thesis have demonstrated a division of labor between DC subsets during influenza virus infection. A critical question which might come up in the field of influenza immunology is why so much different DC subsets are involved in the immune response to influenza virus infection and if it makes sense to divide DCs into functional subsets. For this question we should go back in history and make a comparison to T cell biology. In the late 1980s,

a revolution occurred in the basis understanding of immunology. The discovery that $CD4^+$ T helper cells were not a homogeneous population but could be divided into Th1 and Th2 subsets based on their cytokine profiles caused skeptic reactions. 20 years later the field of T cell subset phenotype and function remains moving with the recently demonstrated existence of T regulatory and Th17 cells adding extra layers of complexity. Nevertheless this division of T cell subsets is by now generally accepted and of big use for the understanding of the immune system. The recent division of DCs into various subsets seems to follow the same route-the more knowledge we obtain on the function of DCs, the more we find out that these functions do not belong to one and the same DC population. Our studies show that DC functionality depends on the environment they reside. Viral infections, like influenza, will alter this environment and trigger DC populations to fulfil a specific role in the immune response. We have clearly shown that this specific role of DCs lies beyond just CTL induction. Either by inducing T cell responses, transporting antigen or affecting chemokine or cytokine levels, DC subsets contribute to various aspects of the immune response against influenza virus infection. Increasing our knowledge on functionality of DC subsets will be useful for the design of improved influenza vaccines and could increase understanding of why particular strains of influenza are more pathogenic than others.

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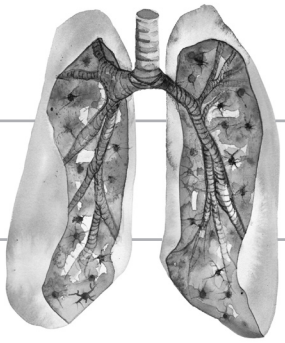


Nederlandse samenvatting

Influenza virus

Influenza virus infectie staat in de volksmond beter bekend als “de griep” en wordt veroorzaakt door het influenza virus. Niet alleen is dit virus de oorzaak van enkele zeldzame pandemiëen uit het verleden, ook de jaarlijkse griep epidemie wordt veroorzaakt door varianten van het influenza virus. Deze jaarlijks terugkerende epidemiëen hebben een grote impact op de maatschappij, zowel vanuit klinisch als economisch perspecief, wat vaak wordt onderschat.

Sinds de identificatie van het virus in 1931 zijn er vele ontwikkelingen geweest om bescherming tegen het virus te bieden. Tot op heden wordt de voorkeur gegeven aan vaccinatie om een influenza virus infectie te voorkomen. Echter, de voortdurende genetische en antigene variatie van het virus maken een continu wereldwijd toezicht (screening) op de ontwikkeling van het virus noodzakelijk. Zo wordt de variatie van het virus gevolgd en kan hierop geanticipeerd worden met de ontwikkeling van het jaarlijkse griep-vaccin. Deze continue screening vraagt veel van de betrokken partijen. Daarom is onderzoek naar verbetering van bestaande vaccinatie strategiëen, danwel het ontwikkelen van nieuwe strategiëen, momenteel een prioriteit in de gezondheidszorg geworden. Voor de ontwikkeling van een breed (cross-protectief) vaccin, dat de noodzaak van continue screening weg zou kunnen nemen, kan gedacht worden aan aanpassingen in de dosis van het vaccin, de manier van toediening, het gebruik van adjuvantia (toevoegingen) of zogenoemde “immuun modulatoren”. Naar welke vorm de voorkeur ook uit gaat, een uitgebreide en gedetaileerde kennis van de immuun respons tegen het virus is essentieel om ontwikkelingen te kunnen maken in het induceren van een gewenste, brede immuun respons tegen influenza virussen.

De immuun respons van het lichaam tegen influenza virussen kan worden onderverdeeld in een aspecifiek (aangeboren) en adaptief (specifiek) deel. De aspecifieke respons in de long is een acute respons gericht tegen een breed scala aan pathogenen. Tijdens deze respons worden veel interferonen geproduceerd door zowel geïnfecteerde epitheliale cellen van de long, alveolaire macrofagen en door dendritische cellen (DCs). Daarnaast worden tijdens deze acute respons natural killer (NK) cellen en DCs aangetrokken naar de infectiehaard. Door deze eerste snelle respons heeft het immuun systeem tijd en signalen gehad om de (specifieke) adaptieve response op gang te krijgen. Deze adaptieve respons kan weer worden onderverdeeld in een B lymfocyten respons en een T lymfocyten

respons. B lymfocyten zijn cellen die antilichamen vormen welke virus partikels kunnen binden en daarmee infectie van nieuwe cellen kunnen voorkomen. De T lymfocyten respons is een cellulaire respons, waarin T lymfocyten het uiteindelijke effect bewerkstelligen. Eerst zal echter het virale antigeen gepresenteerd moeten worden aan T lymfocyten wat gebeurt door DCs. DCs pikken het virale antigeen op, transporteren het en presenteren het aan (naïve) T lymfocyten. Deze T lymfocten zullen na herkenning van het antigeen gaan expanderen in een cytotoxische T cel (CTL) kloon, welke vervolgens direct (via contact door de T cel receptor) geinfecteerde cellen herkennen en elimineren.

De rol van DCs in zowel de aspecifieke en adaptieve immuun respons is in de literatuur beschreven. In dit proefschrift hebben we ons gericht op de functies van DCs tijdens influenza virus infectie; verschillende subtypes DCs in de long zijn geïdentificeerd en we hebben getracht hun rolverdeling in de immuun respons tegen influenza virus bloot te leggen. Een kritische vraag die zou kunnen opkomen is wat nou precies de bijdrage is van een dergelijke onderverdeling aan de influenza immunologie. Voor het beantwoorden van deze vraag zouden we een vergelijking kunnen maken met ontwikkelingen in de T cel biologie. Eind 80-er jaren werd begonnen met het onderverdelen van CD4 T cellen in subtypes, wat aanvankelijk sceptische reacties opriep. Tegenwoordig is de onderverdeling van T cellen (in Th1,Th2,Treg en recentelijk Th17) algemeen aanvaard en is men zich bewust van het nut van deze onderverdeling voor het begrip van het immuun systeem. De recente onderverdeling van DCs in subtypes lijkt een zelfde weg te bewandelen; hoe meer kennis er wordt verkregen over DC functionaliteit in verschillende ziekte modellen, hoe duidelijker het wordt dat deze functies niet aan één en dezelfde DC populatie toebehoren.

Dendritische cellen

DCs zijn 25 jaar geleden voor het eerst beschreven door Steinman en zijn collega's. Aanvankelijk werden ze beschreven als een antigeen presenterende cel populatie in de milt en lymfeklieren van de muis. De jaren daarop is er veel onderzoek verricht naar het fenotype (uiterlijke kenmerken) van deze cellen in verschillende weefsels en hun rol in verschillende ziekte modellen. Om de verschillende DC populaties in de longen te bestuderen hebben wij gebruik gemaakt van de muis als proefdier model, een proefdier dat veel gebruikt wordt in verschillende ziektemodellen.

In de long worden DCs gevonden in de meeste compartimenten, zoals het long parenchym, alveoli, pleura, perivasculair en in de long vaten. Net als de onverderdeling van DCs in lymfoide organen, worden ook in de long verschillende subtypes beschreven. Er wordt een onderverdeling gemaakt in conventionele DCs (cDCs) welke het oppervlakte molecuul CD11c tot expressie brengen (dus $CD11c^+$ zijn), en plasmacytoide DCs (pDCs). Plasmacytoide brengen de marker CD11c middelmatig ($CD11c^{interm}$)tot expressie en worden verder gekenmerkt door andere specifieke oppervlakte markers, zoals 120G8, PDCA-1, Siglec-H en B220.

cDCs in de long worden vervolgens verder onderverdeeld in subpopulaties zijnde $CD11b^+$ en $CD11b^-$. In de trachea en de grotere luchtwegen bestaat in "rust" een uitgebreid netwerk van intra-epitheliale DCs, welke $CD11b^-$ zijn. Deze DCs lijken met hun uitlopers sterk op langerhans cellen in de huid en brengen ook langerin en CD103 tot expressie.

Migratie en antigeen presentatie van DC populaties in de long

Bij een infectie met het influenza virus worden primair de bovenste luchtwegen geinfecteerd. Long cDCs worden door infectie matuur en zullen met het virale antigeen naar de lymfeklieren migreren, alwaar het antigeen wordt gepresenteerd aan T cellen. In hoofdstuk 2 beschrijven we dat de belangrijkste bron van cDCs welke in de lymfeklier aankomen en aldaar het antigeen presenteren, de langerhans-achtige $CD11b^-$ DCs uit de bovenste grote luchtwegen zijn. Nadat deze $CD11b^-$ DCs gemigreerd zijn is er een nieuwe influx van DCs, welke zijn getriggerd door het influenza virus. Dit zijn $CD11b^+$ DCs en worden inflammatoire DCs genoemd.

Het antigeen presenterend vermogen van de verschillende DC populaties in de lymfeklier werd bestudeerd door gebruik te maken van een recombinant virus, waar het ovalbumine (OVA) eiwit is ingebouwd. DC populaties werden na infectie met het recombinant OVA-influenza virus mechanisch gesorteerd uit lymfeklieren van de geïnfecteerde muizen. Vervolgens werden ze in kweek gezet met CD4 of CD8 T cellen specifiek gericht tegen het OVA antigeen. Indien een DC populatie de capaciteit zou hebben om viraal antigeen te presenteren, zouden de T cellen geactiveerd worden waardoor ze zouden gaan delen. De mate van T cel deling is daardoor een maat voor het antigeen presenterend vermogen van de DCs. Een duidelijke taakverdeling werd met deze methode aangetoond. $CD11b^-$ DCs stimuleerden zowel CD4 als CD8 T cellen, terwijl $CD11b^+$ DCs in

alle gevallen slecht het antigeen konden presenteren. Dit duidt erop dat in een enkele CD11b$^-$ DC populatie het virale antigeen kan worden opgepikt, verwerkt wordt en gepresenteerd wordt. Dit is in tegenspraak met wat eerder is aangetoond in de milt en lymfeklieren. CD11b$^-$ DCs kunnen verder worden onderverdeeld in CD8α$^+$ en CD8α$^-$ DCs. CD8α$^+$ DCs zijn resident in de lymfeklier en zullen het antigeen moeten verkrijgen van DCs afkomstig van het geïnfecteerde gebied. Door het aankleuren van viraal antigeen in DC subsets suggereren onze studies dat zowel pDCs als CD11b$^-$CD8α$^-$ DCs uit de hogere luchtwegen het virale antigeen transporteren naar de lymfeklier.

Transgene muis modellen

Naast bovenstaande beschreven methode voor het aantonen van antigeen presenterend vermogen van DC subpopulaties, is er een manier om functionaliteit van DCs te bestuderen. We hebben daarvoor in hoofdstuk 2 gebruik gemaakt van transgene muismodellen. In deze muizen konden DCs specifiek worden gedepleteerd (verwijderd) door gebruik te maken van cel specifieke genetische target-strategiëen. Een receptor voor diphterie toxine (DTR) is in deze muizen geplaatst voor de CD11c promotor. Muizen zijn normaal gesproken niet gevoelig voor diptherie toxine (DT) omdat ze deze receptor niet hebben. In transgene CD11c-DTR muizen zal behandeling met DT echter leiden tot een specifieke depletie van CD11c$^+$ cellen. Een andere transgene muis die we hebben gebruikt is de langerin-DTR muis, welke gebaseerd is op het zelfde principe, maar waarin specifiek depletie van langerin$^+$ cellen wordt bewerkstelligd.

Eerst zijn in de transgene muizen uitvoerige analyses gedaan naar de cel populaties die verdwijnen na lokale toedienening van DT (in de long). Vervolgens zijn de muizen zonder respectievelijk CD11c$^+$ cellen of langerin$^+$ cellen, geïnfecteerd met influenza virus. Het verloop van de infectie werd nauwkeurig gevolgd en de imuunrespons werd in kaart gebracht. In CD11c-DTR werden door DT toediening alle CD11c$^+$ DCs gedepleteerd, maar bleven de CD11b$^-$CD8α$^-$ DCs uit de hogere luchtwegen intact. Infectie van deze muizen verergerde de infectie aanzienlijk, wat ondermeer was af te lezen uit het toegenomen gewichtsverlies van de muizen na infectie. Immuun parameters waren aangetast: een verminderde virus specifieke CTL respons, minder cytokine productie en het virus dat minder snel geklaard werd uit de longen. Om te bewijzen dat dit alles het gevolg was van afwezige CD11c$^+$

DCs (en niet van bijvoorbeeld macrofagen welke ook CD11c tot expressie brengen), werd er ter controle een experiment uitgevoerd waarin na behandeling van DT (en $CD11c^+$ DCs dus weg waren uit de long), $CD11c^+$ DCs werden teruggegeven. Het teruggeven van deze DCs herstelde de imuunrespons en dit toonde de specifieke noodzaak van DCs in de imuunrespons aan.

Zoals beschreven bleven in de CD11c-DTR muizen de $CD11b^-CD8\alpha^-$ DCs intact. Omdat onze eerdere (antigeen presentatie) studies een belangrijke rol voor deze cellen impliceerden, besloten we een model te gebruiken waarin specifiek deze populatie uit de long kon worden gedepleteerd. Daarvoor werden langerin-DTR muizen gebruikt. Analyses in deze muizen toonden een specifieke depletie aan van mucosale $CD11b^-CD8\alpha^-CD103^+langerin^+$ DCs in de hogere luchtwegen, terwijl alle andere populaties intact bleven. Depletie van deze DCs gedurende influenza virus infectie verergerde het verloop van de infectie dramatisch, wat een duidelijke rol voor deze DCs in influenza virus infectie aantoont.

Inflammatoire dendritische cellen

Zoals beschreven worden $CD11b^+$ DCs in de long tijdens en na influenza virus infectie ook wel inflammatoire DCs genoemd. Deze DCs konden het antigeen niet efficiënt presenteren, dus was de vraag wat dan wel de bijdrage van deze DC populatie aan de immuunrespons tegen het influenza virus is. In hoofdstuk 4 hebben we met behulp van immuunhistochemie de $CD11b^+$ DCs gelocaliseerd die, nadat het influenza virus was geklaard, nog in grote getale in de long aanwezig waren. $CD11c^+CD11b^+$ DCs werden gevonden in gebieden van B en T cellen. In de literatuur worden dergelijke B en T cel aggregaten beschreven als iBALT (inducible bronchus associated lymphoid tissue). iBALT is een georganiseerd agregaat van B en T lymfocyten met DCs erin en heeft de structuur van een lymphoide orgaan.

iBALT valt onder de zogenoemde tertiaire lymphoide organen, welke worden geïnduceerd door infectie of locale chronische ontsteking. Hoewel DCs altijd aanwezig zijn in deze structuren was hun exacte rol tot nu toe nog niet bekend. In hoofdstuk 5 hebben wij aangetoond dat $CD11b^+$ DCs in de long nadat influenza virus infectie geklaard is (17 dagen na infectie), zich bevinden in iBALT. Door depletie van deze DCs in CD11c-DTR muizen op 17 dagen na infectie vallen de lymphoide structuren uiteen. Dit heeft effecten op zowel de locale immuniteit als de systemische immuniteit. Naast het feit dat de B cellen in iBALT namelijk

lokaal antilichamen produceren, werden er na het uiteenvallen van iBALT ook systemische veranderingen aangetoond. Virus specifieke imuun globulines in serum namen af en ook virus specifieke plasma cellen in het beenmerg waren verminderd. Deze "long-lived" plasma cellen in het beenmerg zijn een belangrijke bron voor virus specifieke imuun globulines in het serum. Dit impliceert dat enkele weken nadat de infectie geklaard is, de formatie van "long-lived" plasma cellen in het beenmerg afhankelijk is van een continue input van B cellen uit iBALT. De vraag blijft natuurlijk hoe DCs iBALT nu precies controleren. Dit is niet door een continue antigeen presentatie aan omliggende lymfocyten, maar $CD11b^+$ DCs zijn belangrijke producenten van chemokines en cytokines die B cellen op hun plek houden en iBALT organiseren.

Plasmacytoide dendritische cellen

pDCs staan in de literatuur bekend als belangrijke producenten van type 1 interferonen. Hoewel wij deze functie bevestigd hebben tijdens influenza virus infectie, lijkt bij depletie van pDCs (door antilichamen) tijdens influenza virus infectie deze rol overgenomen te worden door andere cel types (als epitheliale cellen en alveolaire macrofagen). Verder migreren pDCs gedurende een influenza virus infectie naar de lymfeklier, maar hun contributie aan het presenteren van antigeen lijkt nihil (hoofstuk 2 en hoofdstuk 5), waarschijnlijk door een defect in de pDC machinerie dat het antigeen verwerkt. Dit alles leidt er toe dat depletie van pDCs tijdens influenza virus infectie het verloop van de infectie niet beïnvloedt. Een opmerkelijke bevinding was echter dat de hoeveelheden virus specifieke antilichamen in muizen waarin pDCs waren gedepleteerd tijdens infectie, lager zijn t.o.v. muizen die wel pDCs hadden. Dit wijst op een mogelijk interactie tussen pDCs en B cellen die nader onderzocht dient te worden.

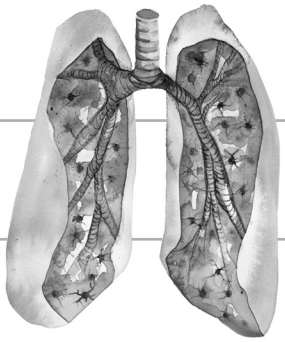
Interferon-producerende dendritische cellen

Interferon producerende dendritische cellen (IKDCs) zijn een zeer recent beschreven subtype van DCs. In hoofdstuk 3 tonen wij voor de eerste keer deze DCs aan in de long tijdens influenza virus infectie en we hebben met uitgebreide flowcytometrie kleuringen de verschillen getoond tussen IKDC an andere celtypes tijdens influenza virus infectie. We hebben kunnen bevestigen dat deze IKDCs

zowel functies van NK cellen hebben (het direct doden van virus geïnfecteerde cellen) als DCs (antigeen presentatie). Echter moet worden opgemerkt dat ook "reguliere" NK cellen tijdens influenza virus infectie een antigeen presenterend vermogen verkrijgen.

Slot conclusie

De studies beschreven in dit proefschrift tonen de rolverdeling aan tussen DC subtypes in de long tijdens een influenza virus infectie. De studies demonstreren dat het onderverdelen van DCs in subtypes niet een vorm van "postzegels verzamelen" is, maar een onderverdeling naar functionaliteit. Functionaliteit van DCs lijkt afhankelijk te zijn van de omgeving waarin ze verkeren. Virale infecties door bijvoorbeeld het influenza virus, zullen deze omgeving veranderen en DCs prikkelen om bepaalde specifieke functies te verrichten, zoals antigeen presentatie, transport van antigeen of productie van chemokines en cytokines. De verworven kennis over DC functionaliteit zal toepasbaar zijn bij het ontwikkelen van verbeterde influenza vaccins, maar draagt tevens bij aan het inzicht in pathogeniteit van verschillende influenza virussen.



About the author

Curriculum Vitae

The author of this thesis was born on October 23d, 1977 in Stadskanaal (the Netherlands). As a child she lived in Bangkok (Thailand) where she attended the International School of Bangkok. Back in the Netherlands she finished high school (VWO) at the Winkler Prins Scholengemeenschap in Veendam. She then spent a year in France to study the French language. In 1996 she started her study in Health Sciences at the University of Maastricht (the Netherlands). After having obtained the first two years in 1998 she was admitted to medical school at Erasmus University in Rotterdam (the Netherlands). During medical school she performed clinical internships at Kanti's Children Hospital in Kathmandu (Nepal) and at Centre Hospitalier Intercommunal Annemasse-Bonneville (France). In 2002 she conducted her MSc research project at the department of Virology at Erasmus MC in Rotterdam (the Netherlands) under supervision of Prof. A. Osterhaus. The project on viral respiratory tract infections in children was performed at Chulalongkorn Hospital in Bangkok (Thailand) during 3 months. In 2003 she started her rotations, during her final rotation she spent 4 months at Hôpital Necker - Enfants Malades in Paris (France) to study hereditary immune deficiencies in children (supervised by Dr. N. Hartwig and Prof. A. Fischer). After having obtained her medical degree in 2005 she started her PhD research at the departments of Virology and Pulmonary Medicine at Erasmus MC Rotterdam, resulting in the present thesis. In september 2009 she will start her medical specialist training in medical microbiology-virology at Erasmus MC in Rotterdam.

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List of Publications

GeurtsvanKessel CH, Willart MA, van Rijt LS, Bergen IM, Muskens F, Osterhaus ADME, Hoogsteden HC, Hendriks R, Rimmelzwaan GF and Lambrecht BN. Dendritic cells control tertiary lymphoid tissue formation in the lung of influenza virus infected mice. *Journal of Experimental Medicine, under revision*

GeurtsvanKessel CH, Muskens F, Bergen IM, Kool M, Boon L, Hoogsteden HC, Osterhaus ADME, Rimmelzwaan GF, Lambrecht BN. Both conventional and 'IKDC' natural killer cells have an antigen presenting capacity during *in vivo* influenza infection. *Journal of Virology, under revision*

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PhD Portfolio

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In-depth couses

2009	Biomedical English Writing and Communication
2008	Workshop on Bioinformatic analysis, Tools and Services
2006	Course in Virology. International training course in general virology.
2005	Laboratory animal science (art.12)
2005	MolMed course
2005-present	International seminar series in Virology, Immunology, Cell Biology and Molecular Medicine, provided by the Post-Graduate School Molecular Medicine and the Department of Virology, Erasmus MC.
2005-present	Weekly internal and external presentations at the departments of Virology and Pulmonary Medicine.

International scientific presentations

Okt 2008	DC 2008 Meeting. Kobe, Japan. (poster presentation)
Sept 2008	Third European Influenza Conference. Vilamoura, Portugal. (poster presentation)
Jan 2008	Bangkok International Conference on Avian Influenza 2008 : integration from Knowledge to Control. Bangkok, Thailand. (oral presentation)
Dec 2007	NK versus DC meeting. University Hospital Ghent, Belgium. (oral presentation)
Sept 2007	4th Orthomyxovirus meeting. Woodshole, USA. (oral presentation)

Aug 2007	13th International Congress of Immunology. Session: "Immunity to Viruses". Rio de Janeiro, Brasil. (oral presentation)
May 2007	EMBO Conference "Shaping Immunity in Healthy and Diseased Tissues". Sardinia, Italy. (oral presentation)
April 2007	NK meeting, Institute Gustave Roussy. Paris, France. (oral presentation)
Nov 2006	DC 2006 Meeting. Edinburgh, UK. (poster presentation)
Feb 2006	Keystone Viral Immunity. Steamboat Springs, USA. (poster presentation)

National scientific presentations

Dec 2008	NVVI- Dutch association of Immunology. Noordwijkerhout, the Netherlands. (oral presentation)
Dec 2007	NVVI- Dutch association of Immunology. Noordwijkerhout, the Netherlands. (oral presentation)
June 2007	Molecular Medicine Course Erasmus MC Rotterdam, the Netherlands. (oral presentation)
April 2007	Dutch Organisation for Scientific Research (NWO) Vaccine Meeting. Utrecht, The Netherlands. (oral presentation)
Feb 2007	Molecular Medicine Day Erasmus MC Rotterdam, the Netherlands. (oral presentation)
Dec 2005	NVVI- Dutch association of Immunology. Noordwijkerhout, the Netherlands. (poster presentation)
2005-present	Several oral scientific presentations for VIRGO partners to establish progress following endpoints of the national VIRGO consortium

Awards

- Nominated for Molecular Medicine best paper award 2009 – second price
- Travel grant for DC2008 Kobe, Japan.
- ESWI Young Scientist Grant. Third European Influenza Conference 2008.
- Travel Grant for Bangkok International Conference on Avian Influenza
- EMBO Travel Grant and Voucher for attendance of EMBO Conference
- "Shaping Immunity in Healthy and Diseased Tissues" in Sardinia, Italy.

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