Pulmonary immune responses against influenza A virus infection
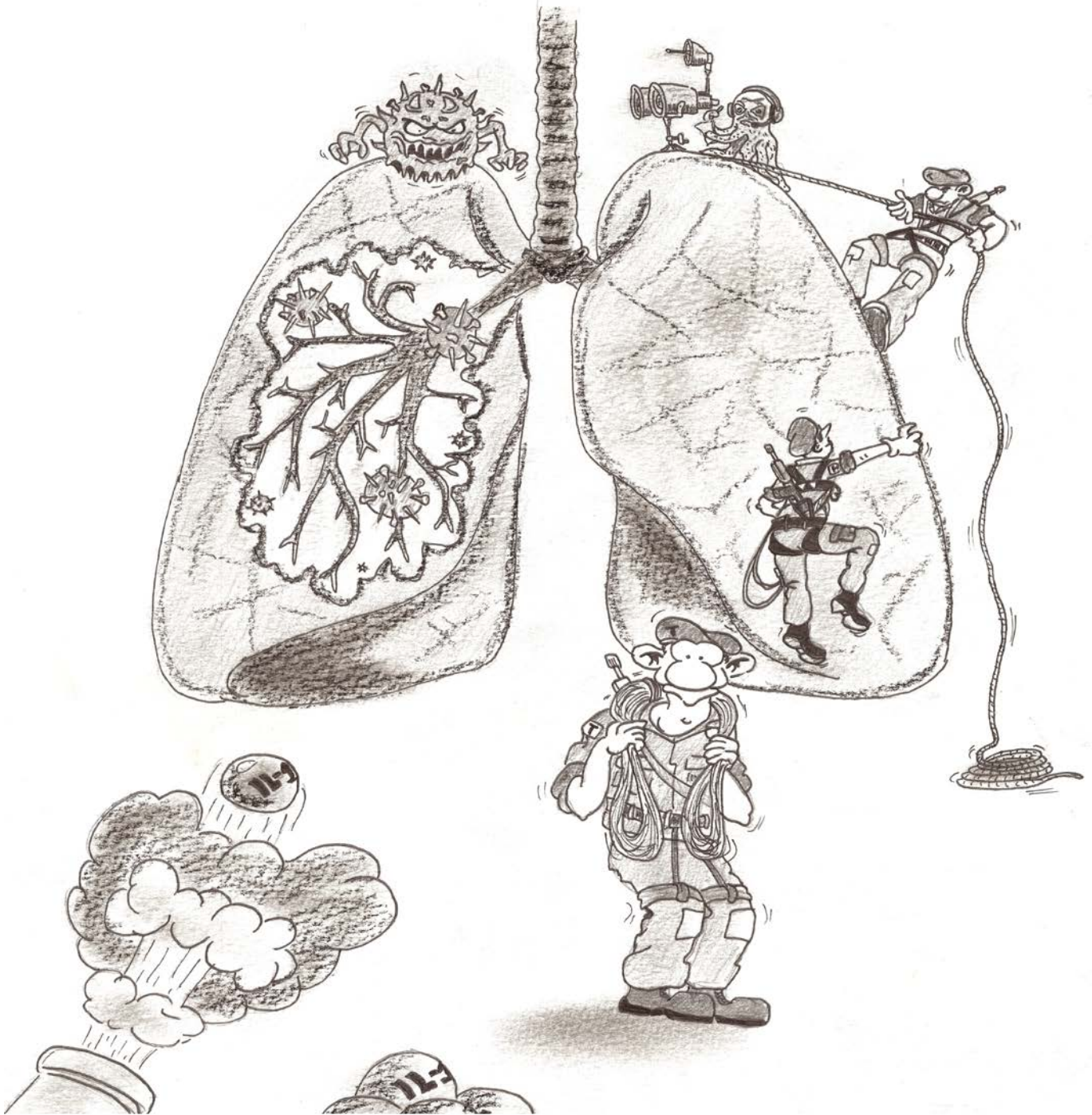

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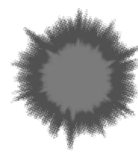Pulmonary immune responses against influenza A virus infection

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny..."

Isaac Asimov

ABSTRACT

Influenza infections usually run a mild course, but pandemics with a more severe outcome can occur when the fragmented genome of the virus undergoes antigenic shift. The continuous threat of a pandemic combined with the ongoing antigenic drift makes that development of a universal protective vaccine is currently impossible. Therefore it is of great interest to thoroughly study the immune response against influenza virus infection as a better understanding of these processes can help to develop new protective or therapeutic strategies.

In this thesis we studied different aspects of the immune system with a focus on the heterogeneity of different cell types and immune reactions. A first study focused on dendritic cell subsets. We identified a maturation status of cDC2s that acquires typical cDC1 functions. This observation draws attention to the fact that a strict delineation of subsets and their function during steady-state conditions is not necessarily applicable to inflammatory settings. In a second study T cell subsets after influenza infection were studied in detail with a focus on the currently weakly defined $CD4^-CD8^-$ double negative T cells. These cells reside in the lung tissue in a preactivated state and are recruited and maintained in a dendritic cell dependent manner. Functionally, double negative T cells were shown to control the balance of dendritic cell subsets. The scope of the last study is the involvement of the early innate IL-1 signal on iBALT formation. We show that the lung is already conditioned 2 days post infection to form iBALT structures whereas these structures are only fully formed 17 days post infection. In this context early IL-1R signaling is crucial for CXCL13 expression and later iBALT formation.

ABBREVIATIONS

AMF	alveolar macrophage
BM	bone marrow
CD	cluster of differentiation
cDC	conventional dendritic cell
CDP	common DC progenitor
cMoP	common monocyte progenitor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dLN	draining lymph node
DN T cell	CD4$^-$CD8$^-$ double negative T cell
Dpi	days post infection
DT	diphtheria toxin
DTR	diphtheria toxin receptor
FDC	follicular dendritic cell
Flt3-L	FMS-like tyrosine kinase 3 ligand
FRC	follicular reticular cell
GC	germinal center
GM-CSF	granulocyte-macrophage colony stimulating factor
HA	hemagglutinin
HEV	high endothelial venule
IAV	Influenza A virus
iBALT	inducible bronchus associated lymphoid tissue
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.n.	intranasal
i.t.	intratracheal
i.v.	intravenous
LTi cell	lymphoid tissue inducer cell
LTo cell	lymphoid tissue organizer cell
MC	monocyte-derived cell
MF	macrophage
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLN	mediastinal lymph node
MoDC	monocyte-derived DC
NA	neuraminidase
NET	neutrophil extracellular trap
NLR	NOD-like receptor
NK cell	natural killer cell
NP	nucleoprotein
PAMP	pathogen-associated molecular pattern
pDC	plasmacytoid dendritic cell
PRR	pattern recognition receptor
RLR	RIG-I-like receptor
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase polymerase chain reaction
SLO	secondary lymphoid organ
SP	surfactant protein

T_{cm} cell	central memory T cell
TCR	T cell receptor
T_{em} cell	effector memory T cell
TF	transcription factor
Tfh cell	follicular helper T cell
Tg	transgenic
Th cell	T helper cell
TLO	tertiary lymphoid organ
TLR	toll-like receptor
Treg cell	regulatory T cell
T_{rm} cell	resident memory T cell
WT	wild type

CONTENTS

Chapter 1

Introduction

Influenza

Influenza, commonly called "the flu", is caused by infection with an influenza virus. Influenza viruses are negative-sense, single-stranded, enveloped RNA viruses with a segmented genome and are classified in the family of *Orthomyxoviridae*. Based on differences in the conserved nucleoprotein (NP) and matrix (M) proteins, influenza viruses are subdivided in 3 genera: influenza A, B and C[1]. Influenza virus A and B have 8 genome segments, whereas influenza C only has 7 genome segments[2, 3]. Influenza A viruses (IAV) are further subdivided into subtypes based on differences in the hemagglutinin (H1-18) and neuraminidase (N1-11) surface proteins (http://www.cdc.gov/flu/avianflu/influenza-a-virus-subtypes.htm).

Epidemiology of influenza A virus infections

Annually 5-10% of adults and 20-30% of children get infected with influenza virus (www.who.int fact sheet N°211). The virus mainly spreads via aerosols caused by coughing and sneezing. The aerosols can infect people directly or reside on the hands of infected people and be passed on via poor hand hygiene. Illness is usually mild and characterized by fever, coughing, headache, muscle pain, malaise, a sore throat and a runny nose. Occasionally, influenza viruses can cause severe illness and even death; especially in the high-risk groups including pregnant women, young children, elderly and people with underlying immune compromising disease.

Structure and genomic organization of influenza A virions

IAV is an enveloped virus with a spherical or filamentous form. The lipid envelope is derived from the host cell membrane during the process of viral budding and consists of only 3 viral surface proteins: hemagglutinin (HA), neuraminidase (NA) and Matrix Protein M2[3](Figure 1). The HA is responsible for binding to the host cell and membrane fusion, NA is involved in viral budding and is necessary to release the newly formed viral particles from the host cell. The M2 protein is a pH-regulating ion channel necessary for viral uncoating. The viral RNA polymerase complex is formed by 3 subunits: Basic Polymerase Protein (PB) 1 and 2 and Acidic Polymerase Protein (PA)[3]. Furthermore, the viral particle consists of the M1 matrix protein, which interacts with the viral RNA, the RNA binding Nucleoprotein (NP), the Nuclear Export Protein (NEP) and the Nonstructural Protein NS1 that regulates gene expression[3].

All viral RNA segments are bound to NP inside the virion and form ribonucleoprotein (RNP) complexes[4]. Historically it was thought that only these 10 proteins are transcribed from the 8 viral RNA segments of the IAV virus[3](Figure 1) but more recently additional splice variants of the polymerase, matrix and non structural protein were defined[5]. Segments 1, 4, 5 and 6 each encode only 1 protein: PB2 (basic polymerase protein 2), HA, NP and NA respectively. Segment 2 encodes PB1 and the accessory protein PB1-F2 and PB1-N40, an N-truncated form of PB1. Segment 3 encodes the acidic polymerase protein (PA) and its related proteins PA-X, PA-N155 and PA-N182. Segment 7 encodes the matrix protein M1, the ion channel protein M2 and M42. Segment 8 encodes NS1,

NEP and NS3, an isoform of NS1. Table 1 summarizes the different proteins that are derived from the 8 gene segments and their respective function.
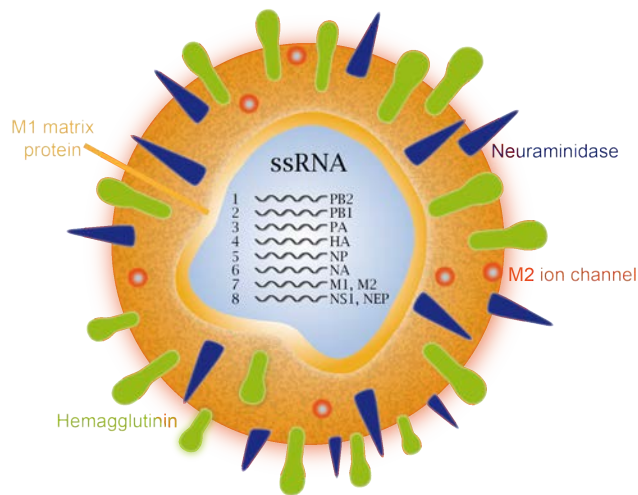
Figure 1- Structure of the influenza A virion

Influenza A virus particles are spherical and consist of a host-derived lipid membrane with three viral surface proteins (neuraminidase, hemagglutinin and the M2 ion channel) that surrounds the 8 ssRNA genome segments

Table 1 - Influenza proteins encoded by the gene segments and their respective function

SEGMENT	PROTEIN	FUNCTION
1	Basic Polymerase Protein 2 (PB2)	Generating cap structure for viral mRNA
2	Basic Polymerase Protein 1 (PB1)	RNA polymerase, RNA elongation
	PB1-F2	Synergistic effect on PA and PB2, regulation of innate immune response, pro-apoptotic, pro-inflammatory
	PB1-N40	Interaction with PB1 and PB1-F2, not PA
3	Acidic Polymerase Protein (PA)	Helicase and ATP-binding
	PA-X	Repression of cellular gene expression by degradation of host mRNA
	PA-N155, PA-N182	No polymerase activity, exact function in replication cycle not defined
4	Hemagglutinin (HA)	Fusion with host cell by binding to sialic acid containing receptors on the cell surface of host cells
5	Nucleoprotein (NP)	Transcription and replication, nuclear import
6	Neuraminidase (NA)	Release of viral particles from infected cells via sialidase activity
7	Matrix Protein 1 (M1)	Separation of RNP from viral membrane, RNA nuclear export regulation, viral budding
	Matrix Protein 2 (M2)	pH regulating ion channel, virus uncoating and assembly
	M42	Similar to M2
8	Nonstructural Protein 1 (NS1)	Regulation of cellular and viral protein expression, interferon antagonist
	Nuclear Export Protein (NEP, NS2)	Nuclear export of RNA
	NS3	Host adaptation (hypothesis)

Viral replication

The composition of IAV viruses is relatively simple as their RNA encodes for a limited set of proteins. As a consequence, they have to use elements of the host transport, replication, transcription and translation machinery in addition to their own proteins to successfully complete their life cycle[6, 7](Figure 2). Viral HA recognizes and binds to sialic acid on the host cell surface. After binding to the host cell, the viral particle is endocytosed. This is a process consisting of two steps: firstly the viral envelope merges with the endosomal membrane; secondly the virion is internally acidified via the M2 ion channel, which allows the release of viral RNPs into the cellular cytoplasm[8].
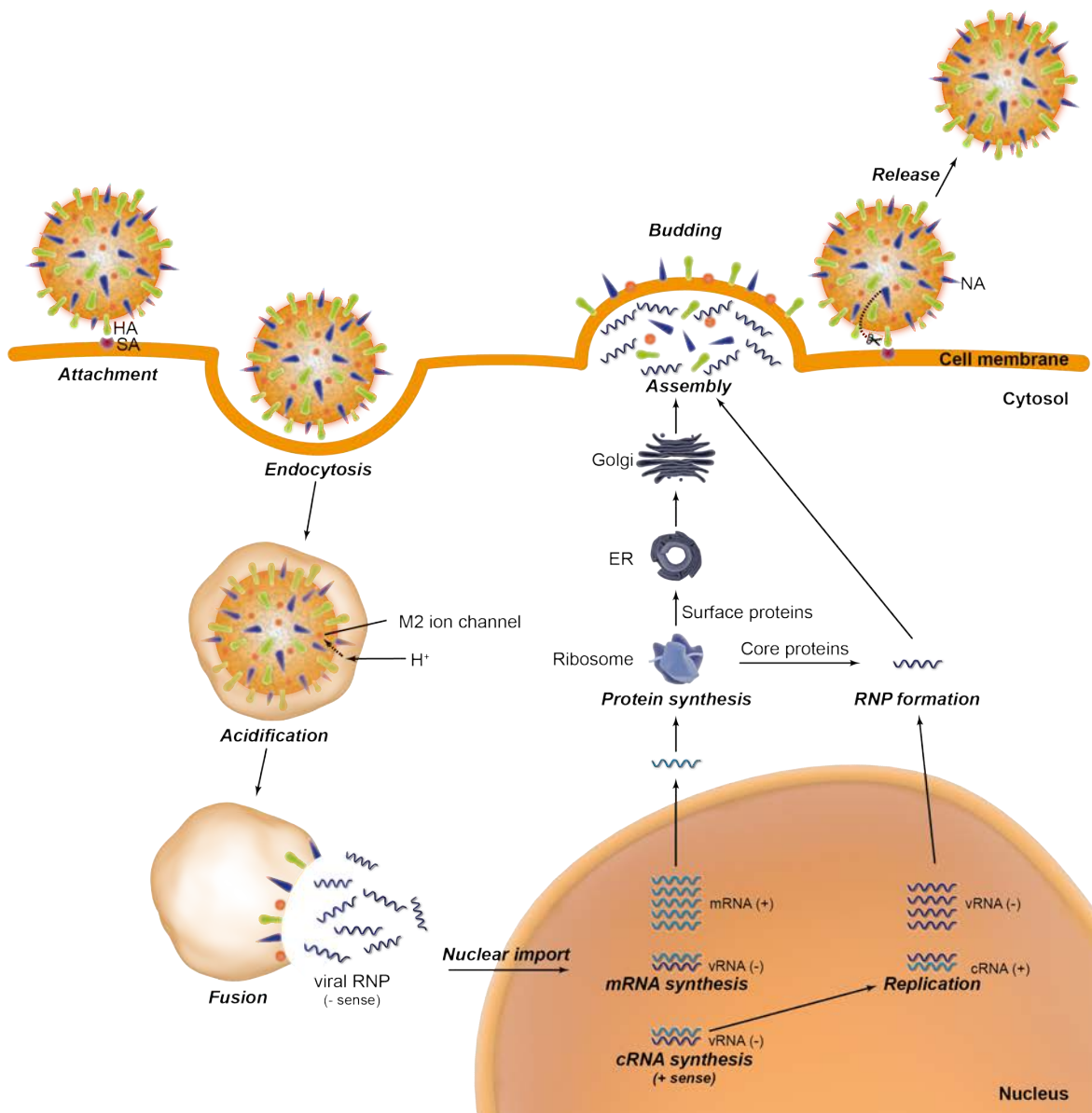

Figure 2 - Schematic representation of the replication cycle of influenza virus
HA = hemagglutinin, NA = neuraminidase, SA = sialic acid, ER = endoplasmic reticulum
vRNA = viral RNA, mRNA = messenger RNA, cRNA = complementary RNA

RNPs are then transported to the nucleus of the cell for RNA synthesis[9]. The negative stranded viral-RNAs (vRNA) serve as a template to synthesize messenger RNA (mRNA) and complementary positive stranded RNA (cRNA) that on its turn serves as a template to transcribe more vRNAs. The host translation machinery in the cytosol translates viral mRNA into proteins. The surface proteins are post-translationally modified in the endoplasmic reticulum and transported to the cell membrane via the Golgi complex. The IAV core proteins interact with the vRNA to form the RNPs. In the cytoplasm, the RNPs are assembled into newly synthesized viral particles that bud from the infected cell, thereby obtaining their viral membrane containing viral surface proteins. NA activity is crucial during this process to release the newly formed viruses that will next use HA to bind sialylated proteins on the host cell membrane[10].

Antigenic shift and drift

The RNA-dependent RNA polymerase mechanism that synthesizes mRNA and cRNA from the negative stranded vRNA template lacks proofreading capacity and therefore random point mutations can be introduced in the viral genome during replication. Owing to this error-prone replication mechanism and the fact that the genome is segmented, these viruses are sensitive to undergo so-called antigenic drift and shift[3](Figure 3).

Antigenic drift is caused by minor genetic changes that arise as a consequence of natural occurring mutations that are not corrected during the replication phase or due to selective pressure under the influence of virus-specific antibodies. This process can result in changes in the antigenic sites of the viral proteins. When the HA or NA surface genes are sufficiently altered, the pre-existing neutralizing antibodies might not be able to recognize the HA or NA epitopes and thereby the virus can escape the prior memory mechanisms of the immune response of the host, causing the yearly influenza epidemics.

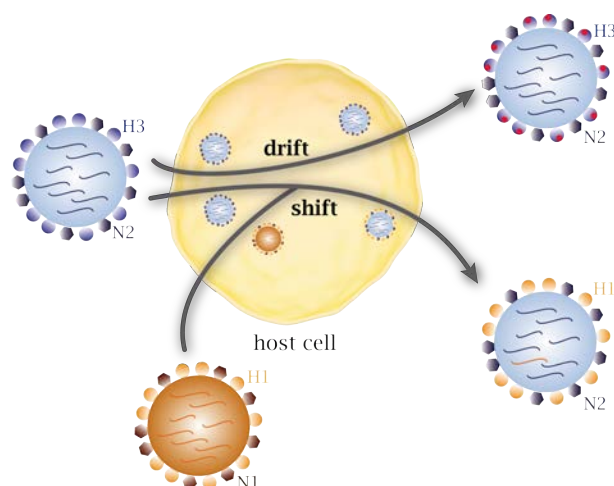Figure 3 - Genetic drift and shift of influenza A virus in the host cell
During genetic drift minor changes are introduced in the influenza A viral genome, which can result in a change in the antigenic sites of the viral proteins. When two different viral strains are present in the same host cell antigenic shift can occur. During this process gene segments are exchanged. H = hemagglutinin, N = neuraminidase

Contrary to the small changes introduced by antigenic drift, a new virus with antigenically unrelated surface proteins can arise when 2 or more viruses co-exist in the same host and exchange viral segments. This process is called antigenic shift. If the newly generated virus is efficiently transmittable from human to human, it can cause influenza pandemics like the Spanish flu in 1918 (H1N1), the Asian flu in 1957 (H2N2), the Hong Kong flu in 1968 (H3N2) and the more recent outbreak of Mexican flu in 2009 (H1N1) since no pre-existing imp munity is present in the majority of the population.

This flexibility of the viral genome is a great challenge for vaccine development. The circulating viruses in humans need to be monitored continuously to follow the genetic changes and predict future changes. Based on these observations the composition of the vaccine is adjusted every year. The vaccine generally consists of the 3 or 4 most relevant circulating viral types: 2 IAV of subtypes H1N1 and H3N2 and one or 2 IBV types. The vaccines are most effective when the vaccine viruses antigenically match the actual circulating viruses, but since antigenic shift and drift are random processes and therefore hard to control and predict, there is never a guarantee for total protection with the current vaccine strategies. In depth knowledge about the molecular and cellular mechanisms of IAV infection is therefore necessary to develop vaccination strategies that overcome the limits of the currently available vaccines[11].

The innate immune response against influenza A virus

Epithelial integrity and antiviral soluble innate immunity

Because of their air-exchange function, the lungs are continuously exposed to particles from the environment such as microbes, allergens, mineral and organic dust particles and pollutants. Therefore a fine balance must be maintained between tolerating non-harmful particles from the environment and mounting a strong and protective immune response against harmful organisms that enter the lungs. To avoid immune reactions in non-harmful conditions, epithelial cells produce suppressive signals. Influenza virus aerosols enter via the respiratory tract and its first target cell is the epithelial cell. To be able to enter and infect the epithelial cells, IAV needs to pass intrinsic protection mechanisms such as the mucus layer and antiviral peptides that can prevent attachment and viral entry. The lining fluid of the lungs that surrounds the cilia of the ciliated bronchial epithelial cells is crucial for the functioning of the mucociliary blanket as this fluid contains numerous soluble molecules that mediate a first line of innate defense. These comprise, lysozyme, lactoferrin, defensins, complement and surfactant proteins A and D. Surfactant protein (SP) A and D play an important role in innate humoral defense mechanisms of the lung as demonstrated in SPA and SPD deficient mice that develop a more severe IAV infection[12, 13]. SPD serves to balance protection against alveolar damage and virus elimination by binding IAV particles, thus preventing infection of epithelial cells and facilitating the clearance of these particles by phagocytic cells[14]. Furthermore binding of viral particles to Galectin-1 decreases susceptibility to infection[15] and viral membranes can be destructed directly by LL-37

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that is released from neutrophil granules and by epithelial cells[16]. Recent genetic studies linked genetic polymorphisms in the complement system proteins to the outcome of IAV infection[17, 18]. Especially component C3 was shown to contribute to protection[19]. In contrast, complement activation was also linked to acute lung injury[20]. Defensins can be constitutively produced by epithelial cells and neutrophils and in response to infection. They inhibit the infection of epithelial cells through direct interactions with the virus or through induction of aggregation of the virus[21-26].

Once the virus breaches this first physical barrier, it can infect the epithelial cells, which results in the loss of epithelial integrity and subsequent loss of homeostatic signals.

Besides suppressive signals that are constitutively produced in the lungs, one of the earliest soluble antiviral proteins that are induced upon recognition of a viral threat are type I interferons (IFN). Type I IFNs consist of 14 IFNα subtypes (only 13 in humans) and one subtype of IFNβ, IFNε, IFNκ and IFNϖ that all bind to the type I IFN receptor composed of an IFNAR1 and an IFNAR2 chain[27, 28]. Type I IFNs are crucial for the defense against most viruses, including respiratory viruses. According to the classical paradigm, type I IFNs induce an antiviral state in neighboring non-infected cells whereby they inhibit protein synthesis in host cells and limit viral replication, a process often referred to as "viral interference"[29]. In recent years, many studies using RNA sequencing and RNA expression arrays have been performed in virus-infected cells, including those lacking type I IFN receptors. From these studies a common "type I interferon signature" has emerged. Genes often induced by type I IFNs are summarized in Table 2.

Table 2 - Interferon stimulated genes and their function

IFN INDUCED GENE	FUNCTION	REFERENCE
IFIT family	Inhibition of cellular translation	30
IFITM1-3	Control of IAV infection by interfering with viral-host receptor binding and entry of RNPs into the cytosol	31, 32
ISG15	Conjugation of NS1, thereby inhibiting its function	33-37
Mx1, Mx2	Interference with viral RNA replication by interacting with RNA helicases and viral NP Susceptibility or resistance to IAV infection is associated with polymorphisms	38-41
OAS1, OASL1	Creates co-factor for RNAseL which cleaves RNA, stopping (viral) replication; cleaved products activate RIG-I providing a positive feedback loop for IFN production	42, 43
PKR	Limiting viral replication by a general block of translation	44
Tetherin	Holds newly produced viral particles at the cell surface; role during IAV infection is questionable	45-48
TRIM protein family	Activation of RIG-I signaling pathway, enhancing the type I IFN response Interaction with NP leads to proteosomal degradation Hampers RNA synthesis	49-51
Viperin	Inhibits budding from the plasmamembrane of infected cells *in vitro*; not confirmed *in vivo*	52, 53

Despite the strong antiviral activities that are initiated by these genes before activation of the adaptive humoral and cellular immune responses, they are not proficient of preventing viral infections as IAV developed evasion strategies to temper the interferon response[54]. In the coming years, it will be important to unravel the precise importance of these individual genes in explaining the antiviral state in more detail.

Almost all cell types can produce type I IFN. As a consequence, the exact source of type I IFN during infections can differ according to the type of virus, the anatomical location and structure and the timing.

Activation of antiviral cellular innate immunity by stimulation of pattern recognition receptors

Innate immune cells have evolved to recognize conserved pathogen-associated molecular patterns (PAMPs) through specific pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). Viral nucleic acids can be recognized by TLRs located in the endosomal compartment: DNA viruses by TLR9, ssRNA by TLR7 and 8 and dsRNA by TLR3. Viral proteins can also bind to TLRs located on the cell surface[55]. The intracellular RLRs RIG-I, MDA5 and LGP2 are present in the cytosol of most cell types and induced by IFN in a positive feedback loop of virus detection. RLRs are essential for induction of innate immune defense mechanisms and type-I IFN production in response to RNA virus infection[56]. RIG-I recognizes RNA with 5′ triphosphates whereas MDA5 recognizes long and stable dsRNA[57]. Apart from the conventional RNA helicases RIG-I and MDA5, a new group of RNA helicases, DEXDc helicases, able to activate innate immune cells has been described[58-62]. It is currently unknown if lung immune cells rely solely on these helicases to recognize particular viral infections or certain phases of the replicating virus.

NLRs have a N-terminal effector domain for interaction with adaptor proteins. When PAMPs are detected, large molecular complexes, called inflammasomes, are formed. Activation of inflammasomes leads to the activation of pro-inflammatory genes through NFκB signaling in a two-step process[63, 64]. IAV virus infection predominantly activates the NLRP3 inflammasome[65-68]. In a first step viral RNA is recognized by TLR7, which leads to production of pro-IL-1β or pro-IL-18. Consequently a second signal uses the M2 ion channel to activate the NLRP3 inflammasome[69], leading to activation of pro-caspase 1 to caspase 1[70] and subsequent cleavage of pro-IL-1β or pro-IL-18 into a bioactive form.

The essence of these PRRs is illustrated by the fact that many viruses have evolved evasion mechanisms to circumvent the activation of PRRs. For example, the non-structural proteins of influenza and RSV viruses evade immune recognition via PRRs[71, 72].

Lung resident cells act as sentinels via their PRRs and can initiate the recruitment of various cell types to the site of inflammation. Epithelial cells are the first target cells for IAV infection[73]. The virus can cause productive infection of these epithelial cells resulting in the release of large numbers of infectious virus progeny[74-76]. The PRRs on their surface will recognize viral particles, which eventually will lead to induction of type I IFN and production of cytokines and chemokines such as IL-6, TNF-α, IL-8, CXCL10, CCL2 and CCL5 to coordinate the innate immune defense to prevent spreading of the virus[77-84]. Infection of murine tracheal epithelial cells leads to induction of IFNβ, α4 and α5, but not the other subtypes, in a RIG-I dependent manner[85].

Alveolar macrophages (AMFs) reside in the lung where they produce IL-10 to dampen the immune responses[86-89]. When pathogens enter the respiratory system, the phagocytic AMFs are crucial as a first line of defense against IAV infection as they

constitute over 90% of the cells in the naïve lung[90-95]. Studies in IFNα reporter mice have revealed that AMFs can also be massive producers of type I IFNs after intranasal infection with certain RNA viruses[96, 97]. During influenza infection, epithelial cells produce CCL2 in a type I IFN dependent manner, which attracts CCR2+ monocytes from the blood into the lung[91, 98]. These monocytes can differentiate into macrophages or monocyte-derived dendritic cells and have the duality of being protective and harmful for the lung environment[91, 99]. Furthermore tissue resident macrophages and DCs produce CCL2 for self-amplification of early infiltrated Ly6C^{hi} monocytes upon type I IFN produced from epithelial cells[100]. Recruited monocytic cells can serve as reservoirs for influenza virus replication early following infection[101].

Neutrophils form the phagocytic compartment together with macrophages and are the first white blood cells that are recruited to the sites of inflammation[94, 95]. They are equipped with multiple strategies to eliminate pathogens. Upon encounter with a pathogen, neutrophils phagocytose the organism and kill it by production of reactive oxygen species[102, 103] or by the release of antibacterial peptides from its granules[16, 21, 24, 104-107]. These proteins can also be released directly in the extracellular milieu to kill organisms without phagocytosing them. Whether neutrophils take up viral particles directly[108] or via ingestion of infected epithelial cells[95] remains a matter of debate. In either case, they are not permissive for productive infection[95, 109, 110]. An additional killing mechanism is the formation of neutrophil extracellular traps (NETs). These NETs are composed of a DNA element to which histones, proteins and enzymes that are released from the neutrophil granules are attached. This forms a network where pathogens can be "trapped" to facilitate phagocytosis and prevent their spread[111]. The recruitment of neutrophils during IAV infection reduces the disease severity by limiting viral replication[94, 112-114]. Besides effector cells participating in the early responses, neutrophils can also participate in the adaptive response. They can acquire antigen-presenting functions and thereby contribute to the adaptive immune response and induce CD8+ T cell responses[115-118].

Another cell type responsible for the first-line defense against infections is the natural killer (NK) cell. They reside in the healthy lung, but are further recruited from the blood to the lung by type I IFN of epithelial cells within the first days after infection[100, 119, 120]. Neutrophils are required for NK cell homeostasis and function[121]. During influenza virus infection, type I IFN-signaling on NK cells is necessary for their activation and expression of the cytolytic effector function and production of IFNγ[122-124]. NK cells are involved in regulating viral clearance[125-130] and are also important for epithelial tissue regeneration[131] thereby limiting morbidity and mortality. The NKp46 NK cell receptor interacts with viral HA and this interaction leads to enhanced cytotoxicity against the infected cells[132-136]. NK cells not only limit the viral replication by killing infected cells prior to the full activation of the adaptive immune response[137], they are also involved in initiating this adaptive response[138, 139]. In contrast to the protective roles of NK cells, some studies described an exacerbating role of NK cells on morbidity and pathology[140, 141]. Most likely, the dual role of NK cells depends on the viral dose and pathogenicity of the different influenza subtypes[142]. Because of the import first-line defense mechanisms of NK cells, IAV virus has also evolved mechanisms to evade or escape NK cell recognition[143-146].

Dendritic cells bridge innate and adaptive immune responses

To cope with the continuous exposition to foreign particles, the lung is equipped with an elaborate network of dendritic cells (DCs). DCs are the so-called sentinels of the immune system that link sensing by ancient innate immune system receptors to activation of adaptive immunity. Ralph Steinman identified them in 1973 as cells with processes or "dendrites"[147-149] that are functionally distinct from macrophages by their ability to stimulate naïve T cells in mixed-lymphocyte reactions[150, 151].

Although once identified as a single cell type, it is now clear that DCs have many faces. In the lungs, DC subsets perform different tasks of antigen sampling, recognition of pathogens and allergens, migration to lymph nodes, induction of cellular CD4 or CD8 immunity, and production of inflammatory chemokines. As a result many different subsets have been described by various investigators in the last 40 years. A major pitfall when studying DCs is that the poor consistency in nomenclature that is used to describe different subsets makes it hard to compare studies. To create a more robust nomenclature, a classification system based on two levels was recently proposed[152] and summarized in Table 3. The first level is based on ontogeny and makes a distinction between monocytes and monocyte-derived cells (MCs) that are derived from the common monocyte progenitor (cMoP) and dendritic cells (DCs) that are derived from the common DC precursor (CDP)[153, 154]. The second level subdivides the true DCs into three major subtypes based on the developmental pathway. The conventional DCs (cDC) develop from the CDP via the pre-cDC stage into either cDC1s (BATF3[155-157], Id2[157-159] and IRF8[160-162] dependent, CD103+ and CD8α+ DCs) or cDC2s (IRF4[162-165], ZEB2[166] and KLF4[167, 168] or NOTCH2[169, 170] dependent, CD11b+ and Sirp1α (CD172a)+ DCs). The pre-pDC gives rise to pDCs in an E2-2 dependent manner[171-173]. Next to the pre-pDC origin, a lymphoid origin has been suggested for the pDC population[174-181] but as most of these studies were performed *in vitro* or by transfer of precursors into irradiated mice, the true *in vivo* relevance remains a matter of debate[182, 183].

Dendritic cell subsets in the steady state mouse lung

Any discussion on the biology of DCs needs to consider the heterogeneity of these cells in various tissues and anatomical locations. In the absence of inflammation, lung conventional DCs can be subdivided in three distinct subsets based on the expression of a combination of specific cell surface markers and their ontogeny (Table 3): CD11c^{hi} CD103+ cDC1s that belong to the CD8α-type cDCs, CD11c^{hi} CD11b+ cDC2s and CD11c^{dim} pDCs[184, 185]. Conventional DCs are differentially distributed in the lung (Figure 4). In the epithelial layer of the conducting airways a network of CD103+CD11b- cDC1s can be found[186-188]. They form long cellular extensions that run in between the basolateral space made up of basal epithelial cells. The lamina propria, which is positioned below the basement membrane, contains CD11b+ cDC2s[189-191]. These DCs are CD103-, but upon inflammation some of these cells can acquire a CD103+CD11b+ phenotype as observed in the intestine[192]. Next to conventional DCs, the conducting airways also contain pDCs[186, 193-195]. In the parenchymal compartment of the lung, gas exchange is taking place at the alveolocapillary membrane. Both CD11b+ and CD11b- cDCs and pDCs are found in the alveolar septa of the lung

parenchyma[196]. So-called patrolling monocytes, mainly expressing CX3CR1 and CD11b, can patrol the vessel wall of the pulmonary arterial vasculature and can capture injected embolic material[197, 198]. Little is known about the subpleural DCs that were described in the first articles on lung DCs[199], but it is likely that these are either inside or on their way to afferent lymphatics that are found in the visceral pleura and drain antigen to the regional lymph nodes.

Table 3 - Mapping of the existing dendritic cell subsets according to the new nomenclature

SUBSET	MOUSE SUBSET	HUMAN SUBSET	PRECURSOR	TRANSCRIPTION FACTOR	MARKERS USED FOR IDENTIFICATION
cDC1	CD103+ CD8α+ XCR1+ DC	CD141+ (BDCA3) XCR1+	CDP ↓ Pre-cDC	IRF8 Id2 BATF3 (can be compensated by other BATF factors[157])	CD11c^{hi} CD11b^{low} XCR1+ Clec9a/DNGR-1+ MAR-1- CD64- SIRPa- CD24+ CD103+ CD207+(Langerin) F4-80^{low}
cDC2	CD11b+ DC	CD1c+ (BDCA1)	CDP ↓ Pre-cDC	IRF4 ZEB2 NOTCH2 Klf4	CD11c^{hi} CD11b+ SIRPa+ MAR-1- CD64- CD103$^{+/-}$ (a) CD24$^{+/-}$ (b) CX3CR1^{int} F4-80^{int}
pDC	pDC	BDCA4+ BDCA2+	CDP ↓ Pre-pDC	IRF8 E2-2 ZEB2	CD11c^{dim} CD11b^{low} Siglec H+ MAR-1- CD64- F4-80^{low} Ly6C+ BST-2/PDCA1(120G8)+
MC	Ly6C^{hi} (TipDC) MoDC	CD14+ CD16-	cMoP ↓ monocyte	IRF8 PU.1	CD11c^{hi} CD11b+ Ly6C$^{hi/-}$ (c) F4/80+ MerTk^{int} MAR-1+ CD64+

a: small fraction expresses CD103 upon inflammation in the lung, but this subset is predominant in the gut
b: can be heterogeneous for CD24 – e.g. in heart and skin
c: Ly6C is rapidly downregulated when entering the tissue
cDC = conventional DC, pDC = plasmacytoid DC, MC = monocyte-derived cell
Markers indicated in italic and bold are used to define DC subsets throughout this thesis
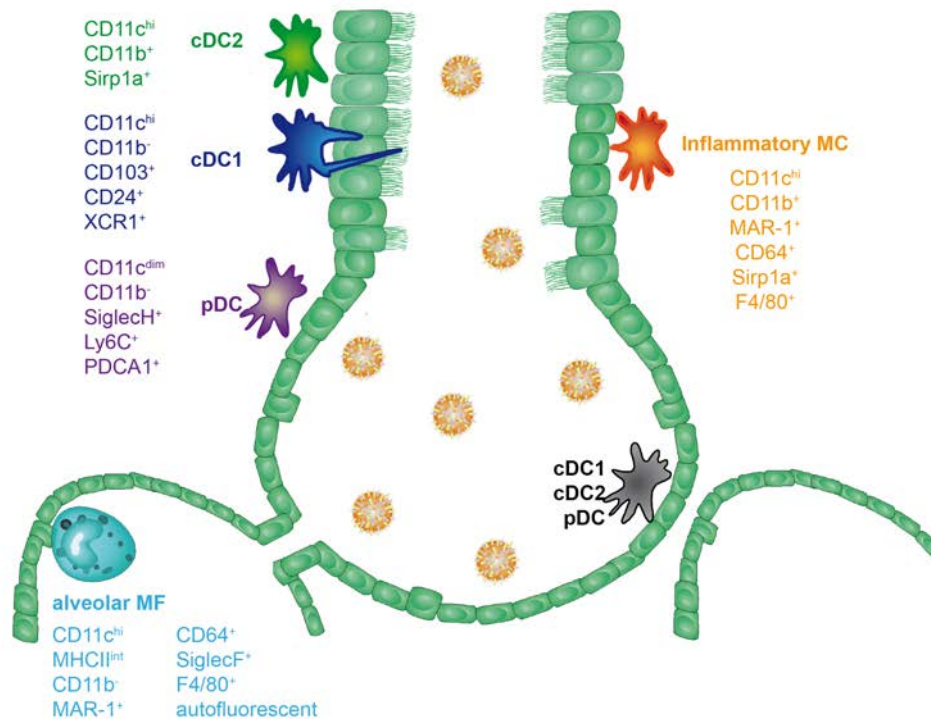
Figure 4 - Dendritic cell subsets in the lung

cDC1s (CD103+ CD11b-) extend their dendrites in between the pulmonary epithelium; cDC2s (CD103-CD11b+) and pDCs (B220+PDCA1+) are located in the lamina propria; alveolar macrophages (MF), cDC1s, cDC2s and pDCs reside in the alveolar septa. During inflammation, inflammatory monocyte-derived cells (MC) are recruited to the lung in addition to cDC1s, cDC2s and pDCs.

Mouse dendritic cell subsets in inflammatory conditions

When the lungs are challenged with foreign antigen, for example during IAV infection, additional CD11b+ monocyte-derived cells (MC) with a DC-like phenotype, historically called MoDCs, are recruited to the conducting airways and lung parenchyma (Figure 4). Although it is clear that monocytes can acquire features typically associated with cDCs (antigen-presentation for example) upon their differentiation during inflammation, it is often difficult to distinguish MCs from cDC2s. This is due to the fact that both MCs and cDC2s express CD11c, MHC II, CD172a and CD11b. As a consequence, it is now clear that many studies have been focusing on a mixture of MCs and cDC2s. Ly6C was for example proposed as a marker to distinguish CD11b+ cDC2s from CD11b+ MCs because it is highly expressed on monocytes[186, 191, 200-202]. One major drawback to the use of Ly6C as discriminative marker is the fact that Ly6C is rapidly downregulated when monocytes differentiate into DC-like or macrophage-like cells[203-205]. Next to the use of Ly6C, caution should also be taken when analyzing the monocyte-origin of cell types based solely on their CCR2-dependency. Ly6C^{hi} monocytes require CCR2 to egress from the bone marrow into the bloodstream and as a result *Ccr2*-deficient mice have much less LyC6^{hi} monocytes in circulation[206, 207]. Therefore the dependency on CCR2 has been typically used as argument to propose the monocytic origin of phagocytes[205, 207-209]. However, it has recently been shown that a fraction of pre-cDC-derived CD11b+ intestinal cDC2s express CCR2 and depend on

this receptor for their development and/or survival[210], indicating that CCR2-expression and CCR2-dependency does not necessarily imply monocytic origin. In my view, a good way to complement CCR2-dependency studies is to transfer the pre-cDC and monocyte progenitors and follow their fate *in vivo* with a cell tracer.

An alternative surface marker that has been proposed to be MC specific is the MAR-1 antibody directed against the α subunit of the high-affinity IgE receptor (FcεRI). Lung DCs recruited in response to viral infection and allergen challenge stain strongly with the MAR-1 antibody, whereas steady-state cDC2s do not[202, 211]. In addition, several groups have described the use of the high affinity IgG receptor, CD64, in discriminating MCs from cDCs. In the skin, muscle, gut and lung CD64 has been proposed to stain MCs and macrophages, but not cDCs[165, 205, 208, 209, 212]. Therefore the combination of both CD64 and MAR-1 expression was proposed as a method to discriminate between MCs and cDCs in the lung and mediastinal lymph node (MLN)[205]. Recent data from the Immgen Consortium (http://www.immgen.org) has also identified CD64 and MerTk as markers of macrophages and MCs but not cDCs.

We have previously found that CD64+ cells are already present in the steady-state lungs, where they account for about 25% of the CD11b+CD11c+MHCII+ lung cells. The fact that MCs are present amongst the CD11b+ population of lung DCs also explains why previous work reporting on the use of LysM-Cre mediated lineage tagging found a high percentage of LysM-Cre tagged cells in the steady state lung DC population whereas only some CD11b+ cDCs express LysM[213]. Recently, both cDC subsets but not MCs were shown to specifically express Zbtb46, a transcription factor that seems highly specific for cDCs and which in the future should be helpful for proper identification of cDCs[214, 215]. Zbtb46 is not required for development of cDCs, but is a negative regulator of DC activation that is downregulated upon TLR stimulation[216]. Zbtb46 reporter mice only show tagging of up to 50% of lung CD11b+ DCs, whereas virtually all CD103+ cDC1s are expressing Zbtb46, indicating the high prevalence of MCs in the CD11b+ gate[215]. The recently developed Zbtb reporter (zDC^GFP) mice and conditional knockout mice (zDC^iCre and zDC^Cre) now provide powerful tools to study cDCs[217, 218]. Moreover, once the cDC phenotype is determined, one can specifically target cDC1s by using the XCR1-DTR[219] or *Karma* mice[220,221]. These observations point out that results obtained by studying CD11b+ DCs should be interpreted with caution as this population is heterogeneously composed of cDCs and MCs when no additional markers are used to separate both cell types. An overview of the markers, transcription factors and developmental signals is shown in Table 3.

What is the true nature of the monocyte-derived cell?

Macrophages (MFs) are generally long-lived and derived from embryonic precursors[222-225]. They are not one uniform cell type throughout the body, but acquire tissue-specific gene expression profiles and specific characteristics based on the imprinting by their micro-environment[226-228]. However, it was recently shown that BM-derived monocytes could also differentiate into self-maintaining MFs that are transcriptionally and functionally equivalent to their embryonic counterparts when they get access to the macrophage niche[229, 230].

As discussed above, monocytes can also acquire DC characteristics and in this case they are often referred to as MoDCs. The standard protocol to culture DCs from bone marrow[231] and peripheral blood monocytes (PBMCs)[232] makes use of GM-CSF. These protocols enabled us to isolate large amounts of DCs for *in vitro* studies. Recent *in vivo* transfer studies showed however that monocytes could develop into DCs when GM-CSF is present but that monocytes are not able to reconstitute the cDC compartment[154, 233-235]. This raised questions about the true nature of these GM-CSF DCs. The total DC population usually consists of an MHCII^{int} and an MHCII^{hi} population, historically considered immature and mature MoDCs respectively. However, recently it was shown that GM-CSF DCs also contain contaminating cDC2s[236]. As a result, MHCII^{hi} cells consist of a mixture of cells derived from CDPs and cMoPs, in contrast the MHCII^{int} cells are mainly derived from cMoPs. Although, not recognized as such at the time, single-cell RNA-Seq also identified two subsets of cells in these cultures with only one of them being very DC-like[237]. Based on micro-array analysis, the CDP-derived DCs in the culture are most closely related to cDC2s. In accordance, their development is controlled by IRF4. In contrast, the cMoP/monocyte-derived cells in the culture were proposed to be macrophage-like cells but did not cluster with any known *in vivo* macrophage population, underlining the importance of tissue imprinting for macrophages. This learns that conclusions made based on *in vitro* cultured GM-CSF DCs should be reconsidered in the light of the fact that these cultures are also monocyte-derived cells but contain contaminating cDC2s.

MCs can simultaneously express macrophage (CD64, MerTK, F4/80) and DC (CD11c, MHCII) markers[205, 208, 209]. They can also display typical DC (present antigen to naïve T cells) and macrophage (excel at phagocytosis) features simultaneously[209, 238]. The difficulty of categorizing MCs can be illustrated by intestinal CX3CR1^{hi} MCs, which have been well studied. These cells have for example been categorized by some labs as DCs due to the fact that they possess transepithelial dendrites and express very high levels of CD11c and MHCII[239, 240], but have also been categorized as MFs by others due to the fact that they excel in phagocytosis and fail to migrate to the intestinal draining lymph nodes[241]. As opposed to these observations, MCs have also been described to be located within the T cell zone of lymph nodes and can efficiently present antigen to naïve T cells in some cases[209].

The CD64$^+$MHCII$^+$CD11c$^+$ MCs in the lung have not been well characterized yet. These cells are of monocytic origin[205] but whether these cells possess typical DC and/or macrophage features is currently unknown. I have therefore decided to label these cells as monocyte-derived cells or MCs in this thesis to avoid that preconceived functions would be attributed to these cells solely based on the fact that they would be categorized as "MoDC" or "macrophage".

During this thesis the recently described gating strategy to separate cDC1s, cDC2s and MCs based on CD64 and MAR-1 expression is used[205]. After gating out the doublets, debris, dead cells, T cells and B cells (CD3$^-$CD19$^-$), CD11c$^+$MHCII$^+$ cells are separated into CD11b$^-$CD103/CD24$^+$MAR-1$^-$CD64$^-$ cDC1s, CD11b$^+$CD103/CD24$^-$MAR-1$^-$CD64$^-$ cDC2s and CD11b$^+$CD103/CD24$^-$MAR-1$^+$CD64$^+$ MCs (Figure 5).
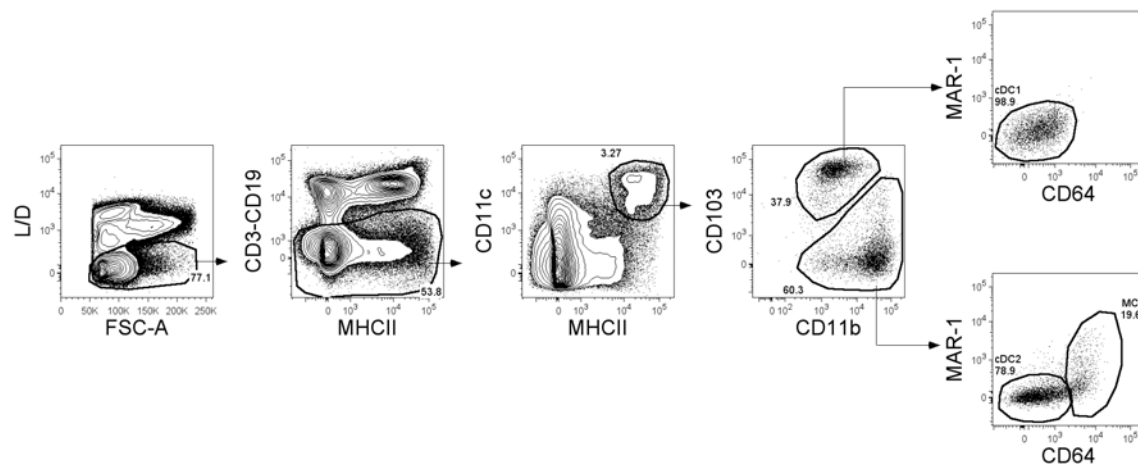
Figure 5 - Flow cytometry gating strategy to define dendritic cell subsets

Lung cells are pregated for single cells and non-debris. The image is a representative image of the lung 4dpi mock infection. L/D = Live/Dead marker

Dendritic cell activation in response to respiratory viruses

Direct activation by viral infection

DCs reside in an immature state in the periphery of the lung, where they are strategically located to detect inhaled particulate and soluble antigen. They express specific pattern recognition receptors (PRRs) for conserved pathogen-associated molecular patterns (PAMPs) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) as discussed above. Immgen array gene expression data have for example shown that mouse lung CD103+ cDC1s mainly express TLR3, whereas CD11b+CD103- cDC2s mainly express TLR2 and TLR7[187].

It has long been enigmatic whether DCs presenting viral antigen to CD8+ T cells were directly infected or acquired viral antigen indirectly via other cells succumbing from viral infection in the respiratory tract, a process called cross-presentation. Alternatively, some DCs might also acquire preformed MHCI-peptide complexes from other cells, a process called cross-dressing[242, 243]. Besides attachment of HA to SA on the cell surface, viral particles can enter into the cell in a SA-independent manner by binding the C-type lectin receptors DC-SIGN and L-SIGN with the mannose-rich glycans on the surface of the virion[244]. DC-SIGN is highly expressed on some DC subsets thus rendering these DCs susceptible for infection. L-SIGN is only expressed in humans. DCs that migrated to the lymph node after IAV can be virus-infected, but this happens only after infection with higher inocula[245, 246] and only the CD103+ cDC1 subset was shown to carry infectious virus to the draining lymph nodes (dLN)[247]. Others, using an elegant strategy with an IAV virus carrying GFP in a non-structural NS1 protein, found that only CD103+ cDC1s carry viral antigens in their endosomal compartments during transport to the mediastinal lymph nodes (MLN), but demonstrated that the virus did not productively infect CD103+ cDC1s[97]. Moreover, this study identified type I IFN as the major mediator of protection of CD103+ cDC1s against infection. This observation was confirmed in a more recent study that showed that IFITM3 is induced in DCs in an IRF7 and IRF3 dependent manner. This inhibits direct infection of DCs, permits migration from lung to

LN and optimal priming of virus-specific T cell[248]. In contrast, it was shown that that type I IFN responses are attenuated in CD103+ cDC1s[247], rendering them more sensitive to infection. Additional studies will be required to settle this issue, but most likely timing, viral strain, infection route and dose play an important role in the outcome.

Indirect activation of dendritic cells in trans

Although direct recognition of viral PAMPS by PRRs is the most likely explanation of how DCs respond to virus, it is now clear that recognition of PAMPs by the nearby bronchial or alveolar epithelial cells is at least as important in activating the lung DC network[191]. Lung epithelial cells can produce the essential chemokines that attract immature cDCs and inflammatory monocytes to the site of antigen exposure: IL-12p80 homodimers are produced by bronchial epithelial cells upon viral and mycobacterial infection of the lung, and IL12p80 recruits DCs to the lung epithelium[249]. Epithelial cells not only make chemokines that attract immature monocytes or pre-cDCs, but they also produce critical maturation cytokines such as IL-1, GM-CSF (Csf2) and TSLP that can activate the recruited monocytes and pre-cDCs to differentiate in DCs and induce their maturation into fully competent antigen-presenting cells. In contrast to the generally accepted dogma that GM-CSF controls the development of inflammatory DCs, it appeared that GM-CSF provides survival signals to CD103+ cDC1s and induces CD8+ T cell immunity[250]. The observation that GM-CSF might be dispensable for CD11b+ cDC2s survival is still under discussion because of the presence of a contaminating MC population that might obscure the results. Additionally, *Csf2-/-* or *Csf2r-/-* mice also lack AMFs and develop alveolar proteinosis that severely alters lung homeostasis and does not allow us to draw firm conclusions about the importance of GM-CSF activation of CD103+ cDC1s in these mice. In fact, reconstitution of *Csf2r-/-* mice with wild-type AMFs was sufficient to restore resistance of these mice to the infection, strongly suggesting that the main effects observed in *Csf2r-/-* mice are due to the lack of AMFs. This does of course not exclude that Csf2r-signaling in cDC1s may be important, and this can be checked by crossing the recently described *Csf2r-floxed* mice[251] onto cDC1-specific Cre mice (for example XCR1-Cre mice[220]).

TLR4 triggering of bronchial epithelial cells induces IL-1α, which acts in an autocrine manner to trigger the release of the DC-attracting chemokines, GM-CSF and IL-33 by epithelial cells[252]. A recent study showed that IL-1 is not only signaling via the epithelium, but that IL-1 can also directly stimulate DCs and that signaling through the IL-1R was required for induction of virus-specific CD8+ T cell responses after influenza virus infection[253]. TSLP has been shown to activate CD11b+ DCs to support local reactivation of CD8+ T cells[254].

Trans-activation of DCs can also be modulated by soluble mediators of the innate immune system, like SPD and complement factors, and other innate immune cells. For example C3 stimulates migration of dendritic cells to the dLN of the lung[255]. Complement C3 and C5 itself is mainly produced by CD103+ cDCs upon influenza infection[256]. Furthermore SPD has been shown to increase the antigen presenting capacity of GM-CSF cultured bone marrow derived DCs *in vitro*[257, 258].

Innate type I IFNs induce an antiviral state, but this is not their sole function. Viruses that strongly activate type I IFN synthesis are also best at inducing adaptive immunity via induction of lung DC maturation[259]. In a model of co-culture of human DCs with airway epithelial cells, it was found that type I IFN from the epithelial cells (together with IL-6) was able to modulate DC maturation[260]. NK cells induce DC migration to the LN and virus uptake by DCs by perforin-mediated lysis of infected cells[122]. Neutrophils that are recruited early in the response to virus infection have also been shown to affect the activation of DCs[261].

Lung dendritic cells and induction of adaptive immunity to respiratory virus infections

Migration to the draining lymph node

Viral infection of DCs induces maturation-related changes in cell surface chemokine receptor expression. Chemokine CCR7 receptor upregulation is crucial for migration of lung DCs to the dLN[262-265]. Activation and migration of cDCs and bone marrow-derived GM-CSF DCs to the dLN can also be enhanced by type I IFN signaling[266-270] and this can lead to enhanced cross-presentation capacity of cDC1s[271-273].

Upon IAV infection, DCs rapidly migrate to the dLN and return to their basal migration level after 48h, despite still ongoing inflammation[274]. This might be explained by the virus that renders the DCs insensitive to migration or by the immune system that has a built-in protective mechanism against too much damage. DC migration is not dose dependent, but defects in respiratory DC migration occur upon ageing. This defect is related to an increased level of PGD2 in the lung. PGD2 inhibits CCR7 upregulation, which renders DCs unresponsive to migratory signals via the CCR7 receptor[275, 276]. The lack of induction of CCR7 by specific viruses like human metapneumovirus (HMPV) and human respiratory syncytial virus (HRSV) can prevent DCs from migrating to the lymph nodes and thereby hamper the induction of protective responses[277, 278].

Role of conventional dendritic cells in antiviral immunity

The role of lung DC subsets in promoting antiviral CD4 and CD8 immunity has been studied best in the context of IAV infection. During IAV infection, CD11b+ cDC2s and CD103+ cDC1s both take up antigen in endocytic vesicles and express high levels of costimulatory molecules and antigen presenting molecules in contrast to MCs and pDCs[279]. Initial studies have demonstrated that antiviral CD8+ T cell responses were induced mainly by a non-migratory resident CD8α+ cDC1 population of the MLN. Antigen was proposed to be carried to this resident population by a migratory lung derived CD11b- (i.e. CD103+) cDC1 population[280]. Our group has demonstrated that CD11b-CD103+ airway derived cDC1s can however also directly present viral derived antigens to CD8 (and CD4) T cells, in addition to the resident CD8α+ cDC1s[186, 281]. Studies using *Batf3*-deficient mice (that lack CD103+ cDC1s and CD8α+ cDC1s) have shown that CD103+ cDC1s were crucial for inducing CD8+ T cell immunity to IAV[97].

The reason why CD103+ cDC1s are so efficient at inducing a protective response to influenza virus could be because they are the only DC subset able to capture and cross-present apoptotic epithelial cells that died from infection[97]. CD103+ cDC1s express

receptors for apoptotic cells along with the machinery to cross-present phagocytosed dead cells[187] and they have enhanced capacity to process and load viral antigens in MHC I molecules[282]. Cross-priming of antigen delivered by dying epithelial cells has been proposed to occur via the dendritic cell receptor DNGR-1 (CLEC9A)[283-285]. Both in human and mouse, the biggest proportion of the DC subset preferentially expressing DNGR-1 belongs to the CD8α+-like/CD103+ cDC1s family[286].

Naïve CD8+ T cells encounter antigen in the LN, get activated and then proliferate in an ordered way. The tempo is regulated by antigen availability, which is determined by antigen delivery by MHCII^{hi}, so called migratory, CD103+ cDC1s[97, 186]. Because CD103+ cDC1 migration is increased for only a short time after infection and T cell proliferation goes on for a longer period, antigen might also be presented by LN resident DCs, which received antigen from migrating DCs via cross-presentation[280]. There is heterogeneity in the proliferation status of T cells; this is caused by sequential migration of circulating naïve T cells into the lymph node, followed by their activation there and exit back to the tissue[287]. MHCII^{lo} DCs display lower levels of costimulatory molecules and therefore are less likely to be the ones inducing proliferation of T cells. In contrast, they have the capacity to induce apoptosis of proliferating CD8+ T cells in an IL-12 induced, FasL dependent manner[246].

At later time points coinciding with the peak of viral infection, CD11b+ DCs accumulate in the LN and represent the predominant DC subset stimulating CD8+ T cells via expression of the costimulatory molecule CD70[288]. This later wave of antigen presentation could potentially be involved in the expansion of activated effector CD8+ T cells in the LN. What is not clear from these studies is whether the CD11b+ DCs also contained MCs, as no discriminating markers were used.

Although most studies on lung DCs in viral infection have been performed in the context of IAV, the importance of DC subsets might differ according to the type of viral infection as well. Despite their good presentation capacity, it is not the CD103+ cDC1 population that predominates the lung upon RSV and IAV infection, but the CD11b+ DC population. During RSV infection, both cDC1s and cDC2s contain RSV RNA and present equally well to CD4+ and CD8+ T cells[289]. In a model of pulmonary vaccinia virus infection, CD103+ cDC1s that migrated from the lung to the LN are the best inducers of CD8+ T cells proliferation compared to CD11b+ migrating DCs and CD103+ MHCII^{lo} cDC1s.

Role of monocyte-derived cells in antiviral immunity
Upon IAV infection, monocytes are recruited to the lung and differentiate rapidly into cells that share features of both DCs and macrophages[99, 290, 291]. This process has been shown to be dependent on type I IFN signaling[292] and CCR2[91]. Human monocyte infection with IAV was shown to be sufficient to differentiate monocytes into type I IFN producing macrophage-like MCs capable of limiting viral replication *in vitro*[99]. These MCs do not upregulate MHC II and costimulatory molecules, nor produce proinflammatory mediators. They are not able to induce T cell proliferation but secrete large amounts of MCP-1 and IP-10, classical monocyte chemoattractants. As a conclusion, they fail to function as conventional antigen presenting cells. The strong type I IFN induction and upregulation of cytosolic RNA sensors RIG-I and MDA5 point

towards a direct antiviral activity. Virus-induced MCs are not the primary DC subset that presents antigen to naïve T lymphocytes. They do provide positive feedback to attract more monocytes to the site of infection via MCP-1 and IP-10 secretion[99, 293]. The lack of antigen-presenting capacity is further supported by the observation that MCs can stimulate naïve CD4+ T cells modestly and CD8+ T cells only minimally after influenza virus infection, although they have the capacity to do so when stimulated with pre-processed peptide[279, 294].

It is not clear if MCs are capable of migrating to the dLN and actively participate to the induction of influenza effector CD8+ T cell responses. On one hand, it was suggested that CD11b+ DCs that massively migrated to the LN upon influenza infection were MCs[288] whereas on the other hand it was argued that the CD11b+ DCs were cDC2s due to their low Ly6C expression[279]. These contradictory views again argue for better discrimination between the two CD11b+ subsets and this is one of the major aims of this thesis.

Although MCs were able to limit viral replication, Ccr2-/- mice or mice treated with a CCR2 antagonist did not show increased viral loads[91] but they did display significantly less effector CD8+ T cells in the lungs during influenza infection[295]. Therefore it is very likely that MCs would be crucial in the interaction with effector T cells locally in the infected tissues, rather than in the induction of effector T cells in the LN[254]. CD8+ memory T cells can in turn license MCs for effective pathogen killing through the secretion of CCL3[296,-297].

Role of plasmacytoid dendritic cells in antiviral immunity
Whereas pDCs are a copious source of type I IFN[298-300], their role during respiratory viral infections remains debatable as they were shown to react differently upon infection with various types of virus and only produce type I IFN upon systemic infection, and not upon respiratory virus infection[96, 301].

These conflicting views regarding the contributions of pDCs to antiviral immunity make it hard to firmly state how pDCs are involved in antiviral immunity in the lung. The importance of pDCs also appears to differ among different viral model systems.

pDCs are increased in the lung and the LN for a long period after RSV infection. When depleted with i.p. injections of 120G8 antibody, it was shown that pDCs play a direct antiviral role by secreting IFNα but also modulate T cell-mediated responses leading to lung pathology characterized by airway hyperresponsiveness, mucus production and inflammation[302, 303] via the TLR7-MyD88 dependent signaling pathway[300, 304]. In contrast, *in vitro* studies have shown that pDCs are unable to express maturation markers whereas they were able to produce IFNα [305]. This suggests that the role of pDCs might be more oriented towards an antiviral response than towards induction of adaptive T cell responses. There is also evidence that pDCs do not productively contribute to IFN production after RSV[306]. In addition MCs and cDCs were also shown to produce IFNα and β and thus exhibit antiviral activity to prevent infection of neighboring cells[307, 308].

pDCs accumulate in the lung and bronchoalveolar space in response to influenza virus infection, but the observation that pDCs can induce a strong influenza specific CD8 response *in vitro*[309-312] does not translate to *in vivo* or *ex vivo* models. *Ikaros*L/L mice that lack pDCs[313] and mice in which DCs were depleted using 120G8 antibody[186]

demonstrate a similar course of disease and did not have differences in the strength of the antiviral type I IFN and CD8^{+} T cell response. pDCs fail to activate either naïve CD4^{+} or CD8^{+} T cells when they are not pulsed with pre-processed peptide or exposed to antigen-specific immunoglobulins[279, 312, 314]. pDCs might even be involved in FasL mediated killing of CD8^{+} T cells during lethal influenza infection[315]. One function that could be attributed to pDCs is formation of HA-specific antibodies[186, 316]. Furthermore it has been suggested that pDCs can provide help for cDCs during antigen presentation via IFN production, but this seems not to be the case. IFN rather appears to serve as a negative feedback loop to control pDC numbers[317].

Using the newly developed BDCA2-DTR mice, pDCs can be depleted conditionally. By infecting these mice with MCMV and VSV it was shown that pDCs were involved in virus-specific NK and CD8^{+} T cell induction. However, it remains to be elucidated if pDCs will react in the same manner during infection with respiratory viruses[318]

These contradictory results regarding the function of pDCs during respiratory infection, might be explained by the observation that in fact 3 distinct pDC populations can be determined based on CD8α and CD8β expression: CD8α[-]β[-], CD8α[+]β[-] and CD8α[+]β^{+} cells[319]. These subsets display different cytokine secretion profiles and functional properties in a model of airway inflammation. CD8α[+]β[-] and CD8α[+]β[+] pDCs can induce tolerance in vivo most likely via induction of regulatory T cells. In contrast, CD8α[-]β[-] pDCs have pro-inflammatory properties. It remains to be determined if respiratory viruses can differentially modulate these three pDC subsets and hence influence the outcome of the infection. However recently it was hypothesized that the different pDC populations are not stable subsets, but that CD8 expression is inducible by TLR stimulation and these subsets rather represent a different activation state[320].

Adaptive immune responses against influenza A virus infection

Long term adaptive T cell memory to respiratory viruses

The recovery from influenza virus infection requires cooperation between CD8^{+} cytotoxic T lymphocytes (CTL) and CD4^{+} T-helper (Th) cells. Naïve T cells migrate to the lymph node by expression of the lymph node homing receptors CD62L and CCR7. Once in the dLN they are stimulated upon triggering of the T cell receptor (TCR) by antigen presented by DCs in a MHC-I or MHC-II dependent manner for CD8^{+} or CD4^{+} T cells respectively[321]. Antigenic stimulation of T cells alone (signal 1) is insufficient to induce effector cells. T cells will become anergic unless a costimulatory signal via CD28 and CD80/CD86 or CD40L and CD40 (signal 2) is given[322] and when inflammatory cytokines stimulate the CD8^{+} T cells or CD4^{+} T cells (signal 3)[323](Figure 6). After their proliferation and differentiation into effector cells, T cells migrate back to the airways by downregulation of CD62L and CCR7 and upregulation of the adhesion molecule CD44 where they can exert their specific function in order to clear the virus from the respiratory tract. To produce high levels of cytokines locally, T cells need to reencounter their cognate antigen in the lung. The proposed antigen-presenting cell for local reactivation of T cells is a CD11b^{+} DC, thus representing cDC2s and/or MCs[324].
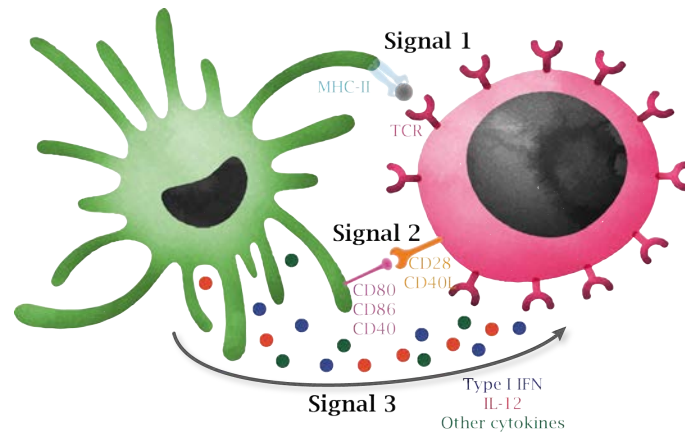
Figure 6 - The 3 steps of T cell activation

Besides antigenic stimulation via the T cell receptor (signal 1), T cells need a costimulatory signal via CD28-CD80/86 or CD40L/CD40 interactions with dendritic cells (signal 2) and a stimulation by inflammatory cytokines (signal 3) for their effective activation.

CD8+ T cells acquire cytolytic activity and upregulate chemokine receptors that allow them to migrate to the site of inflammation. The function of antiviral CD8+ T effector cells depends on the cell type presenting the viral antigen. Firstly, MHCI expression and costimulation via CD80 and CD86 on professional antigen presenting cells are necessary for pro-inflammatory cytokine production (IFNγ, TNFα, MIP-1α etc.), but this cytokine production also contributes to lung pathology and may not be essential for virus clearance since viral clearance is not affected when costimulatory signals are blocked. Secondly, induction of cytotoxicity is induced by MHCI presentation on epithelial cells without the need for costimulatory signals[325, 326]. When cytotoxic T cells detect virally infected epithelial cells, they will kill them indirectly by release of the cytolytic molecules perforin and granzyme or directly via Fas/FasL interactions[327].

CD4+ T cells can differentiate into different types of Th cells from which the functional capacities depend on the environmental context. The most common Th phenotypes are Th1 (driven by IL-12 and IFNγ), Th2 (driven by IL-4 and IL-6), Th17 (driven by IL-6 and TGFβ), T follicular helper (Tfh) (driven by IL-6, IL-1β and TNFα) and regulatory T cell (Treg) (driven by TGFβ) cells[328]. IAV infection is mostly associated with a Th1 response producing IFNγ, TNFα and IL-2; complemented with a Tfh response initiating a robust antibody response providing B cell help and orchestrating the germinal center reaction[329, 330]. Furthermore Th17 might influence the recruitment of neutrophils to the site of infection[331, 332]. Additionally, Treg cells are induced after IAV infection. Treg cells generally dampen antigen-specific responses thereby limiting the corresponding tissue damage[333, 334]. During acute viral infections Treg cells delay the clearance of the virus, but also inhibit the proliferation of infected cells, promote memory formation and influx and efflux of the dLN[335]. Besides the prevention of tissue damage, Treg cells can assist directly in recovery of the tissue damage in an amphiregulin-dependent manner after the virus is cleared[336, 337].

Whereas CD8+ T cells were seen as the typical T cell fighting influenza infection, the role of CD4+ T cells was long underestimated as a helper cell. More and more evidence is

accumulating that point towards a broader function of CD4^{+} T cells[338]. During IAV infection CD4^{+} T cells promote CD8^{+} T cell and B cell responses to influenza virus infection although they are not critical for this process[339-341]. Adoptive transfer studies demonstrated that CD4^{+} T cells are also able to control viral load and exert direct cytotoxic effector functions in the lung environment[342, 343]. Phenotypically, CD4^{+} CTLs resemble Th1 effector cells that additionally express granzyme B and perforin and have direct cytotoxic activity *ex vivo*. CD4^{+} CTLs show perforin-dependent protection against lethal IAV infection in both mouse models and human studies of infection and vaccination[342, 344-347] but the contribution of CD4^{+} T cell cytotoxicity to viral clearance *in vivo* in the lungs is modest[346]. Depletion of either CD4^{+} or CD8^{+} T cells alone still allows recovery from the viral infection, but slower than with fully functional CD4^{+} and CD8^{+} T cells together[343, 348-352] suggesting that cooperation between CD4^{+} and CD8^{+} T cells is necessary for an optimal immune response or that the division between CD4^{+} and CD8^{+} T cells is not absolute and that each cell can acquire some characteristics of the other when necessary.

Non-conventional T cells that express a functional TCR but lack expression of CD4 and CD8 co-receptors (therefore called double negative (DN) T cells), are observed in various disease models –auto-immunity, infection, cancer, post-transplantation tolerance– in human and mice, in which they were attributed different functions ranging from inflammatory cell, to immune suppressive cells and homeostatic cell[353]. The lungs are one of the many tissues where DN T cells were described in steady state[354-357] and following insults to the lung. In an infection model using *Francisella tularensis* DN T cells produced high amounts of IL-17 and IFNγ[358] while they produce predominantly IL-5 in a model of *Toxocara canis*[359]. Furthermore they are also involved in the defense against *Mycobacterium bovis* infection[375, 357, 378, 360]. As DN T cells are defined by exclusion, they are very heterogeneous, arising either from the thymus or extrathymically. Classical DN T cells express intermediate levels of αβTCR, and are different from type I CD1d-restricted invariant natural killer T cells and γδTCR^{+} T cells that are often found to lack CD4 and CD8 expression, and therefore fall under the DN T definition[361, 362]

As acute infections are cleared, effector T cells further differentiate into KLRG-1^{hi}CD127^{lo} short-lived effector cells and CD127^{hi} memory precursor effector cells capable of generating long-lived memory cells[363, 364]. Memory T cells are a heterogeneous population (Table 4). All memory T cells retain expression of CD44 but are heterogeneous in expression of CD62L and CCR7. CCR7^{+}CD62L^{+} cells are lymphoid homing and recirculate via lymphoid organs and are called central memory T cells (T_cm). They do not have immediate effector functions, but in response to a secondary infection they can expand and differentiate into effector cells that subsequently migrate back to the lungs. CD62L^{-}CCR7^{-} memory cells are the effector memory (T_em) cells that patrol in and out of peripheral tissues[365-368] but they can also reside for prolonged periods in the lungs as T resident memory cells (T_rm) that express high levels of CD69, CD11a and/or CD103[364]. Triggered by retained antigen presented by DCs[369], T_rm cells were shown to reside for months in the lungs of IAV infected mice and infected

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volunteers, thus providing immunity from reinfection with the same or heterologous strain of influenza by rapidly acquiring effector functions[364, 370-380].

As for effector CD4 and CD8 T cells, also Treg cells can differentiate into a memory population[381]. Antigen-specific memory Treg cells control the CD8+ recall response upon secondary infection[382].

Table 4 - Different types of memory T cells compared to naïve and effector T cells

CELL TYPE	CHARACTERISTIC						
	CD44	CD62L	CCR7	CD69	CD103	Location	Migratory capacity
Naïve	-	+	+	-	-	Lymphoid tissue	Yes
Effector	+	-	-	+	-	Peripheral tissue	Yes
T_{cm}	int	+	+	-	-	Lymphoid tissue	Yes
T_{em}	+	-	-	-	-	Peripheral tissue	Yes
T_{rm}	+	-	-	+	CD4: - CD8: +	Peripheral tissue	Resident

Cross-reactive cytotoxic T lymphocytes can induce heterosubtypic immunity

Virus-specific T cells increase in frequency and number during infection. They mainly recognize the internal proteins NP, matrix protein and polymerase, which are genetically more conserved than HA and NA[383]. Small mutations in epitopes recognized by T cells due to genetic drift can lead to immune-escape; but generally these mutations occur less in conserved epitopes since this can have a potential functional consequence for the virus[384]. This makes the virus specific T cells against internal proteins ideal candidates to provide heterosubtypic immunity. Although the main role for heterosubtypic immunity is attributed to CD8+ T cells[385, 386], there is evidence that also CD4+ T cells[374, 387] and B cells[388, 389] can contribute to heterosubtypic immunity.

B cells can provide long-term protection by generation of antibodies

The generation of antigen specific B cells is orchestrated in the lymphoid organs or locally in the respiratory tract in the mucosal-associated or bronchus-associated lymphoid tissue in two phases[390]. Dendritic cells carry virus-derived antigens to the dLN and present the antigen not only to T cells but also to B cells. The recognition of antigen by the B cell receptor activates a differentiation process during which the B cells migrate to the edge of the LN and proliferate[391-393]. After recognition through the B cell receptor, the antigen is internalized and processed to be presented by the B cell. CD4+ Tfh cells that recognize antigen presented via MHC II on the B cells provide a CD40-CD40L costimulatory signal that is necessary to induce affinity maturation, isotype switching and memory B-cell development[394, 395]. The B cells enter the germinal center (GC) reaction where they undergo somatic hypermutation, affinity maturation and class-switch recombination (from IgM to IgA or IgG) leading to high-affinity antibody secreting plasma cells and memory B cells[396]. Respiratory B cell activation and the antibody class switching are mediated by innate type I IFN signaling via the IFNAR resulting in a larger amount and better quality of antibody response[397-399]. As IAV infection primarily involves the respiratory mucosa, IgA+ B cells are a major component

of the response to infection. The secreted dimeric IgA that is present at the respiratory mucosa can provide immediate immunity as it can cross link viral particles before it can enter the cells of the host, a process known as immune exclusion[400, 401].

The high-affinity antibody secreting plasma cells produce the early antibody wave after infection, but this population contracts rapidly after clearance of the virus. Memory B cells are in contrast long lived and migrate to the bone marrow and respiratory mucosa where they are maintained several months after infection. Upon reencounter with IAV virus they differentiate into antibody-producing plasma cells rapidly thus providing protection against reinfection[402]. Studies of IAV-infected mice have shown broad dispersion to secondary lymphoid organs and lung localization of virus-specific memory B cells[403]. Strikingly, a recent study showed that B cells are directly infected by IAV virus through BCR-mediated internalization[404]. They subsequently produce viral particles and succumb to infection, particularly in the lung epithelium. This mechanism provides respite for the virus in the lung epithelium. Infection of the rare virus-specific memory B cells impairs the kinetics of the memory response and confers an advantage to the virus, ensuring a window for replication even in the immune host that could promote horizontal transmission.

Virus-specific antibodies can neutralize the virus directly by binding the matching antigen of the virus and thereby inhibiting the function of the bound antigen. Anti-HA antibodies neutralize IAV virus by preventing interactions with sialylated glycoproteins on the surface of the host cell or preventing HA-mediated fusion of virus and host membranes in the endosome, thereby interfering with viral entry. Furthermore antibodies opsonize viral particles by forming antigen-antibody complexes. These complexes can facilitate killing of virus-infected cells (antibody-dependent cell-mediated cytotoxicity) and phagocytosis of virus-infected cells by binding to Fc receptor expressing cells (e.g. macrophages and NK cells) [405, 406]. Antigen-antibody complexes can also activate the complement cascade and kill the cells by complement-dependent cytotoxicity[407].

The HA protein consists of a head region and a stem region. Antibodies that recognize the more conserved stem region of the HA protein often can recognize multiple viral subtypes and provide cross-reactive immunity. However, stem-specific neutralizing antibodies are rare and thus do not provide the main source of cross-reactive immunity. Anti-NA antibodies[408] do not neutralize the viral particles, but limit viral spread by inhibiting the sialidase function of NA and thereby preventing penetration through the mucus layer lining the respiratory epithelium[409] and release of newly produced viral particles from the infected cell[410]. They also facilitate antibody-dependent cell-mediated cytotoxicity, contributing to clearance of infected cells by phagocytes and NK cells[411] and complement-dependent cytotoxicity[407]. They may as well interfere in the attachment of HA to the host cell by steric hindrance[412].

Antibodies against the highly conserved ectodomain of the M2 protein (M2e) have a high potential for long-term protection against multiple subtypes of influenza virus, but M2e has a low immunogenicity and therefore antibodies against M2 are only raised to a limited extent after natural infection[413-417].

The last class of antibodies that provides protection against IAV infection is directed against the conserved NP protein. In contrast to the previous antibodies, this is an intracellular protein and these antibodies can therefore not neutralize viral particles, but they can provide protection in a FcR-dependent manner[418, 419].

B cells are not strictly necessary to clear influenza infections since B cell deficient mice manage to recover from IAV infection[420], mainly through a CD8+ T cell-dependent mechanism. Combining the lack of B cells and CD8+ T cells however leads to lethal infections[421] suggesting that CD4+ T cells alone are not sufficient to clear infections. Since CD4+ T cells are described to have important helper functions during the immune response against IAV infection, it is clear that the optimal immune response requires interaction between CD4+ and CD8+ T cells and B cells.

Innate natural antibodies and antiviral immunity

Although B cells are traditionally seen as parts of the adaptive immune system, some B cells produce natural antibodies that have a broad virus neutralizing capacity. Normally the process of antibody generation by conventional B cells only starts upon encounter with antigens. Nonetheless, antibodies can be found in the serum prior to contact with pathogens and in germ free mice. This antibody pool consists mainly of IgM antibodies and is generated independently of antigen challenge by B-1 cells[422-424]. B-1 cells are fundamentally different from the conventional B-2 cells, which might be the reason for their antigen-independent regulation, however the mechanisms of natural antibody generation are still poorly known[425-427]. B-1 cells are not very abundantly present in lymph nodes, but are the major B cell population in the peritoneal and pleural cavities[425]. Nonetheless, the spleen and bone marrow were shown to be the major source of secreted natural IgM antibodies[428]. Many of the circulating IgM antibodies are directed against self-antigens, which gives them the potential to induce auto-immunity[429-431] but due to their polyreactivity they can also bind PAMPs of microbial[432-435] and viral[436] particles.

Natural antibodies bind antigens with low affinity because they did not undergo affinity maturation during germinal center reactions[437], but due to their pentameric structure they are capable of binding antigens with a high avidity. This specific characteristic provides an important defense mechanism against pathogen replication prior to establishment of specific immune responses[438]. As an example, the airways contain natural IgM antibodies that provide a powerful tool to fight influenza infection since survival is abrogated in the absence of these natural IgM[439]. Natural antibodies can neutralize influenza virus[440] via two possible mechanisms: either transport to the mucosal surface via the poly-Ig receptor[441] or via complement-dependent cytotoxicity[442]. Furthermore, natural IgM can activate complement prior to the development of an effective antibody response[442].

Inducible bronchus associated lymphoid tissue is formed in the lungs after respiratory viral infection

Definition and structure of tertiary lymphoid organs

The immune system has evolved in a way that optimizes the chance of encounter between the rare antigen-specific T and B cells of the adaptive immune system with antigen presenting cells of the innate immune system in organized lymphoid tissues, such as the spleen, lymph nodes and Peyer's patches. These so-called secondary lymphoid organs (SLOs) have three unique features. First, they filter lymph or blood and sample the antigens in these fluids. Second, they allow entry of antigen-loaded DCs and other innate immune cells. Third, they allow recirculating naive T and B cells that have encountered antigen to extravasate, arrest temporarily, proliferate and differentiate[443]. To achieve this goal, the LNs and spleen are connected during embryogenesis to the lymph and bloodstream and are organized into well-defined areas rich in T cells or B cells. These areas are supported by a network of mesenchymal cells that provide the chemokine cues and extracellular matrix on which cells can meaningfully migrate and find each other in such a complex structure[444, 445]. This highly efficient structural organization of antigen encounter and lymphocyte migration and activation can also be recapitulated after birth, in the form of tertiary lymphoid organs (TLOs), particularly when there is continued need for extravasation of leukocytes and a persistent source of antigen, such as seen in zones of infection, transplant rejection, auto-immune attack and cancer (summarized in table 5).

It is well known that when inflammation becomes chronic, plasma cells and lymphocytes gradually increase in number, lymphangiogenesis is induced, and blood vessels acquire characteristics of high endothelial venules (HEV) specialized in allowing extravasation of lymphocytes. In some conditions, the chronic infiltrate organizes into structured lesions, such as those seen in chronic granulomatous inflammation in tuberculosis, Crohn's disease, and sarcoidosis. Sometimes, the chronic infiltrate organizes into distinct T cell-rich and B cell-rich aggregates. Various terms, such as ectopic lymphoid tissue, lymphoid tissue neogenesis and TLOs, have been used to describe the occurrence of these organized structures. These structures can also be named according to their anatomical site, such as inducible bronchus associated lymphoid tissue (iBALT) and vascular associated lymphoid tissue (VALT).

A problem that arises in defining TLO structures is that the difference between a chronic infiltrate and a TLO resides in the degree of its internal organization. According to pathologists, the term TLO can only be used when all the following criteria are fulfilled:

1) The organized infiltrate contains anatomically distinct yet adjacent T cell and B cell compartments
2) The T cell area contains an extensive network of fibroblast reticular cells (FRC)
3) PNAd+ or MECA79+ HEVs are present in the T cell area
4) There is evidence of B cell class switching and GC reactions in the B cell follicles
5) The AID enzyme is present
6) Follicular dendritic cells (FDCs) are present

Often, it appears that strictly speaking not all criteria are fulfilled, while the organized structure can still function as a TLO. Although in most disease models the structures do not fully cover the proposed TLO definition, we use the term TLO because we believe the functional resemblance with SLOs is much more relevant than the structural resemblance.

Table 5 - Diseases and experimental models in which tertiary lymphoid organs have been identified

DISEASE	LOCATION	SPECIES	Ag SPECIFIC?	REF
Microbial causes				
Influenza	iBALT	mouse	yes	446, 447
Vaccinia virus Ankara	lung	mouse	naive T cell activation	448
Intestinal microbiota	isolated lymphoid follicles in intestinal wall	mouse, human	?	449,450, 451
Helicobacter pylori	gastric wall	mouse, human	yes	452
Helicobacter hepaticus	liver	mouse	?	453
Borrelia burgdorferi	multiple sites Lyme's disease	human	?	?
Chlamydia pneumoniae	iBALT	mouse	?	454
Chronic LPS exposure	iBALT	neonatal mice	LPS ?	455
Autoimmune disease				
Common variable immunodeficiency	iBALT	human	Yes: Ko67+ GC	456
Rheumatoid arthritis	synovial space	human mouse	yes	457, 458
	lung interstitium	human	yes	459
Ankylosing spondylitis	Synovium	human	Yes	460
Primary biliary cirrhosis	liver	human	No: absence of any clonal T or B cell proliferation	461
Hashimoto's thyroiditis	thyroid gland	human	thyroglobulin, thyroperoxidase	462
Diabetes	periductally in pancreatic parenchyma	non-obese diabetic mouse	yes, anti-insulin Ab responses	463, 464
Secondary progressive multiple sclerosis	meninges at deep cerebral sulci	mouse (EAE), human	myelin and neuronal antigens: suggested	465, 466
Myasthenia gravis	thymus	human	acetyl choline receptor: correlation of autoantibodies with infiltrates	467
Autoimmune gastritis	gastric lamina propria	mouse	parietal cells	468
SLE	tubulointerstitial nephritis	human	Yes (clonal restriction and mutated antibodies)	469, 470
Chronic transplant rejection				
	Heart, lung, kidney	human mouse	Yes, alloantigens	471-473
Degenerative/environmental causes				
Cigarette smoke	end-stage COPD lung, iBALT and interstitial	human mouse	autoimmune or latent infection	474-476
Inhaled organic dust	interstitial TLO in hypersensitivity pneumonitis	human	fungal spores in moldy hay	459
Diesel particles	iBALT	mouse	?	477
Hypercholesterolemia	atherosclerotic plaques	mouse	?	478
Metal-on-metal prosthetic joints	soft tissues around joints	human	cobalt or nickel ?	479, 480
Protein-cage nanoparticles	iBALT	mice		481
Pristane adjuvant (2,6,10,14-tetramethyl-pentadecane)	peritoneal cavity (milky spots)	mice		470
"Idiopathic" disorders				
Usual interstitial pneumonia, idiopathic lung fibrosis	lung interstitium	human	autoimmune component ?	459, 482
Idiopathic pulmonary arterial hypertension	perivascular lesions of the lung	human rat	likely autoimmune component	483 484
Cancer				
Breast, lung, colorectal, pancreatic, renal, germ cell, skin cancer	Mostly peritumoral Rarely intratumoral	Human, mouse	Tumor-associated antigens	485, 486

Development of organized lymphoid tissues before birth

Before discussing the mechanisms controlling TLO development during chronic inflammation, it is important to summarize organogenesis of SLOs, reviewed in detail elsewhere[444] and summarized in figure 7. SLOs develop in predefined areas in the embryo, often at the crossroads of lymphatics. The lymph node anlagen are first identified in mice on embryonic day 14.5 by the presence of RORγ+ lymphoid tissue inducer (LTi) cells[487]. During SLO development[444] it is crucial that CD3-CD4+CD45+ LTi cells interact with stromal lymphoid tissue organizer (LTo) cells in a process involving lymphotoxin α1β2 (LTa1b2) signaling to the lymphotoxin-β receptor (LTβR). When these cells interact, LTo cells express several adhesion molecules (VCAM1, ICAM1, MADCAM1) and homeostatic chemokines (CCL19, CCL21 and CXCL13). These molecules act as the driving force for recruitment of lymphocytes. In addition, production of lymphangiogenetic growth factors, such as VEGF-C, VEGF-D, FGF-2 and HGF, leads to formation of Lyve-1+ lymphatic vessels[488, 489].

LTo cells develop further into FDCs and FRCs that provide the conduit framework on which T cells and B cells migrate and interact with each other. Before LTi cells can cluster, an $LT\alpha_1\beta_2$-independent instructive signal is given to local fibroblasts to start producing the initial CXCL13 to attract LTi cells. Recent insights indicate that signals derived from the nervous system could provide this first signal for LN development[490, 491]. Together, these studies have highlighted the importance of interactions between hematopoietic cells and stromal cells, and between $LT\alpha_1\beta_2$ signaling and homeostatic chemokines in the complex organization of SLOs.

Induction of tertiary lymphoid structures

Which cytokines and chemokines are involved in TLO formation?

Formation of TLOs at sites of chronic inflammation follows many of the pathways also used by lymphoid organogenesis before birth. Indeed, when LTα, CCL21, or CXCL13 are overexpressed from a tissue specific promoter, ectopic lymphoid tissues are induced before or soon after birth[492-499]. Conversely, in experiments in which either CXCL13, its receptor CXCR5, CCR7 or LTα was genetically absent or neutralized, TLO structures did not develop in various infectious and autoimmune models[452, 458, 478, 500-502]. Like in SLO formation, LTα-seems to instruct stromal cells to develop into FDCs and HEVs, whereas CCL19 and CCL21 acting via CCR7 control the organization of the T cell zones of the TLO[446, 458, 500, 503].

The homeostatic cytokine IL-7 deserves separate mentioning. *Il7-/-* mice lack lymph nodes and Peyer's patches, whereas overexpression of IL-7 induces development of additional lymph nodes[504]. IL-7 is involved in stimulating LTi cells and in maintaining survival of T lymphocytes. IL-7 produced by lymphatic endothelial cells has been linked to the retention of Th2 memory cells in iBALT structures upon allergic airway inflammation[505]. IL-7 expression is augmented in the synovial tissue of RA patients, and there is a strong correlation between IL-7 expression and LTβ expression[506]. In the perivascular TLOs of patients with idiopathic pulmonary hypertension (IPAH) we found strong immunoreactivity for IL-7 very close to ER-TR7+ FRC cells[483].
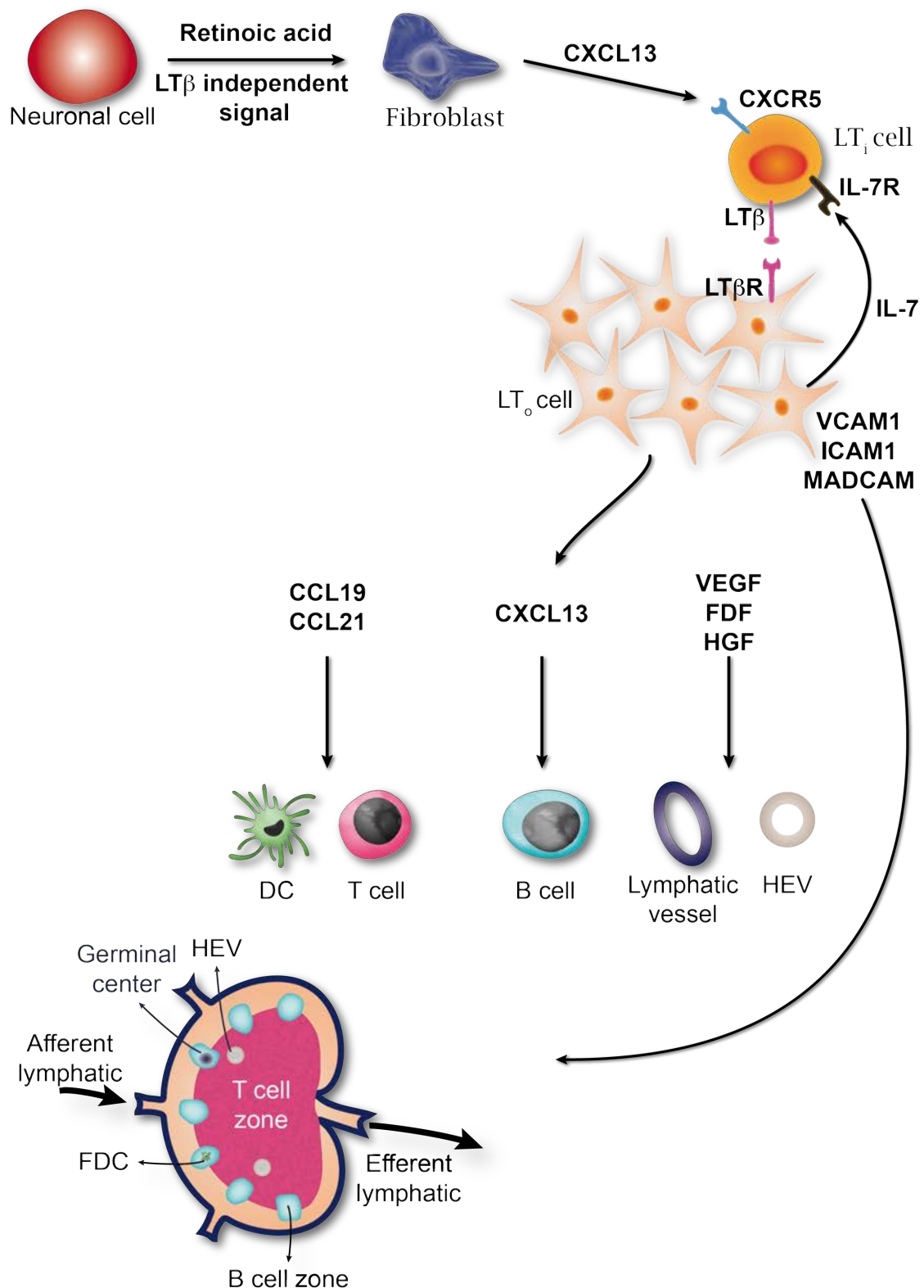
Figure 7 - Development and structure of secondary lymphoid organs

During development of secondary lymphoid organs, the earliest instructive signal is given by a neuronal cell that induces the local fibroblasts to upregulate CXCL13 and thus attract lymphoid tissue inducer cells (LTi). These cells express LTb and instruct the local fibroblasts to become LT organizer cells (LTo) that start producing chemokines for B cells (CXCL13), T cells and DCs (CCL19, CCL21). At the same time, the fibroblasts upregulate cell adhesion molecules to allow LTi cells and recruited T and B cells to stick together. Local angiogenic growth factors are also made to allow the development of high endothelial venules (HEVs), as well as afferent and efferent lymph vessels. As a result, a well-organized structure with a separate B cell (blue) and T cell zone (red) is formed. This allows cell-cell contact at the appropriate time; and entry and exit of lymphocytes and antigen via the HEVs and lymphatics.

Together these data illustrate that homeostatic chemokines and cytokines are both sufficient and necessary for the induction of TLOs. Given this, it has been suggested that inflammation-induced TLOs might have developed during evolution before SLOs did.

Are lymphoid tissue inducer cells necessary for inducing formation of tertiary lymphoid organs?

In contrast to the involvement of CD3⁻CD4⁺CD45⁺ LTi cells in the formation of lymph nodes, their necessity for TLO induction is controversial[444]. The differentiation of LTi cells from lymphoid progenitors involves the transcription factors Id2 and retinoic acid receptor-like orphan receptor RORγt. Mice lacking these factors do not develop most LNs (except nasal associated mucosal tissues, NALT, and tear duct associated lymphoid tissue, TALT)[487, 507-509]. When $Id2^{-/-}$ or $Rorc^{-/-}$ mice lacking LTi cells were infected with influenza virus, they developed normal iBALT structures in the lung[446, 449, 455]. Other phenomena that do not depend on LTi cells are postnatal development of omental milky spots (these are organized lymphomyelopoietic tissue in the peritoneal cavity), isolated lymphoid follicles and TLOs in the intestine caused by DSS-induced colitis, and TLOs in autoimmune thyroiditis or insulitis[449, 495, 510, 511]. The controversy surrounding the role of LTi cells stems from three observations. First, adoptive transfer studies provide evidence that injection of LTi cells into normal skin is sufficient to induce TLO structures[512]. Second, LTi cells have been observed in models of spontaneous TLO development, such as the perivascular TLOs seen in hypercholesterolemic $Apoe^{-/-}$ mice and the iBALT structures seen in neonatal lungs exposed to LPS[455]. Human TLOs, such as those seen in IPAH, also contain adult LTi-like cells that are CD4⁻, cKit⁺ and OX40L positive, and also express RORγt and Id2 [483]. Third, overexpression of homeostatic chemokines under a tissue-specific promoter to induce TLOs (e.g. the rat insulin promoter or the intestine-specific villin promoter driving expression of CXCL13) and transgenic overexpression of IL-7 both resulted in increased numbers of LTi cells and dependence of TLO formation on LTi cells[511]. However, in these systems lymphoid organogenesis starts before birth, which means that the requirement for LTi cells in induction of TLO formation could be overestimated. Nevertheless, most groups agree that LTα1β2 is necessary for TLO induction[500, 502].

Which other cells can act as lymphoid tissue inducer cells in chronic inflammation?

In the models in which LTi cells have been shown to be redundant, the question arises as to which other LTα₁β₂-expressing cell type could be providing the instructive signal to activate stromal organizer cells and initiate TLO formation? It has been proposed that B cells, T cells or DCs could substitute for LTi cells in their inductive function, especially when they are activated and express LTα₁β₂ on their surface[447, 449, 513, 514]. Progression towards mature, fully structured TLOs is dependent on LTβ-sufficient B cells[503] most likely via a positive feedback loop of CXCL13 production and LTβ expression as shown in lungs of COPD patients[515]. According to this hypothesis B cells are activated via TLR signaling, induce expression of LTβ on their surface and interact with LTβ R bearing B cells. This LTβ signaling will induce CXCL13 production and release, which attracts more B cells and upregulates LTβ expression.

T cells can also play a role as LTi cells as was shown in a model of thyroid overexpression of CCL21 that CD3^{+}CD4^{+} activated T cells interacted with DCs at sites of chronic inflammation, and subsequently the activated T cells acted as LTi cells in the absence of Id2 activity[495]. CD4^{+} memory T cells different from Th1, Th2, Treg and Tfh cells isolated from the synovial tissue of patients with rheumatoid arthritis were able to secrete CXCL13 and attract B cells; rendering them a possible candidate to function as LTi cells during ectopic lymphoid structure formation in the synovium[516]. In two recent studies on neonatal mice exposed to endotoxin inhalation and on mice with experimental autoimmune encephalomyelitis respectively, an activated Th17 CD4 T cell population was found to be involved in inducing TLO structures[455, 517]. RORγt[+] IL-17–producing cells were also found inside TLOs of patients with IPAH. In humans, Th17 cells express the CCR6 receptor, and in the bloodstream of IPAH patients circulating CCR6^{+} cells were fewer, while the ligand CCL20 was produced in the perivascular TLOs[483]. However, TLOs seem to develop normally in $Ccr6^{-/-}$ mice[455]. The induction of TLOs by Th17 cells was dependent on expression of podoplanin, but why this is the case remains unknown. One possibility is that podoplanin is required for retention of Th17 cells at sites of TLO formation[455, 517], a process that is inhibited by IL-27[518]. It remains to be seen whether all forms of TLO depend on IL-17 production, and whether IL-17A and/or IL-17F is involved. In this regard, iBALT induced by infection with modified vaccinia virus ankara or influenza virus is not affected by deficiency of IL-17A while *Pseudomonas Aeruginosa*-induced iBALT is dependent on IL-17 signaling[519, 449]. LTβR signaling is not triggered only via LTα1β2; LIGHT (TNFSF14) can also bind the LTβR. In a model of non-obese diabetes, LIGHT expressed on T cells was shown to be important in the development and maintenance of TLO structures[520].

Finally, LTi functions have also been attributed to DCs. In almost all TLO structures that have been described, the T cell area contained antigen-presenting DCs[447, 483]. As DCs activate T cells, it has been suggested that DCs are sufficient for TLO induction[463]. Repeated injection of DCs into the lungs of mice is sufficient for induction of iBALT structures accompanied by induction of myofibroblast differentiation[447, 521]. During formation of Peyer's patches, a CD11c^{+} cell type expressing LTαβ accumulates at the LN anlagen and is necessary for stromal instruction[491]. DCs might also directly instruct stromal cells irrespectively of their effects on T cells. In TLO structures induced in the thymus, DCs were specifically necessary for induction of lymphangiogenesis from stromal cells[522]. How DCs induce TLOs is less clear. In virus-induced iBALT, mainly CD11b^{+} DCs accumulate; these cells express instructive LTα1β2 while also producing the homeostatic chemokines CXCL13 and CCL19/CCL21[447]. However, in some models mostly pDCs accumulate, suggesting a functional role for type I IFN. This is the case in TLOs found in end-stage COPD patients and in a murine SLE model[470, 476]. Three studies have shown that depletion of DCs leads to disappearance of existing TLO structures, suggesting that DCs are necessary for structural organization and maintenance of TLOs, most likely through transpresentation of chemokines, or by provision of a continuous source of antigen presentation to T cells[447, 448, 522].

Overall, there is no strong evidence pointing towards one specialized lymphoid tissue inducer cell type in TLO induction. LTi cells, B cells, T cells and DCs all remain candidate

cell types to instruct organizer cells. Presumably the different cell types are redundant and the model defines which cell type exerts the dominant role of LTi cell.

What is the nature of the stromal organizer cell?

Stromal cells, such as FDCs of the B cell area, FRCs of the T cell area, lymphatic endothelial cells and HEVs, play an important role in the functioning of SLOs by producing cytokines, chemokines and forming the connective tissue conduits on which immune cells interact[523]. It has become clear that TLOs have the same complicated network of stromal cells and conduits, although their function remains undetermined[511]. Upon LTβR triggering by LTi cells or inflammatory cells, LTo cells express cell adhesion molecules and produce chemokines that attract B cells and T cells, produce cytokines that maintain lymphocyte viability (e.g. IL-7), and differentiate into FRCs and FDCs. LTβR signaling from host cells is involved in formation of lymphatic vessels in thyroid TLO[502, 522] and in formation of the reticular network[511]. Several studies have suggested that LTo cells and FRC cells of the T cell zone, like myofibroblasts, stain positive for α-smooth muscle actin and produce desmin[524]. It was elegantly demonstrated that inflammation could induce this program of myofibroblast differentiation in tissue resident fibroblasts, while at the same time inducing the production of the homeostatic chemokines CXCL13 and CCL21, and of lymphangiogenic cytokines. This programming did not require adaptive immunity, innate lymphoid cells, LTi cells, inflammasomes, or pattern recognition receptors, but was dependent on a myeloid cell population, most likely neutrophils. The molecular signal released by these myeloid inflammatory cells is not known[489]. Myofibroblasts are typically seen at sites of scarring and fibrosis, which might explain the occurrence of TLOs in patients with COPD, scleroderma, lung fibrosis and primary biliary cirrhosis[474, 482, 525]. In a model of atherosclerosis, it was shown that mouse aorta smooth muscle cells could differentiate into a cell type that resembles the LTo cell[478, 526].

During neuroinflammation the macrophages of the brain, the microglia, are the main source of CXCL13 in the central nervous system, making them ideal candidates to function as LTo cells during lymphoid follicle formation in multiple sclerosis[527].

In several instances where TLOs have been found in humans, they were also closely associated with c-Kit+ mast cells[483, 506]. Mast cells produce cytokines (e.g. TNFα) and express surface molecules (CD40L) that could help in structuring TLOs. They are also an important source of VEGF-C, which induces lymphangiogenesis.

Finally, our views on TLO formation are even fundamentally changing. It has always been assumed that a cell-cell dependent interaction between LTo cells and LTi cells is necessary for TLO induction. Recently, an interaction via soluble factors has also been proposed in the model of atherosclerotic-related TLO formation[528]. In this model vascular smooth muscle cells are conditioned to become LTo cells in an LTβR-independent manner by M1 type macrophages.

In conclusion, TLO formation (Figure 8) follows the same basic set of rules also implicated in formation of the lymph nodes and Peyer's patches (Figure 7). Controversies remain however on the precise nature of the LTi and LTo cell, and how they communicate with each other. Most likely the nature of the inducer and organizer
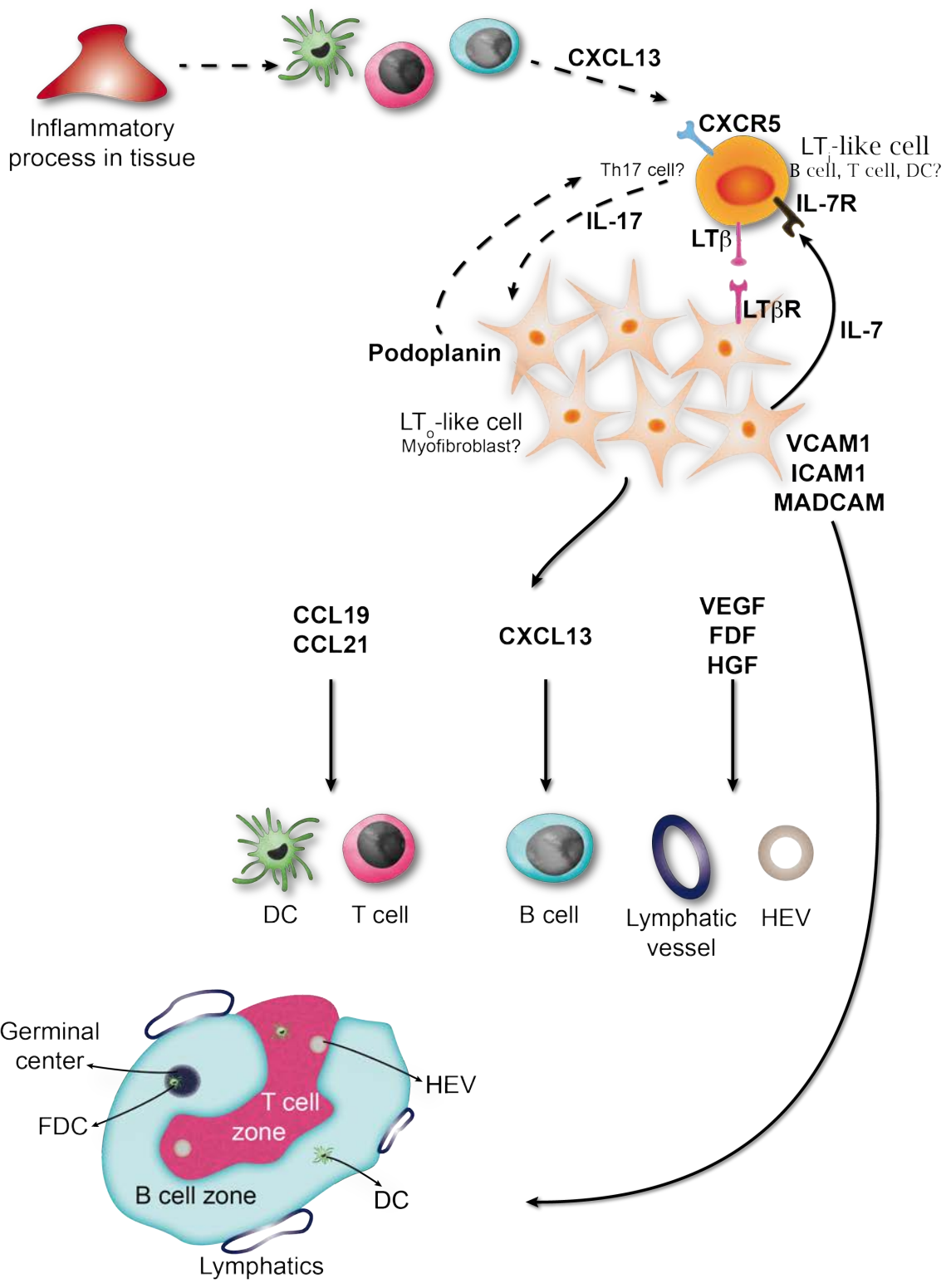
Figure 8 - Development and structure of tertiary lymphoid structures

During chronic immune responses or transplant rejection, DCs continuously present antigens to T cells and B cells. Activated B cells express LTb and can act as potent LTi-like cells, to induce an LTo phenotype in local myofibroblasts. Alternatively, chronic antigen presentation by DCs might also lead to induction of a Th17 cell response that can also induce TLOs through unclear mechanisms. Th17 cells are held in place via interactions with podoplanin. As in SLOs, TLOs are divided into discrete B (blue) and T (red) cell areas. Although, fully formed TLO structures often contain only a single T cell area and a larger B cell area, in which germinal center reactions (GC) can be seen. These also contain DCs and follicular DCs. At the periphery, an elaborate network of lymphatics (Lyve1+ and Prox1+) is commonly found but it is currently unknown if these are afferent or efferent lymphatics.

cell depends on the anatomical environment where TLOs develop as well as the initiating antigen.

The function of tertiary lymphoid organs

TLOs develop in areas of chronic immune stimulation. However, although different infectious and immune or inflammatory triggers exhibit the same degree of chronic inflammation and inflict similar damage, their capacity to induce TLOs can vary widely. Moreover, tertiary lymphoid tissues develop more easily in neonates[455]. There is accumulating evidence that TLOs represent an adaptation of the body to increased demand for a localized immune response. The evolutionary pressure could be the constant equilibrium between commensals and the mucosal immune system, such as occurs typically in the gut and the lung. Isolated lymphoid follicles (ILF) are a specialized type of TLO that forms postnatal in the intestinal wall, through epithelial recognition of NOD1 ligands derived from commensals[529]. Although the deeper lung was once considered sterile, there is now evidence that it is not, particularly following viral infection or chronic cigarette smoking, typical triggers of TLO formation in the lung[530]. Here again, commensals could be a driving force for TLO development.

Tertiary lymphoid organs as immune inductive sites for protective immunity to (re-)infection
Just like SLOs that bring together naive T cells, B cells and DCs, TLOs have been shown to allow the activation of recirculating naive T cells and the activation of B cells within germinal centers. One caveat is that these studies were performed in mice lacking LNs, which could lead to overestimation of the potential of TLOs[446]. However, recent work has suggested that TLOs can be immune inductive even in mice with normal LN anatomy and a fully functional immune system. Dendritic cells in TLOs often have an activated phenotype, suggesting that they are the predominant antigen presenting cells stimulating immunity[470]. The function of DCs in TLO biology has been studied mainly in iBALT structures induced by modified vaccinia ankara virus or influenza virus[447, 448]. Preformed iBALT structures collect DCs that are injected into the trachea, but it is unclear how these DCs reach the T cell area of iBALT[448]. One possibility is that iBALT structures are connected to afferent lymphatics located immediately underneath the epithelial basement membrane[525]. In many instances, the TLOs are found immediately next to lymphatics, but no study so far has been able to determine if these lymphatics are afferent (bringing antigen to the TLO) or efferent (exporting lymphocytes). It is also possible that the remodeling around blood vessels and the subepithelial areas leads to formation of a conduit system that provides a path for the encountered antigens to reach the TLO. This is supported by the close resemblance between the type of extracellular matrix found in atherosclerosis-associated or IPAH-associated TLOs compared with the one found in the FRC network and conduit system of LNs[478, 483]. Whatever the mechanism, TLO structures do allow the extravasation of naive T cells and their differentiation into effector T cells upon contact with DCs[448].
Major constituents of TLOs are the B cells that accumulate on the FDC network. Almost all TLOs described exhibit signs of B cell class switching, as exemplified by the presence

of high amounts of the AID enzyme and the presence of germinal center reactions[447, 483]. Such B cells provide an important source of memory B cells that are activated upon reinfection[447, 459]. Plasma cells are found in the immediate vicinity of the TLOs and secrete antibodies specific for the pathogen that induced the TLO, such as influenza virus or Helicobacter species[447, 453]. Although some of the B cells generated in the TLO also seem to reach the bone marrow, where they could reside as long lived plasma cells, the iBALT system that forms after influenza virus infection and the ILFs that form in response to intestinal commensals mainly serve to produce local mucosal IgA[447, 475, 531].

It is not known why some infections leave behind TLO structures whereas others do not. In a model of influenza, for T cell immunity to persist, there is continued dependency on lung DCs that take viral antigens from the lung to the MLN, even long after the virus has been cleared, for presentation to memory T cells. As CD11b$^+$ DCs could be found mainly in iBALT in the lung long after virus was cleared, it is possible that they capture retained viral antigens from the FDC network[532].

It remains to be determined if persistence of T_{rm} cells after IAV infection depends on the induction of TLOs as a sanctuary for TLO survival. In the skin at least, T_{rm} cells accumulate in organized clusters resembling TLOs.

Therapeutic applications of tertiary lymphoid organs

Exploiting the induction of TLOs by vaccines could be a valuable option in promoting long-lasting, local antimicrobial immunity in the lung and gut[533]. Mice pre-treated with protein-caged nanoparticle (PCN) adjuvants in the absence of any specific viral antigens were protected against both sub-lethal and lethal doses of two different influenza viruses, a mouse-adapted SARS-coronavirus, and a mouse pneumovirus. Treatment with PCN significantly increased survival, enhanced viral clearance, accelerated induction of virus-specific antibody production, and significantly decreased morbidity and lung damage; these changes were attributed to prior development of iBALT[481]. A deeper understanding of the mechanism of action of this protection is needed before we can rationally design adjuvants that induce TLOs.

As TLOs are found in many chronic diseases with an autoimmune component and in chronic transplant rejection, interfering with their induction or maintenance could be developed into a novel therapeutic approach. One possibility is to interfere with the LTβR. In a model of autoimmune diabetes in NOD mice, disrupting TLOs by antagonizing the action of LIGHT with LTβR inhibited formation of autoaggressive T cells and progression to diabetes[520]. Similarly, disruption of TLOs in the salivary glands that develop in the NOD model of Sjögren's disease also led to partial restoration of salivary function[501]. Another approach could be to target crucial TLO chemokines or their receptors, such as CXCL13 or the CXCR5 receptor. In a model of rheumatoid arthritis, Cxcr5$^{-/-}$ mice had significantly reduced joint destruction[458]. In a model of diabetes in NOD mice, neutralization of CXCL13 led to disorganized TLOs, yet there was no clear effect on diabetes incidence[534]. Clearly, more preclinical studies on these compounds are needed before we can envisage an intervention study in humans.

Given the fact that the function of TLOs are similar to those of SLOs, the basic question for the field is why exactly our defense system mounts this at first sight redundant response. The capacity of TLOs to generate of tissue-directed autoantibodies, argues that targeting TLO formation could be beneficial in autoimmune diseases. On the other hand, stimulating the formation of TLOs can be beneficial in the fight against viral and bacterial infections.

Pulmonary immune responses: a fine balance between protection and immunopathology

To efficiently eliminate a viral infection from the lungs, the activation of both the innate and adaptive immune system is necessary. As a consequence, this results in the release of several cytokines, proteins and reactive oxygen species that have beneficial effects in defending the body against the insult, but may also induce tissue damage.

Type I IFNs counteract a viral infection by inducing an antiviral state, inducing apoptosis and interfering with the viral replication cycle. These antiviral actions can be responsible for side effects and pathological damage. The apoptotic TRAIL[535, 536] and FAS-FASL[537] pathway are induced to inhibit the spread of IAV but if the apoptosis-inducing proteins are expressed on the surface of structural cells, tissue damage can be induced. This mechanism can be linked to the susceptibility to IAV infection. More pathogenic strains were found to induce a stronger and more sustained type I IFN signal that leads to successive upregulation of TRAIL expression by monocytes and the TRAIL-ligand DR5 on epithelial cells[538].

Besides induction of apoptosis, type I IFNs also lead to the production of pro-inflammatory cytokines and chemokines such as CCL2 that recruit monocyte-derived DCs and macrophages to the site of infection, resulting in infection-associated pulmonary pathology and mortality as they are extensive cytokine secretors and can mediate TRAIL-induced cell death of the respiratory epithelium[91, 535]. In contrast to these pathology-inducing characteristics of type I IFN, it has also been described that type I IFNs have the potency to limit tissue inflammation by induction of the immune regulatory cytokine IL-10 and by inhibiting neutrophil recruitment and affecting monocyte differentiation[539, 540].

One of the first cell types recruited to the lungs after IAV infection is the neutrophil. Improper or prolonged activation of neutrophils leads to an increased exposure to reactive oxygen species[541] and the proteolytic enzymes on the NETS[92, 93, 542], which can lead to tissue injury and can cause pneumonia and acute respiratory distress syndrome[543, 544]. The host inflammatory response is of greater impact on the lethality of IAV infection than the effect of the pathogen itself. For example, a reduced infiltration of neutrophils mostly correlates with reduced pathology and lethality[545-547]. More specifically gene-expression analysis learned that lethality correlates better with neutrophil activation than with viral load[548]. In contrast to the possible severe side effects of neutrophil products, they are beneficial as their antibacterial peptides can prevent secondary bacterial superinfection[549-551].

CD8^{+} T cells are highly associated with lung injury caused by the pulmonary inflammation. Excess T cell infiltration into the virus-infected lung compromises lung function and compliance. One of the main factors that contribute to this inflammation is TNFα. Inhibition of TNFα can significantly reduce lung damage, but TNFα was shown to be essential for proper viral clearance as inhibition of TNFα results in a delayed viral clearance[552]. To protect the host against the detrimental effects, interaction of CD8^{+} T cells with NK cells activates a negative feedback loop to limit TNFα production[553].

Lung dendritic cells and virus-induced immunopathology

After activation in the lung, DCs can play dual roles during the immune response against respiratory viral infections. On the one hand, they are necessary to mount the antiviral CD8 cytotoxic T cell response in the draining lymph node (LN) that leads to viral clearance. On the other hand, the inflammation induced by DCs contributes to disease severity and even death from acute lung injury[554]. This implies that an unbalanced immune response can have detrimental effects on systemic illness and survival.

When effector cells return to the lung after being generated in the MLN, there is evidence that DCs are also necessary locally for boosting effector function, which could eventually lead to immunopathology[326, 555]. MCs (or TIP-DCs) have been proposed to be the predominant cause of immune pathology caused by influenza and RSV virus infection[554, 556]. High pathogenic strains induce more TNF and iNOS producing (TIP)-DC recruitment to the lung. They promote immune-induced pathology, but their complete depletion is detrimental[295]. This might be related to the fact that iNOS-derived nitric oxide also has direct antiviral activity to IAV[557].

The final function of antiviral CD8^{+} T cell effector cells is instructed by the phenotype of the cell type presenting the viral antigen. MHC class I expression and costimulation via CD80 and CD86 on professional antigen presenting cells are necessary for pro-inflammatory cytokine production, whereas induction of cytotoxicity is induced by MHCI presentation on epithelial cells without the need of costimulatory signals. This cytokine production also contributes to lung pathology and may not be essential for viral clearance since viral clearance is not affected when costimulatory signals are blocked[325, 326].

Tertiary lymphoid organs in immunopathology

The presence of TLOs in models of chronic autoimmunity and in chronic transplant rejection (see Table 5) and their association with tissue destruction[449, 465, 466, 472, 473, 558] has led to the suggestion that they are important inductive sites for self-reactive T lymphocytes and antibodies that contribute to pathology. However, the evidence for this is rather sparse. In the TLOs associated with diabetes in NOD mice, T lymphocytes are detected that destroy islets and plasma cells with specificity for insulin[464, 520]. The expansion of B cells inside an individual TLO around islets is often oligoclonal, suggesting the presence of an autoantigen[559]. In the lungs of patients with rheumatoid arthritis, B cells in the TLO were found to produce rheumatoid factor and antibodies against citrullinated proteins, typical self-antigens in this disease[525]. In human autoimmune

thryroiditis, most B cells inside ectopic GCs bound the autoantigens thyroglobulin and thyroidal peroxidase[462].

However, it is less clear which antigens the lymphocytes might react to in more severely degenerative diseases such as COPD induced by cigarette smoke and atherosclerosis induced by western diet. Although it has been proposed that there is an autoimmune component in many chronic tissue-destructive diseases such as COPD, another explanation could be colonization of the lung by latent adenovirus or unexpected commensals in the lung[560]. Until we know the antigen-specificity of the B cell and T cell responses in TLOs, we can only speculate on the nature of the antigens presented in diseases such as IPAH and cirrhosis. In any case, the extensive formation of myofibroblast-like cells that constitute part of the stromal organizing network might contribute to local fibrosis that could compromise organ function.

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Chapter 2

Aims and outline of the thesis

As was pointed out in the introduction, IAV infection evokes a complex immune response. A common theme of the thesis is that the heterogeneity and complexity of the immune system and its components are taken into account to understand the immunology of IAV infection. By using a mouse model for mild seasonal IAV infection (X31 virus, H3N2) three diverse aspects of the immune response against IAV infection were studied:

- What is the precise functional contribution of the recently described subsets of cDCs and MCs?
- What is the precise contribution of a rare population of CD4$^-$CD8$^-$ "double negative" T cells?
- Is there a role for the innate immune response in driving organized lymphoid tissues (iBALT) development in response to infection?

First, even if DCs have already been studied in the context of IAV, It is important to realize that each lung DC subset can activate a different functional program in response to viral infections. Most studies on viral lung infection thus far have not incorporated DC heterogeneity into the conceptual framework. On the other hand, the same DC subset can react differently depending on the type of virus that is detected. Therefore in chapter 3 we are focusing on the different dendritic cell subsets during influenza infection. More specifically DC subsets in the lung and MLN during IAV infection are reexamined with more precision by using a gating strategy including CD64 and MAR-1. By applying this gating strategy the origin, kinetics and the migratory capacity of the different subsets after infection are defined and the previously never described MAR-1$^+$ cDC$_2$ is characterized in detail.

Secondly, the same degree of heterogeneity and complexity is also seen in T cells. Traditionally, T cells have been subdivided in CD4 and CD8 T cells and this is also how T cells have been mainly studied in IAV infection. In chapter 4 the T cell response after influenza infection is described and more specifically the often-ignored CD4$^-$CD8$^-$ T cells. These cells are a rather small part of the total population of T cells, but our experiments showed that these cells can also exert immunoregulatory functions. The new concept that some T cells become resident memory cells is incorporated and the relationship between DCs and conventional and CD4$^-$CD8$^-$ T cells is studied.

Finally in chapter 5 we study in greater detail how TLO formation is initiated following IAV infection. We describe that IL-1 plays a role and provide evidence on how innate cytokines like IL-1 that are produced very early after infection can modulate the lung environment and condition it to promote iBALT development, an event that is only fully completed after viral clearance.

To end, the findings of this thesis are extensively discussed and put in perspective in chapter 6.

IFNAR signaling induces a functional IRF8 module in type 2 conventional dendritic cells during influenza infection

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Abstract

Dendritic cells play a crucial role during the immune response against influenza A virus infection as they can take up viral antigen and present it to T cells to induce a T cell response. Historically several subsets have been introduced and many different functions were attributed to these different subsets. It is generally accepted that CD103$^+$ cDC1 DCs have the best cross-presenting capacities, but an important antigen presenting function was also attributed to CD11b$^+$ cDC2s. Recent insights in dendritic cell biology provide potential to study the functional specialization of DC subsets during influenza A virus infection. We applied a novel gating strategy implementing CD64 and MAR-1 antibodies to clearly differentiate between conventional and monocyte-derived cells and study the DC functions on well-defined populations. By applying this gating strategy on a mouse model for mild seasonal influenza infection, a conventional cDC2 population that acquired expression of the monocyte markers MAR-1 and CD64 and expression of IRF8, the terminal selector of the cDC1 lineage, was identified. Functionally this MAR-1$^+$ cDC2 has a more mature phenotype than MAR-1$^-$ cDC2s and has better cross-presenting capacities. This population is induced in a type 1 interferon dependent manner but is not dependent of IRF8 for its development. Rather, IRF8 controls a functional module in these cells and confers increased antigen cross-presentation and increased T cell stimulating capacities.

Introduction

Influenza A virus (IAV) is a respiratory pathogen that causes seasonal infections that usually run a mild course. Pandemics with a more severe outcome can occur when the fragmented genome of the virus undergoes antigenic shift. Combined with the continuous antigenic drift it is currently not possible to develop a long-term effective vaccine. Therefore it is important to understand the immune response against IAV infection thoroughly to find new protective or therapeutic strategies.

Dendritic cells (DC) are the sentinels of the immune system that activate the adaptive immune system after sensing viral particles by innate immune receptors. Within the DC compartment, IRF8 and IRF4 are two transcription factors involved in driving the development towards the CD103+ cDC1 lineage or the CD11b+ cDC2 lineage respectively[1-9]. Functionally, IRF4 and IRF8 are involved in DC maturation, IL-12 production, migration and induction of the MHCI and MHCII presentation machinery genes[2, 9, 10]. Both directly or indirectly, these transcription factors can regulate T cell responses, like the acquisition of helper functions in CD4 T cells and induction of CD8 cytotoxic T cells[3, 4, 11-20]. During the course of primary IAV infection, DCs can acquire viral antigens through direct infection or through the acquisition of exogenous antigens by phagocytosis of dying infected cells or viral particles and prime CD8+ T cells by a process called MHC-I presentation or cross-presentation respectively. In the past, several studies were undertaken to define which DC subsets induce T cell proliferation and how this process is induced. Generally CD103+CD11b- DCs were shown to have the best cross-presenting capacity during IAV infection[14, 21-23]. Other groups described that CD11b+ DCs and late arriving presenting cells (LAPC) were the major antigen-presenting DC subsets controlling adaptive CD8 and CD4 T cell immunity to IAV[24, 25].

The observed inconsistencies in the results of these early studies on the function of various DC subsets have generated a lot of discussion on why results could be so diametrically different. Recent insights in DC biology now warrant further exploration and offer some explanations. Firstly, the paradigm that cDC1s are the exclusive cross-presenting cells that activate and induce cytotoxic CD8+ T cells (CTL) and that cDC2 are the antigen presenting cells that activate CD4+ T cells to acquire a Th phenotype has recently been questioned. It was shown that also cDC2s can induce CTL responses under the condition that antigen is seen in the presence of a TLR signal, that might differ from the cDC1 subset[26]. For cDC1 DCs, TLR3 triggering (dsRNA) is necessary whereas for cDC2 DCs TLR7 triggering (ssRNA) is crucial. The exact molecular mechanism driving the acquisition of cross-presenting potential by cDC2s is currently unknown. Presumably, a gene module switch is induced by a transcriptional network downstream of TLR7. Secondly, the lack of robust identification markers to distinguish pure cDC subpopulations has obscured the results of many experiments, as many markers that define a lineage in steady state can be profoundly upregulated when cells get activated by cytokines or PRR signals[27]. In this paper, we apply the recently described gating method based on CD64 and MAR-1[28] to identify the DC subsets during mild IAV infection, greatly improving the correct identification of cDCs, as contaminating macrophages and monocyte-derived cells (MCs) are gated out. This led to the identification of a maturation state of conventional cDC2s that expressed the

monocyte-marker MAR-1 and thus called MAR-1$^+$ cDC2 DC. MAR-1 expression on cDC2s was induced in a type 1 IFN dependent manner and these cells were more mature than conventional cDC2s and acquired expression of the cDC1-associated transcription factor IRF8. Functionally, IRF8 controlled a functional module in cDC2s, responsible for maturation and induction of a CD8$^+$ T cell response rather than a CD4$^+$ T cell response. These findings show that the transcription factor IRF8, traditionally associated with cDC1 development, is co-opted by cDC2s to drive a functional module associated with cDC1 functions.

Results

A population of MAR-1$^+$ CD11b$^+$ DCs is present in the lung and MLN during Influenza A virus infection

We have recently described that dendritic cells (DCs) in the lung consist of both conventional DCs (cDCs) and monocyte-derived DCs (recently termed MCs, to reflect their origin from circulating monocytes)[28, 29]. In the steady-state lung and MLN, cDCs, which express CD11c and MHCII but lack expression of CD64 and MAR-1, can be subdivided into two subsets, cDC1s and cDC2s that are distinguishable by CD103 and CD11b expression[28]. MCs express CD11c, MHCII, CD11b, CD64 and MAR-1. Thus inclusion of CD64 and MAR-1 in the gating strategy is required to distinguish cDC2s from MCs. Unfortunately, many papers describing the function of DCs during IAV infection discriminate the DC subsets based on expression of CD11b and CD103 only. Thus we first aimed to reexamine the DC subsets present in the lung and MLN during IAV infection with more precision using a gating strategy including CD64 and MAR-1. Similar to steady-state mice, this analysis revealed three DC subsets to be present in the lungs of mock-infected mice (Fig 1A): CD103$^+$ cDC1s, CD11b$^+$ cDC2s which made up almost half of the DC population, and CD11b$^+$ CD64$^+$ MCs which were the smallest fraction of pulmonary DCs (Fig 1B). At 4 days post infection (dpi) with IAV, the proportion of cDC1s and cDC2s was decreased while the proportion of MCs was significantly increased (Fig 1B). However, also a previously undescribed DC population within the CD11b$^+$CD11c$^+$MHCII$^+$ cells expressing intermediate levels of CD64 (CD64^{int}) and MAR-1 (MAR-1$^+$) was identified (Fig 1A), which we termed MAR-1$^+$ DCs. Following viral clearance all DC populations returned to baseline and MAR-1$^+$ DCs were no longer identifiable in the lung (Fig 1C).

To assess if this MAR-1$^+$ DC population migrated to the draining lymph node, the same gating strategy was applied to the MHCII^{hi} DCs of the MLN. After mock-infection, analysis of the MLN revealed a slight increase in cDC2s compared with cDC1s while no MCs or MAR-1$^+$ DCs were found (Fig 1D,E). However, at 4dpi with IAV, while again no MCs were identified, there was a significant increase in both cDC1s and cDC2s and a substantial population of MAR-1$^+$ DCs. Indeed these MAR-1$^+$ DCs represented over half of the total DC population at 4dpi (Fig 1D,E). Similar to the situation in the lung, cDC1s, cDC2s and MAR-1$^+$ DCs returned to baseline levels following viral clearance (Fig 1F).

Interestingly, the generation of MAR-1⁺ DCs was not unique to infection with IAV. When mice were infected with a low dose of the murine paramyxovirus pneumonia virus of mice (PVM), MAR-1⁺ DCs in the lung and MLN were similarly detected(Fig 1G) from 8dpi with PVM onwards.
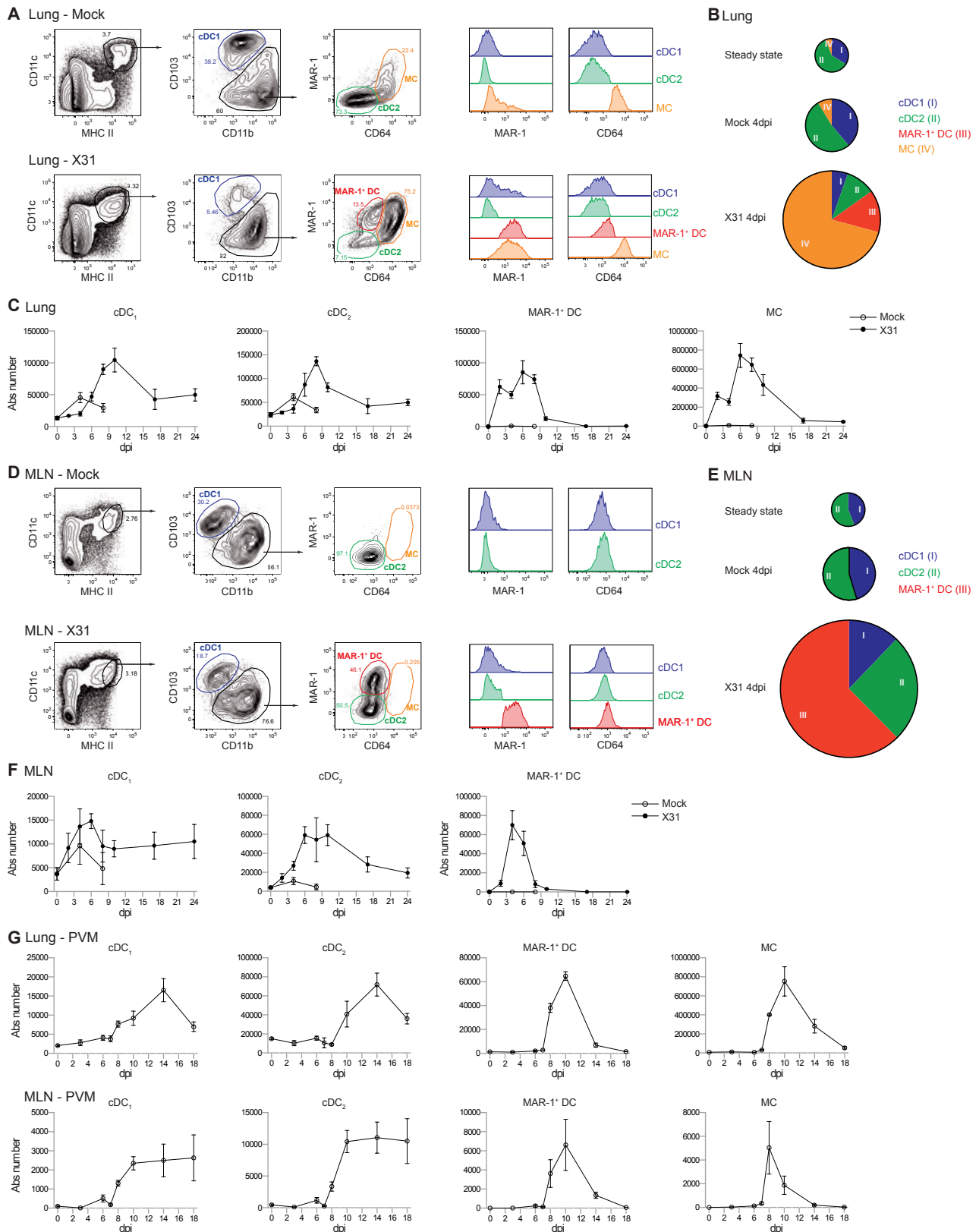
Figure 1 - MAR-1+ CD11b+ DCs are induced after influenza infection
(A) Representative flow cytometry plots showing gating of lung CD11c+ MHCII+ DC subsets 4dpi with X31 virus (bottom panel) compared with mock-infected controls (upper panel). Plots are pre-gated on single, live, CD3-CD19- cells. Histograms show expression of MAR-1 and CD64 on the different CD11c+ MHCII+ DC subsets. (B) Pie charts showing distribution of lung DC subsets in steady state conditions and 4dpi with mock or X31 virus as percentage of living DCs. The size of the pie is proportional to the total amount of DCs in the lung. (C) Kinetics of different lung DC subset numbers after mock (white circles) or X31 (black circles) infection. (D) Representative flow cytometry plots showing the gating strategy used to define MHCII[hi] DC subsets in the mediastinal lymph node (MLN) in mock (upper panel) and X31 virus (bottom panel) infected mice at 4dpi. Plots are pre-gated on single, live, CD3-CD19- cells. Histograms show expression of MAR-1 and CD64 on the different CD11c+ MHCII[hi] DC subsets. (E) Pie charts showing distribution of MHCII[hi] DC subsets in steady state and 4dpi with mock or X31 virus in the MLN as percentage of living MHCII[hi] DCs. The size of the pie is proportional to the absolute number of MHCII[hi] DCs in the MLN. (F) Kinetics of different MHCII[hi] DC subset numbers in the MLN after mock (white circles) or X31 (black circles) infection. (G) Kinetics of different lung (upper panel) and MHCII[hi] MLN (lower panel) DC subset numbers after PVM infection. Data are representative of at least two independent experiments with 4-6 mice per group.

MAR-1+ DCs are bona fide cDCs

As MAR-1 is a marker typically used to define monocyte-derived cells, including the cells historically known as MoDCs[28], we next questioned whether the MAR-1+ DC population was of monocytic or pre-cDC origin, like cDC1s and cDC2s. To examine this directly, pre-cDCs were sorted from the bone marrow of wild-type CD45.2 mice, labeled with a cell tracker dye and transferred into CD45.1/CD45.2 mice at 1dpi with IAV. The expression of the congenic marker within the DC subsets was analyzed 4 days later (Fig S1, Fig 2A). The transferred pre-cDCs gave rise to cDC1s, cDC2s and the MAR-1+ DC population, but not to the CD64+MAR-1+ MC population (Fig 2A). To determine if the origin of MAR-1+ DCs was restricted to cDC-committed progenitors, we next examined the dependence of this population on the chemokine receptor CCR2 using *Wt:Ccr2-/-* mixed BM chimeras. CCR2 is required on monocytes for the egress from the BM to the blood and hence CCR2 dependency is often used to define monocyte origin. Analysis of the DC populations in these chimeras revealed MAR-1 DCs to be CCR2 dependent but this dependence was less pronounced than for the MC population (Fig S2). As it has recently been reported that some cDCs can also be CCR2-dependent[30] we next assessed whether MAR-1+ DCs could also derive from monocytes. Monocytes were sorted from the bone marrow of CD45.1 wild-type mice, labeled with a cell tracker dye and transferred into CD45.2 *Ccr2-/-* mice at 1dpi. The expression of the congenic markers on DC subsets was analyzed 4 days later (Fig S1, Fig 2B). *Ccr2-/-* mice were used as hosts to give the transferred monocytes a competitive advantage over the endogenous population aiding their identification following transfer. Four days after the monocyte transfer, the transferred cells were present exclusively in the CD11b+ gate in the lung and within this gate only in the CD64+ MAR-1+ MC gate (Fig 2B). Thus MAR-1+ DCs generated during IAV infection are strictly derived from pre-cDCs and hence represent a bona fide population of cDCs. No transferred monocyte-derived cells could be identified in the MLN indicating that MCs do not migrate to the draining LN at this time point after infection.
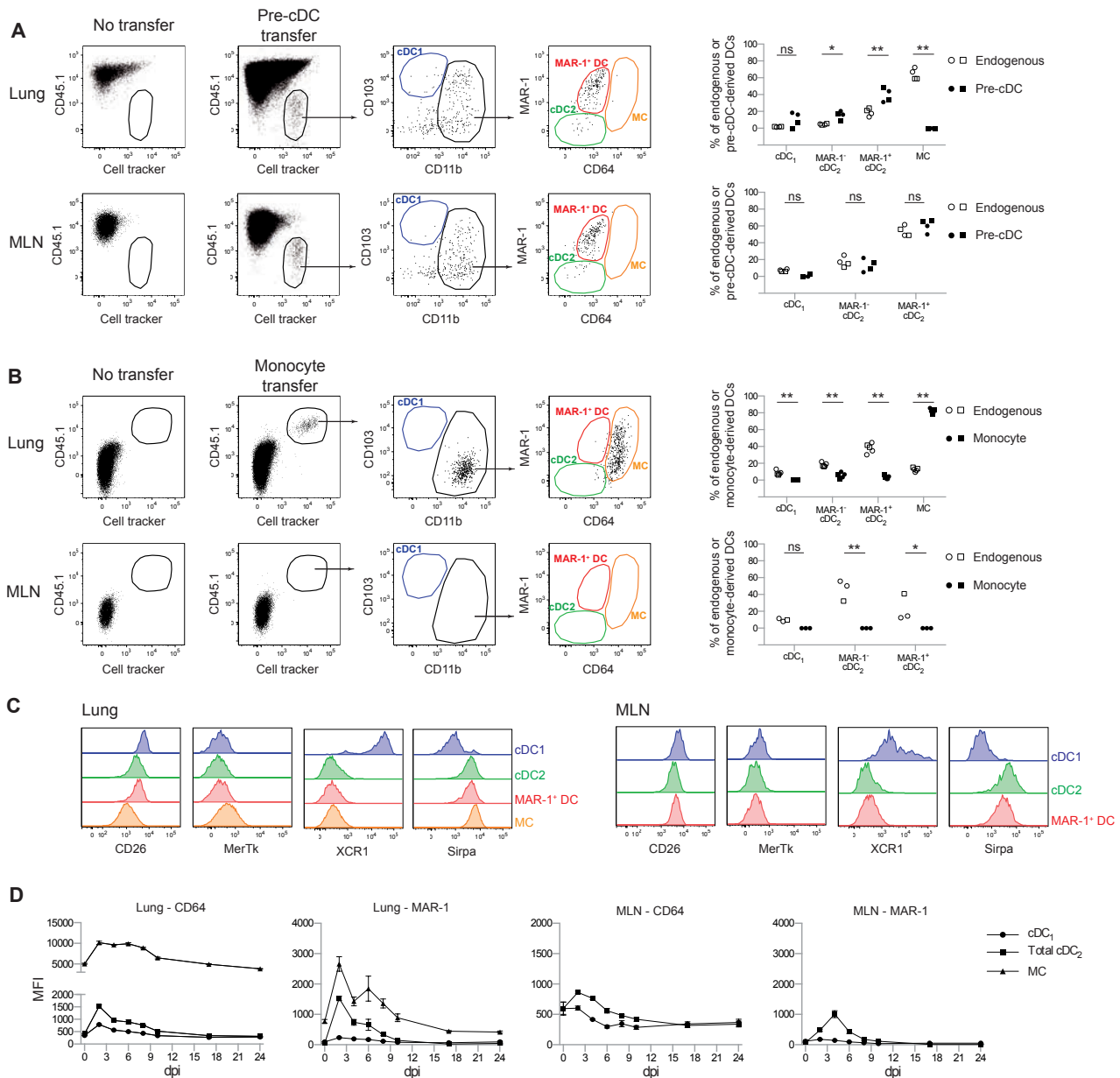
Figure 2 - MAR-1+ CD11b+ DCs are of conventional origin

(A) CD45.2 pre-cDCs were sorted from eFl450+ cell tracker labeled bone marrow and transferred i.v. into CD45.1/CD45.2 wild-type recipient mice at 1dpi with IAV. 4 days later donor cells were identified in the lung (upper panel) and MLN (lower panel) as cell tracker+ CD45.1- cells and were subsequently examined for CD103 and CD11b expression and for CD64 and MAR-1 expression. The graphs represent the distribution of the different DC subsets in the endogenous population (white) and the pre-cDC derived population (black). Data are pooled from 2 independent experiments (circles and squares) with 4 transferred mice in total are analyzed with a Two-way ANOVA with Sidak correction for multiple comparisons. The plots showing transferred pre-cDCs in the MLN is a pooled sample of two transferred mice. (B) CD45.1/CD45.2 monocytes were sorted from eFl450+ cell tracker labeled bone marrow and transferred i.v. into CD45.2 CCR2-/- recipient mice at 1dpi. 4 days later donor cells were identified in the lung (upper panel) and MLN (lower panel) as cell tracker+ CD45.1+ cells and were subsequently examined for CD103 and CD11b expression and for CD64 and MAR-1 expression. The graphs represent the distribution of the different DC subsets in the endogenous population (white) and the monocyte-derived population (black). Data are from 2 independent experiments (circles and squares) with 5 transferred mice in total are analyzed with a Two-way ANOVA with Sidak correction for multiple comparisons. (C) Representative histograms showing expression of CD26, MerTk, XCR1 and SIRPα on lung and MLN DC subsets at 4dpi with IAV. Data are representative of 2 independent experiments with 4-6 mice per group. (D) Expression of CD64 and MAR-1 on lung and MLN DC subsets over time, expressed as MFI.

* = p ≤ 0,05; ** ≤ 0,01; ns = not statistically significant.

As CD26 and MerTk have recently been described by the Immgen consortium to be conserved in cDCs and macrophages respectively[31-33] we next stained for these markers in the lung and MLN during IAV infection to assess their expression on MAR-1+ cDCs compared with the other DC populations. Correlating with their pre-cDC origin, MAR-1+ cDCs expressed high levels of CD26 and low levels of MerTk, similar to cDC1s and cDC2s (Fig 2C). Conversely and fitting with their distinct origin, MCs expressed intermediate levels of both CD26 and MerTk (Fig 2C). Expression of CD11b is not enough to categorize DCs as cDC2s as CD11b can be induced on DC subsets, like pDCs as part of an activation module[34]. XCR1 and SIRPα (CD172a) have been identified as conserved surface markers that can be used to identify cDC1s and cDC2s respectively throughout different tissues and species[29, 33, 35, 36]. MAR-1+ cDCs in the lung and MLN expressed SIRPα but did not express XCR1 identifying them as members of the cDC2 lineage and further described as MAR-1+ cDC2s (Fig 2C). As MAR-1 expression, and to a lesser extent CD64 expression, was induced over time on the total cDC2 population (i.e. MAR-1- cDC2s and MAR-1+ cDC2s) and on the MCs and returned back to baseline levels once the infection is cleared, this suggests that MAR-1 and CD64 may represent temporal activation markers of cDC2s (Fig 2D).

MAR-1+ cDC2s represent a hyper activated state of cDC2s

To further characterize the MAR-1+ cDC2s, DC subsets were sorted 4 days post mock and IAV infection and were subjected to a micro-array analysis. By performing a principal component analysis (PCA, Fig 3A), the first principal component (PC1) corresponded to the ontogeny of the DCs whereas the second principal component (PC2) corresponded the activation status (mock versus IAV). The MAR-1+ cDC2s appeared to be in a more extreme activation state than MAR-1- cDC2s at 4dpi IAV infection. This was also confirmed when the 30 most upregulated genes and the 30 most downregulated genes between mock cDC2s and MAR-1+ cDC2s were plotted on a heat map (Fig S3A). MAR-1- cDC2s from IAV infected mice always showed an intermediate gene expression profile between MAR-1- cDC2s from mock infected mice and MAR-1+ cDC2s from IAV infected mice. In a next step we sought to identify unique genes that were expressed in the different DC subsets, using a novel triwise comparison algorithm[37]. Therefore the gene expression profiles from the 3 different subsets were plotted on a triwise plot representing total gene expression under mock or IAV condition (Fig 3B). Even in mock condition hardly any cDC2 specific genes could be defined, while *Xcr1*, *Clec9a* and *Irf8* were highly specific for cDC1s and *Mertk*, *Emr1* (F4/80) and *Fcgr1* were specific for MCs. The cDC2s rather shared most genes with either cDC1s (*Dpp4* (CD26) and *Zbtb46*) or MCs (*Sirpa*). Furthermore cDC1s shared almost no genes with MCs whereas they did share genes with their conventional cDC2 counterparts.

To determine which genes are differentially expressed upon IAV infection, and which genes remained reliable identifiers of cDC subsets, a pairwise comparison of cDC1s, cDC2s and MCs in mock versus X31 condition was made and the differentially expressed genes were plotted on a triwise plot representing the total gene expression profile 4dpi with IAV (Fig 3C). Only a very low number of genes were upregulated in
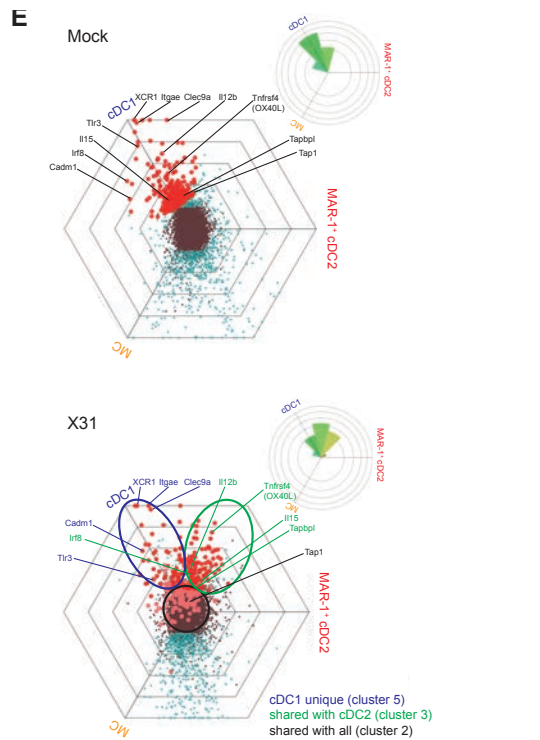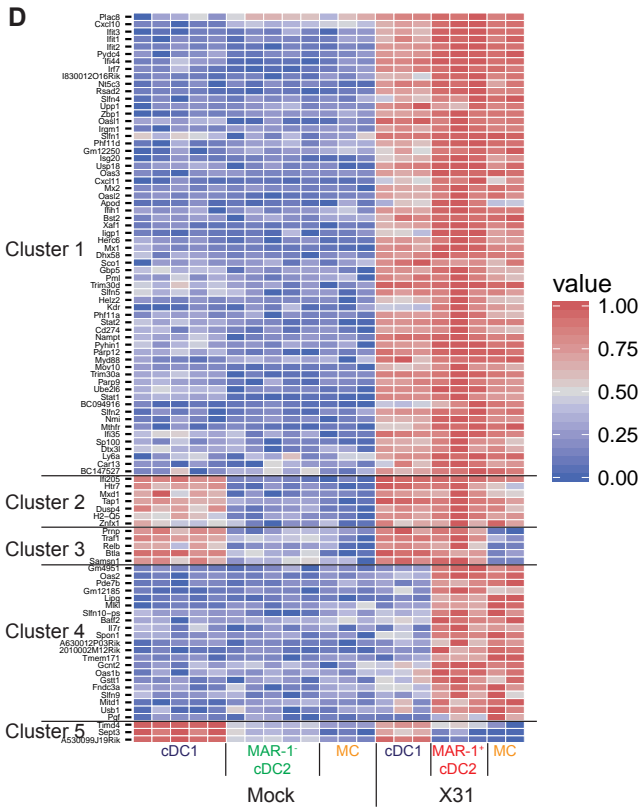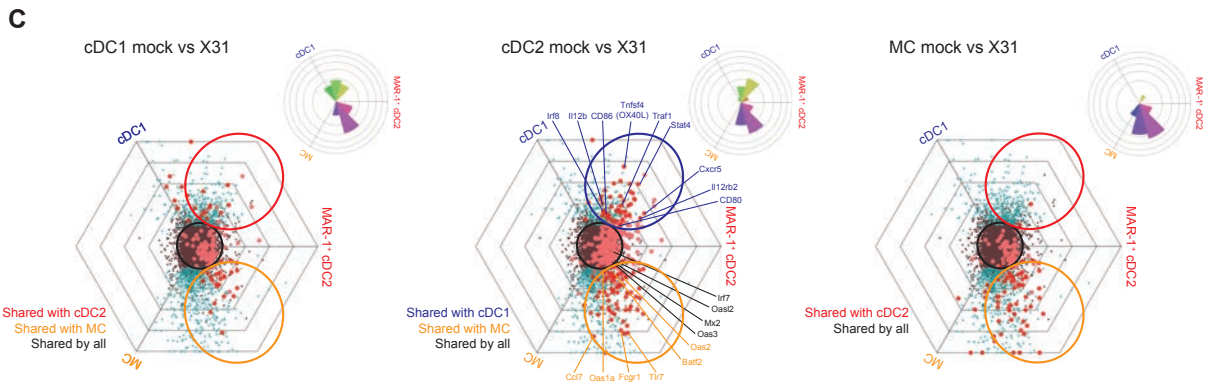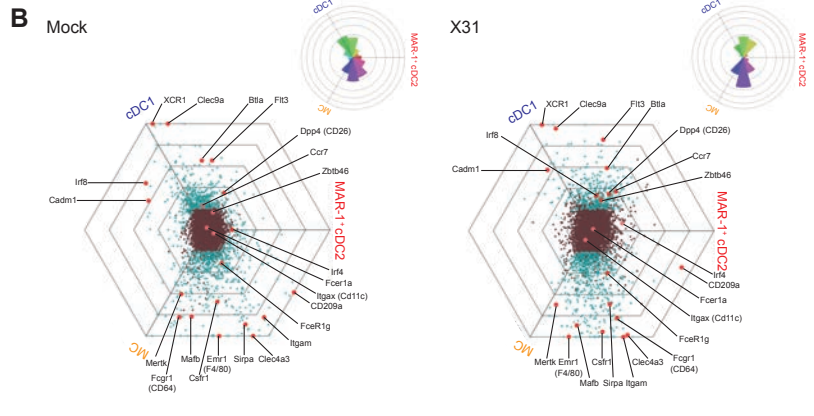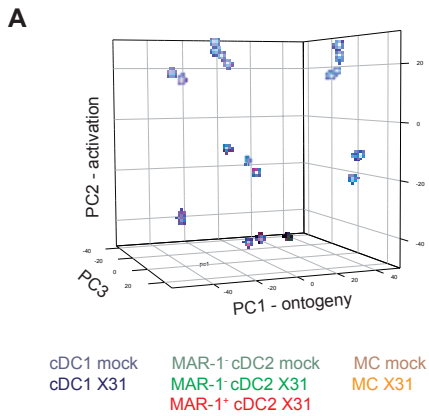
only one cellular subset. The genes upregulated in cDC1s were mostly shared with cDC2s and MCs during IAV infection. For cDC2s the upregulated genes were either shared by cDC1s (e.g. *Irf8, Il12b, Il12rb, Cd80, Cd86, Tnfsf4*), MCs (e.g. *Cxcl10, Cxcl11, Fcgr1, Ccl7, Tlr7, Batf2*) or all subsets. The genes shared with cDC1s were classified by Ingenuity pathway analysis (IPA) to belong to the canonical pathways involved with T helper cell differentiation, DC maturation and IL-12 signaling (Fig S3B). In contrast, the shared genes with MCs were classified in the canonical pathways linked with recognition of viruses by pattern recognition receptors (PRR), IFN induction, and activation of IRF by cytosolic PRR and RIG-I like receptors (Fig S3C). This suggests that MAR-1+ cDC2s acquired a hybrid phenotype sharing typical cDC1 and MC gene modules. The differentially expressed genes in MCs were shared with all the subsets or shared with cDC2 DCs but not with cDC1s. However, most of the genes that were upregulated in the different subsets upon infection were mostly shared by all the subsets. This common "influenza signature" in the DCs consists mainly of type I IFN genes (e.g. *Ifit* family, *Irf7, Oas* family, *Mx2, Stat1*) (Fig 3D, cluster 1).

Another cluster of genes represented the genes that are specifically expressed in mock cDC1s, but are acquired by cDC2 and/or MCs but caution should be taken as these genes are separated into 3 different clusters upon IAV infection (Fig 3E): only a small fraction of surface markers remained cDC1 specific (e.g. *Xcr1, Cadm1, Clec9a, Tlr3* and *Itgae*; cluster 5). The remainders of genes were either also expressed by cDC2s (e.g. *Irf8, Il12b, Il15, Stat4* and *Tnfrsf4*; cluster 3) or by both cDC2s and MCs and became shared by all DC subsets (e.g. *Tap1, Bcl2l1, Slfn1*; cluster 2). This indicates that caution should be taken when studying DCs during inflammatory conditions.

Despite the fact that MAR-1+ cDC2s were FACS-purified on the basis of expression of the MAR-1 epitope of FcεR1, the gene, *Fcer1a*, was not identified as being differentially expressed between MAR-1- and MAR-1+ cDC2s and also appeared not to be specifically expressed in MCs (Fig 3B). As we found this quite surprising, we next sought to investigate if the MAR-1 antibody was indeed specifically staining FcεR1 on DCs and MCs. To this end, we infected WT and *Fcεr1*-/- mice with IAV and examined MAR-1 expression by the DC subsets. Despite lacking FcεR1, MAR-1+ cDC2s were readily identifiable in the lung and MLN of *Fcεr1*-/- mice (Fig S4). Furthermore, there was no difference in either their proportion or number compared with WT controls. This demonstrates that the MAR-1 antibody also recognizes another surface protein than

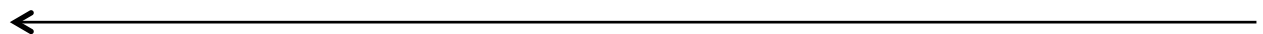←———————————————————————————————————————

Figure 3 - Gene expression changes dramatically upon IAV infection

(A) PCA analysis of lung DC subsets 4dpi mock (lighter colors) or IAV (darker color) infection. (B) To visualize differential gene expression between the DC subsets after mock (left) and IAV infection (right) each gene was plotted in a hexagonal triwise diagram in which the direction of a point represents an up-regulation in ore or two populations, whereas the distance from the origin represents the magnitude of this up-regulation. Genes that are >32 fold differentially expressed are plotted on the outer grid line. Rose diagrams (top right) show the percentage of genes in each orientation. Brown dots represent genes that are not differentially expressed. Green dots represent statistically significant differentially expressed genes. (C) Triwise diagrams of the gene expression after IAV infection. Red dots represent the upregulated genes in cDC1s (left), cDC2s (middle) and MCs (right) after IAV infection. Rose diagrams (top right) show the percentage of the red dots in each orientation. (D) Heat maps showing relative expression of differentially expressed genes between DC subsets from mock and IAV infected mice. (E) Triwise diagrams of the gene expression after mock (upper diagram) and IAV (lower diagram) infection. The red dots represent the genes that are cDC1 specific in mock conditions. Rose diagrams (top right) show the percentage of the red dots in each orientation.

the FcεR1 expressed on mast cells and basophils. The precise nature of this epitope in cDC2s and MC remains enigmatic.

One major observation in the micro-array analysis was that *Irf8* expression, which was previously shown to be a terminal selector of the cDC1 lineage[38], shifted during IAV infection towards a shared expression pattern with cDC2s (Fig 3B,E). We next aimed to confirm these differences at the protein level. To this end, intracellular IRF4 and IRF8 were stained in all DC subsets in the lung and MLN during IAV infection. This analysis confirmed that both MAR-1^{-} and MAR-1^{+} cDC2s upregulated IRF8 after IAV infection while retaining IRF4 expression (FIG 4A), but the MAR-1^{+} cDC2s expressed higher levels of IRF8 than their MAR-1^{-} cDC2 counterparts. Other than in the lung, where the expression of IRF8 in MAR-1^{+} cDC2s remained modest compared to the expression in cDC1s (Fig 4A), IRF8 expression by MAR-1^{+} cDC$_2$ DCs in the MLN was further increased and almost equaled the IRF8 expression level of cDC1s (Fig 4B).

As the transcription factor IRF8 is induced in cDC2s during IAV, we next addressed whether IRF8 drove the expression of MAR-1 on cDC2s. *Irf8*^{fl/fl} x *Cd11c* Cre mice that lack IRF8 specifically in CD11c-expressing cells were infected with IAV and DC subsets were analyzed at 4dpi. The transgenic mice indeed showed a loss of cDC1s consistent with previously published data[1, 38-42], however, no reduction in the MAR-1^{-} or MAR-1^{+} cDCs in the lung and MLN compared with Cre[-] littermate controls was observed (Fig 4C,D). Thus, unlike cDC1s, MAR-1^{+} cDC2s are not dependent upon IRF8 for their development.

MAR-1[+] cDC2s acquire expression of IRF8 in a type I IFN dependent manner
Contrary to cDC1s requiring IRF8 for their development, cDC2s have been shown to require IRF4 for their terminal differentiation, survival and migration[4, 5, 43-46]. Thus we also examined the prevalence of the distinct DC subsets in *Irf4*^{fl/fl} x *Cd11c* Cre mice, which lack IRF4 specifically in CD11c-expressing cells. While lack of IRF4 in CD11c^{+} cells resulted in a reduction of CD24^{+} cDC2s in the lung and a severe reduction of migratory cDC2s in the MLN in the steady state[1], this dependence on IRF4 was overcome during the inflammatory setting of IAV infection where the proportion and absolute numbers of all of the conventional DC subsets was similar to that in Cre[-] littermate control mice (Fig 4E and F). Thus IRF4 is dispensable for development, survival and migration of cDC2s during inflammation.

Based on the micro-array data, IPA analysis suggested that IRF8, IFNAR, STAT1, IRF3 and IRF7 were potential upstream regulators of the differential gene expression between cDC2s in mock condition and MAR-1^{+} cDC2. A deeper analysis of the canonical pathways linking these upstream regulators showed that IFNAR signaling could be the initiating factor (Fig S3E). To validate this, *Ifnar*^{-/-} mice were infected with IAV and DC subsets were quantified at 4dpi. This analysis revealed that MAR-1^{+} cDC2s were almost completely absent in *Ifnar*^{-/-} mice (Fig 5A) demonstrating their dependence on type 1 IFN signaling. Additionally, the intensity of MAR-1 expression was found to be decreased within the MC population, showing that IFNAR-signaling is involved in inducing MAR-1 expression on all DC subsets, also those derived from monocytes and sharing macrophage characteristics (Fig 5A). Conversely, MAR-1^{-} cDC2s were increased in both
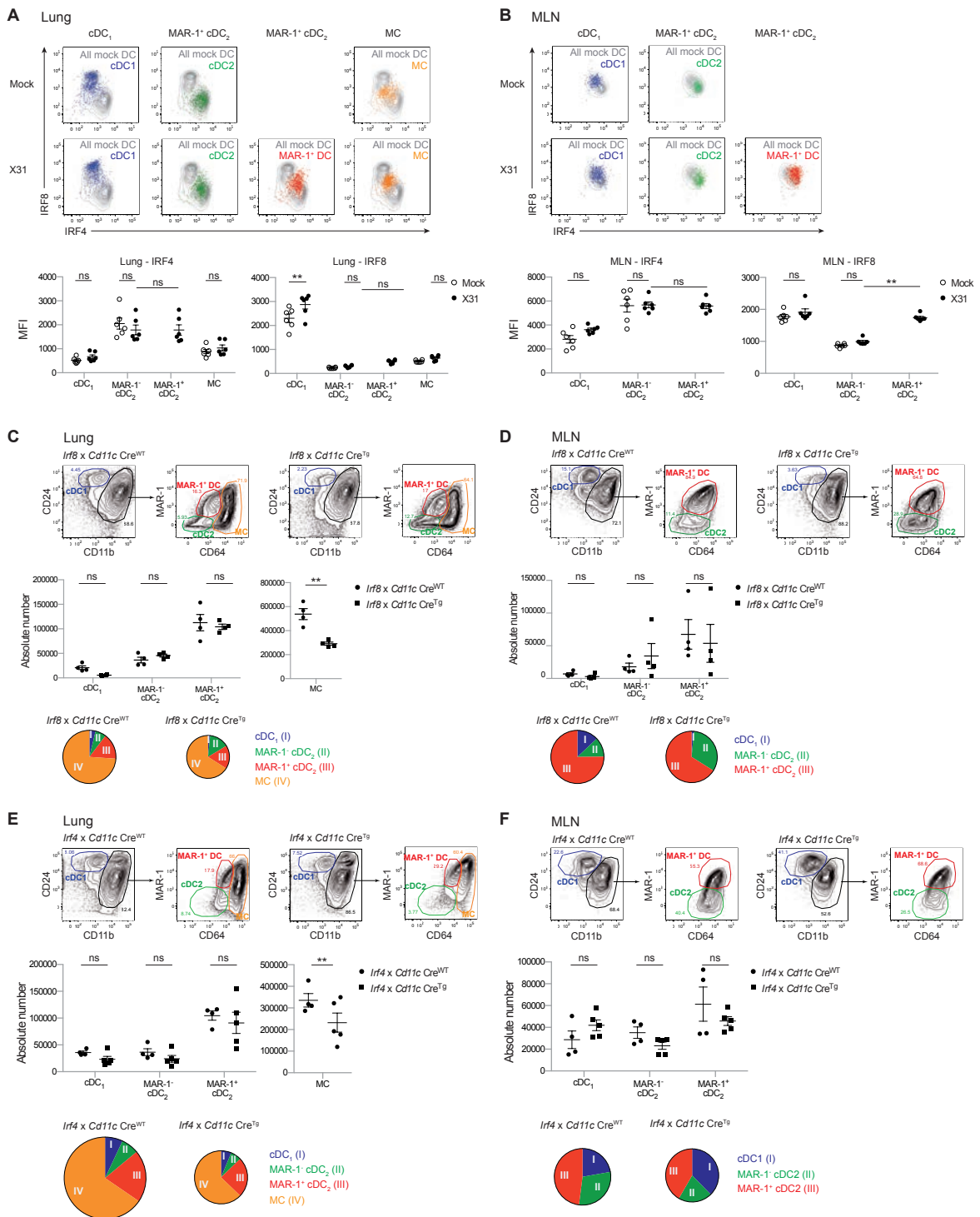
Figure 4 - MAR-1+ cDC2s express IRF8 but do not depend on IRF8 or IRF4 for their induction

(A,B) Representative flow cytometry plots showing IRF4 and IRF8 expression (upper panel) and pooled MFIs (bottom panel) in (A) lung and (B) MLN DC subsets at 4dpi with IAV compared with mock infected controls. Data are representative for 3 independent experiments with 3-6 mice per group and are analyzed with a Two-way ANOVA with Tukey correction for multiple comparisons. (C,D) Representative flow cytometry plots (upper panel), absolute numbers (middle panel) and distribution of DC subsets as a % of live DCs (lower panel) in the (C) lungs and (D) MLN of *Irf8* x *Cd11c* Cre[WT] and *Irf8* x *Cd11c* Cre[Tg] mice 4dpi with IAV. The size of the pie chart is proportional to the total amount of DCs. Data are representative for 2 independent experiments with 4-5 mice per group. (E,F) Representative flow cytometry plots (upper panel), absolute numbers (middle panel) and distribution of DC subsets as a % of live DCs (lower panel) in the (E) lungs and (F) MLN of *Irf4* x *Cd11c* Cre[WT] and *Irf4* x *Cd11c* Cre[Tg] mice 4dpi with IAV. The size of the pie chart is proportional to the total amount of DCs. Data are representative for 2 independent experiments with 3-6 mice per group and are analyzed with a Two-way ANOVA with Sidak correction for multiple comparisons.

* = p ≤ 0,05; ** ≤ 0,01; ns = not statistically significant.
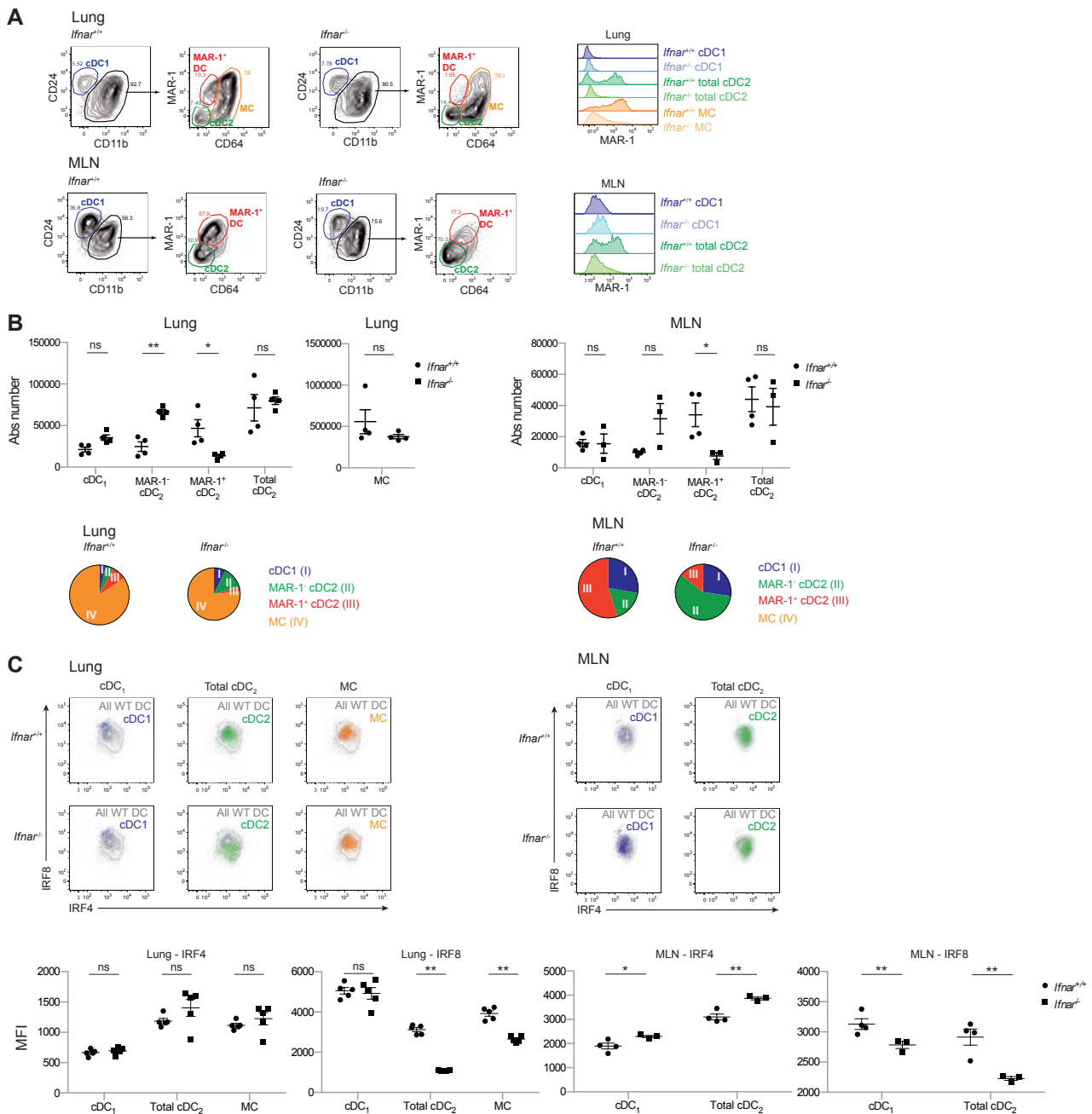
Figure 5 - IFNAR signaling is necessary for the generation of MAR-1⁺ cDC2s and their expression of IRF8

(A) Representative flow cytometry plots showing identification of DC subsets in the lung (upper panel) and MLN (lower panel) in *Ifnar⁺/⁺* and *Ifnar⁻/⁻* mice 4dpi with IAV. Representative histograms showing expression of MAR-1 on *Ifnar⁺/⁺* and *Ifnar⁻/⁻* DC subsets 4dpi with IAV. Data are representative for 3 independent experiments with 4-5 mice per group. (B) Absolute number (upper panel) and distribution as a percentage of live DCs (lower panel) of DC subsets lung and MLN of *Ifnar⁻/⁻* and *Ifnar⁺/⁺* mice 4dpi with IAV. Data are representative for 3 independent experiments with 4-5 mice per group. (C) Expression of IRF4 and IRF8 by DC subsets in lung and MLN of *Ifnar⁻/⁻* and *Ifnar⁺/⁺* mice 4dpi with IAV. Upper panel shows representative flow cytometry plots. Bottom panel shows MFIs. Data are representative for 2 independent experiments with 4-5 mice per group and are analyzed with a Two-way ANOVA with Sidak correction for multiple comparisons. * = p ≤ 0,05; ** ≤ 0,01; ns = not statistically significant.

proportion and number in the lung and MLN which lead to an unchanged total amount of cDC2s (Fig 5B). Additionally, we confirmed that type I IFN signaling is an upstream regulator of IRF8, as IRF8 expression was significantly reduced in the cDC2s in *Ifnar⁻/⁻* mice compared with WT controls (Fig 5C).

MAR-1+ cDC2s gain the capacity to stimulate CD8 T cells

Given the observation that expression of *Cd80*, *Cd86*, *Il12* and *Irf8* was induced in MAR-1+ cDC2 DCs during IAV infection (Fig 3C) and that these genes are typically linked to antigen presentation, we sought to address the functional capacity of the IRF8^{hi} MAR-1+ cDC$_2$ DCs. Therefore the WSN-OVA virus, encoding the OVA$_{257-264}$ Kb restricted MHC I epitope in the neuraminidase gene, was used to infect wildtype mice. DC subsets were sorted from the MLN at 4dpi and put in coculture with CFSE-labeled CD8+ OT-I T cells. After 4 days of coculture, the cells were restimulated for 5 hours and CFSE dilution and production of IFNγ and Granzyme B were examined by flow cytometry. In the MLN, cDC1s were found to induce strong proliferation of OT-I T cells. Strikingly, MAR-1+ cDC2s also induced proliferation of OT-I T cells, whereas MAR-1- cDC2s only induced a modest degree of proliferation (Fig 6A). The same results were obtained with lung DC subsets, but MCs did not induce T cell proliferation (data not shown). These results were further reflected in the cytokine production of responding T cells; MAR-1+ cDC2 DCs had the tendency to induce stronger IFNγ and Granzyme B production than MAR-1- cDC2 DCs, although to a lesser extent than cDC$_1$ DCs (Fig 6B). In contrast to an enhanced presentation to OT-I CD8+ T cells, the capacity of cDC2s to present antigen to CD4+ OT-II cells was diminished when they acquired the MAR-1+ state (Fig 6C). These results suggest that MAR-1+ cDC2s acquire an intermediate phenotype in which they can acquire cDC1 functions depending on the environmental milieu.

Examination of expression of the T cell costimulatory molecules CD80 and CD86 at protein level by flow cytometry showed that all DC subsets were activated by IAV infection and moreover that MAR-1+ cDC2s are in a hyper activated state compared to classical MAR-1- cDC2s potentially explaining their enhanced ability to cross-present antigen to naïve OT-I cells (Fig 6C). In addition to the increased expression of maturation markers, a significant increase was observed in the proportion of MAR-1+ cDC2s producing IL-12 compared with MAR-1- cDC2s after 5 hours of ex vivo restimulation (Fig 6C).

Other genes involved in presentation in the MHCI pathway such as *Tapbpl* were induced in MAR-1+ cDC2 after IAV, whereas this is a gene normally expressed higher in cDC1s (Fig 3E). To delineate the underlying signaling cascade responsible for this hyper mature state of MAR-1+ cDC2s we assessed the maturation state and IL-12 production capacity in *Ifnar-/-* and *Irf8 x Cd11c* Cre mice. This revealed that in addition to inducing MAR-1 expression on cDC2s, IFNAR signaling was also required to induce the hyper-mature state of cDC2s, with MLN cDC2s from *Ifnar-/-* mice expressing less CD80, CD86 and IL-12 compared with WT controls (Fig 6E). Similarly, the increased IRF8 expression observed on MAR-1+ cDC2s was also essential to induce this hyper mature state as this was not induced in MAR-1+ cDC2s from *Irf8 x Cd11c* Cre MLNs (Fig 6F).

Thus taken together, these data demonstrate that type 1 IFN signaling during IAV infection leads to the generation of a MAR-1+ cDC2 which upregulates IRF8 causing them to be hyper-activated and acquiring an enhanced ability to induce CD8+ T cell proliferation rather than CD4+ T cell proliferation.
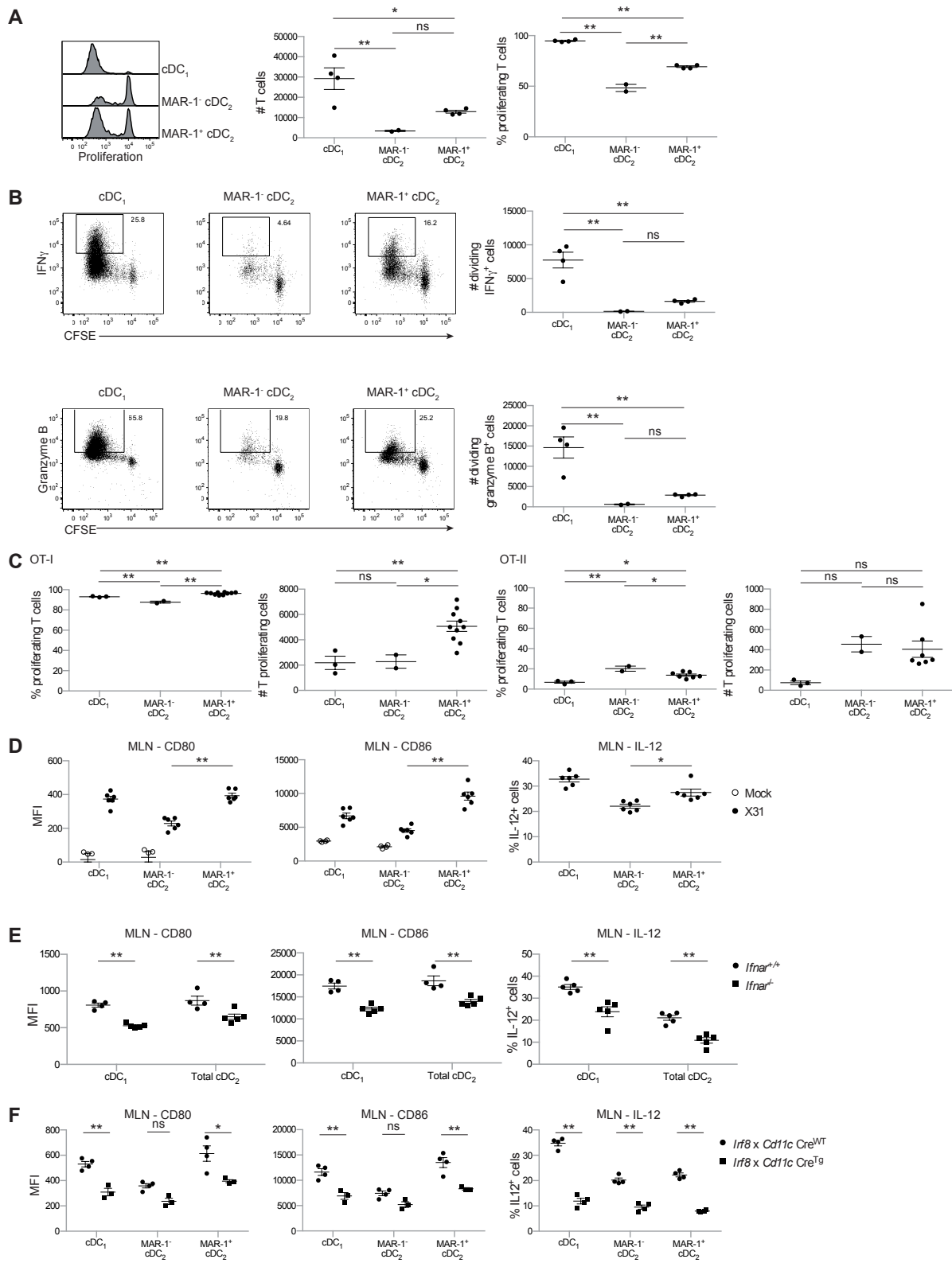
Figure 6 - MAR-1+ cDC2s represent a hyper-activated cDC subset

(A) Proliferation profile (left) and absolute number of T cells (right) following 4 days of coculture of CFSE-labeled OT-I T cells and DC subsets sorted from the MLN of WSN-OVA infected mice. (B) Representative flow cytometry plots showing IFNγ (upper row) and Granzyme B (lower row) expression by OT-I T cells following 4 days of coculture with DC subsets from MLN. Data are representative for 2 independent experiments with DC subsets sorted from 10-15 mice per group. Graphs with amount of cells show the individual replicates from 1 experiment. Data are analyzed by a One-way ANOVA and Tukey correction for multiple comparisons. (C) Percentage and absolute number of proliferating T cells following 5 days of coculture of eFl450 proliferation dye labeled OT-I (left) or OT-II (right) cells and DC subsets sorted from the MLN of X31 infected mice together with 10ug/ml ovalbumin. Data are analyzed by a One-way ANOVA and Tukey correction for multiple comparisons. (D) Expression of CD80 and CD86 expressed as MFI on MHCIIhi DC subsets from the MLN and % of MHCII hi MLN DCs expressing IL-12 after 6h of restimulation in X31 virus infected mice compared with mock-infected controls at 4dpi. (E-F) Same analysis for Ifnar+/+ and Ifnar-/- (panel E) and Irf8 x Cd11c CreWT and CreTg mice (panel F). Data are representative for 2 independent experiments with 3-4 mice per group and are analyzed with a Two-way ANOVA with Sidak correction for multiple comparisons. * = p ≤ 0,05; ** ≤ 0,01; ns = not statistically significant.

Discussion

Previously published data about the division of labor between different DC subsets during IAV infection often led to results that are inconclusive and hard to compare. The variability between the used strains, the infectious dose, the route of administration and the timing of the read-outs is one major influencing factor. Next to this, the strategy to define the different DC subsets lacked consistency between different reports[14, 21, 23, 24, 47]. In addition, until recently no flow cytometry staining strategy was available to study the different subsets without contamination, as CD11b+ DCs were a mixture of conventional and monocyte-derived DCs due to the lack of good monocyte markers. Therefore, we applied a new gating strategy based on expression of MAR-1 and CD64 to define the monocyte-derived compartment of the CD11b+ gate (MC). MCs expanded during infection and became the majority of the pulmonary DC population 4dpi with IAV while they were absent in the MHCIIhi DCs in the MLN, suggesting that they do not migrate from the lung to the MLN. Remarkably, we also identified a CD11b+ DC with intermediate expression of CD64 and MAR-1 in mice infected with IAV and PVM. It has been shown that infection with Sendai virus also induces expression of MAR-1 on lung DCs, and MAR-1 was proposed by these authors to stain specifically the alpha chain of the FcεRI receptor[47]. In our hands however, MAR-1 staining was also observed on lung MCs and cDC2s of Fcerl-/- mice. Which protein is causing the cross-reaction with the MAR-1 antibody on DCs and MCs is a matter of intense study in our lab.

Transfer of pre-cDCs and monocytes pointed out that MAR-1+ CD64int DCs represented bona fide cDCs as they developed from pre-cDCs but not monocytes and they shared the expression pattern of CD26 with MAR-1- cDC2 DCs, while lacking expression of MerTk found on macrophages and MCs. Despite the fact that MAR-1+ DCs were pre-cDC derived, they were partially CCR2 dependent. Together with the description of a conventional DC in the gut that is partially CCR2 dependent[30] this shows that caution should be taken when Ccr2:Wt mixed chimeras are used to define the monocyte origin of a cell population. MAR-1+ cDC2s expressed Sirpα on their surface, but no XCR1 just like MAR-1- cDC2s. MAR-1+ cDC2s can thus be seen as a special activation status of the cDC2 population.

The induction of MAR-1 is dependent on IFNAR signaling since no MAR-1+ population could be detected within the cDC2 gate in Ifnar-/- mice, as was previously reported by others as well[47]. Analysis of the gene expression data obtained by micro-array pointed

out that a strong type I IFN signature was induced upon IAV infection in the 3 different DC subsets. It is however not clear which cell type is the source of the IFN as many cells can produce it. Given the anatomical structure of the lung environment, epithelial cells are one of the first targets of IAV infection[48]. *In vitro* infection of murine tracheal epithelial cells leads to induction of IFNβ, α4 and α5, but not other subtypes, rendering epithelial cells ideal candidate sources of type I IFN that on its turn can induce MAR-1 upregulation on cDC2s[49]. But amongst many other cell types, DCs can also produce type I IFNs themselves. In the blood of infected people, DCs appeared to be the highest producers of IFN[50]. Although it was previously thought that pDCs are responsible for much of the type I IFN production[51], more recent studies showed that cDCs also produce considerable amounts of type I IFNs[52, 53]. As we were limited to the use of full *Ifnar* deficient mice and could not deplete *Ifnar* in specific cell types, it remains to be elucidated if IFNAR signaling induces MAR-1 on cDC2s in a direct or indirect manner. In cDCs, type I IFN production after viral infection seems to occur in two distinct waves. The first wave is driven directly by viral recognition acting on TLR3, TLR7 or RIG-I like receptors. The second wave is driven by a feedback mechanism whereby type I IFN signaling induces IRF8, which subsequently further boosts type I IFN production[54].

Gene expression profiling suggested that transcription factor IRF8 was a possible upstream regulator of the differentially expressed genes when comparing mock cDC2s and IAV cDC2s. Furthermore, IRF8 got upregulated in cDC2s and acquired an expression pattern shared with cDC1s whereas in mock conditions IRF8 is specific for cDC1s. IRF4 and IRF8 are structurally very similar and therefore can compete for binding the same gene elements[55]. Potentially the ratio of IRF4 and IRF8 expression can determine a switch in cell development or differentiation. This concept of IRF4 and IRF8 being the driving forces behind the switch of phenotypes has been described before in other cell types. IRF4 is involved in the polarization of macrophages towards a M2 rather than a M1 phenotype[56, 57] and IRF4 is essential for the acquisition of Th1, Th2, Th9, Th17, Tfh and Treg phenotypes[15-20, 58]. In this process of polarization, IRF4 and IRF8 can also have counteracting functions. For example IRF4 regulates Rorγt, thereby promoting Th17 cell differentiation[11, 12] whereas IRF8 silences the Th17 differentiation by interfering with Rorγt and repression of IL-17 associated genes[13]. In B cell development expression of IRF8 and IRF4 mutually counteracts each other[59]. IRF4 and IRF8 expression is also involved in the cDC1-cDC2 lineage decision during DC development. IRF8 is expressed already in the common DC progenitor[60] whereas IRF4 expression only comes up in the pre-cDC stage and cDC2s only develop when IRF4 expression is induced. The final numbers of cDC2s and cDC1s most likely depend on the levels of IRF4 and IRF8 in the tissues[1].

In this paper, we describe that the expression of IRF4 and IRF8 are not limited to respectively cDC2s and cDC1s. We observed that IRF8 could be acquired by cDC2s in an inflammatory situation. This observation questions on its turn the dogma that different DC subsets are specialized in particular functions. According to this dogma, cDC1s are specialized in induction of CTLs, whereas cDC2s are specialized in induction of CD4 Th cells[61, 62]. We showed that by the acquisition of IRF8 a functional module leading to enhanced CD8+ T cell stimulation and proliferation was induced in cDC2s. This enhanced MHCI-dependent antigen presentation capacity was induced by an

induction of the maturation status of the MAR-1^{+} cDC2 as these DCs expressed higher levels of the activation markers CD80 and CD86 on their surface, and by acquisition of processing machinery necessary for MHCI presentation. Recently it was already argued that cDC2s are capable to induce CD8^{+} T cell responses on the condition that they are provided with a strong TLR7 signal[26]. Our micro-array data indicated that *Tlr7* is upregulated in cDC2s upon IAV infection, which can equip the cDC2s with a more MHCI prone machinery. Furthermore they have an enhanced capacity to produce IL-12 upon *ex vivo* restimulation. These changes in activity status are induced in a DC-intrinsic IRF8-dependent manner. Type I IFN signaling and IRF8 expression in cDC1s licenses them to produce IL-12 and thereby promote Th1 immune responses[63-68] while IRF4 can inhibit IL-12 production and skew the responses towards a Th2 profile[69-72]. Based on these data, it was somewhat surprising that we observed a decreased ability to present antigen to OT-II CD4^{+} T cells *in vitro* and to induce Th cell proliferation whereas the ability to present antigen to OT-I CD8^{+} T cells and to induce CTL proliferation was enhanced. Nevertheless, IL-12 has also been described to contribute to the proliferation and IFNγ production of cytotoxic T cells during influenza infection, but not their cytolytic activity[73, 74] and for memory CTL induction[75] which supports our observations in the *in vitro* cocultures. Recently it was shown that IRF4 is superior to IRF8 in providing DCs with the machinery to process antigen and present it in a MHCII-dependent manner to initiate T helper cell responses[2]. Given the capacity of IRF4 and IRF8 to bind the same gene elements, it can be hypothesized that the induction of IRF8 in cDC2s results in a competition with IRF4 for their binding partners resulting in less IRF4 able to activate the genes driving MHCII antigen presentation and thereby favoring IRF8 to activate the genes necessary for CTL induction.

All together, we have shown that during IAV infection cDC2s evolve towards a hybrid DC type that combines the capacity of cDC1s for CTL induction with the capacity of MCs for inducing an antiviral response. This shows the extraordinary flexibility of cDC2s, and their potential to react to environmental cues like type I interferons, and exploiting a gene module that is mainly used for development and function of cDC1s. It is possible that this flexibility is required during later phases of infection, when cDC1s tend to be depleted by IAV infection. Future studies will have to unravel the precise genes bound by IRF8 in cDC1s and activated cDC2s, to understand the full functional implications of our observations. We finally want to caution against the use of IRF8 as a universal lineage defining transcription factor for cDC1s that could be used to knock out this lineage. Our data clearly show that genetic deficiency of IRF8 selectively in DCs will greatly impact also on cDC2 function.

Materials and methods

Ethics statement
All experiments were approved by the independent animal ethical committee "Ethische Commissie Dierproeven – faculteit Geneeskunde en Gezondheids-wetenschappen Universiteit Gent" (identification number: ECD 14/49). Animal care and used protocols adhere to the Belgian Royal Degree of May 29th 2013 for protection of experimental animals which incorporates European guideline 2010/63/EU.

Mice
C57Bl/6 mice (6-10w) were purchased from Harlan Laboratories. CD45.1/2, OT-I Tg, OT-II Tg, $Fc\varepsilon RI^{-/-}$ and $IRF8^{fl/fl}$ x CD11c Cre mice were bred and housed in specific pathogen-free conditions at the animal facility of Ghent University. $Ifnar^{-/-}$ mice were kindly donated by Prof. Claude Libert.

Influenza virus infection
Mice were infected intranasally with 10^5 $TCID_{50}$ H3N2 X31 influenza A virus, 10^3 $TCID_{50}$ WSN influenza A virus encoding the $OVA_{257-264}$ Kb restricted MHC I epitope in the neuraminidase gene (Topham et al. J Immunol 2001 - Doherty) or mock virus (allantoic fluid of uninfected eggs); all diluted in 50µl PBS. Weight loss was monitored daily.
-
PVM virus infection
Mice were infected intratracheally with 35pfu PVM virus (J3666) diluted in 80µl PBS. Weight loss was monitored daily.

Isolation of lung and MLN cells
Mice were sacrificed and the MLN and lung were isolated and mechanically cut. Single cell suspensions were prepared by digestion in collagenase/DNase (Roche) solution; lungs were digested for 30 minutes at 37°C, MLNs were digested 15 minutes at 37°C. After digestion, the suspension was filtered over an 100µm filter and red blood cells in the lung suspension were lysed with osmotic lysis buffer.

Flow cytometry and cell sorting
Staining of DC subsets was done by staining with MAR-1 (conjugated to biotin, eBioscience) combined with streptavidin (conjugated to PE-CF594, BD biosciences), CD3 (conjugated to PE-Cy5, eBioscience), CD19 (conjugated to PE-Cy5, eBioscience), CD11c (conjugated to PE-Cy7, eBioscience), MHC II (conjugated to APC-Cy7, Biolegend), CD103 (conjugated to PE, BD biosciences or Pacific Blue, Biolegend), CD11b (conjugated to BV605 or Horizon V450, BD biosciences), CD64 (conjugated to AF647 or PE, BD biosciences), CD24 (conjugated to eFl450, eBioscience) and a fixable live/dead marker in eFl506 (eBioscience). Following additional markers were used: XCR1 (conjugated to PE, Biolegend), Sirpa (conjugated to PerCp-eFl710, eBioscience), CD26 (conjugated to Fitc, BD biosciences), MerTk (Unlabeled, R&D systems) combined with a donkey-anti-goat (conjugated to AF647, Invitrogen), CCR2 (conjugated to PE, R&D systems), CD45.1 (conjugated to BV605, Biolegend), CD45.2 (conjugated to AF700, eBioscience), IRF4 (Unlabeled, Santa Cruz) combined with an donkey-anti-goat (conjugated to AF647, Invitrogen), IRF8 (conjugated to PerCp-Efl710, eBioscience), CD40 (conjugated to PE, BD biosciences), CD80 (conjugated to PerCp-Cy5.5, BD biosciences), CD86 (conjugated to PE-Cy7, Biolegend).

Acquisition of 12-color samples was performed on a Fortessa cytometer equipped with FACSDiva software (BD98 biosciences). Final analysis and graphical output were performed using FlowJo software (Tree Star, Inc.).

For sorting of DC subsets, cells were stained as described and cell sorting was performed on a FACSAria II (BD biosciences).

Pre-cDC transfer

To expand pre-cDCs, CD45.2 wildtype mice were injected every other day with 10μg Flt3L (PSF, VIB). Mice were killed 8 days after the first treatment and bone marrow was isolated. Red blood cells were lysed by osmotic lysis buffer. Cells were labeled with eFl450 cell proliferation dye (eBioscience) and pre-cDCs were sorted as CD45$^+$, Lin$^-$ (CD3, CD19, MHC II, CD49b, CD11b, B220), CD11c^{int}, Sirpa^{int}. 8×10^5 cells were injected i.v. into CD45.1/2 wildtype mice 1dpi. Lung and MLN cells were analyzed 4 days later (5dpi).

Monocyte transfer

Bone marrow cells were isolated from CD45.1/2 wildtype mice. Red blood cells were lysed by using osmotic lysis buffer. Cells were labeled with eFl450 cell proliferation dye (eBioscience) and monocytes were sorted as Lin$^-$ (CD3, CD19, MHC II, Siglec F, Ly6G), CD11c$^-$, c-kit$^-$, CD11b^{hi} and Ly6C^{hi}. 8×10^5 cells were injected i.v. into CD45.2 CCR2$^{-/-}$ mice 1dpi. Lung and MLN cells were analyzed 4 days later (5dpi).

Micro-array

10 000 cells of each of the DC subsets were isolated from the lung and MLN 4dpi as described above. RNA was obtained with an RNEasy Plus Micro Kit according to the manufacturer's instructions (Qiagen) and RNA integrity was assessed with a Bioanalyser 2100 (Agilent). With a WT Expression Kit (Ambion) 50ng of total RNA per sample 'spiked' with bacterial poly(A) RNA positive control (Affymetrix) was converted to double-stranded cDNA in a reverse-transcription reaction. Samples were fragmented and labeled with biotin in a terminal labeling reaction according to the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridization controls (Affymetrix) was hybridized on a GeneChip Mouse Gene 1.0 ST Array (Affymetrix), followed by staining and washing in a GeneChip fluidics station 450 according to the manufacturer's procedures (Affymetrix). For analysis of raw probe signal intensities, chips were scanned with a GeneChip scanner 3000 (Affymetrix). Samples were subsequently analyzed with software of the R project for statistical computing (Bioconductor). All samples passed quality control, and the robust multiarray average procedure was used for normalization of data within arrays (probe-set summarization, background correction and log2 transformation) and between arrays (quantile normalization). In a subsequent step, probe sets that either mapped to multiple genes or had low variance were filtered out. The final analysis of the obtained data was performed by using Ingenuity Pathway Analysis (IPA, Qiagen).

DC–OT-I T cell coculture

Mice were infected with WSN influenza virus encoding the OVA$_{257-264}$ Kb restricted MHC I epitope in neuraminidase. OT-1 transgenic T cells were isolated from spleens and LN of OT-1 transgenic mice, enriched by MACS purification with a CD8 T cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec) and labeled with CFSE (Invitrogen). 10 000 sorted DCs were co-cultured with T cells in a 1:10 DC:T cell ratio for 4 days. T cells were restimulated for 5h with a cell stimulation cocktail containing PMA,

ionomycin, Brefeldin A and monensin (eBioscience). T cell divisions and cytokine production were measured by flow cytometry.

DC–OT-II T cell coculture
Mice were infected with the X31 influenza virus. OT-II transgenic T cells were isolated from spleens and LN of OT-II transgenic mice, enriched by MACS purification with a CD4 T cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec) and naive T cells were isolated by FACS purification. The cells were labeled with cell proliferation dye eFl450 (eBioscience). 5 000 sorted DCs were co-cultured with T cells in a 1:10 DC:T cell ratio for 5 days in the presence of 10ug/ml ovalbumine (endograde, Hyglos). T cells were restimulated for 5h with a cell stimulation cocktail containing PMA, ionomycin, Brefeldin A and monensin (eBioscience). T cell divisions and cytokine production were measured by flow cytometry.

Statistical analysis
All experiments were performed using 3-6 animals per group. All experiments were performed at least two to three times. Statistical analysis was performed with Prism version 6 (GraphPad Software, Inc.). Data are depicted as mean +/- SEM. Differences were considered significant when $p<0,05$. * = $p \leq 0,05$; ** $\leq 0,01$; ns = not statistically significant.

Supplementary material
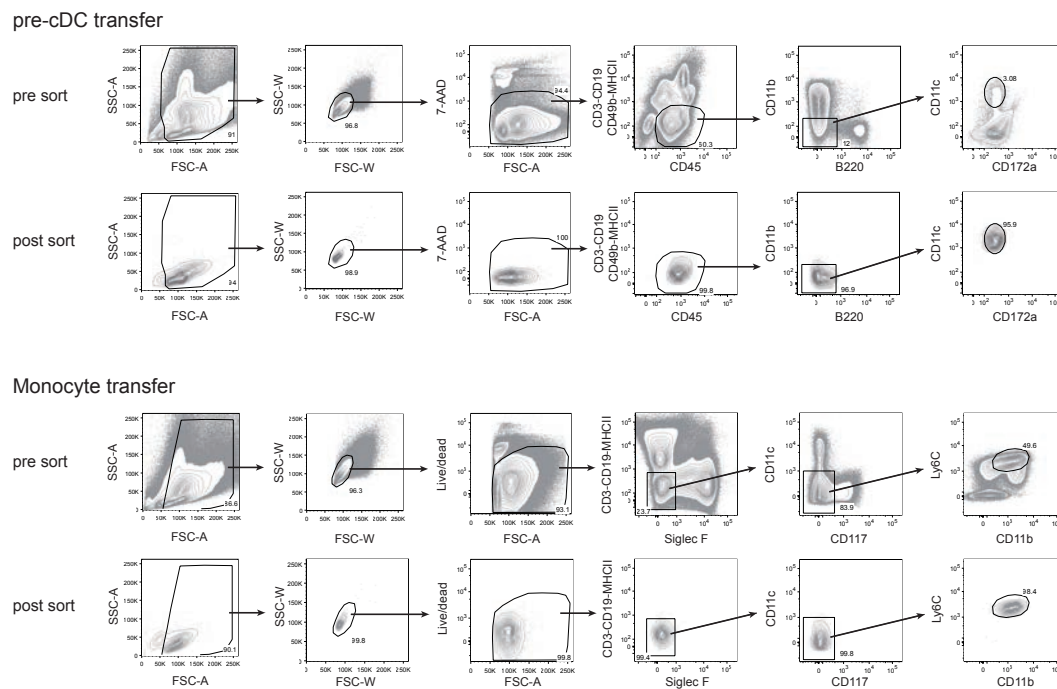
Figure S1

Flow cytometry plots illustrating the gating strategy to FACS purify pre-cDCs and monocytes (upper row) and the purity after the sort (lower row).
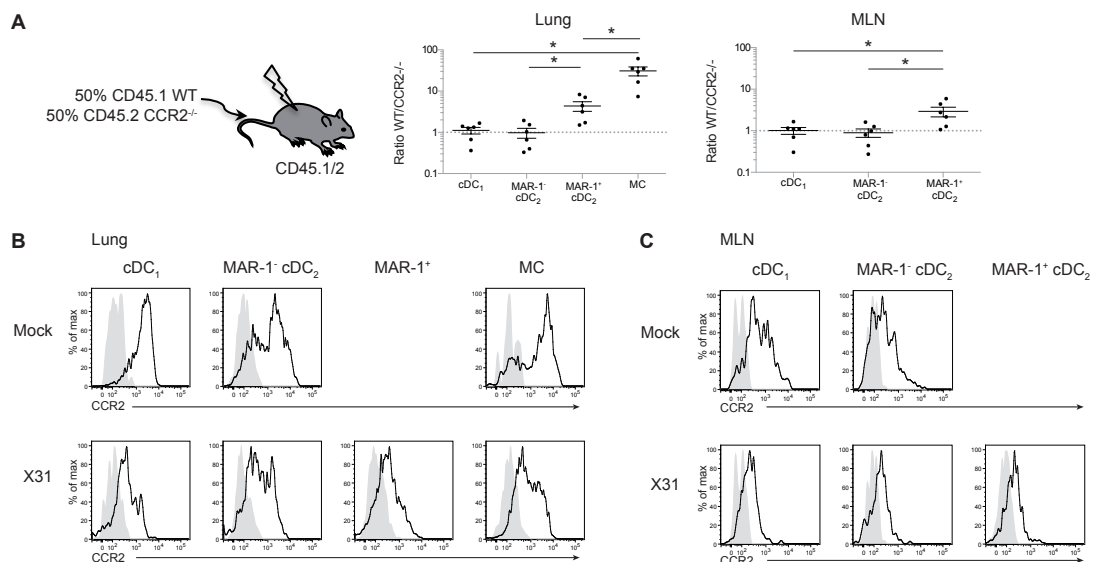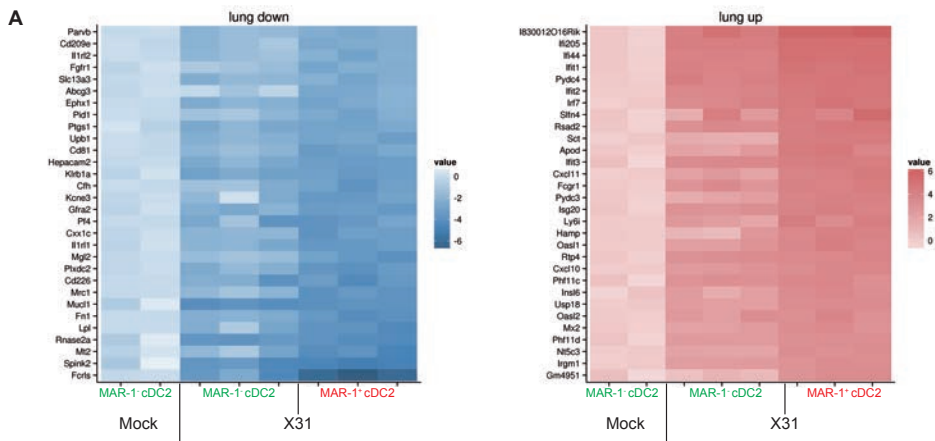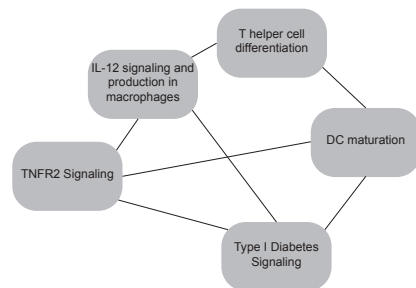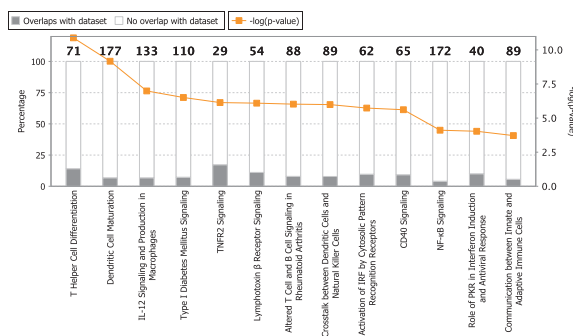

Figure S2

(A) CD45.1/2 wildtype recipient mice were irradiated and reconstituted with mixed CD45.1 WT and CD45.2 CCR2$^{-/-}$ bone marrow in a 50:50 ratio. 3 months after irradiation, the proportion of DC subsets from wildtype and CCR2$^{-/-}$ origin were defined. Graphs show the ratio of wildtype over CCR2$^{-/-}$ origin of DC subsets 4dpi in lung (left) and MLN (right). (B) CCR2 expression on lung DC subsets in mock (upper row) or X31 virus (lower row) infected animals at 4dpi. (C) CCR2 expression on MHCII^{hi} DC subsets in the MLN of mock (upper row) or X31 virus (lower row) infected animals at 4dpi.

For irradiation experiments 9 mice were irradiated in two independent rounds and analyzed in 3 different independent experiments (and also 10 for mock infection, but not shown).
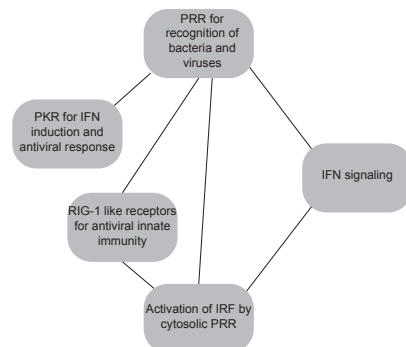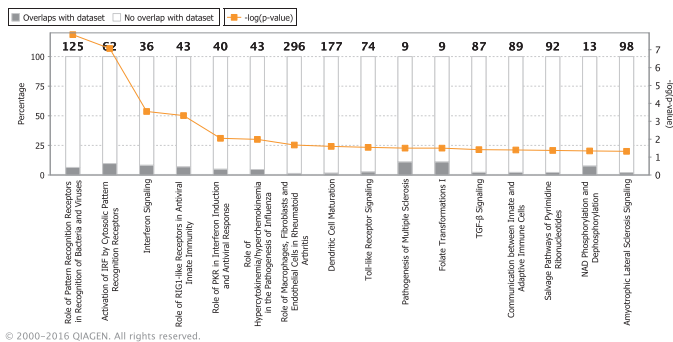
Figure S3

(A) Heat maps showing the relative expression of the top 30 downregulated (left) and upregulated (right) genes in lung DC subsets after mock and IAV infection. (B) IPA analysis for the potential canonical pathways driving the genes that are upregulated upon IAV infection in cDC2 DCs that are shared with cDC1s. (C) Same analysis for genes shared with MCs.

E

| Upstream Regulator	Log Ratio	Molecule Type	Predicted Activ...	Activation z-score	p-value of overlap	Target molecules in...	Mechanistic Net...
IRF8	↑2.950	transcription regulato	Activated	2.097	1.30E-07	↑BCL2L1, ↑... ...all 9	59 (12)
IFNAR1		transmembrane rece	Activated	2.767	1.21E-06	↑CCL5, ↑C... ...all 9	61 (12)
Ifnar		group	Activated	2.948	1.10E-06	↑CCL5, ↑C... ...all 9	57 (9)
IFNB1		cytokine	Activated	2.767	1.82E-08	↑CCL5, ↑... ...all 14	56 (9)
IFN alpha/beta		group	Activated	2.563	3.39E-09	↑CCL5, ↑... ...all 10	55 (7)
IFNG		cytokine	Activated	2.393	3.94E-13	↑BATF2, ↑... ...all 34	54 (12)
STAT1		transcription regulato	Activated	3.582	3.64E-11	↑BATF2, ↑... ...all 17	53 (9)
TICAM1		other	Activated	2.207	4.80E-12	↑ACSL1, ↑... ...all 16	51 (10)
TLR3		transmembrane rece	Activated	2.359	7.87E-04	↑CCL5, ↑C... ...all 7	33 (8)
IRF3		transcription regulato	Activated	2.495	2.66E-05	↑CCL5, ↑FA... ...all 8	27 (3)
BAK1		other	Activated	2.588	6.09E-08	↑CCL5, ↑H... ...all 7	25 (3)
DOCK8		other	Activated	2.121	2.63E-06	↑BBX, ↑CD40 ...all 8	
SASH1		other	Activated	2.121	3.27E-06	↑BBX, ↑CD40 ...all 8	
IRF7		transcription regulato	Activated	2.573	5.58E-05	↑FAM26F, ↑... ...all 7	
FADD		other	Activated	2.373	1.48E-04	↑ASB13, ↑... ...all 6	
Interferon alpha		group	Activated	2.200	4.81E-03	↑IFI16, ↑IL15 ...all 5	
ITK		kinase	Activated	2.000	1.90E-02	↑CCL5, ↑H... ...all 4	
KDM5B		transcription regulato	Activated	2.000	4.00E-02	↓CDCA3, ↑... ...all 4	
CEBPA		transcription regulato	Activated	2.219	1.82E-01	↑ACSL1, ↓C... ...all 5	

F
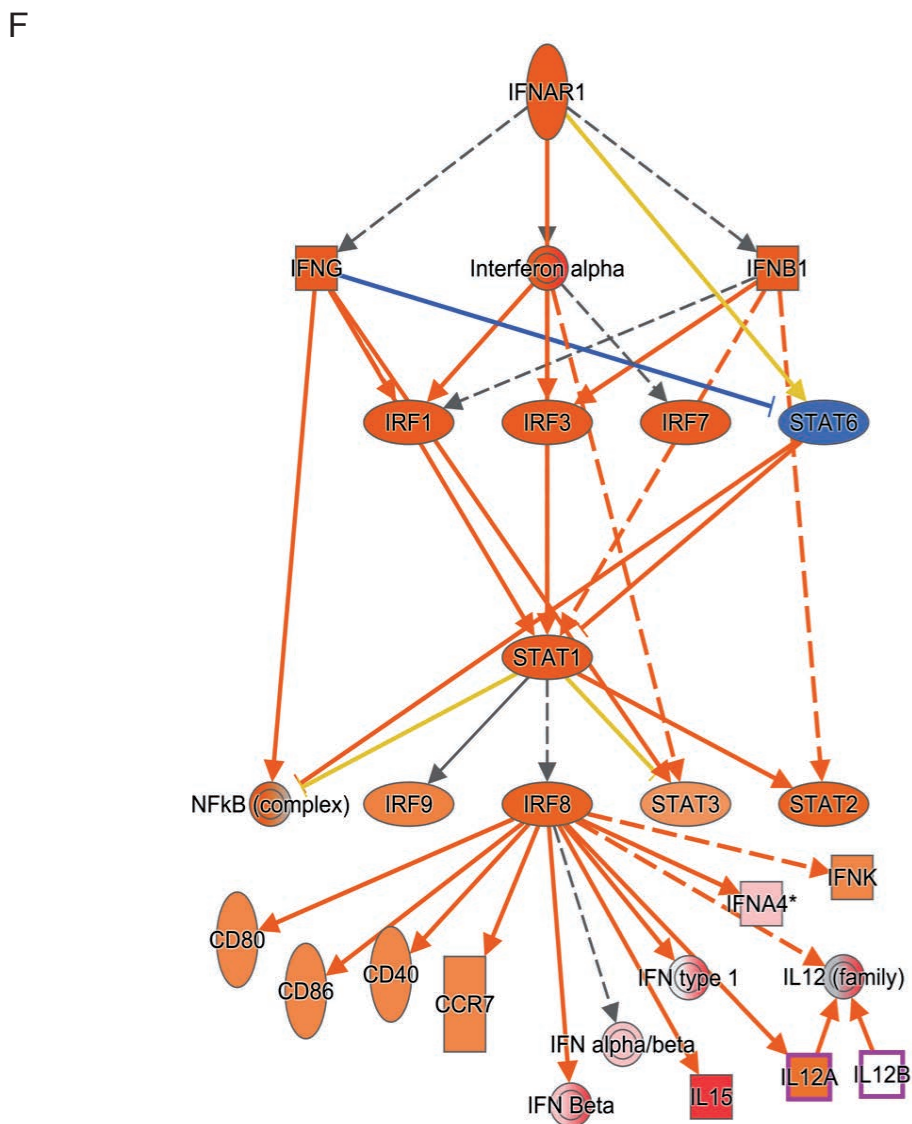
Figure S3
(E) IPA analysis for the possible upstream genes regulating the differential gene expression between mock cDC2s and MAR-1+ cDC2s. (F) Possible hierarchical clustering of the proposed upstream regulators of the differential gene expression between mock cDC2s and MAR-1+ cDC2s.
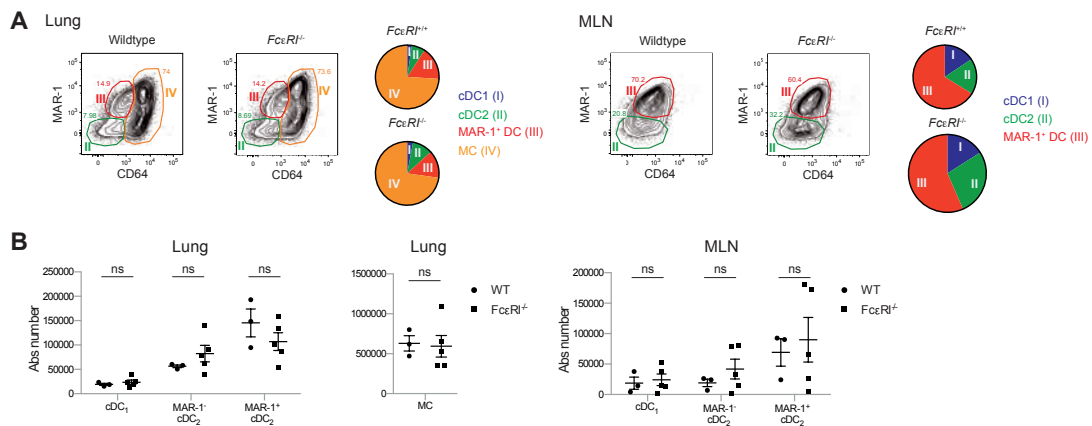
Figure S4

(A) Representative flow cytometry plots showing DC subsets in the lung (left) and MLN (right) of wild-type and *FcεRI*-/- mice at 4dpi with IAV and distribution of DC subsets as percentage of live DCs in the lung and MLN in WT and *FcεRI*-/- mice 4dpi with IAV. The size of the pie is proportional to the total amount of DCs. (B) Absolute number of each DC subset in the lung and MLN of WT and *FcεRI*-/- mice at 4dpi with IAV. Data are representative for 2 independent experiments with 4-6 mice per group.

Acknowledgements

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Double-negative T resident memory cells of the lung react to influenza virus infection via CD11c^{hi} dendritic cells

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Abstract

Immunity to Influenza A virus (IAV) is controlled by conventional TCR$\alpha\beta^+$ CD4$^+$ and CD8$^+$ T lymphocytes that mediate protection or cause immunopathology. Here, we addressed the kinetics, differentiation and antigen specificity of CD4$^-$CD8$^-$ double negative (DN)T cells. DN T cells expressed intermediate levels of TCR/CD3 and could be further divided in $\gamma\delta$ T cells, CD1d-reactive type I NKT cells, NK1.1$^+$ NKT-like cells and NK1.1$^-$ DN T cells. NK1.1$^-$ DN T cells had a separate antigen specific repertoire in the steady-state lung, and expanded rapidly in response to IAV infection, irrespectively of the severity of infection. Up to 10% of DN T cells reacted to viral nucleoprotein. Reinfection experiments with heterosubtypic IAV revealed that viral replication was a major trigger for recruitment. Unlike conventional T cells, the NK1.1$^-$ DN T cells were in a preactivated state, expressing memory markers CD44, CD11a, CD103 and the cytotoxic effector molecule FasL. DN T cells resided in the lung parenchyma, protected from intravascular labeling with CD45 antibody. The recruitment and maintenance of CCR2$^+$ CCR5$^+$ CXCR3$^+$ NK1.1$^-$ DN T cells depended on CD11c^{hi} dendritic cells. Functionally, DN T cells controlled the lung DC subset balance, suggesting they might act as immunoregulatory cells. In conclusion, we identify activation of resident memory NK1.1$^-$ DN T cells as an integral component of the mucosal immune response to IAV infection.

Introduction

Mucosal tissues such as the lung are frequently exposed to pathogens that can cause life threatening pulmonary infections. These infectious agents like influenza virus (IAV) must be quickly and efficiently controlled by the immune system, without causing overt damage to the gas exchange apparatus of the lung[1]. Upon IAV infection, CD103+ and CD11b+ dendritic cells (DCs) take up and process viral particles and migrate to the mediastinal lymph node where they encounter naïve CD4+ and CD8+ T cells[2]. These T cells undergo a stepwise process of activation, proliferation and differentiation towards a helper or cytotoxic phenotype respectively, and migrate back to the lung as effector cells in a process requiring the chemokine receptors CCR2, CCR5 and CXCR3[3, 4]. CD8 effector T cells are crucial for viral clearance, but their effector functions need tight regulation since they can also cause immunopathology and damage to the lung microenvironment[5,6]. CD4 T cells promote CD8 T cell and B cell responses to IAV infection although they are not critical for this process[7-10]. Adoptive transfer studies demonstrated that CD4 T cells are also able to control viral load and exert direct cytotoxic effector functions in the lung environment[11, 12], yet the contribution of CD4 T cell cytotoxicity to viral clearance *in vivo* in the lungs is modest[13].

As acute infections are cleared, effector CD8+ T cells further differentiate into KLRG-1hiCD127lo short-lived effector cells and CD127hi memory precursor effector cells capable of generating long-lived memory CD8+ T cells, and a similar process occurs in CD4 T cells[14, 15]. Long lived memory cells can recirculate via lymphoid organs as T central memory cells (T_{cm}), patrol in and out peripheral tissues as T effector memory (T_{em}) cells or reside for prolonged periods in the lungs as T resident memory cells (T_{rm}), that express high levels of CD69, CD11a and/or CD103[15]. Triggered by retained antigen presented by DCs, CD4 T_{rm} and CD8 T_{rm} cells were shown to reside for months in the lungs of IAV-infected mice and -infected volunteers, thus providing immunity against reinfection with the same or heterologous strain of influenza[15-22].

Non-conventional T cells that express a functional T cell receptor (TCR) but lack expression of CD4 and CD8 co-receptors (therefore called double negative (DN) T cells), can be observed in various disease models in human and mice in which they were attributed different functions[23]. The lungs are one of the many tissues where DN T cells were described in steady state and following insults to the lung[24-30]. As DN T cells are defined by exclusion, they are very heterogeneous, arising either from the thymus or extrathymically. Classical DN T cells express intermediate levels of αβTCR, and are different from type I CD1d-restricted invariant natural killer T cells and γδTCR+ T cells that are often found to lack CD4 and CD8 expression, and therefore fall under the DN T definition[31, 32].

The involvement of the different DN T cells in IAV infection is currently unknown. We therefore carefully addressed the phenotype, origin, antigen specificity and TCR repertoire, kinetics of recruitment and activation, and acquisition of effector and memory markers of αβTCR+ DN T cells and conventional T cells during and following infection with the H3N2 X31 influenza A virus (IAV) strain, or reinfection with the heterosubtypic H1N1 PR8 IAV strain. We observed a predominant accumulation of NK1.1- αβTCR+ DN T cells in the lung after primary influenza infection, but not after heterosubtypic infection and these cells had characteristics of T_{rm} cells situated in the

lung interstitium. The induction and maintenance of the NK1.1- DN T cell response was dependent on lung DCs that caused DN T accumulation through recruitment. Functionally these cells may act as immunoregulatory cells by controlling the lung DC subset balance.

Results

Influenza infection induces accumulation of unconventional CD4-CD8- double negative T cells in the lung

Studies on T cell responses to airway infection with influenza A virus (IAV; H3N2, strain X31) have mainly focused on MHCI-restricted CD8+ and MHCII-restricted CD4+ conventional T cells, which can be easily identified within the αβTCR+ CD3+ cell population of a lymphocyte gate (FSClo SSClo) on dispersed lung cells (Fig 1A;A and B respectively). Within these αβTCR+ lymphocytes, a CD4-CD8- double negative (DN) population can be consistently observed. As this population of DN T cells is defined mainly by exclusion of CD4 and CD8 expression, we sought to further define it using multi-color flow cytometry[26]. A significant proportion of CD3+ DN T cells expressed a γδ TCR receptor (Fig 1A; population C), consistent with the notion that pulmonary γδ T cells often lack expression of CD4 and CD8. Another well-known population of unconventional T cells are NKT cells, sharing some phenotypic markers with NK cells (NK1.1 expression in C57Bl/6 mice), variably expressing CD4 depending on tissue residence, and many of which can be identified by staining with α-galactosylceramide (α-GalCer) loaded CD1d tetramers (TM). Based on CD1d TM binding and NK1.1 expression, lung DN T cells could be further classified as DN type I NKT cells (Fig 1A; population D). After gating out γδ T cells and type I NKT cells, the remaining lung DN T cells could be further divided into NK1.1- CD1d TM- αβTCR+ DN T cells (Fig 1A; population E) and NK1.1+ CD1d TM- αβTCR+ DN T cells (Fig 1A; population F). Whether NK1.1 expression represents an activation state of some lymphocytes or a truly different cell population of DN NKT-like cells remains a matter of debate[33, 34]. Up to 15% of NK1.1- CD1d TM- αβTCR+ DN T expressed B220, a marker previously found on peripheral DN T cells (data not shown). All DN T cells, including the NK1.1- CD1d TM- αβTCR+ DN T cells expressed intermediate TCR levels compared with conventional CD4+ or CD8+ T cells, a finding previously also reported for other DN T cells (Fig 1A, histograms)[24]. We next analyzed the relative distribution and kinetics of accumulation of all DN T subsets following IAV or mock infection. Both in mock and IAV infected mice, CD4+ and CD8+ conventional T cells represented the majority of T cells in the lung 9 days post infection (dpi), unconventional T cells each representing less than 2.5% of T cells (Fig 1B). When absolute numbers of DN T cells were studied over time (Fig 1C), only the population of NK1.1- CD1d TM- αβTCR+ DN T cells expanded significantly following infection, in a kinetic that closely resembled the expansion of CD4 and CD8 conventional T cells (Fig 1E). The more than 20 fold expansion of NK1.1- αβTCR+ DN T cells at the peak of the response (8dpi) was followed by a steep contraction phase also seen in conventional T cells. Since the NK1.1- CD1d TM- αβTCR+ population is the only one that is induced after infection, this is the population that was studied in further detail and will be called NK1.1- DN T cells throughout the paper.
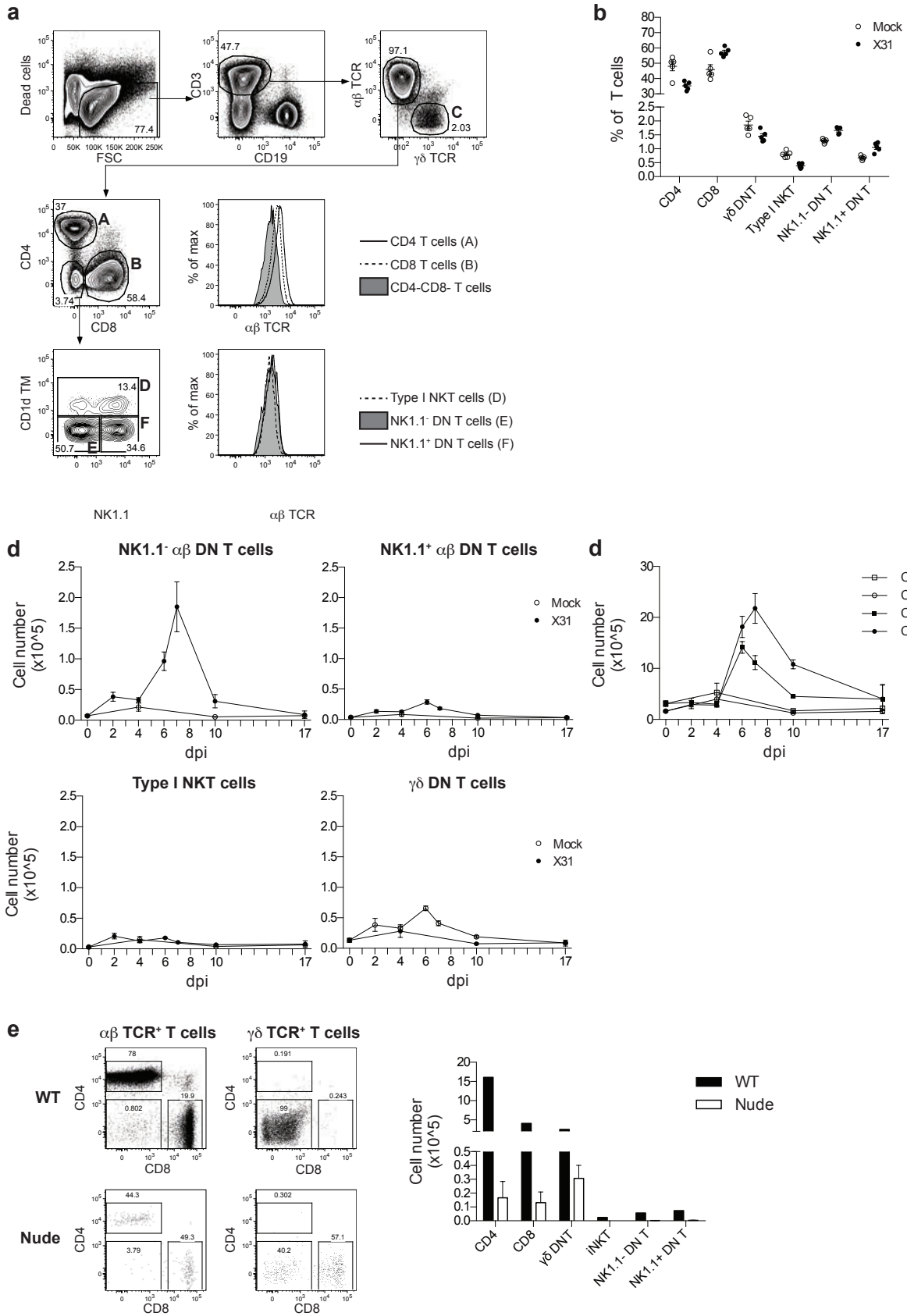
a

b

d NK1.1⁻ αβ DN T cells

d

NK1.1⁺ αβ DN T cells

Type I NKT cells

γδ DN T cells

e

Figure 1 - **αβ** TCR[int] double negative T cells accumulate in the lungs of influenza virus infected mice

(A) Gating strategy used to subdivide the T cell populations into conventional CD4+ (population A) and CD8+ (population B) T cells and non-conventional T cells: γδ TCR+ DN T cells (population C), αβ TCR+ CD1d TM+ DN T cells (type I NKT, population D), αβ TCR+ CD1d TM- NK1.1- DN T cells (population E) and αβ TCR+ CD1d TM- NK1.1+ DN T cells (population F). As an example, plots were generated 9dpi. Histograms show αβ TCR expression intensity on lymphocyte subsets: CD4+ T cells (black), CD8+ T cells (black dashed line) and the total CD4-CD8- T cells population (red) on the upper panel, type I NKT (black dashed line), NK1.1- DN T cells (gray filled line) and NK1.1+ DN T cells (black) (B) Distribution of conventional and non-conventional T lymphocyte subsets in the lungs 9dpi after X31 (black) or mock (white) virus infection, expressed as % of total CD3+ alive T cells. (C) Kinetics of accumulation of non-conventional T cells in the lungs of X31 (black) or mock (white) virus infected mice. (D) Kinetics of accumulation of conventional CD4+ (squares) and CD8+ (dots) T cells in the lungs of X31 (black) or mock (white) virus infected mice. (E) CD4 and CD8 expression profile of αβ TCR+ T cells (left) and γδ TCR+ T cells (right) in the lungs of athymic nude mice (lower row) compared to 1 wild-type mouse (upper row) at 2dpi and absolute cell number of conventional and non-conventional T cells. Cells were pregated as singlets, alive, CD19- and CD3+. All experiments were performed at least twice and figures are representative for each separate experiment.
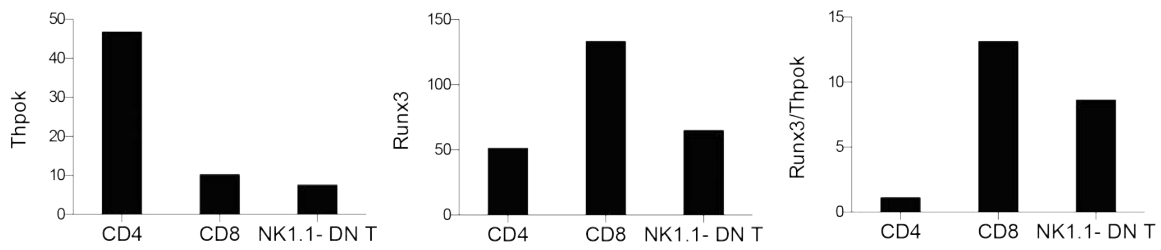
Origin of lung DN T cells

We next addressed the origin of the DN T cells of the lungs, which can develop like classical T cells in the thymus or outside of the thymus. DN T cells of the gut have indeed been described in thymectomized mice, but the origin of lung DN T cells is less clear[35-37]. We therefore infected athymic nude-Foxn1[nu] mice and defined T cell subsets 2dpi. Some remaining αβ and γδ T cells could be observed, and there was a shift towards more CD8+ γδ T cells in athymic nude-Foxn1[nu] mice, consistent with the notion that many γδ T cells develop extrathymically. Some lung CD4+ and CD8+ αβTCR+ T cells were still present, indicative of extrathymic development (Fig 1D). Unexpectedly, lung DN T cells were almost completely lacking in Foxn1[nu] mice. These observations point towards a thymic origin of the type I NKT, NK1.1- and NK1.1+ DN T cell populations during IAV infection.
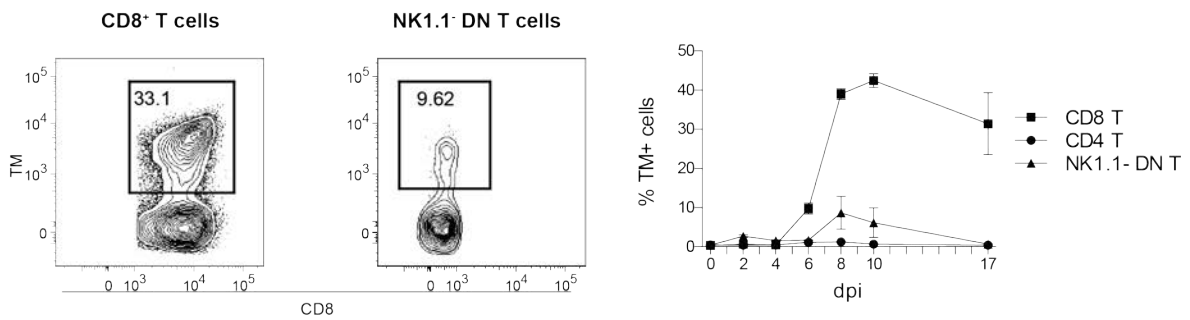
NK1.1- DN T cells resemble CD8+ T cells

As DN T cells are defined by lack of CD4 and CD8, and as the kinetics of accumulation, and the thymic origin closely resembled those of conventional T cells, we questioned whether some of the DN T cells represent revertant conventional T cells, losing surface expression of CD4 and/or CD8 after ligation of the TCR, as previously described[38]. NK1.1- DN T cells and conventional T cells were therefore sorted from lungs 9dpi. T cell lineage determination is molecularly controlled by the balance between Thpok (promoting CD4 T cell differentiation) and Runx3 (promoting CD8+ T cell differentiation) transcription factors[39]. Like CD8+ conventional T cells, NK1.1- DN T cells had low expression of the CD4 lineage transcription factor Thpok by qPCR and were negative for the transcription factor Rorγt that is typical for Th17 and some subsets of γδ T cells (data not shown). Expression of the CD8 lineage transcription factor Runx3 was lower in NK1.1- DN T cells than in conventional CD8+ T cells but higher than in CD4+ T cells. Although the ratio of Runx3 over Thpok suggests that NK1.1- DN T cells are transcriptionally more related to CD8+ T cells than to CD4+ T cells (Fig 2A), these results do not show a clear bias towards CD4 or CD8 lineage imprinting for the entire NK1.1- DN T cell population. Intracellular staining for CD8 and CD4 revealed that 10% of the NK1.1- DN T cells had intracytoplasmic CD8 (but not CD4) expression (Fig S1) to the same extent as

conventional CD8+ T cells, indicating that at least part of the DN T cells might indeed be revertant CD8+ T cells.

a

b

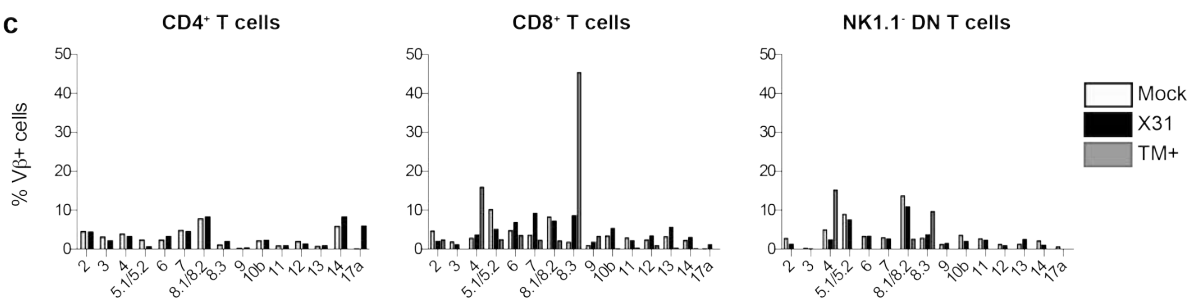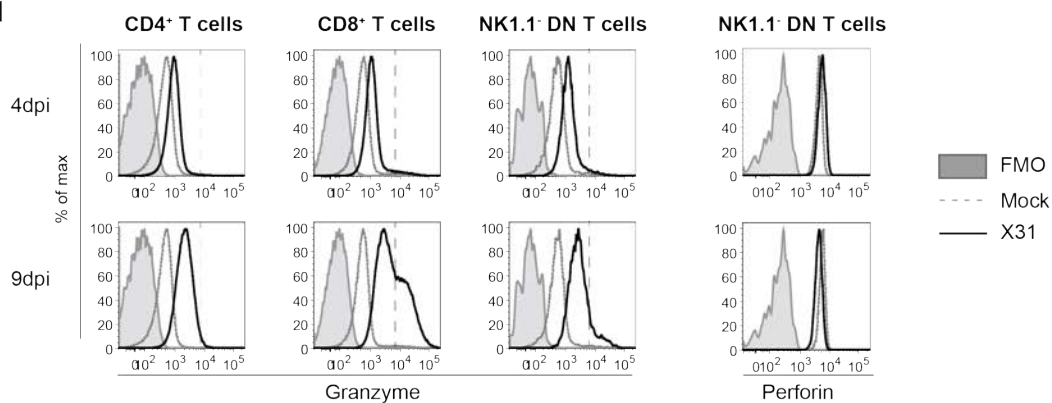CD8+ T cells NK1.1- DN T cells

c

CD4+ T cells CD8+ T cells NK1.1- DN T cells

Mock
X31
TM+

d

CD4+ T cells CD8+ T cells NK1.1- DN T cells NK1.1- DN T cells

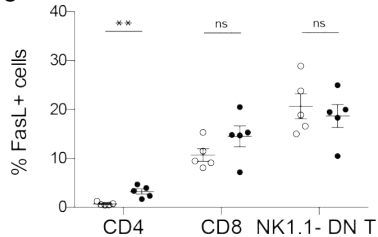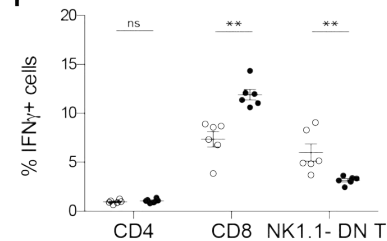Granzyme Perforin

FMO
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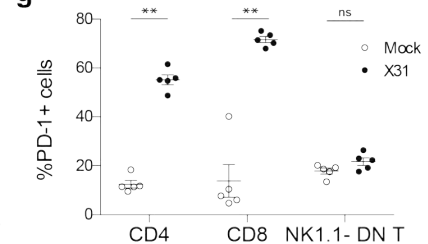
e

f

g

Mock
X31

Figure 2 - NK1.1⁻ DN T cells are transcriptionally related to CD8⁺ T cells, yet have a different T cell repertoire (A) Quantification of the expression level of *Runx3* (CD8 lineage) and *Thpok* (CD4 lineage) by Q-PCR on sorted CD4, CD8 and NK1.1⁻ DN T cells from the lungs of X31 virus infected mice 8dpi. Expression levels were normalized to expression of the housekeeping gene *hprt*. B) NP tetramer staining (Kᵇ-ASNENMETM) of CD8 and NK1.1⁻ DN T cells on lungs of X31 infected mice 8dpi and kinetic of TM⁺ T cells expressed as percentage of CD4 (dots), CD8 (squares) or αβ TCR⁺ CD1d TM⁻ NK1.1⁻ DN T cells (triangles). (C) Screening of the T cell receptor Vβ repertoire of CD4, CD8 and NK1.1⁻ DN T cells in the pooled lungs of 6 mock (white bars) and 6 X31 (black bars, TM⁺ cells: gray bars) infected mice 8dpi expressed as % of CD4, CD8 or NK1.1⁻ DN T cells. (D) Quantification of granzyme B (left) and perforin (right) expression on CD4, CD8 and NK1.1⁻ DN T cells in the lungs of X31 (black line) or mock-infected (grey line) mice 4dpi (upper panel) and 9dpi (lower panel). The FMO staining for granzyme and perforin is indicated as a filled gray line (E) Quantification of FasL expression on CD4, CD8 and NK1.1⁻ DN T cells in the lungs of X31 (black) or mock-infected (white) mice 9dpi. (F) Quantification of IFNγ production by CD4, CD8 and NK1.1⁻ DN T cells in the lungs of X31 (black) or mock-infected (white) mice. Lung cells were isolated 9dpi and restimulated for 4 hours with NP_ASNENMETM peptide in the presence of Golgi Stop before staining for IFNγ. (G) Expression of exhaustion marker PD-1 was determined on CD4⁺ and CD8⁺ T cells and NK1.1⁻ DN T cells 9 days after mock (white) or X31 (black) infection. All experiments were performed at least two times and figures are representative for every separate experiment. ** = p < 0,01, * = p < 0,05, ns = not statistically significant different.

TCR repertoire of NK1.1⁻ DN T cells

Following influenza infection, conventional CD8⁺ T cells react to a restricted set of immunodominant epitopes derived from various antigens, and these CD8⁺ T cells undergo oligoclonal expansion. Indeed, at 8dpi, close to 40% of the lung conventional CD8⁺ T cells had a receptor specific for the IAV nucleoprotein (NP) peptide ASNENMETM, as revealed by TM staining using the Kᵇ-ASNENMETM tetramer (Fig 2B).

A considerable proportion of NK1.1⁻ DN T cells also stained for this tetramer, but at the peak of the response, this fraction represented only 10% of DN T cells followed by a slow contraction phase (Fig 2B).

To further delineate if there would be oligoclonal expansion of DN T cells resembling the one seen in CD8+ T cells, we performed a more elaborate profiling of TCR Vβ usage at T cell population level in subsets of lung T cells (Fig 2C). In mock-infected animals, Vβ usage was broad across conventional

CD4⁺ and CD8⁺ T cells, whereas in NK1.1⁻ DN T cells, there was an overrepresentation of Vβ 8.1/8.2 cells to 14% of the repertoire. As previously reported, the entire influenza specific CD8⁺ T cell pool has a TCR Vβ repertoire skewed towards TCR Vβ8.3, Vβ4 and Vβ7[40] and type I NKT express an oligoclonal TCR repertoire (Vα 14)[41, 42] combined with one of three Vβ chains (Vβ2, Vβ7, Vβ8.2). Whereas in CD8⁺ T cells there was enrichment for Vβ7 and Vβ8.3 in the total pool of CD8⁺ T cells following influenza infection, there was no further enrichment in NK1.1⁻ DN T cells post infection and TCR Vβ8.1/8.2 and 5.1/5.2 remained the most prominently expressed TCR Vβ in the total NK1.1⁻ DN T population. In NP_ASNENMETM-reactive CD8⁺ T cells, there was strong enrichment for Vβ4 and Vβ8.3 usage, and the same phenomenon was seen in NP_ASNENMETM-reactive NK1.1⁻ DN T cells.

Effector functions of NK1.1⁻ DN T cells

As at least some NK1.1⁻ DN T cells were transcriptionally related to CD8⁺ T cells and shared NP-reactivity with CD8⁺ T cells, we measured some of the effector molecules involved in CD8 function. An increase in Granzyme B content of CD4⁺, CD8⁺ and NK1.1⁻ DN T cells was observed in reaction to IAV infection already 4dpi, compared with mock-infected mice. The difference in mean fluorescence intensity (MFI) between

mock and virus infected mice was 441, 648 and 773 for CD4⁺, CD8⁺ and NK1.1⁻ DN T cells respectively (Fig 2D). At 9dpi however, the Granzyme B content was further increased in all cell types. Conventional CD8⁺ T cells showed the largest increase in Granzyme B content with a difference in MFI of 5018 compared to mock-infected mice, whereas the difference in MFI for CD4⁺ T cells and NK1.1⁻ DN T cells was 1631 and 2242 respectively (Fig 2D). Perforin expression did not change dramatically upon virus infection compared to mock infected mice (Fig 2D).

Conventional CD8⁺ T cell mediated cell killing is not only induced via release of intracellular Granzyme B, it can also be mediated via surface expression of FasL (CD95L). In mock-infected animals, NK1.1⁻ DN T cells expressed the highest level of FasL, followed by CD8⁺ conventional T cells that expressed significantly more FasL than CD4⁺ T cells. After IAV infection FasL was upregulated further, but only in CD4 T cells this reached statistical significance (Fig 2E).

Conventional cytotoxic T cells are a major source of IFNγ during infection. The IFNγ production was indeed increased in CD8⁺ T cells after IAV infection. In NK1.1⁻ DN T cells however the capacity to produce IFNγ was reduced upon IAV infection (Fig 2F). This suppression of cytokine production might indicate that the NK1.1⁻ DN T cells have an exhausted phenotype after IAV infection. One of the signs of T cell exhaustion is expression of the co-inhibitory B7 family receptor PD-1 on the cell surface. Upon IAV infection, PD-1 was expressed on about 60% of CD4 T cells and 70% of CD8⁺ T cells. In contrast, the percentage of PD-1 expression on NK1.1⁻ DN T cells was low (20%) and did not increase upon infection (Fig 2G). Therefore NK1.1⁻ DN T cells are unlikely to become exhausted upon IAV infection.

Lung NK1.1⁻ DN T cells display an activated phenotype of resident memory T cells

The high levels of surface FasL and intermediate levels of intracellular IFNγ present already in mock-infected mice suggested that lung NK1.1⁻ DN T cells might be in a pre-activated state prior to infection. To address this issue further, we employed a panel of T cell activation markers. In mock-infected animals, up to 20% of lung conventional T cells expressed the memory/effector T cell marker CD44, whereas close to 80% of NK1.1⁻ DN T cells expressed CD44 (Fig 3A). At 4dpi, CD44 expression was further induced on CD8⁺ T cells, and by 9dpi, when the virus was cleared, 60-80% of conventional T cells expressed CD44 (Fig 3B). Expression of CD44 on NK1.1⁻ DN T cells remained high at 9dpi. The early activation marker CD69 was induced on all studied T cells after IAV infection; 25% of NK1.1⁻ DN T cells expressed CD69, whereas only 15% of CD4⁺ T cells and 5% of CD8⁺ T cells expressed CD69 at 4dpi (Fig 3C). Even at 9dpi, the levels of CD69 were still elevated on all subsets (Fig 3D). During the clearance of respiratory virus infection, conventional effector T cells can give rise to different cell fates, either giving rise to immediate and short lived effector cells, or to effector cells with the potential to generate long lived memory cells[43]. The phenotype and fate of CD44ʰⁱ effector T cells can be studied in more detail by using the markers KLRG1 and CD127[14] (Fig 3E). Within the CD8⁺CD44⁺ effector memory population (population A), short-lived effector cells (SLEC) are enriched in the KLRG1⁺CD127⁻ cells (population D), whereas memory
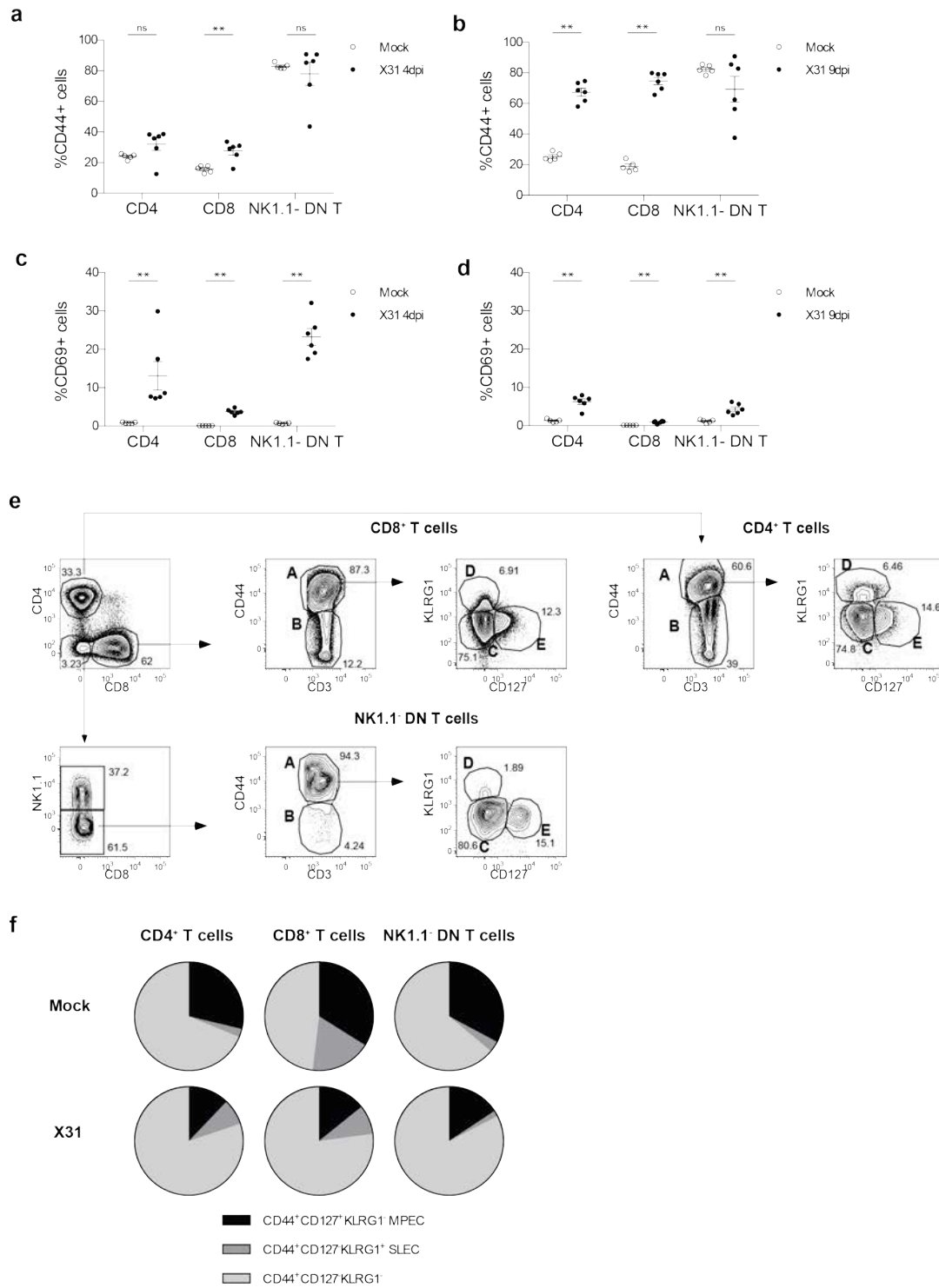
Figure 3 - NK1.1⁻ double negative T cells display a pre-activated memory phenotype and expand as KLRG1⁻ CD127⁻ cells

(A) Mice were infected with X31 (black) or mock (white) virus, at 4dpi the expression of the memory marker CD44 was measured in conventional and NK1.1⁻ DN T cells, gated as in Fig1. (B) Identical analysis at 9dpi. (C) Identical analysis for CD69 at 4dpi. (D) Identical analysis for CD69 at 9dpi. (E) Gating strategy for studying the phenotype of memory T cells. T cells were gated as CD3⁺ CD19⁻ αβ TCR⁺ cells. Memory T cells were gated as CD44⁺ (population A), naïve cells were gated as CD44⁻ (population B). On memory cells, KLRG1⁻CD127⁻ cells were effector cells (population C, Teff), KLRG1⁺CD127⁻ cells were identified as short-lived effector memory cells (population D, SLEC), and KLRG1⁻CD127⁺ cells are memory precursor effector cells (population E, MPEC). (F) Distribution of the memory populations (MPEC: black, SLEC: dark grey and KLRG1⁻CD127⁻ cells: light grey) of CD4, CD8 and NK1.1⁻ DN T cells the lungs of X31 or mock-infected mice 9dpi expressed as percentage of CD44⁺ cells. All experiments were performed at least twice and figures are representative for every separate experiment. ** = p < 0,01, * = p < 0,05, ns = not statistically significant different.

precursor effector cells (MPEC) are enriched in the KLRG1^{-}CD127^{+} population of cells (population E) (Fig 3E). Whereas this staining has mainly been employed to follow the fate of CD8^{+} T cells, we also employed it to CD4^{+} and NK1.1^{-} DN T cells (Fig 3E). In mock-infected cells, very few NK1.1^{-} DN T cells expressed KLRG1 indicative of immediate effector potential, whereas a major population of CD127^{+} memory cells was observed. Viral infection mainly led to expansion of KLRG1^{-}CD127^{-} early effector cells (population C; Fig 3E), of which the ultimate fate is hard to predict.

Memory cells can reside in the central lymphoid organs (as T central memory cells, T_{cm}) and recirculate via the blood to other lymphoid tissues. Alternatively, a considerable part of antiviral memory T cells reside in peripheral tissues as T resident memory (T_{rm}) cells[44]. T_{rm} cells have been identified by expression of various markers including CD69, CD103 and CD11a[15, 44]. In the lung, T_{rm} cells are hard to discriminate from recirculating blood T_{cm} or naïve T cells that firmly adhere to lung capillaries, even after extensive flushing of the lung capillary bed. To delineate intravascular DN T cells and conventional T cells simultaneously, we injected an AF700-labeled antibody to the pan leukocyte marker CD45 intravenously, and obtained blood and lung homogenates 5 minutes after injection. Using this labeling protocol, 100% of circulating peripheral blood CD3^{+} T cells was readily labeled with AF700-CD45 (Fig 4A). In mock-infected cells (Fig 4B), the majority of lung CD4 and CD8^{+} T cells were labeled with CD45, demonstrating that most lung lymphocytes were still in the lung vascular pool, even after extensive exsanguination and flushing. The majority of lung NK1.1[-] DN T cells were protected from CD45 *in vivo* labeling already in mock-infected mice (Fig 4B), identifying these cells as tissue resident cells. At 9dpi, up to 90% of lung conventional CD4^{+} and CD8^{+} T cells were protected from CD45 labeling and these cells also expressed CD69 (data not shown), as previously described[3, 15]. Tissue resident lymphocytes express various levels of CD11a and/or CD103[3, 15]. Like CD4 and CD8 T_{rm} cells, 10-15% of CD45^{-}NK1.1^{-} DN T cells co-expressed CD11a and CD103 and around 60-70% expressed CD11a but not CD103 (Fig 4C).

Reinfection with homologous or heterologous virus does not trigger NK1.1[-] DN T accumulation

Primary infection with IAV led to induction of an immune response of antigen-specific conventional T cells and NK1.1[-] DN T cells, which acquired T_{em} and T_{rm} memory characteristics, and conventional T cells have been shown to control heterosubtypic immunity to re-exposure with a heterologous virus[45]. We therefore set up primary infections using X31 (H3N2) followed by reinfection with the same X31 or the PR8 (H1N1) virus to test the reactivity of DN T cells to reinfection with the same or heterologous virus. X31 usually causes a mild and self-limiting viral infection, whereas PR8 leads to progressive infection that ultimately leads to death. We therefore used a much lower inoculum of PR8 virus to reinfect (5 $TCID_{50}$ compared with $1x10^5$ $TCID_{50}$ for the X31 virus). In the mice that first received a mock infection, comparisons between X31 and PR8 primary infection were possible. Due to the low inoculum, PR8 infection initially led to less weight loss compared with the higher inoculum of X31, but nevertheless caused more weight loss when infection advanced to 8dpi. The amount of NK1.1[-] DN T cells
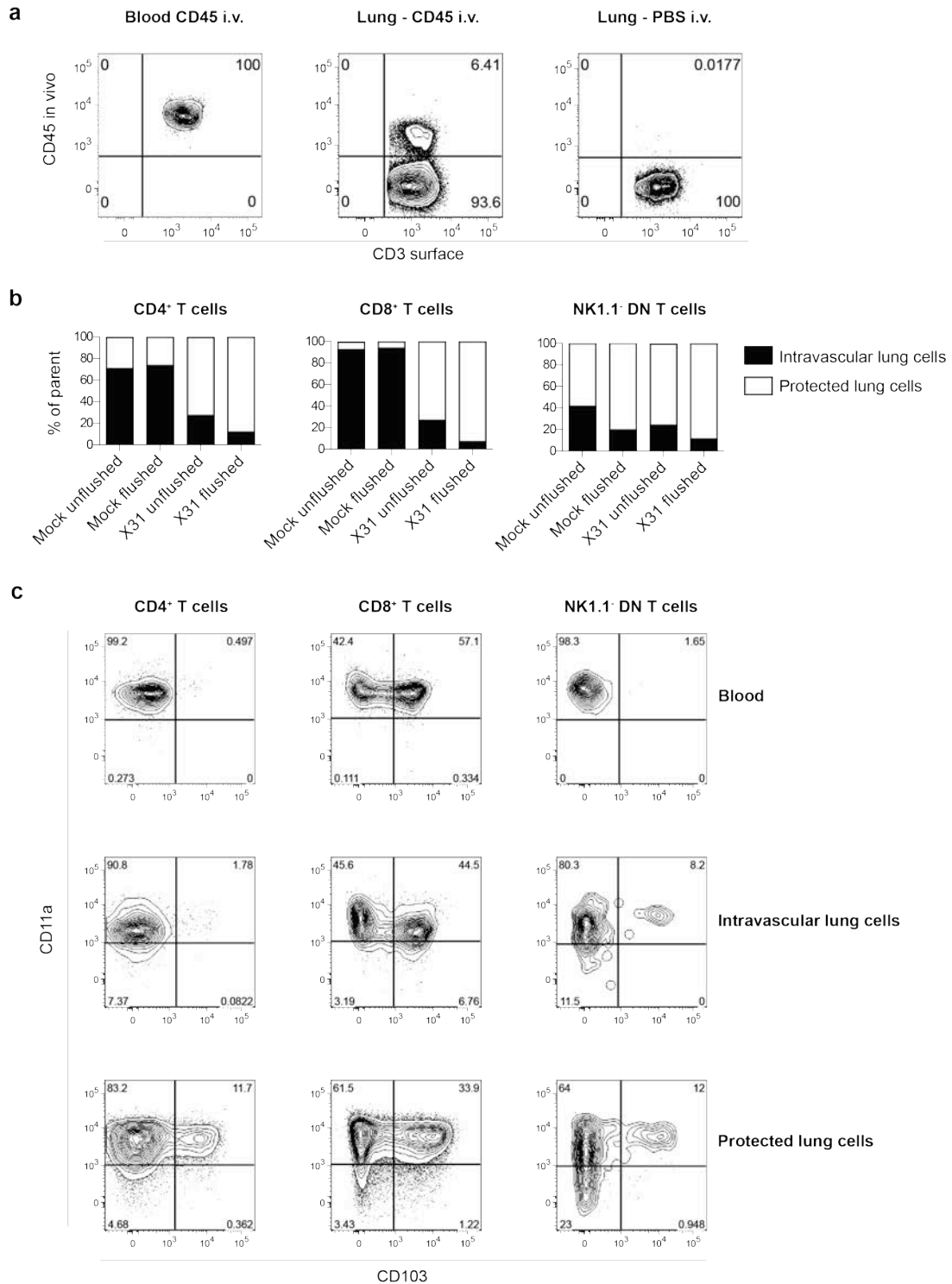
Figure 4 - NK1.1⁻ DN T cells are protected from intravenous CD45 staining and have a T_rm phenotype

(A) In vivo labeling of circulatory T cells by intravenous injection of an AF700 labeled antibody against CD45 or PBS 6dpi combined with an in vitro staining of surface CD3 on blood and lung cells. Gated on living CD3⁺ αβ TCR⁺ cells. (B) Proportion of protected CD45⁻ cells (white boxes) and intravascular CD45⁺ cells (black boxes) within conventional CD4⁺ or CD8⁺ T cells and NK1.1⁻ DN T cells. (C) CD11a and CD103 expression on CD45⁺ blood cells (upper panel), intravascular CD45⁺ lung cells (middle panel) and protected CD45⁻ lung cells (lower panel). Cells were gated as in Fig1, but images were acquired from mechanically dispersed lung cells, after performing bronchoalveolar lavage. All experiments were performed twice and figures are representative for every separate experiment.

obtained after infection with the X31 virus at 8dpi was not significantly different from the amount obtained after infection with the PR8 (H1N1) virus, despite the observed difference in weight loss at 8dpi (Fig 5A and B, mock-X31 versus mock-PR8). As expected, when mice were first infected with X31, re-infection with X31 did not cause weight loss, as replication and infection was prevented due to antibody-mediated sterilizing immunity. There was however an increased accumulation of CD8+ and CD4+ conventional T cells, whereas NK1.1- DN T cells failed to expand (X31-X31 versus X31-mock). Boosting of cellular immunity was most likely due to enhanced presentation of opsonized viral antigens, as antibodies to H3 and N2 are induced in these mice. Upon reinfection with the heterologous PR8 virus, heterosubtypic CD8+ T cell-mediated immunity has been described to protect mice from becoming sick[46] and consequently mice did not loose weight (X31-PR8 versus mock-PR8). In these mice, there was no boosting of conventional CD4+, CD8+ nor NK1.1- DN T cells. When we studied NP-specific T cells, re-infection of mice led to strong increases in NP-specific CD8+ conventional T cells in mice reinfected with X31 and PR8, but no such increase was seen in NK1.1- DN T cells (Fig 5C). Together, these observations suggested that viral replication and/or a strong inflammatory signal is needed to induce NK1.1- DN T cells. In contrast to conventional CD8+ T cells, NK1.1- DN T cells did not mount a recall response upon the mere presentation of viral antigens.

Induction and maintenance of the NK1.1- DN T response depends on chemokine production by conventional DCs

The increased numbers of NK1.1- DN T cells in the lungs of primary infected, but not reinfected, mice could be due to increased local proliferation or local recruitment of T cells with T_{em} or T_{rm} phenotype. To address this, we injected BrdU and measured instantaneous cell division by measuring BrdU uptake in conventional and DN T cells 3,5h later. Whereas 14 and 24% of conventional CD4+ and CD8+ T cells respectively were dividing within the 3,5h pulse-chase experiment at 6dpi, only a minority of NK1.1- DN T cells incorporated BrdU (Fig 6A), indicating that local proliferation is unlikely to be the explanation for the increase in NK1.1- DN T cell numbers. We next infected mice and measured the amount of NK1.1- DN T cells per 100µl of whole blood every other day following infection. A drop early after infection followed by an increase suggested that increased recruitment from the bloodstream is causing the increase in pulmonary NK1.1- DN T cells (Fig 6B) after infection.

As previously reported, administration of DT efficiently depleted all hematopoietic CD11c+ cells from the lungs (Fig S2)[48]. DT was administered either 1 day prior to infection, 7dpi or at both time points and numbers of conventional CD4+ and CD8+ T cells, as well as NK1.1- DN T cells were analyzed 2 days after the last treatment (9dpi). As shown in Fig 6C, the accumulation of cytotoxic CD8+ T cells was strongly reduced in infected DT treated mice, when treatment was given before or after primary infection. Likewise, the accumulation of NK1.1- DN T cells was strongly reduced in animals given DT early and late in infection. However, CD4+ T cells were not reduced by DT treatment early in infection, and only minor reductions of CD4+ T cells were seen when DT was given late in infection.
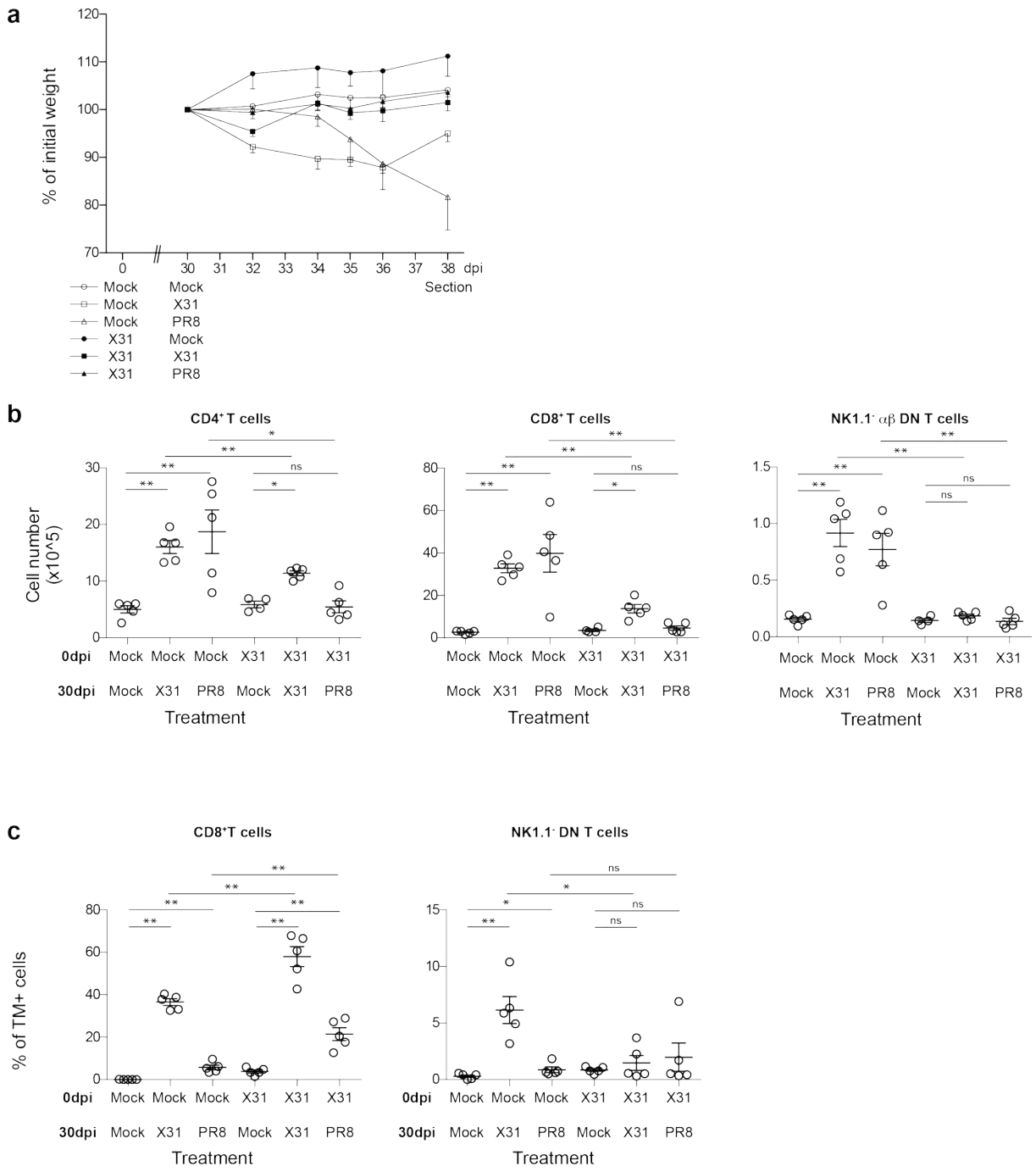
Figure 5 - Viral replication triggers accumulation of NK1.1⁻ double negative T cells irrespective of severity of infection

(A) Weight loss after second infection expressed as % of initial weight: Mock (dots), X31 (squares) and PR8 (triangles) after mock (white) or X31 (black) infection. (B) Quantification of conventional and NK1.1⁻ DN T cells after primo infection or reinfection with the same or with a heterologous virus, carrying shared nucleoprotein T cell antigens, yet lacking overlapping hemagglutinin and neuraminidase to which neutralizing antibodies are generated. Mice were first infected with mock or X31 (H3N2) virus and reinfected with mock, X31 (H3N2) or PR8 (H1N1) virus 30dpi. Lungs were analyzed 38 days after initial infection. (C) Proportion of TM+ conventional CD8+ T cells and NK1.1⁻ DN T cells 38dpi. All experiments were performed at least two times and figures are representative for every separate experiment. ** = p < 0,01, * = p < 0,05, ns = not statistically significant different.

Lung CD8$^+$ T cells are recruited by DCs to the lung interstitium via production of CCL3, CCL4, CCL5 and CXCL10, acting on chemokine receptors CCR2, CCR5 and CXCR3. To investigate which DC-derived chemokines could signal to NK1.1$^-$ DN T cells to attract or maintain them in the lung, CD4$^+$, CD8$^+$ and NK1.1$^-$ DN T cells were sorted from the lung 8dpi. Like conventional CD8$^+$ T cells, NK1.1$^-$ DN T cells expressed CCR2, CCR5 and CXCR3, suggesting that DCs might induce recruitment and retention of these cells via these chemokine receptor interactions (Fig 6D).

NK1.1$^-$ DN T cells balance the ratio of dendritic cell subsets
We finally wanted to address the potential function of NK1.1$^-$ DN T cells recruited to the lungs by CD11c^{hi} cells. In transplantation and autoimmunity models it has been suggested that DN T cells have an immunoregulatory capacity by controlling DCs[51], and the fact that CD11c^{hi} cells attracted these cells, led us to study the impact of NK1.1$^-$ DN T cells on DCs. We therefore performed an experiment in which we sorted lung DCs (carefully excluding CD11c^{hi} macrophages) and NK1.1$^-$ DN T cells from the lungs of infected mice at 9dpi and cocultured them for 36h. We observed that the presence of DN T cells stimulated the survival of lung DCs in culture, whereas in the absence of sorted DN T more apoptotic and dead cells were present in the culture (Fig 6E). CD11c^{hi} cells of the lungs can be divided in CD103$^+$ cDC$_1$, CD11b$^+$ cDC$_2$ and CD64$^+$ monocyte derived cells. Within the total population of CD11c^{hi} lung cells, only CD11b$^+$ DCs and monocyte-derived cells had a survival benefit.

Discussion

Prior to the discovery of NKT cells, CD4$^-$CD8$^-$ double negative T cells were found as a major fraction of lung lymphocytes, expressing an intermediate level of TCR, and representing up to 20-60% of all lung CD3$^+$ cells[52]. However, since NKT cell and $\gamma\delta$ TCR specific antibodies have been used in combination with the αGalCer CD1d tetramer in multi-color flow cytometry, the frequency of classical TCR^{int} DN T cells was found to be much lower, in the range of 1-2% of lung CD3$^+$ T lymphocytes[28, 32]. We found that the only population of DN T cells that accumulated following IAV infection with X31 or PR8 infection was characterized by intermediate expression of $\alpha\beta$TCR, yet lacking expression of NK1.1. Analysis of αGalCer CD1d tetramers showed that these cells were not type I NKT cells. A minor contamination of NKT-like cells or type II NKT cells in the CD1d TM$^-$NK1.1$^-$ DN T cell gate cannot be excluded since those cells can also lose expression of NK1.1[53, 54].

The precise origin of these cells has been unclear, but it has been suggested that they originate from the thymus by escaping negative selection[55, 56]. The fact that the numbers of TCR^{int} DN T cell are unaffected in the lungs of athymic nude mice, led to the suggestion that these cells might also arise extrathymically, very similar to the intraepithelial lymphocytes of the lamina propria of the gut[27, 28]. However, in our hands the number of DN T cells in the lungs was severely reduced in athymic mice. This suggests that NK1.1$^-$ DN T cells develop via the thymus. In the context of immune
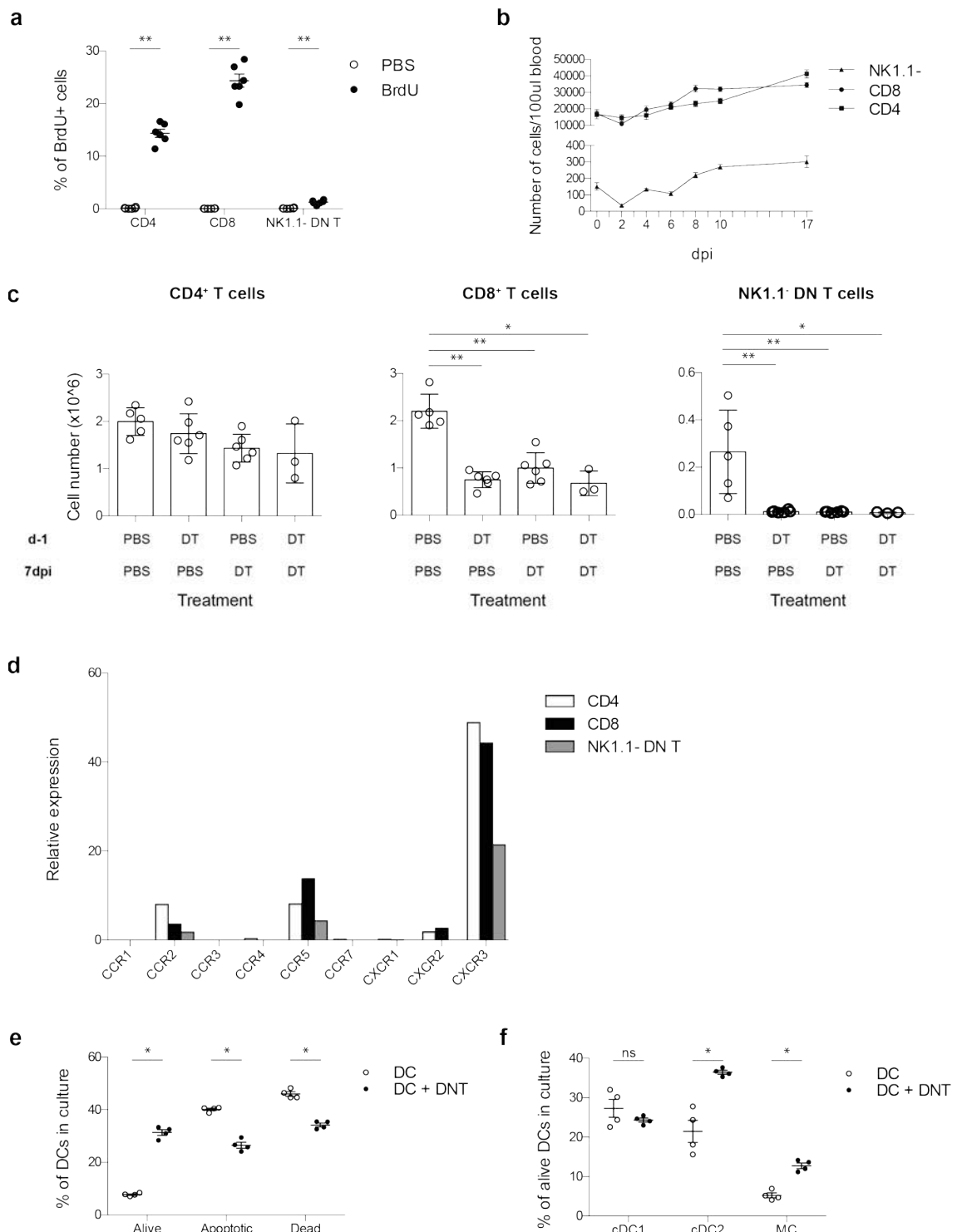
Figure 6 - NK1.1⁻ DN T cells are recruited from the blood in a DC-dependent manner and control the DC subset balance

(A) BrdU expression on conventional CD4 and CD8⁺ T cells and NK1.1⁻ DN T cells 3,5h after i.v. injection of BrdU (black) or PBS (white). (B) Kinetics of conventional CD4 (squares) and CD8 (circles) T cells and NK1.1⁻ DN T cells (triangles) per 100ul blood after X31 infection. (C) CD11c DTR chimeric mice were injected with PBS or DT 1 day before infection with X31 virus or 7 dpi. CD4, CD8 and NK1.1⁻ DN T cells in the lungs were quantified 9dpi. (D) Q-PCR analysis of the chemokine receptor repertoire of sorted CD4 (white bars), CD8 (black bars) and NK1.1⁻ DN T (grey bars) cells from the lungs of mice infected with X31 8dpi; expression levels were normalized for expression of the housekeeping gene *hprt*. (E) Proportion of alive, apoptotic (Annexin V⁺) and dead (7AAD⁺) pulmonary DCs in culture after 36h of coculture with (black) or without (white) sorted DN T cells. (F) Proportion of the alive pulmonary DC subsets in culture after 36h of coculture with (black) or without (white) DN T cells. All experiments were performed two times and figures are representative for every separate experiment. ** = p < 0,01, * = p < 0,05, ns = not statistically significant different

activation, some T cells might downregulate TCR expression after cognate ligand-MHC recognition and downregulate CD8 membrane expression, which could also lead to a very similar phenotype of TCR^{int} DN T cells[38]. Intracytoplasmic staining for CD4 and CD8 did however not reveal evidence for selective downregulation of membrane CD4 expression and only a small fraction of NK1.1$^-$ DN T cells showed intracellular CD8 expression. The Vβ repertoire of the NK1.1$^-$ NKT cells was distinct from the Vβ repertoire of CD4$^+$ and CD8$^+$ T cells. Furthermore, the Vβ repertoire was not skewed towards a NKT[57, 58] or MAIT cell[58, 59] usage. However, staining with a K^b-NP tetramer did reveal some MHCI-restricted antigen-specificity shared with conventional CD8 cytotoxic T cells. Lineage specific transcription factor analysis also demonstrated that a part of the NK1.1$^-$ DN T cells were more related to CD8 than to CD4 T cells. Together, these data suggest that some 10% of NK1.1$^-$ DN T cells represent antigen specific CD8$^+$ T cells that have lost surface expression of CD8, while maintaining it in the cytoplasm. Studies in human systemic lupus erythematosus patients have shown that DN T cells can originate from CD8$^+$ T cells by upregulation of the CREMα transcription factor that in turn represses expression of the CD8A and CD8B gene[60, 61]. As we found residual cytoplasmic expression of CD8 in 10% of DN T cells, this is an unlikely scenario. One clear difference between lung CD8$^+$ and NK1.1$^-$ DN T cells was the steady-state activation state in the lung. Indeed, the majority of lung NK1.1$^+$ DN T cells were CD44^{hi}, whereas a majority of CD8$^+$ T cells was CD44^{neg} in the mock-infected lung. Studying lymphocytes in the lung is not straightforward, as the lung is a highly vascularized organ and houses a major reservoir of recirculating naïve or T$_{cm}$ lymphocytes in the lung capillaries. These lymphocytes cannot always be removed by flushing the lung vasculature with PBS via the pulmonary artery. One way of reliably studying conventional T$_{rm}$ cells is to in vivo label these cells by intravenous injection of antibodies to CD4 or to CD8, labeling mainly the intravascular pool of lymphocytes, followed by ex vivo staining for other surface markers, labeling all lymphocytes[3, 62]. Due to their tissue residence around large airways, T$_{rm}$ cells are protected from labeling by i.v. injected antibody. These studies have been performed using antibodies to CD4 or CD8, but these antibodies were not useful for identifying DN T cells in vivo. To delineate intravascular DN T cells and conventional T cells simultaneously, we developed an in vivo labeling method employing the pan leukocyte marker CD45, effectively labeling 100% of circulating peripheral blood CD3$^+$ T cells and a majority of lung CD4 and CD8$^+$ T lymphocytes, demonstrating that most of the lung conventional lymphocytes in the resting lung are in the lung vascular pool, even after extensive exsanguination and flushing. Only after IAV infection, 90% of lung conventional CD4 and CD8$^+$ T cells were protected from in vivo CD45 labeling and these cells also variably expressed CD69, CD11a and CD103, as previously described for T$_{rm}$ cells and thus validating the use of CD45 labeling[3, 15]. On the contrary, the majority of lung NK1.1$^-$ DN T cells were already protected from CD45 in vivo labeling in the steady-state mock-infected lung, and expressed high levels of CD11a identifying these cells as T$_{rm}$ cells. This is also the reason why the levels of CD44 were so different between CD8$^+$ and NK1.1$^-$ DN T cells, as they were representing the differences between naïve and memory cells respectively. The activated phenotype was previously also reported in human patients with cutaneous leishmaniasis[63] and tuberculosis[64]. The memory profile of NK1.1$^-$ DN T cells argues against a MAIT cell

phenotype or contamination since MAIT cell are reported to have a mostly naïve phenotype in mice[65] and MAIT cell activation is not observed in *in vitro* viral infection models[66].

Heterosubtypic immunity to different strains of IAV that differ in hemagglutinin and neuraminidase is poorly understood but very desirable if we are to develop a universal IAV vaccine. It is generally believed to be mediated by T lymphocytes that reside in the lung as T_{rm} cells, a phenotype also seen in NK1.1⁻ DN T cells prior to and following infection. One striking finding in our study however was that reinfection with heterosubtypic virus did not lead to expansion of lung NK1.1⁻ DN T cells, despite the fact that these cells expressed a phenotype of CD44^{hi}, CD11a^{hi} T_{rm} cells, and some had specificity for viral nucleoprotein. A lack of further expansion upon reinfection with heterologous virus does not prove that these cells play no role in mediating heterosubtypic immunity. A study using depleting antibodies is however very difficult to design as NK1.1⁻ DN T cells are defined by lack of expression of markers. We initially set up experiments in athymic nude mice so that we could use depleting anti-CD3 antibodies to deplete DN T cells. Unfortunately however, lung DN T cells were already depleted in athymic mice.

Previous studies on the function of DN T cells in lung immunity have led to conflicting results, possibly due to differences in models used. In a passive transfer model of DN T cells to immunodeficient mice, there was no protection offered against respiratory infection with *Rhodococcus equi*[67]. However, in a model of *Francisella tularensis* respiratory infection, DN T cells were found to be a prominent source of IFNγ and IL-17 early, but not late after infection[30]. In our hands NK1.1⁻ DN T cells made IFNγ but no IL-17 after restimulation with NP$_{ASNENMETM}$ peptide (data not shown) and IFNγ was downregulated by IAV infection. We have purified NK1.1⁻ DN T cells and adoptively transferred them to other mice in an attempt to study the function of these cells that were recruited to the lungs after IAV infection (data not shown). Unfortunately, the numbers of cells were too low to perform conclusive adoptive transfer studies. We can therefore only speculate on the potential role of NK1.1⁻ DN T cells in IAV, guided by experiments from the past.

An important consideration is that NK1.1⁻ DN T cells might have immunoregulatory capacity as they closely resemble the DN Tregs that control allograft rejection by specifically killing Ag specific effector T cells with the same specificity or by killing DCs in a FasL dependent manner[51, 68]. One striking observation was that 20% of the lung NK1.1⁻ DN T cells expressed high levels of FasL in steady state lung. However, when we cultured lung NK1.1⁻ DN T cells together with lung DCs, we found that the presence of DN T cells did not kill DCs, but rather led to a higher percentage of DCs in the culture, mainly attributable to an increased survival of CD11b⁺ cDC$_2$ DCs and monocyte derived cells, while CD24⁺ cDC$_1$ DCs were not affected by the presence or absence of DN T cells. Thus, interaction of DN T cells with certain DC subsets turns them less sensitive to apoptosis. As DN T cells were previously described to interact with other cell types such as CD8 and CD4 T cells, B cells, macrophages and NK cells[69], it remains an interesting topic to study the interaction of DN T cells with several types of immune cells and to determine if they can exert different functions depending on the cell type they interact with.

In human studies, DN T cells are often reported to be correlated with progression or severity of disease. DN T cells decrease upon HIV disease progression[70] and are inversely correlated with disease activity in rheumatoid arthritis[71]. In contrast, DN T cells are increased during severe *M. tuberculosis* infection compared to non-severe *M. tuberculosis* infections[64] and during active Sjögren's syndrome[72] in which the level of DN T cells correlates with the degree of tissue inflammation[73]. Although the fact that DN T cells contract quickly after viral clearance (8dpi) and thus correlate with the kinetic of disease, we could not confirm a relationship with severity of infection since there was no significant difference between the amounts of DN T cells after X31 or PR8 infection, that cause different degrees of weight loss. Future experiments will have to address whether this subset of lung DN T cells has an influence on pulmonary immunity and regulates the severity of immunopathology to variants of IAV.

In conclusion, we have carefully characterized a subset of NK1.1$^-$ DN T cells that resides as a preactivated T$_{rm}$-like cell in the lung parenchyma, protected from i.v. labeling. This population rapidly expands in response to IAV infection in a process requiring CD11c^{hi} DCs, and has the capacity to balance the ratio of DC subsets. Future studies, in which these cells might be depleted selectively using genetic tools will have to address whether these cells are beneficial or harmful to the outcome of IAV infection.

Materials and methods

Mice

C57Bl/6 and athymic nude-Foxn1^{nu} mice (8-10w) were purchased from Harlan Laboratories. CD11c-DTR Tg (H2-D^b) mice were bred and housed in specific pathogen-free conditions. All experiments were performed on 4-6 mice per group, unless mentioned otherwise.

Ethics statement

All experiments were approved by the independent animal ethics committees "Ethische Commissie Dierproeven – faculteit Geneeskunde en Gezondheids-wetenschappen Universiteit Gent" (identification number: ECD 13/05) and "Ethische Commissie Proefdieren – faculteit Wetenschappen Universiteit Gent en VIB-site Ardoyen" (identification number: EC 2013_002). Animal care and used protocols adhere to the Belgian Royal Degree of May 29th 2013 for protection of experimental animals. European guideline 2010/63/EU is incorporated in this Belgian legislation.

Influenza virus infection

Mice were infected intranasally with 10^5 TCID$_{50}$ H3N2 X-31 influenza virus, 5 TCID$_{50}$ H1N1 PR8 influenza virus (Medical Research Council, Cambridge, England) or mock (allantoic fluid of uninfected eggs); all diluted in 50µl PBS.

For reinfection experiments, mice were infected with 10^5 TCID$_{50}$ X-31 or mock virus and were reinfected 30 days later with 3x10^5 TCID$_{50}$ X-31, 5 TCID$_{50}$ PR8 or mock virus diluted in 50µl PBS. Weight loss was monitored daily.

Isolation of lung cells

Mice were sacrificed and bronchoalveolar lavage was performed by injecting 3 times 1ml EDTA-containing PBS through a tracheal catheter before isolating the lungs. For

some experiments lungs were additionally flushed with 20ml PBS through the right heart ventricle before isolation. Single cell lung suspensions were prepared by digestion in collagenase/DNase solution for 30 minutes at 37°C. After digestion, the suspension was filtered over an 100μm filter and red blood cells were lysed with osmotic lysis buffer.

Flow cytometry and cell sorting
T cell staining was done by using CD3 (PE-Cy7 and eFl450, eBioscience; APC, BD biosciences), CD4 (conjugated to PE-TxR, Invitrogen; PE-Cy5 and FITC, eBioscience), CD8a (conjugated to efluor450 and PE-Cy7, eBioscience; PerCp, Biolegend; PE-Cy5, BD biosciences), CD19 (conjugated to APC, BD biosciences; AF700 and PE-Cy5, eBioscience), NK1.1 (conjugated to BV605, Biolegend; PE-Cy7, BD biosciences), CD1d tetramer (conjugated to PE and APC, NIH tetramer core facility), αβTCR (conjugated to APC-Cy7, Biolegend), γδTCR (conjugated to FITC, BD biosciences), NP tetramer (conjugated to PE, loaded with ASNENMETM peptide, Pelimer, Sanquin) and a fixable live/dead marker in eFl506 (eBioscience). Following additional extracellular markers were used: B220 (conjugated to PE, BD biosciences; AF700, eBioscience), CD44 (conjugated to AF700, BD biosciences), CD127 (conjugated to PE-CF594, BD biosciences), KLRG1 (conjugated to APC, eBioscience), CD69 (conjugated to PerCp-Cy5.5, BD biosciences), CD103 (conjugated to PE, eBioscience), FasL (conjugated to PE-Cy7, eBioscience), CD11c (conjugated to PE-TxR, Invitrogen), PD-1 (conjugated to PE-Cy7, Biolegend), annexin V (conjugated to PE, BD biosciences) and 7-AAD (BD biosciences). Granzyme B (conjugated to PE, Life Technologies Europe) and perforin (conjugated to APC, eBioscience) was stained intracellularly. The TCR repertoire was analyzed by using the mouse Vβ TCR screening panel (conjugated to FITC, BD biosciences) staining Vβ 2, 3, 4, 5.1 + 5.2, 6, 7, 8.1 + 8.2, 8.3, 9, 10b, 11, 12, 13, 14 and 17a. DC subsets were defined by using CD3 (conjugated to PE-Cy5, Tonbo Bioscience), CD19 (conjugated to PE-Cy5, eBioscience), CD11c (conjugated to PE-Cy7, eBioscience), MHCII (conjugated to APC-Cy7, Biolegend), CD11b (conjugated to BV605, BD bioscience), CD24 (conjugated to eFl450, eBioscience), FcεRI (conjugated to biotin, eBioscience) combined with SAV (conjugated to CF594, BD bioscience) and a fixable live/dead marker in eFl506 (eBioscience).
Acquisition of 12-color samples was performed on a LSR II or Fortessa cytometer equipped with FACSDiva software (BD biosciences). Final analysis and graphical output were performed using FlowJo software (Tree Star, Inc.).
For soring of T cells, cells were stained as described and cell sorting was performed on a FACSAria II (BD biosciences). The purity of sorted populations was >95%.

Cytokine staining – in vitro restimulation
Lung single cell suspensions were restimulated with NP$_{ASNENMETM}$ peptide (10μg/ml, Anaspec) 5 hours at 37°C in the presence of Golgi stop (BD biosciences, 1/1500) at a concentration of 5x10^6 cells/ml. After restimulation, cells were washed and stained extracellular, washed with PBS and fixed with 2% PFA, permeabilized with 0,5% saponin and stained intracellularly for IFNγ (Conjugated to PerCp-Cy5.5, eBioscience).

In vivo CD45 labeling
3μg anti-CD45 antibody (AF700, eBioscience) was injected i.v. Mice were killed 5 minutes later, blood was collected immediately before performing bronchoalveolar lavage. To remove blood from the capillary bed of the lungs, the lungs were flushed by injecting 20ml PBS through the right ventricle. To protect the *in vivo* CD45 staining, lungs were dispersed mechanically instead of enzymatically by smashing them through an 40μm filter before lysis of red blood cells.

BrdU incorporation assay

Mice were injected i.p. with 200µl of 10µg/ml BrdU (Sigma, 2µg total/mouse) 6dpi and were killed 3,5 hours after BrdU treatment. Lung cells were isolated as described above. Extracellular stained T cells were fixed and permeabilized by using the BrdU Flow Kit (BD biosciences) according to the manufacturer's protocol in combination with an eFl450 labeled anti-Brdu antibody (eBioscience).

Depletion of CD11c^{hi} cells

C57Bl/6 mice were irradiated sublethally (9Gy) and reconstituted with 2x10^6 bone marrow cells i.v. from CD11c DTR transgenic donor mice 4 hours after reconstitution. Mice were used for experiment at least 10 weeks after reconstitution. CD11c DTR chimeric mice were injected intraperitoneally with 200ng diphteria toxin (DT) diluted in 200µl PBS or with PBS 24h before infection or 7 dpi. Lungs were analyzed 9 dpi.

Real-time quantitative RT-PCR.

Quantitative RT-PCR for *Thpok*, *Runx3*, *Ccr1*, *Ccr2*, *Ccr3*, *Ccr4*, *Ccr5*, *Ccr7*, *Cxcr1*, *Cxcr2* and *Cxcr3* were performed on cDNA samples obtained from sorted lung T cell subsets. Total RNA was extracted using Tripure reagent (Roche) according to the manufacturer's protocol. RNA was resuspended in Diethyl-polycarbonate (DEPC, Sigma) treated water. A total of 1µg RNA was used for reverse transcription using the Transcriptor High Fidelity Reverse Transcriptase kit (Roche) according to the manufacturer's protocol.

The subsequent target amplification on triplicates of each cDNA sample was performed using the Universal Probe Library system from Roche (that contains fluorescent hydrolysis probes of 8 loked nucleic acids (LNA). Primers were designed with the help of the web-based application Probefinder (https://qpcr.probefinder.com) and a minimum of 2 primer pairs per target were analyzed. Primers were validated first using the LC480 SybrGreenl Master (Roche) with melting curve analysis (TM calling) in the LC480 Software and then using the LC480 Probes Master. Aspecific primer pairs were discarded. Table 1 shows a comprehensive view of the primer/probe combinations chosen. PCR conditions were: 5' pre-incubation at 95°C followed by 45 amplification cycles of 10" at 95°C, 10" at 60°C and 20" at 72°C using a Lighcycler 480 (Roche). PCR amplifications for the housekeeping genes encoding *Hprt* or *L27* were performed during each run for each sample to allow normalization between samples.

Pulmonary DC-DN T cocultures

Pulmonary DCs were sorted from the lungs of IAV infected mice at 9dpi as lineage⁻, alive, CD11c⁺ MHCII⁺ cells. DN T cells were sorted from lungs of infected mice at 9dpi as previously described and cocultured with DCs in a 3,5:1 ratio in cell culture medium containing 10% FCS for 36 hours.

Statistical analysis.

All experiments were performed using 4-6 animals per group, unless mentioned otherwise. All experiments were performed at least two to three times. The difference between groups was calculated using the Mann-Whitney U test for unpaired data (Prism version 6; GraphPad Software, Inc.). Data are depicted as mean +/- SEM. Differences were considered significant when $P<0,05$.

Supplementary material
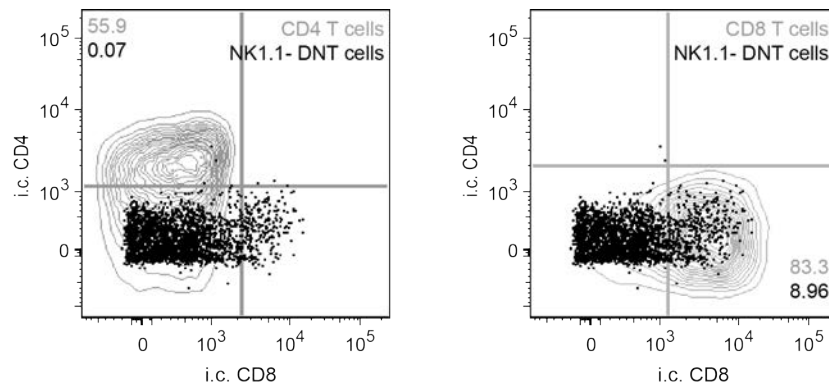
Figure S1

Intracellular expression of CD4 and CD8 in NK1.1⁻ DN T cells, that stained negative for extracellular CD4 and CD8, compared with T cells that have expression of extracellular CD4 (left) and CD8 (right). DN T cells are represented as black dots, CD4+ and CD8+ T cells are represented as a grey contour plot.
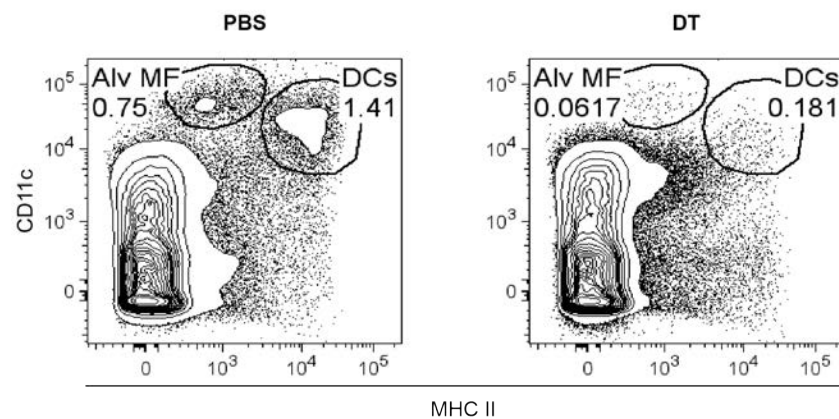

Figure S2

Depletion check in CD11c DTR chimeric mice. Mice received PBS (left) or DT (right) i.t. and lung DCs were analyzed one day later.

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Early IL-1 signaling promotes iBALT induction after influenza virus infection

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Abstract

Inducible Bronchus Associated Lymphoid Tissue (iBALT) is a long lasting tertiary lymphoid tissue that can be induced following influenza A virus (IAV) infection. Previous studies have shown that iBALT structures containing germinal center (GC) B cells protect against repeated infection by contributing locally to the cellular and humoral immune response. If we are to exploit this in vaccination strategies, we need a better understanding on how iBALT structures are induced. One hypothesis is that the strength of the initial innate response dictates induction of iBALT. In the present study, we investigated the role of IL-1 and IL-1R signaling on iBALT formation.

Mice lacking the IL-1R, had a delayed viral clearance and thus a prolonged exposure to viral replication, leading to increased disease severity compared to wild type mice. Contradictorily, iBALT formation following clearance of the virus was heavily compromised in $Il1r1^{-/-}$ mice. Quantification of gene induction after IAV infection demonstrated induction of IL-1α and to a much lesser extent of IL-1β. Administration of recombinant IL-1α to the lungs of wild type mice early but not late after IAV infection, led to more pronounced iBALT formation and an increased amount of GC B cells in the lungs. Bone marrow chimeric mice identified the stromal compartment as the crucial IL-1 responsive cell for iBALT induction. Mechanistically, Q-PCR analysis of lung homogenates revealed a strongly diminished production of CXCL13, a B cell attracting chemokine, in $Il1r^{-/-}$ mice during the early innate phase of IAV infection.

These experiments demonstrate that appropriate innate IL-1α - IL-1R signaling is necessary for IAV clearance and at the same time instructs the formation of organized tertiary lymphoid tissues through induction of CXCL13 early after infection. These findings are discussed in the light of recent insights on the pathogenesis of TLO formation in the lung in various diseases where the IL-1 axis is hyperactive such as rheumatoid arthritis and COPD.

Introduction

Influenza A virus (IAV) is a respiratory pathogen that causes seasonal or pandemic outbreaks with severe outcome in elderly and immune compromised patients. Epithelial cells are the first target cells for IAV infection[1, 2] and also coordinate the innate immune defense to prevent spreading of the virus, via production of type I interferons (IFNs). Interleukin (IL)-1α and IL-1β are among the first cytokines that are secreted by epithelial cells and macrophages at sites of IAV replication[3]. Secretion of IL-1β requires activation of the Nlrp3 inflammasome that leads to activation of caspase-1 and cleavage of pro-IL-1β into IL-1β. Infection with IAV leads to activation of the Nlrp3 inflammasome in a process requiring the type I IFN induced RNAse L/OAS system, while the virus actively suppresses IL-1β production and Nlrp3 activation via the NS1 protein[4-6]. IL-1 induces the expression of endothelial adhesion molecules that promote the entry of innate inflammatory cells like neutrophils, NK cells, dendritic cells (DCs) and monocytes resulting in a double effect on the host. On the one hand it promotes survival by killing virus infected cells, clearing debris and alarming the adaptive immune response. On the other hand, overzealous neutrophil recruitment can also cause inflammatory pathology that can ultimately lead to diffuse alveolar damage and death[6-10]. Not surprisingly, the outcome of genetic deficiency of key components in IL-1 generation or signaling has been very different depending on the severity of the IAV infection[2, 9, 11].

Simultaneously with the activation of the innate immune response, adaptive immune responses are initiated in the draining lymph nodes by antigen presenting migratory DCs. The architecture of lymph nodes promotes contact between antigen presenting DCs and rare antigen-specific T cells and B cells of the adaptive immune system to maximize the immune response against a certain antigen. Antigen specific T lymphocytes undergo clonal proliferation upon encounter with antigen presented by antigen presenting cells and migrate back to the site of inflammation as T effector memory (T_{EM}) cells or become central memory T cells (T_{CM}) or T resident memory (T_{RM}) cells[12]. Antibody production is initiated from B-lymphocytes that differentiate into plasmablasts immediately, or become plasma cells after going through a germinal center (GC) reaction that promotes somatic hypermutation and affinity maturation of B cells[13].

The coordinated events of T and B cell activation induced by virus-laden DCs mainly occur in secondary lymphoid organs (SLOs) like lymph nodes and spleen that develop during embryogenesis at predefined areas, often at the crossroads of lymphatic vessels[14]. However, highly organized structures of T and B cells can also be formed in the lung after birth as an adaptation to the increased demand for a localized immune response. Various names such as lymphoid tissue neogenesis, ectopic lymphoid tissue and tertiary lymphoid structures have been used to describe these structures. Furthermore they are often named after the anatomical region in which they occur. In the lungs for example, lymphoid aggregations can often be found in close proximity to bronchi, and hence these are called inducible bronchus associated lymphoid tissues (iBALT). As they resemble SLOs anatomically and functionally, yet only develop after birth as a result of chronic immune stimulation, they can also be called tertiary lymphoid organs (TLOs), even when found within the boundaries of another organ.

TLOs have been implicated in protection against IAV. Mice that lack secondary lymphoid organs can mount a rapid CD8 T cell response during IAV infection due to the induction iBALT[15], induced after clearance of IAV infection. Such TLO structures are generally formed in close proximity to bronchi after IAV infection, but can also be observed in the lung interstitium and are fully formed 17dpi. iBALT structures that are induced in mice with functional lymph nodes can serve as an additional priming site for T cells[16] and can also contribute to the humoral immune responses[17]. Once formed, iBALT structures can mount high affinity immune responses to other antigenic stimuli than the initiating stimulus due to the presence of germinal centers that allow somatic hypermutation and affinity maturation complementing the immune response in the draining lymph nodes[16, 18]. Furthermore, iBALT structures could be the perfect environment for depots of viral antigen that were described long after viral clearance and recovery from viral infection, and possibly related to the induction or maintenance of virus-specific T_{RM} cells[19, 20].

Despite morphological and functional similarities between SLOs and TLOs, the pathways that control formation and maintenance of TLOs are less clear. Generally, the molecular pathway that organizes T and B cells in discrete areas resembles the highly regulated inductive pathway for SLO development. Production of CCL19, CCL21, CXCL12 and CXCL13 by stromal cells and B cells helps to organize and retain T and B cells in discrete areas[21-26]. Also IL-7 seems required for TLO formation, e.g. in joints of rheumatoid arthritis patients and mouse models[27-30] and lungs of idiopathic pulmonary hypertension (IPAH) patients[31]. It is more controversial which cells give the initial instruction for stromal cells to produce these chemokines. During SLO development in the fetal period, this is the distinct task of lymphoid tissue inducer (LTi) cells, a cell type that develops from Flt3+ and Flt3- a_4b_7 integrin positive common progenitors that also forms innate lymphoid cells (ILC), and get expanded in response to Flt3L injections[32, 33]. During formation of the lymph nodes and spleen, LTi cells provide signals for lymphoid organogenesis like lymphotoxin-beta (LTβ) acting on the LTβR on stromal cells, but the precise signals might differ from organ to organ. *Flt3l*^{-/-} mice for example have reduced LTi cells and lack Peyer's patches but still have lymph nodes[33]. However, research in *Id2*^{-/-} and *Rorc*^{-/-} mice, which lack LTi cells, showed that LTi cells were dispensable for the initial TLO induction after IAV infection or other forms of TLO induction [15, 34-37 38]. Although LTi cells seem not strictly necessary for TLO induction, an instructive LTβ-LTβR signal remains essential for proper TLO development[25, 39]. B cells, T cells and DCs are heavily induced during inflammatory processes and all express LTα1β2 on their cell surface[17, 35, 40, 41] therefore they are perfect candidates to function as a substitute for LTi cells.

Whatever the precise molecular mechanisms of TLO induction might be, these TLO structures are virtually always seen at sites of inflammation. Yet, which inflammatory cytokines contribute to TLO induction is currently unknown. After many insults to the lung, including viral or bacterial infection, interleukin (IL)-1α and IL-1β are among the first cytokines to be secreted[3]. IL-1 secretion induces the expression of endothelial adhesion molecules that promote the entry of innate and adaptive immune cells, and could thus promote TLO formation. On the other hand, it is also known that IL-1R^{-/-} mice have a

delayed viral clearance and thus a longer exposure to viral particles[7]. Chronic immune stimulation is often assumed to lead to TLO formation[42]. If IL-1 limits viral replication, it could reduce the trigger for TLO induction.

In this paper we addressed the role of IL-1 and IL-1R in TLO formation in the lung. We show that the iBALT-inducing events are initiated early after infection, long before the virus is cleared. More specifically we show that early IL-1R signaling is necessary for proper IAV-associated iBALT and germinal center (GC) induction and that prolonged viral presence does not automatically lead to iBALT induction.

Results

Il1r1^{-/-} mice have prolonged viral load but are unable to induce iBALT

To assess the immune response to IAV infection in mice lacking signaling via IL-1R, we infected *Il1r1^{-/-}* mice and monitored weight loss and viral load in the lungs. Wild type mice showed maximum weight loss around 6dpi and bodyweight was fully restored around 10dpi. *Il1r1^{-/-}* mice lost weight with slower and prolonged kinetics, and reaching a nadir at 8dpi. Like wild type mice they did manage to gain weight again, but did not recover to their starting bodyweight before 17dpi (Fig 1A). This difference in the weight loss curve was also reflected in the viral load in the lungs (Fig 1B). Viral load in wild type mice peaked around 6dpi yet was cleared at 8dpi. *Il1r1^{-/-}* mice had systematically higher viral loads during the entire course of infection and did not clear the infection completely at 8dpi as an estimated remaining titer of 100 000 viral particles is detected at this time point.

Viral clearance depends on induction of adaptive immunity by dendritic cells that activate CD8 and CD4 T cells and a humoral immune response by B cells. Total numbers and kinetics of increase of T and B cells and conventional dendritic cells (DCs) were not altered in *Il1r1^{-/-}* mice in response to IAV infection. Lung conventional DCs consist of various subsets that have different functions and can be discriminated based on cell surface markers CD11b and CD103[43, 44]. As soon as there is inflammation in the lung, monocytes can also be recruited and these can rapidly differentiate into a MHCII^{+}CD11c^{+} cell type (so called monocyte-derived cells, MC) that also expresses the macrophage marker CD64[45]. In contrast to the conventional CD103^{+} and CD11b^{+} DCs, the accumulation of CD11c^{+}CD64^{+} MCs was reduced in the lungs of *Il1r1^{-/-}* mice (Fig 1C).

To evaluate the effect of the higher and prolonged viral exposure on IAV-associated iBALT formation, we visualized iBALT structures in the lungs by hematoxilin staining. Generally clusters of cells near the bronchi were more readily detected in wild type mice than in *Il1r1^{-/-}* mice (Fig 1D). Because a hematoxilin stain did not allow us to evaluate if the inflammatory clusters of cells were organized and immunologically active iBALT structures, we stained frozen lung sections for B cells, T cells, DCs and GC B cells and analyzed them by confocal microscopy. In wild type mice we could easily detect organized structures composed of B cells, T cells and DCs and B cell aggregates that contain GC B cells, but we were unable to detect similar infiltrates in *Il1r1^{-/-}* mice (Fig 1E). To quantify the presence or absence of iBALT structures we measured the proportion of GC B cells in the lungs at 17dpi by flow cytometry as a measure for

biologically active iBALT. As GL7+ GC B cells are not found in the lungs of mice in the absence of iBALT, we believe this is a good approximation of the amount of iBALT formed[17]. Wild type mice showed an induction of GC B cells upon IAV infection, but this induction was absent in *Il1r1-/-* mice (Fig 1F). Taken together, these data suggest that although *Il1r1-/-* mice had a higher and prolonged exposure to viral particles in the lung, they were unable to form organized iBALT structures in the lung, pointing to an essential role for IL-1 cytokines in TLO induction following influenza virus infection.
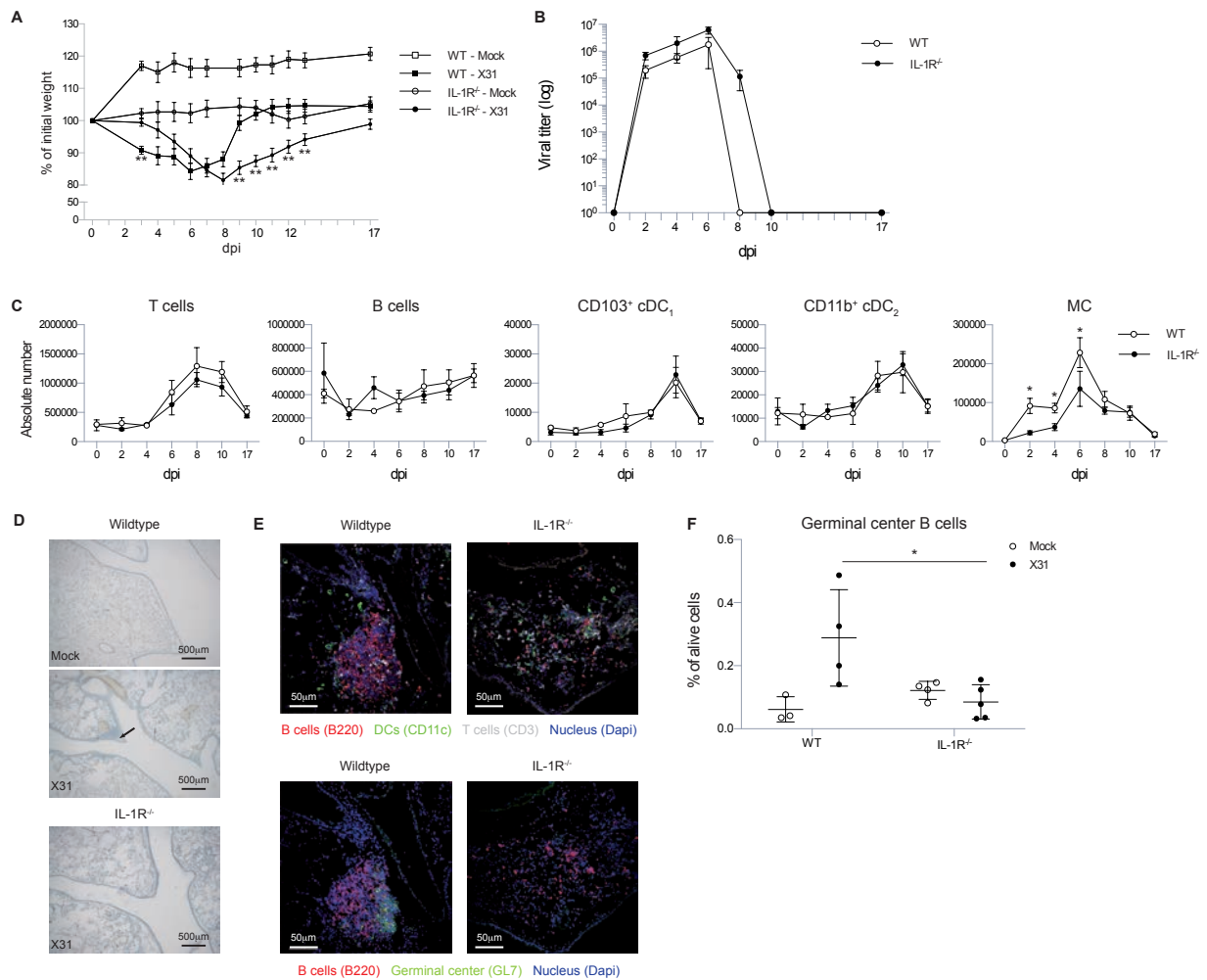

Figure 1 - *Il1r1-/-* mice have a prolonged viral load in the lungs, but do not develop iBALT structures
(A) Weight loss curve as percentage of the initial bodyweight for wild type (squares) and *Il1r1-/-* mice (circles) that have been infected with mock (white) or X31 (black) virus. (B) Viral titers in the lung of wild type (white) or *Il1r1-/-* (black) mice determined by hemagglutination assay after culture with MDCK cells. (C) Numbers of T and B cells, CD103+ cDC$_1$ and CD11b+ cDC$_2$ DCs and monocyte derived cells (MC) in the lungs of wild type (white) and *Il1r1-/-* mice (black). (D) Hematoxylin-stained lung sections 17dpi of wild type and *Il1r1-/-* mice. Images are representative for at least 3 mice per group. (E) Confocal images of lung sections of wild type and *Il1r1-/-* mice at 17dpi. Sections were stained with either B220 (red), CD11c (Green), CD3 (Grey) and dapi (blue) or B220 (red), GL7 (Green) and dapi (Blue). Shown images are representative for 5 mice per group. (F) Proportion of germinal center B cells (GL7+) in the lungs of wild type and *Il1r1-/-* mice infected with mock (white) or X31 (black) virus at 17dpi determined by flow cytometry. All experiments were performed at least twice with 4-6 mice per group. * = p<0,05

IL-1α administration promotes induction iBALT structures in the lung
As the mere presence of viral particles was not enough to trigger the iBALT initiation and IL-1R signaling was necessary we quantified expression of IL-1α and IL-1β, which signal

both via IL-1R, in the lungs after IAV infection. Both cytokines were induced after infection in a bimodal curve with a first peak around 4dpi and a second, but smaller, peak around 10dpi (Fig 2A). In general, the induction of mRNA for IL-1α was more pronounced compared with IL-1β.

We next addressed if administration of IL-1 cytokine would be enough to further boost iBALT induction in IAV infected mice. Since IL-1α and IL-1β have similar effects on the IL-1R, and as induction of IL-1α was more pronounced after IAV infection, we chose to only administer IL-1α. When recombinant IL-1α was administered i.t. 2 days post IAV infection in wild type mice, clustering of inflammatory cells could be detected more readily on lung sections compared to PBS treated IAV infected mice (Fig 2B). To quantify biologically active iBALT we again quantified the proportion of GC B cells by flow cytometry. Administration of recombinant IL-1α at 2dpi resulted in a higher proportion of GC B cells in the lungs 17dpi compared to PBS administration (Fig 2C). However, when the administration of recombinant IL-1α was only initiated at 10dpi, no differences in the proportion of GC B cells could be observed between IL-1α and PBS treated groups (Fig 2C). This suggests that early, but not late IL-1R signaling is necessary and sufficient to promote GC B cell positive iBALT structures.
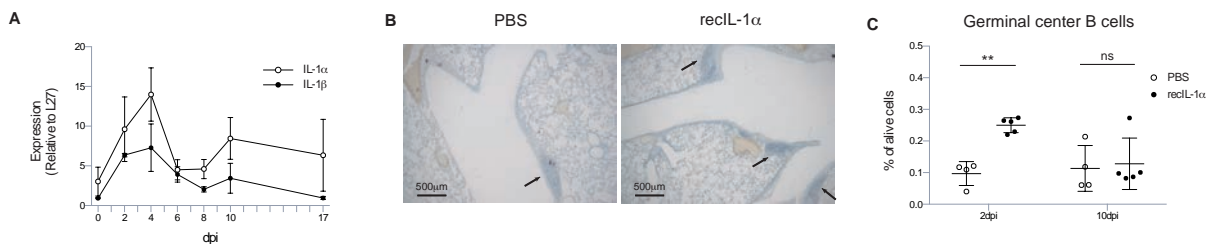

Figure 2 - Early IL-1α signaling is sufficient and necessary for iBALT induction
(A) mRNA expression of IL-1α (white) and IL-1β (black) relative to the housekeeping gene L27 in the lungs of wild type mice. (B) Hematoxylin-stained lung sections 17dpi of mice treated with PBS or recIL-1α 2dpi. Images are representative for at least 3 mice per group. (C) Proportion of germinal center B cells 17dpi in the lungs of wild type mice treated with PBS (white) or recombinant IL-1α (black) i.t. 2 or 10dpi.
All experiments were performed at least twice with 3-5 mice per group. ns = not significant, ** = p<0,01

IL-1R signaling on stromal cells is necessary to induce GC B cells

As early IL-1R signaling is necessary to induce iBALT structures in the lung, we sought to identify the cell type that is responsive to IL-1 signals. Therefore we constructed bone marrow chimeric mice in which either the radiosensitive hematopoietic or the radioresistant stromal compartment was deficient for *Il1r1*. As a control we also reconstituted *Il1r1*-/- mice with *Il1r1*-/- bone marrow as a substitute for intact *Il1r1*-/- mice and control for irradiation effects. *Il1r1*-/- mice that received wild type bone marrow followed a weight loss curve characterized by a longer weight loss and slower recovery, as observed in *Il1r1*-/- mice. In contrast, wild type mice that were reconstituted with *Il1r1*-/- bone marrow cells followed a weight loss curve that resembled the one observed in wild type mice with a maximum of approximately 15% weight loss. Surprisingly, *Il1r1*-/- mice that were reconstituted with IL-1R sufficient bone marrow had a tendency to lose more weight than the *Il1r1*-/- mice that were reconstituted with IL-1R-deficient bone marrow (Fig 3A). We also counted the proportion of GC B cells in the lung. Wild type

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mice with IL-1R-deficient hematopoietic cells were able to induce a higher proportion of GC B cells than *Il1r1⁻/⁻* mice with normal hematopoietic cells, suggesting that IL-1 boosts the formation of iBALT structures by signaling to radioresistant stromal cells.
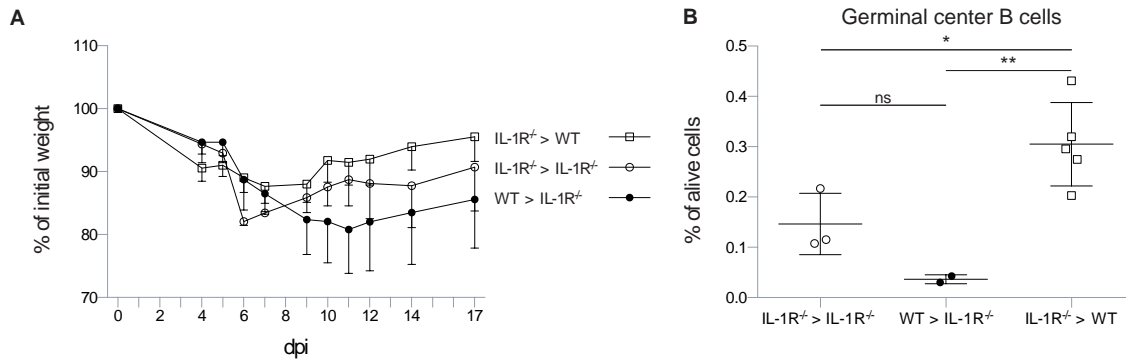

Figure 3 - IL-1R signaling on stromal cells is necessary to induce germinal center B cells in the lung
(A) Weight loss curve after infection with X31 virus as percentage of the initial bodyweight for wild type (squares) and IL-1R⁻/⁻ (circles) mice reconstituted with wild type (black) or IL-1R⁻/⁻ bone marrow (white). (B) Proportion of germinal center B cells 17dpi in the lungs of wild type (squares) and IL-1R⁻/⁻ (circles) mice reconstituted with wild type (black) or IL-1R⁻/⁻ bone marrow (white). ns = not significant, * = p<0,05, ** = p<0,01

CXCL13 expression is reduced in Il1r1⁻/⁻ mice
To define which downstream iBALT-instructive signals are induced by IL-1R signaling in radioresistant cells, we measured the expression level of the chemokines CXCL12, CXCL13, CCL19 and CCL21 which all have been implicated in iBALT formation. The chemokines CCL19 and CCL21 instruct the organization of T cell zones in iBALT structures, but were not impaired in *Il1r1⁻/⁻* mice (Fig 4A). The chemokine CXCL12 is important for B cell lymphopoiesis but expression of this chemokine was only slightly reduced early after infection in *Il1r1⁻/⁻* mice. The chemokine CXCL13 functions as an LTᵢ and B cell chemoattractant and its expression was reduced 4dpi in *Il1r1⁻/⁻* mice (Fig 4A). None of the chemokines involved in iBALT formation and organization was differentially expressed at 17dpi, when iBALT had fully developed in wild type mice, showing that the instructive chemokine signals are given early after infection when the virus is not yet cleared from the lungs. Although CXCL13 expression was impaired early after infection, the total amount of B cells in the lungs was not significantly altered at the time iBALT was present (Fig 1C), suggesting that it is not recruitment of B cells to the lungs that is impaired in *Il1r1⁻/⁻* mice, but that the B cells in the lung fail to cluster into organized iBALT structures. Whether this is a direct or indirect effect of defective IL-1R signaling on stromal cells remains a subject for future experiments.

Discussion and review of the literature
The IL-1 axis has been described previously to be responsible for inflammatory pathology in the lung, resulting in increased mortality, increased viral titers and neutrophil recruitment following IAV infection[7]. *Il1r1⁻/⁻* mice indeed suffered more from the mild X31 IAV infection and displayed a tendency to higher viral titers in the lung, but
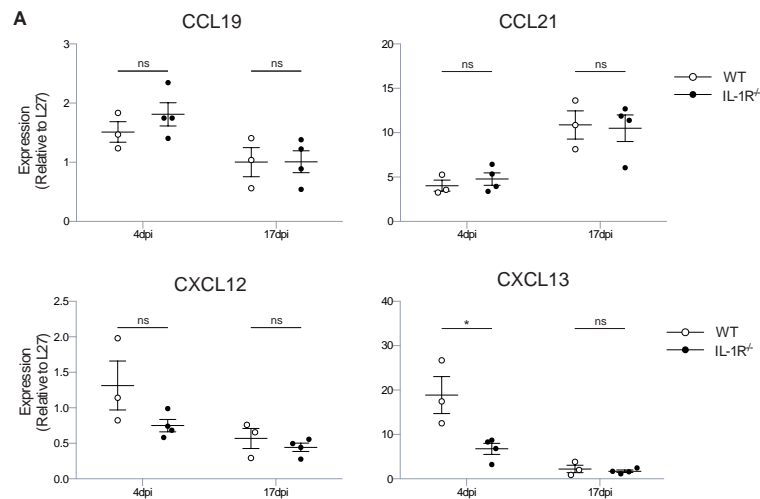
Figure 4 - *Il1r1-/-* mice have a defect in the production of the iBALT-inducing chemokine CXCL13

(A) mRNA expression levels of the chemokines involved in iBALT induction: CXCL12, CXCL13, CCL19, CCL21 relative to the expression of housekeeping gene L27.
The experiment was performed twice with 3-5 mice per group. ns = not significant, * = p<0,05.

did manage to clear infection with delayed kinetics, leading to a presumably higher viral exposure over time. Despite this increased viral exposure, they hardly formed iBALT structures in the lung. Conversely, when recombinant IL-1 was administered early after IAV infection to wild type mice, the formation of iBALT structures was facilitated.

Mechanistically, we found impaired CXCL13 chemokine induction early after infection in *Il1r1-/-* mice. Later, at the time when iBALT was fully formed in wild type mice, we could not detect any differences in CXCL13 levels, suggesting that the instructive signals that condition the lung for clustering adaptive immune cells are given very early (2-4dpi) after infection. By studying iBALT formation in chimeric mice, we found that IL-1R expression on stromal cells is necessary for proper iBALT formation. The exact cell type of stromal cells that is needed to induce GC B cells however still needs to be defined. Whether this stromal cell type is directly responsible for the CXCL13 production needed to initiate iBALT formation or an intermediate cell type is involved remains a matter of debate.

As we observed a decreased induction in MCs and these cells are previously described as being major cytokine and chemokine producers[45], it is a possibility that these cells are involved in the CXCL13 induction. Alternatively, LTβ-sufficient B cells can support the progression towards mature, fully structured TLOs[35, 46] most likely via a positive feedback loop of CXCL13 production and LTβ expression[24]. According to this hypothesis B cells are activated via TLR signaling, induce expression of LTβ on their surface and interact on its turn with LTβR bearing B cells. This LTβ signaling will induce CXCL13 production and release, which attracts more B cells and upregulates LTβ expression. It is an attractive hypothesis that IL-1 might also induce LTβ expression on B cells, although this is hard to reconcile with our observation that a radioresistant cell type responds to IL-1 in our model.

We can only speculate about the source of IL-1α. Previous research has shown that IL-1α can be released by dying cells[47]. In this respect virus-infected lung epithelial cells might

be a possible source of IL-1α as it has been observed that IL-1α can feedback on epithelial cells and induce a second cytokine and chemokine wave during innate immune responses in the lung[3, 48]. We have only measured the mRNA for IL-1β. Secretion of bioactive IL-1β requires activation of the Nlrp3 inflammasome that leads to activation of caspase-1 and cleavage of pro-IL-1β into IL-1β. Infection with IAV leads to activation of the Nlrp3 inflammasome in a process requiring the type I IFN induced RNAse L/OAS system, while the virus actively suppresses IL-1β production and Nlrp3 activation via the NS1 protein[4, 5]. Although others have shown that the NLRP3 inflammasome controls severity of infection[9, 11, 49, 50], future studies will have to address if lack of key components of this inflammasome also leads to reduced iBALT formation.

Generally TLOs are absent in the lungs of healthy adults[51], but bronchus associated lymphoid tissue can be observed in the lungs of children that are frequently infected by respiratory viruses[52] and in the lungs of adults that suffer from rheumatoid arthritis[53, 54], transplant rejection[55], COPD[56] and IPAH[31]. We can only speculate that IL-1 might also be involved in the formation of these TLO structures. IL-1 is certainly a cytokine that has been implicated in the pathogenesis of rheumatoid arthritis, and targeting the IL-1 pathway via IL1RA (anakinra) has been used as an alternative biological treatment in patients failing therapy on TNFα blockade. A very common risk factor for rheumatoid arthritis development is smoking, which also leads to COPD. End stage COPD is also accompanied by TLO formation in the lungs, and these can be sites of production of antibodies to citrullinated antigens, typical of RA patients. In a preclinical model of smoking induced TLO formation, the production of autoantibodies and TLO structures was reduced in $Il1r1^{-/-}$ mice, accompanied by a reduced CXCL13 production in the lungs[57].

During development neuronal cells give an LTα1β2-independent instructive signal to local fibroblasts to produce CXCL13 and hereby attract CD3$^-$CD4$^+$CD45$^+$ LT$_i$ cells[58, 59]. The crucial step for SLO development is the interaction of LT$_i$ cells with stromal lymphoid tissue organizer (LT$_o$) cells. This process happens via interaction of LTβ expressed on LT$_i$ cells and the LTβR expressed on LT$_o$ cells. Upon this interaction, LT$_o$ cells produce homeostatic chemokines that drive the recruitment of lymphocytes. T cells and DCs are attracted by chemokine CC ligand (CCL)19 and CCL21; B cells are attract by chemokine CXC ligand (CXCL)13. Expression of vascular cell adhesion molecule (VCAM)1, intercellular adhesion molecule (ICAM)1, mucosal addressin cell adhesion molecule (MADCAM)1 allow the attracted cells to cluster together. IL-1 has been very well known for its effects of stimulating adhesion molecules on endothelial cells[60]. It is tempting to speculate that the effects of IL-1 on radioresistant cells is via induction of the crucial adhesion molecules that initially tether a LT$_i$ like cell to the circulation and subsequently to initiate a communication between stromal cells and lymphoid cells, that initiates the CXCL13 production.

The LT$_i$-potential of T cells was first addressed in a model of thyroid overexpression of CCL21, where it was shown that CD3$^+$CD4$^+$ activated T cells interacted with DCs at sites of chronic inflammation, and subsequently acted as LT$_i$ cells in the absence of Id2

activity[37]. It has also been suggested that IL-17 signaling is involved during the initiation phase of iBALT formation by inducing CXCL13, but this role for IL-17 remains controversial[34]. In two studies on neonatal mice exposed to endotoxin inhalation and on mice with experimental autoimmune encephalomyelitis respectively, an activated Th17 CD4 T cell population was found to be involved in inducing TLO structures[34, 61]. RORC+ IL-17–producing cells were also found inside lung TLOs of patients with IPAH. In humans, Th17 cells express the CCR6 receptor, and in the bloodstream of IPAH patients circulating CCR6+ cells were fewer, while the ligand CCL20 was produced in the perivascular TLOs[31]. However, TLOs seem to develop normally in Ccr6-/- mice [34]. The induction of TLOs by Th17 cells was dependent on expression of podoplanin, but why this is the case remains unknown. One possibility is that podoplanin is required for retention of Th17 cells at sites of TLO formation[34, 61]. The role of Th17 as LTi-like cells is still under debate and it remains to be seen whether all forms of TLO depend on IL-17 production, and whether IL-17A and/or IL-17F is involved. In this regard, iBALT induced by infection with modified vaccinia virus Ankara or influenza virus is not affected by deficiency of IL-17A while *Pseudomonas Aeruginosa*-induced iBALT is dependent on IL-17 signaling[35, 62]. As IL-17 production by γδ T cells and Th17 cells can be induced by IL-1[63-66] we also considered the possibility that IL-17 is part of the cascade leading to IL-1α driven iBALT formation. In our hands, IAV infection indeed gave rise to a higher amount of IL-17+ CD4 T cells, but treatment with IL-1α 2dpi could not increase the amount of IL-17+ CD4 T cells in the lung, and experiments in which we administered IL-1 to IAV infected *Il17ra-/-* mice were inconclusive (data not shown). This suggests that, in contrast to SLO formation, the instructive signals can differ depending on the source of initiating antigen or the inflammatory stimulus that is elicited by the used model.

In almost all TLO structures that have been described, the T cell area contained antigen-presenting DCs[17, 31]. As DCs activate T cells, it has been suggested that DCs are sufficient for TLO induction[67]. This hypothesis is supported by the observation that repeated injection of DCs into the lungs of mice is sufficient for induction of iBALT structures accompanied by induction of myofibroblast differentiation[17, 68]. During formation of Peyer's patches, a CD11c+ cell type expressing LTαβ accumulates at the LN anlagen and is necessary for instruction of stromal cells[59]. DCs might also directly instruct stromal cells irrespectively of their effects on T cells. In TLO structures induced in the thymus, DCs were specifically necessary for induction of lymph angiogenesis from stromal cells [69] but how DCs induce TLOs is less clear. In virus-induced iBALT, mainly CD11b+ DCs or monocyte-derived cells accumulate; these cells express instructive LTα1β2 while also producing the homeostatic chemokines CXCL13 and CCL19/CCL21[17]. However, in some models mostly pDCs accumulate, suggesting a functional role for type I IFN. As is the case in TLOs found in end-stage COPD patients and in a murine SLE model[56, 70]. Three studies have shown that depletion of DCs leads to disappearance of existing TLO structures, suggesting that DCs are necessary for structural organization and maintenance of TLOs, most likely through transpresentation of chemokines, or by providing a continuous source of antigen presentation to T cells[16, 17, 69]. We did observe reduced numbers of monocyte-derived DCs in *Il1r1-/-* mice, but have not performed experiments in which only DCs lacked Il-1R to study if the effects of IL-1 were cell-intrinsic

or resulting from effects of IL-1 on epithelial cells. Indeed, IL-1R triggering on lung epithelial cells is a very well known trigger for the production of GM-CSF, one of the major cytokines driving activation of monocytes to adopt a DC-like phenotype[48].

In conclusion, we have described a novel role for early IL-1 production in IAV infection to control the formation of iBALT structures via induction of CXCL13 in a stromal cell compartment. Future studies will have to address if this effect of one of the best-known proinflammatory and innate cytokines is a general feature of TLO formation at sites of acute and chronic immune stimulation such as infectious disease and autoimmune pathologies and if this can be exploited to induce iBALT formation as part of a mucosal vaccination strategy[71].

Materials and methods

Ethics statement
All experiments were approved by the independent animal ethics committee "Ethische Commissie Proefdieren – faculteit Wetenschappen Universiteit Gent en VIB-site Ardoyen" (identification number: EC 2013_070). Animal care and used protocols adhere to the Belgian Royal Degree of May 29th 2013 for protection of experimental animals. European guideline 2010/63/EU is incorporated in this Belgian legislation.

Mice
C57Bl/6 mice (8-10w) were purchased from Harlan Laboratories. IL-1R$^{-/-}$ mice were bred in-house and housed in specific pathogen-free housing.
To create chimeric mice, IL-1R$^{-/-}$ or wild type acceptor mice were irradiated sublethally (9Gy) and reconstituted with $2x10^6$ bone marrow cells i.v. from wild type or IL-1R$^{-/-}$ donor mice respectively 4 hours after irradiation. Mice were used for experiment at least 10 weeks after reconstitution.

Influenza virus infection
Mice were infected intranasally with 10^5 TCID50 H3N2 influenza virus X-31 (Medical Research Council) or mock (allantoic fluid of uninfected eggs); diluted in 50µl PBS. Weight loss was monitored daily. For suppletion assays, mice were treated intratracheally with 80µg carrier free recombinant IL-1α (R&D) at 2 or 10 dpi.

TCID50 assay viral titers
Lungs were homogenized with a tissue homogenizer in 1ml PBS and centrifuged (5min, 400g) to remove cellular debris before storage at -80°C. Titers of infectious virus were determined in triplicate by titration on MDCK cells in serum-free TPCK-treated trypsin-containing medium. Viral titers were determined by measuring chicken red blood cell agglutination activity in the cell supernatant after 7 days of infection of MDCK cells by using the calculation method of Reed and Muench.

Isolation of lung cells
Mice were sacrificed and the lungs were removed. Single cell suspensions were prepared by digestion in collagenase/DNase solution for 30 minutes at 37°C. After digestion, the suspension was filtered over a 100µm filter and red blood cells were lysed with osmotic lysis buffer (10mM $KHCO_3$, 155mM NH_4Cl, 0,1mM EDTA in ddH_2O).

Flow cytometry

Lung cells were stained extracellularly with anti-CD3 (17A2, conjugated to AF700, eBioscience), anti-CD19 (1D3, conjugated to APC, BD Bioscience), anti-IgM (R6-60.2, conjugated to PerCp-Cy5.5, BD Bioscience), anti-IgD (11-26c.2a, conjugated to PE, BD Bioscience), anti-CD95 (Jo2, conjugated to PE-Cy7, BD Bioscience), anti-GL7 (GL7, conjugated to Fitc, BD Bioscience) and a fixable live-dead marker (conjugated to eFl506, eBioscience).

Acquisition of samples was performed on a LSR II or Fortessa cytometer equipped with FACSDiva software (BD biosciences). Germinal center B cells were defined as CD3$^-$CD19$^+$IgM$^-$IgD$^-$CD95$^+$GL7$^+$. Final analysis and graphical output were performed using FlowJo software v9 (Tree Star, Inc.).

Real-time quantitative RT-PCR.

Quantitative RT-PCR for IL-1α, IL-1β, LTβ, CCL19, CCL21, CXCL12 and CXCL13 were performed on RNA obtained from whole lung homogenates. Total RNA was extracted using Tripure reagent (Roche) according to the manufacturer's protocol. RNA was resuspended in Diethyl-polycarbonate (DEPC, Sigma) treated water. A total of 1μg RNA was used for reverse transcription using the Transcriptor High Fidelity Reverse Transcriptase kit (Roche) according to the manufacturer's protocol. The subsequent target amplification on triplicates of each cDNA sample was performed using the Universal Probe Library system from Roche (that contains fluorescent hydrolysis probes of 8 locked nucleic acids (LNA). Primers were designed with the help of the web-based application Probefinder (https://qpcr.probefinder.com) and a minimum of 2 primer pairs per target were analyzed. Primers were validated first using the LC480 SybrGreenI Master (Roche) with melting curve analysis (TM calling) in the LC480 Software and then using the LC480 Probes Master. Aspecific primer pairs were discarded. Table 1 shows a comprehensive view of the primer/probe combinations chosen. PCR conditions were: 5' pre-incubation at 95°C followed by 45 amplification cycles of 10″ at 95°C, 10″ at 60°C and 20″ at 72°C using a Lightcycler 480 (Roche). PCR amplifications for the housekeeping genes encoding *Hprt* or *L27* were performed during each run for each sample to allow normalization between samples.

Histology

Lungs were inflated with 1ml 1:1 PBS-OCT (Tissue Tec), snap frozen in liquid nitrogen and stored at -80°C. Frozen 8μm sections were fixed in 4% PFA and blocked in a 1% blocking buffer (Roche).

Immunofluorescence staining was performed by staining for B220 (RA3-6B2, Rat-a-mB220 conjugated to PE, BD Bioscience) + Goat-a-Rat conjugated to AF555, Invitrogen), CD4 (RM4-5, Rat-a-CD4 conjugated to Fitc, eBioscience + Rab-a-Fitc conjugated to AF488, Invitrogen), CD8 (53-6.7, Rat-a-CD8 conjugated to Fitc, BD bioscience + Rab-a-Fitc conjugated to AF488, Invitrogen), CD11c (N418, Hamster-a-mCD11c conjugated to AF647, eBioscience), GL7 (GL7, Rat-a-mGL7 Fitc, BD bioscience + a-Fitc conjugated to HRP, Jackson + a-HRP conjugated to Fitc, Jackson). Where necessary, slides were incubated with 10% normal rat serum to prevent aspecific binding of antibodies. Slides were counterstained with Dapi and digitized on a LSM710 microscope (Zeiss). All depicted pictures are representative of at least five mice per group. Images were analyzed using Imaris software (Bitplane).

Statistical analysis

All experiments were performed using 3-6 animals per group. All experiments were performed at least two times. The difference between groups was calculated using the student t test for unpaired data (Prism version 6; GraphPad Software, Inc.). Data are depicted as mean +/- SEM. Differences were considered significant when p<0,05.

Analysis of the repeated relative body weight data was performed using the residual maximum likelihood (REML) as implemented in Genstat v17 (Payne, 2013). The following linear mixed model (random terms underlined) was fitted to the data:

$Y_{ijkt} = \mu + genotype_j + treatment_k + time_t + (genotype.treatment)_{jk} + (genotype.time)_{jt} + (treatment.time)_{kt} + (genotype.treatment.time)_{jkt} + \underline{(mouse.time)_{it}} + \underline{residual}_{ijkt}$, where Y_{ijkt} is the relative body weight of *i*-th mouse having genotype *j*, *k*-treated and measured at time point *t* (*t* = 3 – 17 days; unequally spaced), and μ is the overall mean calculated for all mice considered across all time points. A first order antedependence covariance structure was used to model the within-subject correlation. The significance of the comparison between WT-X31 and KO-X31 across time was assessed by an F-test.

A log-linear model (Poisson distribution and log link) was fitted to the amount of viral particles measured by hemagglutination inhibition assay. The dispersion parameter was set as free. Significance of main effects GENOTYPE and TIME and the GENOTYPE.TIME interaction effect was assessed by an F test.

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Chapter 6

Summarizing discussion and future perspectives

Every winter period many people are affected by an influenza infection. While for the majority of people infected this does not cause severe illness or death, it does have a significant economical impact due to the associated medical costs and days lost from work or school. The threat of influenza pandemics is constantly present. Due to the genetic variability of influenza virus strains, the circulating viruses need to be monitored continuously and the vaccine composition needs to be adapted yearly. Currently no general vaccination strategy exists. Therefore it is crucial to enhance the knowledge about the molecular mechanisms behind genetic variability but also about the immune response against influenza infections.

The main immune responses against influenza virus infection are well understood. The epithelial layer provides a first natural barrier against infection, once this barrier is broken, influenza viruses can infect these epithelial cells. Innate immune cells like NK cells and neutrophils will recognize these foreign antigens and provide a first non-specific defense mechanism. Dendritic cells will sample viral antigens and present it to CD4$^+$ and CD8$^+$ T cells in the lung-draining lymph nodes (LNs). Antigen specific T cells will be activated, proliferate and migrate back to the lung environment where a second encounter with their cognate antigen will activate them to exert their effector functions: cytotoxic killing by CD8 T cells and Th1-mediated. Also in the LNs, antigen-specific B cells will be activated and will produce virus-specific antibodies to kill infected cells or limit viral spread. Once the infection is cleared, memory T and B cells will remain in the tissue and the secondary lymphoid organs that provide a rapid defense mechanism by reinfection with a homologous or heterologous virus.

In this thesis, different aspects of the immune response to influenza A virus infection were addressed – DC-mediated immunity, T cell immunity and iBALT formation – by using a mouse model for mild seasonal influenza infection and with the main focus on a better understanding of the heterogeneity of the different arms of the immune system.

In chapter 3 we examined the different populations of dendritic cells (DCs) present in the lung and LNs during influenza infection. Dendritic cells are the crucial link between the innate and adaptive immune responses, sampling antigen during the innate response and initiating the adaptive response by presenting such antigens to naïve T cells in the LN. As DCs are a crucial link to shift from a non-specific innate immune response towards a very specific adaptive immune response, better understanding of the DC biology can aid to improve or accelerate the generation of a powerful and more optimal T cell response during vaccination or treatment strategies. Our analysis of the DC subsets revealed that in addition to the presence of cDC1s, cDC2s and monocyte derived cells (MCs), which are also found in the lung during homeostasis, mild seasonal influenza infection (X31 virus, H3N2) resulted in the presence of a new subset of cDCs. These cDCs resembled cDC2s, however they also expressed the prototypical monocyte-marker MAR-1. We termed these novel cDC2s MAR-1$^+$ cDC2s. We found that MAR-1 expression was induced in a type 1 IFN dependent manner and that these cells were more mature than conventional cDC2s. Additionally, and somewhat surprisingly, these MAR-1+ cDC2s also acquired expression of the cDC1-related transcription factor IRF8. Functionally, IRF8 induction controls a functional module in cDC2s responsible for maturation and antigen presentation. During the process of defining the ontogeny of this MAR-1$^+$ DC, we came across some limitations

of widely used techniques for dissecting DC ontogeny. We first attempted to use *Flt3I*^{-/-} and *Ccr2*^{-/-} mice to define the pre-cDC or monocyte-derived cells respectively[1, 2] but this was not that straightforward as several bystander effects occurred in these transgenic mice. Flt3I^{-/-} mice also have a defect in lymphocyte, NK cell and neutrophil development[3] that can influence the course of infection and obscure the results. CCR2 dependency is typically used to identify cells of monocytic origin. However, we noted a significant reduction in cDC1s and cDC2s upon inflammation in *Ccr2*^{-/-} mice which would theoretically indicate that these cells are of monocytic origin however, we know this is not the case as it is well established that cDCs arise from progenitors committed to the cDC lineage namely the pre-cDCs. Additionally, the WT:CCR2^{-/-} competitive chimeras that we generated suggested that the MAR-1^{+} DCs were CCR2-dependent and hence one would normally conclude that these are thus of monocytic origin. However, when we performed adoptive transfer of monocytes or pre-cDCs, it became apparent that these MAR-1^{+} DCs arose from pre-cDCs and not monocytes demonstrating them to be a cDC population. Thus adoptive transfer of progenitors appears to be the most valid method of investigating and confirming DC ontogeny. Importantly, this is not the first time that CCR2-dependency has been described in a cDC population. In the gut it has previously been reported that a subset of CD103^{-} CD11b^{+} cDC2s express CCR2 and are dependent upon it[4]. Additionally, in our lab it has recently been shown that cDCs in the heart also express CCR2 and these are also reduced in *Ccr2*^{-/-} mice (Van Der Borght *et al.* submitted for publication). This is the first time that a typical monocyte marker is described to be expressed on conventional DCs. This questions of course the classification of MAR-1 as a monocyte marker. Together with the analysis of gene expression data, we showed that caution should be taken when analyzing DC subsets in inflammatory models since the expression levels of many genes and surface markers change upon inflammation, which can influence the classification system. Gene expression analysis showed that XCR1 and SIRPα are good markers to separate cDC1s and cDC2s respectively as XCR1 expression remains cDC1 specific upon IAV infection and the expression of SIRPα remains shared between MCs and cDC2s. As SIRPα expression is shared with MCs, a good monocyte and conventional marker remains necessary to split up MCs and cDC2s. Two possible candidates we identified in this project are MerTk and CD26. This confirms the gating strategy that can be applied on human, mouse and macaque DC subsets that was empirically developed in our lab by Dr. Charlotte Scott and Prof. Martin Guilliams in collaboration with the labs of Prof. Bernard Malissen and Prof. Florent Ginhoux which they described as "a global key" to define DC subsets (Guilliams *et al.*, Immunity, in press).

As mentioned above MAR-1^{+} cDC2s were found to express the transcription factor (TF) IRF8. TFs such as IRF8 can induce and/or repress a substantial amount of genes and therefore we hypothesized that the upregulation of IRF8 in cDC2s would have major effects. Indeed, we found that both the maturation status (signal 2 for T cell activation) and the capacity to produce IL-12 (signal 3 for T cell activation) were increased in MAR-1^{+} cDC2s compared with MAR-1^{-} cDC2s. This led us to investigate the antigen presenting potential of these cells. Remarkably, MAR-1^{+} cDC2s acquire a good capacity to present antigen to CD8^{+} T cells and to induce their proliferation, a function

that is mostly attributed to cDC1s during IAV[5, 6]. Nevertheless, some research groups also attributed this function to CD11b^{+} DCs, consisting of both true cDCs and MCs[7]. Retrospectively, the ratio of MCs and cDCs in this population might be influencing the outcome of antigen presentation assays as we have shown that MCs do not fall in the MHCII^{hi} DC population of the LN (suggesting that they do not migrate from the lung to the LN) and that MCs of the lung are not capable of inducing CD8^{+} T cell proliferation. Therefore, when they are highly present in the sorted population, they might dilute the antigen presenting capacity of the cDCs in the population. The contrary is also true, antigen presentation capacity of cDCs will only be picked up in a model where the induction of MAR-1^{+} cDC2s is high compared to the MAR-1^{-} cDC2s as these are poor antigen presenters in a MHCI context and thus can also dilute the effect of the MAR-1^{+} cDC2s in antigen presentation assays.

In conclusion, the correct identification of the DC subsets is crucial and only once the distinct subsets are evaluated can the consequences of different viral strains, infectious doses and routes of administration be compared. Thus we believe that the gating strategy presented in this thesis provides a significant step forward in the field as it allows the distinct subsets present during IAV to be comprehensively evaluated. The next step will be to see if this holds true in humans so that once we understand the specific functions of the cells we can aim to boost the DC response of the most powerful subset to aid in viral elimination.

Philosophically we can wonder why the immune system would instruct a cell to acquire a hybrid phenotype (MAR-1^{+} cDC2) when a specialized cell (cDC1) is already present to perform a certain function. One hypothesis is that in the context of inflammation and more specifically IAV infection, the pressure on the immune system to limit the spread of the virus is high. We and others have previously made the observation that the amount cDC1s in the lungs is not strongly increased shortly after IAV infection and cDC1 DCs disappear from the trachea at that time, which might limit the strength of the CTL response[8, 9]. It is possible that induction of the IRF8-dependent module is an emergency rescue for allowing additional CTL induction by DC subsets that normally do not (cross)present viral antigens to CD8 T cells, driven by type I interferons[10]. One can assume that in this way a maximum capacity of antigen transport to the draining lymph nodes is maintained as cDC2s migrate proficiently also later in infection. Additionally, it would be beneficial to the host to send cells to the lymph node that can combine different immune functions. In this way, the MAR-1^{+} cDC2 acquire a capacity to induce the CTL response (a function traditionally attributed to cDC1) while maintaining the capacity to mount a strong antiviral state (a feature shared with MCs and cDC1). As there are also genes that are specifically induced by IAV only in MAR-1^{+} cDC2s and MCs (cluster 4 genes), we speculate that closer study of those genes might offer explanations why DCs adopt a hybrid phenotype.

New tools are urgently needed to address the exact *in vivo* role of the MAR-1^{+} cDC2s. The use of CD11c Cre mice to create conditional knock-out mice has the disadvantage that the whole DC population is targeted. Additionally, as alveolar macrophages also express CD11c these are also targeted in these mice. Recently, two interesting new mice lines have been created. *Zbtb46* Cre mice target the conventional DCs (i.e. cDC1 and cDC2) specifically while the *Xcr1* DTR mice allow the

specific removal of cDC1s through administration of diphtheria toxin. Crucially, however, a cDC2 specific mouse line is still missing and considering the fact that cDC2s share all their genes with either cDC1s or MCs, it will be almost impossible to develop such a line. The MAR-1 antibody that we used to define MCs and that is also expressed on a subset of cDC2s is generated to react specifically to an antigenic site of the FcεRI. By studying the DC subsets in FcεRI$^{-/-}$ mice we found that the amount and distribution of the different subsets, nor the intensity of MAR-1 staining on DCs is affected in these mice, while expression of FcεRI on mast cells and basophils is indeed absent. This means that the antibody potentially has a cross reactivity with other proteins. Hence, if we can identify the gene product that is targeted by our MAR-1 antibody, we could develop new mouse tools. First, we could develop a mouse line lacking the MAR-1 epitope based on Cre-Lox technology (floxing the gene of interest, encoding the MAR-1 epitope) and crossing it to the Zbtb46 Cre mice. In this way, we will create a mouse that is unable to express the unknown MAR-1 epitope on the cDCs, while still allowing expression on MCs. Hence we can address the effect of lack of MAR-1 induction in the cDC2 population on for example CD8$^+$ T cell responses in vivo. A tool to specifically deplete the MAR-1$^+$ cDC2 subset would involve creating a knock in of loxSTOPlox-DTR in the "MAR-1" gene, and subsequent crossing of these mice to the Zbtb46-Cre line. This should allow specific targeting of MAR-1$^+$ cDC2s, while sparing MCs.

Besides improving murine experimental tools, it is also necessary to develop tools to investigate whether MAR-1$^+$ cDC2s can also be detected in humans. Currently there is no human MAR-1 antibody available. This means that the MAR-1$^+$ cDC2s can until now only be identified as an IRF8 expressing population within the total cDC2 population. If human cDC2s also segregate into two different activation states (being an IRF8 expressing population and an IRF8$^-$ population), it would be of great interest of the DC vaccination field to screen for correlation between disease severity, duration of the disease and induced direct antiviral and memory responses.

As described above, cDCs are crucial in the initiation of adaptive immune responses through the presentation of antigen to naïve T cells. Thus having investigated the distinct DC populations during IAV, in chapter 4, we next turned our attention to the T cell response during IAV infection. Specifically, we focused on the CD4$^-$CD8$^-$ double negative (DN) T cell population, as these cells are often neglected from analysis, and compared their phenotype with the already intensively described CD4$^+$ and CD8$^+$ T cells. DN T cells are true T cells as they express the T cell receptor and are not NKT cells, as they don't react with the αGalCer CD1d tetramers. They are thymically derived and in contrast to the conventional T cells, they reside in the lung parenchyma rather than in the lung vasculature already in steady-state conditions where they have a T$_{rm}$ phenotype. Although they had a memory phenotype, they did not react to reinfection with a heterologous virus, showing that viral replication is necessary for their expansion. In the field of transplant rejection it is believed that DN T cells are activated CD4 T cells that downregulate the expression of the CD4 coreceptor[11]. We were not able to confirm that during IAV infection classical CD4$^+$ or CD8$^+$ T cells give rise to the DN T cells population. Our arguments against this hypothesis are that the Vβ repertoire of the TCR is not similar to that of either CD4$^+$ or CD8$^+$ T cells and that intracellular staining for CD4

and CD8 could not reveal significant expression of CD4 and CD8 inside DN T cells. Moreover it was recently shown that thymically-derived DN T cells from the gut epithelium have a different TCR repertoire than other T cells, with a specificity different from the currently existing MHCI- and MHCII-restricted specificities[12]. These observations led to the hypothesis that DN T cells are playing a non-redundant role in the protection against invading pathogens especially under conditions of immune evasion as well-developed conventional pathways are often the target of immune evasion strategies. This hypothesis is well applicable to the IAV infection model as influenza viruses developed several immune evasion strategies based on protection mechanisms at different levels of the immune response[13]. Therefore it would be interesting to study the TCR repertoire in more detail, e.g. with the cloning strategies used by the Cheroutre group. Functionally, DN T cells have immunoregulatory capacities as they enhanced the survival of CD11b+ cDC2 and MCs during *in vitro* cocultures. The inhibition of apoptosis in part of the DC subsets indicates that DN T cells can prolong the survival of certain cell types when immune pressure is present. A substantial part of the DN T cells expressed high levels of FasL, a protein involved in cell dead induction. Therefore it was surprising that DN T cells actually prolonged the survival of certain DC subsets *in vitro*. This suggests that DN T cells might either have different functions or that they represent a mixed population composed of cells with opposite functions. In the first scenario, DN T cells might exert a different function depending on the cells they interact with. Previously, they have been described to suppress B cells[14] and T cells[15]. Therefore it might be of interest to coculture them with different cell types and compare the effect of presence of DN T cells with the cells to address whether they react differently depending on the interacting cell type.

As these cells are defined by negative markers, it is difficult to do functional *in vivo* studies, as we cannot deplete them with depleting antibodies. Therefore it would be interesting to perform single-cell RNA sequence analysis to evaluate if this population is homogenous or a mixture of different cell types. Further, we can use these data to define a discriminating marker to facilitate *in vivo* functional studies on these cells.

Long after the virus has been cleared from the lung by specific T cell and B cell responses, iBALT structures can be observed in the lungs. These tertiary lymphoid structures often form at the branching of the bronchi and are composed of B cells, T cells and DCs, similar to classical secondary lymphoid organs such as the spleen and lymph nodes. Consequently they can be a local induction site for protective immune responses in case of viral or bacterial infections. Therefore it is of great interest to have an in-depth knowledge about the developmental mechanisms and signals to sustain iBALT structures in the lung. The development of TLOs is very similar to SLOs, but in contrast to the well-defined developmental pathway of SLOs, the development of TLOs is subjected to more variation depending on the model. Especially the early initiating steps are still unclear. For example, the typical lymphoid tissue inducer cells are dispensable for iBALT formation, but the true nature of the cell or signal that is necessary to induce iBALT is still under debate. In chapter 5 we questioned whether the intensity of the early innate response is influencing the outcome of iBALT formation. We have shown that the instructive signals for iBALT formation are already given very early (2dpi)

after infection and that it is the innate cytokine IL-1α that can boost germinal center formation in the lung. Before, it was shown that IL-17 plays an important role in iBALT induction during IAV infection[16]. As there is a link between IL-1 signaling and IL-17 production[17-20], we also questioned whether IL-17 is involved in our model. However, studies in *Il17r*^{-/-} mice could not reveal a difference in iBALT formation and the administration of recombinant IL-1 did not influence the magnitude of the Th17 response in our model. We deliberately chose a mild seasonal influenza virus infection (X31, H3N2) whereas mostly PR8 (H1N1), a more pathological virus strain, is used to study iBALT induction. Additionally, the Randall group already primed neonatal mice with LPS before influenza infection at 6 weeks of age. Given the big amount of LPS to which the lungs are exposed we can assume that the natural environment of the lung is already changed before the actual influenza infection. In our lab, Martijn Schuijs studied the effect of LPS exposure on asthma development. LPS pretreatment appeared protective for later asthma development and histological analysis of the lungs revealed a major presence of iBALT structures whereas the normal house dust mite-driven model of asthma development generally does not lead to iBALT formation. So it remains a question if the Randall group rather studied LPS-driven iBALT formation rather than influenza-driven iBALT formation. The group of Förster has confirmed that iBALT formation caused by different respiratory infections can have different developmental mechanisms as infection with Vaccinia Ankara virus leads to iBALT formation in an IL-17 independent manner whereas *Pseudomonas*-induced iBALT does require IL-17 signals[21]. We have previously shown that repeated injections of GM-CSF cultured bone marrow DCs can induce iBALT formation[22]. As we noticed that MCs are decreased during the course of IAV infection in IL-1R^{-/-} mice and that MCs were previously described to be major chemokine producers, it might be that IL-1 signaling results in MC recruitment and these MCs might be the producers of CXCL13. We also observed a decrease in MAR-1^{+} cDC2 induction (data not shown), so it would be informative to address the iBALT-inducing capacity of injected MCs and MAR-1^{+} cDC2s and compare them with macrophages as it was recently shown that GM-CSF DCs are a mixture of cDC2s and a macrophage-like monocyte-derived cell[23].

The fact that germinal centers are present in these structures enables them to mount specific immune responses to invading pathogens. Therefore, they are of interest to be exploited during vaccination[24]. Our study identified an innate cytokine that boosts iBALT formation. It would be interesting to see if repeated exposure of the lungs to IL-1α without the presence of a pathogen also induces iBALT. Secondly, iBALT structures are present in the lungs of children with recurrent respiratory infections[25], but are generally absent in the lungs of healthy adults[26] suggesting that they disappear over time when there are not enough insults. Thus it would not only be interesting to study if IL-1α can induce iBALT but also if repeated exposure after formation of iBALT structures can prevent the disappearance.

Before iBALT can be used as a vaccination strategy, it is however crucial to investigate how we can prevent that, as a side effect, iBALT structures can also generate autoantibodies and thus induce auto-immune diseases. Another issue that is hampering translation to humans is the fact that invasive techniques are essential to assess the presence of iBALT structures in the lungs.

All together, we generated several insights that can improve future research to the immune response against influenza virus infection. Firstly, we identified a maturation status of cDC2s that acquires typical cDC1 functions and might be a strategy of the immune response to optimize the immune response by combining the most necessary immune functions in one cell type, a finding that could be exploited to create novel vaccination strategies, if we could find vaccines that specifically elicit IRF8$^+$MAR-1$^+$ cDC2s. In addition, this research has raised the consciousness about the fact that caution should be taken when using cell subset "specific" markers or transcription factors – generally regarded as the gold standards of lineage definition – in inflammatory models. Secondly, we gained more insight in the phenotype of DN T cells. More in-depth analysis of the TCR specificity of these cells will however be necessary to evaluate if these cells exert an antigen-specific function or not. Moreover single-cell analysis will be crucial to exclude that these cells are a heterogeneous population. Lastly, we showed that innate immune signals condition the lung for iBALT formation after viral clearance, creating the awareness that the first nonspecific immune response is crucial for later adaptive immune reservoirs. Again, better understanding of such innate signals that boost the formation of iBALT structures that are emerging as important structures in long lasting immune protection could greatly improve vaccine design in the future.

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Chapter 7

Nederlandse samenvatting

Elke winter worden heel wat mensen getroffen door de griep, veroorzaakt door infectie met een influenza virus. Voor de meerderheid leidt griep infectie gelukkig niet tot ernstige ziekte of zelfs de dood. Toch heeft dit een aanzienlijke economische impact door de medische kosten die het met zich mee brengt en de afwezigheid op het werk of school. Bovendien is er de continue dreiging dat er een pandemie ontstaat. Doordat het genoom van influenza virussen zeer variabel is moeten de circulerende virussen constant gescreend worden en moet de samenstelling van de vaccins voor het komende griepseizoen op basis van voorspellingen aangepast worden. Daardoor is er nooit een volledige zekerheid dat het vaccin perfect afgestemd zal zijn op de virussen die daadwerkelijk mensen gaan infecteren. Bovendien is er nog geen uitzicht op een universele vaccinatiestrategie die de variabiliteit van het influenza virus omzeilt. Daarom is het belangrijk om de kennis over de moleculaire mechanismes die de genetische variabiliteit drijven en over de afweerreactie tegen influenza virussen uit te bouwen.

De algemene principes van de afweerreactie tegen influenza virus infectie zijn al goed gekarakteriseerd. Het epitheel van de longen vormt een eerste natuurlijke barrière, maar eens deze barrière omzeild is, kan het virus de epitheelcellen infecteren. Aangeboren (innate) afweercellen zoals NK cellen en neutrofielen zullen de vreemde partikels herkennen en een eerste aspecifieke afweerreactie induceren. Dendritische cellen nemen stukjes van het virus op en presenteren dit antigen aan de T cellen in de lymfeknopen. Op deze plaats zullen T cellen die deze stukjes herkennen geactiveerd worden, migreren naar de long en daar hun functies uitoefenen. Veder zullen er in de lymfeknopen ook B cellen geactiveerd worden en deze cellen zullen uiteindelijk antilichamen produceren om geïnfecteerde cellen te doden of om de verspreiding van het virus tegen te gaan. Eenmaal de infectie geklaard is zullen T en B cellen in de weefsels en lymfoïde organen achterblijven die een "geheugen" hebben over hun functie en specificiteit en hierdoor zullen zij een snelle maar heel specifieke afweerrespons kunnen induceren wanneer het lichaam getroffen wordt door een reïnfectie met hetzelfde of een sterk gelijkend virus.

In dit proefschrift hebben we verschillende takken van de afweerrespons tegen influenza virussen bestudeerd: de heterogeniteit van dendritische cellen en T cellen en de vorming van iBALT structuren.

In hoofdstuk 3 werden de verschillende types dendritische cellen (DCs) bestudeerd in de long en de lymfeknopen die de long draineren. Omdat DCs een cruciale link vormen tussen de overgang van een aspecifieke aangeboren afweerreactie naar een specifieke afweerrespons, is het belangrijk om de biologie van de DCs goed te begrijpen. Deze kennis kan immers bijdragen tot het verbeteren of versnellen van een optimale T cel respons tijdens vaccinatie- of behandelingsstrategieën. Tijdens onze onderzoeken hebben we een type 2 DC geïdentificeerd die de oppervlakte merkers van een monocyte-afgeleide cel (MC) verkrijgt, maar ook functies kan uitvoeren die voornamelijk aan type 1 DCs werden toegewezen. Dit zou een strategie van het afweersysteem kunnen zijn om de meest noodzakelijke functies in één celtype te combineren. Deze bevindingen kunnen gebruikt worden om te onderzoeken of we vaccins kunnen ontwikkelen die specifiek dit celtype induceert. Tijdens dit onderzoek

hebben we er ook op gewezen dat het gebruik van merkers en transcriptiefactoren die eerder beschreven werden als specifiek voor een bepaald type cel vaak geen stand houdt tijdens inflammatoire reacties.

Aangezien DCs verantwoordelijk zijn voor het induceren van een specifieke (adaptieve) afweerreactie door antigenen te presenteren aan T cellen, zijn we in hoofdstuk 4 dieper ingegaan op de T cel respons tegen influenza virus infectie. We hebben hierbij voornamelijk de focus gelegd op de zogenaamde "dubbel negatieve T cellen" die heel vaak niet geanalyseerd worden wanneer men de verschillende types T cellen bestudeert. Als eerste hebben we aangetoond dat dit weldegelijk echte T cellen zijn aangezien ze de T cel receptor op hun oppervlakte dragen. Verder hebben we het fenotype van deze cellen grondig vergeleken met dat van de CD4$^+$ en CD8$^+$ T cellen. Hieruit bleek dat deze cellen al in basale condities een geheugen fenotype hebben en eerder in het weefsel dan in de bloedvaten van de long verblijven. Functioneel hebben we aangetoond dat deze cellen het overleven van type 2 DCs en MCs, maar niet van type 1 DCs, tijdens *in vitro* culturen verlengen. Verder onderzoek moet uitwijzen of deze cellen een homogene populatie vormen of een samenstelling zijn van functioneel verschillende types T cellen.

Na het klaren van de infectie blijven er vaak iBALT structuren achter in de long. Deze lymfoïde structuren liggen dicht bij de vertakkingen van de van de luchtpijp. Hun samenstelling is sterk vergelijkbaar met die van de lymfeknopen en functioneel kunnen ze ook een plaats zijn waar T en B cel responsen geïnduceerd worden. In hoofdstuk 5 hebben we bestudeerd of de intensiteit van de vroege aangeboren afweerreactie invloed heeft op latere iBALT vorming. Uit de proeven bleek dat de signalen voor iBALT vorming al heel vroeg na infectie moeten gegeven worden en dat het cytokine IL-1 het ontstaan van iBALT structuren kan bevorderen. Een experimenteel model waarin gekeken wordt of IL-1 toediening enerzijds de vorming van iBALT structuren kan induceren zonder blootstelling aan pathogenen en anderzijds de al gevormde iBALT structuren in stand kan houden zou een interessante onderzoekslijn zijn om de mogelijke toepassing van iBALT als vaccinatiestrategie te bepalen. Echter, een belangrijke kanttekening bij het potentieel van iBALT structuren als vaccinatiestrategie is het feit dat deze structuren naast een nuttige afweerrespons tegen virale infecties ook een afweerrespons zou kunnen induceren tegen lichaamseigen eiwitten en zo auto-immuun ziektes zou kunnen veroorzaken.

About the author

Curriculum Vitae

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A strong interest in understanding the immune response leading to allergic diseases and the possible genetic predispositions was the reason why I started my master thesis on mast cells and asthma in the lab of prof. Lambrecht at Ghent University. After my thesis, I was offered the opportunity to start up an influenza research line in the lab to study tertiary lymphoid organ formation after influenza virus infection. Six years later, after successfully implementing viral assays and read-outs in our lab and several side-step projects, I am finishing my PhD thesis based on several review articles and three primary research articles on dendritic cell and T cell subsets biology and iBALT formation. A specific interest in translating basic research into vaccine or drug development inspired me to work as an industrial liaison within the Lambrecht lab to coordinate and perform research assignments from pharmaceutical and biotech companies like GSK, ALK, Thrombogenics and Argen-x.

Education

2009 - 2016   PhD in biomedical sciences
                     "Pulmonary immune responses after influenza virus infection"
                     Promotor: Prof. Dr. Bart Lambrecht
                     VIB Inflammation Research Center
                     FWO Aspirant Fellowship

2007 - 2009   Master in biomedical sciences
                     "The role of transcription factor GATA-1 in mast cell development and function"
                     Promotor: Prof. Dr. Bart Lambrecht
                     Ghent University

2004 - 2007   Bachelor in biomedical sciences
                     Ghent University

2002 - 2004   Secondary school: science-mathematics
                     Sint-Lievenscollege Gent

1998 - 2002   Secondary school: Latin-mathematics
                     De La Salle College Gent

PhD portfolio

Publications in A1 journals

Neyt K, GeurtsvanKessel C, Deswarte K, Hammad H, Lambrecht B. 2016. Early IL-1α signaling is required and sufficient for iBALT induction after influenza virus infection. Frontiers in Immunlogy 7:312.

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Oral presentations
NVVI Annual Meeting 2013, Noordwijkerhout – The Netherlands
The innate cytokine IL-1alpha is necessary and sufficient for formation of tertiary lymphoid tissues following influenza A virus infection

The Innate Immune Response in the Pathogenesis of Infectious Disease, Keystone Symposium 2013, Ouro Preto - Brasil
The innate cytokine IL-1alpha is necessary and sufficient for formation of tertiary lymphoid tissues following influenza A virus infection
Keystone Scholarship

VIB seminar 2013, Blankenberge – Belgium
The innate cytokine IL-1alpha is necessary and sufficient for formation of tertiary lymphoid tissues following influenza A virus infection

NVVI Winterschool 2012, Noordwijkerhout – The Netherlands
The innate cytokine IL-1alpha is necessary and sufficient for formation of tertiary lymphoid tissues following influenza A virus infection

4th ESWI influenza conference 2011, Malta
The role of CD4-CD8- double negative T cells during influenza virus infection
Young scientist award

Poster presentations
The Innate Immune Response in the Pathogenesis of Infectious Disease, Keystone Symposium 2013, Ouro Preto - Brasil
The innate cytokine IL-1alpha is necessary and sufficient for formation of tertiary lymphoid tissues following influenza A virus infection

NVVI Annual Meeting 2011, Noordwijkerhout – The Netherlands
The role of CD4-CD8- double negative T cells during influenza virus infection

Additional courses and workshops
Course in virology; *Erasmus MC Rotterdam*
Entrepreneurship in life sciences; *VIB*
Microscopy summer school; *VIB*
Quality research skills; *doctoral schools UGent*
Leadership foundation; *doctoral schools UGent*
Project management; *doctoral schools UGent*
Effective scientific communication; *doctoral schools UGent*
Populair-wetenschappelijk schrijven; *doctoral schools UGent*
Advanced academic English, writing skills; *doctoral schools UGent*
Advanced academic English, academic posters; *doctoral schools UGent*
Statistics (basics of statistical interference, ANOVA), *doctoral schools UGent*

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