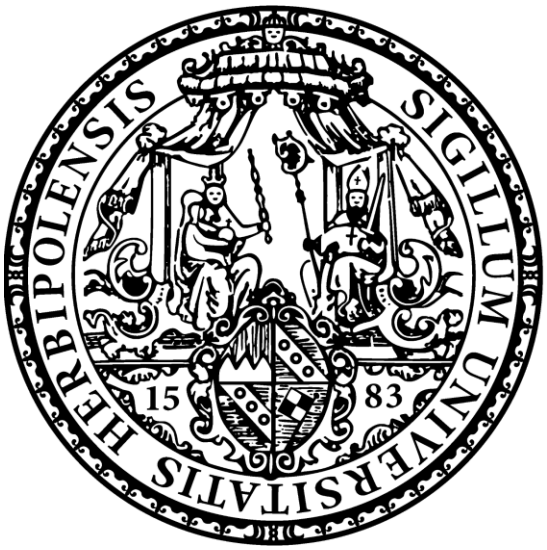


Kinetics and timing of IL-12 production by dendritic cells for Th1 polarization *in vivo*

Kinetik und zeitlicher Ablauf der IL-12-Produktion durch Dendritische Zellen für die Th1 Polarisierung *in vivo*

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,

Section Infection and Immunity

Submitted by
DiyaaEldin Ashour

From:
Cairo, Egypt

Würzburg 2019

Submitted on:

……..……..

Office stamp

Members of the *Promotionskomitee*:

Chairperson: Prof. Dr. med. Georg Gasteiger

Primary Supervisor: Prof. Dr. Manfred Lutz

Supervisor (Second): PD Dr. Friederike Berberich-Siebelt

Supervisor (Third): Prof. Dr. Dr. Andreas Beilhack

Supervisor (Fourth):

…….

(If applicable)

Date of Public Defence: ……

Date of Receipt of Certificates:……..

ACKNOWLEDGEMENTS

I am really grateful to my supervisor Prof. Manfred Lutz for his continuous support and guidance throughout my thesis. His passion towards Immunology has encouraged me to pursue my own scientific ideas within the project and made the completion of this work possible.

I am also very thankful to the members of my PhD-committee: Prof. Andreas Beilhack and Dr. Friederike Berberich-Siebelt for their time and the discussions especially during the annual meetings and for the critical reading and evaluation of this manuscript.

I am really grateful to my wife who has continuously supported me during difficult times and put up with my really late nights of work. I would also like to thank my parents and siblings who, even without being here, have supported me a lot. To my family, I could never make it without you.

I am very grateful to the Wilhelm Sander-Foundation for the generous support, the Graduate School of Life Sciences (GSLS), University of Würzburg, and the DAAD STIBET program for supporting the project. I thank Prof. Gottfried Alber and Prof. Wolfgang Kastenmüller for providing mice. I am also grateful to Dr. Nora Müller for her help with the confocal microscope and Sarah Eickhoff for help with the Imaris analyses.

Last but not least, I would like to thank all the members of AG Lutz for their continuous support, the lively atmosphere in the lab, and being always there whenever I needed help. I believe I was very lucky to have such great lab mates. Many thanks to Vini John, Ina Eckert, Emilia Vendelova, Marion Heuer, Eliana Ribechini, Cinthia Silva Vilches and Szymon Matus.

Table of Contents

CONTENTS

SUMMARY

Dendritic cell (DC) based vaccines rely on the quality of DC maturation to induce antigen presentation, co-stimulation, lymph node migration and the release of heterodimeric IL-12p70 in case of T helper type-1 cell (Th1) polarization. In contrast, DCs that cannot secrete IL-12p70 (e.g. after cytokine cocktail maturation) readily induce Th1 cells when injected into mice and humans. Since it was also previously suggested that DCs are capable of activating other DCs in a bystander fashion, we tested here for the DC source of IL-12p70 for Th1 polarization in a murine DC vaccination model. Migration of the injected murine bone marrow-derived DCs (BM-DCs) was essential for antigen delivery to the lymph node. However, they contributed only partially to antigen presentation, and induced a non-polarized Th0 state of the cognate T cells producing IL-2 but no IFN-γ. Instead, endogenous dermal migratory XCR1$^+$ cDC1s underwent re-programming by the injected BM-DCs to acquire bystander antigen presentation and IL-12 release for Th1 polarization in the lymph node. Genetic deficiency of migratory DCs and specifically of XCR1$^+$ migratory DCs completely abolished Th1 priming. The kinetic of cell interactions in the draining lymph nodes appeared step-wise as i) injected DCs with cognate T cells, ii) injected DCs with bystander XCR1$^+$ DCs, and iii) bystander XCR1$^+$ DCs with T cells. The transcriptome of the bystander DCs showed a down-regulation of Treg and Th2/Th9 inducing genes, and up-regulation of genes required for Th1 instruction. Together, these data show that injected mature lymph node migratory BM-DCs direct T cell priming and bystander DC activation, but not Th1 polarization which is mediated by endogenous IL-12p70$^+$ XCR1$^+$ migratory bystander DCs. Our results are of importance for clinical DC-based vaccinations against tumors where endogenous DCs may be functionally impaired by chemotherapy.

ZUSAMMENFASSUNG

Auf Dendritische Zellen (DCs) basierende Vakzinen hängen von der Qualität der DC-Reifung ab, um Antigenpräsentation, Kostimulation, Lymphknotenmigration und, im Faller einer T-Helfer-1 (Th1) Polarisierung, die Freisetzung von IL-12 zu induzieren. Die Herstellung des heterodimeren IL-12p70 durch injizierte DC wurde klassisch als Schlüsselfaktor beschrieben, der für die Erzeugung einer polarisierten Th1 Immunreaktion erforderlich ist. Dennoch induzieren DCs, die IL-12 nicht ausscheiden können (z. B. nach Reifung des Cytokin-Cocktails), Th1 polarisierte Immunantwortenin Mäusen und Menschen. Da zuvor auch beschrieben wurde, dass DCs in der Lage sind, andere DCs auf Bystander-Weise zu aktivieren, haben wir hier die DC-Quelle der IL-12 Produktion für die Th1-Polarisation in einem murinen DC-Vakzinemodell untersucht. Die Migration der injizierten, aus murinem Knochenmark generierten DCs (BM-DCs) war für den Antigentransport in den Lymphknoten wesentlich. Sie trugen jedoch nur teilweise zur Antigenpräsentation bei und induzierten nur einen nicht polarisierten Th0-Zustand der T-Zellen, die IL-2 produzierten, aber kein IFN-γ. Stattdessen deuten die Daten daraufhin, dass endogene dermale migrierende $XCR1^+$ DCs als Bystander-DCs zur Antigenpräsentation beitragen und IL-12 für die Th1 Polarisation bereitstellten. Die genetische Ablation von migrierenden DCs und speziell von $XCR1^+$ migrierenden DCs hebt das Th1 Priming vollständig auf, Die Kinetik der Wechselwirkungen in den drainierenden Lymphknoten erfolgt schrittweise, indem i) injizierte DCs mit verwandten T-Zellen, ii) injizierte DCs mit Bystander $XCR1^+$ DCs und iii) Bystander $XCR1^+$ DCs mit T-Zellen in Kontakt treten. Das Transkriptom der Bystander-DCs zeigte eine Herunterregulierung von Treg- und Th2/Th9-induzierenden Genen und eine Hochregulierung der für die Th1- Induktion erforderlichen Gene. Zusammen zeigen diese Daten, dass injizierte reife migrierende BM-DCs das T-Zell-Priming und die Bystander-DC-Aktivierung steuern, nicht jedoch die Th1-Polarisation, die durch endogene IL-12p70$^+$ $XCR1^+$ Bystander-DCs vermittelt wird. Unsere Ergebnisse sind von Bedeutung für klinische Studien mit Vakzine-DCs, bei denen endogene DCs durch eine Chemotherapie funktionell beeinträchtigt werden können.

1 INTRODUCTION

1.1 Innate immune response

The innate immune response acts as the first line of defense protecting us from invading pathogens. It consists of cellular and biochemical defense mechanisms that can detect and destroy microbial invaders within minutes or hours. This is done using a limited number of secreted proteins and cell-associated receptors that are encoded by intact genes inherited through the germline to detect infection and to distinguish between pathogens and host tissues. These receptors are called pattern recognition receptors (PRRs). They can recognize structures exclusively present on and shared by microbes, which are called pathogen-associated molecular patterns (PAMPs), and endogenous danger-associated molecular patterns (DAMPs) which are molecules released by stressed cells undergoing necrosis. These act as danger signals promoting and exacerbating the inflammatory response. They are often essential structures for survival of the microbes, thus limiting the capacity of microbes to evade detection by mutating or losing expression of these molecules. PRRs include receptors present on the cell surface and in endosomes such as Toll-like receptors (TLRs), recognizing a wide variety of ligands, including bacterial cell wall components and microbial nucleic acids. Also, cytoplasmic pattern recognition receptors that recognize microbial molecules such as the RIG-like receptors (RLRs), which recognize viral RNA, and the NOD-like receptors (NLRs), which activate the inflammasome and are involved in the response against several pathogens, inflammatory diseases, cancer, metabolic and autoimmune disorders (Takeuchi and Akira, 2010).

The principal components of innate immunity are (1) anatomic barriers including physical and chemical barriers, such as cilia, and mucus membranes. Also epithelial cells secreting antimicrobial peptides such as defensins that directly lyse bacterial cell membranes, or enzymes such as lysozyme that can digest bacterial cell wall; (2) phagocytes such as neutrophils, macrophages, dendritic cells, natural killer (NK) cells, and lymphocytes with invariant antigen receptors that act directly by engulfing and digesting invading microorganisms; (3) blood proteins, including members of the complement system that targets pathogens both for lysis and for phagocytosis by cells of the innate immune system; and (4) cytokines that regulate and coordinate many of the activities of the innate immune system (Turvey and Broide, 2010). Such cytokines include:

TNF and IL-1 that activate endothelial cells, stimulate chemokine production, and increase neutrophil production by the bone marrow (BM). IL-1 and TNF induce IL-6 production, and all three cytokines mediate systemic effects, including fever and acute-phase protein synthesis by the liver (Heinrich et al., 1990). IL-12 and IL-18 stimulate production of the macrophage activating cytokine IFN-γ by NK cells and T cells (Tominaga et al., 2000). These cytokines function in innate immune responses to different classes of microbes and modify adaptive immune responses that follow the innate immune response. Adaptive immunity can recognize a much broader range of substances and, unlike innate immunity, displays memory of antigen encounter and specialization of effector mechanisms.

1.2 Adaptive immune response

Almost all types of infections require the concerted action of both innate and adaptive immunity. There are two types of adaptive immune responses, which are humoral immunity and cell-mediated immunity. Both are mediated by different components of the immune system and cooperate in eliminating different types of microbes (Bonilla and Oettgen, 2010).

The humoral immune response serves to protect the extracellular spaces, where antibodies produced by B cells destroy extracellular microorganisms and their products and prevent the spread of intracellular infections. Antibodies can bind to pathogens and prevent them from entering and infecting cells, thus they are said to neutralize the pathogen; they also bind bacterial toxins to prevent their action or their entry to the cell. Another function they serve is opsonization which is the uptake of the pathogens by phagocytes. This happens by binding to Fc receptors on phagocytes through their constant regions (C regions). Finally, they can activate proteins of the classical pathway of the complement system when they are bound to pathogens. This enhances the opsonization process by other complement proteins placed on the surface of pathogens, recruit phagocytes to the infection site, and activate the so-called membrane-attack complex, which directly lyse certain pathogens by inducing pore-formation on their surface. The humoral immunity chooses which effector mechanism to use based on the heavy-chain isotype of the produced antibodies (Lu et al., 2018).

Cell-mediated immunity is initiated upon encounter of naive T cells with a specific antigen on the surface of antigen-presenting cells (APCs) in the T-cell zones of secondary lymphoid organs (SLOs). In most cases, APCs responsible for activating naive T cells and inducing their clonal expansion, are dendritic cells that express the co-stimulatory molecules B7-1/CD80, and B7-2/CD86 (Freeman et al., 1993). This activates T cells and make them produce IL-2 (Freeman et al., 1993), which is an important cytokine modulating early proliferation and differentiation of T cells (Ross and Cantrell, 2018). After recognizing a specific antigen and receiving co-stimulatory signals, the T cells are in a stage termed Th0, previously observed in mouse T cell clones, where IL-2 production by the T cells can be measured but no fully polarized Th cytokine restriction is observed (Firestein et al., 1989; Openshaw et al., 1995). T cells are classified based on the expression of two surface markers, which are CD4 and CD8 (Miceli and Parnes, 1991). CD8 cytotoxic T cells (CTLs) kill target cells upon the recognition of the specific foreign antigen, which their T cell receptor (TCR) can bind to, presented on the major histo-compability class I molecule (MHC I) of virus or bacteria-infected cells. They also recognize tumor cells expressing tumor-induced neoantigens on MHC I molecules (Zhang and Bevan, 2011). CD4 T cells enhance the effector functions of CTLs but also of the innate immune response and are thus called helper T cells. They can recognize foreign antigens presented on MHC class II molecules which are resent exclusively on professional APCs such as dendritic cells (Zhu and Paul, 2008). Additionally, a distinct subset of CD4 T cells, termed regulatory T cells (Tregs), can serve to help control and limit immune responses by suppressing immunogenic T-cell activity (Vignali et al., 2008; Zhu and Paul, 2008).

1.3 Dendritic cells

DCs are BM-derived or embryo-derived cells that are found in blood and lymphoid organs (Banchereau and Steinman, 1998; Wang et al., 2012). They were originally identified by Ralph Steinman and Zanvil Cohn in mouse spleen based on their unique stellate morphology, which distinguished them from macrophages (Steinman and Cohn, 1973). Subsequently, they were found to be the most potent stimulators of naive T cells in mixed lymphocyte reactions, this set the foundation for decades of research that showed the importance of DCs in initiating and propagating adaptive immune responses (Steinman et al., 1983). Their main function is to bridge the innate and adaptive immune systems. In

this sense they act as central regulators of the entire immune response, responsible for both sensing the nature of the threats faced and activating the precise combination of effector mechanisms to eliminate such threats. DCs are innate immune cells because they recognize and respond to PAMPs and DAMPs, thus shaping the acute inflammatory response. The way they bridge to the adaptive immunity is by processing extracellular and intracellular proteins and present them as peptide antigens in the context of MHC molecules to prime naive T cells. Also, they are considered as critical modulators of both thymic and peripheral immune tolerance. This was demonstrated in several transgenic mouse models, where eliminating DCs was sufficient to break immune tolerance and induce autoimmune pathologies (Audiger et al., 2017; Hasegawa and Matsumoto, 2018). Based on the different cytokines and transcription factors that drive the development of DCs and on the surface markers they express; DCs are a heterogenous mixture of cells that share a common origin but differ in their development and function (Dudziak et al., 2007; Guilliams et al., 2016; Merad et al., 2013). Upon completing their differentiation, they are all characterized by the expression of the integrin CD11c and MHC II molecules. These are considered as universal markers for DC identification, though their expression level varies depending on the subset. DCs that were originally identified by Steinman in the spleen are termed conventional DCs (cDCs) as they were the first DCs identified (Steinman and Cohn, 1973). Later a population of cells that morphologically resemble plasma cells but produce large amounts of IFN-α upon viral stimulation were identified and are thus termed plasmacytoid DCs (pDCs) (Colonna et al., 2004). Another subset arises from monocytes under inflammatory conditions and is termed monocyte derived DCs (MoDCs) (Lutz et al., 2017).

1.4 Dendritic cell lineage and development

cDCs arise from hematopoietic lineages that are different from other immune cells. The first early committed progenitors defined in mice and human are common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) (Akashi et al., 2000; Kondo et al., 1997). The identification of progenitors of certain immune cells from other hematopoietic lineages relied mainly on adoptive transfer studies of irradiated animals that carry elevated levels of circulating cytokines. Many groups are now validating such studies using genetic fate-mapping of these clonogenic progenitors in the steady state.

The adoptive transfer of CLPs and CMPs into irradiated animals mainly produced cDCs and pDCs (Traver et al., 2000). Similar potential was also found for human CLPs and CMPs cultured in vitro. The maintenance of cDC developmental potential from hematopoietic progenitors was strictly linked to the expression of the tyrosine kinase receptor Fms-like tyrosine kinase 3 (Flt3), the ability of the progenitors to respond to its ligand, Flt3L, and the downstream signaling factor STAT3 (Naik et al., 2005). Flt3L or Flt3 deficient mice showed a dramatic loss of pDCs and cDCs in the spleen and lymph nodes (LNs), and in peripheral tissue (McKenna et al., 2000). Later it was shown that CLPs and CMPs are heterogenous for Flt3 expression and only $Flt3^+$ fractions were capable of generating DCs (Karsunky et al., 2003). Most of the steady-state cDCs however are of myeloid origin as it was shown that CMPs vastly outnumber CLPs. CMPs are defined by being ($CD117^+$) $cKit^{hi}$, $CD34^+$, $CD16/32^+$, $Sca1^-$. They give rise to monocyte and DC committed precursors (MDPs) (Fig 1) that lose their capacity to differentiate to granulocytes (Fogg et al., 2006). MDP failure to give rise to granulocytes depends on expression of the transcription factor interferon (IFN) regulatory factor 8 (IRF8) (Becker et al., 2012). These progenitors are Lin^-, Sca^-, Kit^{hi}, $Flt3^+$, ($M\text{-}CSFR^+$) $CD115^+$, $CX3CR1^+$ and lack lymphoid, megakaryocyte, and granulocyte development potential but can differentiate to monocytes, macrophages and DCs. MDPs give rise to the common dendritic progenitor (CDP) (Fig 1) which remain $CD115^+$ ($M\text{-}CSFR^+$), $CD135^+$ ($Flt3^+$), and express high levels of IRF8 which is critical for their survival and continued development. CDPs lack monocyte differentiation capacity and subsequently move towards a pDC or cDC fate (Liu et al., 2009; Onai et al., 2007). A CDP-derived clonogenic pre-DC population that gives rise to cDCs was identified first in lymphoid tissues, and later found in blood, which populates lymphoid organs via the circulation and gives rise to cDCs in both lymphoid and nonlymphoid tissues. This population differentiates in the BM and can be classified into four different subsets along their development axis based on the expression of the surface markers Ly6C which is a GPI-linked surface protein and Siglec H which is a CD33-related siglec family member or alternatively CD117 and transcription factor Zbtb46 expression. Siglec H^+ $Ly6C^-$ pre-DCs are the developmentally earliest cells differentiating from CDPs. If they are Siglec H^+ and$Ly6C^+$ they lose their potential to develop into pDCs and leave the BM giving rise eventually to cDCs (Fig 1) (Schlitzer et al., 2015). If they stay in the BM, they develop into pre-pDCs which are $Flt3^+$ but lack M-CSFR expression and eventually give rise to mature IFNα-secreting pDCs (Fig 1) that

depend on the helix-loop-helix transcription factor E2-2 (also known as TCF4) for their development (Cisse et al., 2008; Onai et al., 2013). pDCs leave the BM to go to the lymphoid organs and peripheral blood upon completing development (O'Keeffe et al., 2003). cDC precursors that leave the BM travel through the blood and enter SLOs and other tissue, differentiate under yet-unknown cues into two subsets: pre-cDC1 which are Siglec H^- and $Ly6C^-$ and give rise to conventional type 1 DCs (cDC1) characterized by CD8α or CD103 and the chemokine XC receptor (XCR1) expression, and pre-cDC2 which are Siglec H^- $Ly6C^+$ and are dedicated precursors to conventional type 2 DCs (cDC2) characterized by CD4 and CD11b expression (Fig 1) (Merad et al., 2013). This allows an additional level of tissue-specific regulation of DCs that enables organ- and niche-specific functional adaptation. cDC subsets will be described in more details in a subsequent chapter. cDCs are generally short-lived and are constantly being replaced by precursors from the BM every 3 to 6 days in a Flt3L-dependent manner (Guilliams et al., 2016; Merad et al., 2013). The transcription factors Ikaros (Wu et al., 1997), PU.1, Gfi1 (Rathinam et al., 2005) and CbfB (Satpathy et al., 2014) were also shown to control the development of DC lineages. PU.1 drives Flt3 expression at later stages of DC development and is involved in expression of IRF8 as early as the CMP or MDP (Carotta et al., 2010). Recently, the transcription factor Bcl11a was shown to also drive Flt3 expression and is required for all DC subsets development (Ippolito et al., 2014). Bcl11a is required for IL-7 receptor expression which is not only required for T and B cell development but also for DC development (Vogt et al., 2009).

MDPs can also differentiate to common monocyte progenitors (cMoPs) characterized by being Lin^-, $CD117^+$ ($c\text{-}Kit^+$), $CD115^+$ ($M\text{-}CSFR^+$), $CD135^-$ ($Flt3^-$), $Ly6C^+$, which strictly derive monocytes. Under inflammatory conditions monocytes can give rise to monocyte derived DCs (MoDCs) which can be developed using IL-4 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig 1) (Sallusto and Lanzavecchia, 1994) and lack the expression of the transcription factor ZBTB46 and is characterized by histone H4K16 acetylation that does not take place in monocytes and macrophages (Lutz et al., 2017; Nicholas et al., 2015). In addition to cDCs and MoDCs, Langerhans cells (LCs) are another subset of DCs that has been described as the prototypical DC. They are sentinel tissue-resident DCs that populate the epidermal skin layer and migrate to skin-draining LNs to activate naive T cells upon activation. Unlike cDCs, LC development is independent of Flt3L but requires ($M\text{-}CSFR^+$) engagement though not by Csf1, but rather

its alternative ligand IL-34 and arise from embryonic monocytic precursor cells (Fig 1) (Wang et al., 2012). This caused an intense debate about whether LCs are true DCs or are more closely related to tissue-resident macrophages (Lutz et al., 2017).

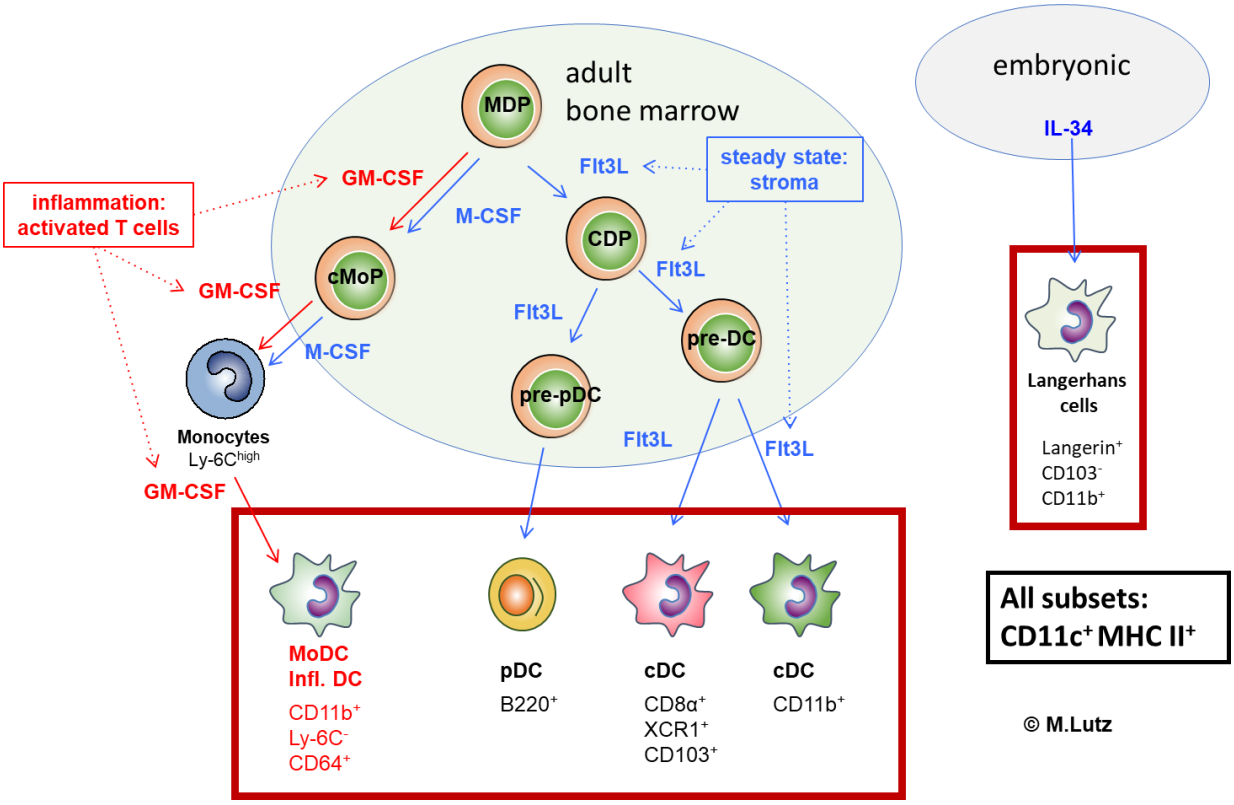


Figure 1: The development and defining markers for the different DC subsets including cDC1s, cDC2s, pDCs, LCs, and MoDCs

1.5 DC migration

DCs have been called professional antigen-presenting cells because of their potent ability to prime naive T cells. Much of this ability stems from their unique migratory pattern. Unlike most innate sentinel cells, upon encountering a viral or bacterial pathogen, activated DCs leave the infected area and migrate towards the draining LNs. As naive T cells can only access SLOs, they rely on this sentinel function of DCs for responding to the presence of an infection. The migratory pattern of DCs from peripheral tissues to and within SLOs has been well studied for decades, but a coherent set of rules for how these migratory journeys impact the most important roles for DCs, T cell priming and differentiation, is only now becoming clear (Calabro et al., 2016).

DCs are strategically positioned at ports entering the organs such as the splenic marginal zone and at different physical body barriers such as the skin or mucosa. Efficient directional migration of cDCs toward T cell zones within lymphoid organs or towards

distant draining LNs is required to ensure efficient stimulation of naive T cells. Peripheral cDC migration via afferent lymphatics depends on the chemokine receptor CCR7 and utilize CCL19 and CCL21 which are the same migratory cues that T cells use to enter the LN (Forster et al., 1999; Teijeira et al., 2014). Immobilized CCL21 on lymphatic endothelium plays a critical role in facilitating the chemotaxis and arrest of migrating tissue DCs to enter the afferent lymphatics (Tal et al., 2011). Complementary roles in cDC migration have been suggested for other chemokine receptors such as CXCR4 and its ligand CXCL12, but CCR7 seems to be the major player (Kabashima et al., 2007). Intra-tissue migration of lymphoid organ-resident cDCs, although seemingly also CCR7 dependent, remains less well understood. It is important to note that *Ccr7*$^{-/-}$ mice have an abnormal development and compartmentalization of immune cells within the LN (Ohl et al., 2003). Other chemokines have been described for the migration of DCs within lymphoid and non-lymphoid tissues. For example, splenic CD11b^{+} cDC2s are recruited to the bridging channels of the marginal zone by the chemotactic receptor EBI2 (Gatto et al., 2013), while the CCR6-CCL20 axis controls the migration of DCs toward the mucosal surfaces within the Peyer's patch (Cook et al., 2000). XCR1 expression on cross-presenting DCs has been shown to orchestrate their intra-tissue positioning in the thymus (Lei et al., 2011) and more recently , their recruitment to CD8^{+} T cells expressing the XCR1 ligand XCL1 during viral immune response (Brewitz et al., 2017). On the other hand, the egress of monocytes from the BM under inflammatory conditions and the subsequent arrival into the skin dermis where they can be converted into MoDCs is mediated by CCR2 together with the help of CCR5 (Nagao et al., 2012). While the migration of DCs from peripheral tissues to draining LNs are extensively studied, the molecular cues required for the immigration of DCs from the BM and from blood to lymphoid and non-lymphoid tissues is unfortunately still poorly understood.

1.6 Dendritic cell subsets

1.6.1 CD103+ and CD8+ dendritic cells

cDC1s can be found in both the spleen and LNs but also as tissue-resident DCs that migrate to draining LNs upon receiving a pathogen or a danger signal, and they also migrate under steady-state conditions to deliver self-antigens for tolerance induction. Both subsets are defined by their unique expression of XCR1 but are distinguished from each other by the expression of CD11c and MHC II molecules and by the integrin marker

CD103 (ITGAE) and $CD8^+$ expression. Lymphoid resident cDC1s are $CD8^{high}$ $CD11c^{high}$ $CD103^{low}$ and MHC II^{int}, while migratory cDC1s are $CD8\alpha^{low}$ $CD11c^{int}$ $CD103^{high}$ and MHC II^{high}. $CD8\alpha^+$ lymphoid resident DCs express the CD8α transcript and protein, but not CD8αβ, which is most commonly expressed by $CD8^+$ T cells (Shortman and Heath, 2010). $CD103^+$ cDC1s that reside in the dermis also express the C-type lectin langerin (CD207) which is involved in the formation of the intracytoplasmic Birbeck granules (Kissenpfennig et al., 2005).

cDC1s represent 20–40% of spleen and LN cDCs. In contrast, they constitute most of the thymic cDC population and are generated locally from early thymocyte progenitors. They express distinct lectin and TLRs compared to $CD11b^+$ cDC2s. Lectin receptors include CD205, and Clec9A, and langerin which are expressed mostly by cDC1s (Jiang et al., 1995), in contrast to DC immunoreceptor 2 (DCIR2) expressed exclusively by $CD11b^+$ DCs. These receptors have been exploited for targeted delivery of antigens to specific DC subsets. They also lack macrophage markers such as CD11b, CD115, CD172a, F4/80, and CX3CR1 (Ginhoux et al., 2009). Until now cDC1s are considered the best-characterized cDC subset, both by their phenotype and their gene expression signature, and they also appear to be an evolutionary conserved subset. They are distinguished by being the most efficient cross-presenters of foreign antigens such as viral and tumor antigens on MHC I molecules to $CD8^+$ T cells, though unlike in mice, $CD11b^+$ DCs were shown to also possess cross-presentation capacity in humans (Segura et al., 2013).

TLR stimulation on $CD8a^+$ and $CD103^+$ cDC1s induces prominent secretion of the bioactive IL12p70 (Reis e Sousa et al., 1997). It is worth noting that in contrast to macrophages, IL-12 production by $CD8\alpha^+$ and $CD103^+$ cDC1s requires c-Rel (Grumont et al., 2001). Their development is orchestrated by the same set of transcription factors: IRF8; which is required for the development of all cDCs from their BM precursors as discussed before, inhibitor of DNA binding 2 (Id2), basic leucine zipper ATF-like 3 transcription factor (BATF3), and the nuclear factor interleukin 3 regulated (NFIL3). Knockout models of either of these genes results in a severe developmental defect of $CD8\alpha^+$ and $CD103^+$ cDC1s, but not $CD11b^+$ cDC2s. The hierarchy and sequential involvement of these specific transcription factors within the CD8α and CD103 cDC lineage is emerging (Murphy et al., 2016). While IRF8 seems obligatory for the development of Id2-expressing DC precursors, BATF3 is induced at later stages of CD8α and CD103 cDC1 maturation (Hildner et al., 2008). Indeed, cDC1 development can be

observed in BATF3-deficient mice infected with intracellular pathogens or treated with IL-12, this appears to be dependent on compensatory BATF and BATF2 expression (Tussiwand et al., 2012). Additionally, short-term development of cDC1s was observed in the absence of Id2, NFIL3, and BATF3, which collectively suggests that IRF8 is the master regulator of CD8a and CD103 cDC1 development (Seillet et al., 2013).

1.6.2 CD11b$^+$ dendritic cells

CD11b$^+$ cDCs (cDC2s) are the most abundant cDCs in lymphoid organs except for the thymus, representing 50-60 % of spleen and LN cDCs, and can also be found as migratory cells in nonlymphoid tissue. They are separated by the differential expression of CD11c and MHC II as described for CD8α and CD103 cDC1s. However, in contrast to CD8α and CD103 DC1s, the population currently defined as CD11b$^+$ cDC2s is heterogeneous and remains less well characterized. Like cDC1s, cDC2s proliferate in situ in response to Flt3L, and are reduced in Flt3-and Flt3L-deficient mice, though to a lesser extent compared to cDC2s. They can be identified by expression of surface markers CD11b, DCIR2 (by staining with the antibody 33D1), CD301b (MGL2), CD4 or signal regulatory protein-α (SIRPα), depending on the tissue investigated (Suzuki et al., 2004). The transcription factors controlling general CD11b$^+$ cDC2 development include RelB (Briseño et al., 2017), NOTCH2 (Lewis et al., 2011), RBP-J (Caton et al., 2007), IRF2 (Ichikawa et al., 2004), and IRF4 (Suzuki et al., 2004). It is worth noting that IRF4 also controls functional aspects of CD11b$^+$ DCs, such as their MHC presentation capacity (Vander Lugt et al., 2014) and migration (Bajana et al., 2012; Gao et al., 2013). However, deficiencies of IRF4 and NOTCH2 only partially impair this compartment and this impairment is variable in different tissues (Bajana et al., 2012; Lewis et al., 2011). Given the heterogeneity of this subset, it is not surprising that assigning specific functions to cDC2s is still a daunting task, and in most of the cases, CD11b$^+$ DCs are still defined by absence of cDC1 associated-functions. For instance, their inability to cross-present and produce specific cytokines, such as IL-12 compared to cDC1s. It was observed though that cDC2s are superior in the induction of CD4$^+$ T cell immunity compared to cDC1s, potentially due to their superior of MHC-II presentation capacity (Dudziak et al., 2007; Lewis et al., 2011). They are also characterized by a distinct cytokine secretion profile, such as IL-6 (Persson et al., 2013) and IL-23 (Schlitzer et al., 2013b). Also, splenic cDC2s were shown to be major producers of proinflammatory chemokines such as CCL3, CCL4,

and CCL5 (Proietto et al., 2004) after TLR ligand exposure. The *in vivo* relevance of these activities is yet to be determined.

cDC2s in the spleen can be sub-classified based on the differential expression of the endothelial cell–specific adhesion molecule (ESAM). $ESAM^{hi}CD11b^{+}$ DCs seem to depend of Notch2 expression and are defined by higher CD4, CD11c, and Flt3 levels and lower Csf-1R, Csf-3R, and CCR2 levels than do $ESAM^{lo}CD11b^{+}$ DCs. $ESAM^{hi}CD11b^{+}$ splenic DCs derive from DC-restricted precursors and are dependent on Notch2 signaling (Lewis et al., 2011), whereas $ESAM^{lo}CD11b^{+}$DCs are thought to derive from circulating monocytes and are dependent on Kruppel Like Factor 4 (Klf4) signaling (Tussiwand et al., 2015).

1.6.3 $CD8^{-}$ $CD11b^{-}$ (DN) dendritic cells

Another DC subset that remains poorly characterized are $CD8^{-}$ $CD11b^{-}$ (DN) DCs (Iwasaki and Kelsall, 2001; Proietto et al., 2004). They constitute around one-third of all DCs in Peyer's patch, around 30% in the mesenteric LN and only a minor and indistinct population in the spleen (9%) and peripheral lymph nodes (13%) (Iwasaki and Kelsall, 2001). Among splenic cDCs, they were shown to express moderate amounts of CCR6 (Iwasaki and Kelsall, 2000) and, under inflammatory conditions they upregulate the chemokines CCL3, CCL4, and CCL5 (Proietto et al., 2004). They responded specifically to R848 (a TLR-7 agonist) and CpG (a TLR-9 agonist) among a panel of tested TLR stimuli (Proietto et al., 2004). *In vitro* stimulation of DN DCs was shown to induce DEC-205 expression, but they remain negative for the CD8 and CD11b markers indicating that this subset does not represent an immature stage of cDC1s or cDC2s (Iwasaki and Kelsall, 2001). In human DCs, $CD141^{+}$ DCs correspond to mouse $CD8^{+}$ cDC1s and $CD1c^{+}$ DCs correspond to mouse $CD11b^{+}$ cDC2s. A $CD141^{-}$ $cDC1^{-}$ DC subset is also identified in human peripheral blood mononuclear cells (PBMCs) (Villani et al., 2017). More recently, they were also shown to be significantly higher in squamous cell tonsillar cancer biopsies compared to their counterparts in benign tonsils (Abolhalaj et al., 2018)

1.6.4 Plasmacytoid dendritic cells

pDCs are blood-circulating DCs that are found only as a small population throughout the periphery and in lymphoid organs. They characteristically have a highly developed secretory compartment and are recognized by the expression of CD45R (B220), Ly6C,

and the transmembrane glycoprotein PDCA-1 (CD317) in mice and CD123, CD303/BDCA2, and CD304/BDCA4 in humans. They have a distinct morphology but they share many cDC characteristics such as Flt3L dependency (Kingston et al., 2009), and an ability to prime T cells though in a more restricted manner (Sapoznikov et al., 2007). Also, their transcription profile overlaps with cDCs but is quite distinct (Robbins et al., 2008). They selectively express Toll-like receptor 7 (TLR7) and TLR9, and as such their most important function is thought to be producing large quantities of type 1 IFNs in response to single-stranded viral RNA and DNA, a direct consequence of their constitutive IRF7 expression (Honda et al., 2005). E2-2 transcription factor that is required for their development mediates that by direct suppression of Id2 expression that is required for CD8 and CD103 cDC1 development (Ghosh et al., 2010). This has been confirmed by early deletion of E2-2, which leads to a complete absence of pDCs both in human and mouse (Cisse et al., 2008). While excision of the E2-2 gene in mature pDCs initiated an alternative cDC transcription program shifting them towards CD8α development (Ghosh et al., 2010). Such ‘‘converted’’ CD8α^+ pDCs bear D-J rearrangement indicative of their pDC past and are discriminated from CD8α^+ cDC1s by expression of CX3CR1 among other markers (Bar-On et al., 2010), though the contribution of these ‘‘ex-pDCs’’ to the immune response is yet to be shown. pDCs have the potential to act as APCs, since they express MHC II and co-stimulatory molecules; but their ability to phagocytose dead cells and present cell-associated antigen is yet to be clearly established, also their ability to cross-present exogenous antigen on MHC class I. Single-cell RNA-sequencing analysis of blood DCs coupled with functional characterization indicates that human pre-DCs contaminated the classically defined pDC gate and this contamination can be responsible for the previous misrepresentation of pDCs’ “T cell-activating” property (Villani et al., 2017). In tumors, pDCs seem to correlate with poor prognosis of both breast and ovarian cancers (Conrad et al., 2012), but they are also studied as potential therapeutic targets to elicit IFN-α release and antigen presentation by cDCs (Kranz et al., 2016; Treilleux et al., 2004).

1.6.5 Langerhans cells

LCs are described as the prototypical DCs that were first discovered in the 19th century and gained special attention after splenic DC discovery. They are a unique population of mononuclear phagocytes that are restricted to the epidermal skin layer. They were shown

to express high levels of MHC II and could stimulate an MLR after in vitro culture with T cells. Moreover, studying LCs was the first clue to understanding the distinct functional stages of DC maturation (Schuler and Steinman, 1985). They constitute 3–5% of epidermal cells, with approximately 700 LCs/mm2 (Merad et al., 2008) and are different from other tissue cDCs by having unique ontogeny and homeostatic properties (Ginhoux and Merad, 2010). As described earlier, LCs arise from fetal liver-derived monocytes (Wang et al., 2012), and self-renew under physiological steady-state conditions without replacement by blood-borne precursors (Merad et al., 2002). LCs are characterized by high levels of Langerin expression similar to dermal cDC1s, but they are differentiated from dermal cDC1s by the lack of expression of CD103, XCR1, and DEC-205 (Ginhoux et al., 2007).

Murine LCs also express a specific epithelial cell adhesion molecule (EpCAM). The deletion of this molecule was shown to reduce mobilization of LCs and inhibit their tolerance induction capacity in a contact hypersensitivity mouse model (Gaiser et al., 2012). They play important roles both in immunity and tolerance induction upon mobilizing to the skin draining lymph nodes. They secrete IL-6 which is essential for protecting mice against *Candida albicans* infection by promoting Th17 cell responses (Igyarto et al., 2011). They were also shown to suppress protective immunity during *Leishmania major* infection (Kautz-Neu et al., 2011). In human LCs expression of high levels of CD1a was described (Ito et al., 1999). CD1c is a member of the group 1 CD1 proteins (CD1a, CD1b, and CD1c). This group of proteins has been shown to have the capacity to present lipid antigens to T cells (Hunger et al., 2004).

1.6.6 Monocyte-derived DCs (MoDCs)

MoDCs arise as a consequence of inflammation or infection and can be found in lymphoid and non-lymphoid organs (Serbina et al., 2003). They are derived from the monocyte influx induced by inflammation and as such termed ‘‘monocyte-derived DCs’’ (MoDCs) or ‘‘inflammatory DCs’’ (iDCs) (Segura and Amigorena, 2013). The study of BM culture-derived MoDCs using IL-4 and GM-CSF has yielded many of our insights into DC biology over the past decades. MoDCs are characterized by being more versatile and can perform functions of different DC subsets. In tissues, they can perform typical DC functions such as antigen presentation to effector T cells, pathogen clearance, migration to SLOs and cytokine production. Since they are of monocytic origin they express CD64,

the Fc-gamma receptor 1 (FcgRI) but also Ly6C and typical DC markers including CD11c, high levels of MHC II and CD11b (Lutz et al., 2017; Min et al., 2018). Though their gene expression profiles differ considerably from cDCs (Xu et al., 2007), they are still very useful in many DC studies owing to their powerful antigen presentation capacity and robust cytokine production. TIP DCs that appear during pathogen-associated inflammation (Serbina et al., 2003) have been considered the prototypic MoDCs. However, their pronounced proinflammatory signature and their Csf2-independent development might suggest that they are activated effector monocytes rather than cDC-like cells (Greter et al., 2012). *In vivo* MoDCs are also DC-SIGN (CD209a)-positive DCs as described by (Cheong et al., 2010) where they appeared in LNs after TLR ligand challenge. MoDCs also appear to be more closely related to $CD11b^+$ cDC2s. Thus, understanding MoDCs *in vivo* will probably contribute to our understanding of the heterogeneous $CD11b^+$ cDC compartment. MoDCs were shown to be recruited from blood into lymph nodes and are differentiated from monocytes by LPS and live or dead gram-negative bacteria. Their mobilization requires TLR4 and its CD14 co-receptor and Trif. *In vivo* MoDCs were shown to be as capable as cDCs of antigen presentation. This included cross-presentation of proteins and live gram-negative bacteria on MHC I similar to cDC1s. After their full differentiation they required L-selectin and CCR7 for their migration to the T cell areas (Cheong et al., 2010).

1.7 Spatiotemporal organization of DCs in LNs

After CCR7-dependent migration from tissues, migratory DCs end up at the subcapsular sinus (SCS) of the LN and cross the SCS floor in a CCR7-independent manner (Braun et al., 2011). Migratory cDC1s and cDC2s then enter the interfollicular zone (IFZ) and home to the different areas of the LN (Schumann et al., 2010). An early study observed segregation of cDC1s and cDC2s, cDC1s were located in the deep T cell zone (TCZ) and cDC2s were in the T cell–B cell border (Ingulli et al., 2002). A langerin reporter mouse model that marks both $CD103^+$ cDC1s and LCs migrating from the skin showed that both populations migrate into the deep TCZ (Kissenpfennig et al., 2005). On the other hand, $CD11b^+$ migratory DCs localized to the outer paracortex (Gerner et al., 2015; Krishnaswamy et al., 2017). Studies using mice with knock-in photoconvertible fluorescent proteins to track DC migration dynamics showed that migratory cDC1s arrive to the skin draining LNs within 1 day after immunization but require another 24 hours to

reach the deep TCZ, where they are mixed with LN-resident cDC1s (Braun et al., 2011; Tomura et al., 2014).

This result was also similar when imaging LNs by intravital microscopy following adoptive transfer of either BM-derived or splenic DCs. These transferred cells were located in the T cell–B cell border, proximal to HEVs, and therefore this might serve as a zone for DC scanning by incoming naive T cells from the circulation (Bajenoff et al., 2003; Mempel et al., 2004; Miller et al., 2004). Also, in lung-draining LNs; migratory cDC2s localize to the T cell–B cell border, while migratory cDC1s were located in the deep TCZ (Krishnaswamy et al., 2017). Therefore, it appears that this distribution pattern of cDC1s and cDC2s is conserved across a variety of tissues both at steady state and upon immunization. This was also mirrored in an elegant study that was conducted in human LNs; where cDC2s are found at the T cell–B cell border, and cDC1s at the TCZ (Granot et al., 2017).

Studies indicate that both LN-resident cDC1s and cDC2s can acquire antigen through sampling of lymph after footpad or intra-auricular injection (Gerner et al., 2015). Whether this occurs at cortical conduits in the outer TCZ or lymphatic sinuses at the cortico-medullary junction which is adjacent to peripheral follicles is still unknown. It was previously shown that LN-resident $CD11b^{+}$ DCs sample small antigens that are transported to LN conduits after injection in the outer LN T cell–B cell border (Sixt et al., 2005). On the other hand, another study identified the same subset sitting at lymphatic sinuses on the medullary side of the LN, and they can capture large particulate antigens from lymph (Gerner et al., 2015). It is still unclear whether these are two separate subsets or the same migrating to different locations in the lymph node.

Apart from its importance in mediating the migration of DCs from tissues towards the draining LN, CCR7 is also important for DCs to locate the TCZ (Ato et al., 2002; Braun et al., 2011). The TCZ of both LNs and the spleen are areas high in CCL19 and/or CCL21 (Ato et al., 2002; Luther et al., 2000). and since cDC1s show higher expression of CCR7 than cDC2s, they follow the CCL19 and CCL21 gradient and preferentially localize to the deep TCZ (Krishnaswamy et al., 2017). XCR1 selective expression by cDC1s potentially helps in establishing this niche since CD8+ T cells secrete its ligand XCL1 during early activation (Brewitz et al., 2017).

On the other hand, cDC2s express higher levels of CXCR5 compared to CCR7 which causes them to be positioned at the T cell–B cell border after migration to the LN (Krishnaswamy et al., 2017). The chemotactic receptor Epstein–Barr virus induced gene

2 (EBI2) was recently shown to guide cDC2s to oxysterols at the T cell–B cell border in the spleen (Li et al., 2016) while cDC1s express enzymes that degrade oxysterols and therefore create a T cell area relatively devoid of EBI2 ligands (Lu et al., 2017). Also, selective expression of sphingosine-1-phosphate (S1P) receptor 3 (S1PR3) by cDC2s was observed and potentially results in sustained responsiveness to S1P gradients. S1P is concentrated in cortical sinusoids in the paracortex, which largely overlaps with the T cell–B cell border and the region with highest concentration of migratory cDC2s (Grigorova et al., 2010).

1.8 Dendritic cell control of T cell response

(CD11c)-DTR mice showed that lack of $CD11c^+$ cells led to the loss of CD8 T cell priming to cell-associated antigens and to intracellular pathogens such as Listeria monocytogenes and malaria (Jung et al., 2002). Later, Zbtb46-DTR mice lacking only cDCs resulted in a complete inability to prime CD8 or CD4 T cells against soluble antigen (Meredith et al., 2012) and a failure to prime CD4 T cells against Mtb (Samstein et al., 2013). Additionally, cDC-specific deletion of MHCII expressing cells using Zbtb46-cre led to a complete reduction in CD4 T cell priming to soluble antigen (Loschko et al., 2016). Furthermore, antigen targeting to specific DC subsets indicated the functional specialization of each subset, with cDC1s preferentially priming CD8 T cells and the only subset efficiently carrying out cross-presentation of exogenous antigens on MHC class I, while cDC2s were more efficient in priming CD4 T cells (Dudziak et al., 2007). Depleting cDC1s using the *Xcr1*-DTRvenus strain also abrogated CD8 T cell priming against soluble and cell-associated antigen as well as against Listeria infection (Yamazaki et al., 2013). CD4 T cells could still be primed against soluble antigen in these mice. Future work with specific depletion of cDC2s is needed to confirm their unique role in priming CD4 T cells.

1.8.1 Control of type 1 immune response

Immune responses that are induced against intracellular pathogens and require IFN-γ activated macrophages and cytotoxic CD8 T cells for their clearance are termed type 1 immune responses. In the early phases of the response, NK cells and innate lymphoid cells type 1 (ILC1s) are the major source of IFN-γ while antigen-specific Th1 and CD8 T cells produce this cytokine at later stages. The bioactive IL-12p70 is the major cytokine

required for activation of type 1 responses, as it drives NK cells and ILC1s to produce IFN-γ, and is responsible for polarizing naive T cells to type 1 helper (Th1) T cell (Macatonia et al., 1995). Over the years, many studies proved that cDC1s are the major non-redundant source of IL-12 and is responsible for mounting type 1 responses by T cells. Using the Karma mouse strain where cDC1s are specifically depleted completely abolishes IL-12 production in response to soluble Toxoplasma antigen and a significant reduction in IFN-γ levels (Alexandre et al., 2016). Also, *Batf3*$^{-/-}$ mice showed reduced IFN-γ production from NK cells during Toxoplasma infection, which indicates that IL-12 from cDC1s is critical for NK cell activation and reduced IFN-γ production (Askenase et al., 2015). Additionally, using *Itgax*-DTR mice where all CD11c cells can be conditionally depleted (Neuenhahn et al., 2006), *Batf3*$^{-/-}$ mice (Edelson et al., 2011), *Ly75*-DTR (Fukaya et al., 2012), and *Xcr1*-DTRvenus (Yamazaki et al., 2013) mice where cDC1s are specifically depleted all showed reduced CD8 T cell responses in Listeria infection models. Interestingly, many of these models showed reduced Listeria burden in the spleen and in *Batf3*$^{-/-}$ mice increased resistance to the infection was even observed. This is because cDC1 infection by Listeria in the splenic marginal zone is important for them to spread and proliferate in the lymphoid areas of the spleen (Neuenhahn et al., 2006). Another role the cDC1s play in promoting the survival and activation of NK cells depend on their trans-presentation of IL-15. cDC1s express the non-signaling receptor chain IL-15Rα and binds IL-15, then it presents it to the full IL-15αβγ receptor on NK cells (Burkett et al., 2004; Mortier et al., 2008).

Control of viral infections rely on CD8^{+} cytotoxic lymphocytes (CTLs), their activation requires the engagement of nucleic acid sensors by APCs leading to the production of type 1 interferons and IL-12. Both CD103^{+} DCs migratory as well as CD8α^{+} lymphoid-resident DCs were shown to be capable of cross-presenting viral antigens and activating CD8^{+}T cells (Waithman et al., 2013). Also, during a viral response, CD4 T cell help provided to CD8 T cells appears to be mediated through ‘‘licensing” of cDC1s. Analysis of immune responses suggests that CD4 T cells are primed by DCs earlier than CD8 T cells, this is followed by a three-way clustering of 1) CD4 T cells with; 2) cDC1s in order to license them, then with; 3) CD8 T cells which then get primed by the cDC1s that cross-present the viral antigen to the CD8 T cells (Eickhoff et al., 2015; Hor et al., 2015).

1.8.2 Control of type 2 immune response

Type 2 responses are mounted against multicellular parasites at barrier surfaces in order to aid in their expulsion. Several cytokines have been described that induce this response including IL-4 that polarizes naive T cells to Th2 cells(Le Gros et al., 1990), and IL-25 and IL-33 that activate innate lymphoid cells type 2 (ILC2s) and push them towards production of effector cytokines such as IL-4, IL-5, and IL-13 (Sonnenberg and Artis, 2015). Depletion of $CD11c^+$ cells in *Itgax*-DTR mice abrogated type 2 responses to inhaled house dust mite (HDM) allergen (Hammad et al., 2010) and an FcεRI expressing $CD11c^+$ $MHCII^+$ cell population was shown to be the major subset responsible for the response. This was confirmed to be the cDC2 subset by a study which showed that conditional deletion of Irf4 with *Itgax*-Cre (Gao et al., 2013) diminished Th2 priming in response to papain, which is a model allergen inducing Th2 response, and *Nippostrongylus* infection. Also, depletion of a subset of cDC2s, that depend on Klf4 for their development, using $Klf4^{f/f}$ *Itgax*-Cre strain increased susceptibility to *Schistosoma mansoni* infection and diminished allergic inflammation after intranasal HDM challenge (Tussiwand et al., 2015). The production of cytokines by DCs was not established in any of these models, so whether they control type 2 responses by this mechanism or some other is yet to be determined. Other studies suggested that ILC2s might be the source of cytokines acting to induce type 2 responses (Halim et al., 2014). It is possible that ILC2s and DCs cooperate in Th2 priming, though this is yet to be studied. Of note, in humans, Langerhans cells induce the secretion of Th2 cell cytokines in mixed-leukocyte reactions more efficiently than other skin DC subsets do (Klechevsky et al., 2008; Segura et al., 2012). This suggests that different skin DC subsets can carry out the priming of Th2 cells in mice and humans depending on the context.

1.8.3 Control of Th17 immune response

Th17 immune responses is mainly carried out at barrier surfaces such as the lungs and intestines to control infections by extracellular bacteria and fungi. Cytokines including IL-23 and IL-6, and TGF-β are required for this response and IL-6, and TGF-β initiate T helper 17 (Th17) cell polarization (Bettelli et al., 2006). On the other hand, IL-23 increases the survival and expansion of committed Th17 cells (Veldhoen et al., 2006). This cytokine is also critical for innate responses activating ILC3s to produce IL-22. This promotes

production of bactericidal lectins such as RegIII-γ from small intestinal epithelial cells (Kinnebrew et al., 2012; Sonnenberg and Artis, 2015). *Citrobacter rodentium* is a famous mouse pathogen used to study Th17 immune responses. It requires IL-23 and IL-22 for its clearance (Zheng et al., 2008); (Basu et al., 2012); (Mundy et al., 2005). Notch2-dependent cDC2s were shown to be the critical source of IL-23 in defense against this pathogen (Satpathy et al., 2013) as demonstrated by conditional knockout of Notch2-dependent cDC2s. Studies have also implicated cDC2s in various other Th17 immune responses. The depletion of $CD103^+$ $CD11b^+$ intestinal cDC2s, using *Notch2*$^{f/f}$ *Itgax*-cre, *Irf4*$^{f/f}$ *Itgax*-cre, or *CD207*-DTA mice, showed fewer small intestinal Th17 cells at steady state (Lewis et al., 2011); (Schlitzer et al., 2013a); (Welty et al., 2013). This was also the case for Th17 cells in the small intestine lamina propria and mesenteric LNs at steady state and less Th17 polarized cells after immunization with antigen plus αCD40 and LPS (Persson et al., 2013). Another study also showed that cDC2s must produce IL-23 to activate IL-17 secretion from dermal γδ-TCR T cells during cutaneous *Candida albicans* infection (Kashem et al., 2015). In conclusion, the depletion of cDC2s was clearly responsible for defects in Th17 responses to several pathogens, such as *Citrobacter* (Satpathy et al., 2013), *Streptococcus* (Linehan et al., 2015), and Candida (Kashem et al., 2015); (Trautwein-Weidner et al., 2015). On the other hand, Th17 responses were intact in mice lacking cDC1s which again underscores the functional specialization of different cDC subsets and the non-redundant roles they play in host defense.

1.8.4 Interleukin-12 and its role in type 1 immune responses

Interleukin 12 (IL-12) belongs to type I cytokines and has a four α-helical bundle structure. IL-12 acts in a form of a heterodimeric protein (IL-12-p70; IL-12-p35/p40) consisting of two covalently linked p35 and p40 subunits. Following the discovery of IL-12, three other members (IL-23, IL-27, and IL-35) have been added to the IL-12 family and shown to play critical roles in Th1 cell functions. IL-12 is a ligand of a receptor composed of two amino acid chains, IL-12R-β1 and IL-12R-β2. IL-12 receptor (e.g., The IL-12R-β1 chain is expressed in a constitutive manner in B cells (Pistoia et al., 2009), while IL-12R-β2 is expressed in an inducible manner in a variety of immune cells, including NK cells, T, and B lymphocytes. Ligand-bound IL-12R-β2 becomes phosphorylated on tyrosines, which provides harboring sites for two kinases, JAK2 and TYK2. Among the STAT family of

transcription factors, STAT4 is considered the most specific mediator of cellular responses elicited by IL-12 (Thierfelder et al., 1996). In human T cells, both IFN-α and IL-12 activate STAT4, yet IFN-α cannot substitute for the loss of IL-12 function *in vivo*, as judged by the fact that loss of IL-12R expression in humans results in a failure to induce protective Th1-mediated immune responses (Jong et al., 1998), (Altare et al., 1998). In addition to forming heterodimers with p35, both mouse and human p40 are secreted in large excess as free p40 monomers and can also form homodimers (p402), which exhibit biological activities antagonistic to heterodimeric IL-12p70 (Gillessen et al., 1995),(Ling et al., 1995). The production of immunosuppressive IL-12p40 homodimers was also induced in DCs and macrophages exposed to ultraviolet radiation (Schmitt and Ullrich, 2000). Surprisingly, Jana et al. found that IL-12p70, $p40_2$ (the p40 homodimer) and p40 (the p40 monomer) all induced the production of TNF-α in BV-2 microglial cells and in mouse primary microglia and peritoneal macrophages.

IL-12p70 is released in response to intracellular bacterial and parasitic infections and though the relative roles of IL-12p70 and IFN-γ in Th1-cell priming is to a significant extent pathogen-dependent, in most infections IL-12p70 regulates the magnitude of the IFN-γ response at the initiation of infection, thus potentiating natural resistance, favoring Th1-cell development, and inhibiting Th2 responses. Treatment of animals with IL-12p70, either alone or as a vaccine adjuvant, has been shown to prevent diseases caused by many of the same infectious agents, by stimulating innate resistance or promoting specific reactivity.

Additionally, IL-12p70 plays a major role in tumor control and rejection (Eisenring et al., 2010; Segal et al., 2002). Recombinant human IL-12p70 has been studied as a single agent for systemic treatment of various types of cancer in patients. A strong CTL response was observed in patients with advanced melanoma after IL-12p70 administration. The number of tumor-specific CTL increased in the circulation, and influx of specific memory $CD8^+$ T cells into metastasized lesions was demonstrated (Lasek et al., 2014).

1.9 Bone marrow-derived dendritic cells

Inaba and colleagues were the first to successfully generate large quantities of DC from mouse BM precursors supplied with GM-CSF (Inaba et al., 1992). About 5×10^6 DC at 70% purity could be obtained per mouse after 6 days of culture. These monocyte-derived

cultured DC exhibited characteristic morphology and phenotype of DC, including the presence of small cytoplasmic protrusions and the expression of CD11c, high levels of MHC molecules and costimulatory molecules, and have a strong ability to initiate mixed leukocyte reaction (MLR). This method was further improved by our lab to generate higher yields and purities of DCs (Lutz et al., 1999). The major modifications include: the avoidance of any active depletion of BM cell subsets, reduction of GM-CSF concentration, a lower seeding density of BM cells and a prolonged culture period. With the modified method, a higher yield of $1–3\times10^8$ immature and mature DC per mouse at nearly 95% purity can be achieved (Lutz et al., 1999). Further maturation of DC could be induced by high doses of LPS or TNFα, where 50–70% of the non-adherent fraction represented mature DC expressing high levels of CD86 and CD40. Such matured BM-derived dendritic cells (BM-DCs) closely resemble the inflammation induced MoDCs (Inaba et al., 1990; Lutz et al., 2017).

Surprisingly, abrogation of GM-CSF or its receptor in mice did not affect MoDC generation and activation of $CD8^+$ T cell responses. Conversely, deficiency of M-CSFR impaired inflammatory MoDC recruitment and CD80/CD86 surface expression (Greter et al., 2012). Thus, the role of GM-CSF in the generation of inflammatory MoDCs *in vivo* is yet to be determined.

BM-DCs can also be generated using GM-CSF BM cultures supplemented with IL-4 both from mouse BM or peripheral blood monocytes (Schreurs et al., 1999), (León et al., 2004). Compared with DC differentiated in the presence of GM-CSF alone, additional supplementation of IL-4 significantly enhanced DC differentiation, leading to an intermediate degree of maturation (Labeur et al., 1999), and induced DC growth and maturation. IL-4 was also proposed to inhibit macrophage colony formation (Hiasa et al., 2009).

BM-DCs supplemented with IL-15 together with GM-CSF have similar phenotype and functions to that of GM-CSF/IL-4-induced DC (Pulendran et al., 2004). Notably, the cultured IL-15 DC displayed superior Th1 polarization and cytotoxic T lymphocyte (CTL) induction ability. They appear to also acquire cytotoxic capabilities upon LPS activation and exhibit direct tumoricidal function via expression of iNOS (Pulendran et al., 2004);(Hanke et al., 2014).

Unlike cDCs which show a specific and restricted functional profile; BM-DCs appear more versatile in their functions and have been shown to present and cross-present

equivalently well (Cheong et al., 2010). We and others have shown that they can be instructed by pathogens or inflammatory signals to induce Th1, Th2, and Th17 responses depending on the quality and magnitude of the stimulation. BM-DCs that were stimulated with TNF or Variant surface glycoprotein (VSG) from *Trypanosoma* induced an inflammatory response that correlates with Th2 polarizing capacity. This signature was changed significantly with a different quality and quantity of gene responses after LPS treatment of BM-DCs and they were shifted towards a Th1 inducing profile (Pletinckx et al., 2011). On the other hand, the use of high doses of cholera toxin induced a Th17-polarizing BM-DC secreting Th17 characteristic cytokines such as IL-1β, IL-6 and IL-23 (Silva-Vilches et al., 2017).

1.10 Dendritic cell vaccination

Due to the efficient protocols to generate large amounts of human MoDCs, the large body of evidences from studies using BM-DCs that show their strong anti-tumor activities in mouse models, and their possibility to direct both CD4 and CD8 T cell responses, they were the prime candidates for adoptive DC vaccine trials in tumor patients (Nestle et al., 1998). Currently, peripheral blood mononuclear cells (PBMCs) are the most common source of cells for vaccine preparation (Reichardt et al., 2004). Since DCs circulate in peripheral blood with low frequency, they are usually differentiated from monocyte precursors that comprise up to 10% of PBMCs. PBMCs are usually obtained by leukapheresis and enriched for monocytes by their adherence to plastic and CD14 selection. Differentiation of monocytes into immature DCs is then achieved by culture with cytokines, most often granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-4. Alternative cytokine cocktails, including GM-CSF/IL-15 (Anguille et al., 2009) and IFN-β/IL-3 (Trakatelli et al., 2006), have also been used to optimize vaccine preparation, although these are not as well studied in the clinic. DC maturation is typically achieved by a standard cytokine consisting of GM-CSF, IL-4, IL-1β, IL-6, TNF-α and prostaglandin (PGE)-2 (Jonuleit et al., 1997), or from Toll-like receptor (TLR) ligands such as dsRNA or CpG motifs. Several dosing forms have been used in clinical trials including intradermal, intramuscular, intravenous or intratumoral. (Anguille et al., 2009; Jonuleit et al., 1997; Reichardt et al., 2004; Ridgway, 2003; Trakatelli et al., 2006). The most characteristic feature of DC vaccination is their very high tolerability by most of the patients and a low toxicity profile (Mitchell et al., 2015b),(Krishnadas et al., 2015). However, the clinical

picture so far of most DC vaccine clinical trial do not show a very high response rate. Only one vaccine DC has gained an FDA approval so far which is Sipuleucel-T, and it has shown sufficient efficacy in a Phase III clinical trials (Kantoff et al., 2010). The immunosuppressive milieu of tumors is one of the major contributing factors to the low responsiveness to DC vaccination and many studies try to combine DC vaccines with agents that counter the immunosuppression. This included PD-L1 combination, which showed high IL-12 production capacity by DCs and better T cell responses. Also, a reduction in tumor size was observed in a breast cancer model (Ge et al., 2013). Other studies combined DC vaccines with IL-10 blocking antibodies, which shows better NK responses, increased tumor shrinkage, and increased survival in a murine breast cancer model (Rossowska et al., 2015). Another strategy that was used is the repeated injection of DCs or pre-injection of the DC injection site with TNF. This was shown to dramatically enhance the migration of injected DCs (MartIn-Fontecha et al., 2003). Also, pretreatment of the injection site with tetanus/diphtheria toxoid was shown to dramatically improve the vaccine efficacy (Mitchell et al., 2015a). The use of low doses of GM-CSF was also shown to be beneficial (Parmiani et al., 2007).

1.11 In vitro GM-CSF DCs as a source of IL-12

The introduction so far shows that the induction of Th1 responses by DCs relies on three distinct stimuli coming from DCs. The combination of signal 1 by MHC peptide complexes ligating the TCR with signal 2 by CD80/CD86 co-stimulation will lead to T cell activation and proliferation, reaching the Th0 stage, previously observed in mouse T cell clones, (Openshaw et al., 1995). A major signal 3 for the induction of Th1 responses has been identified as the heterodimeric IL-12p70 cytokine (Fig 2) (Macatonia et al., 1995). IL-12 production by DCs can be induced by different pathogen signals but not pro-inflammatory cytokines (Reis e Sousa et al., 1997).

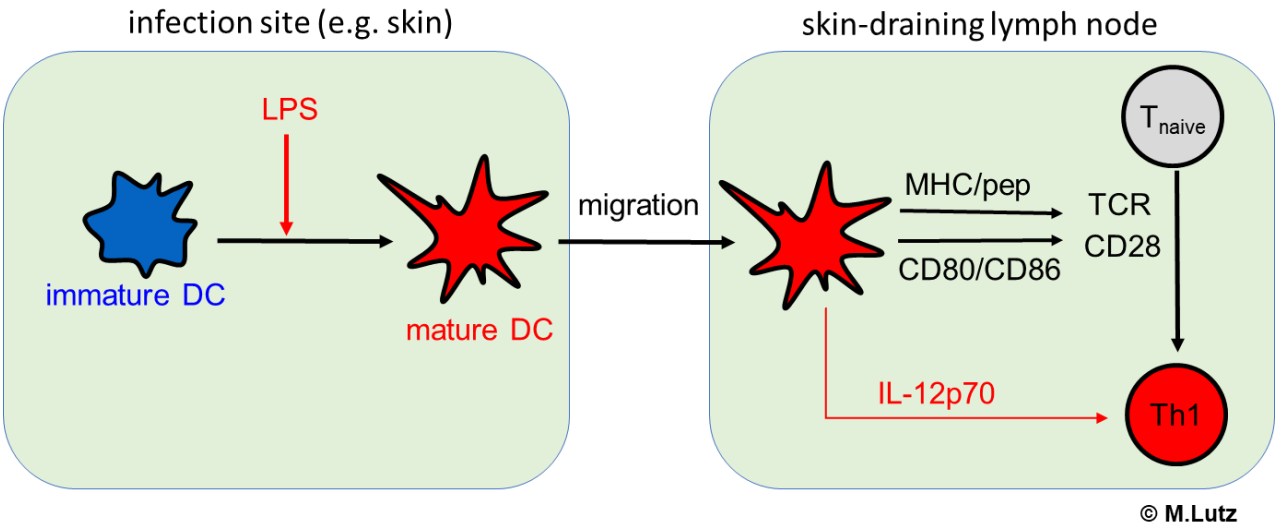


Figure 2: The three-signal theory of T cell priming by DCs. A single DC primes the naive T cell towards Th1 cell type by providing 3 signals: 1) Peptide presented on MHC II, 2) Co-stimulation by the CD80/CD86 molecules, 3) IL-12p70 required for Th1 polarization

Surprisingly, in the DC vaccination setup, several findings question the common believe that injected vaccine MoDCs do provide all signals 1, 2 and 3 for Th1 priming, which is the classical model of DC priming of T cells (Kapsenberg, 2003).

Several DC vaccination studies in tumor patients use the cytokine cocktail consisting of IL-1β/TNFα/IL-6/PGE_2 (Jonuleit et al., 1997) for maturation of MoDCs, (Bol et al., 2016); (Gross et al., 2017). Although tumor therapy with this cocktail matured MoDCs has proven to be successful in melanoma patients and is in fact the most common used approach, MoDCs generated with this cocktail unexpectedly are unable to produce IL-12 (Lee et al., 2002). Despite readily being able to induce Th1 responses in these patients (Gross et al., 2017); this cocktail was compared to synthetic double stranded RNA (poly I:C), soluble CD40 ligand trimer as maturation cues. And while all maturation stimuli induce a mature dendritic cell phenotype, the IL-1β/TNFα/IL-6/PGE_2 cocktail was the most efficient despite the lack of induction of IL-12p70.

In another study that used BM-DCs from IL-12-deficient mice for vaccination against *Leishmania major* infection indicated that the development of Th1 responses relied on an undetermined source of IL-12 production by the recipient mice, not the injected DCs (Ramirez-Pineda et al., 2004), The immunization of mice with a single dose of BM-DCs that were pulsed with *Leishmania major* Ag and activated with CpG ex vivo was enough to confer a very high level of protection against a normally lethal challenge with L. major. Unexpectedly though, the level of IL-12 released by BM-DCs did not correlate with their

capacity to mediate protection against leishmaniasis, since they did not express high amounts of IL-12. The LmAg-induced reduction of the IL-12 production by CpG-activated BM-DCs was a consistent finding. This was supported by the finding that the protective efficacy of Ag-loaded BM-DCs from *Il12a*$^{-/-}$ or *Il12b*$^{-/-}$ mice was virtually identical with that of BM-DCs obtained from WT mice, excluding also a role of the related cytokine IL-23, which shares the p40 subunit with IL-12. Rather, IL-12-deficient mice with CpG-activated BMDC delivering LmAg did not result in protection, demonstrating that IL-12 released by recipient cells is required. Interestingly, the same group previously showed that when Ag-pulsed Langerhans cells from IL-12-deficient mice were as the source of DC, they completely failed to mediate protection against L. major as opposed to those from WT mice (Berberich et al., 2003). This underscores the importance of the type of DC used for vaccination approaches.

Additionally, previous data from our group showed that injected BM-DCs reaching the draining lymph node lack IL-12 production among other cytokines, and they rather induce cytokine production by host endogenous DCs. LPS + CD40-matured BM-DCs that were secondarily matured with TNF in vitro activated their cytokine production. However, upon reaching the lymph nodes they were negative for intracellular TNF, IL-6, IL-12p40, IL-10 cytokines (Voigtlander et al., 2006). On the other hand, endogenous DC population stained positive for all these cytokines with higher levels for TNF, IL-6, and IL-12p40 after LPS + CD40-matured BM-DC injection as compared with TNF-matured BM-DC injection. In this study GM-CSF without IL-4 was used for differentiating the BM-DCs. IL-4 in these GM-CSF cultures promotes the Langerhans cell-like phenotype (Menges et al., 2005). And since no IL-4 was used in the GM-CSF cultures here, this data is in agreement with the reports from the *Leishmania major* model (Berberich et al., 2003; Ramirez-Pineda et al., 2004). Such a model of BM-DCs being incapable of cytokine production upon migrating to the lymph nodes and rather induce cytokine production from other DCs is in agreement with data from Luft and colleagues (Luft et al., 2004), that showed that CCR7 dependent migration of MoDCs and their ability to produce IL-12 are mutually exclusive events.

1.12 DC-DC cross talk for propagation of the immune response

Emerging evidences from the literature indicated that dendritic cells from different subsets communicated with each other and one DC can possibly induce the activation of another

DC in a bystander fashion under certain pathological conditions (Brewitz et al., 2017; De Koker et al., 2017; Plantinga et al., 2013). One study showed that communication of pDCs with XCR1$^+$ cDC1s is required to induce optimal CTL responses in a vaccinia virus setup (Brewitz et al., 2017). Also, the production of IL-12 by MoDCs under CpG vaccination conditions required the coordinated action of cDCs and MoDCs (De Koker et al., 2017). Also in a house dust allergy model, it was shown that MoDCs were mainly responsible for proinflammatory cytokine production in the lungs while CD11b$^+$ migratory cDC2s were responsible for antigen presentation and inducing Th2 responses in the LN, suggesting the requirement of their concerted actions (Plantinga et al., 2013).

The possibility of BM-DCs communicating with other endogenous DCs in the draining lymph nodes is suggested by the studies described in the previous section. However, there are no concrete evidences for which endogenous DC subset is being activated by BM-DCs in a bystander fashion and carry out the IL-12 production and Th1 priming function. Nothing is also known about the possible molecular patterns involved in this presumed crosstalk.

One possible mechanism for this communication between DCs is via the interaction of the tumor necrosis factor receptor family member 9 (TNFRSF9 or 4-1BB) with its ligand TNFSF9 or 4-1BBL. 4-1BB was first thought to only be an important mediator of survival signaling, particularly in CD8$^+$ T cells. Later however it was shown that they are more broadly expressed on regulatory T cells (Tregs) (Gavin et al., 2002), (McHugh et al., 2002), follicular dendritic cells (DCs) (Pauly et al., 2002), DCs (Futagawa et al., 2002), (Wilcox et al., 2002a), differentiating myeloid-lineage cells (Lee et al., 2008), monocytes ((Langstein et al., 1998), immunoglobulin E (IgE)-stimulated mast cells (Nishimoto et al., 2005), eosinophils (Heinisch et al., 2001), neutrophils (Heinisch et al., 2000), (Lee et al., 2005)), activated natural killer T cells (NKTs) (Vinay et al., 2004), and activated NK cells (Melero et al., 1998), (Wilcox et al., 2002b). 4-1BBL is strictly induced on activated APCs (Futagawa et al., 2002), (Goodwin et al., 1993) and is also expressed on myeloid progenitors and hematopoietic stem cells (Lee et al., 2008), (Jiang et al., 2008a), (Jiang et al., 2008b). Its expression appears to be tightly regulated *in vivo*, such that its expression during an ongoing immune response *in vivo* is difficult to detect at the protein level (Lin et al., 2009). However, during chronic and inflammatory conditions 4-1BBL is more readily detectable at the mRNA or protein level (Tan et al., 2000), (Lin et al., 2009),

(Seko et al., 2001), (Mack et al., 2008). On macrophages, 4-1BBL message is induced by LPS in a Toll-like receptor 4 (TLR4), nuclear factor kappaB (NF-κB)-dependent manner (Futagawa et al., 2002), (Goodwin et al., 1993), (Kang et al., 2007). Its expression is rapid and transient, with peak mRNA expression on macrophages at 4h and returning to baseline by 12h (Kang et al., 2007)). 4-1BBL is also upregulated on B cells and DCs by CD40 signaling (DeBenedette et al., 1997), (Diehl et al., 2002). The interaction of 4-1BBL on mature DC with 4-1BB on co-cultured immature DCs has been shown to induce IL-12 production by the immature DCs (Futagawa et al., 2002). Additionally, a more recent study showed that upon injecting lentiviral vectors (LV) expressing 4-1BBL and influenza nucleoprotein (NP) separately; they conferred superior CD8 T cell activation capacity than LV co-expressing 4-1BBL and NP, i.e. to target CD8 T cells that recognize the influenza antigen and receive further activation signals from the 4-1BBL. This suggested that 4-1BBL is more effective when expressed in trans, acting on adjacent DCs and thus activating them in a bystander fashion (Macdonald et al., 2014).

1.13 Aim of the study

In this study we aimed to clarify the cellular sources of IL-12p70 production after subcutaneous BM-DC vaccine injection in the skin draining lymph node, and to study the possibility of bystander activation of different endogenous DC subsets upon interacting with the injected BM-DCs. We generated a chimeric situation by injection of different gene-modified BM-DCs into different strains of gene-modified recipient mice. This allowed us to identify the separate functional contributions of injected versus endogenous DCs for Th1 polarization.

The study attempts to answer 3 main questions:

1- Which DC subset is the source of IL-12p70 needed for Th1 induction during BM-DC vaccination?

2- Are BM-DCs communicating with endogenous DCs to induce bystander DCs in the draining lymph nodes?

3- If so, how and when are they communicating and what signals are communicated to the bystander DCs?

2 MATERIALS AND METHODS

2.1 Reagents

2.1.1 Chemical reagents

Product	Purchased from
Agarose	Roth (Karlsruhe, Germany)
Ammonium Chloride (10% solution)	Applichem (Darmstadt, Germany)
β-mercaptoethanol	Sigma-Aldrich (Deisenhofen, Germany)
Brefeldin A (from *Penicillium brefeldianu*)	Sigma-Aldrich (Deisenhofen, Germany)
BSA	Roth (Karlsruhe, Germany)
Complete Freund's Adjuvant (CFA)	Sigma-Aldrich (Deisenhofen, Germany)
CellTrace™ Violet Cell Proliferation Dye	Invitrogen (Darmstadt, Germany)
CFSE (carboxyfluorescein diacetate succinimidyl ester)	Invitrogen (Darmstadt, Germany)
Chloroform	Applichem (Darmstadt, Germany)
Complete Freund's Adjuvant (CFA)	Sigma-Aldrich (Deisenhofen, Germany)
CpG ODN	Sigma-Aldrich (Deisenhofen, Germany)
DEPC (diethyl pyrocarbonate)	Roth (Karlsruhe, Germany)
DMSO (dimethyl sulfoxide)	Sigma-Aldrich (Deisenhofen, Germany)
Diphtheria Toxin	Sigma-Aldrich (Deisenhofen, Germany)
dNTPs	Fermentas (St. Leon-Rot, Germany)
EDTA	Applichem (Darmstadt, Germany)
eFluor™ 670 Cell Proliferation Dye	Invitrogen (Darmstadt, Germany)
Ethanol	Applichem (Darmstadt, Germany)
Ethidium Bromide	Roth (Karlsruhe, Germany)
Fetal Calf Serum (FCS)	PAA Laboratories (Pasching, Austria)
Fluoromount-G	Serva Electrophoresis (Germany)
Formaldehyde (37%)	Roth (Karlsruhe, Germany)
Hydrogen Peroxide (30%)	Applichem (Darmstadt, Germany)
IC Fixation buffer	Affymetrix eBioscience
Ionomycin calcium salt (from Streptomyces)	Sigma-Aldrich (Deisenhofen, Germany)
Isopropanol	Applichem (Darmstadt, Germany)
L-Glutamine	PAA Laboratories (Pasching, Austria)
L-Lysine	Sigma-Aldrich (Deisenhofen, Germany)
LPS (*E. coli* 0127:B8)	Sigma-Aldrich (Deisenhofen, Germany)

Methanol	Applichem (Darmstadt, Germany)
Paraformaldehyde	Sigma-Aldrich (Deisenhofen, Germany)
Penicillin/Streptomycin	PAA Laboratories (Pasching, Austria)
PMA (Phorbol 12-myristate 13-acetate)	Sigma-Aldrich (Deisenhofen, Germany)
Sodium azide (NaN_3)	Roth (Karlsruhe, Germany)
Sodium (meta)periodate	Sigma-Aldrich (Deisenhofen, Germany)
Sodium phosphate dibasic	Sigma-Aldrich (Deisenhofen, Germany)
Sodium phosphate monobasic	Sigma-Aldrich (Deisenhofen, Germany)
Sucrose ≥99.5%	Sigma-Aldrich (Deisenhofen, Germany)
TRIzol	(Darmstadt, Germany) Sigma-Aldrich
Tris	Applichem
Tissue-tek	Sakura
Triton X-100	Sigma-Aldrich (Deisenhofen, Germany)
Trypan blue	Sigma-Aldrich (Deisenhofen, Germany)

Table 1. List of chemical reagents used throughout experimental set-up

2.1.2 Primary Antibodies

2.1.2.1 Primary antibodies directed against surface or intracellular markers

Antigen	Clone	Dilution	Fluorochrome	Purchased from
CD4	GK 1.5	1:150	APC	Biolegend
CD4	GK 1.5	1:150	FITC	Biolegend
CD8	53-6.7	1:150	Pacific Blue	Biolegend
CD11b	M1/70	1:300	PerCP-Cy5.5	Biolegend
CD11c	N418	1:100	APC	Biolegend
CD11c	N418	1:100	PE-Cy7	Biolegend
CD27	LG3A10	1:100	Pacific Blue	Biolegend
CD45R (B220)	RA3-6B2	1:200	APC	Biolegend
CD64	X54-5/7.1	1:100	PE	Biolegend
CD70	FR70	1:100	PE	Biolegend
CD90.1 (Thy1.1)	OX-7	1:150	PerCP-Cy5.5	Biolegend
CD90.1 (Thy1.1)	HIS51	1:500	biotinylated	BD biosciences
CD103	2E7	1:100	PerCP-Cy5.5	Biolegend
CD103	2E7	1:100	biotinylated	Biolegend

CD137 (4-1BB)	17B5	1::100	PE	BD biosciences
CD137L (4-1BBL)	TKS-1	1:100	PE	BD biosciences
F4/80	BM8	1:100	FITC	Biolegend
I-A/I-E (MHC II)	M5A/114.15.2	1:600	Alexa Fluor 700	Biolegend
Ly-6C	HK1.4	1:200	Brilliant Violet 510™	Biolegend
XCR1	ZET	1:100	APC	Biolegend

Table 2. List of antibodies directed against surface markers, intracellular markers or transcription factors

2.1.2.2 Primary antibodies directed against cytokines

Antigen	Clone	Dilution	Fluorochrome	Purchased from
IFN-γ	XMG1.2	1:100	PE	Biolegend
IL-2	JES6-5H4	1:200	PE	Biolegend
IL-12p35	4D10p35	1:100	PE	eBioscience
IL-12p40	C11.5	1:00	PE	BD biosciences
IL-12p70	R2-9A5	1:100	unconjugated	Bio X Cell
IL-13	85BRD	1:300	PE	BD biosciences

Table 3. List of antibodies directed against cytokines

2.1.2.3 Secondary antibodies or conjugates

Antigen	Host	Dilution	Fluorochrome	Purchased from
anti-rat IgG, κ light chain	Goat	1:200	PE	BD biosciences
Streptavidin	x	1:500	PE-Cy7	Biolegend
Streptavidin	x	1:300	Brilliant Violet 510™	Biolegend
Streptavidin	x	1:500	Cy3	Biolegend

Table 4. List of secondary antibodies or conjugates

2.2 Buffers, media and solutions

For preparation of buffers and solutions, ultrapure Milli-Q water was obtained from Milli-Q water purification systems (Millipore, Schwalbach/Ts, Germany)

Buffers	**Composition**
PBS (phosphate buffered saline)	0.2g KCl 8.0 g NaCl KH_2PO_4 1.15 g 1.15 g Na_2HPO_4 Fill up to 1l Milli-Q water
RPMI 1640 complete medium	500ml RPMI 1640 (PAA Paching Austria) 10% heat-inactivated sterile filtered FCS(PAA) 100U/ml penicillin(PAA) 100µg/ml streptomycin (PAA) 2mM L-glutamine (PAA) 50mM β-mercaptoethanol (Sigma-Aldrich)
DC preparation buffer	45ml PBS 5ml FCS 100µg EDTA (0.5 M)
FACS buffer	500ml PBS 0.1% BSA (Roth) 0.1% NaN_3 (Roth)
Fixation buffer (2% FA)	35ml PBS 2ml Formaldehyde (37%) (Roth)
Perm buffer	PBS 0.1% BSA (Roth) 0.1% NaN3 (Roth) 0.5% Saponin (Sigma-Aldrich)
HL1 complete medium	500ml HL1 (Lonza, Verviers Belgium) 100U/ml penicillin (PAA) 100µg/ml streptomycin (PAA) 2mM L-glutamine (PAA) 50mM-mercaptoethanol (Sigma-Aldrich)
MACS Buffer	PBS 0.5% FCS (PAA) 2mM EDTA (Applichem)
PLP fixation buffer	1% paraformaldehyde 0.075M L-Lysin 0.01M Sodium (meta)periodate
Di-basic buffer	7.098g Sodium phosphate dibasic 50ml ddH_2O

Mono-basic buffer	6g Sodium phosphate dibasic 50ml ddH_2O
Phosphate buffer	75% Di-basic buffer 25% Mono-basic buffer
Tris-acetate-EDTA	242g Tris 57.1ml Acetic acid 100ml (0.5M) EDTA Fill up to 1l DEPC-treated water

Table 5. List of secondary antibodies or conjugates

2.3 Mice

C57BL/6 were purchased from Charles River (Sulzfeld, Germany) and bred in house, OT-II mice were kindly provided by Francis Carbone, Melbourne, Australia and were crossed with congenic C57BL/6 Thy1.1 mice. *Il12a*$^{-/-}$ (Mattner et al., 1996) *and* Yet40 reporter mice (Reinhardt et al., 2006) were kindly provided by Gottfried Alber. *Ccr7*$^{-/-}$ mice (Forster et al., 1999) were obtained from Martin Lipp and Reinhold Förster. *Yet40.Ccr7*$^{-/-}$ and *Il12a.Ccr7*$^{-/-}$ mice were bred in house. *Xcr1*-DTR-venus mice (Yamazaki et al., 2013) were kindly provided by Wolfgang Kastenmüller, and MHCII$^{-/-}$ mice (Madsen et al., 1999) were provided by Andreas Beilhack. All genetically-modified mice were on a C57BL/6 background. All mice were bred in our own animal facilities at Würzburg, kept under specific pathogen-free conditions, and used at an age of 6 to 12 weeks. All animal experiments were performed according to the German animal protection law as well as after approval and under control of the local authorities.

2.4 Primary cell techniques

2.4.1 Handling of cells

All procedures were performed under sterile conditions in class II biological safety cabinets (Thermo Scientific, Langenselbold, Germany) and using sterile plastic and glass ware. Cells were incubated in an incubator set at 37°C with 7 % CO2 atmosphere. Before use, culture medium was prewarmed to 37°C in a water bath. Centrifugation was performed at 1200 rpm for 5 minutes at RT unless indicated.

2.4.2 Counting cells

To determine cell concentration, 5 to 10 µl of a cell suspension was diluted in a trypan blue solution (Sigma-Aldrich, Deisenhofen, Germany) for visualization of non-viable cells

and counted by using a Neubauer counting chamber (Hartenstein, Wuerzburg, Germany) and an Axiostar plus microscope (with A-Plan objective 10x/0.25 Ph1, Carl Zeiss MicroImaging, Goettingen, Germany). Cell concentration was determined using the following formula: [(number live cells large quadrant 1 + number live cells opposing large quadrant 2) / 2] *dilution *10^4 = cells/ml

2.4.3 Generation of GM-CSF cell supernatant

Culture supernatant for BM-DC generation was obtained from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line kindly provided by B. Stockinger (London, UK). The GM-CSF transfected cell line was thawed according to standard procedure and left for 2 days in a T75 cell culture flask (Greiner Bio-One, Frickenhausen, Germany). Then, 107 cells were harvested and transferred to a T182 cell culture flask (Greiner Bio-One) in ca. 90ml complete RPMI 1640 (PAA, Pasching, Austria). After 3-4 days, cell line reached cell growth confluence of ca. 90% in T182 cell culture flask after which culture supernatant was harvested and centrifuged at 1000rpm for 10 minutes. Harvested culture supernatant was sterile-filtered and frozen at -20°C until usage in generation of BM-DCs.

2.4.4 Isolation of bone marrow (BM) cells

BM-DC were generated as previously described (Lutz et al., 1999). Briefly, hind limbs were removed from 6 to 12-week-old mice and bones were relieved from surrounding muscle tissue by rubbing with unsterile paper tissues. Then, intact bones were soaked for 1-2min in sterile 10cm petri dishes (#664102, Greiner Bio-One, Frickenhausen, Germany) filled with an ethanol-propanol solution (Terralin® liquid, Schülke & Mayr, Norderstedt, Germany) for disinfection. Remaining alcohol was evaporated by air. A minimal fraction of both ends of the tibiae or femurs was cut by scissors and bone marrow was flushed out with a PBS-filled sterile 10ml tuberculin syringe (Pentaferte, Campli, Italy) using a Neoject® 27G or 0.40mm diameter needle (Dispomed Witt, Gelnhausen, Germany). Bone marrow was washed once by centrifugation at 1000rpm for 10 minutes and clusters of the BM cell suspension was disrupted by vigorous pipetting. About 5 to 7 x 10^7 BM cells could be obtained from one mouse.

2.4.5 Generation of GM-CSF derived BM-DCs

At day 0, BM cells were seeded at 3 x 10^6 in sterile 10cm petri dishes (#664102, Greiner Bio-One, Frickenhausen, Germany) containing 10 ml complete RPMI 1640 medium

supplemented with 10% culture supernatant from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line. At day 3, another 10 ml complete RPMI 1640 medium containing 10% GM-CSF culture supernatant was added to the plates. At day 6, BM cells were fed by gently removing 10ml old RPMI 1640 medium and adding 10ml fresh complete RPMI 1640 medium containing 10% GM-CSF culture supernatant. The non-attached cells were harvested and used at day 8. Procedure typically yields 60-80% $CD11c^{high}$ expressing cells as determined by flow cytometry.

2.4.6 Generation of single cell suspension from spleen and lymph nodes

Skin-draining lymph nodes (popliteal, inguinal, and cervical) and spleens were isolated from 6-12-week-old mice under sterile conditions and transferred in 5cm petri dishes (Greiner Bio-One, Frickenhausen, Germany) containing ice-cold sterile PBS. Organs were cut into small pieces using forceps and digested for 20 min at RT with 1 mg/ml DNase I (Roche) and 1 mg/ml collagenase IV (Worthington) in DC preparation buffer. A single cell suspension was obtained by mashing spleen/ Lymph nodes with tuberculin syringe. Then, cell suspension was filtered through a 0.70µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) positioned on a 5cm petri plate. Cells were then washed by centrifugation at 1000rpm for 5 minutes at 25°C. For spleen cell suspension, an erythrocyte lysis was performed by resuspending cell pellet in a 1:1 solution of PBS and 1.67% ammonium chloride (NH_4Cl) buffer followed by incubation for 3 minutes in 37°C pre-warmed water bath. To remove ammonium chloride, splenocytes were washed by centrifugation at 1000rpm for 5 minutes at 1200rpm at 4°C prior to determination of cell count number as described in previous section.

2.5 Standard immunological/molecular techniques

2.5.1 Flow cytometry

2.5.1.1 Surface staining

Cells were stained in FACS buffer containing ice-cold PBS supplemented with 0.1% BSA and 0.1% sodium azide. To avoid unspecific antibody binding, cells were incubated with supernatant derived from the 2.4G2 hybridoma cell line (anti-Fc-gamma-RII/III; ATCC, Wesel, Germany). Typically, 5 x 10^5 to 10^6 cells were stained in 50µl FACS buffer supplemented with antibodies directed against surface markers in a particular dilution (see table) for 20-30 minutes at 4°C in the dark. To remove unbound antibodies, cells

were washed by centrifugation at 1200rpm for 5 minutes at 4°C. Samples were stored in 100µl of a 1:1 solution containing 1 part FACS buffer and 1 part 2% Formaldehyde until samples were acquired at a FACS LSR II flow cytometer (BD Biosciences, Heidelberg, Germany) provided with BD FACSDIVA™ Software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, USA).

2.5.1.2 Intracellular cytokine staining

For intracellular cytokine detection, cells were stained for surface markers as described prior to fixation in a 2% formaldehyde solution during 20-30 minutes at 4°C in the dark. Then, cells were washed with FACS buffer by centrifugation on 1200rpm for 5 minutes at 4°C followed by permeabilization in Perm Buffer for 30 minutes at 4°C in the dark. Staining of intracellular cytokines was performed using antibodies diluted in 50 µl Perm buffer for 30 minutes at 4°C in the dark followed by washing with Perm buffer to remove unbound antibodies and analysis on flow cytometer.

2.5.2 BM-DC labelling

Day 8 BM-DCs were labeled with either CFSE for *in vitro* studies, eFluor™ 670 Cell Proliferation Dye for injection into YET40 mice, or with CellTrace™ Violet Cell Proliferation Dye for other *in vivo* experiments. For CFSE labelling, cells were labelled by adjusting to $2*10^7$ cells/ml in PBS followed by CFSE addition in a final concentration of 3µM. Cells were labelled at room temperature for 10 min in a light-protected environment followed by extensive washing. For eFluor™ 670 labelling, cells were resuspended at 10^7 cells/ml and were resuspended in 10 ml PBS + 3.5 µl of eFluor™ 670. Cells were incubated in the dark for 15 minutes, 3 ml of complete RPMI 1640 (PAA, Pasching, Austria) was added to stop the reaction. Cells were then centrifuged at 1000rpm for 10 minutes and the cell pellet, which was blue indicative of successful staining, was resuspended in PBS at $4* 10^7$ cells/ml to be injected. For CTV labelling, cells were pelleted and resuspend at $1*10^7$ cells/ml in CTV stain solution (CTV 1:1000 in PBS). Cells were incubated for 6 minutes at room temperature, followed by adding 0.5 ml of FCS and 10 ml of complete RPMI 1640 (PAA, Pasching, Austria). Cells were incubated again in a water bath prewarmed to 37°C. Cells were then centrifuged at 1000rpm for 10 minutes and the cell pellet, which was blue indicative of successful staining, was resuspended in PBS at $4* 10^7$ cells/ml to be injected.

2.6 Specialized immunological techniques

2.6.1 BM-DC maturation

For *in vitro* experiments, BM-DCs were harvested at day 8 and re-plated at a density of 10^6 cells per ml in a 24-well plate (non-tissue culture treated; Greiner Bio-One, Frickenhausen, Germany). For maturation analysis by surface marker expression or cytokine production, BM-DC were cultured for 20-24 hours (unless otherwise indicated) in the presence of 0.5 µg/ml LPS (Sigma-Aldrich, Deisenhofen, Germany) or 5 nmol/ml CpG (Sigma-Aldrich, Deisenhofen, Germany). As a control cells were left untreated for the same period. For s.c. injections BM-DCs were harvested at day 8 and centrifuged at at 1000rpm for 10 minutes and the cell pellet was resuspended in the same 50 ml Falcon tube (Greiner Bio-One, Frickenhausen, Germany) in 5 ml of complete RPMI 1640 (PAA, Pasching, Austria) and 0.5 µg/ml LPS (Sigma-Aldrich, Deisenhofen, Germany) or 5 nmol/ml CpG (Sigma-Aldrich, Deisenhofen, Germany) were added. In case of additional injection of OT-II Thy1.1$^+$ cells, the BM-DCs were incubated with OVA-peptide$_{327\text{-}339}$ in addition to LPS or CpG. Cells were incubated at 37°C for 4 hours and were placed in a tilted position and shaken every 1 hour to prevent their attachment to the plastic. 45 ml of PBS were added to the cells and they were centrifuged at at 1000rpm for 10 minutes. This process was repeated twice to remove any remaining attached LPS. Cells were then labelled as described in the previous section.

2.6.2 BM-DC vaccination

LPS or CpG matured BM-DCs were injected into the footpad or into the flanks of mice. Popliteal or Inguinal lymph nodes were collected at the given time points, respectively. In case of adoptive OT-II.Thy1.1$^+$ T cell transfer, single cell suspensions of lymph nodes (pooled skin-draining and mesenteric lymph nodes) and spleens from OT-II.Thy1.1$^+$ mice were prepared as described above. Cells were labeled with CellTrace™ Violet Cell Proliferation dye (Sigma-Aldrich, Deisenhofen, Germany) and 1x10^7 cells were injected into the lateral tail vein of recipient mice. The cells were allowed to circulate in the mice for one day. On the next day, the OVA-peptide$_{327\text{-}339}$ loaded, LPS or CpG matured BM-DCs were injected into the footpad or into the flanks of mice, 2x10^6 cells were injected on each side. Popliteal or Inguinal lymph nodes were collected at the given time points, respectively.

2.6.3 Yet40 immunization by Complete Freund's adjuvant

Yet40 mice were injected s.c. on both flanks with 100µg of Complete Freund's adjuvant (CFA; Sigma-Aldrich, Deisenhofen, Germany) further enriched with 5mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, USA) by using Sterican® 23G or 0.60mm diameter needles (Braun, Melsungen, Germany) and a 1ml Injekt®-F syringe (Braun). Inguinal, cervical lymph nodes, and spleens were collected 24h after the injection and analyzed by flow cytometry.

2.6.4 Ex vivo stimulation and cytokine staining of cells from lymph nodes and spleen

Single cell suspensions from skin-draining lymph nodes or from spleen were prepared as described above and resuspended at a density of $2x10^6$ cells per ml in HL1 complete medium (Lonza, Verviers Belgium), this medium does not contain FCS to avoid unspecific T cell reactivation. Intracellular cytokine detection was performed by restimulation with 0.01µg/ml PMA (Sigma-Aldrich, Deisenhofen, Germany) and 1µg/ml ionomycin calcium salt (Sigma-Aldrich) in the presence of 5µg/ml Brefeldin A (from Penicillium brefeldianu, Sigma-Aldrich) followed by standard intracellular staining procedures as described in flow cytometry section.

2.6.5 Depletion of XCR1+ DCs in *Xcr1*-DTR-venus mice

Xcr1-DTR-venus mice have a human diphtheria toxin (DT) receptor (DTR) transduced into the *Xcr1* gene locus, followed by a fluorescent protein, venus, under the control of the *Xcr1* gene promoter (Yamazaki et al., 2013). Upon injecting DT, targeted deletion of *Xcr1* expressing cells is achieved. In our experimental setup, transgenic mice were treated with 0.5 µg diphtheria toxin (DT) i.p. (Sigma-Aldrich, Deisenhofen, Germany) on the same day when OT-II.Thy1.1$^+$ cells were injected i.v. (day -1). On the following day the same dose was injected together with 10^6 of OVA-peptide$_{327-339}$ loaded, LPS matured BM-DCs (day 0). On day 3 and day 5 0.25 µg of DT was injected. Popliteal lymph nodes were collected on day6 for subsequent experiments.

2.6.6 Detection of IL-12p40-YFP by immunofluorescence staining

For detection of IL-12p40-YFP by BM-DCs, day 8 BM-DCs that were further matured with LPS or left untreated, as described before, were used for Cytospins. Briefly, d 200ul of each cell suspension were added to a Cytospin slide chamber. The cytospins were centrifuged at 600rpm for 10 minutes using a Cytospin Universal centrifuge (Hettich,

Tuttlingen, Germany). Cytospins were fixed with 4% Paraformaldehyde (Sigma-Aldrich, Deisenhofen, Germany) for 15 minutes, washed twice with PBS. This was followed by blocking using 5% BSA for 30 minutes, the excess liquid was tipped off the slide. CD11c-APC (N418, Biolegend) was diluted in 1% BSA and the cytospins were stained overnight. Next day, slides were washed 3 times with PBS and drop of Fluoromount-G (Serva Electrophoresis) was added per slide. Samples were stored at 4 °C until images were acquired using LSM 780 confocal microscope (Carl Zeiss Microimaging). For imaging of lymph nodes, popliteal lymph nodes were fixed using the PLP fixation protocol. Briefly, fresh lymph nodes were directly immersed in PLP fixation buffer and were left to rotate at 4 °C overnight. Next day, they were washed with phosphate buffer three times for 5 minutes and passed through a Sucrose gradient. First using 10% Sucrose, followed by 20% then 30%. Lymph nodes were then washed once with phosphate buffer then placed in tissue-tek (Sakura) and kept at -80 °C till staining. Endogenous avidin and biotin in lymph node sections were blocked using Avidin/Biotin Blocking Kit (Vector Labs) and sections were stained with antibodies diluted in 2% FCS/PBS. Imaging was performed using LSM 780 (Carl Zeiss Microimaging). Quantitative image analysis was done using the Imaris software tools. The different labeled cells were localized using Imaris spot function and the relative distance was calculated using Excel software calculating the minimal distance in the X and Y planes.

2.7 RNA sequencing of lymph node DC subsets

Mice were injected with LPS/BM-DCs into the footpad and the popliteal and inguinal lymph nodes were collected after 48h. T and B cells were depleted by Dynabeads™ Biotin Binder (Invitrogen™) using Biotin-B220, CD3, and CD4 according to the manufacturer's protocol. The negatively selected cells were sorted for different migratory DC subsets using a BD FACS Aria III (precision: single-cell; nozzle: 100 µm). 100 cells for every population for in total 3 replicates were sorted into individual wells of a 96-well plate (Brand) filled with 4 µl lysis buffer (Takara). Cells were spun down, immediately chilled to 4°C and stored at −80°C. All the following experimental steps were performed using the SMART-Seq® v4 Ultra® Low Input RNA Kit (Takara) with a quarter of the recommended reagent volumes. The PCR amplification was performed according to the manual using 21 cycles. Libraries were quantified by QubitTM 3.0 Fluometer (ThermoFisher) and quality was checked using 2100 Bioanalyzer with High Sensitivity DNA kit (Agilent). 0.5

ng of each library was subjected to a tagmentation-based protocol (Nextera XT, Illumina) using a quarter of the recommended reagent volumes, 10 min for tagmentation at 55°C and 1 min extension time during PCR for multiplexing. After PCR, the libraries were purified using AMPure XP beads and eluted in 15 µl of resuspension buffer. Libraries were pooled and sequenced in paired-end mode on the NextSeq500 sequencer (IIIumina) using the Mid Output 2×75 cycle kit.

2.8 RNA sequencing data analysis

Base calling was done by the internal software of the NextSeq 500 sequencer "NextSeq Control/RTA v2" and bcl2fastq2 Conversion Software v.2 was used to demultiplex the pooled libraries and to convert the bcl files generated by the sequencer to standard fastq files for downstream analysis. The generated raw reads were processed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) for assessing read quality, number of duplicates and presence of adapter sequences. After this, the Illumina Nextera adaptors were cleaved using cutadapt (Martin, 2011) (version 1.16) and resulting reads were further trimmed keeping a quality drop value below a mean of Q20. Further, the processed sequences were mapped to the mouse genome using the short-read aligner STAR (Dobin et al., 2013) (version-2.5.2b) with genome and annotation files retrieved from GENCODE (July 2017, GRCh38.p5, M16). For all the studied samples, the proportion of reads uniquely mapped to the mouse reference genome ranged between 83% and 87% in total. The sequences aligning to specific genes were quantified using bedtools (Quinlan and Hall, 2010) subcommand intersect (version 2.15.0). Next, the differentially expressed genes were identified using DESeq2 (Love et al., 2014) (version 1.18.1). Only the genes having a Benjamini-Hochberg corrected p-value below 0.05 were classified as significantly differentially expressed (DEGs). The data were visualized as MA plot using DESeq2's function plotMA. The RNA-Seq data presented in this work has been deposited at the NCBI Gene Expression Omnibus (Edgar et al., 2002) and can be accessed through GEO series accession number GSE124677. For pathway analysis, the enrichment scores for pathways up or downregulated by endogenous migratory XCR1^{+} DCs was calculated using GOrilla analysis tool (Gene Ontology enRIchment anaLysis and visuaLizAtion tool). The data were plotted as FDR q-value after Benjamini and Hochberg correction of p-values. The threshold of significance was set at FDR q-value (0.05).

2.9 Quantitative real-time PCR

cDNA libraries generated by the SMART-Seq® v4 Ultra® Low Input RNA Kit from the sorted endogenous migratory DC subsets were used. Quantitative real-time PCR was performed on cDNA using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Reactions were run on a real-time quantitative PCR system (Roche, LightCycler® 96) and 18S was used as housekeeping gene. Relative expression differences were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Primer sequences were as follows:

Primer	**Sequence (5' to 3')**
18S Fwd	GTAACCCGTTGAACCCCATT
18S Rev	CGCTACTACCGATTGGATGG
Il12a Fwd	AGCTCCTCTCAGTGCCGGTC
Il12a Rev	GGTCTTCAGCAGGTTTCGGG
Il12b Fwd	AGCAGTAGCAGTTCCCCTGA
Il12b Rev	AGTCCCTTTGGTCCAGTGTG

Table 6. List of primer sequences used for Real-Time PCR.
All primer sequences were synthesized by Sigma-Aldrich (Deisenhofen, Germany) and oligo's were desalted and removed from truncated sequences by the manufacturer. (Fwd = forward primer, Rev = reverse primer)

2.10 Statistical analysis

Statistical analyses were performed using Prism 6.0 software (GraphPad Prism). The unpaired, two-tailed Student's t-test was used, if data sets of two independent groups were normally distributed. The Wilcoxon signed-rank test was performed to analyze the relative distances between cells in lymph node sections generated from the Imaris software tool.

3 RESULTS

3.1 XCR1^{+} CD103^{+} migratory DCs are the major source of IL-12 production in draining lymph nodes in a DC vaccination setup

3.1.1 YFP production by Yet40 mice is equivalent to IL-12p40 by in vitro generated BM-DCs

We first wanted to test whether the production of YFP from the Yet 40 mice was a reliable measure of IL-12p40 production by DCs. To test that, we generated BM-DCs from Yet40 mice using 10% GM-CSF and the cells were treated at day 8 with 100ng/ml LPS and left in culture for another 24h or left without stimulation as a control. Likewise, cells that were generated from C57BL/6 mice were stimulated with the same dose of LPS or left untreated. Following that the cells were stained for CD11c as a classical marker for DCs and MHC II as a marker for their maturation (Fig 3A). YFP was detected in the FITC channel without any further staining and the C57BL/6 cells were stained for IL-12p40 production intracellularly. The cells were analyzed by flow cytometry and the production of YFP by the Yet40-BM-DCs was observed after 24h LPS-stimulation compared to untreated controls (Fig. 3B), which was of comparable levels to the IL-12p40 production by the C57BL/6 WT cells (Fig. 3C). The YFP production was also observed only after LPS treatment using immunofluorescence microscopy (Fig 3D). Thus, we decided to use the Yet40 mice for the analysis of endogenous IL-1240 production by the different DC subsets in the draining LNs and in spleen.

3.1.2 Endogenous migratory dendritic cells are the major producers of IL-12p40 in the draining lymph nodes at steady state

Next, we analyzed the popliteal, inguinal, auxiliary, cervical skin draining lymph nodes and the gut-draining mesenteric lymph node for production of IL-12p40. Using the same gating strategy, we found that only the CD11c^{int} MHC II^{high} cells which are the migratory DCs were positive for YFP production at steady-state conditions in the skin draining and in mesenteric lymph nodes. The CD11c^{high} MHC II^{int} resident DCs, CD11c^{int} MHC II^{low} macrophages, CD11c^{-} MHC II^{high} B cells, and the cells negative for both markers which

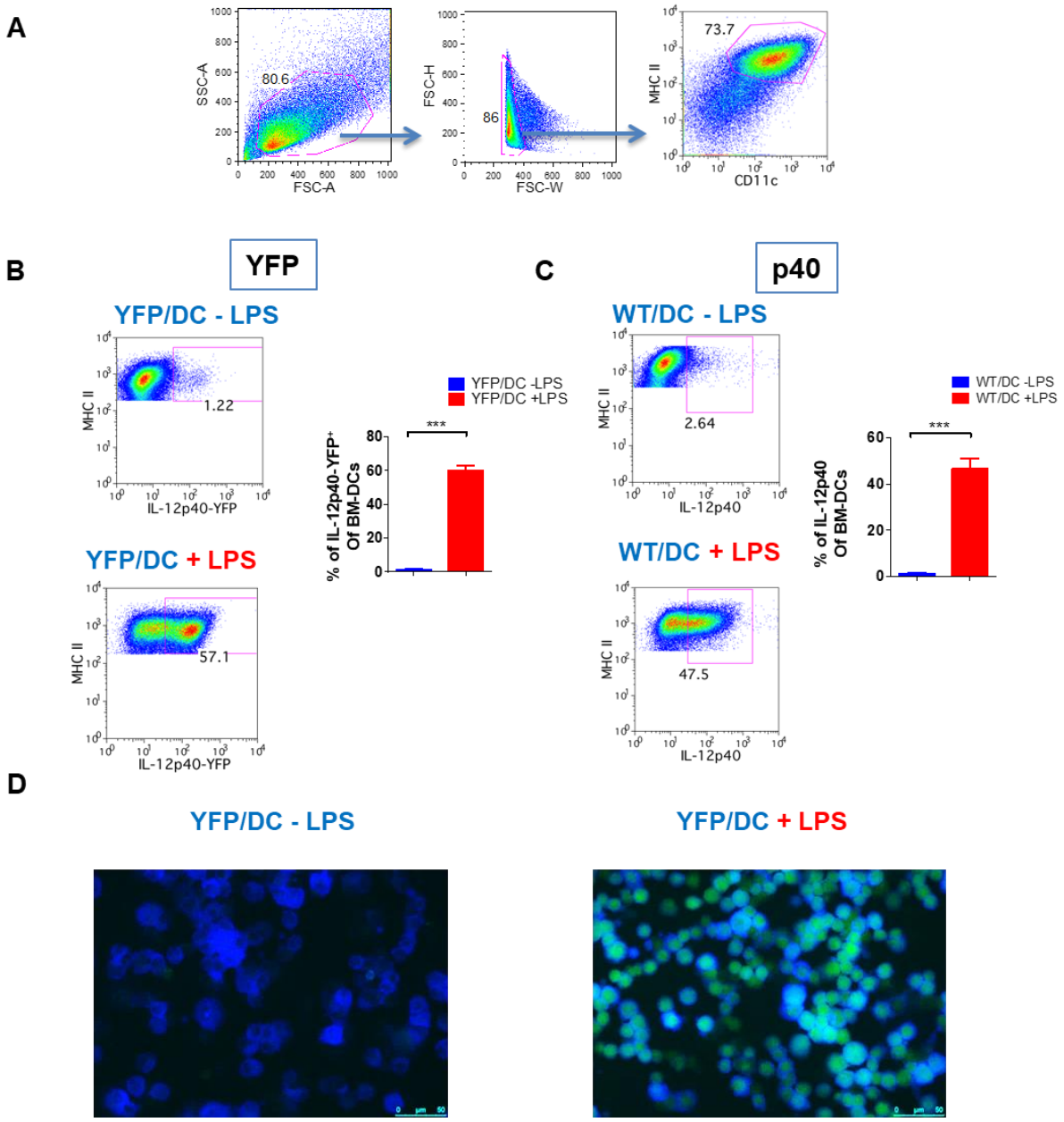


Figure 3 : YFP production by Yet40 mice is comparable to p40 intracellular staining. **A.** Representative flow cytometry plots of generated BM-DCs on day 8 from C57BL/6 or from Yet40 mice. Gated on live cells followed by doublet exclusion, then gated on CD11c^{+} MHC II^{high} DCs **B,C.** Representative flow cytometry plots (left) and graphs comparing % of cells producing (right) IL-12p40-YFP **(B)** or IL-12p40 intracellularly (right) **(C)** by BM-DCs treated for 24h + or - LPS 0.5µg/ml. **D.** Confocal microscopy images of CD11c+ (blue) untreated BM-DCs (left) or LPS treated DCs (right) to detect YFP production (green). Data are representative of three independent experiments. ***P < 0.0001, **p < 0.001, *p < 0.05.

are the T cells and other cells in the lymph nodes were all negative for YFP production (Fig 4A). To confirm that these are indeed migratory cells, the same populations were analyzed in a Yet40.*Ccr7*$^{-/-}$ mouse. In such mice, only a minor population of CD11c^{int} MHC II^{high} population was present. This population showed a small fraction of IL-12p40-YFP positive cells (Fig 4B). Interestingly, in the spleen the same CD11c^{int} MHC II^{high} population

was also positive for YFP production in YFP.*Ccr7*$^{+/+}$ mice and it was still present in the Yet40.*Ccr7*$^{-/-}$ mice (Fig 4C), indicating that this subset is not of migratory origin.

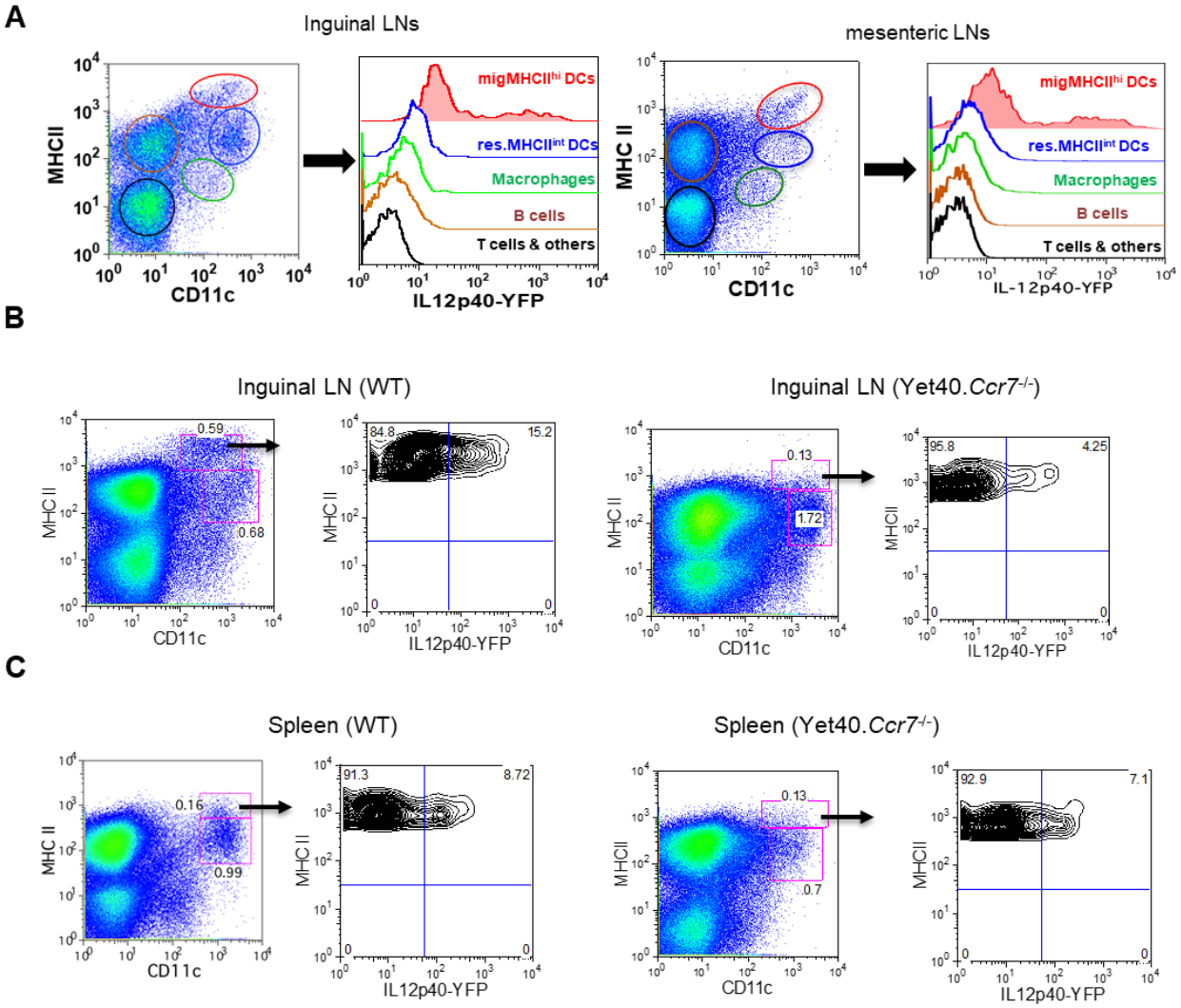


Figure 4 : YFP production at steady-state in skin draining LNs and in spleen **A.** Representative flow cytometry plots of inguinal lymph node (left) and mesenteric lymph node (right) subpopulations gated based on their expression of CD11c and MHC II in a Yet40 reporter mouse. Right side of the black arrows are histogram plots of IL-12p40-YFP production by each subpopulation. **B, C.** Representative flow cytometry plots of inguinal lymph node **B.** or spleen **C.** subpopulations gated based on their expression of CD11c and MHC II in a Yet40 reporter mouse or a Yet40.*Ccr7*$^{-/-}$ mouse. Right side of the black arrows are plots showing % of cells producing IL-12p40-YFP from the CD11c^{int} MHC II^{high} subpopulation

3.1.3 XCR1^{+} CD103^{+} Langerin^{+} dermal DCs are the major subset producing steady-state IL-12p40-YFP in draining lymph nodes

Migratory DCs subsets in the skin draining lymph nodes are comprised of three subsets: 1) Langerin^{+} CD103^{-} CD11b^{-} Langerhans cells that migrate from the epidermis. 2) Langerin^{+} CD103^{+} CD11b^{-} dermal cDC1s (dDCs) and 3) Langerin^{-} CD103^{-} CD11b^{+} dermal cDC2s. We checked for the production of IL-12p40-YFP by the three different

subsets. The Langerin^{+} CD103^{+} XCR1^{+} CD11b^{-} dermal DCs were the major YFP producers (Fig 5A), in line with what Reinhardt and colleagues found out (Reinhardt et al., 2006). That was also the case in the mesenteric lymph nodes, while the CD103 CD11b double positive population that was mainly implicated in tolerogenic functions did not produce any YFP (Fig 5B). In the spleen the CD11c^{int} MHC II^{high} YFP producing population that we identified were of the CD8^{+} CD11b^{-} subset (Fig5 3C).

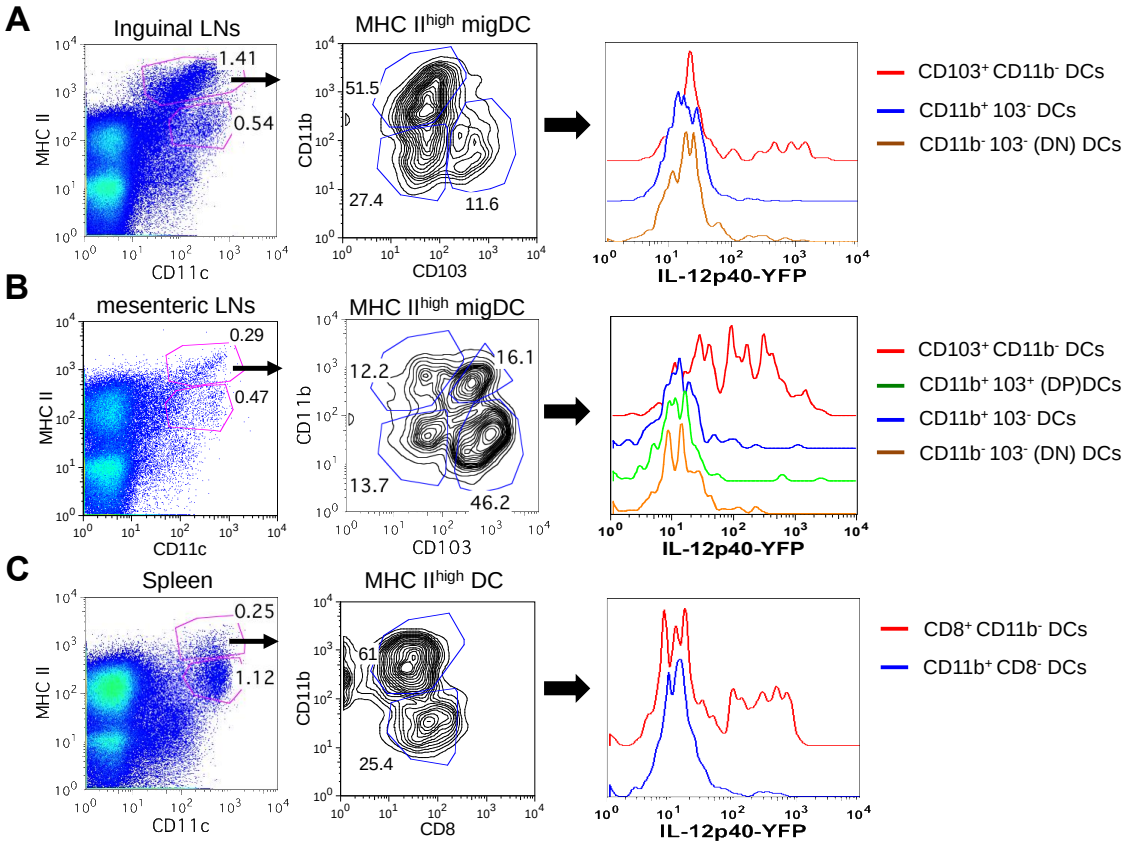


Figure 5 : YFP production by different DC subsets in LNs and in spleen **A, B, C.** Representative flow cytometry plots of inguinal lymph node **A.** mesenteric lymph node **B.** and spleen **C.** subpopulations gated based on their expression of CD11c and MHC II in a Yet40 reporter mouse (Left panels). CD11c^{int} MHC II^{high} cells were sub-gated based on CD103 (CD8a for spleen) and CD11b expression (Middle panels). Histogram plots of IL-12p40-YFP production by subpopulations based on CD103 (CD8a for spleen) and CD11b expression are shown (right panel)

3.1.4 CFA injection augments IL-12p40-YFP production by CD103^{+} dDCs in the skin draining lymph nodes

Since IL-12 is the most important cytokine for polarizing T cells towards a T helper 1 response (Macatonia et al., 1995), we tested for IL-12p40-YFP production *in vivo* upon CFA injection which skews the T cell response towards a Th1 phenotype (Shibaki and Katz, 2002). The different DC subsets from the draining inguinal LN were analyzed 24h

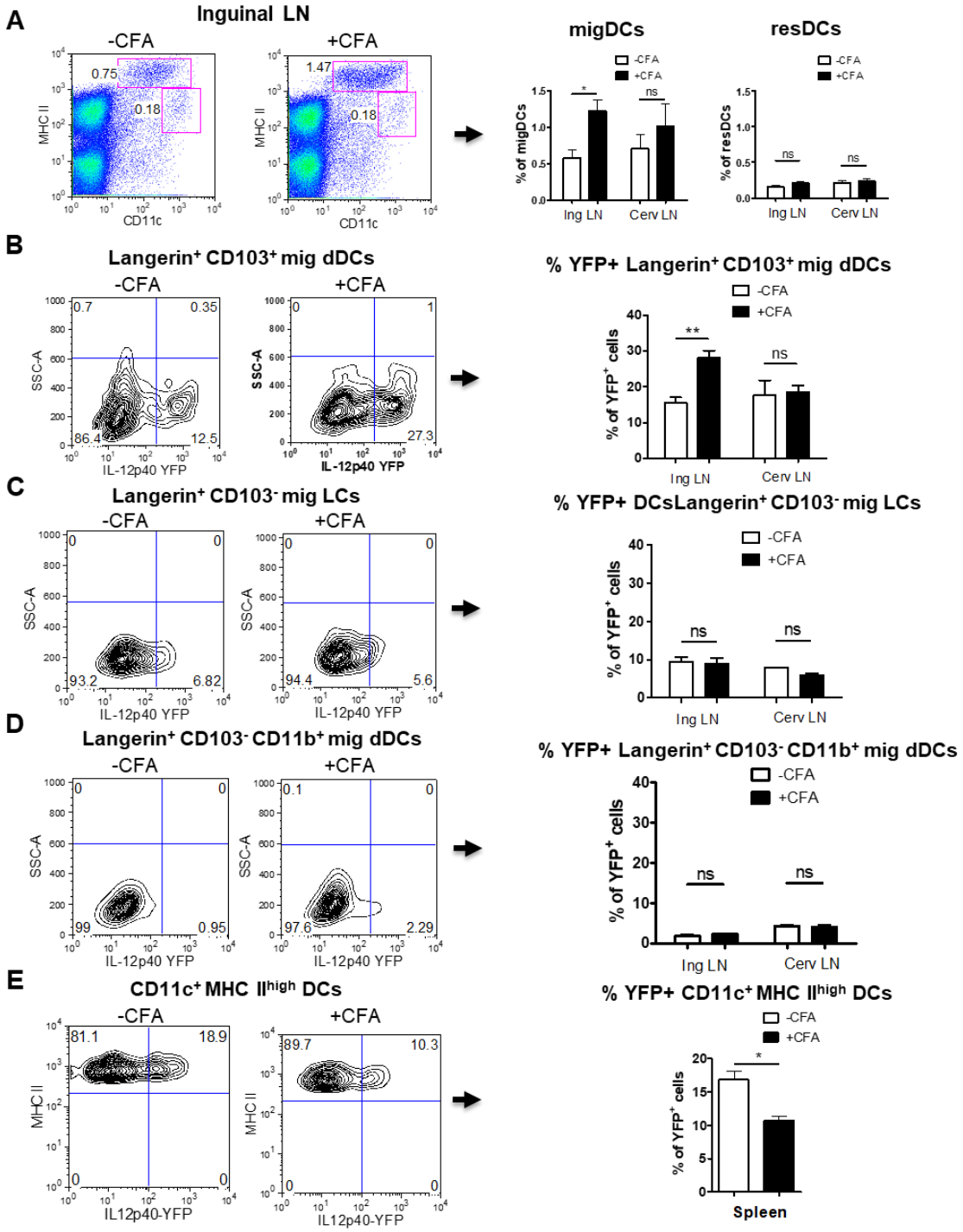


Figure 6 : CD103^{+} migratory dDCs are the major IL-12p40-YFP producers after CFA injection **A.** Representative flow cytometry plots of inguinal lymph nodes subpopulations gated based on their expression of CD11c and MHC II (left) and graphs showing % of CD11c^{int} MHC II^{high} migratory DCs and CD11c^{high} MHC II^{int} LN resident DCs in a Yet40 reporter mouse before and 24h after 50 µl CFA s.c. injection. **B,C,D,E.** Representative flow cytometry plots (left) and bar graphs (right) of % of cells producing IL-12p40-YFP from CD103^{+} mig dDCs **B.** LCs **C.** CD11b^{+} mig DCs **D.** in inguinal or cervical LNs, and CD11c^{+} MHC II^{high} DCs in spleen **E.** before and after 50 µl CFA s.c. injection. Data are representative of three independent experiments. **$p < 0.001$, *$p < 0.05$.

after CFA injection s.c. in the flank of the mouse, and cervical LNs were used as a non-draining LN control. The spleen subsets were also analyzed for any systemic response.

We observed an increase in CD11c^{int} MHC II^{high} migratory DC population indicating their increased mobilization from the skin in response to CFA injection, this was not observed by resident DCs and not in cervical LNs (Fig 6A). The CD11c^{int} MHC II^{high} Langerin^{+} CD103^{+} migratory dDCs were the major subset that increased YFP production after CFA injection (Fig 6B). This was only the case in the inguinal lymph node in comparison to the non-draining cervical LN (Fig 6B). The Langerin^{+} CD103^{-} migratory LCs and Langerin^{-} CD103^{-} CD11b^{-} migratory dDCs showed no such increase both in inguinal and cervical LNs (Fig 6C, D). Interestingly, in the spleen we noticed that the % of IL-12p40-YFP producing population was reduced (Fig 6E), this might indicate that this CCR7-independent population in the spleen is rather tolerogenic and is reduced under inflammatory conditions.

3.1.5 LPS-matured BM-DC injection enhances IL-12p40-YFP production by CD103^{+} dDCs in a time dependent fashion

The fact that CD103^{+} dDCs were the major producers of IL-12p40 after CFA injection prompted us to test for IL-12p40 production after BM-DC injection where they are assumed to provide all the three signals required for Th1 induction; including IL-12 production (Elster et al., 2016). BM-DCs that were stimulated with LPS for 4h and labeled with eFluor670 to distinguish them from endogenous DC subsets (Fig 7A) were injected s.c. in the flank and the YFP production was analyzed after 24h, 48h, or 72h from draining inguinal LNs. The spleen was analyzed also for any systemic response. LN cellularity was significantly increase compared to CFA injection (Fig 7B) and all migratory DC subsets increased in their migration compared to CFA (Fig 7C, D, E). YFP production was significantly increased only from CD103^{+} migratory dDCs over the three time points, and the production peaked at 72h (Fig 7B). The CD11b^{+} migratory and resident DCs did not increase their YFP production in contrast to stimulation of LN cells with LPS *in vitro*, indicating no direct effect of LPS attached to the injected BM-DCs on the endogenous DC subset of the recipient mice (Fig 7C). The Langerin^{+} CD103^{-} LCs also showed no significant increase in YFP production (Fig 7D). Interestingly, unlike what we previously observed (Voigtlander et al., 2006), YFP production was observed from the injected BM-DCs over the indicated time points (Fig 8A). One possible explanation is that the YFP half-life is longer than IL-12p40 (Lorang et al., 2001), and thus the YFP signal observed

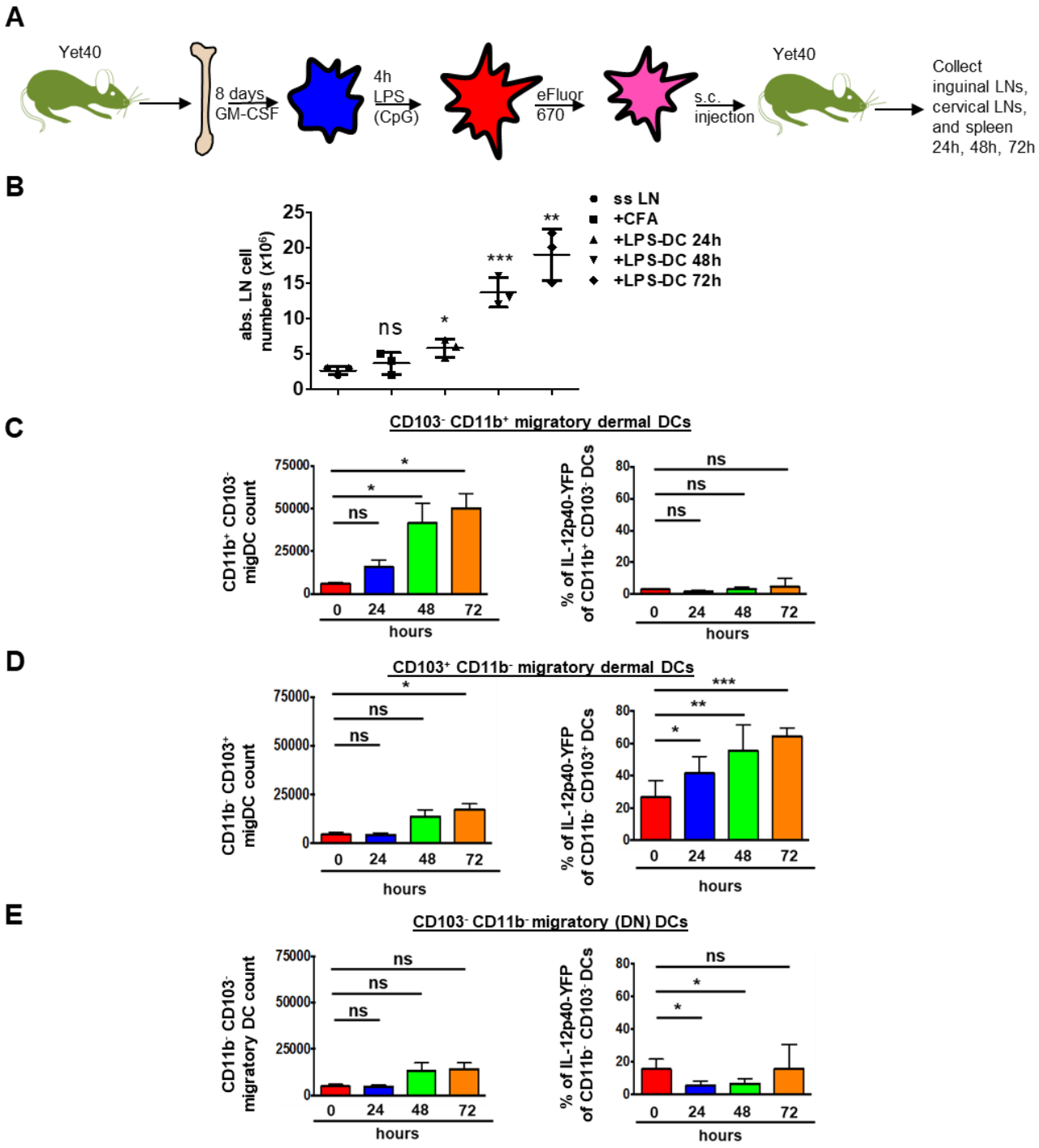


Figure 7 : CD103⁺ migratory dDCs are the major IL-12p40-YFP producers after LPS-matured BM-DC injection **A.** Inguinal LNs, cervical LNs and spleen were analyzed after LPS (0.5 µg/ml)-matured, eFlour670 labeled.BM-DC s.c. flank injection **B.** Graph showing inguinal LN cell count before or 24h after CFA injection compared to 24h, 48h, 72h, after LPS.BM-DC injection **C,D,E.** Graphs showing absolute counts of migratory CD11c⁺ MHC II^{high} DCs (left panels) and % of IL-12p40-YFP producing cells from CD11b⁺ mig dDCs **C.** CD103⁺ mig dDCs **D.** and DN mig DCs **E.** (right panels) after s.c. injection of WT.LPS/DC into *Yet40* recipient mice (24, 48, 72h timepoints). Data are representative of three independent experiments. ***P < 0.0001, **p < 0.001, *p < 0.05.

from BM-DCs is residual from the LPS stimulation before injection. The injected-BM-DCs did not reach the cervical LNs (Fig 8B), nor did they reach the spleen (Fig 8C) since no

eFluor670-labeled cells were observed. This indicates that the migration of the injected BM-DCs is limited to the draining LN nearest to the injection site.

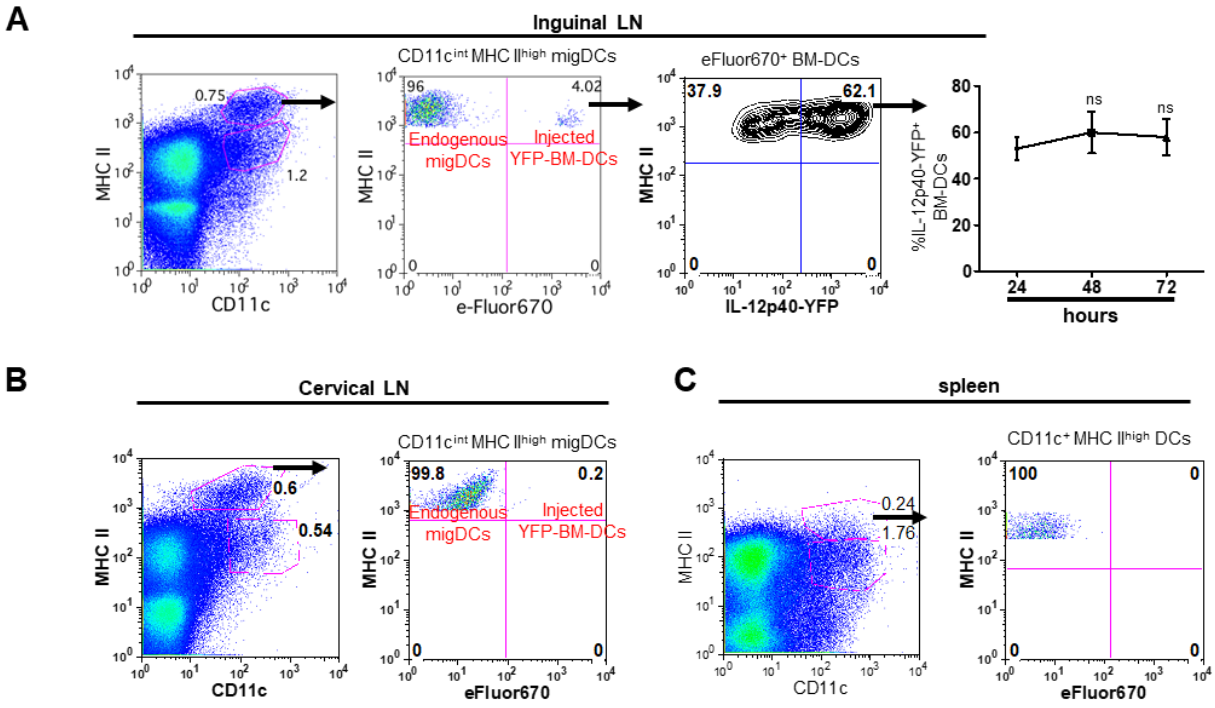


Figure 8 : Injected BM-DCs producing residual IL-12p40-YFP are restricted to the draining LN closest to the injection site **A.** Representative flow cytometry plots of inguinal lymph node subpopulations gated on CD11c and MHC II in a Yet40 reporter mouse (Left), CD11c^{int} MHC II^{high} migDCs are either endogenous (eFluor670^{-}) or injected BM-DCs (eFluor670^{+}) (middle), Graph showing % of IL-12p40 YFP producing cells in the eFluor670^{+} BM-DC injected gate 24h, 48h, 72h, after s.c. flank injection in inguinal LN (right). **B, C.** Representative flow cytometry plots of inguinal lymph node subpopulations gated on CD11c and MHC II in a Yet40 reporter (left) then sub gated on either: CD11c^{int} MHC II^{high} migDCs in **(B)** control cervical LN or CD11c^{+} MHC II^{high} DCs in **(C)** spleen for eFluor670^{+} cells (right). Data are representative of three independent experiments. ns= non-significant.

3.1.6 Monocyte-derived DCs do not produce IL-12p40-YFP after LPS-BM-DC injection

Previous studies have suggested that the major source of IL-12 production after CpG-adjuvanted vaccine injection are monocyte-derived DCs (MoDCs) that migrate to lymph nodes in a CCR2-dependent fashion (De Koker et al., 2017). Using the same setup described in Fig 8A, we analyzed the CD11c^{int} MHC II^{high} migratory and CD11c^{high} MHC II^{int} resident DC subsets in the skin draining LNs for the development of MoDCs and for their production of IL-12p40-YFP. MoDCs were defined as CD11c^{int} MHC II^{high} CD11b^{+} CD103^{-} CD64^{+} and they down-regulate Ly6C expression (Zigmond et al., 2012) (Fig 9A).

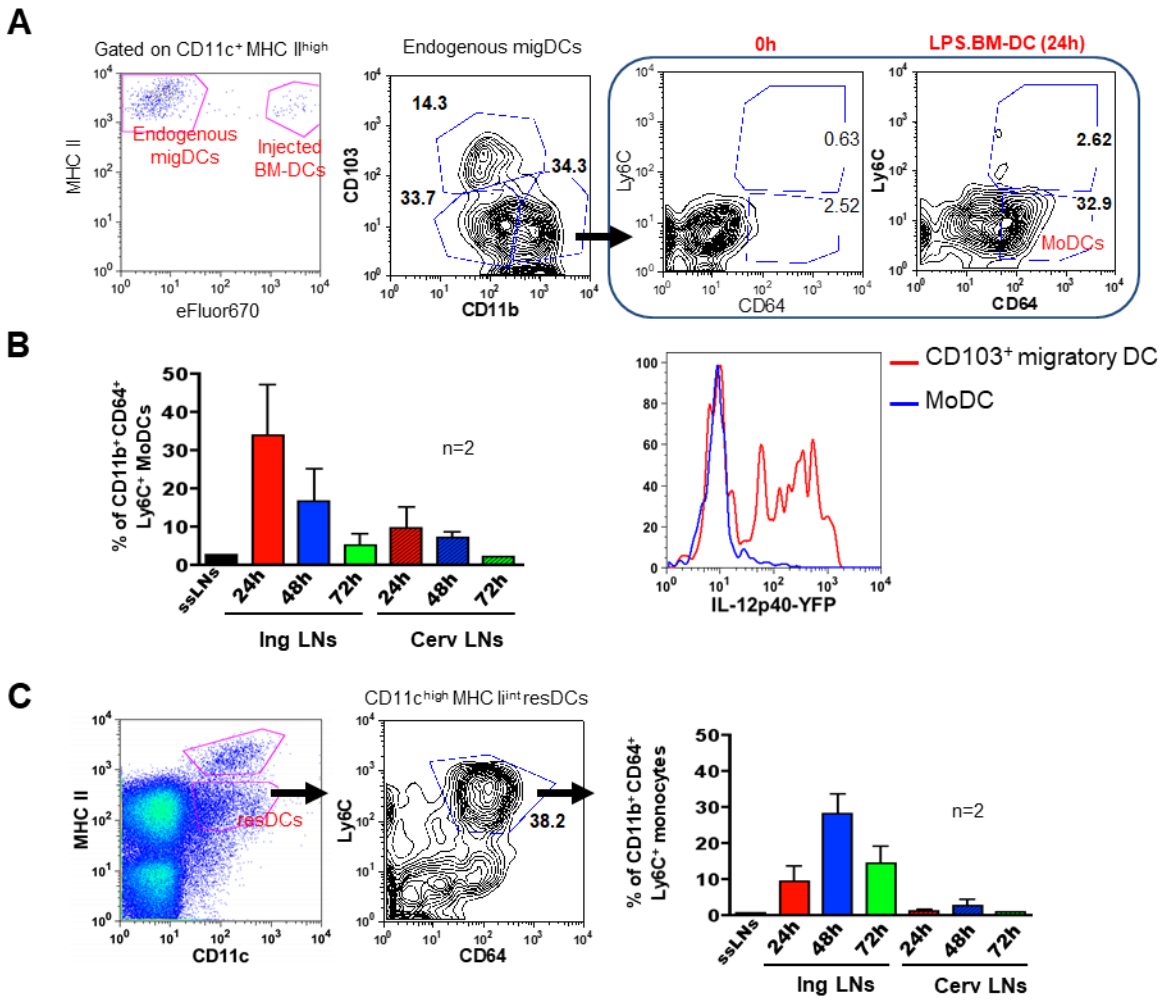


Figure 9 : moDCs are not the source of IL-12p40-YFP after LPS-matured BM-DC injection **A.** Gating strategy for MoDCs in inguinal LN where CD11c^{+} MHC II^{high} eFluor670^{-} endogenous migDCs (left) were sub gated based on CD103 and CD11b (middle). MoDCs are CD11b^{+} CD103^{-} CD64^{+} and Ly6C^{-} and appear only after LPS. BM-DC s.c. injection (right) **B.** Graph showing % of IL-12p40 YFP^{+} cells within the MoDCs in inguinal LN compared to cervical LN at steady state compared to 24h, 48h, 72h after LPS. BM-DC injection (left) and flow cytometry showing IL-12p40 YFP^{+} cells within the MoDC gate (right). **C.** Gating strategy of CD11c^{+} MHC II^{int} Ly6C^{+} CD64^{+} monocytes that appear after s.c. BM-DC injection (left), and graph showing their % in inguinal LN compared to cervical LN at steady state compared to 24h, 48h, 72h after LPS. BM-DC injection. Data are representative of two independent experiments.

They appeared in the CD11c^{int} MHC II^{high} migratory compartment in the 24h and 48h time points post-BM-DC injection and started disappearing in the 72h time point (Fig 9B). No IL-12p40-YFP production was observed by MoDCs (Fig 9B), indicating the difference in the source of IL-12 in our BM-DC injection setup from CpG immunizations. Also, within the CD11c^{high} MHC II^{int} resident gate, a monocytic population that was CD11b^{+} CD103^{-} CD64^{+} Ly6C^{+} appeared in the inguinal LNs next to the injection site, and not in the non-draining cervical LNs. The presence of this population peaked at 48h then declined again at 72h but no IL-12p40-YFP production was observed (Fig 9C).

3.1.7 Increased production of IL-12p40-YFP by XCR1^{+} CD103^{+} dDCs is similar for LPS- or CPG- matured BM-DCs

Residual LPS remaining on the surface of the injected BM-DCs might be responsible for the increased IL-12p40-YFP production by XCR1^{+} CD103^{+} dDCs (Schwarz et al., 2014).

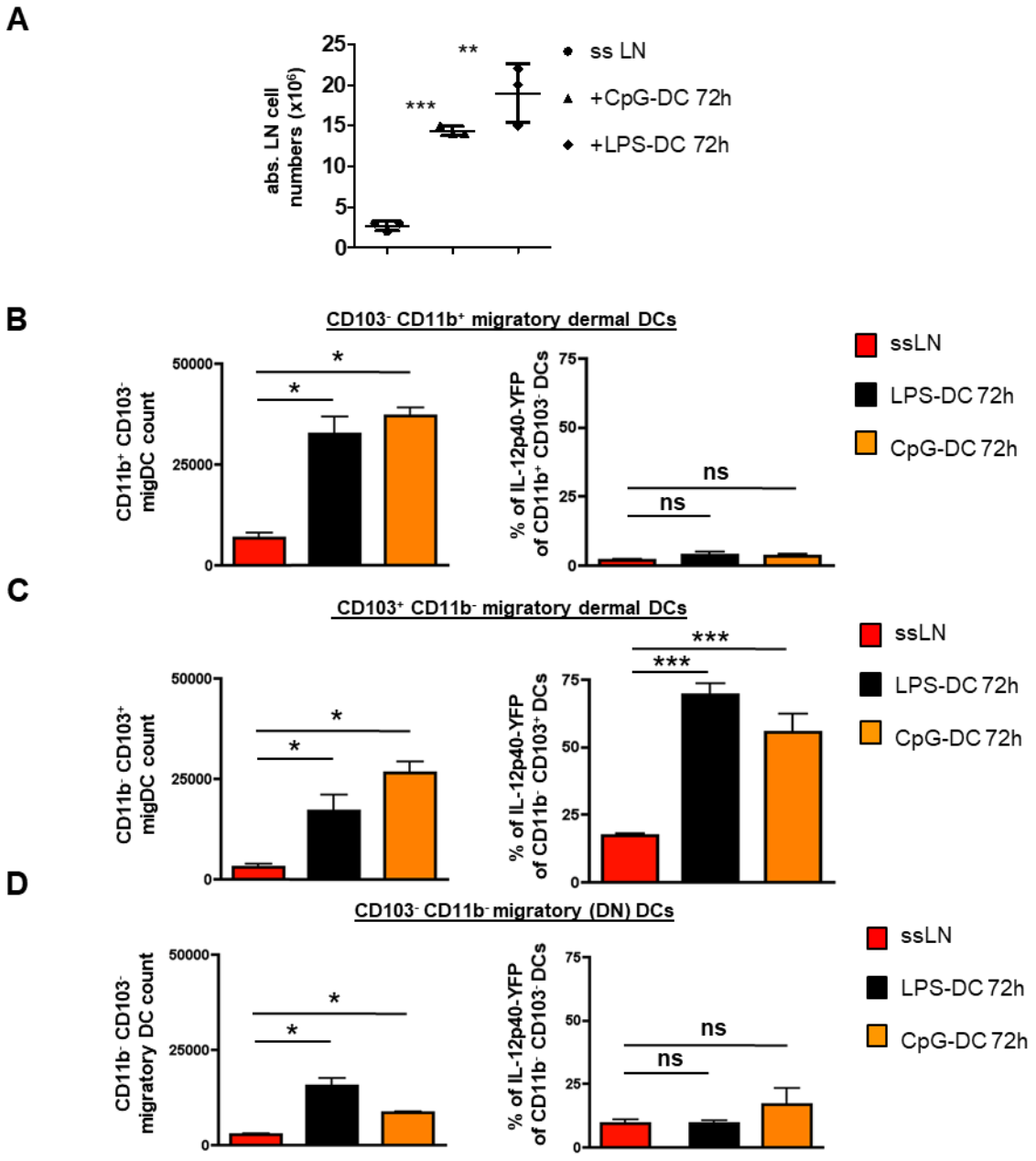


Figure 10 CpG matured BM-DCs induce IL-12p40 YFP production by CD103^{+} migratory dDCs similar to LPS matured BM-DCs **A.** Graph showing inguinal LN cell count at steady state, 72h after CpG.BM-DC s.c. injection, or 72h after LPS.BM-DC s.c. injection **B,C,D.** Graphs showing absolute counts of migratory CD11c^{+} MHC II^{high} DCs (left panels) and % of IL-12p40-YFP producing cells from CD11b^{+} mig dDCs **C.** CD103^{+} mig dDCs **D.** and DN mig DCs **E.** (right panels) after s.c. injection of LPS.BM-DC or CpG.BM-DC into *Yet40* recipient mice (72h). Data are representative of three independent experiments. ***P < 0.0001, **p < 0.001, *p < 0.05.

To rule out that this is the only possibility in our system, we tested the YFP production at 72h by endogenous DC lymph node subsets after the injection of eFluor670-labeled CpG-matured BM-DCs. Unlike the surface Toll-like receptor 4 that recognizes LPS, CpG acts on the intracellular Toll-like receptor 9 thus test for the surface LPS problem. Similar to LPS-BM-DC injection, LN cellularity significantly increased in a similar fashion as the 72h timepoint after LPS-BM-DC injection (Fig 10A). The migration of all CD11c^{int} MHCII^{high} DC subsets was increased, while IL-12p40-YFP production was significantly induced only by the CD103^{+} migratory dDCs (Fig 10B, C, D). This indicates that LPS attached to the injected BM-DCs is not the only mechanism that induces IL-12p40-YFP production by CD103^{+} migratory dDCs.

3.1.8 IL-12p35 subunit and IL-12p70 was not detectable by flow cytometry

IL-12p40-YFP production was observed at steady state from CD103^{+} XCR1^{+} migratory dDCs, CD103 CD11b double positive migratory DCs in skin-draining and mesenteric LNs respectively, and from CD11c^{int} MHCII^{high} CD8^{+} DCs in the spleen. And as mentioned

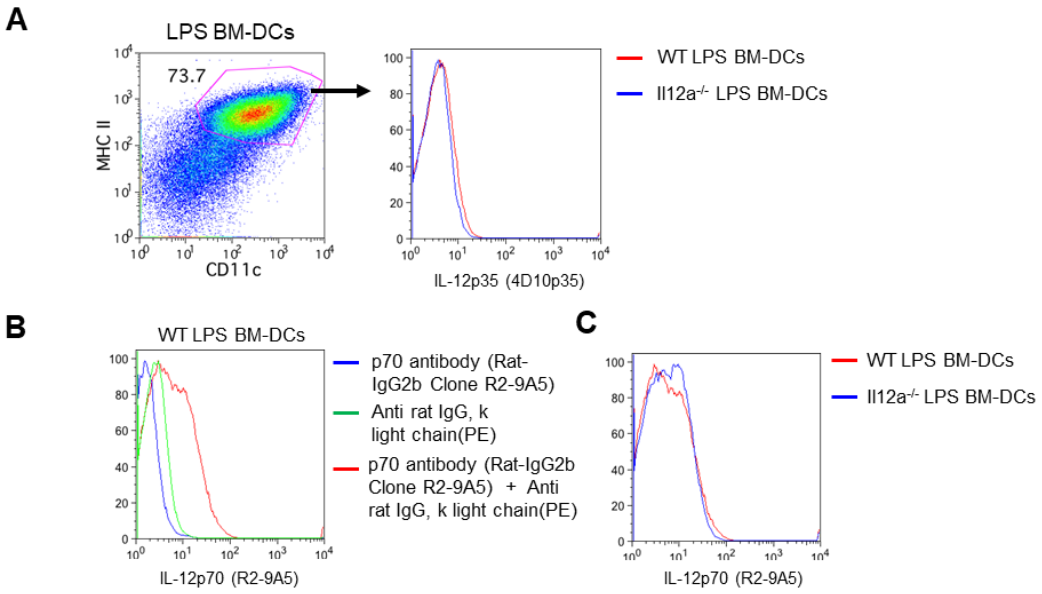


Figure 11 : Flow cytometry staining of IL-12p35 and IL-12p70 proved difficult **A.** Representative flow cytometry plot of day 8 BM-DCs sub gated on CD11c^{+} MHC II^{high} mature DCs. Right side of the black arrow is a histogram plot of IL-12p35 production (clone 4D10p35) by WT LPS. BM-DCs compared to Il12a$^{-/-}$ LPS.BM-DCs. **B.** Histogram plot for IL-12p70 production (clone R2-9A5) gated on CD11c^{+} MHC II^{high} LPS-matured BM-DCs **C.** Histogram plot of IL-12p70 production (clone R2-9A5) by WT LPS. BM-DCs compared to Il12a$^{-/-}$ LPS.BM-DCs.

before, studies have shown that the p40 molecule can form homodimers that acts as an IL-12 antagonist (Gillessen et al., 1995). The p35 subunit (*Il12a*) of the Th1 functional IL-12p70 molecule proved difficult to detect by flow cytometry from *in vitro* LPS-matured BM-

DCs (Fig 11A). And while the staining with the IL-12p70 (rat-IgG2b clone R2-9A5) antibody appeared to be positive on LPS-matured BM-DCs (Fig 11B), the same positive population appeared in LPS-matured BM-DCs from *Il12a*$^{-/-}$ mice (Fig 11C). This indicated that this antibody was nonspecifically binding to the IL-12p40 molecule. Therefore, we decided to switch to a system where we can study the dynamics of Th1 induction by a transgenic T cell population and monitor the dependency of such a response on IL-12 production from either the injected BM-DCs or the endogenous migratory and resident DC subsets.

3.2 XCR1$^+$ CD103$^+$ migratory DCs and not vaccine DC are the source of IL-12 for Th1 polarization and partly for antigen presentation

3.2.1 LPS-matured antigen-loaded BM-DCs induce Th1 responses by CD4$^+$ T cells in draining lymph nodes and in spleen

To study the dynamics of IL-12p70 requirement for Th1 induction after BM-DC injections, we first injected T cells intravenously into C57BL/6 WT mice that have a transgenic TCR which only recognizes the OVA peptide from chicken egg white presented on MHC II molecules on APCs (Barnden et al., 1998). The OT-II cells also carried the congenic Thy1.1 (CD90.1) marker to be traceable in C57BL/6 WT mice with Thy1.2 background. Next day, OVA-loaded, LPS-matured BM-DCs (OVA-LPS/BM-DC) were injected s.c. in the flank or in the footpad and the inguinal or popliteal LNs were collected respectively as the closest draining LNs on day 6. Spleens were also collected to analyze the systemic T cell response (Fig 12A). Th1 polarization was tested by analyzing the lymphoblast gate for the presence of CD4$^+$ OT-II. Thy1.1 T cells. These cells were analyzed for the production of IL-2, IFN-γ and IL-13 by intracellular staining (Fig 12B). We first observed that an effective T cell response required the injection of at least 10^6 BM-DCs per flank. Injecting $2*10^5$ BM-DCs caused a significant increase in LN cellularity but were not enough to induce antigen specific T cell expansion (Fig 12C) as indicated by CD4$^+$ OT-II. Thy1.1 T at day 6 which was similar to injecting T cells and no BM-DCs and by their minimal IL-2 production (Fig 12C). This was also the case after double injections of $2*10^5$ BM-DCs (Fig 12C). Upon injecting 10^6 BM-DCs per flank, we noticed a significant increase in LN cellularity and in CD4$^+$ OT-II. Thy1.1 expansion in the skin draining LNs

after 6 days when compared to injecting OT-II. Thy1.1 cells only, where the injected population dies out at day 6 (Fig 12C).

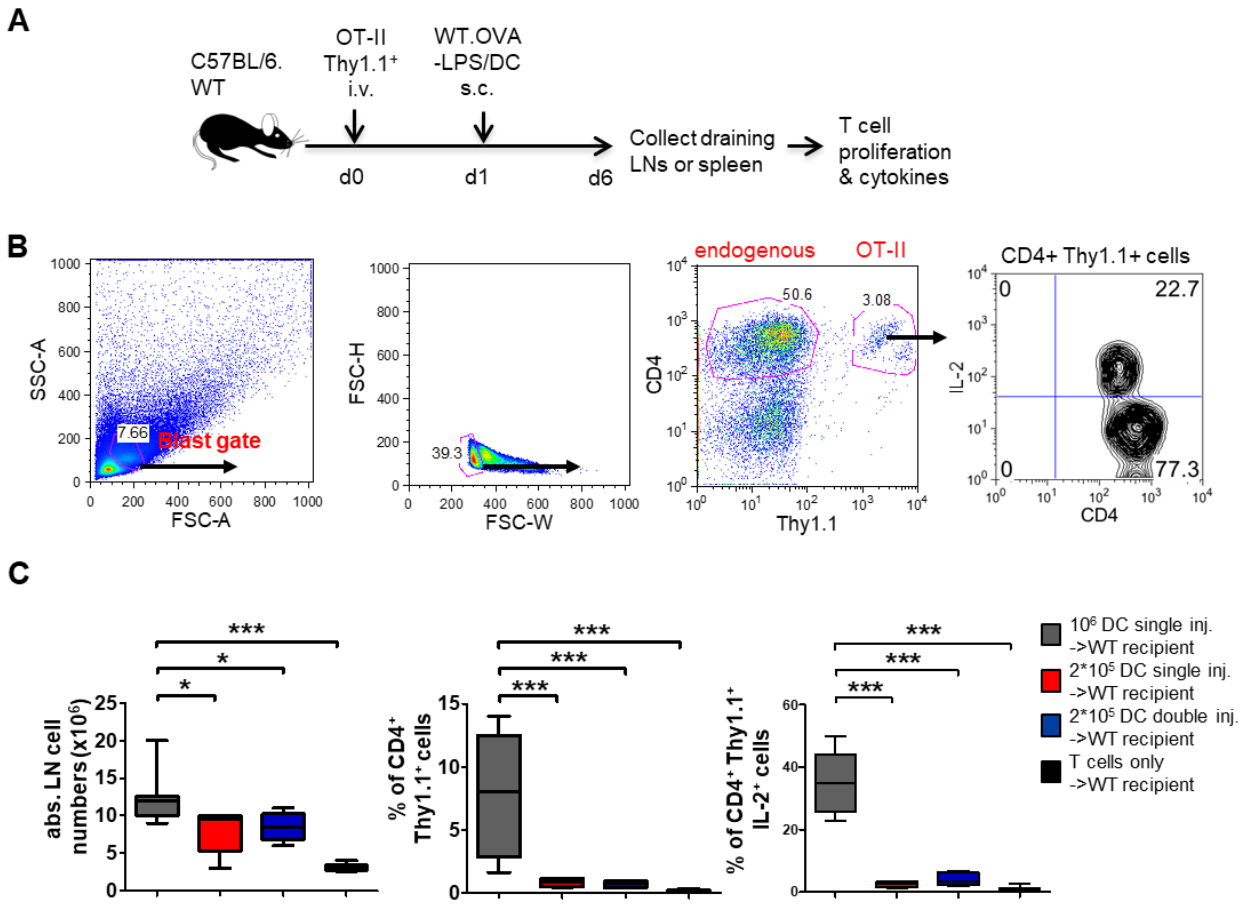


Figure 12 : LPS-OVA/BM-DCs induce CD4$^+$ OT-II Thy1.1$^+$ T cell expansion in draining lymph node **A.** OT-II$^+$.Thy1.1$^+$ T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of OVA (10 µM)-loaded, LPS (0.5 µg/ml)-matured BM-DC (OVA-LPS/DC) s.c. footpad or flank injection. **B.** Gating strategy of CD4$^+$ OT-II Thy1.1$^+$ T cells, gated on live blasts followed by doublet exclusion, then gated on CD4$^+$ Thy1.1$^+$ T cells, IL-2 producing CD4$^+$ Thy1.1$^+$ T cells are shown on the right. **C.** Graphs comparing lymph node cell counts (left), frequency of injected OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells (middle) and percentage of IL-2 producing cells (right) after s.c. injection of 10^6 OVA-LPS/DC single injection (grey bars), $2*10^5$ OVA-LPS/DC single injection (red bars), or $2*10^5$ OVA-LPS/DC double injection, compared to T cell injection alone (black bars) into C57BL/6.WT recipient mice. Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

The proliferation of these cells was also traced by CTV dilution and they showed a significant increase in proliferation when compared to CTV labeled CD4$^-$ injected cells. This indicates their expansion in an antigen-dependent manner (Fig 13A). IL-2 and IFN-γ production was analyzed by intracellular flow cytometry and only the CTV negative population of CD4$^+$ OT-II. Thy1.1 T cells were producing both cytokines, indicating their polarization to a Th1 profile (Fig 13B). Also, only a minor population was producing IL-13 indicating no shift towards Th2 T cell response (Fig 13B).

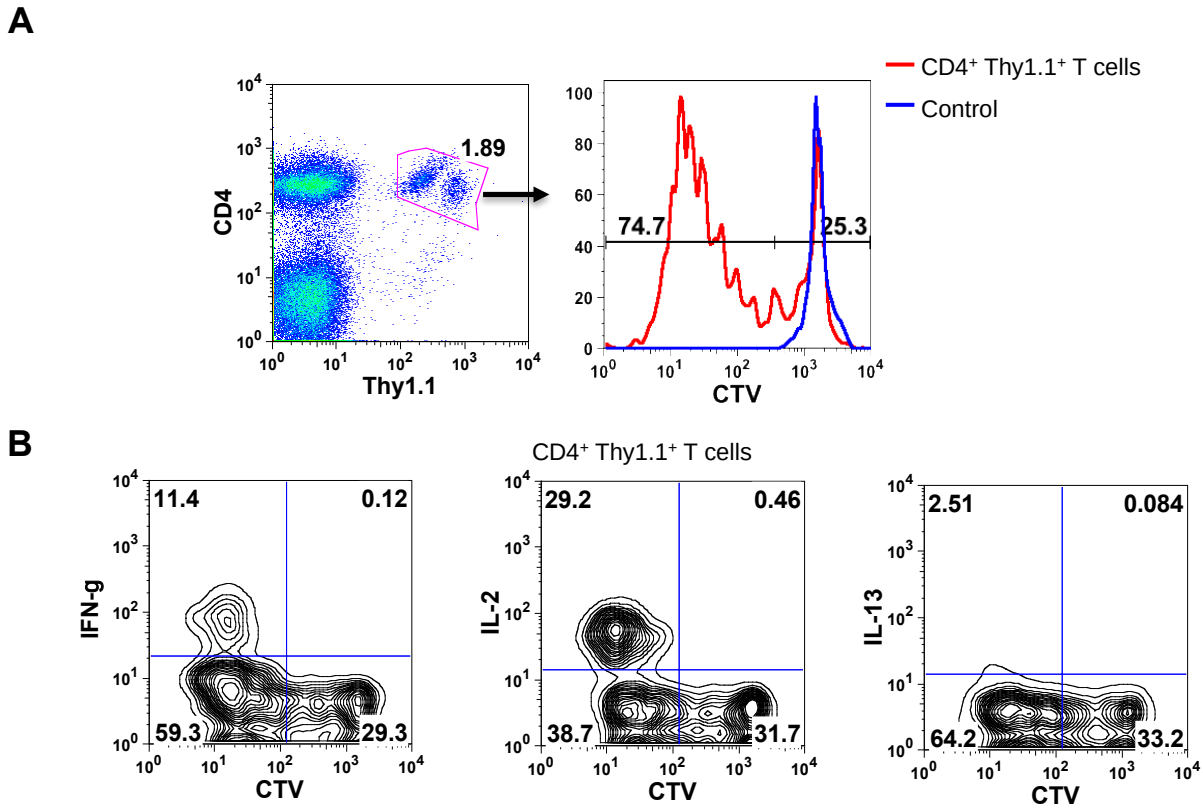


Figure 13 : Proliferation and cytokine production by CD4$^+$ OT-II Thy1.1$^+$ T cells after LPS-OVA/BM-DC injection **A.** Representative flow cytometry plot of OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells in popliteal LN (left), and histogram of their proliferation based on CellTrace™ Violet (CTV) dye dilution by CD4$^+$ OT-II Thy1.1$^+$ T cells (red line) compared to other CTV-labeled cells (blue line) (right). **B.** Representative flow cytometry plots of IFN-γ, IL-2, or IL-13 producing OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells versus CTV labeling

Surprisingly, we also observed a significant population of proliferating CD4$^+$ OT-II. Thy1.1 T cells in the spleen even though no injected BM-DCs arrive in the spleen as indicated in Fig 9C, indicated by their CTV dilution profile (Fig 14A). This population expanded equally as CD4$^+$ OT-II. Thy1.1 T cells in the draining lymph nodes (Fig 14B) and showed more Th1 polarization indicated by their significantly increased IL-2 and IFN-γ production compared to the CD4$^+$ OT-II. Thy1.1$^+$ T cell population in the draining lymph node (Fig 14C). No substantial levels of IL-13 producing cells were observed (Fig 14C). Additionally, these cells showed a higher level of proliferation as indicated by the % of CTV^{-ve} cells and higher ratio of effector T cells/ naive T cells based on expression of the adhesion molecules CD44 and CD62L (Fig 14D). The cellular source responsible for antigen presentation to induce such robust Th1 response by the CD4$^+$ OT-II. Thy1.1 T cells in the spleen is yet to be determined.

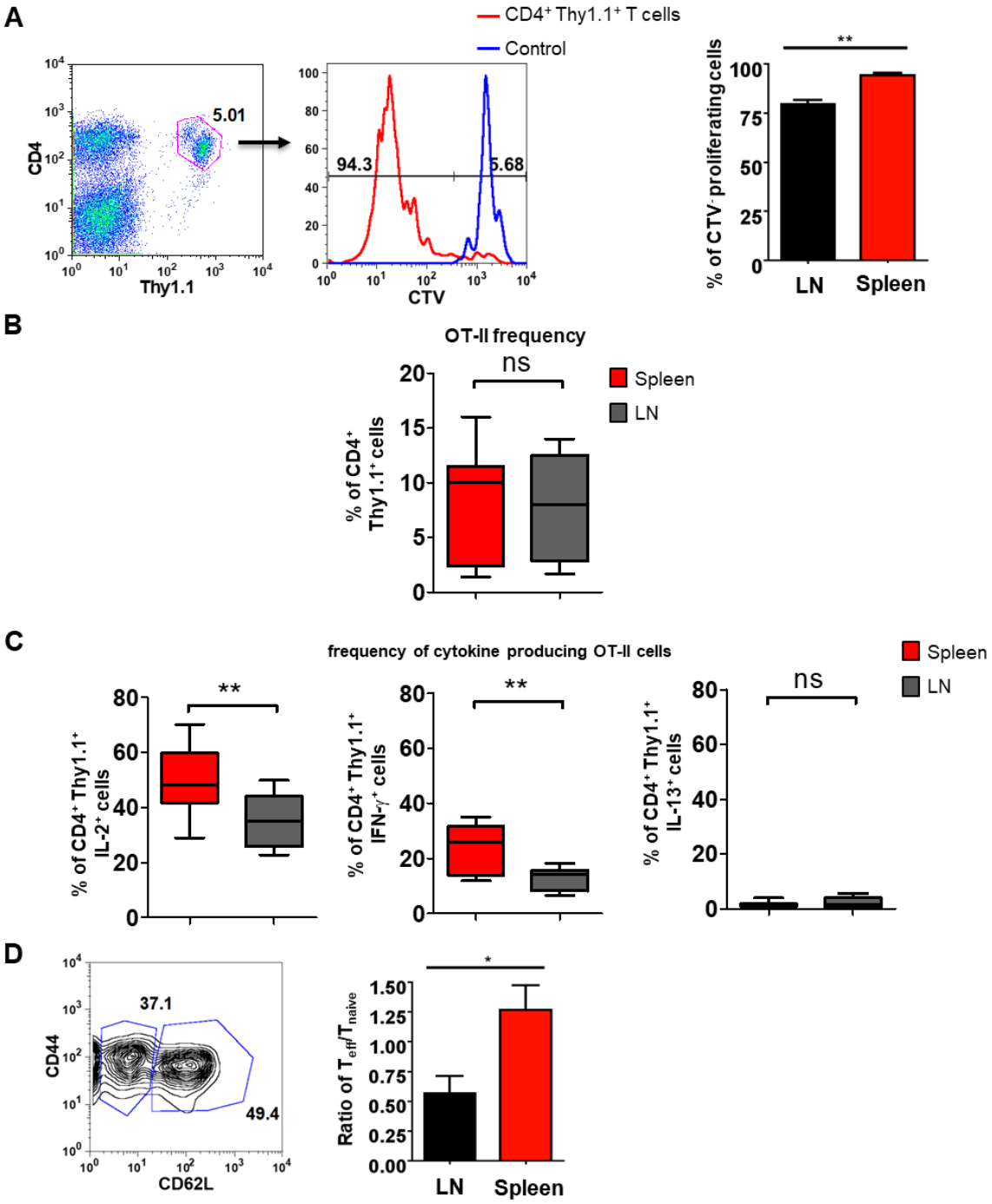
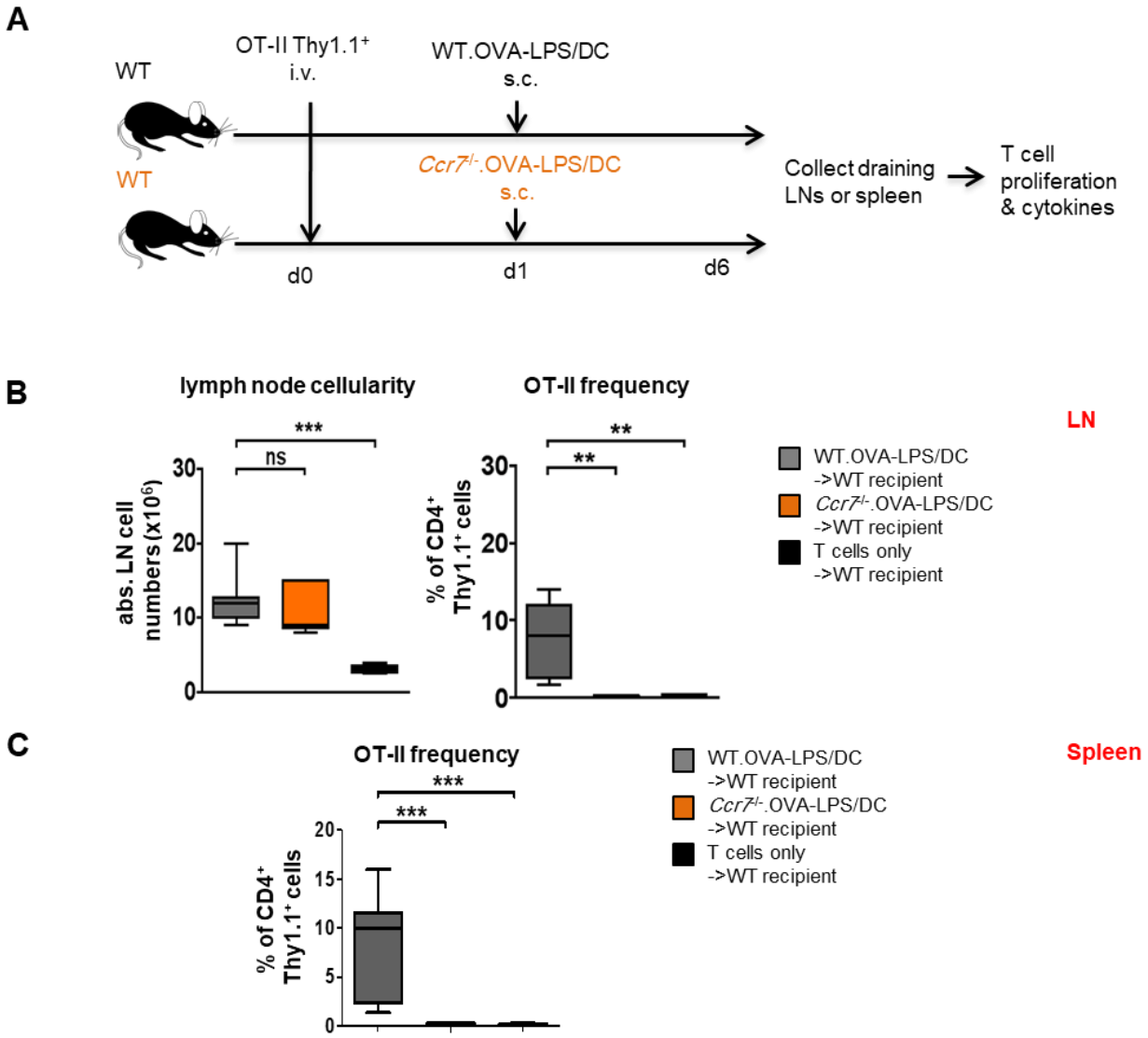


Figure 14 : CD4$^+$ OT-II Thy1.1$^+$ T cell expansion and cytokine production in the spleen **A.** Representative flow cytometry plot of OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells in the spleen (left), histogram of their proliferation based on CellTrace™ Violet (CTV) dye dilution by CD4$^+$ OT-II Thy1.1$^+$ T cells (red line) compared to other CTV-labeled cells (blue line) (middle), and graph comparing the % of proliferating CD4$^+$ OT-II Thy1.1$^+$ T cells in the popliteal LN to the spleen (right). **B,C.** Graphs showing the percentage of CD4$^+$ OT-II Thy1.1$^+$ T cells **(B)** and of IFN-γ, IL-2, or IL-13 producing OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells **(C)** in the popliteal or inguinal LN (grey bars) compared to the spleen (red bars). **D.** Representative flow cytometry plot of CD62L$^+$ CD44^{low} naive T cells and CD62$^-$ CD44^{low} effector T cells within the OT-II$^+$.Thy1.1$^+$CD4$^+$ T cell gate (left), and graph comparing the ratio of naive to effector T cells in the LNs (black bar) to the spleen (red bar) (right) Data are representative of three independent experiments analyzing at least 5 mice per group. **p < 0.001, *p < 0.05.

3.2.2 Migration of antigen-loaded BM-DCs is required for T cell expansion

The arrival of injected BM-DCs was directly related with IL-12p40-YFP production and no increase in YFP production was observed in the distant LNs where no BM-DCs arrived. Therefore, we tested whether the migratory capacity of injected BM-DCs is



.

Figure 15 : Injected BM-DC migration is required for antigen-dependent T cell expansion and polarization **A.** OT-II$^+$.Thy1.1$^+$ T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of WT.OVA-LPS/DC or *Ccr7*$^{-/-}$.OVA-LPS/DC s.c. footpad or flank injection into C57BL/6.WT recipient mice . **B.** Graphs comparing lymph node cell counts and frequency of injected OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells in inguinal or popliteal LNs after s.c. injection of WT.OVA-LPS/DC (grey bars) or *Ccr7*$^{-/-}$.OVA-LPS/DC (orange bars) compared to T cell injection alone (black bars) . **C.** Same as **(B)** but showing only frequency of injected OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells in spleen. Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

mandatory for antigen presentation or if they can hand over antigens to other migratory subsets in the skin. Using BM-DCs generated from *Ccr7*$^{-/-}$ mice that cannot migrate to the draining lymph node and remains trapped in the injection site, we used the setup described in Fig 12A to test for OT-II. Thy1.1 T cell response (Fig 15A). On day 6, the LN cellularity was significantly increased compared to no BM-DC injection and to a comparable level to injecting WT BM-DCs (Fig 15B). This can be attributed to the local inflammation at the injection site. On the other hand, no expansion of OT-II Thy1.1 T cells was observed similar to no BM-DC injection, indicating that antigen presentation by injected BM-DCs takes place only when they reach the draining LN (Fig 15B). We also observed no OT-II Thy1.1 T cells in the spleens (Fig 15C), which indicates that the response does not disseminate systemically unless the T cells get primed first in the draining LN.

3.2.3 IL-12p70 production by injected BM-DCs is not required for a Th1 response

As mentioned, DCs in clinical trials that are matured in the presence of IL-1β/TNFα/IL-6/PGE_2-containing cytokine cocktail show desirable anti-tumor TH1 responses despite the fact that they have a reduced ability to produce IL-12p70 (Lee et al., 2002; Schuler-Thurner et al., 2002). Therefore, we decided to test whether IL-12 lacking BM-DCs would be capable of inducing a proper Th1 response in our system. To this end, again we used the same setup described previously in Fig12A but injected *Il12a*$^{-/-}$ BM-DCs instead of WT BM-DCs to check for IL-12 requirement (Fig 16A). Surprisingly, the exogenous IL-12 production was not needed for Th1 induction. As shown with WT BM-DC injections, the LN cellularity and the $CD4^+$ OT-II. Thy1.1 T cell expansion was of comparable levels on day 6 (Fig 16B). Also, there was no significant reduction in IL-2 and IFN-γ production and no increase in IL-13 production compared to WT BM-DCs injection (Fig 16C). The robust Th1 response previously observed in the spleen also remained unchanged with significantly higher levels of IL-2 and IFN-γ cytokine production compared to LNs (Fig 16D).

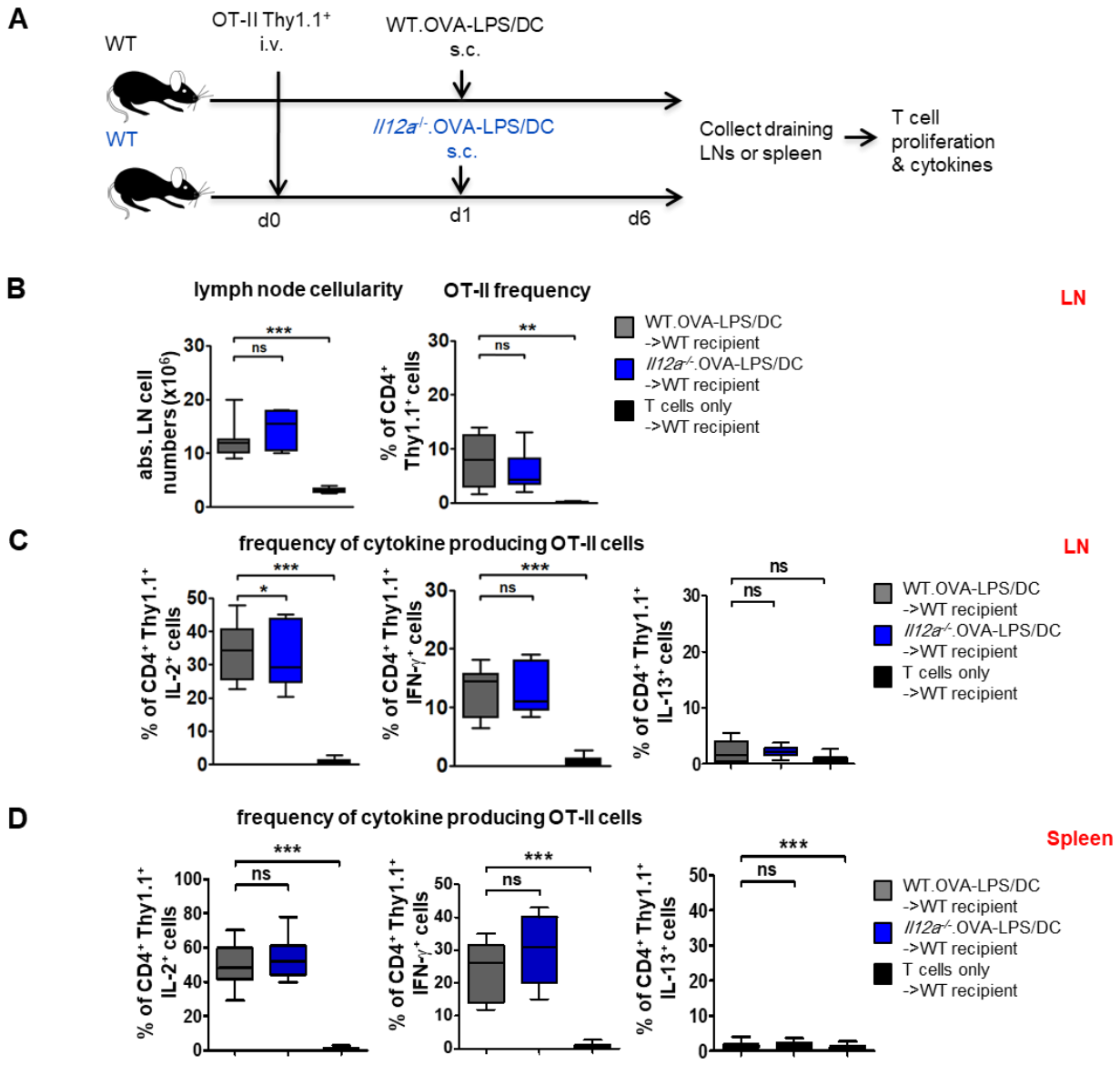


Figure 16 : IL-12p70 production by endogenous recipient DCs is required for required for antigen-dependent T cell expansion and Th1 polarization **A.** OT-II^{+}.Thy1.1^{+} T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of WT.OVA-LPS/DC or *Il12a*$^{-/-}$.OVA-LPS/DC s.c. footpad or flank injection into C57BL/6.WT recipient mice . **B.** Graphs comparing lymph node cell counts and frequency of injected OT-II^{+}.Thy1.1^{+}CD4^{+} T cells in inguinal or popliteal LNs after s.c. injection of WT.OVA-LPS/DC (grey bars) or *Il12a*$^{-/-}$.OVA-LPS/DC (blue bars) compared to T cell injection alone (black bars). **C,D.** Graphs comparing percentage of OT-II^{+}.Thy1.1^{+}CD4^{+} IFN-γ, IL-2, or IL-13 producing cells in LNs **(C)** or spleen **(D)** after s.c. injection of WT.OVA-LPS/DC (grey bars) or *Il12a*$^{-/-}$.OVA-LPS/DC (blue bars). Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

3.2.4 Endogenous IL-12p70 production is required for Th1 polarization

Since IL-12 production by the injected BM-DCs was not required for Th1 polarization, we proceeded by switching to a system lacking IL-12 production by the endogenous recipient

mice. We used the same setup from Fig 12A but using *Il12a*$^{-/-}$ mice as recipients. The injected BM-DCs were generated from either WT mice or from *Il12a*$^{-/-}$ mice (Fig 17A). On day 6, the LN cellularity was significantly increased because of BM-

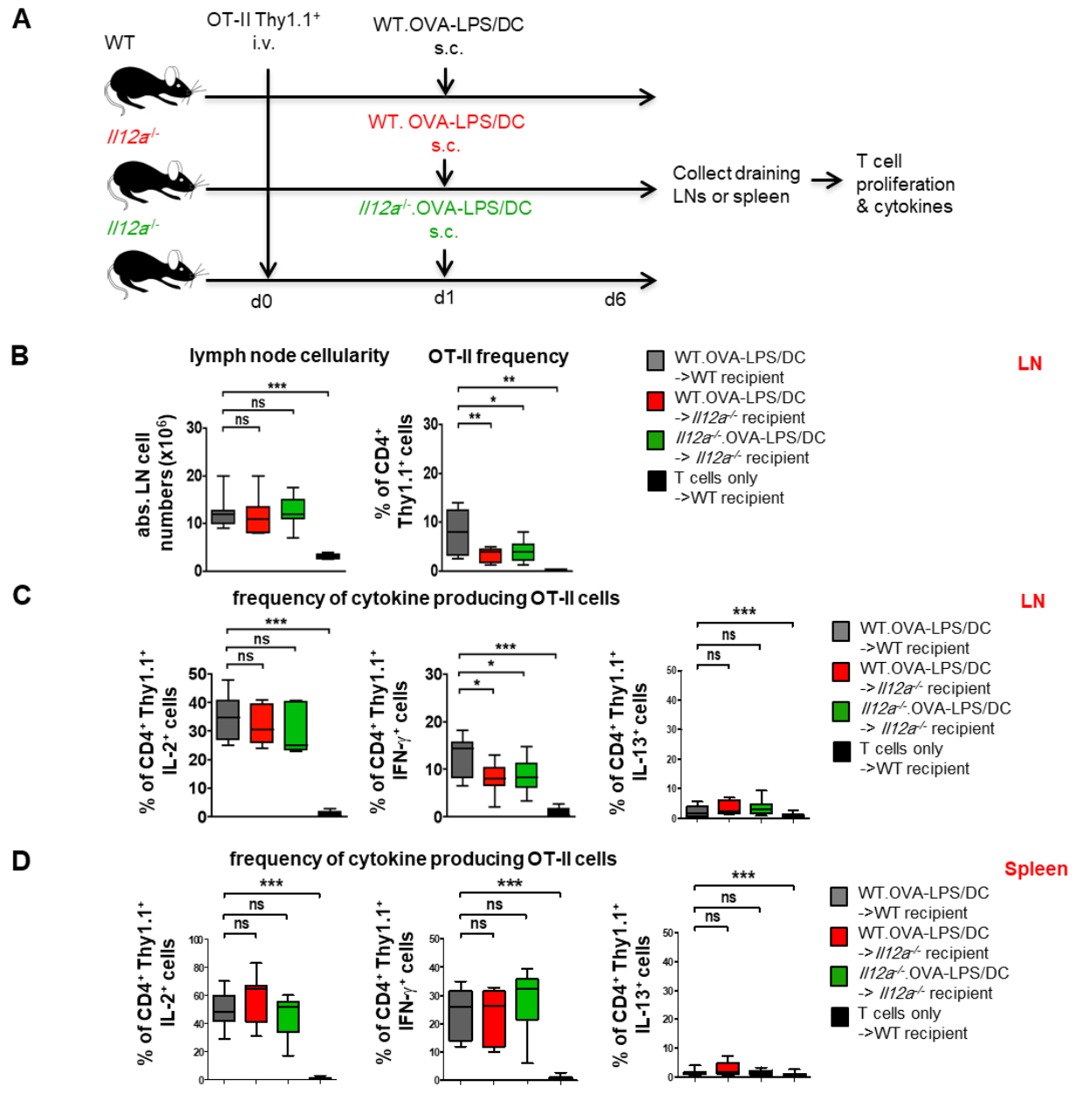


Figure 17 : IL-12p70 production by endogenous recipient DCs is required for required for antigen-dependent T cell expansion and Th1 polarization **A.** OT-II^{+}.Thy1.1^{+} T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of WT.OVA-LPS/DC s.c. injection into C57BL/6.WT recipient mice, WT.OVA-LPS/DC or *Il12a*$^{-/-}$.OVA-LPS/DC s.c. injection into *Il12a*$^{-/-}$ recipient mice. **B.** Graphs comparing lymph node cell counts and frequency of injected OT-II^{+}.Thy1.1^{+}CD4^{+} T cells in inguinal or popliteal LNs after s.c. injection of WT.OVA-LPS/DC (grey bars) into C57BL/6.WT recipient mice, WT.OVA-LPS/DC (red bars) or *Il12a*$^{-/-}$.OVA-LPS/DC (green bars) s.c. injection into *Il12a*$^{-/-}$ recipient mice compared to T cell injection alone (black bars). **C,D.** Graphs comparing percentage of OT-II^{+}.Thy1.1^{+}CD4^{+} IFN-γ, IL-2, or IL-13 producing cells in LNs **(C)** or spleen **(D)** after the same injections described in **(B)**. Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

DC injection similar to WT recipients, and this was the case regardless of whether the BM-DCs were generated from WT or *Il12*$^{-/-}$ mice. However, the expansion of CD4^{+} OT-II. Thy1.1 T cells was significantly reduced only in *Il12*$^{-/-}$ recipients compared to WT recipients (Fig 17B), indicating the requirement of endogenous IL-12 for T cell expansion. The CD4^{+} OT-II. Thy1.1 T cells showed a reduced polarization towards a Th1 response, indicated by a significantly reduced IFN-γ production, the similar levels of IL-2 production, and a non-significant increased tendency for IL-13 production as seen by intracellular staining (Fig 17C). However, this reduction in Th1 response was not observed in the spleens of *Il12*$^{-/-}$ recipients. This was indicated by no significant reduction of IL-2 or IFN-γ production similar to spleens of WT recipient mice (Fig 17D). This suggests that the yet unexplained Th1 response in the spleen is most likely propagated in an IL-12 independent manner.

3.2.5 A migratory CCR7^{+} DC provides the third signal for a Th1 response

Since IL-12 production from endogenous cells of the recipient mice were required for an optimum Th1 polarization, we wanted to pin down which cellular subset is producing it. We first tested whether the endogenous subset is of migratory or of lymph node-resident origin. Using the same setup from Fig12A, *Il12a*.*Ccr7*$^{-/-}$ recipient mice that lack both IL-12 production and endogenous migratory capacity towards the draining LNs were injected with BM-DCs generated from *Il12a*$^{-/-}$ animals (Fig 18A).

In this setup, the LN cellularity was significantly increased as observed before. Interestingly though, CD4^{+} OT-II. Thy1.1 T cells expanded in a similar fashion as WT recipients (Fig 18B). Even though *Il12a*$^{-/-}$ recipient mice showed a reduction in CD4^{+} OT-II. Thy1.1 T cells before (see Fig 17B). Nevertheless, the CD4^{+} OT-II. Thy1.1 T cells were incapable of adopting any T cell polarization phenotype. This is indicated by a complete abolishment of IFN-γ and IL-13 production and a minor fraction of IL-2 producing cells (Fig 18C). Also, only a minor population of CD4^{+} OT-II. Thy1.1 T cells was present in the spleens of *Il12a*.*Ccr7*$^{-/-}$ mice with significantly reduced IL-2 and IFN-γ production compared to *Il12a*$^{-/-}$ recipient mice (Fig 18D). However, this reduction was less significantly pronounced in the spleen compared to the LN, with significant IFN-γ production observed in the spleen (Fig 18D).

3.2.6 Endogenous migratory CCR7^{+} cells substantially contribute to antigen presentation

One possible explanation for the observed reduction in IFN-γ and IL-2 production in *Il12a*.*Ccr7*$^{-/-}$ recipient mice compared to mice lacking *Il12a* only is that endogenous migratory DCs are required for antigen presentation also. We used MHC class II$^{-/-}$ recipient mice and injected WT BM-DCs to study this (Fig 18A). Due to a general lack of CD4^{+} T cells in these mice, the LN cellularity was significantly reduced compared to all

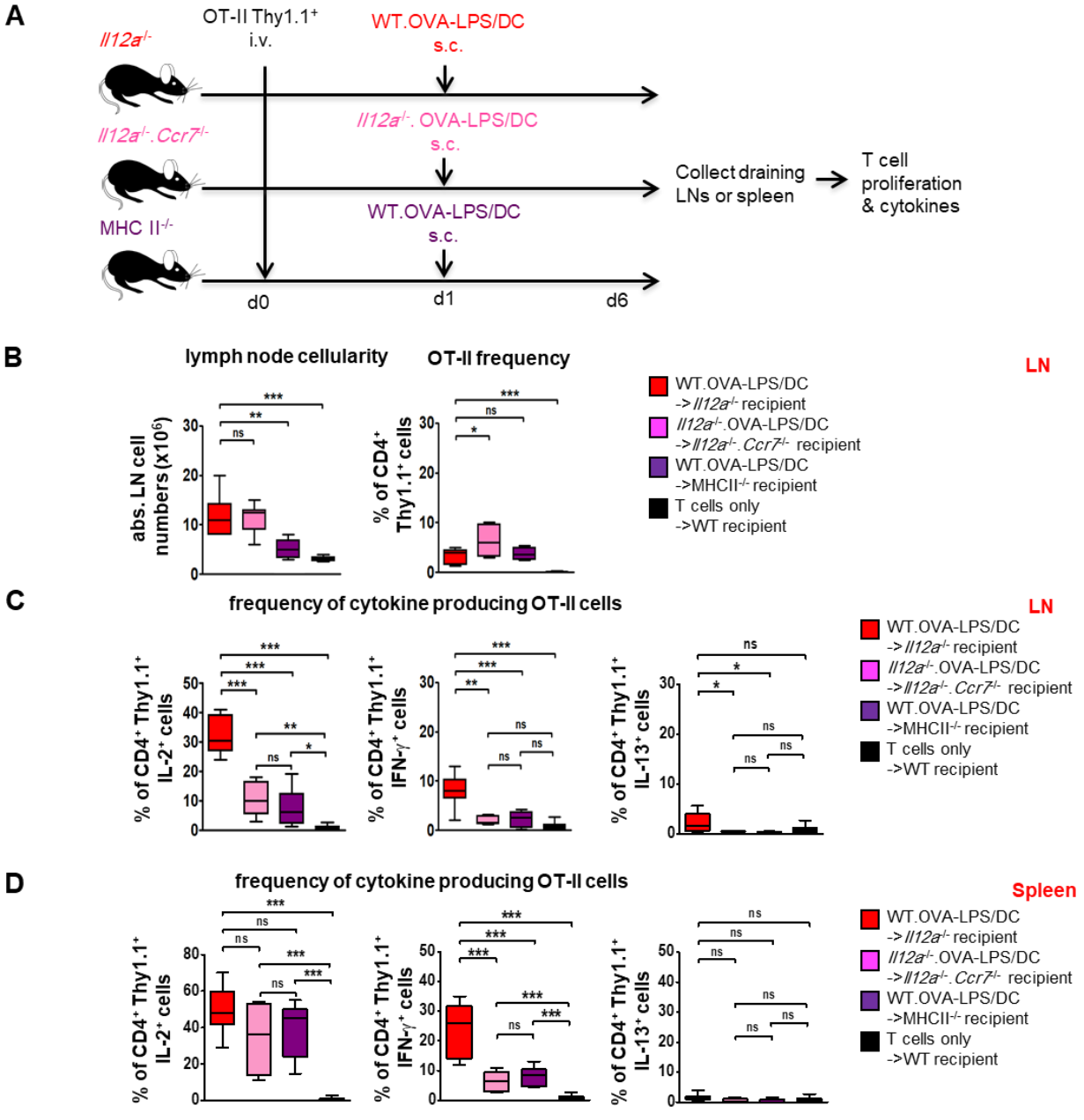


Figure 18 : An endogenous migratory CCR7+ DC contributes to antigen presentation and provides the third signal for a Th1 response **A.** OT-II^{+}.Thy1.1^{+} T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of *Il12a*$^{-/-}$.OVA-LPS/DC into *Il12a*$^{-/-}$.*Ccr7*$^{-/-}$ recipient mice or WT.OVA-LPS/DC into MHC II$^{-/-}$ recipient mice compared to WT.OVA-LPS/DC into *Il12a*$^{-/-}$recipient mice. **B.** Graphs comparing lymph node cell counts and frequency of injected OT-II^{+}.Thy1.1^{+}CD4^{+} T cells in inguinal or popliteal LNs after s.c. injection of WT.OVA-LPS/DC into MHC II$^{-/-}$ recipient mice (purple bars) or *Il12a*$^{-/-}$.OVA-LPS/DC into *Il12a*$^{-/-}$.*Ccr7*$^{-/-}$ recipient mice (pink bars) compared to WT.OVA-LPS/DC into *Il12a*$^{-/-}$recipient mice (red bar) and to T cell injection alone (black bars). **C,D.** Graphs comparing percentage of OT-II^{+}.Thy1.1^{+}CD4^{+} IFN-γ, IL-2, or IL-13 producing cells in LNs **(C)** or spleen **(D)** after the same injections described in **(B)**. Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

the previous setups at d6, and the CD4^{+} OT-II. Thy1.1^{+} T cell expansion was equally reduced as in Il12a$^{-/-}$ recipient mice (Fig 18B), no IFN-γ and IL-13 production was observed and only a minor fraction of cells produced IL-2 similar to *Il12a*.*Ccr7*$^{-/-}$ recipient mice (Fig 18C). Similarly, the population of CD4^{+} OT-II. Thy1.1 T cells present in the spleen showed significantly reduced IFN-γ production compared to *Il12a*$^{-/-}$ recipient mice (Fig 18D). Unexpectedly, a significant percentage of cells were producing IL-2 in this case (Fig 18D). These results suggest that endogenous migratory DCs substantially contribute to antigen presentation. Considering the results shown in Fig 16B, that T cell priming strictly depends on BM-DCs migrating to the lymph nodes and transfer of antigen in the skin can be excluded, our data suggest that transfer of antigen or MHC II/peptide complexes from BM-DCs to endogenous migratory bystander DCs occurs in the lymph node. This is in agreement with data showing that antigen transfer to an endogenous DC subset is required to enhance T cell response during DC vaccination (Kleindienst and Brocker, 2003). This antigen transfer substantially contributes to Th0 priming and is essentially required for Th1 polarization.

3.2.7 XCR1^{+} CD103^{+} dDCs provide the third signal for Th1 induction after BM-DC injection in the draining lymph node

So far, all the evidences point to XCR1^{+} CD103^{+} dDCs as the major producer of IL-12 for Th1 polarization in the BM-DC injection setup. This is based on YFP production by the Yet40 mice and by the exclusive requirement of IL-12 from an endogenous *Ccr7*-dependent migratory DC source. To conclusively show that, we used *Xcr1*-Venus-DTR mice, where the XCR1^{+} CD103^{+} dDC subset can be conditionally depleted upon diphtheria toxin (DTX) injection (Yamazaki et al., 2013) (Fig 19A). We confirmed the depletion of this subset specifically in the draining lymph nodes and in the spleen after

DTX injection. In these mice, there was a slight reduction in LN cellularity 6 days after BM-DC injection while the CD4$^+$ OT-II. Thy1.1 T cells expanded in a similar fashion as WT mice (Fig 19B). The production of IL-2 however was significantly reduced indicating an impairment in T cell priming and IFN-γ and IL-13 were not detected suggesting the dependency of T cells on XCR1$^+$ CD103$^+$ dDCs for Th1 polarization (Fig 19C). Interestingly, in the spleens of these mice the expanded CD4$^+$ OT-II. Thy1.1 T cells showed a strong Th1 polarization profile indicated by robust production of IL-2, IFN-γ and no IL-13 This suggested that Th1 polarization in the spleen was not dependent on the XCR1$^+$ CD8$^+$ DC subset unlike the situation in the lymph node.

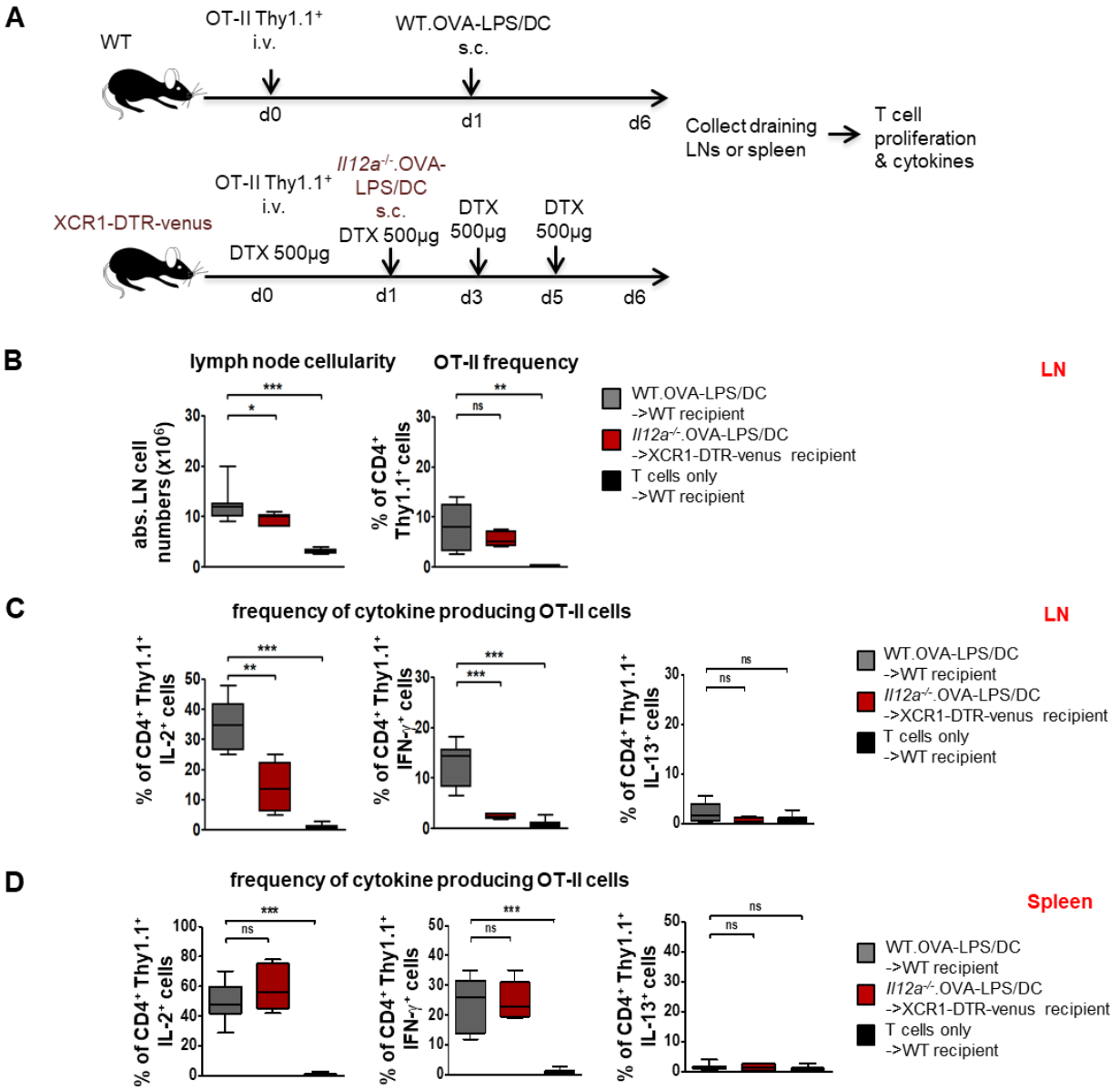


Figure 19 : XCR1+ CD103+ dDCs provide the third signal for Th1 induction after BM-DC injection only in the lymph node A. OT-II^{+}.Thy1.1^{+} T cell priming analyzed in popliteal or inguinal LNs and in spleen after 6 days of *Il12a*$^{-/-}$.OVA-LPS/DC into *Xcr1*-DTR-venus recipient mice compared to WT.OVA-LPS/DC into C57BL/6.WT recipient mice. B. Graphs comparing lymph node cell counts and frequency of injected OT-II^{+}.Thy1.1^{+}CD4^{+} T cells in inguinal or popliteal LNs after s.c. injection of *Il12a*$^{-/-}$.OVA-LPS/DC into *Xcr1*-DTR-venus recipient mice (dark red bars) compared to WT.OVA-LPS/DC into C57BL/6.WT recipient mice (grey bar) and to T cell injection alone (black bars). C,D. Graphs comparing percentage of OT-II^{+}.Thy1.1^{+}CD4^{+} IFN-γ, IL-2, or IL-13 producing cells in LNs (C) or spleen (D) after the same injections described in (B). Data are representative of three independent experiments analyzing at least 5 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

3.2.8 CpG maturation of BM-DCs induce Th1 polarization similar to LPS-BM-DCs

The activation of bystander DCs by injected BM-DCs for IL-12p70 production and contribution to antigen presentation suggests their interaction in the draining lymph

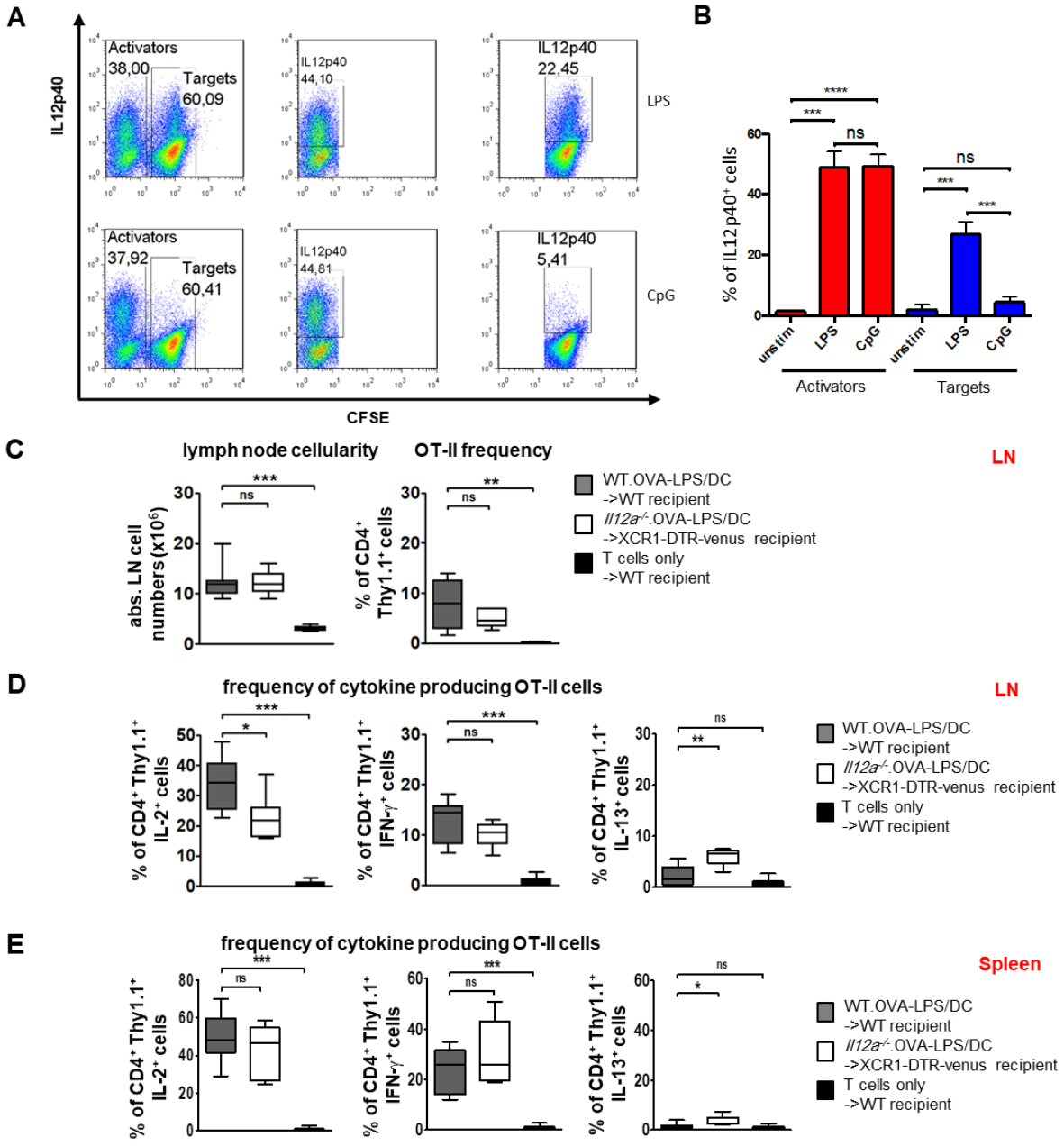


Figure 20: Presentation of surface bound antigen is not the cause of bystander DC activation. **A.** Representative flow cytometry plots for $CFSE^-$ donor DCs (Activators) and $CFSE^+$ bystander activated DCs (Targets) and the % of IL-12p40 producing cells upon using LPS (top), or CpG (bottom)-matured activators. Graphs showing the % of IL-12p40$^+$ cells from untreated, LPS or CpG-matured activator DCs and their respective bystander activated target DCs in three independent experiments. **B.** Graphs comparing lymph node cell counts and frequency of injected OT-II$^+$.Thy1.1$^+$CD4$^+$ T cells in inguinal or popliteal LNs after s.c. injection of WT.OVA-LPS/DC (grey bars) or WT.OVA-CpG/DC (white bars) compared to T cell injection alone (black bars). **C,D.** Graphs comparing percentage of OT-II$^+$.Thy1.1$^+$CD4$^+$ IFN-γ, IL-2, or IL-13 producing cells in LNs **(C)** or spleen **(D)** after s.c. injection of WT.OVA-LPS/DC (grey bars) or WT.OVA-LPS/DC (white bars). Data are representative of three independent experiments analyzing at least 5 mice per group. ****P < 0.00001 ***P < 0.0001, **p < 0.001, *p < 0.05.

nodes. One possibility of DC-DC interaction leading to bystander activation is that LPS bound to TLR4 on the migrated BM-DCs is 'presented' to other DCs in the lymph node. Previous *in vitro* data from our lab indicated that LPS-matured BM-DCs can activate co-cultured immature BM-DCs to secrete IL-12p40, while CpG matured BM-DCs were unable to show this effect (Fig 20A, B). These data suggest that LPS remains bound to surface TLR4 and can be 'presented' to bystander DCs, while CpG seems to be efficiently internalized by DEC-205/CD205 (Lahoud et al., 2012) to bind TLR9 within intracellular vesicles. We showed in (Fig 10C) that CpG matured BM-DCs induce bystander IL-12p40 production similar to LPS matured BM-DCs. We next wanted to test whether CpG matured BM-DCs can also induce Th1 polarization *in vivo*. The use of CpG-matured, OVA-loaded BM-DCs (OVA-CpG/DC) provoked a similar lymph node swelling, OT-II$^+$.Thy1.1$^+$ CD4$^+$ T cell expansion (Fig 20C) and IFN-γ frequencies as observed after OVA-LPS/DC injection, while the frequency of IL-2$^+$ OT-II cells was slightly reduced, and substantial amounts of IL-13 was observed (Fig 20D). These data indicate that injected BM-DCs possess additional mechanisms of bystander DC activation beyond the 'presentation' of surface bound pathogen. Similar to OVA-LPS/DC injections, the robust Th1 response previously observed in the spleen also remained unchanged with significantly higher levels of IL-2, IFN-γ and negligible IL-13 production compared to LNs (Fig 20E).

3.2.9 CD27-CD70 interactions might be involved in IL-12 independent Th1 priming

BM-DCs generated from *Il12a*$^{-/-}$ mice induced similar Th1 polarization as WT BM-DCs. Also, *IL12a*$^{-/-}$ recipient mice showed a significant reduction in Th1 polarization however there was a residual Th1 polarization that was not present in *Il12a*.*Ccr7*$^{-/-}$, MHC II$^{-/-}$, and

Xcr1.venus-DTR mice. This prompted us to check for possible IL-12 independent Th1 priming pathways. One of the reported pathways that are involved in providing the third signal for T cells is the interaction of the TNF receptor family member CD27 on T cells and its ligand on APCs namely the CD70 molecule (Sanchez and Kedl, 2012; Soares et al., 2007).

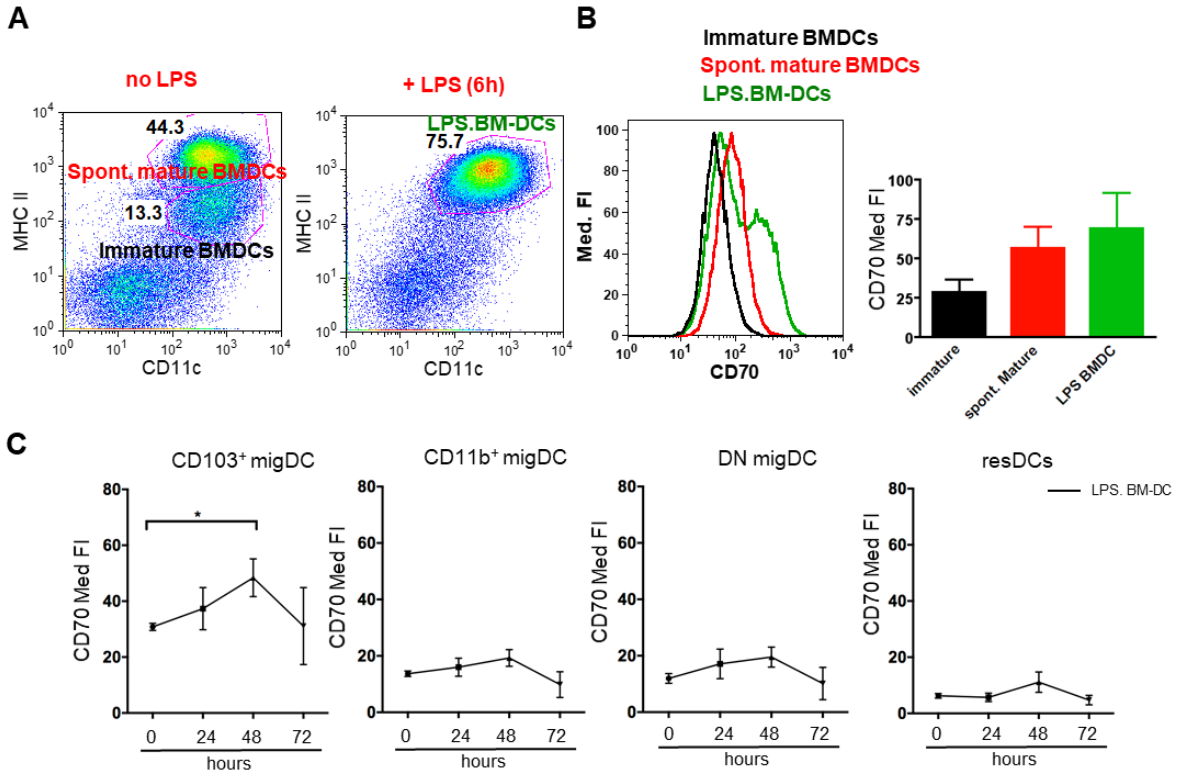


Figure 21 : CD27-CD70 interactions might be involved in IL-12 independent Th1 priming. A. Flow cytometry plot of day 8 C57BL/6.WT BM-DCs before and after 6h of LPS treatment showing CD11c^{+} MHC II^{int} immature DCs (black), CD11c^{+} MHC II^{high} spontaneously matured DCs (red) in no LPS cultures and CD11c^{+} MHC II^{high} LPS matured DCs (green). **B.** Representative histogram (left) and graph of three independent experiments (right) of CD70 median FI in the populations described in **(A). C.** Graphs from inguinal LNs of C57BL/6.WT mice of CD70 median FI in CD103^{+}, CD11b^{+}, DN migratory DCs, or LN resident DCs at steady-state or 24h, 48h, or 72h after LPS. BM-DC injection. Data are representative of three independent experiments. *$p < 0.05$.

First, we tested the expression of CD70 on generated BM-DCs that were stimulated for 6h with LPS or left untreated as a control. We found an upregulation of CD70 on both the CD11c^{high} MHC II^{high} fully LPS-matured DCs and on the CD11c^{high} MHC II^{int} spontaneously-matured DCs (Fig 21A, B). On the other hand, within the CD11c^{high} MHC II^{int} lymph node resident DCs and the CD11c^{int} MHC II^{high} migratory DCs; the XCR1^{+} CD103^{+} dDC subset expressed the highest levels of CD70 after LPS-BM-DC injection. This peaked 48h after injection and was decreased at the 72h time point (Fig 21C). These data may point to a role of CD70 on XCR1^{+} DCs for Th1 priming.

3.3 Vaccine DC communication with XCR1$^+$ CD103$^+$ migratory DCs induce their bystander activation and shifts them to a Th1 polarizing profile

3.3.1 Kinetics of BM-DC entry and T cell expansion in the skin draining LN

The fact that endogenous XCR1$^+$ CD103$^+$ dDC were the main subset responsible for providing signal three for Th1 polarization entails that the injected BM-DCs communicate with the endogenous DCs during the T cell priming process. We first wanted to study the dynamics of entry of the injected DCs and the kinetics of expansion of CD4$^+$ OT-II. Thy1.1 T cells in the draining lymph node. To this end OT-II. Thy1.1 T cells were injected i.v. and CTV-labeled LPS-BM-DCs generated from *Il12a*$^{-/-}$ mice were injected next day into the footpads of the mice. The popliteal LNs were collected and analyzed for the percentage of injected BM-DC and CD4$^+$ OT-II. Thy1.1 T cells at 24h, 48h, and 72h time points respectively (Fig 22A). The entry of CTV-labeled LPS-BM-DCs following their injection peaked at 48h (Fig 22B), while expansion of T cells started at 48h and reached its peak levels at 72h (Fig 22C). This was evident also using confocal microscopy of popliteal LNs of *Yet40* mice upon quantifying CTV-labeled BM-DCs and OT-II. Thy1.1$^+$ T cells over the different timepoints in the T cell area of the lymph node. On the other hand, the number of IL-12p40$^+$ cells remained constant, showing only a trend of higher frequencies at 48h (Fig 22D, 22E). We noticed that injected CTV-labeled BM-DCs were located in the T cell area and distinct clusters of OT-II. Thy1.1 cells were observed at the 24h time point (Fig 22D, 22E). This is in line with what was previously observed by (Mempel et al., 2004) where during this period, T cells form long-lasting stable conjugates with DCs. At 48h and 72h more CTV-labeled BM-DCs entered the LNs and OT-II. Thy1.1 T cell expansion was observed peaking at 72h. However, less clustering of BM-DCs with T cells was evident indicating the more motile, proliferative phase of the expanding T cells (Fig 22D, 22E).

3.3.2 YFP$^+$ endogenous DCs show patterns of communication with injected BM-DCs at later time points

We next wanted to study whether there are specific interactions between the injected-BM-DCs and endogenous DCs of the recipient mice, presumably with the XCR1$^+$ migratory DCs that were shown to provide the third signal for Th1 proliferation in a

bystander fashion. Using the same setup described in Fig 22A, we analyzed the relative distance of YFP$^+$ endogenous DCs to the injected BM-DCs 24h, 48h, and 72h after their

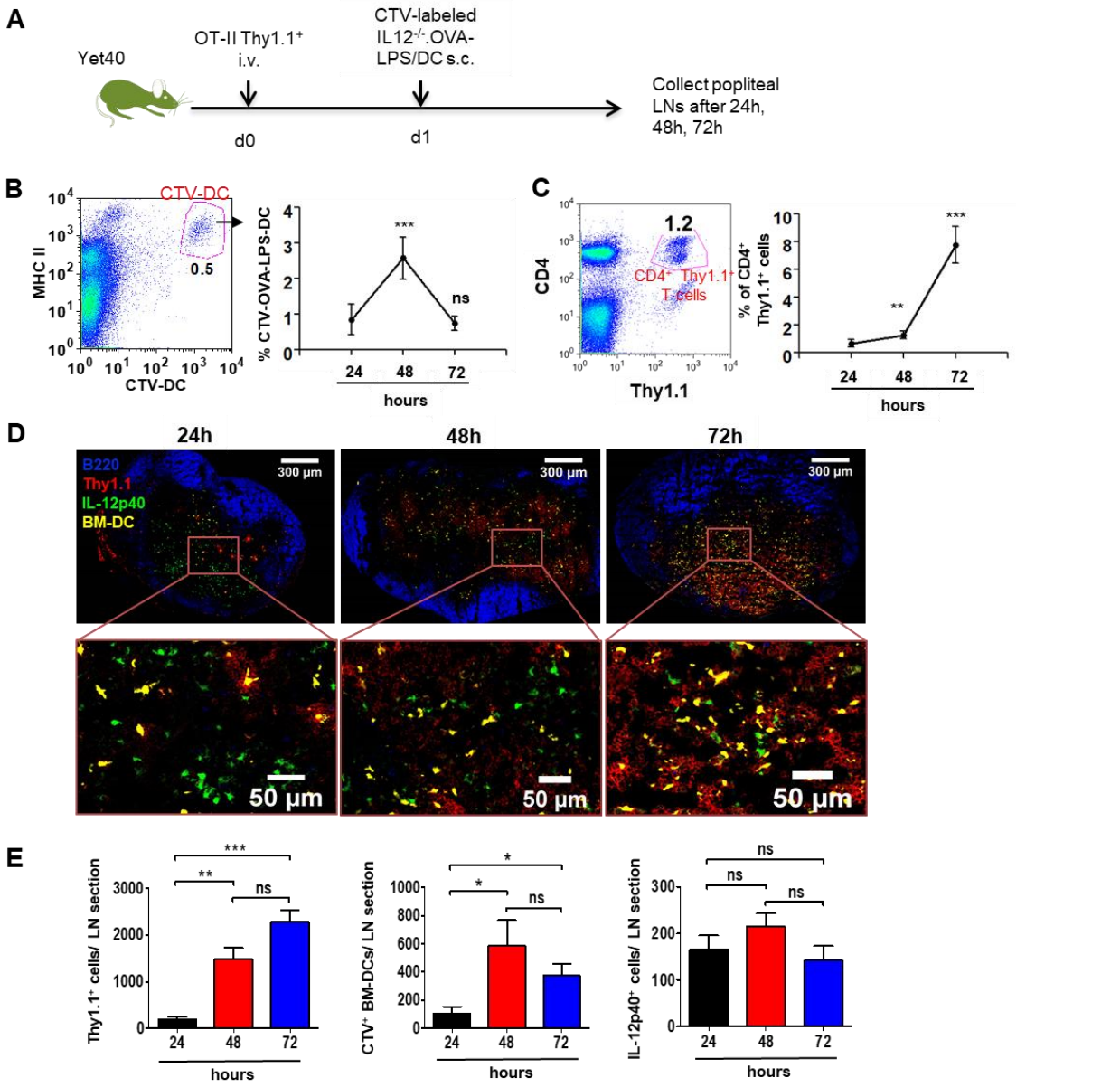


Figure 22 : Kinetics of BM-DC entry into the draining lymph node and T cell expansion. **A.** Popliteal LNs were analyzed by FACS and confocal microscopy. OT-II.Thy1.1$^+$ cells were injected i.v., next day OVA (10 µM)-loaded, LPS (0.5 µg/ml)-matured CTV-lalebeld *Il12a*$^{-/-}$.BM-DCs (CTV-OVA-LPS.DC) were injected s.c. into the footpad of Yet 40 recipients, mice were collected after 24h, 48h, or 72h. **B.** Flow cytometry plot (left) and graph of three independent experiments (right) of percentage of CTV-OVA-LPS.DC in the LN 24h, 48h, and 72h after injection. **C.** Flow cytometry plot (left) and graph of three independent experiments (right) of percentage of CD4$^+$ OT-II.Thy1.1$^+$ cells in the LN 24h, 48h, and 72h after injection. **D.** Representative immunofluorescence microscopy images of whole popliteal lymph nodes sections (upper row) and magnification of the T cell area after of OT-II$^+$.Thy1.1$^+$ T cell injection (red) + CTV labeled *Il12a*$^{-/-}$.OVA-LPS/DC s.c. injection (yellow) into IL-12p40-YFP mice (green cells) (24, 48, or 72h after injection) **E.** Graphs showing number of OT-II$^+$.Thy1.1$^+$ T cells, CTV-labeled OVA-LPS/DC, and YFP$^+$ endogenous Cs/popliteal lymph node cut 24, 48, 72h after DC injection. Data are representative of two independent experiments analyzing at least 4 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

injection within the T cell area of popliteal draining lymph nodes. As a comparison, the relative distance between total XCR1+ endogenous dDCs and injected-BM-DCs which includes the presumably bystander-activated CD103$^+$ XCR1$^+$ DCs and the tolerance inducing ones (Muzaki et al., 2016). Also, the relative distance of the unrelated CD11b$^+$ DCs to injected-BM-DCs was used as a YFP$^-$ XCR1$^-$ endogenous DC control subset. The relative distance between CD11b$^+$ and total XCR1$^+$ endogenous DCs to injected DCs showed a tendency to increase at the 48h and 72h time points when compared to the 24h time point (Fig 23A). This is attributed to their random movement in the lymph node that gets enlarged at the 48h and 72h time point. On the other hand, the relative distance between YFP$^+$ endogenous DCs and injected BM-DCs was significantly reduced at both 48h and 72h time points compared to 24h (Fig 23A). This strongly points to a specific interaction between the YFP$^+$ XCR1$^+$ endogenous DCs with the injected BM-DCs that is initiated during the T cell proliferation phase.

3.3.3 Cognate T cells and YFP$^+$ endogenous DCs show patterns of communication at later time points

If the YFP$^+$ endogenous DCs are indeed receiving signals from the injected BM-DCs at later time points, we expected them to provide the third signal to T cells in a bystander fashion also at later time points during the T cell expansion phase. This was already indicated by the fact that IL-12p40-YFP peaked at the 72h (Fig 7C), indicating its requirement later in the expansion phase. To confirm that, we measured the relative distance of OT-II. Thy1.1$^+$ T cells to YFP$^+$ endogenous DCs and used the relative distance between OT-II. Thy1.1$^+$ T cells and injected BM-DCs as a control representing cognate antigen recognition. The relative distance between T cells and BM-DCs showed a tendency to increase after 48h, which increased significantly at 72h, compared to the 24h time point (Fig 23B). This is due to the increase in LN size indicated before. On the other hand, the relative distance between T cells and YFP$^+$ endogenous DCs was significantly reduced after 48h and 72h as compared to the 24h time point (Fig 23B). From these findings we concluded that a time-dependent three-way communication i) between the injected BM-DCs and cognate T cells, ii) the BM-DCs with YFP$^+$ XCR1$^+$ endogenous bystander cDC1s, and iii) YFP$^+$ XCR1$^+$ endogenous bystander cDC1s with the primed Th0 cells for further polarization into Th1 cells.

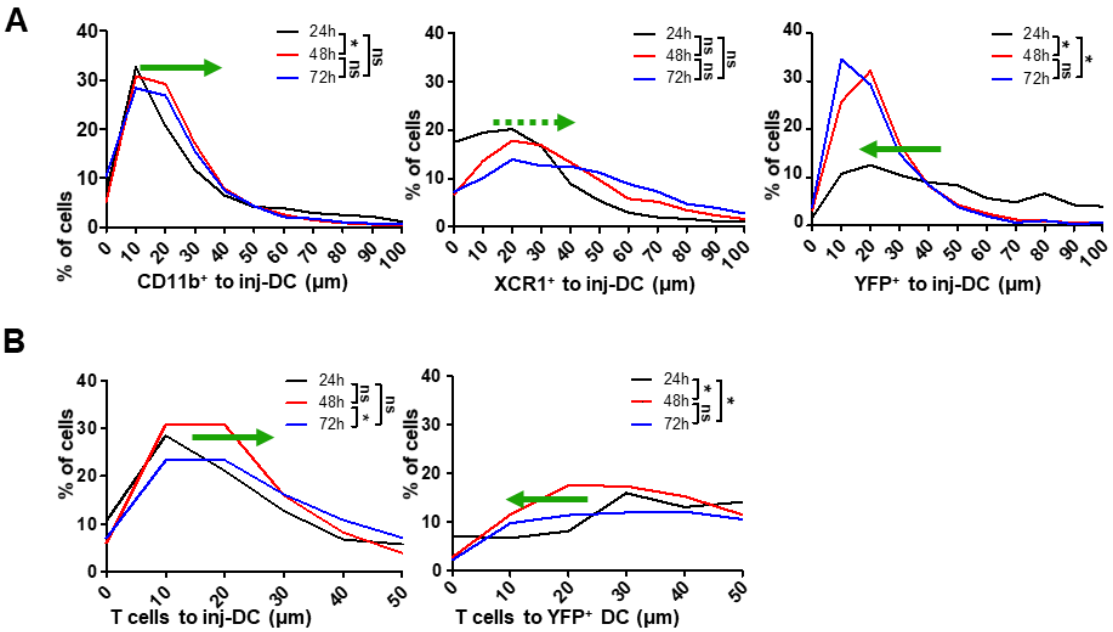


Figure 23 : YFP$^+$ XCR1$^+$ DCs interact with injected DCs and antigen-specific T cells at later time points. **A.** Graphs showing the relative distance of CD11b$^+$ cells, XCR1$^+$ cells, and YFP+ cells to CTV-labeled OVA-LPS/DC in the peripheral lymph nodes 24, 48, or 72h after DC injection **B.** Graphs showing the relative distance of OT-II$^+$.Thy1.1$^+$ T cells to CTV-labeled OVA-LPS/DC or to YFP$^+$ endogenous DCs in the peripheral lymph nodes 24, 48, or 72h after DC injection. Green arrow indicates the distance shift at 48h and 72h compared to 24h. Data are representative of two independent experiments analyzing at least 4 mice per group. ***P < 0.0001, **p < 0.001, *p < 0.05.

3.3.4 4-1BB and 4-1BBL interactions as a candidate signal driving DC-DC communication

As mentioned earlier, one of the possible signals promoting DC-DC crosstalk is the interaction between the TNF receptor family member 4-1BB and its ligand 4-1BBL (Futagawa et al., 2002). We showed previously that both molecules are expressed on matured BM-DCs. This was confirmed on day 8 BM-DCs that were treated with LPS for 6h compared to unstimulated control.

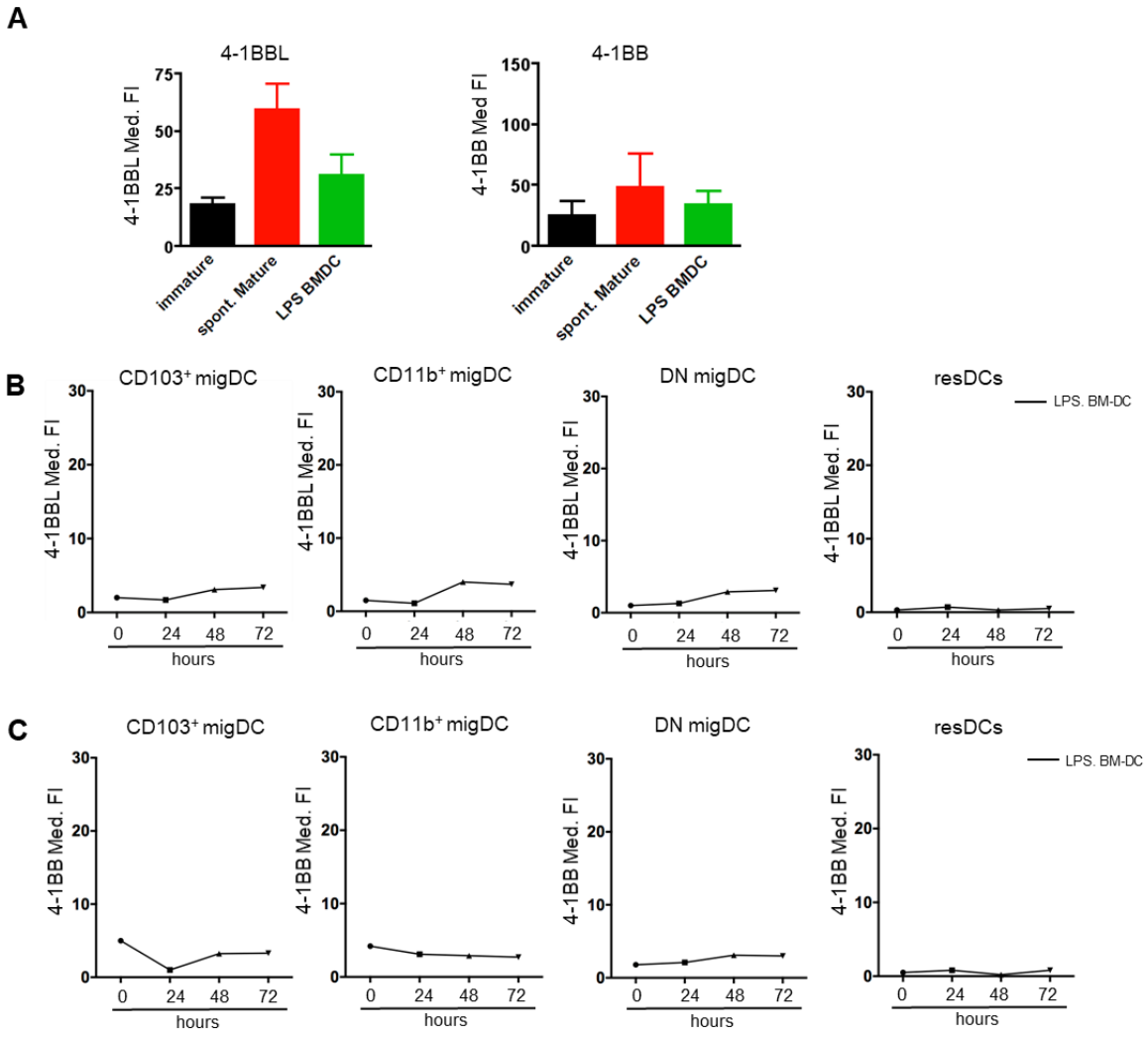


Figure 24 : 4-1BB and 4-1BBL interactions as a candidate signal driving bystander DC activation . **A.** Graphs of three independent experiments of 4-1BBL (left) or 4-1BB (right) median FI showing CD11c^{+} MHC II^{int} immature DCs (black), CD11c^{+} MHC II^{high} spontaneously matured DCs (red) in no LPS cultures and CD11c^{+} MHC II^{high} matured DCs after 6h LPS treatment (green). **B,C.** Graphs from inguinal LNs of C57BL/6.WT mice of 4-1BBL **(B)** or 4-1BB **(C)** median FI in CD103^{+}, CD11b^{+}, DN migratory DCs, or LN resident DCs at steady-state or 24h, 48h, or 72h after LPS. BM-DC injection. Data are representative of three independent experiments.

A percentage of 4-1BB and 4-1BBL positive BM-DCs was observed on untreated control cells and 4-1BB was upregulated on the CD11c^{high} MHC II^{int} spontaneously-matured DCs and upon LPS treatment. Such upregulation was not observed by 4-1BBL (Fig 24A). Unfortunately, no 4-1BB positive population was detected within CD11c^{high} MHC II^{int} lymph node resident DCs and the CD11c^{int} MHC II^{high} migratory DCs. And this did not change after LPS-BM-DC injection 24h, 48h,72h following the injection (Fig 24B). This was also the case when analyzing 4-1BBL within the lymph node DC subsets (Fig 24C).

3.3.5 Distinct transcriptional shifts are observed in migratory DC populations after LPS-BM-DC injection

Apart from 4-1BB and 4-1BBL interactions as a possible signal for communication between injected BM-DCs and the different endogenous migratory DC subset, we wanted to test for the global transcriptional shifts that occur within these subsets and identify other possible interaction partners. We made use of next-generation sequencing technologies

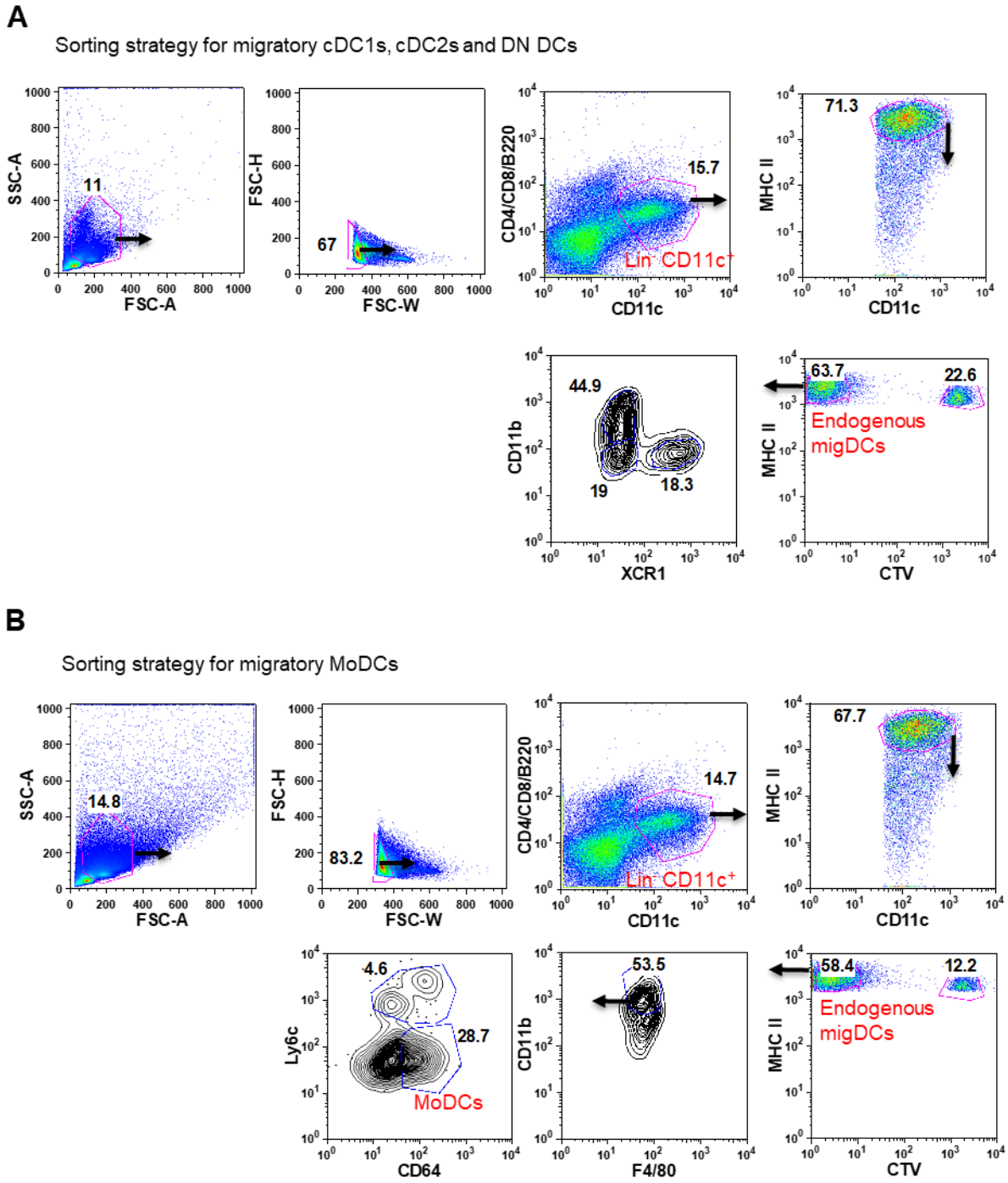
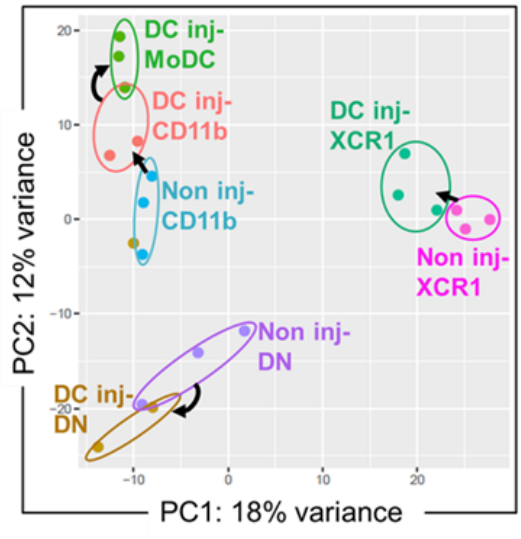


Figure 25 : Sorting strategy for cDCs and MoDCs RNA sequencing. **A.** Gating strategy for migratory cDC1s, cDC2s and DN DCs in popliteal and inguinal LNs. CD11c^{+} MHC II^{high} Lin^{-} (CD4^{-} CD8^{-} B220^{-}) CTV^{-} endogenous migDCs were sub gated based on XCR1 and CD11b into XCR1^{+} CD11b^{-} cDC1s, CD11b^{+} XCR1^{-} cDC2s, and XCR1^{-} CD11b^{-} DNs. **B.** Gating strategy for MoDCs from CD11c^{+} MHC II^{high} Lin^{-} (CD4^{-} CD8^{-} B220^{-}) CTV^{-} endogenous migDCs. MoDCs were CD11b^{+} F4/80^{-} CD64^{+} and Ly6C^{-} and appear only after LPS. BM-DC s.c. injection (right).

to study these changes. Briefly, we sorted CD11c^{int} MHCII^{high} migratory XCR1^{+} CD11b^{-} dDCs, CD11b^{+} XCR1^{-} dDCs, and CD11b^{-} XCR1^{-} DCs (DN) at steady-state and 48h after injecting CTV-labeled LPS.BM-DC (Fig 25A). Also, CD11b^{+} CD64^{+} Ly6C^{low} inflammation-induced MoDCs that we described in (Fig 9C) were also sorted at 48h (Fig 25B). Since MoDCs could not be detected in naive mice, we were lacking a direct related control for this population. Cells were sorted from pooled popliteal and inguinal LNs that were isolated from C57BL/6 mice after footpad injections. We chose the 48h time point based on the observed change in the relative distance between injected BM-DCs, endogenous YFP^{+} DCs and cognate Thy1.1^{+} T cells which started only at 48h, indicating that bystander activation of endogenous migratory DCs starts at this time point. RNA sequencing was performed on 100 sorted cells from each population. Principle component analysis (PCA) segregated the samples into three distinct groups. Each of the three cDC subsets clustered differently and shifted to a different direction after their bystander activation. MoDCs clustered close to CD11b^{+} dDCs and appeared further distant from bystander activated CD11b^{+} dDCs indicating a close relation (Fig. 26A). Gene ontology (GO) and pathway enrichment analysis for the differentially regulated genes in the XCR1^{+} dDC subset indicated a down-regulation of nucleosome organization, cellular development and, cellular differentiation pathways. Interestingly, genes promoting Th2 induction or Treg induction by DCs were included in these down-regulated pathways, together with genes involved in DC migration and genes that modulates the antigen presentation capacity of DCs. (Fig. 26B). We compared the differentially regulated genes from activated XCR1^{+} dDCs to steady-state XCR1^{+} dDCs, and likewise for CD11b^{+} dDCs and DN DCs. MoDCs were compared to activated CD11b^{+} dDCs. A total of 112 genes were significantly up- or down-regulated in at least one of the comparisons. The clusters of genes that were regulated in each comparison were very different for each DC subset (Fig. 26C). This may indicate that the enlarged lymph nodes deliver distinct signals to individual DC subsets that cannot be explained simply by a general inflammatory situation that should activate similar transcriptional responses in each DC subset. Genes such as

A



B

DCs inducing Th2 and Treg

Ahr	DC instruction of Treg induction (Nguyen et al., 2010)
Atf3	Downregulation of IL-6 and IL-12 (Whitmore et al., 2007)
Il3ra	Promotes Th2-polarizing DCs (Ebner et al., 2002)
Il7r	DC control of T cell proliferation (Guimond et al., 2009)
Jag2	Promotes Th2-polarizing DCs (Amsen et al., 2004)
Klf2	Negative regulation of DCs for subsequent T cell activity (Alberts-Grill et al., 2016)
Pirb	Tolerogenic signals for DCs (van der Touw et al., 2018).
Stat5b	Promotes Th2-polarizing DCs (Bell et al., 2013)
Stat6	Promotes Th2-polarizing DCs (Arima et al., 2010)
Tgfbr2	TGF-β signaling and Treg induction (Ramalingam et al., 2012)

DC migration

Cxcr4	DC migration(Kabashima et al., 2007)
L1cam	Promotes DC transendothelial migration (Maddaluno et al., 2009)
Nfkb2	Steady-state migration of DCs (Azukizawa et al., 2011)
Nr4a3	Promotes CD103⁺ DC migration (Park et al., 2016)

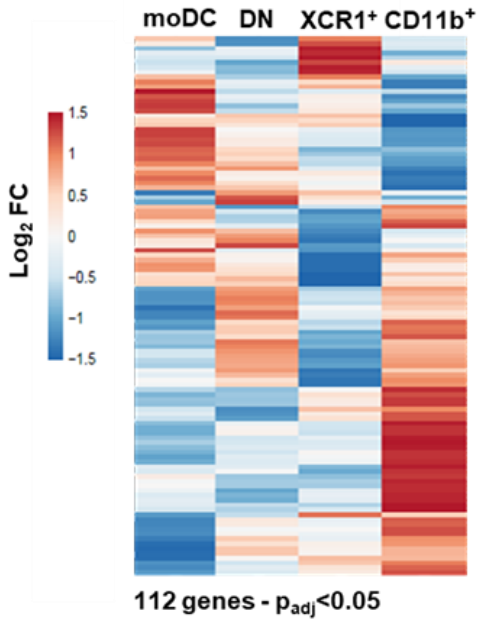
DC antigen presentation

Prdm1	Modulates MHC II presentation on DCs (Kim et al., 2017)
Pirb	Prevents cross-priming (Endo et al., 2008)

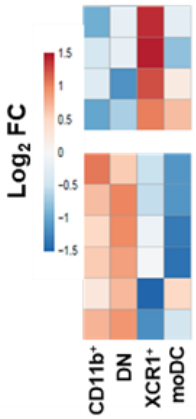
Other genes

Cd8a	DC lineage marker
Itgb7	Gut homing (Wang et al., 2010)
Runx1	Flt3⁺ DC development (Satpathy et al., 2014)
Runx2	pDC development and migration (Sawai et al., 2013)

C



D



Genes	Association
Vmp1	autophagy
Tmem79	exocytosis regulation
H2-M2	MHC class 1b
Slamf7	cell adhesion molecule
Cd109	TGFβ binding
CD274	Impaired T cell function
CD80	T cell co-stimulation
il9r	Th2/Th9 immunity
Bco1	retinoic acid pathway
s1004a	NF-κB signaling

E

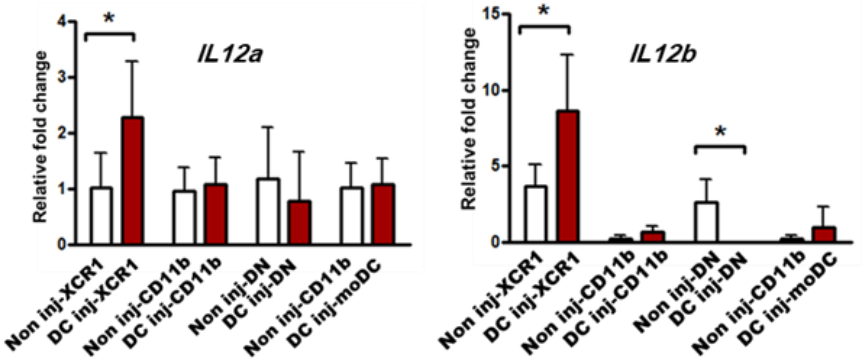


Figure 26 : Endogenous migratory DC subsets have distinct transcriptional changes after bystander activation by BM-DC injection. **A.** PCA for XCR1$^+$, CD11b$^+$ and XCR1$^-$ CD11b$^-$ endogenous migratory DCs from popliteal lymph nodes nodes before and 48h after immunization, and CD11b$^+$ CD64$^+$ Ly6C$^-$MoDCs compared to CD11b$^+$ DCs after immunization. **B.** Genes downregulated in XCR1$^+$ DCs 48h after immunization according to the GOrilla analysis tool. Only pathways with a Benjamini and Hochberg corrected p-value below 0.05 were considered. Green color: down in XCR1$^+$ DCs only; orange color: down in XCR1$^+$ DCs and MoDCs. **C.** Heatmap plot of the 112 genes that are at least 1.5-fold differentially expressed in one comparison (red: upregulated; blue: downregulated). Plotting was done using Clustvis web tool, Clustering was performed using Pearson's correlation and average linkage. **D.** Heat map showing differential expression of selected immune-related genes before and after immunization; heat map was generated as described in **C.** **E.** qPCR analysis of *Il12a* and *Il12b* expression in the all sorted DC subsets before and after immunization N=3±s.e.m.

H2-M2 which is a TAP-independent surface-expressed MHC class Ib molecule (Moore et al., 2004), Slamf7 which is a signaling lymphocytic activation molecule family member known to activate NK cells in a homotypic fashion (Cruz-Munoz et al., 2009), Vmp1 involved in autophagy (Zhao et al., 2017), and Tmem79, a transmembrane protein involved in the lamellar granules secretory system and skin barrier function (Sasaki et al., 2013), were specifically up-regulated on XCR1$^+$ bystander dDCs which might be involved in cell-cell communication. The bystander DCs also down-regulated genes not related to Th1 induction, such as TGF-β signaling required for Treg induction or promoting Th2 and Th9 immunity (Fig. 26D). *Il12a* and *Il12b* did not appear up-regulated by XCR1$^+$ dDCs in the RNA-seq analysis. Nevertheless, the up-regulation of *Il12a* and *Il12b* on bystander activated XCR1$^+$ dDCs was confirmed with real-time PCR and both genes were found to be specifically up-regulated on the designated XCR1$^+$ subset and not on any of the other bystander activated DC subsets (Fig. 26E). We assume that not all endogenous migratory XCR1$^+$ DCs are activated to become bystander DCs since they may carry out their tolerogenic functions (Muzaki et al., 2016). This may cause a dilution of transcriptional changes serving as bystander signals such as the IL-12 signal and only strongly regulated genes become visible. Together, the transcriptional profiling of XCR1$^+$ migratory bystander cDC1s is characterized by a down-regulation of genes involved in other polarizations than Th1 and the up-regulation of genes such as *Il12a* and *Il12b* required for IL-12p70-mediated Th1 polarization.

4 DISCUSSION

DCs are the dominant immune cells to induce T cell priming *in vivo*. Also, the instruction of T helper cell responses by DCs providing polarizing signals has become generally accepted. Among the Th1 instructing cytokines, IL-12p70 plays a prominent role. Here we addressed, whether the priming capacity and polarizing IL-12p70 signals are derived from injected vaccine DCs. We employed s.c. injection of BM-DCs into mice as a model to test different chimeric situations where injected BM-DCs and recipient mouse strains were bearing different genetic deficiencies. Our model is close to clinical studies where human MoDC vaccines are tested against tumors, due to the fact that GM-CSF generated BM-DCs are monocyte-derived (Lutz et al., 2017).

Our data revealed that s.c. injected vaccine BM-DCs only partially contribute to antigen presentation at an early stage (24h) and they do not contribute to Th1 polarization. A major part of antigen presentation for Th0 induction and the entire capacity for Th1 polarization is mediated by endogenous XCR1$^+$ migratory bystander cDC1s at later time points (48-72h). However, BM-DCs migration to the draining lymph node is strictly required and bystander activation for IL-12 production seems to occur in the lymph node. Our findings argue for a step-wise process of priming naive T cells into an IL-2$^+$ IFN-γ- Th0 phenotype by the injected DCs, followed by a communication between injected BM-DCs and XCR1$^+$ bystander cDC1s. Bystander contact includes transfer of antigen and initiation of IL-12p70 production. This period is followed by contacts of activated IL-12p70$^+$ XCR1$^+$ bystander cDC1s with the Th0 cells to continue antigen presentation and conversion into Th1 polarized cells. RNA sequencing allowed the identification of transcriptional changes during the conversion of endogenous migratory XCR1$^+$ cDC1s into XCR1$^+$ bystander cDC1s. Among those, DC genes known to polarize naive T cells into Treg or Th2/Th9 immune responses or to counteract IL-12 production were down-regulated, while Th1 supporting genes were induced.

4.1 Impact of the study on dendritic cell vaccination strategies

The optimization of DC vaccination protocols has focused mainly on enhancing the activation of generated DCs (Morse et al., 2005; Tsang et al., 2005), their cytokine production profile (Okada et al., 2005), and their migration capacity (Turnis et al., 2010). Other studies attempted to combine the vaccine injection with adjusting the

immunosuppressive milieu of the tumor microenvironment to a more immunogenic one, for example by blocking inhibitory receptors such as PD-1/PD-L1 (Soares et al., 2015).

In this study, we find that endogenous DCs are critically required to induce polarized Th1 responses and enhance Th0 priming by vaccine DCs. We were able to identify $XCR1^+$ endogenous migratory dDCs (cDC1s) as communication partners that take up the message delivered by the injected vaccine DC and are responsible for promoting full-blown Th1 responses. This opens a new level of complexity when considering new strategies for vaccine DC optimization. The requirement of endogenous DCs for optimal anti-tumor DC vaccination is of clinical importance since these patients are treated with immuno-suppressive chemotherapy and are subjected to γ-irradiation that will affect endogenous DC populations. In contrast, the use of CTLA-4 and PD-1 targeted checkpoint inhibitors would not negatively affect endogenous DCs.

This study also sheds light on the question why the IL-1β/TNFα/IL-6/PGE_2 matured vaccine DCs are successful in Th1 priming, despite a lack of IL-12 producing capacity (Gross et al., 2017; Lee et al., 2002; Schuler-Thurner et al., 2002). The addition of PGE_2 is known to improve the yield and function of human DCs matured with TNF-α, IL-1β, and IL-6, and skews them towards a Th1 phenotype (Jonuleit et al., 1997). In vitro experiments however showed that this cocktail skewed T cells towards Th2 responses (Kaliński et al., 1997), and this was attributed to PGE_2 blocking the formation of the bioactive IL-12p70 heterodimer (Kalinski et al., 1998). This always contradicted the obvious clinical outcome of such a maturation protocol.

The fact that Th1 priming is not dependent on IL-12 secretion by monocyte-derived DC immunizations has been reported in pathologies other than tumor; such as in *L. major* challenged mice (Ramirez-Pineda et al., 2004). In our study, IL-12 production strongly supported Th1 polarization, and the $XCR1^+$ migratory cDC1s were the mediators of this function. These cells appear to take over the Th1 polarization function from the injected BM-DCs at a later stage of the T cell response, when the initial antigen presentation phase is terminated and T cells enter their proliferative phase (Mempel et al., 2004; Miller et al., 2004).

4.2 Steady-state production of IL-12p40 by CD103+ migratory DCs

Several studies have shown before that $CD103^+$ migratory DCs were capable of producing IL-12p40 (*Il12b*) at steady-state in the skin draining LNs (Reinhardt et al.,

2006), in mesenteric and hepatic LNs (Everts et al., 2016), and in mediastinal LNs (Conejero et al., 2017). This was clearly demonstrated also in our study by FACS, RNA sequencing and real time PCR. Two of these reports suggested that this steady-state *Il12b* production serves as a suppressor of Th2 and Th17 derived immune responses in a helminth infection model (Everts et al., 2016) and in a house dust mite allergy model (Conejero et al., 2017). These studies however only looked at the *Il12b* subunit using Yet40 mice (Reinhardt et al., 2006) and not at the bioactive IL-12p70 molecule (Macatonia et al., 1995). Homodimers formed from the *Il12b* subunit $(p40)_2$ have been reported by several groups to have immunosuppressive effects and act as an antagonist to the Th1 inducing IL-12p70 by binding to the IL-12 receptor and blocking its activity (Gillessen et al., 1995; Ling et al., 1995). Another group showed that UV irradiated DCs and macrophages failed to produce IL-12p70 and induce Th1 cells but rather produced p40 homodimers and failed to induce Th1 responses while maintaining Th2 priming capabilities (Schmitt and Ullrich, 2000). The fact that $CD103^+$ cDC1s secrete *Il12b* at the steady-state and not the IL-12p70 heterodimer as indicated by our data suggests that *Il12b* is rather required for maintaining the tolerogenic functions of this DC subset (Coombes et al., 2007; Idoyaga et al., 2013) rather than preventing Th2 and Th17 induction as claimed by these studies (Conejero et al., 2017; Everts et al., 2016). The expression profile of $CD103^+$ migratory cDC1s in the Immgen database shows a similar profile with high levels of *Il12b* expression specifically by this subset and not by other LN resident and migratory DC subsets (Miller et al., 2012), and no expression of the *Il12a* molecule. Upon activation of migratory cDC1s under an immunizing condition, they produce *Il12a* which forms the IL-12p70 required for Th1 priming. It is also possible that such *Il12b* steady-state production maintains migratory cDC1s in a “ready” state for IL-12p70 production and Th1 priming. Interestingly, we see steady-state production of *Il12b* by $CD8\alpha^+$ spleen resident cDC1s which further supports the tolerogenic function of *Il12b* since they were shown to be responsible for iTreg induction in a TGF-β dependent manner (Yamazaki et al., 2008).

4.3 Control of immune response by resident XCR1+ compared to migratory XCR1+ cDC1s

While both resident and migratory cDC1s share the expression of XCR1 (Crozat et al., 2011) and were shown to have a unique cross presenting capability to CD8 T cells

(Hildner et al., 2008), the distinction of their functional capabilities is still unclear. In the context of viral infection, migratory cDC1s were shown to be required for priming of CD8 T cells to occur (Allan et al., 2006). The antigen is then transferred to resident cDC1s which appear to cross present viral antigens at later stages of the response (Allan et al., 2006); (Eickhoff et al., 2015). Imaging the viral response in the draining lymph node revealed that CD4 T cells and CD8 T cells were initially primed at different time points and cDC1s serve as platforms for CD4 T cell augmentation of CD 8 T cell response (Brewitz et al., 2017; Eickhoff et al., 2015).

On the other hand, recent studies showed that the migratory cDC1s and not resident cDC1s were uniquely required for promoting tumor immunity. This included their active transport of tumor antigens to tumor draining lymph nodes and also their robust ability to activate naïve CD8 T cells ex vivo following sorting of the various cDC subsets (Roberts et al., 2016; Salmon et al., 2016). Migratory cDC1s appear to transfer a fraction of the tumor antigen they are bearing to other migratory and lymph node resident DCs. However, despite the transfer, isolation of the draining lymph node and testing the different DC subsets *ex vivo* showed that only migratory cDC1s and not resident cDC1s, have the capacity to stimulate naive $CD8^+$ T cells against tumor-associated model antigens (Roberts et al., 2016; Salmon et al., 2016).

In our setup, we find that migratory cDC1s and not resident cDC1s are responsible for promoting Th1 responses later in the immune response, which suggests that unlike in viral responses, BM-DC immunization promotes an immune response resembling the tumor situation in migratory cDC1s. What mechanistically differentiates the antiviral from the antitumor response is yet to be determined.

4.4 Transfer of antigens from vaccine DCs to XCR1+ migratory DCs

Apart from their requirement for promoting Th1 polarization, we also find that $CD103^+$ $XCR1^+$ migratory cDC1s were required for antigen presentation later in the T cell response, indicating antigen transfer from the injected BM-DCs. This is not caused by handover of antigen to $CD103^+$ $XCR1^+$ migratory cDC1s in the skin such as observed for injected apoptotic DCs (Desch et al., 2011; Inaba et al., 1998), since no T cell priming or polarization occurred when antigen-loaded *Ccr7*$^{-/-}$ BM-DC were used for immunization. Antigen transfer in the lymph node was reported to occur mainly from migratory DCs to $CD8^+$ resident DCs after viral infections (Allan et al., 2006; Eickhoff et al., 2015) or via the

conduit system for particulate antigens (Gerner et al., 2015; Sixt et al., 2005). Other reports suggest that migratory DCs also, and especially CD103$^+$ XCR1$^+$ migratory cDC1s, can capture archived antigens from lymphatic endothelial cells (LEC) in the draining lymph nodes when the antigen is cell-associated (Kedl et al., 2017). A similar scenario can possibly account for antigen transfer from vaccine DCs to CD103$^+$ XCR1$^+$ migratory cDC1s. The requirement for endogenous DCs to support optimal CD4$^+$ T cell responses by DC-DC contacts in lymph nodes has been observed before, but the endogenous DC subset was not identified and further bystander function for Th1 polarization was not investigated (Kleindienst and Brocker, 2003). Several mechanisms of antigen transfer between different DC subsets have been suggested, for example: via trogocytosis (cross-dressing) (Zhang et al., 2008), or via exosomes (Segura et al., 2007), both pathways have been implicated in the transfer of peptide-bound MHC molecules and also co-stimulatory molecules. MHC I peptide transfer has been show to involve trogocytosis, where direct cell to cell contact between the donor and the recipient cells, and not exosome exchange was observed (Wakim and Bevan, 2011). Since the study from Kleindienst and colleagues showed that direct cell to cell contact is required for antigen transfer; trogocytosis is the more probable mode of antigen transfer (Kleindienst and Brocker, 2003). Our data suggest that migrated BM-DCs transfer antigen and Th1 polarizing information specifically to XCR1$^+$ bystander DCs in the lymph node. In contrast to IL-12 production, the process of antigen transfer may not require transcriptional changes in the bystander DCs.

4.5 IL-12 independent priming by XCR1+ migratory cDC1s

We observed residual Th1 polarization capacity from XCR1$^+$ migratory cDC1s even when they lacked IL-12 production capabilities as indicated by our *Il12a*$^{-/-}$ recipient experiments. The residual Th1 polarization was completely abolished when migratory DCs were absent in the *Il12a*.*Ccr7*$^{-/-}$model, and specifically when the XCR1$^+$ migratory cDC1s are lacking in *Xcr1*-venus-DTR mice. Although we were not able to identify the mechanism involved in such IL-12 independent priming, CD27-CD70 interactions is still a possible mechanism (Soares et al., 2007). CD70 expression was specifically upregulated on migratory cDC1s 48h after LPS BM-DC injection compared to steady-state by flow cytometry. A study that used activating or blocking CD70 antibodies that were specifically targeted to the uptake receptor DEC-205 on cDC1s showed that CD70 promotes IL-12 independent IFN-γ

production by CD4 T cells. This effect was not seen on DCIR2 expressing CD11b^{+} cDC2s (Soares et al., 2007). It is possible that following antigen transfer from injected BM-DCs to migratory cDC1s, T cells receive IL-12 independent CD27-CD70 signals during recognition of the transferred antigen on the migratory cDC1s during the later phase of the response (48h). The fact that we see CD70 upregulation only at this timepoint might support this hypothesis. CD27-CD70 interactions might explain several models where IL-12 absence did not abolish IFN-γ production (Jankovic et al., 2002; Yang et al., 1999), such as *Il12b*$^{-/-}$ mice that still showed substantial IFN-γ production after *Toxoplasma gondii* infections (Jankovic et al., 2002). This also might explain why patients with genetic deficiencies in IL-12 or IL-12 receptor are still able to produce IFN-γ and avoid infection with most intracellular pathogens (Fieschi et al., 2003). Another group showed that OX40, which is another TNF receptor family member, can augment the function of CD70 and this also is independent of IL-12 production by cDC1s (Sanchez and Kedl, 2012).

4.6 CCR7 knockout as a model to study migratory DC functions

Using the *Il12a*.*Ccr7*$^{-/-}$ mouse model, we noticed that despite the lack of Th1 priming and significantly lower IL-2 production capacity of T cells, there was a significant population of proliferating T cells. This can be explained by the abnormal LN structure and the perturbations of immune homeostasis observed in these mice (Forster et al., 1999). These mice are characterized by the lack of normal distribution of B cell follicles to the outer cortex as well as a paracortical T cell–rich area. They rather display an irregular distribution of B and T cells within the paracortex. Also, B cell follicles with considerably enlarged germinal centers were observed within the paracortex. It is possible that such an abnormal organization of the LN structure results in an increased likelihood of the “more fit” BM-DCs to meet their antigen specific T cell, where they are capable of providing signal 1 and 2 for the initial period of the T cell response. However, since the migratory cDC1s are lacking in this model, the response cannot be propagated and is blocked at the Th0 phase (Openshaw et al., 1995). The Th0 response is also limited since migratory cDC1s are partly required for antigen presentation.

4.7 How is the systemic response propagated?

One finding that we were not able to explain in this study is the robust Th1 polarized response observed in the spleen, even though injected BM-DCs do not reach the spleen neither do they reach LNs that are far from the injection site, which indicates a confined immunization process. Thus, the source of antigen that causes such a response in the spleen remains unclear. Interestingly though, this response is not affected by the absence of *Il12a*, unlike the response in the LN, but is almost absent in MHC $II^{-/-}$ mice, which indicates that endogenous antigen presentation is initiated in the lymph node and is propagated later to the spleen. It is also absent in the spleens of *Il12a*.*Ccr7*$^{-/-}$ mice. This can be attributed to the fact that CCR7 is also required for a normal spleen structure. In these mice, T cells were spread throughout the marginal sinuses and the red pulp in large clusters (Forster et al., 1999), and are incapable of entering the periarteriolar lymphoid sheath (PALS) (Sharma et al., 2015). On the other hand, T cell response in the spleens of *Xcr1*-venus-DTR mice showed a robust Th1 polarization, which points out that a subset other than $CD8\alpha^{+}$ resident cDC1s in the spleen is taking over the antigen presentation and Th1 priming functions. It is possible that this is mediated by an unknown cellular subset that carries the apoptotic remains of the injected DCs from the draining LN through the blood stream to be internalized by the splenic marginal zone (Morelli and Larregina, 2010; Morelli et al., 2003)

4.8 Bystander activation of XCR1 migratory DCs by vaccine DCs

While we didn't identify the molecular interactions responsible for cDC1 communication with BM-DCs following immunization, 4-1BB and 4-1BBL interactions are still a likely candidate. A recent study reported that bystander activation of one DC to another *in trans* via 4-1BB to 4-1BBL interactions may play a role in the immune response to infection or vaccination, and required cell to cell contact (Macdonald et al., 2014). The use of 4-1BB (Kwon et al., 2002) or 4-1BBL (DeBenedette et al., 1999) deficient mice would allow further studies on the role of this interaction in the context of vaccine DC immunization.

Another possible candidate that came up from the RNA sequencing dataset is Slamf7, a signaling lymphocytic activation molecule (SLAM) family member (Cannons et al., 2011), which mediates homotypic interactions with itself on adjacent cell surfaces (Li et al., 2013). We found that it was specifically upregulated on $XCR1^{+}$ migratory DCs after immunization. A recent report showed Slamf7 as an interacting partner for the integrin

CD11b (Chen et al., 2017), which is highly expressed by our injected BM-DCs suggesting that Slamf7-CD11b interaction is a possible route of DC-DC communication.

We provided evidence that direct pathogen 'presentation' of LPS can induce bystander BM-DC activation, while CpG matured BM-DCs did not trans-activate other DCs *in vitro*. CpG activation of TLR9 within intracellular vesicles is precedes surface binding of GpG to DEC-205 and internalization as shown for splenic $CD8^+$ cDC1s (Lahoud et al., 2012). Nevertheless, both LPS-or CpG matured BM-DCs While LPS may remain attached to the cell surface and could be "presented" to bystander DCs, this is largely excluded for CpG oligonucleotides, which are incorporated to trigger the vesicular TLR9. Since also our BM-DCs express DEC-205 as detected by the NLDC-145 antibody (Lutz et al., 1999) the lack of bystander activation in vitro cannot account for a DEC-205 defect but indicates that other bystander mechanisms exist besides trans-activation by pathogen.

4.9 Functional switch of XCR1 migratory DCs after bystander activation

Here we compared the transcriptomes of steady state migratory DCs with bystander DCs. Steady state migratory DCs appear as a semi-mature stage with up-regulated RelB and surface MHC II, CD86, CD40 and CCR7 molecules but lower as compared with pathogen or inflammation matured migratory DCs (Azukizawa et al., 2011; Idoyaga et al., 2013; Ohl et al., 2004), which further up-regulate MHC and costimulation and also produce pro-inflammatory cytokines (Ardouin et al., 2016; Voigtlander et al., 2006). The dermal steady state migratory cDC1 subset (ssm-cDC1), identified by expression of XCR1, CD103 and Langerin, has been characterized transcriptionally and revealed a matured phenotype with expression of RelB, IL-12p40 and CCR7 (Ardouin et al., 2016). Functionally, we found earlier that the ssm-cDC1s converted naive $CD4^+$ T cells into $Foxp3^+$ iTregs (also called pTregs) in a TGF-β dependent manner in the skin-draining lymph nodes (Azukizawa et al., 2011). This TGF-β signature was later confirmed at the transcriptome level (Ardouin et al., 2016). Also, integrin $\alpha v\beta 8$-dependent activation of latent TGF-β contributes to iTreg conversion support peripheral tolerance against the self-antigens presented by ssm-DCs and thereby prevent auto-immunity (Travis et al., 2007). We found that immunogenic bystander cDC1s did not markedly up-regulate typical RNA signatures or markers for DC maturation over the tolerogenic ssm-cDC1 comparison. However, the transcriptional TGF-β signature decreased. This indicates that functionally tolerogenic $XCR1^+$ ssm-cDC1s may be re-programmed in the lymph node to become immunogenic

bystander DCs. Functional plasticity of ssm-Langerhans cells had been shown before by their continued capacity to internalize antigens after migration into the draining lymph nodes (Ruedl et al., 2001). Our previous data showed that BM-DCs that were matured with the inflammatory stimulus TNF maintained maturation plasticity since they could be further stimulated by LPS *in vitro* to release IL-12p70 or *in vivo* by endogenous stimuli after s.c. injection to polarize for Th1 responses (Voigtlander et al., 2006) instead of inducing tolerance by i.v. injection (Menges et al., 2002). Together, the transcriptional changes in bystander cDC1s indicate that they down-regulate steady-state functions and become activated to induce Th1 responses. The data provide evidence that XCR1$^+$ steady state migratory cDC1s can undergo functional re-programming into bystander matured DCs by LPS from the migrated BM-DCs or other bystander signals sensed in the lymph node that appeared inflamed with increased cellularity.

Transcriptional profiling of the XCR1$^+$ migratory DCs showed a signature that shifts away from their capacity to direct Th2/Th9 and Treg polarization after BM-DC immunization. This was specific only for XCR1$^+$ DCs and not observed in other migratory DC subsets. Interestingly, genes involved in DC migration were also down-regulated, which may indicate that the bystander activated DCs terminated migration to stimulate Th1 induction. The RelB binding partner p52 (*Nfkb2*) was among these genes, which we showed previously to be a hallmark of ssm-DCs (Ardouin et al., 2016; Azukizawa et al., 2011; Dohler et al., 2017). Among the up-regulated genes is Tmem79 which might be involved in exocytosis (Sasaki et al., 2013). The secretion by exocytosis of IL-12 is mediated by the SNARE family member VAMP7 (Chiaruttini et al., 2016).

The significant increase in *IL12a* and *IL12b* gene expression by qPCR that we detected specifically in XCR1$^+$ migratory DCs after BM-DC immunization was not observed in by RNA sequencing, we also detected a non-significant increase in IL-1β and CD80 expression by RNA sequencing. While the low number of DCs used for sequencing can attribute to such a discrepancy, it is also possible that the bystander activation signal was diluted by the remaining steady-state XCR1$^+$ migratory cDC1s that still carry out their tolerogenic functions. Such a heterogeneity has been observed by single cell sequencing among LPS-stimulated spleen cells where the DCs clustered differently when compared to the existing marker-based classification (Jaitin et al., 2014). Using our defined transcriptional signature for bystander activated XCR1$^+$ migratory cDC1s, it might be

possible to distinguish them from their steady-state counterpart and specifically target them for enhancing DC vaccination protocols.

In conclusion, our data suggest that Th1 priming by DC vaccination requires endogenous bystander DCs for antigen presentation and IL-12p70 production. The time kinetic experiments suggest a strict requirement for the injected BM-DCs for Th0 priming preceding the bystander DC activation and IL-12 production. Only the $CCR7^+$ migratory but not resident fraction of the $XCR1^+$ cDC1 subset acquired bystander function. These findings are of translational importance for human DC vaccination studies in immuno-compromised tumor patients where the bystander DC activity may be impaired. The dissemination of the systemic response and the exact molecular mechanisms driving the endogenous bystander migratory $XCR1^+$ cDC1 activation are two important points that need further studying.

5 REFERENCES

1. Abolhalaj, M., D. Askmyr, C.A. Sakellariou, K. Lundberg, L. Greiff, and M. Lindstedt. 2018. Profiling dendritic cell subsets in head and neck squamous cell tonsillar cancer and benign tonsils. *Scientific reports* 8:8030.
2. Akashi, K., D. Traver, T. Miyamoto, and I.L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404:193.
3. Alexandre, Y.O., S. Ghilas, C. Sanchez, A. Le Bon, K. Crozat, and M. Dalod. 2016. XCR1+ dendritic cells promote memory CD8+ T cell recall upon secondary infections with Listeria monocytogenes or certain viruses. *The Journal of experimental medicine* 213:75-92.
4. Allan, R.S., J. Waithman, S. Bedoui, C.M. Jones, J.A. Villadangos, Y. Zhan, A.M. Lew, K. Shortman, W.R. Heath, and F.R. Carbone. 2006. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 25:153-162.
5. Altare, F., A. Durandy, D. Lammas, J.-F. Emile, S. Lamhamedi, F. Le Deist, P. Drysdale, E. Jouanguy, R. Döffinger, F. Bernaudin, O. Jeppsson, J.A. Gollob, E. Meinl, A.W. Segal, A. Fischer, D. Kumararatne, and J.-L. Casanova. 1998. Impairment of Mycobacterial Immunity in Human Interleukin-12 Receptor Deficiency. *Science (New York, N.Y.)* 280:1432-1435.
6. Anguille, S., E.L. Smits, N. Cools, H. Goossens, Z.N. Berneman, and V.F. Van Tendeloo. 2009. Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. *Journal of translational medicine* 7:109.
7. Ardouin, L., H. Luche, R. Chelbi, S. Carpentier, A. Shawket, F. Montanana Sanchis, C. Santa Maria, P. Grenot, Y. Alexandre, C. Gregoire, A. Fries, T.P. Vu Manh, S. Tamoutounour, K. Crozat, E. Tomasello, A. Jorquera, E. Fossum, B. Bogen, H. Azukizawa, M. Bajenoff, S. Henri, M. Dalod, and B. Malissen. 2016. Broad and Largely Concordant Molecular Changes Characterize Tolerogenic and Immunogenic Dendritic Cell Maturation in Thymus and Periphery. *Immunity* 45:305-318.
8. Askenase, M.H., S.J. Han, A.L. Byrd, D. Morais da Fonseca, N. Bouladoux, C. Wilhelm, J.E. Konkel, T.W. Hand, N. Lacerda-Queiroz, X.Z. Su, G. Trinchieri, J.R. Grainger, and Y. Belkaid. 2015. Bone-Marrow-Resident NK Cells Prime Monocytes for Regulatory Function during Infection. *Immunity* 42:1130-1142.
9. Ato, M., S. Stager, C.R. Engwerda, and P.M. Kaye. 2002. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nature immunology* 3:1185-1191.
10. Audiger, C., M.J. Rahman, T.J. Yun, K.V. Tarbell, and S. Lesage. 2017. The Importance of Dendritic Cells in Maintaining Immune Tolerance. *Journal of immunology (Baltimore, Md. : 1950)* 198:2223-2231.
11. Azukizawa, H., A. Dohler, N. Kanazawa, A. Nayak, M. Lipp, B. Malissen, I. Autenrieth, I. Katayama, M. Riemann, F. Weih, F. Berberich-Siebelt, and M.B. Lutz. 2011. Steady state migratory RelB+ langerin+ dermal dendritic cells mediate peripheral induction of antigen-specific CD4+ CD25+ Foxp3+ regulatory T cells. *European journal of immunology* 41:1420-1434.
12. Bajana, S., K. Roach, S. Turner, J. Paul, and S. Kovats. 2012. IRF4 promotes cutaneous dendritic cell migration to lymph nodes during homeostasis and inflammation. *J Immunol* 189:3368-3377.
13. Bajenoff, M., S. Granjeaud, and S. Guerder. 2003. The strategy of T cell antigen-presenting cell encounter in antigen-draining lymph nodes revealed by imaging of initial T cell activation. *The Journal of experimental medicine* 198:715-724.
14. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
15. Bar-On, L., T. Birnberg, K.L. Lewis, B.T. Edelson, D. Bruder, K. Hildner, J. Buer, K.M. Murphy, B. Reizis, and S. Jung. 2010. CX3CR1+ CD8alpha+ dendritic cells are a steady-state population related to plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A* 107:14745-14750.
16. Barnden, M.J., J. Allison, W.R. Heath, and F.R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunology and cell biology* 76:34-40.

17. Basu, R., D.B. O'Quinn, D.J. Silberger, T.R. Schoeb, L. Fouser, W. Ouyang, R.D. Hatton, and C.T. Weaver. 2012. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity* 37:1061-1075.
18. Becker, A.M., D.G. Michael, A.T. Satpathy, R. Sciammas, H. Singh, and D. Bhattacharya. 2012. IRF-8 extinguishes neutrophil production and promotes dendritic cell lineage commitment in both myeloid and lymphoid mouse progenitors. 119:2003-2012.
19. Berberich, C., J.R. Ramirez-Pineda, C. Hambrecht, G. Alber, Y.A. Skeiky, and H. Moll. 2003. Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. *J Immunol* 170:3171-3179.
20. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
21. Bol, K.F., G. Schreibelt, W.R. Gerritsen, I.J. de Vries, and C.G. Figdor. 2016. Dendritic Cell-Based Immunotherapy: State of the Art and Beyond. *Clinical cancer research : an official journal of the American Association for Cancer Research* 22:1897-1906.
22. Bonilla, F.A., and H.C. Oettgen. 2010. Adaptive immunity. *The Journal of allergy and clinical immunology* 125:S33-40.
23. Braun, A., T. Worbs, G.L. Moschovakis, S. Halle, K. Hoffmann, J. Bolter, A. Munk, and R. Forster. 2011. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nature immunology* 12:879-887.
24. Brewitz, A., S. Eickhoff, S. Dahling, T. Quast, S. Bedoui, R.A. Kroczek, C. Kurts, N. Garbi, W. Barchet, M. Iannacone, F. Klauschen, W. Kolanus, T. Kaisho, M. Colonna, R.N. Germain, and W. Kastenmuller. 2017. CD8(+) T Cells Orchestrate pDC-XCR1(+) Dendritic Cell Spatial and Functional Cooperativity to Optimize Priming. *Immunity* 46:205-219.
25. Briseño, C.G., M. Gargaro, V. Durai, J.T. Davidson, D.J. Theisen, D.A. Anderson, 3rd, D.V. Novack, T.L. Murphy, and K.M. Murphy. 2017. Deficiency of transcription factor RelB perturbs myeloid and DC development by hematopoietic-extrinsic mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* 114:3957-3962.
26. Burkett, P.R., R. Koka, M. Chien, S. Chai, D.L. Boone, and A. Ma. 2004. Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *The Journal of experimental medicine* 200:825-834.
27. Calabro, S., D. Liu, A. Gallman, M.S. Nascimento, Z. Yu, T.T. Zhang, P. Chen, B. Zhang, L. Xu, U. Gowthaman, J.K. Krishnaswamy, A.M. Haberman, A. Williams, and S.C. Eisenbarth. 2016. Differential Intrasplenic Migration of Dendritic Cell Subsets Tailors Adaptive Immunity. *Cell reports* 16:2472-2485.
28. Cannons, J.L., S.G. Tangye, and P.L. Schwartzberg. 2011. SLAM family receptors and SAP adaptors in immunity. *Annual review of immunology* 29:665-705.
29. Carotta, S., A. Dakic, A. D'Amico, S.H. Pang, K.T. Greig, S.L. Nutt, and L. Wu. 2010. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity* 32:628-641.
30. Caton, M.L., M.R. Smith-Raska, and B. Reizis. 2007. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *The Journal of experimental medicine* 204:1653-1664.
31. Chen, J., M.C. Zhong, H. Guo, D. Davidson, S. Mishel, Y. Lu, I. Rhee, L.A. Perez-Quintero, S. Zhang, M.E. Cruz-Munoz, N. Wu, D.C. Vinh, M. Sinha, V. Calderon, C.A. Lowell, J.S. Danska, and A. Veillette. 2017. SLAMF7 is critical for phagocytosis of haematopoietic tumour cells via Mac-1 integrin. *Nature* 544:493-497.
32. Cheong, C., I. Matos, J.-H. Choi, D.B. Dandamudi, E. Shrestha, M.P. Longhi, K.L. Jeffrey, R.M. Anthony, C. Kluger, G. Nchinda, H. Koh, A. Rodriguez, J. Idoyaga, M. Pack, K. Velinzon, C.G. Park, and R.M. Steinman. 2010. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143:416-429.

33. Chiaruttini, G., G.M. Piperno, M. Jouve, F. De Nardi, P. Larghi, A.A. Peden, G. Baj, S. Muller, S. Valitutti, T. Galli, and F. Benvenuti. 2016. The SNARE VAMP7 Regulates Exocytic Trafficking of Interleukin-12 in Dendritic Cells. *Cell reports* 14:2624-2636.
34. Cisse, B., M.L. Caton, M. Lehner, T. Maeda, S. Scheu, R. Locksley, D. Holmberg, C. Zweier, N.S. den Hollander, S.G. Kant, W. Holter, A. Rauch, Y. Zhuang, and B. Reizis. 2008. Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development. *Cell* 135:37-48.
35. Colonna, M., G. Trinchieri, and Y.J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nature immunology* 5:1219-1226.
36. Conejero, L., S.C. Khouili, S. Martínez-Cano, H.M. Izquierdo, P. Brandi, and D. Sancho. 2017. Lung CD103+ dendritic cells restrain allergic airway inflammation through IL-12 production. *JCI insight* 2:e90420.
37. Conrad, C., J. Gregorio, Y.H. Wang, T. Ito, S. Meller, S. Hanabuchi, S. Anderson, N. Atkinson, P.T. Ramirez, Y.J. Liu, R. Freedman, and M. Gilliet. 2012. Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. *Cancer research* 72:5240-5249.
38. Cook, D.N., D.M. Prosser, R. Forster, J. Zhang, N.A. Kuklin, S.J. Abbondanzo, X.D. Niu, S.C. Chen, D.J. Manfra, M.T. Wiekowski, L.M. Sullivan, S.R. Smith, H.B. Greenberg, S.K. Narula, M. Lipp, and S.A. Lira. 2000. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 12:495-503.
39. Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of experimental medicine* 204:1757-1764.
40. Crozat, K., S. Tamoutounour, T.-P. Vu Manh, E. Fossum, H. Luche, L. Ardouin, M. Guilliams, H. Azukizawa, B. Bogen, B. Malissen, S. Henri, and M. Dalod. 2011. Cutting Edge: Expression of XCR1 Defines Mouse Lymphoid-Tissue Resident and Migratory Dendritic Cells of the CD8α⁺ Type. 187:4411-4415.
41. Cruz-Munoz, M.E., Z. Dong, X. Shi, S. Zhang, and A. Veillette. 2009. Influence of CRACC, a SLAM family receptor coupled to the adaptor EAT-2, on natural killer cell function. *Nature immunology* 10:297-305.
42. De Koker, S., L. Van Hoecke, A. De Beuckelaer, K. Roose, K. Deswarte, M.A. Willart, P. Bogaert, T. Naessens, B.G. De Geest, X. Saelens, B.N. Lambrecht, and J. Grooten. 2017. Inflammatory monocytes regulate Th1 oriented immunity to CpG adjuvanted protein vaccines through production of IL-12. *Scientific reports* 7:5986.
43. DeBenedette, M.A., A. Shahinian, T.W. Mak, and T.H. Watts. 1997. Costimulation of CD28- T lymphocytes by 4-1BB ligand. *The Journal of Immunology* 158:551.
44. DeBenedette, M.A., T. Wen, M.F. Bachmann, P.S. Ohashi, B.H. Barber, K.L. Stocking, J.J. Peschon, and T.H. Watts. 1999. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J Immunol* 163:4833-4841.
45. Desch, A.N., G.J. Randolph, K. Murphy, E.L. Gautier, R.M. Kedl, M.H. Lahoud, I. Caminschi, K. Shortman, P.M. Henson, and C.V. Jakubzick. 2011. CD103+ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *The Journal of experimental medicine* 208:1789-1797.
46. Diehl, L., G.J. van Mierlo, A.T. den Boer, E. van der Voort, M. Fransen, L. van Bostelen, P. Krimpenfort, C.J. Melief, R. Mittler, R.E. Toes, and R. Offringa. 2002. In vivo triggering through 4-1BB enables Th-independent priming of CTL in the presence of an intact CD28 costimulatory pathway. *J Immunol* 168:3755-3762.
47. Dobin, A., C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T.R. Gingeras. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* 29:15-21.

48. Dohler, A., T. Schneider, I. Eckert, E. Ribechini, N. Andreas, M. Riemann, B. Reizis, F. Weih, and M.B. Lutz. 2017. RelB(+) Steady-State Migratory Dendritic Cells Control the Peripheral Pool of the Natural Foxp3(+) Regulatory T Cells. *Frontiers in immunology* 8:726.
49. Dudziak, D., A.O. Kamphorst, G.F. Heidkamp, V.R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H.-W. Lee, C.G. Park, R.M. Steinman, and M.C. Nussenzweig. 2007. Differential Antigen Processing by Dendritic Cell Subsets in Vivo. 315:107-111.
50. Edelson, B.T., T.R. Bradstreet, K. Hildner, J.A. Carrero, K.E. Frederick, W. Kc, R. Belizaire, T. Aoshi, R.D. Schreiber, M.J. Miller, T.L. Murphy, E.R. Unanue, and K.M. Murphy. 2011. CD8alpha(+) dendritic cells are an obligate cellular entry point for productive infection by Listeria monocytogenes. *Immunity* 35:236-248.
51. Edgar, R., M. Domrachev, and A.E. Lash. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* 30:207-210.
52. Eickhoff, S., A. Brewitz, M.Y. Gerner, F. Klauschen, K. Komander, H. Hemmi, N. Garbi, T. Kaisho, R.N. Germain, and W. Kastenmuller. 2015. Robust Anti-viral Immunity Requires Multiple Distinct T Cell-Dendritic Cell Interactions. *Cell* 162:1322-1337.
53. Eisenring, M., J. vom Berg, G. Kristiansen, E. Saller, and B. Becher. 2010. IL-12 initiates tumor rejection via lymphoid tissue-inducer cells bearing the natural cytotoxicity receptor NKp46. *Nature immunology* 11:1030-1038.
54. Elster, J.D., D.K. Krishnadas, and K.G. Lucas. 2016. Dendritic cell vaccines: A review of recent developments and their potential pediatric application. *Human vaccines & immunotherapeutics* 12:2232-2239.
55. Everts, B., R. Tussiwand, L. Dreesen, K.C. Fairfax, S.C.-C. Huang, A.M. Smith, C.M. O'Neill, W.Y. Lam, B.T. Edelson, J.F. Urban, K.M. Murphy, and E.J. Pearce. 2016. Migratory CD103⁺ dendritic cells suppress helminth-driven type 2 immunity through constitutive expression of IL-12. 213:35-51.
56. Fieschi, C., S. Dupuis, E. Catherinot, J. Feinberg, J. Bustamante, A. Breiman, F. Altare, R. Baretto, F. Le Deist, S. Kayal, H. Koch, D. Richter, M. Brezina, G. Aksu, P. Wood, S. Al-Jumaah, M. Raspall, A.J. da Silva Duarte, D. Tuerlinckx, J.-L. Virelizier, A. Fischer, A. Enright, J. Bernhöft, A.M. Cleary, C. Vermylen, C. Rodriguez-Gallego, G. Davies, R. Blütters-Sawatzki, C.-A. Siegrist, M.S. Ehlayel, V. Novelli, W.H. Haas, J. Levy, J. Freihorst, S. Al-Hajjar, D. Nadal, D. de Moraes Vasconcelos, O. Jeppsson, N. Kutukculer, K. Frecerova, I. Caragol, D. Lammas, D.S. Kumararatne, L. Abel, and J.-L. Casanova. 2003. Low Penetrance, Broad Resistance, and Favorable Outcome of Interleukin 12 Receptor β1 Deficiency. *Medical and Immunological Implications* 197:527-535.
57. Firestein, G.S., W.D. Roeder, J.A. Laxer, K.S. Townsend, C.T. Weaver, J.T. Hom, J. Linton, B.E. Torbett, and A.L. Glasebrook. 1989. A new murine CD4+ T cell subset with an unrestricted cytokine profile. 143:518-525.
58. Fogg, D.K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D.R. Littman, A. Cumano, and F. Geissmann. 2006. A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells. 311:83-87.
59. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99:23-33.
60. Freeman, G., J. Gribben, V. Boussiotis, J. Ng, V. Restivo, L. Lombard, G. Gray, and L. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. 262:909-911.
61. Fukaya, T., R. Murakami, H. Takagi, K. Sato, Y. Sato, H. Otsuka, M. Ohno, A. Hijikata, O. Ohara, M. Hikida, B. Malissen, and K. Sato. 2012. Conditional ablation of CD205+ conventional dendritic cells impacts the regulation of T-cell immunity and homeostasis in vivo. *Proc Natl Acad Sci U S A* 109:11288-11293.
62. Futagawa, T., H. Akiba, T. Kodama, K. Takeda, Y. Hosoda, H. Yagita, and K. Okumura. 2002. Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *International immunology* 14:275-286.

63. Gaiser, M.R., T. Lammermann, X. Feng, B.Z. Igyarto, D.H. Kaplan, L. Tessarollo, R.N. Germain, and M.C. Udey. 2012. Cancer-associated epithelial cell adhesion molecule (EpCAM; CD326) enables epidermal Langerhans cell motility and migration in vivo. *Proc Natl Acad Sci U S A* 109:E889-897.
64. Gao, Y., S.A. Nish, R. Jiang, L. Hou, P. Licona-Limon, J.S. Weinstein, H. Zhao, and R. Medzhitov. 2013. Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* 39:722-732.
65. Gatto, D., K. Wood, I. Caminschi, D. Murphy-Durland, P. Schofield, D. Christ, G. Karupiah, and R. Brink. 2013. The chemotactic receptor EBI2 regulates the homeostasis, localization and immunological function of splenic dendritic cells. *Nature immunology* 14:446-453.
66. Gavin, M.A., S.R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nature immunology* 3:33-41.
67. Ge, Y., H. Xi, S. Ju, and X. Zhang. 2013. Blockade of PD-1/PD-L1 immune checkpoint during DC vaccination induces potent protective immunity against breast cancer in hu-SCID mice. *Cancer letters* 336:253-259.
68. Gerner, Michael Y., P. Torabi-Parizi, and Ronald N. Germain. 2015. Strategically Localized Dendritic Cells Promote Rapid T Cell Responses to Lymph-Borne Particulate Antigens. *Immunity* 42:172-185.
69. Ghosh, H.S., B. Cisse, A. Bunin, K.L. Lewis, and B. Reizis. 2010. Continuous Expression of the Transcription Factor E2-2 Maintains the Cell Fate of Mature Plasmacytoid Dendritic Cells. *Immunity* 33:905-916.
70. Gillessen, S., D. Carvajal, P. Ling, F.J. Podlaski, D.L. Stremlo, P.C. Familletti, U. Gubler, D.H. Presky, A.S. Stern, and M.K. Gately. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *European journal of immunology* 25:200-206.
71. Ginhoux, F., M.P. Collin, M. Bogunovic, M. Abel, M. Leboeuf, J. Helft, J. Ochando, A. Kissenpfennig, B. Malissen, M. Grisotto, H. Snoeck, G. Randolph, and M. Merad. 2007. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *The Journal of experimental medicine* 204:3133-3146.
72. Ginhoux, F., K. Liu, J. Helft, M. Bogunovic, M. Greter, D. Hashimoto, J. Price, N. Yin, J. Bromberg, S.A. Lira, E.R. Stanley, M. Nussenzweig, and M. Merad. 2009. The origin and development of nonlymphoid tissue CD103+ DCs. *The Journal of experimental medicine* 206:3115-3130.
73. Ginhoux, F., and M. Merad. 2010. Ontogeny and homeostasis of Langerhans cells. *Immunology and cell biology* 88:387-392.
74. Goodwin, R.G., W.S. Din, T. Davis-Smith, D.M. Anderson, S.D. Gimpel, T.A. Sato, C.R. Maliszewski, C.I. Brannan, N.G. Copeland, N.A. Jenkins, and et al. 1993. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *European journal of immunology* 23:2631-2641.
75. Granot, T., T. Senda, D.J. Carpenter, N. Matsuoka, J. Weiner, C.L. Gordon, M. Miron, B.V. Kumar, A. Griesemer, S.H. Ho, H. Lerner, J.J.C. Thome, T. Connors, B. Reizis, and D.L. Farber. 2017. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. *Immunity* 46:504-515.
76. Greter, M., J. Helft, A. Chow, D. Hashimoto, A. Mortha, J. Agudo-Cantero, M. Bogunovic, Emmanuel L. Gautier, J. Miller, M. Leboeuf, G. Lu, C. Aloman, Brian D. Brown, Jeffrey W. Pollard, H. Xiong, Gwendalyn J. Randolph, Jerry E. Chipuk, Paul S. Frenette, and M. Merad. 2012. GM-CSF Controls Nonlymphoid Tissue Dendritic Cell Homeostasis but Is Dispensable for the Differentiation of Inflammatory Dendritic Cells. *Immunity* 36:1031-1046.
77. Grigorova, I.L., M. Panteleev, and J.G. Cyster. 2010. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure. *Proc Natl Acad Sci U S A* 107:20447-20452.
78. Gross, S., M. Erdmann, I. Haendle, S. Voland, T. Berger, E. Schultz, E. Strasser, P. Dankerl, R. Janka, S. Schliep, L. Heinzerling, K. Sotlar, P. Coulie, G. Schuler, and B. Schuler-Thurner. 2017. Twelve-year survival and immune correlates in dendritic cell-vaccinated melanoma patients. *JCI insight* 2:e91438.

79. Guilliams, M., C.-A. Dutertre, Charlotte L. Scott, N. McGovern, D. Sichien, S. Chakarov, S. Van Gassen, J. Chen, M. Poidinger, S. De Prijck, Simon J. Tavernier, I. Low, Sergio E. Irac, Citra N. Mattar, Hermi R. Sumatoh, Gillian Hui L. Low, Tam John K. Chung, Dedrick Kok H. Chan, Ker K. Tan, Tony Lim K. Hon, E. Fossum, B. Bogen, M. Choolani, Jerry Kok Y. Chan, A. Larbi, H. Luche, S. Henri, Y. Saeys, Evan W. Newell, Bart N. Lambrecht, B. Malissen, and F. Ginhoux. 2016. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity* 45:669-684.
80. Halim, T.Y., C.A. Steer, L. Matha, M.J. Gold, I. Martinez-Gonzalez, K.M. McNagny, A.N. McKenzie, and F. Takei. 2014. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40:425-435.
81. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M.A. Willart, M. Kool, F. Muskens, and B.N. Lambrecht. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *The Journal of experimental medicine* 207:2097-2111.
82. Hanke, N.T., C.J. LaCasse, C.B. Larmonier, D. Alizadeh, M. Trad, N. Janikashvili, B. Bonnotte, E. Katsanis, and N. Larmonier. 2014. PIAS1 and STAT-3 impair the tumoricidal potential of IFN-γ-stimulated mouse dendritic cells generated with IL-15. *European journal of immunology* 44:2489-2499.
83. Hasegawa, H., and T. Matsumoto. 2018. Mechanisms of Tolerance Induction by Dendritic Cells In Vivo. *Frontiers in immunology* 9:350-350.
84. Heinisch, I.V., C. Bizer, W. Volgger, and H.U. Simon. 2001. Functional CD137 receptors are expressed by eosinophils from patients with IgE-mediated allergic responses but not by eosinophils from patients with non-IgE-mediated eosinophilic disorders. *The Journal of allergy and clinical immunology* 108:21-28.
85. Heinisch, I.V., I. Daigle, B. Knopfli, and H.U. Simon. 2000. CD137 activation abrogates granulocyte-macrophage colony-stimulating factor-mediated anti-apoptosis in neutrophils. *European journal of immunology* 30:3441-3446.
86. Heinrich, P.C., J.V. Castell, and T. Andus. 1990. Interleukin-6 and the acute phase response. *The Biochemical journal* 265:621-636.
87. Hiasa, M., M. Abe, A. Nakano, A. Oda, H. Amou, S. Kido, K. Takeuchi, K. Kagawa, K. Yata, T. Hashimoto, S. Ozaki, K. Asaoka, E. Tanaka, K. Moriyama, and T. Matsumoto. 2009. GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF-α converting enzyme (TACE). *Blood* 114:4517-4526.
88. Hildner, K., B.T. Edelson, W.E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B.U. Schraml, E.R. Unanue, M.S. Diamond, R.D. Schreiber, T.L. Murphy, and K.M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science (New York, N.Y.)* 322:1097-1100.
89. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772.
90. Hor, J.L., P.G. Whitney, A. Zaid, A.G. Brooks, W.R. Heath, and S.N. Mueller. 2015. Spatiotemporally Distinct Interactions with Dendritic Cell Subsets Facilitates CD4+ and CD8+ T Cell Activation to Localized Viral Infection. *Immunity* 43:554-565.
91. Hunger, R.E., P.A. Sieling, M.T. Ochoa, M. Sugaya, A.E. Burdick, T.H. Rea, P.J. Brennan, J.T. Belisle, A. Blauvelt, S.A. Porcelli, and R.L. Modlin. 2004. Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *The Journal of clinical investigation* 113:701-708.
92. Ichikawa, E., S. Hida, Y. Omatsu, S. Shimoyama, K. Takahara, S. Miyagawa, K. Inaba, and S. Taki. 2004. Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. *Proc Natl Acad Sci U S A* 101:3909-3914.
93. Idoyaga, J., C. Fiorese, L. Zbytnuik, A. Lubkin, J. Miller, B. Malissen, D. Mucida, M. Merad, and R.M. Steinman. 2013. Specialized role of migratory dendritic cells in peripheral tolerance induction. *The Journal of clinical investigation* 123:844-854.

94. Igyarto, B.Z., K. Haley, D. Ortner, A. Bobr, M. Gerami-Nejad, B.T. Edelson, S.M. Zurawski, B. Malissen, G. Zurawski, J. Berman, and D.H. Kaplan. 2011. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 35:260-272.
95. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *The Journal of experimental medicine* 176:1693-1702.
96. Inaba, K., J.P. Metlay, M.T. Crowley, M. Witmer-Pack, and R.M. Steinman. 1990. Dendritic cells as antigen presenting cells in vivo. *International reviews of immunology* 6:197-206.
97. Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, M. Albert, N. Bhardwaj, I. Mellman, and R.M. Steinman. 1998. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *The Journal of experimental medicine* 188:2163-2173.
98. Ingulli, E., D.R. Ulman, M.M. Lucido, and M.K. Jenkins. 2002. In situ analysis reveals physical interactions between CD11b+ dendritic cells and antigen-specific CD4 T cells after subcutaneous injection of antigen. *J Immunol* 169:2247-2252.
99. Ippolito, G.C., J.D. Dekker, Y.H. Wang, B.K. Lee, A.L. Shaffer, 3rd, J. Lin, J.K. Wall, B.S. Lee, L.M. Staudt, Y.J. Liu, V.R. Iyer, and H.O. Tucker. 2014. Dendritic cell fate is determined by BCL11A. *Proc Natl Acad Sci U S A* 111:E998-1006.
100. Ito, T., M. Inaba, K. Inaba, J. Toki, S. Sogo, T. Iguchi, Y. Adachi, K. Yamaguchi, R. Amakawa, J. Valladeau, S. Saeland, S. Fukuhara, and S. Ikehara. 1999. A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. J Immunol 163:1409-1419.
101. Iwasaki, A., and B.L. Kelsall. 2000. Localization of Distinct Peyer's Patch Dendritic Cell Subsets and Their Recruitment by Chemokines Macrophage Inflammatory Protein (Mip)-3α, Mip-3β, and Secondary Lymphoid Organ Chemokine. 191:1381-1394.
102. Iwasaki, A., and B.L. Kelsall. 2001. Unique Functions of CD11b⁺, CD8α⁺, and Double-Negative Peyer's Patch Dendritic Cells. 166:4884-4890.
103. Jaitin, D.A., E. Kenigsberg, H. Keren-Shaul, N. Elefant, F. Paul, I. Zaretsky, A. Mildner, N. Cohen, S. Jung, A. Tanay, and I. Amit. 2014. Massively Parallel Single-Cell RNA-Seq for Marker-Free Decomposition of Tissues into Cell Types. 343:776-779.
104. Jankovic, D., M.C. Kullberg, S. Hieny, P. Caspar, C.M. Collazo, and A. Sher. 2002. In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting. *Immunity* 16:429-439.
105. Jiang, D., Y. Chen, and H. Schwarz. 2008a. CD137 induces proliferation of murine hematopoietic progenitor cells and differentiation to macrophages. *J Immunol* 181:3923-3932.
106. Jiang, D., P.S. Yue, D. Drenkard, and H. Schwarz. 2008b. Induction of proliferation and monocytic differentiation of human CD34+ cells by CD137 ligand signaling. *Stem cells (Dayton, Ohio)* 26:2372-2381.
107. Jiang, W., W.J. Swiggard, C. Heufler, M. Peng, A. Mirza, R.M. Steinman, and M.C. Nussenzweig. 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* 375:151-155.
108. Jong, R.d., F. Altare, I.-A. Haagen, D.G. Elferink, T.d. Boer, P.J.C. van Breda Vriesman, P.J. Kabel, J.M.T. Draaisma, J.T. van Dissel, F.P. Kroon, J.-L. Casanova, and T.H.M. Ottenhoff. 1998. Severe Mycobacterial and Salmonella Infections in Interleukin-12 Receptor-Deficient Patients. *Science (New York, N.Y.)* 280:1435-1438.
109. Jonuleit, H., U. Kuhn, G. Muller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A.H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *European journal of immunology* 27:3135-3142.

110. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E.G. Pamer, D.R. Littman, and R.A. Lang. 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 17:211-220.
111. Kabashima, K., N. Shiraishi, K. Sugita, T. Mori, A. Onoue, M. Kobayashi, J. Sakabe, R. Yoshiki, H. Tamamura, N. Fujii, K. Inaba, and Y. Tokura. 2007. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *The American journal of pathology* 171:1249-1257.
112. Kaliński, P., C.M. Hilkens, A. Snijders, F.G. Snijdewint, and M.L. Kapsenberg. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. 159:28-35.
113. Kalinski, P., J.H. Schuitemaker, C.M. Hilkens, and M.L. Kapsenberg. 1998. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol* 161:2804-2809.
114. Kang, Y.J., S.O. Kim, S. Shimada, M. Otsuka, A. Seit-Nebi, B.S. Kwon, T.H. Watts, and J. Han. 2007. Cell surface 4-1BBL mediates sequential signaling pathways 'downstream' of TLR and is required for sustained TNF production in macrophages. *Nature immunology* 8:601-609.
115. Kantoff, P.W., C.S. Higano, N.D. Shore, E.R. Berger, E.J. Small, D.F. Penson, C.H. Redfern, A.C. Ferrari, R. Dreicer, R.B. Sims, Y. Xu, M.W. Frohlich, and P.F. Schellhammer. 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *The New England journal of medicine* 363:411-422.
116. Kapsenberg, M.L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nature reviews. Immunology* 3:984-993.
117. Karsunky, H., M. Merad, A. Cozzio, I.L. Weissman, and M.G. Manz. 2003. Flt3 Ligand Regulates Dendritic Cell Development from Flt3⁺ Lymphoid and Myeloid-committed Progenitors to Flt3⁺ Dendritic Cells In Vivo. 198:305-313.
118. Kashem, S.W., M.S. Riedl, C. Yao, C.N. Honda, L. Vulchanova, and D.H. Kaplan. 2015. Nociceptive Sensory Fibers Drive Interleukin-23 Production from CD301b+ Dermal Dendritic Cells and Drive Protective Cutaneous Immunity. *Immunity* 43:515-526.
119. Kautz-Neu, K., M. Noordegraaf, S. Dinges, C.L. Bennett, D. John, B.E. Clausen, and E. von Stebut. 2011. Langerhans cells are negative regulators of the anti-Leishmania response. *The Journal of experimental medicine* 208:885-891.
120. Kedl, R.M., R.S. Lindsay, J.M. Finlon, E.D. Lucas, R.S. Friedman, and B.A.J. Tamburini. 2017. Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nature Communications* 8:2034.
121. Kingston, D., M.A. Schmid, N. Onai, A. Obata-Onai, D. Baumjohann, and M.G. Manz. 2009. The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. *Blood* 114:835-843.
122. Kinnebrew, M.A., C.G. Buffie, G.E. Diehl, L.A. Zenewicz, I. Leiner, T.M. Hohl, R.A. Flavell, D.R. Littman, and E.G. Pamer. 2012. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity* 36:276-287.
123. Kissenpfennig, A., S. Henri, B. Dubois, C. Laplace-Builhe, P. Perrin, N. Romani, C.H. Tripp, P. Douillard, L. Leserman, D. Kaiserlian, S. Saeland, J. Davoust, and B. Malissen. 2005. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22:643-654.
124. Klechevsky, E., R. Morita, M. Liu, Y. Cao, S. Coquery, L. Thompson-Snipes, F. Briere, D. Chaussabel, G. Zurawski, A.K. Palucka, Y. Reiter, J. Banchereau, and H. Ueno. 2008. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity* 29:497-510.
125. Kleindienst, P., and T. Brocker. 2003. Endogenous dendritic cells are required for amplification of T cell responses induced by dendritic cell vaccines in vivo. *J Immunol* 170:2817-2823.

126. Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell* 91:661-672.
127. Kranz, L.M., M. Diken, H. Haas, S. Kreiter, C. Loquai, K.C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Husemann, A. Selmi, A.N. Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R.A. Jabulowsky, S. Heesch, J. Hassel, P. Langguth, S. Grabbe, C. Huber, O. Tureci, and U. Sahin. 2016. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* 534:396-401.
128. Krishnadas, D.K., S. Shusterman, F. Bai, L. Diller, J.E. Sullivan, A.C. Cheerva, R.E. George, and K.G. Lucas. 2015. A phase I trial combining decitabine/dendritic cell vaccine targeting MAGE-A1, MAGE-A3 and NY-ESO-1 for children with relapsed or therapy-refractory neuroblastoma and sarcoma. *Cancer immunology, immunotherapy : CII* 64:1251-1260.
129. Krishnaswamy, J.K., U. Gowthaman, B. Zhang, J. Mattsson, L. Szeponik, D. Liu, R. Wu, T. White, S. Calabro, L. Xu, M.A. Collet, M. Yurieva, S. Alsen, P. Fogelstrand, A. Walter, W.R. Heath, S.N. Mueller, U. Yrlid, A. Williams, and S.C. Eisenbarth. 2017. Migratory CD11b(+) conventional dendritic cells induce T follicular helper cell-dependent antibody responses. *Science immunology* 2:
130. Kwon, B.S., J.C. Hurtado, Z.H. Lee, K.B. Kwack, S.K. Seo, B.K. Choi, B.H. Koller, G. Wolisi, H.E. Broxmeyer, and D.S. Vinay. 2002. Immune responses in 4-1BB (CD137)-deficient mice. *J Immunol* 168:5483-5490.
131. Labeur, M.S., B. Roters, B. Pers, A. Mehling, T.A. Luger, T. Schwarz, and S. Grabbe. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* 162:168-175.
132. Lahoud, M.H., F. Ahmet, J.-G. Zhang, S. Meuter, A.N. Policheni, S. Kitsoulis, C.-N. Lee, M. O'Keeffe, L.C. Sullivan, A.G. Brooks, R. Berry, J. Rossjohn, J.D. Mintern, J. Vega-Ramos, J.A. Villadangos, N.A. Nicola, M.C. Nussenzweig, K.J. Stacey, K. Shortman, W.R. Heath, and I. Caminschi. 2012. DEC-205 is a cell surface receptor for CpG oligonucleotides. 109:16270-16275.
133. Langstein, J., J. Michel, J. Fritsche, M. Kreutz, R. Andreesen, and H. Schwarz. 1998. CD137 (ILA/4-1BB), a member of the TNF receptor family, induces monocyte activation via bidirectional signaling. *J Immunol* 160:2488-2494.
134. Lasek, W., R. Zagożdżon, and M. Jakobisiak. 2014. Interleukin 12: still a promising candidate for tumor immunotherapy? *Cancer immunology, immunotherapy : CII* 63:419-435.
135. Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of experimental medicine* 172:921-929.
136. Lee, A.W., T. Truong, K. Bickham, J.F. Fonteneau, M. Larsson, I. Da Silva, S. Somersan, E.K. Thomas, and N. Bhardwaj. 2002. A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. *Vaccine* 20 Suppl 4:A8-a22.
137. Lee, S.-W., Y. Park, T. So, B.S. Kwon, H. Cheroutre, R.S. Mittler, and M. Croft. 2008. Identification of regulatory functions for 4-1BB and 4-1BBL in myelopoiesis and the development of dendritic cells. *Nature immunology* 9:917-926.
138. Lee, S.C., S.A. Ju, H.N. Pack, S.K. Heo, J.H. Suh, S.M. Park, B.K. Choi, B.S. Kwon, and B.S. Kim. 2005. 4-1BB (CD137) is required for rapid clearance of Listeria monocytogenes infection. *Infection and immunity* 73:5144-5151.
139. Lei, Y., A.M. Ripen, N. Ishimaru, I. Ohigashi, T. Nagasawa, L.T. Jeker, M.R. Bosl, G.A. Hollander, Y. Hayashi, W. Malefyt Rde, T. Nitta, and Y. Takahama. 2011. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *The Journal of experimental medicine* 208:383-394.
140. León, B., G. Martínez del Hoyo, V. Parrillas, H.H. Vargas, P. Sánchez-Mateos, N. Longo, M. López-Bravo, and C. Ardavín. 2004. Dendritic cell differentiation potential of mouse monocytes: monocytes

represent immediate precursors of CD8⁻ and CD8⁺ splenic dendritic cells. *Blood* 103:2668-2676.
141. Lewis, K.L., M.L. Caton, M. Bogunovic, M. Greter, L.T. Grajkowska, D. Ng, A. Klinakis, I.F. Charo, S. Jung, J.L. Gommerman, Ivanov, II, K. Liu, M. Merad, and B. Reizis. 2011. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* 35:780-791.
142. Li, J., E. Lu, T. Yi, and J.G. Cyster. 2016. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature* 533:110-114.
143. Li, Y., G. Cao, X. Zheng, J. Wang, H. Wei, Z. Tian, and R. Sun. 2013. CRACC-CRACC interaction between Kupffer and NK cells contributes to poly I:C/D-GalN induced hepatitis. *PloS one* 8:e76681.
144. Lin, G.H., B.J. Sedgmen, T.J. Moraes, L.M. Snell, D.J. Topham, and T.H. Watts. 2009. Endogenous 4-1BB ligand plays a critical role in protection from influenza-induced disease. *J Immunol* 182:934-947.
145. Linehan, J.L., T. Dileepan, S.W. Kashem, D.H. Kaplan, P. Cleary, and M.K. Jenkins. 2015. Generation of Th17 cells in response to intranasal infection requires TGF-beta1 from dendritic cells and IL-6 from CD301b+ dendritic cells. *Proc Natl Acad Sci U S A* 112:12782-12787.
146. Ling, P., M.K. Gately, U. Gubler, A.S. Stern, P. Lin, K. Hollfelder, C. Su, Y.C. Pan, and J. Hakimi. 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J Immunol* 154:116-127.
147. Liu, K., G.D. Victora, T.A. Schwickert, P. Guermonprez, M.M. Meredith, K. Yao, F.F. Chu, G.J. Randolph, A.Y. Rudensky, and M. Nussenzweig. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science (New York, N.Y.)* 324:392-397.
148. Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* 25:402-408.
149. Lorang, J.M., R.P. Tuori, J.P. Martinez, T.L. Sawyer, R.S. Redman, J.A. Rollins, T.J. Wolpert, K.B. Johnson, R.J. Rodriguez, M.B. Dickman, and L.M. Ciuffetti. 2001. Green fluorescent protein is lighting up fungal biology. *Applied and environmental microbiology* 67:1987-1994.
150. Loschko, J., H.A. Schreiber, G.J. Rieke, D. Esterhazy, M.M. Meredith, V.A. Pedicord, K.H. Yao, S. Caballero, E.G. Pamer, D. Mucida, and M.C. Nussenzweig. 2016. Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. *The Journal of experimental medicine* 213:517-534.
151. Love, M.I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15:550.
152. Lu, E., E.V. Dang, J.G. McDonald, and J.G. Cyster. 2017. Distinct oxysterol requirements for positioning naive and activated dendritic cells in the spleen. *Science immunology* 2:
153. Lu, L.L., T.J. Suscovich, S.M. Fortune, and G. Alter. 2018. Beyond binding: antibody effector functions in infectious diseases. *Nature reviews. Immunology* 18:46-61.
154. Luft, T., E. Maraskovsky, M. Schnurr, K. Knebel, M. Kirsch, M. Görner, R. Skoda, A.D. Ho, P. Nawroth, and A. Bierhaus. 2004. Tuning the volume of the immune response: strength and persistence of stimulation determine migration and cytokine secretion of dendritic cells. 104:1066-1074.
155. Luther, S.A., H.L. Tang, P.L. Hyman, A.G. Farr, and J.G. Cyster. 2000. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc Natl Acad Sci U S A* 97:12694-12699.
156. Lutz, M.B., N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *Journal of immunological methods* 223:77-92.
157. Lutz, M.B., H. Strobl, G. Schuler, and N. Romani. 2017. GM-CSF Monocyte-Derived Cells and Langerhans Cells As Part of the Dendritic Cell Family. *Frontiers in immunology* 8:1388.

158. Macatonia, S.E., N.A. Hosken, M. Litton, P. Vieira, C.S. Hsieh, J.A. Culpepper, M. Wysocka, G. Trinchieri, K.M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 154:5071-5079.
159. Macdonald, D.C., A. Hotblack, S. Akbar, G. Britton, M.K. Collins, and W.C. Rosenberg. 2014. 4-1BB ligand activates bystander dendritic cells to enhance immunization in trans. *J Immunol* 193:5056-5064.
160. Mack, D.G., A.K. Lanham, B.E. Palmer, L.A. Maier, T.H. Watts, and A.P. Fontenot. 2008. 4-1BB enhances proliferation of beryllium-specific T cells in the lung of subjects with chronic beryllium disease. *Journal of immunology (Baltimore, Md. : 1950)* 181:4381-4388.
161. Madsen, L., N. Labrecque, J. Engberg, A. Dierich, A. Svejgaard, C. Benoist, D. Mathis, and L. Fugger. 1999. Mice lacking all conventional MHC class II genes. *Proceedings of the National Academy of Sciences of the United States of America* 96:10338-10343.
162. MartIn-Fontecha, A., S. Sebastiani, U.E. Hopken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2003. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *The Journal of experimental medicine* 198:615-621.
163. Martin, M.J.E.j. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. 17:pp. 10-12.
164. Mattner, F., J. Magram, J. Ferrante, P. Launois, K. Di Padova, R. Behin, M.K. Gately, J.A. Louis, and G. Alber. 1996. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. *European journal of immunology* 26:1553-1559.
165. McHugh, R.S., M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, and M.C. Byrne. 2002. CD4+CD25+ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity* 16:311-323.
166. McKenna, H.J., K.L. Stocking, R.E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C.R. Maliszewski, D.H. Lynch, J. Smith, B. Pulendran, E.R. Roux, M. Teepe, S.D. Lyman, and J.J. Peschon. 2000. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 95:3489-3497.
167. Melero, I., J.V. Johnston, W.W. Shufford, R.S. Mittler, and L. Chen. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cellular immunology* 190:167-172.
168. Mempel, T.R., S.E. Henrickson, and U.H. Von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427:154-159.
169. Menges, M., T. Baumeister, S. Rossner, P. Stoitzner, N. Romani, A. Gessner, and M.B. Lutz. 2005. IL-4 supports the generation of a dendritic cell subset from murine bone marrow with altered endocytosis capacity. *Journal of leukocyte biology* 77:535-543.
170. Menges, M., S. Rößner, C. Voigtländer, H. Schindler, N.A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M.B. Lutz. 2002. Repetitive Injections of Dendritic Cells Matured with Tumor Necrosis Factor α Induce Antigen-specific Protection of Mice from Autoimmunity. 195:15-22.
171. Merad, M., F. Ginhoux, and M. Collin. 2008. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nature reviews. Immunology* 8:935-947.
172. Merad, M., M.G. Manz, H. Karsunky, A. Wagers, W. Peters, I. Charo, I.L. Weissman, J.G. Cyster, and E.G. Engleman. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nature immunology* 3:1135-1141.
173. Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. 31:563-604.
174. Meredith, M.M., K. Liu, G. Darrasse-Jeze, A.O. Kamphorst, H.A. Schreiber, P. Guermonprez, J. Idoyaga, C. Cheong, K.H. Yao, R.E. Niec, and M.C. Nussenzweig. 2012. Expression of the zinc finger

transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *The Journal of experimental medicine* 209:1153-1165.

175. Miceli, M.C., and J.R. Parnes. 1991. The roles of CD4 and CD8 in T cell activation. *Seminars in immunology* 3:133-141.

176. Miller, J.C., B.D. Brown, T. Shay, E.L. Gautier, V. Jojic, A. Cohain, G. Pandey, M. Leboeuf, K.G. Elpek, J. Helft, D. Hashimoto, A. Chow, J. Price, M. Greter, M. Bogunovic, A. Bellemare-Pelletier, P.S. Frenette, G.J. Randolph, S.J. Turley, M. Merad, C. the Immunological Genome, E.L. Gautier, C. Jakubzick, G.J. Randolph, A.J. Best, J. Knell, A. Goldrath, J. Miller, B. Brown, M. Merad, V. Jojic, D. Koller, N. Cohen, P. Brennan, M. Brenner, T. Shay, A. Regev, A. Fletcher, K. Elpek, A. Bellemare-Pelletier, D. Malhotra, S. Turley, R. Jianu, D. Laidlaw, J. Collins, K. Narayan, K. Sylvia, J. Kang, R. Gazit, D.J. Rossi, F. Kim, T.N. Rao, A. Wagers, S.A. Shinton, R.R. Hardy, P. Monach, N.A. Bezman, J.C. Sun, C.C. Kim, L.L. Lanier, T. Heng, T. Kreslavsky, M. Painter, J. Ericson, S. Davis, D. Mathis, and C. Benoist. 2012. Deciphering the transcriptional network of the dendritic cell lineage. *Nature immunology* 13:888.

177. Miller, M.J., O. Safrina, I. Parker, and M.D. Cahalan. 2004. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *The Journal of experimental medicine* 200:847-856.

178. Min, J., D. Yang, M. Kim, K. Haam, A. Yoo, J.-H. Choi, B.U. Schraml, Y.S. Kim, D. Kim, and S.-J. Kang. 2018. Inflammation induces two types of inflammatory dendritic cells in inflamed lymph nodes. *Experimental &Amp; Molecular Medicine* 50:e458.

179. Mitchell, D.A., K.A. Batich, M.D. Gunn, M.N. Huang, L. Sanchez-Perez, S.K. Nair, K.L. Congdon, E.A. Reap, G.E. Archer, A. Desjardins, A.H. Friedman, H.S. Friedman, J.E. Herndon, 2nd, A. Coan, R.E. McLendon, D.A. Reardon, J.J. Vredenburgh, D.D. Bigner, and J.H. Sampson. 2015a. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature* 519:366-369.

180. Mitchell, D.A., E.J. Sayour, E. Reap, R. Schmittling, G. DeLeon, P. Norberg, A. Desjardins, A.H. Friedman, H.S. Friedman, G. Archer, and J.H. Sampson. 2015b. Severe adverse immunologic reaction in a patient with glioblastoma receiving autologous dendritic cell vaccines combined with GM-CSF and dose-intensified temozolomide. *Cancer immunology research* 3:320-325.

181. Moore, Y.F., D. Lambracht-Washington, P. Tabaczewski, and K. Fischer Lindahl. 2004. Murine MHC class Ib gene, H2-M2, encodes a conserved surface-expressed glycoprotein. *Immunogenetics* 56:1-11.

182. Morelli, A.E., and A.T. Larregina. 2010. Apoptotic cell-based therapies against transplant rejection: role of recipient's dendritic cells. *Apoptosis : an international journal on programmed cell death* 15:1083-1097.

183. Morelli, A.E., A.T. Larregina, W.J. Shufesky, A.F. Zahorchak, A.J. Logar, G.D. Papworth, Z. Wang, S.C. Watkins, L.D. Falo, and A.W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. 101:611-620.

184. Morse, M.A., T.M. Clay, A.C. Hobeika, T. Osada, S. Khan, S. Chui, D. Niedzwiecki, D. Panicali, J. Schlom, and H.K. Lyerly. 2005. Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11:3017-3024.

185. Mortier, E., T. Woo, R. Advincula, S. Gozalo, and A. Ma. 2008. IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *The Journal of experimental medicine* 205:1213-1225.

186. Mundy, R., T.T. MacDonald, G. Dougan, G. Frankel, and S. Wiles. 2005. Citrobacter rodentium of mice and man. *Cellular microbiology* 7:1697-1706.

187. Murphy, T.L., G.E. Grajales-Reyes, X. Wu, R. Tussiwand, C.G. Briseno, A. Iwata, N.M. Kretzer, V. Durai, and K.M. Murphy. 2016. Transcriptional Control of Dendritic Cell Development. *Annual review of immunology* 34:93-119.

188. Muzaki, A.R., P. Tetlak, J. Sheng, S.C. Loh, Y.A. Setiagani, M. Poidinger, F. Zolezzi, K. Karjalainen, and C. Ruedl. 2016. Intestinal CD103(+)CD11b(-) dendritic cells restrain colitis via IFN-gamma-induced anti-inflammatory response in epithelial cells. *Mucosal immunology* 9:336-351.

189. Nagao, K., T. Kobayashi, K. Moro, M. Ohyama, T. Adachi, D.Y. Kitashima, S. Ueha, K. Horiuchi, H. Tanizaki, K. Kabashima, A. Kubo, Y.H. Cho, B.E. Clausen, K. Matsushima, M. Suematsu, G.C. Furtado, S.A. Lira, J.M. Farber, M.C. Udey, and M. Amagai. 2012. Stress-induced production of chemokines by hair follicles regulates the trafficking of dendritic cells in skin. *Nature immunology* 13:744-752.
190. Naik, S.H., A.I. Proietto, N.S. Wilson, A. Dakic, P. Schnorrer, M. Fuchsberger, M.H. Lahoud, M. O'Keeffe, Q.X. Shao, W.F. Chen, J.A. Villadangos, K. Shortman, and L. Wu. 2005. Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* 174:6592-6597.
191. Nestle, F.O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nature medicine* 4:328-332.
192. Neuenhahn, M., K.M. Kerksiek, M. Nauerth, M.H. Suhre, M. Schiemann, F.E. Gebhardt, C. Stemberger, K. Panthel, S. Schroder, T. Chakraborty, S. Jung, H. Hochrein, H. Russmann, T. Brocker, and D.H. Busch. 2006. CD8alpha+ dendritic cells are required for efficient entry of Listeria monocytogenes into the spleen. *Immunity* 25:619-630.
193. Nicholas, D., H. Tang, Q. Zhang, J. Rudra, F. Xu, W. Langridge, and K. Zhang. 2015. Quantitative proteomics reveals a role for epigenetic reprogramming during human monocyte differentiation. *Molecular & cellular proteomics : MCP* 14:15-29.
194. Nishimoto, H., S.-W. Lee, H. Hong, K.G. Potter, M. Maeda-Yamamoto, T. Kinoshita, Y. Kawakami, R.S. Mittler, B.S. Kwon, C.F. Ware, M. Croft, and T. Kawakami. 2005. Costimulation of mast cells by 4-1BB, a member of the tumor necrosis factor receptor superfamily, with the high-affinity IgE receptor. *Blood* 106:4241-4248.
195. O'Keeffe, M., H. Hochrein, D. Vremec, B. Scott, P. Hertzog, L. Tatarczuch, and K. Shortman. 2003. Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c⁺ DC1 precursors. 101:1453-1459.
196. Ohl, L., G. Henning, S. Krautwald, M. Lipp, S. Hardtke, G. Bernhardt, O. Pabst, and R. Förster. 2003. Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *The Journal of experimental medicine* 197:1199-1204.
197. Ohl, L., M. Mohaupt, N. Czeloth, G. Hintzen, Z. Kiafard, J. Zwirner, T. Blankenstein, G. Henning, and R. Forster. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 21:279-288.
198. Okada, N., S. Iiyama, Y. Okada, H. Mizuguchi, T. Hayakawa, S. Nakagawa, T. Mayumi, T. Fujita, and A. Yamamoto. 2005. Immunological properties and vaccine efficacy of murine dendritic cells simultaneously expressing melanoma-associated antigen and interleukin-12. *Cancer gene therapy* 12:72-83.
199. Onai, N., K. Kurabayashi, M. Hosoi-Amaike, N. Toyama-Sorimachi, K. Matsushima, K. Inaba, and T. Ohteki. 2013. A Clonogenic Progenitor with Prominent Plasmacytoid Dendritic Cell Developmental Potential. *Immunity* 38:943-957.
200. Onai, N., A. Obata-Onai, M.A. Schmid, T. Ohteki, D. Jarrossay, and M.G. Manz. 2007. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nature immunology* 8:1207-1216.
201. Openshaw, P., E.E. Murphy, N.A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *The Journal of experimental medicine* 182:1357-1367.
202. Park, K., Z. Mikulski, G.Y. Seo, A.Y. Andreyev, P. Marcovecchio, A. Blatchley, M. Kronenberg, and C.C. Hedrick. 2016. The transcription factor NR4A3 controls CD103+ dendritic cell migration. *The Journal of clinical investigation* 126:4603-4615.
203. Parmiani, G., C. Castelli, L. Pilla, M. Santinami, M.P. Colombo, and L. Rivoltini. 2007. Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. *Annals of oncology : official journal of the European Society for Medical Oncology* 18:226-232.

204. Pauly, S., K. Broll, M. Wittmann, G. Giegerich, and H. Schwarz. 2002. CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers. *Journal of leukocyte biology* 72:35-42.
205. Persson, E.K., H. Uronen-Hansson, M. Semmrich, A. Rivollier, K. Hagerbrand, J. Marsal, S. Gudjonsson, U. Hakansson, B. Reizis, K. Kotarsky, and W.W. Agace. 2013. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 38:958-969.
206. Pistoia, V., C. Cocco, and I. Airoldi. 2009. Interleukin-12 receptor beta2: from cytokine receptor to gatekeeper gene in human B-cell malignancies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27:4809-4816.
207. Plantinga, M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad, and B.N. Lambrecht. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38:322-335.
208. Pletinckx, K., B. Stijlemans, V. Pavlovic, R. Laube, C. Brandl, S. Kneitz, A. Beschin, P. De Baetselier, and M.B. Lutz. 2011. Similar inflammatory DC maturation signatures induced by TNF or Trypanosoma brucei antigens instruct default Th2-cell responses. *European journal of immunology* 41:3479-3494.
209. Proietto, A.I., M. O'Keeffe, K. Gartlan, M.D. Wright, K. Shortman, L. Wu, and M.H. Lahoud. 2004. Differential production of inflammatory chemokines by murine dendritic cell subsets. *Immunobiology* 209:163-172.
210. Pulendran, B., S. Dillon, C. Joseph, T. Curiel, J. Banchereau, and M. Mohamadzadeh. 2004. Dendritic cells generated in the presence of GM-CSF plus IL-15 prime potent CD8+ Tc1 responses in vivo. *European journal of immunology* 34:66-73.
211. Quinlan, A.R., and I.M. Hall. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics (Oxford, England)* 26:841-842.
212. Ramirez-Pineda, J.R., A. Frohlich, C. Berberich, and H. Moll. 2004. Dendritic cells (DC) activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. *J Immunol* 172:6281-6289.
213. Rathinam, C., R. Geffers, R. Yucel, J. Buer, K. Welte, T. Moroy, and C. Klein. 2005. The transcriptional repressor Gfi1 controls STAT3-dependent dendritic cell development and function. *Immunity* 22:717-728.
214. Reichardt, V.L., P. Brossart, and L. Kanz. 2004. Dendritic cells in vaccination therapies of human malignant disease. *Blood reviews* 18:235-243.
215. Reinhardt, R.L., S. Hong, S.J. Kang, Z.E. Wang, and R.M. Locksley. 2006. Visualization of IL-12/23p40 in vivo reveals immunostimulatory dendritic cell migrants that promote Th1 differentiation. *Journal of immunology (Baltimore, Md. : 1950)* 177:1618-1627.
216. Reis e Sousa, C., S. Hieny, T. Scharton-Kersten, D. Jankovic, H. Charest, R.N. Germain, and A. Sher. 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *The Journal of experimental medicine* 186:1819-1829.
217. Ridgway, D. 2003. The first 1000 dendritic cell vaccinees. *Cancer investigation* 21:873-886.
218. Robbins, S.H., T. Walzer, D. Dembele, C. Thibault, A. Defays, G. Bessou, H. Xu, E. Vivier, M. Sellars, P. Pierre, F.R. Sharp, S. Chan, P. Kastner, and M. Dalod. 2008. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome biology* 9:R17.
219. Roberts, E.W., M.L. Broz, M. Binnewies, M.B. Headley, A.E. Nelson, D.M. Wolf, T. Kaisho, D. Bogunovic, N. Bhardwaj, and M.F. Krummel. 2016. Critical Role for CD103(+)/CD141(+) Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma. *Cancer cell* 30:324-336.

220. Ross, S.H., and D.A. Cantrell. 2018. Signaling and Function of Interleukin-2 in T Lymphocytes. *Annual review of immunology* 36:411-433.
221. Rossowska, J., N. Anger, J. Kicielinska, E. Pajtasz-Piasecka, A. Bielawska-Pohl, J. Wojas-Turek, and D. Dus. 2015. Temporary elimination of IL-10 enhanced the effectiveness of cyclophosphamide and BMDC-based therapy by decrease of the suppressor activity of MDSCs and activation of antitumour immune response. *Immunobiology* 220:389-398.
222. Ruedl, C., P. Koebel, and K. Karjalainen. 2001. In vivo-matured Langerhans cells continue to take up and process native proteins unlike in vitro-matured counterparts. *J Immunol* 166:7178-7182.
223. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of experimental medicine* 179:1109-1118.
224. Salmon, H., J. Idoyaga, A. Rahman, M. Leboeuf, R. Remark, S. Jordan, M. Casanova-Acebes, M. Khudoynazarova, J. Agudo, N. Tung, S. Chakarov, C. Rivera, B. Hogstad, M. Bosenberg, D. Hashimoto, S. Gnjatic, N. Bhardwaj, A.K. Palucka, B.D. Brown, J. Brody, F. Ginhoux, and M. Merad. 2016. Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* 44:924-938.
225. Samstein, M., H.A. Schreiber, I.M. Leiner, B. Susac, M.S. Glickman, and E.G. Pamer. 2013. Essential yet limited role for CCR2(+) inflammatory monocytes during Mycobacterium tuberculosis-specific T cell priming. *eLife* 2:e01086.
226. Sanchez, P.J., and R.M. Kedl. 2012. An alternative signal 3: CD8(+) T cell memory independent of IL-12 and type I IFN is dependent on CD27/OX40 signaling. *Vaccine* 30:1154-1161.
227. Sapoznikov, A., J.A. Fischer, T. Zaft, R. Krauthgamer, A. Dzionek, and S. Jung. 2007. Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. *The Journal of experimental medicine* 204:1923-1933.
228. Sasaki, T., A. Shiohama, A. Kubo, H. Kawasaki, A. Ishida-Yamamoto, T. Yamada, T. Hachiya, A. Shimizu, H. Okano, J. Kudoh, and M. Amagai. 2013. A homozygous nonsense mutation in the gene for Tmem79, a component for the lamellar granule secretory system, produces spontaneous eczema in an experimental model of atopic dermatitis. *The Journal of allergy and clinical immunology* 132:1111-1120.e1114.
229. Satpathy, A.T., C.G. Briseno, X. Cai, D.G. Michael, C. Chou, S. Hsiung, D. Bhattacharya, N.A. Speck, and T. Egawa. 2014. Runx1 and Cbfbeta regulate the development of Flt3+ dendritic cell progenitors and restrict myeloproliferative disorder. *Blood* 123:2968-2977.
230. Satpathy, A.T., C.G. Briseno, J.S. Lee, D. Ng, N.A. Manieri, W. Kc, X. Wu, S.R. Thomas, W.L. Lee, M. Turkoz, K.G. McDonald, M.M. Meredith, C. Song, C.J. Guidos, R.D. Newberry, W. Ouyang, T.L. Murphy, T.S. Stappenbeck, J.L. Gommerman, M.C. Nussenzweig, M. Colonna, R. Kopan, and K.M. Murphy. 2013. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nature immunology* 14:937-948.
231. Schlitzer, A., N. McGovern, P. Teo, T. Zelante, K. Atarashi, D. Low, A.W. Ho, P. See, A. Shin, P.S. Wasan, G. Hoeffel, B. Malleret, A. Heiseke, S. Chew, L. Jardine, H.A. Purvis, C.M. Hilkens, J. Tam, M. Poidinger, E.R. Stanley, A.B. Krug, L. Renia, B. Sivasankar, L.G. Ng, M. Collin, P. Ricciardi-Castagnoli, K. Honda, M. Haniffa, and F. Ginhoux. 2013a. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* 38:970-983.
232. Schlitzer, A., N. McGovern, P. Teo, T. Zelante, K. Atarashi, D. Low, A.W.S. Ho, P. See, A. Shin, P.S. Wasan, G. Hoeffel, B. Malleret, A. Heiseke, S. Chew, L. Jardine, H.A. Purvis, C.M.U. Hilkens, J. Tam, M. Poidinger, E.R. Stanley, A.B. Krug, L. Renia, B. Sivasankar, L.G. Ng, M. Collin, P. Ricciardi-Castagnoli, K. Honda, M. Haniffa, and F. Ginhoux. 2013b. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* 38:970-983.
233. Schlitzer, A., V. Sivakamasundari, J. Chen, H.R.B. Sumatoh, J. Schreuder, J. Lum, B. Malleret, S. Zhang, A. Larbi, F. Zolezzi, L. Renia, M. Poidinger, S. Naik, E.W. Newell, P. Robson, and F. Ginhoux.

2015. Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. *Nature immunology* 16:718.
234. Schmitt, D.A., and S.E. Ullrich. 2000. Exposure to ultraviolet radiation causes dendritic cells/macrophages to secrete immune-suppressive IL-12p40 homodimers. *J Immunol* 165:3162-3167.
235. Schreurs, M.W.J., A.A.O. Eggert, A.J. de Boer, C.G. Figdor, and G.J. Adema. 1999. Generation and functional characterization of mouse monocyte-derived dendritic cells. *European journal of immunology* 29:2835-2841.
236. Schuler-Thurner, B., E.S. Schultz, T.G. Berger, G. Weinlich, S. Ebner, P. Woerl, A. Bender, B. Feuerstein, P.O. Fritsch, N. Romani, and G. Schuler. 2002. Rapid Induction of Tumor-specific Type 1 T Helper Cells in Metastatic Melanoma Patients by Vaccination with Mature, Cryopreserved, Peptide-loaded Monocyte-derived Dendritic Cells. 195:1279-1288.
237. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *The Journal of experimental medicine* 161:526-546.
238. Schumann, K., T. Lammermann, M. Bruckner, D.F. Legler, J. Polleux, J.P. Spatz, G. Schuler, R. Forster, M.B. Lutz, L. Sorokin, and M. Sixt. 2010. Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity* 32:703-713.
239. Schwarz, H., M. Schmittner, A. Duschl, and J. Horejs-Hoeck. 2014. Residual endotoxin contaminations in recombinant proteins are sufficient to activate human CD1c+ dendritic cells. *PloS one* 9:e113840-e113840.
240. Segal, J.G., N.C. Lee, Y.L. Tsung, J.A. Norton, and K. Tsung. 2002. The role of IFN-gamma in rejection of established tumors by IL-12 : source of production and target. *Cancer research* 62:4696-4703.
241. Segura, E., and S. Amigorena. 2013. Inflammatory dendritic cells in mice and humans. *Trends in immunology* 34:440-445.
242. Segura, E., M. Durand, and S. Amigorena. 2013. Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. *The Journal of experimental medicine* 210:1035-1047.
243. Segura, E., C. Guérin, N. Hogg, S. Amigorena, and C. Théry. 2007. CD8⁺ Dendritic Cells Use LFA-1 to Capture MHC-Peptide Complexes from Exosomes In Vivo. 179:1489-1496.
244. Segura, E., J. Valladeau-Guilemond, M.H. Donnadieu, X. Sastre-Garau, V. Soumelis, and S. Amigorena. 2012. Characterization of resident and migratory dendritic cells in human lymph nodes. *The Journal of experimental medicine* 209:653-660.
245. Seillet, C., J.T. Jackson, K.A. Markey, H.J.M. Brady, G.R. Hill, K.P.A. MacDonald, S.L. Nutt, and G.T. Belz. 2013. CD8α⁺ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. 121:1574-1583.
246. Seko, Y., N. Takahashi, H. Oshima, O. Shimozato, H. Akiba, K. Takeda, T. Kobata, H. Yagita, K. Okumura, M. Azuma, and R. Nagai. 2001. Expression of tumour necrosis factor (TNF) ligand superfamily co-stimulatory molecules CD30L, CD27L, OX40L, and 4-1BBL in murine hearts with acute myocarditis caused by Coxsackievirus B3. *The Journal of pathology* 195:593-603.
247. Serbina, N.V., T.P. Salazar-Mather, C.A. Biron, W.A. Kuziel, and E.G. Pamer. 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19:59-70.
248. Sharma, N., A.P. Benechet, L. Lefrançois, and K.M. Khanna. 2015. CD8 T Cells Enter the Splenic T Cell Zones Independently of CCR7, but the Subsequent Expansion and Trafficking Patterns of Effector T Cells after Infection Are Dysregulated in the Absence of CCR7 Migratory Cues. *Journal of immunology (Baltimore, Md. : 1950)* 195:5227-5236.
249. Shibaki, A., and S.I. Katz. 2002. Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant. *Experimental dermatology* 11:126-134.
250. Shortman, K., and W.R. Heath. 2010. The CD8+ dendritic cell subset. *Immunological reviews* 234:18-31.

251. Silva-Vilches, C., K. Pletinckx, M. Lohnert, V. Pavlovic, D. Ashour, V. John, E. Vendelova, S. Kneitz, J. Zhou, R. Chen, T. Reinheckel, T.D. Mueller, J. Bodem, and M.B. Lutz. 2017. Low doses of cholera toxin and its mediator cAMP induce CTLA-2 secretion by dendritic cells to enhance regulatory T cell conversion. *PloS one* 12:e0178114.
252. Sixt, M., N. Kanazawa, M. Selg, T. Samson, G. Roos, D.P. Reinhardt, R. Pabst, M.B. Lutz, and L. Sorokin. 2005. The Conduit System Transports Soluble Antigens from the Afferent Lymph to Resident Dendritic Cells in the T Cell Area of the Lymph Node. *Immunity* 22:19-29.
253. Soares, H., H. Waechter, N. Glaichenhaus, E. Mougneau, H. Yagita, O. Mizenina, D. Dudziak, M.C. Nussenzweig, and R.M. Steinman. 2007. A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *The Journal of experimental medicine* 204:1095-1106.
254. Soares, K.C., A.A. Rucki, A.A. Wu, K. Olino, Q. Xiao, Y. Chai, A. Wamwea, E. Bigelow, E. Lutz, L. Liu, S. Yao, R.A. Anders, D. Laheru, C.L. Wolfgang, B.H. Edil, R.D. Schulick, E.M. Jaffee, and L. Zheng. 2015. PD-1/PD-L1 blockade together with vaccine therapy facilitates effector T-cell infiltration into pancreatic tumors. *Journal of immunotherapy (Hagerstown, Md. : 1997)* 38:1-11.
255. Sonnenberg, G.F., and D. Artis. 2015. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nature medicine* 21:698-708.
256. Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of experimental medicine* 137:1142-1162.
257. Steinman, R.M., B. Gutchinov, M.D. Witmer, and M.C. Nussenzweig. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *The Journal of experimental medicine* 157:613-627.
258. Suzuki, S., K. Honma, T. Matsuyama, K. Suzuki, K. Toriyama, I. Akitoyo, K. Yamamoto, T. Suematsu, M. Nakamura, K. Yui, and A. Kumatori. 2004. Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. *Proc Natl Acad Sci U S A* 101:8981-8986.
259. Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805-820.
260. Tal, O., H.Y. Lim, I. Gurevich, I. Milo, Z. Shipony, L.G. Ng, V. Angeli, and G. Shakhar. 2011. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *The Journal of experimental medicine* 208:2141-2153.
261. Tan, J.T., J. Ha, H.R. Cho, C. Tucker-Burden, R.C. Hendrix, R.S. Mittler, T.C. Pearson, and C.P. Larsen. 2000. Analysis of expression and function of the costimulatory molecule 4-1BB in alloimmune responses. *Transplantation* 70:175-183.
262. Teijeira, A., E. Russo, and C. Halin. 2014. Taking the lymphatic route: dendritic cell migration to draining lymph nodes. *Seminars in immunopathology* 36:261-274.
263. Thierfelder, W.E., J.M. van Deursen, K. Yamamoto, R.A. Tripp, S.R. Sarawar, R.T. Carson, M.Y. Sangster, D.A. Vignali, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171-174.
264. Tominaga, K., T. Yoshimoto, K. Torigoe, M. Kurimoto, K. Matsui, T. Hada, H. Okamura, and K. Nakanishi. 2000. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *International immunology* 12:151-160.
265. Tomkinson, A., C. Duez, M. Lahn, and E.W. Gelfand. 2002. Adoptive transfer of T cells induces airway hyperresponsiveness independently of airway eosinophilia but in a signal transducer and activator of transcription 6-dependent manner. *The Journal of allergy and clinical immunology* 109:810-816.
266. Tomura, M., A. Hata, S. Matsuoka, F.H. Shand, Y. Nakanishi, R. Ikebuchi, S. Ueha, H. Tsutsui, K. Inaba, K. Matsushima, A. Miyawaki, K. Kabashima, T. Watanabe, and O. Kanagawa. 2014. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Scientific reports* 4:6030.

267. Trakatelli, M., M. Toungouz, D. Blocklet, Y. Dodoo, L. Gordower, M. Laporte, P. Vereecken, F. Sales, L. Mortier, N. Mazouz, M. Lambermont, S. Goldman, P. Coulie, M. Goldman, and T. Velu. 2006. A new dendritic cell vaccine generated with interleukin-3 and interferon-beta induces CD8+ T cell responses against NA17-A2 tumor peptide in melanoma patients. *Cancer immunology, immunotherapy : CII* 55:469-474.
268. Trautwein-Weidner, K., A. Gladiator, F.R. Kirchner, S. Becattini, T. Rulicke, F. Sallusto, and S. LeibundGut-Landmann. 2015. Antigen-Specific Th17 Cells Are Primed by Distinct and Complementary Dendritic Cell Subsets in Oropharyngeal Candidiasis. *PLoS pathogens* 11:e1005164.
269. Traver, D., K. Akashi, M. Manz, M. Merad, T. Miyamoto, E.G. Engleman, and I.L. Weissman. 2000. Development of CD8α-Positive Dendritic Cells from a Common Myeloid Progenitor. 290:2152-2154.
270. Travis, M.A., B. Reizis, A.C. Melton, E. Masteller, Q. Tang, J.M. Proctor, Y. Wang, X. Bernstein, X. Huang, L.F. Reichardt, J.A. Bluestone, and D. Sheppard. 2007. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* 449:361-365.
271. Treilleux, I., J.Y. Blay, N. Bendriss-Vermare, I. Ray-Coquard, T. Bachelot, J.P. Guastalla, A. Bremond, S. Goddard, J.J. Pin, C. Barthelemy-Dubois, and S. Lebecque. 2004. Dendritic cell infiltration and prognosis of early stage breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10:7466-7474.
272. Tsang, K.Y., C. Palena, J. Yokokawa, P.M. Arlen, J.L. Gulley, G.P. Mazzara, L. Gritz, A.G. Yafal, S. Ogueta, P. Greenhalgh, K. Manson, D. Panicali, and J. Schlom. 2005. Analyses of recombinant vaccinia and fowlpox vaccine vectors expressing transgenes for two human tumor antigens and three human costimulatory molecules. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11:1597-1607.
273. Turnis, M.E., X.T. Song, A. Bear, A.E. Foster, S. Gottschalk, M.K. Brenner, S.Y. Chen, and C.M. Rooney. 2010. IRAK-M removal counteracts dendritic cell vaccine deficits in migration and longevity. *J Immunol* 185:4223-4232.
274. Turvey, S.E., and D.H. Broide. 2010. Innate immunity. *The Journal of allergy and clinical immunology* 125:S24-S32.
275. Tussiwand, R., B. Everts, G.E. Grajales-Reyes, N.M. Kretzer, A. Iwata, J. Bagaitkar, X. Wu, R. Wong, D.A. Anderson, T.L. Murphy, E.J. Pearce, and K.M. Murphy. 2015. Klf4 expression in conventional dendritic cells is required for T helper 2 cell responses. *Immunity* 42:916-928.
276. Tussiwand, R., W.L. Lee, T.L. Murphy, M. Mashayekhi, W. Kc, J.C. Albring, A.T. Satpathy, J.A. Rotondo, B.T. Edelson, N.M. Kretzer, X. Wu, L.A. Weiss, E. Glasmacher, P. Li, W. Liao, M. Behnke, S.S. Lam, C.T. Aurthur, W.J. Leonard, H. Singh, C.L. Stallings, L.D. Sibley, R.D. Schreiber, and K.M. Murphy. 2012. Compensatory dendritic cell development mediated by BATF-IRF interactions. *Nature* 490:502-507.
277. Vander Lugt, B., A.A. Khan, J.A. Hackney, S. Agrawal, J. Lesch, M. Zhou, W.P. Lee, S. Park, M. Xu, J. DeVoss, C.J. Spooner, C. Chalouni, L. Delamarre, I. Mellman, and H. Singh. 2014. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nature immunology* 15:161-167.
278. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
279. Vignali, D.A.A., L.W. Collison, and C.J. Workman. 2008. How regulatory T cells work. *Nature reviews. Immunology* 8:523-532.
280. Villani, A.C., R. Satija, G. Reynolds, S. Sarkizova, K. Shekhar, J. Fletcher, M. Griesbeck, A. Butler, S. Zheng, S. Lazo, L. Jardine, D. Dixon, E. Stephenson, E. Nilsson, I. Grundberg, D. McDonald, A. Filby, W. Li, P.L. De Jager, O. Rozenblatt-Rosen, A.A. Lane, M. Haniffa, A. Regev, and N. Hacohen. 2017. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science (New York, N.Y.)* 356:

281. Vinay, D.S., B.K. Choi, J.S. Bae, W.Y. Kim, B.M. Gebhardt, and B.S. Kwon. 2004. CD137-deficient mice have reduced NK/NKT cell numbers and function, are resistant to lipopolysaccharide-induced shock syndromes, and have lower IL-4 responses. *J Immunol* 173:4218-4229.
282. Vogt, T.K., A. Link, J. Perrin, D. Finke, and S.A. Luther. 2009. Novel function for interleukin-7 in dendritic cell development. 113:3961-3968.
283. Voigtlander, C., S. Rossner, E. Cierpka, G. Theiner, C. Wiethe, M. Menges, G. Schuler, and M.B. Lutz. 2006. Dendritic cells matured with TNF can be further activated in vitro and after subcutaneous injection in vivo which converts their tolerogenicity into immunogenicity. *Journal of immunotherapy (Hagerstown, Md. : 1997)* 29:407-415.
284. Waithman, J., D. Zanker, K. Xiao, S. Oveissi, B. Wylie, R. Ng, L. Togel, and W. Chen. 2013. Resident CD8(+) and migratory CD103(+) dendritic cells control CD8 T cell immunity during acute influenza infection. *PloS one* 8:e66136.
285. Wakim, L.M., and M.J. Bevan. 2011. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature* 471:629.
286. Wang, Y., K.J. Szretter, W. Vermi, S. Gilfillan, C. Rossini, M. Cella, A.D. Barrow, M.S. Diamond, and M. Colonna. 2012. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nature immunology* 13:753-760.
287. Welty, N.E., C. Staley, N. Ghilardi, M.J. Sadowsky, B.Z. Igyarto, and D.H. Kaplan. 2013. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism. *The Journal of experimental medicine* 210:2011-2024.
288. Wilcox, R.A., A.I. Chapoval, K.S. Gorski, M. Otsuji, T. Shin, D.B. Flies, K. Tamada, R.S. Mittler, H. Tsuchiya, D.M. Pardoll, and L. Chen. 2002a. Cutting edge: Expression of functional CD137 receptor by dendritic cells. *J Immunol* 168:4262-4267.
289. Wilcox, R.A., K. Tamada, S.E. Strome, and L. Chen. 2002b. Signaling Through NK Cell-Associated CD137 Promotes Both Helper Function for CD8⁺ Cytolytic T Cells and Responsiveness to IL-2 But Not Cytolytic Activity. *The Journal of Immunology* 169:4230.
290. Worthington, J.J., A. Kelly, C. Smedley, D. Bauche, S. Campbell, J.C. Marie, and M.A. Travis. 2015. Integrin alphavbeta8-Mediated TGF-beta Activation by Effector Regulatory T Cells Is Essential for Suppression of T-Cell-Mediated Inflammation. *Immunity* 42:903-915.
291. Wu, L., A. Nichogiannopoulou, K. Shortman, and K. Georgopoulos. 1997. Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity* 7:483-492.
292. Xu, Y., Y. Zhan, A.M. Lew, S.H. Naik, and M.H. Kershaw. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* 179:7577-7584.
293. Yamazaki, C., M. Sugiyama, T. Ohta, H. Hemmi, E. Hamada, I. Sasaki, Y. Fukuda, T. Yano, M. Nobuoka, T. Hirashima, A. Iizuka, K. Sato, T. Tanaka, K. Hoshino, and T. Kaisho. 2013. Critical roles of a dendritic cell subset expressing a chemokine receptor, XCR1. *J Immunol* 190:6071-6082.
294. Yamazaki, S., D. Dudziak, G.F. Heidkamp, C. Fiorese, A.J. Bonito, K. Inaba, M.C. Nussenzweig, and R.M. Steinman. 2008. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *Journal of immunology (Baltimore, Md. : 1950)* 181:6923-6933.
295. Yang, J., T.L. Murphy, W. Ouyang, and K.M. Murphy. 1999. Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. *European journal of immunology* 29:548-555.
296. Zhang, N., and M.J. Bevan. 2011. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35:161-168.
297. Zhang, Q.J., X.L. Li, D. Wang, X.C. Huang, J.M. Mathis, W.M. Duan, D. Knight, R. Shi, J. Glass, D.Q. Zhang, L. Eisenbach, and W.A. Jefferies. 2008. Trogocytosis of MHC-I/peptide complexes derived from tumors and infected cells enhances dendritic cell cross-priming and promotes adaptive T cell responses. *PloS one* 3:e3097.

298. Zhao, Y.G., Y. Chen, G. Miao, H. Zhao, W. Qu, D. Li, Z. Wang, N. Liu, L. Li, S. Chen, P. Liu, D. Feng, and H. Zhang. 2017. The ER-Localized Transmembrane Protein EPG-3/VMP1 Regulates SERCA Activity to Control ER-Isolation Membrane Contacts for Autophagosome Formation. *Molecular cell* 67:974-989.e976.
299. Zheng, Y., P.A. Valdez, D.M. Danilenko, Y. Hu, S.M. Sa, Q. Gong, A.R. Abbas, Z. Modrusan, N. Ghilardi, F.J. de Sauvage, and W. Ouyang. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature medicine* 14:282-289.
300. Zhu, J., and W.E. Paul. 2008. CD4 T cells: fates, functions, and faults. 112:1557-1569.
301. Zigmond, E., C. Varol, J. Farache, E. Elmaliah, A.T. Satpathy, G. Friedlander, M. Mack, N. Shpigel, I.G. Boneca, K.M. Murphy, G. Shakhar, Z. Halpern, and S. Jung. 2012. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity* 37:1076-1090.

6 LIST OF TABLES

7 LIST OF FIGURES

8 ABBREVIATIONS

°C	Degrees Celsius
APC	Antigen presenting cell
BM	Bone marrow
BM-DC	Bone marrow-derived dendritic cell
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCR7	C-C chemokine receptor 7
CD	Cluster of differentiation
cDC	Conventional DC
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CO2	Carbon dioxide
Ct	Cycle threshold
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
ddH2O	Double-distilled water
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphate
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBI2	Epstein-Barr virus induced 2
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence acquired cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3-L	Fms-like tyrosine kinase 3 ligand
FSC	Forward scatter
Fwd	Forward
g	Gram
GATA-3	GATA binding protein 3
GEO	Gene Expression Omnibus
GM-CSF	Granulocyte/macrophage colony-stimulating factor
h	Hours
i.p.	intraperitoneal

i.v.	intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-12R	IL-12 receptor
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minutes
ml	Millilitre
moDC	Monocyte-derived dendritic cell
mRNA	Messenger RNA
neg	Negative
NFκB	Nuclear factor κB
NK	Natural killer
NLR	Nod-like receptors
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed cell death-Lignad1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PGE_2	Prostaglandin E2
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	subcutaneous
SA	Streptavidin
SD	Standard deviation
sec	Second

SIRPα	Signal regulatory protein alpha
SSC	Sideward scatter
SSC	Side scatter
ssm	Steady state migratory
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
Th1	T helper 1
Tip DC	TNF and iNOS producing DC
TLR	Toll-like receptors
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
wt	Wild type
YFP	Yellow Fluorescent Protein
µg	Microgram
µl	Microliter
µM	Micromolar

9 LIST OF OWN PUBLICATIONS

1.Low doses of cholera toxin and its mediator cAMP induce CTLA-2 secretion by dendritic cells to enhance regulatory T cell conversion.
Silva-Vilches C, Pletinckx K, Lohnert M, Pavlovic V, Ashour D, John V, Vendelova E, Kneitz S, Zhou J, Chen R, Reinheckel T, Mueller TD, Bodem J, Lutz MB.
PLoS One. 2017 Jul 31;12(7): e0178114.

2.Tolerogenic Transcriptional Signatures of Steady-State and Pathogen-Induced Dendritic Cells.
Vendelova E, Ashour D, Blank P, Erhard F, Saliba AE, Kalinke U, Lutz MB.
Front Immunol. 2018 Feb 28; 9:333.

3. Th1 priming by vaccine dendritic cells depends on IL-12 production by endogenous migratory XCR1+ dendritic cells. In preparation
Ashour D, Arampatzi P, Förstner K, Kaisho T, Beilhack A, Erhard F, Lutz MB
In preparation

10 CURRICULUM VITAE

Diyaa El din Zakaria Ashour

Gutenbergstr. 2 | Würzburg, Germany. P.O.97080

EDUCATION

10.2013-03.2015	**Würzburg University, Graduate School of Life Sciences,** Würzburg, Germany FOKUS Life Sciences Master's Program **Grade:** 1.1
09.2005-05.2010	**Cairo University, Faculty of Pharmacy,** Cairo, Egypt Bachelor of Pharmaceutical Sciences **Grade:** Very Good

RESEARCH EXPERIENCE

04.2015-Present	**Kinetics and timing of IL-12 production by dendritic cells for Th1 priming in vivo** PhD thesis project, AG Lutz, Institut für Virologie und Immunbiologie, Würzburg, Germany
01.2015-02.2015	**Genomic and functional analysis of the lncRNA LIPCAR** Internship, AG Dandekar, Bioinformatik, Würzburg, Germany
03.2014-09.2014	**Investigation of two novel long non-coding RNAs that are differentially expressed in microbially stimulated human monocytes** Master's thesis project, AG Vogel, Institute for Molecular Infection Biology (IMIB), Würzburg, Germany
01.2014-02.2014	**Analysis of a novel long non-coding RNA locus in human monocytes**: Internship, AG Vogel, Institute for Molecular Infection Biology (IMIB), Würzburg, Germany

PROFESSIONAL EXPERIENCE

08.2010-03.2013	**Pharmacist and Manager at Pharma Care drug distribution company (Family Business)** The company is mainly responsible for the storing and, distribution of medications to community pharmacies in Cairo, Egypt
05.2010-0.8.2010	**Community Pharmacist** at Salamtak Pharmacy, Ciaro, Egypt

CONFERENCES

01.2018	**Oral presentation:** The Dendritic Cell Symposium 2018 in Mainz, Germany

09.2017 **Poster presentation:** at the 47th Annual Meeting of the German Society for Immunology in Erlangen, Germany

03.2017 **Oral and poster presentation:** at the World Immune Regulation Meeting XI-2017 in Davos, Switzerland

Würzburg,
Place, Date **Signature**

11 AFFIDAVIT

I hereby confirm that my thesis entitled ‘Kinetics and timing of IL-12 production by Dendritic cells for Th1 priming *in vivo*’ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,
Place, Date **Signature**

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation ‘Kinetics and timing of IL-12 production by Dendritic cells for Th1 priming *in vivo*’ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg,
Ort, Datum **Unterschrift**