Dynamics of homeostatic maturation and migration of intestinal dendritic cells

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

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Tag der mündlichen Prüfung: 19.04.2024

Abstract

Dendritic cells (DCs) are the most important antigen-presenting cells (APCs) in the body that play a pivotal role in the induction of both immunogenic as well as tolerogenic adaptive immune responses. Central to these functions is their ability to sample their environment and subsequently migrate from peripheral tissues, via lymph, to lymph nodes, where they present peripherally acquired antigens to T cells. While DC migration can be induced by inflammatory stimuli, DCs continually migrate in the absence of overt inflammation, which is essential for the maintenance of peripheral tolerance. This has been best studied in the context of the intestine, where homeostatic DC migration is indispensable for the induction of tolerance to intestinal antigens, thereby preventing exaggerated immune responses such as chronic intestinal inflammation or food allergies. However, the signals and mechanisms that drive homeostatic DC migration remain largely unknown. Here, we examined the life cycle of small intestinal DCs using a range of approaches, including single-cell transcriptomics, photoconversion-based *in vivo* cell tracking, and multiparameter flow cytometry, to address how the maturation and subsequent migration of intestinal DCs is regulated. Using *in vivo* DC tracking combined with EdU incorporation, we show that small intestinal DCs proliferate *in situ* and, interestingly, are induced to proliferate upon tissue entry. Thereafter, DCs progressively lose their proliferative capacity along their maturation program, which is itself characterized by a gradual increase in surface MHCII as well as costimulatory molecules such as CD40 and CD86. Notably, we found that in the final stages of maturation, DCs share a common transcriptional program that is characterized by the upregulation of CCR7, apoptosis-associated genes and cell cycle arrest, regardless of their tissue of origin or subset. These findings suggest that the induction of migration is inherently linked to the DC maturation program and may therefore be regulated by highly conserved intrinsic pathways. Furthermore, our detailed quantification of the migration kinetics demonstrates that steady-state intestinal DC migration leads to an almost complete turnover of the migratory DC compartment of the mesenteric lymph node (MLN) every day. In addition, we utilize a bone marrow chimera system to reveal that the migration of intestinal cDC1s is inhibited by a cell-intrinsic lack of the metalloprotease ADAM10. Furthermore, we show that the migration of small intestinal CD103+ cDC2s is reduced by administration of the S1PR inhibitor FTY720, demonstrating that these pathways are selectively used by distinct DC subsets for the migration process. Taken together, our data provide novel insights into both the homeostatic maturation and migration of intestinal DCs, while also establishing key experimental systems for the analysis of molecular mechanisms regulating DC migration kinetics. Therefore, the data and tools presented here may aid in the development of drugs and vaccines aimed at enhancing the tolerogenic homeostatic migration of DCs to prevent or treat deleterious inflammatory responses, including allergies and autoimmune diseases.

Zusammenfassung

Dendritische Zellen (DCs) sind die wichtigsten antigen-präsentierenden Zellen (APCs) im Körper, die eine zentrale Rolle bei der Auslösung sowohl immunogener als auch tolerogener adaptiver Immunantworten spielen. Von zentraler Bedeutung für diese Funktionen ist ihre Fähigkeit von peripheren Geweben über die Lymphe zu den Lymphknoten zu wandern, wo sie den T-Zellen die peripher aufgenommenen Antigene präsentieren. Während die Migration von DCs durch Entzündungsreize ausgelöst werden kann, wandern DCs auch dauerhaft, ohne erkennbare Entzündungsbedingungen, was für die Aufrechterhaltung der peripheren immunologischen Toleranz unerlässlich ist. Dieser Zusammenhang wurde besonders im Darm erforscht, wo gezeigt wurde, dass die homöostatische DC-Migration für die Induktion immunologischer Toleranz gegenüber intestinalen Antigenen unerlässlich ist, was überschießende Immunreaktionen wie chronische Darmentzündungen oder Nahrungsmittelallergien verhindert. Die Signale und Mechanismen, die die homöostatische DC-Migration steuern, sind jedoch weitestgehend unerforscht. In dieser Thesis haben wir den Lebenszyklus von DCs im Dünndarm mit verschiedensten Methoden, darunter „single-cell" Transkriptomanalyse, *in vivo* Zellverfolgung mittels Photokonversion und Multiparameter-Durchflusszytometrie untersucht, um herauszufinden, wie die Reifung und anschließende Migration von DCs im Darm reguliert wird. Mithilfe der Photokonversion in Kombination mit EdU-Inkorporationen zeigen wir, dass DCs im Dünndarm *in situ* proliferieren und interessanterweise beim Eintritt in das Gewebe zur Proliferation angeregt werden. Im Laufe ihres Reifungsprogramms verlieren die DCs schrittweise ihre Proliferationsfähigkeit. Dieser Prozess ist durch eine allmähliche Zunahme der MHCII Expression sowie der Expression der ko-stimulatorischen Moleküle CD40 und CD86 gekennzeichnet. Insbesondere haben wir festgestellt, dass DCs in den letzten Stadien der Reifung ein gemeinsames Transkriptionsprogramm aufweisen, das durch die Hochregulierung von CCR7, Apoptose-assoziierten Genen und einem Zellzyklus-Stillstand gekennzeichnet ist, unabhängig von ihrem Ursprungsgewebe oder ihrer Untergruppe. Diese Ergebnisse deuten darauf hin, dass die Migrationsinduktion eng mit dem DC-Reifungsprogramm verbunden ist und daher möglicherweise durch hochkonservierte intrinsische Signalwege reguliert wird. Darüber hinaus zeigt unsere detaillierte Quantifizierung der Migrationskinetik, dass die homöostatische Migration intestinaler DCs bereits nach einem Tag zu einem fast vollständigen Austausch des migratorischen DC-Kompartiments im mesenterischen Lymphknoten führt. Des Weiteren nutzen wir Knochenmarkschimären, um zu zeigen, dass die Migration von intestinalen cDC1s durch eine Zell-intrinsische Deletion der Metalloprotease ADAM10 gehemmt wird. Zudem zeigen wir, dass die Migration von CD103+ cDC2s aus dem Dünndarm durch die Verabreichung des S1PR-Inhibitors FTY720 reduziert wird, was beweist, dass diese Signalwege selektiv von verschiedenen DC-Untergruppen für den Migrationsprozess genutzt

werden. Insgesamt bieten unsere Daten neue Einblicke in die homöostatische Reifung und Migration intestinaler DCs und schaffen gleichzeitig wichtige experimentelle Systeme für die Analyse molekularer Mechanismen zur Regulierung der DC-Migrationskinetik. Daher können die hier vorgestellten Daten und Methoden bei der Entwicklung von Medikamenten und Impfstoffen helfen, die auf eine Verbesserung der tolerogenen homöostatischen Migration von DCs abzielen, um Entzündungsreaktionen, sowie Allergien und Autoimmunkrankheiten, zu verhindern oder zu behandeln.

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1. Introduction

1.1. Dendritic cells

Dendritic cells (DCs) were first described in 1973 by Steinman et. al. who also named the cells after their characteristic morphology as observed under the microscope (Steinman et al. 1973). This discovery paved the way for an extensive research field that has expanded to better understand the ontogeny, phenotype and functions of these cells. DCs are now recognized as the most effective professional antigen presenting cells (APCs) in the body, uniquely able to prime naïve T cells. Like all leukocytes, DCs originate from hematopoietic stem cells in the bone marrow. Their immediate precursors, pre-DCs, are able to enter the blood and seed both lymphoid and non-lymphoid tissues where they differentiate into immature DCs (Naik et al. 2006; Liu et al. 2009) (Figure 1). Equipped with a variety of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectins (CLECs), DCs are able to sense their environment and recognize potential pathogens and tissue damage. In addition, DCs constantly sample their environment for antigens, enabling them to convey information about the immunological state of the tissue in which they reside in to naïve T cells, which are restricted to the secondary lymphoid tissues such as the lymph nodes (LNs). In order to transmit the information acquired in the periphery, DCs have the unique ability to migrate out of peripheral tissues to the tissue-draining lymph nodes via lymphatic vessels. This process is ultimately dependent on the expression of the CC-chemokine receptor 7 (CCR7) and the active movement towards its ligands CC-chemokine ligand 21 (CCL21) and CCL19, guiding cues that are available at increasing levels in afferent lymphatic vessels and the lymph nodes, respectively (Worbs et al. 2017). Once in a lymph node, DCs migrate into T-cell zones where they can interact with naive CD4+ and CD8+ T cells by presenting processed peptides on the major histocompatibility complexes II (MHCII) and MHCI, respectively. In the LNs, DCs excel at priming naïve T cells, as they are not only able to present antigens but also provide co-stimulation and cytokine production which dictate the differentiation and polarization of T cells. Depending on the signals encountered in the periphery, DCs can direct T cell differentiation toward immunogenic effectors such as T helper type 1 (Th1), Th2, Th17 or cytotoxic T lymphocytes (CTLs), as well as toward tolerogenic regulatory T cells (Tregs) (Kapsenberg 2003; Agalioti et al. 2018). With these unique capabilities, DCs represent an important link between the innate and the adaptive immune systems and have therefore come into focus as promising target cells to improve the efficacy of cancer immunotherapy and vaccines, as well as to dampen excessive immune responses such as those seen in allergies or autoimmune diseases.

This thesis will only consider classical or conventional dendritic cells (cDCs) which are associated with the afore mentioned characteristics: cDCs develop from dedicated fms-like

tyrosine kinase 3 ligand (Flt3L)-dependent precursors, are able to migrate via lymph and have the ability to generate *de novo* immune responses by priming naïve T cells. Other cells that share ontogenic, morphologic or phenotypic properties, and were therefore often referred to as DCs, will be mentioned shortly in a later section, highlighting the differences to cDCs. It is important to note that due to the complexity of the life cycle of cDCs, which includes migration between tissues and interaction with other cells, most of the studies on cDCs are based on the mouse as a model system. Therefore, most of the references and markers mentioned refer to the situation in mice. However, whenever human data are referenced, it is explicitly stated.

1.2. DC heterogeneity

Across both murine and human tissues, cDCs can generally be identified by the common, but not exclusive expression of both MHCII and the integrin CD11c (encoded by the *Itgax* gene). cDCs are divided into two major subsets, termed conventional DCs type 1 (cDC1) and cDC2, which are functionally and phenotypically distinct.

1.2.1. cDC1s

cDC1s are a homogeneous and evolutionarily conserved subset of cDCs that can be identified by the specific expression of XC-Motif Chemokine Receptor 1 (XCR1) across different tissues and species (Crozat et al. 2010). The development of cDC1s is dependent on an interplay of transcription factors that include Interferon regulatory factor 8 (IRF8), Basic Leucine Zipper ATF-like Transcription Factor 3 (Batf3) and the inhibitor of DNA binding 2 (Id2) (Jaiswal et al. 2013). Central to cDC1 identity is the level of IRF8 expression, as this transcription factor maintains cDC1-associated chromatin accessibility. Loss of IRF8 in cDC1s results in a loss of cDC1 identity and a switch to a cDC2-like phenotype, while their survival is not affected (Lanca et al. 2022). cDC1s also uniquely express the C-type lectin DNGR-1 (encoded by *Clec9a*) that allows for efficient uptake of apoptotic and dead cells (Schraml et al. 2013). Together with high expression of MHCI pathway genes, cDC1s excel at presenting exogenous antigens on MHCI molecules, a process called cross presentation. Cross presentation is a critical step in the induction of anti-tumor and anti-viral immunity, as it enables the presentation of exogenous cell associated antigen to CD8+ T cells, which is crucial for the generation of CTLs (Embgenbroich et al. 2018; Cerovic et al. 2015). The importance of this process was evidenced by experiments with transporter associated with antigen processing (TAP)-deficient chimeric mice whose hematopoietic compartment lacked MHCI antigen presentation function. These mice were unable to elicit CTL responses against viruses or tumors, emphasizing the role of cDC1 cross presentation ability in these responses (Sigal et al. 1999; Huang et al. 1994). Additionally, cDC1s have been shown to be important for the generation of Th1 immune responses against intracellular pathogens such as *Toxoplasma* due to their non-redundant role

in the production of interleukin 12 (IL-12) (Mashayekhi et al. 2011). Importantly, cDC1s have also been shown to drive tolerogenic responses by generating Tregs in response to orally administered antigens (Esterházy et al. 2016). Furthermore, it was also shown that cDC1s are able to generate Foxp3+ CD8+ T cells that themselves promote tolerance to autoantigens (Joeris et al. 2021), emphasizing the importance of cDC1s for both immunogenic and tolerogenic immune responses.

1.2.2. cDC2s

Compared to cDC1s, cDC2s are a much more heterogeneous subset. While cDC2s can generally be differentiated from cDC1s by the expression of the signal regulatory protein α (Sirpα) and the integrin αM (CD11b), many cDC2 markers are shared with monocytes and macrophages. Therefore, it is important to exclude macrophages using expression of markers such as CD64 and F4/80 or MerTK for accurate cDC2 identification (Backer et al. 2023). In general, cDC2s have many different tissue-specific adaptations which influence their phenotype and function, depending on the niche and microenvironment in which they reside in. Well characterized examples include the Esam+ splenic cDC2s, CD11b- cDC2s in the skin and CD103+ intestinal cDC2s (Merad et al. 2013). While the development of cDC2s is generally dependent on the transcription factor IRF4, it was shown that the interplay of other signal mediators such as Krüppel-like factor 4 (Klf4) and Notch are important for the development of different cDC2 subsets in different tissues (Bosteels et al. 2020b). Additionally, T-bet and RORγt expression has been used to differentiate between different cDC2 subsets termed as cDC2A and cDC2B, respectively (Shin et al. 2020). Functionally, cDC2s are specialized for the presentation of peptides on MHCII and the induction of CD4+ Th cells. For instance, cDC2s are involved in the generation of Th2 cells both in skin and intestine in response to infection with *Nippostrongylus* or *Schistosoma*, respectively (Mayer et al. 2021; Tussiwand et al. 2015). Furthermore, IRF4- as well as Notch-2-dependent cDC2s also produce IL-23 which is necessary for the induction of Th17 both in steady state and in infection models (Schlitzer et al. 2013*)*. Additionally, cDC2s have also been shown to be involved in tolerance induction by generating Tregs (Nutsch et al. 2016; Price et al. 2015). For instance, cDC2s are the main APC that induce Foxp3+ Tregs in response to sublingual delivered antigen (Tanaka et al. 2017). Similarly, dermis derived CD103- cDC2s in the cutaneous LN are capable to induce Foxp3+ Tregs in an *in vitro* setting (Guilliams et al. 2010).

1.2.3. DC3s

Another DC subset that has been described in human blood is termed DC3, which shows features of both cDC2 and monocyte transcriptome and surface phenotype (Villani et al. 2017). DC3s develop from a fraction of granulocyte-monocyte and DC progenitors (GMDPs) that appear to rely on both GM-CSF and Flt3L for their differentiation and maturation (Bourdely et

al. 2020; Dutertre et al. 2019). In humans, DC3s share the expression of CD1c with cDC2s and the expression of CD14 and CD163 with monocytes and macrophages. Depending on the experimental setup, DC3s can induce Th1 responses under inflammatory conditions (Dutertre et al. 2019) and Th17 responses under steady-state conditions (Bourdely et al. 2020). The validation of the existence of DC3s in mice has been challenging due to lack of knowledge of the developmental origin. Recently, Lui et al. described DC3s and their development in the bone marrow (BM), blood and spleen of mice. These murine DC3s share similarities in the transcriptome with human DC3s, derive from Ly6C+ monocyte-dendritic cell progenitors (MDPs), are Flt3L dependent while also expressing CSF1R (Liu et al. 2023). Whether they are also found in peripheral and other lymphoid organs remains an open question and is dependent on the generation of suitable models such as a DC3-specific Cre-recombinase system to unambiguously track this lineage and propose novel markers that will be needed for their characterization.

1.2.4. cDC distinction in lymph nodes depending on tissue of origin

Since peripheral cDCs eventually upregulate CCR7 and migrate into LNs in order to present acquired antigen, LN cDCs can be classified into two compartments, migratory and resident, based on the entry route. Peripheral cDCs that enter LNs via afferent lymph are referred to as **migratory** and can be identified by high expression of MHCII and intermediate expression of the integrin CD11c. Like peripheral tissues, lymph nodes are also seeded by pre-cDCs via blood. These blood-borne cDCs are referred to as **resident** DCs and are characterized by intermediate levels of MHCII and high levels of CD11c. It is important to note that while resident cDCs do not migrate via afferent lymphatics, they are still able to express CCR7 and to migrate into T cell zones, thereby acquiring an activated phenotype similar to migratory cDCs that originate from peripheral tissues (Ugur et al. 2023). Additionally, the distinction of migratory and resident cDCs in lymph nodes is applicable for both cDC1s and cDC2s, since both subsets seed the LN directly via blood and are able to migrate in lymph (Cerovic et al. 2013).

1.2.5. Other APCs

Several different cell types have functional, phenotypic of developmental features that are related to cDCs.

Plasmacytoid dendritic cells (pDCs) express Flt3 and, similar to cDCs, depend on Flt3L for their development (Gilliet et al. 2002). However, the ontogeny of these cells is still a matter of debate. Early studies indicated that pDCs can be generated from both lymphoid- and myeloid precursors as demonstrated by transfer experiments (D'Amico et al. 2003). More recently, this view has been challenged by single-cell and fate tracking experiments suggesting a mainly lymphoid rather than myeloid origin (Dress et al. 2019; Herman et al. 2018). However, depletion of lymphoid progenitors did not affect pDC numbers or perturb their development

(Harman et al. 2006). Similarly, using a DNA barcoding approach, Feng et al. reported that pDCs share ontogeny with cDC1s, but not with lymphoid cells, leaving the exact developmental origin of pDCs an open question (Feng et al. 2022).

In general, pDCs express high levels of the nucleic acid sensors TLR7 and TLR9 and are specialized to sense and react to viral infections (Gilliet et al. 2008). After activation, pDCs respond with massive secretion of type I Interferons (IFNs) that are important mediators in the host defense against viral infections (Reizis 2019). In contrast to cDCs, pDCs are not able to migrate in lymph and are associated with poor antigen presentation capacity (Yrlid et al. 2006a; Villadangos et al. 2008). Taken together, pDCs appear to primarily function as potent interferon producing cells, rather than professional antigen presenting cells, which makes them functionally distinct from cDCs, while the exact developmental origin and relation with cDCs remains to be determined.

Monocyte-derived dendritic cells (moDCs) are a subset of antigen-presenting cells that have been proposed to develop from monocytes and independently of Flt3L dependent pre-cDCs. They have mainly been characterized in inflammatory settings and show substantial phenotypic overlap with cDC2s as they also express MHCII, CD11c, Sirpα and CD11b but can be distinguished by the expression of monocyte markers such as CX3CR1, CD64, CD16 and others. However, the role of moDCs in antigen presentation, and particularly their ability to migrate from periphery to lymph nodes, are still largely controversial. For example, it has been suggested that the transport of *Salmonella* from the LP to the MLN can be carried out by CX3CR1 expressing cells of monocytic origin (Diehl et al. 2013). However, two independent studies that used CCR2- and CCR7-knockout mice showed that, in inflammation, moDCs enter the lymph nodes via blood rather than via lymph, challenging the notion of moDCs transporting peripheral antigens to lymph nodes (Nakano et al. 2009; Gautier et al. 2013). Moreover, a later study showed that CX3CR1-hi cells do not migrate in intestinal lymph while *Salmonella* can also travel to the MLN by cell independent carriage (Bravo-Blas et al. 2019). Interestingly, CD103- cDC2s present in the SI LP and gut lymph share many of the proposed features of moDCs, such as expression of CX3CR1 and partial dependency on CCR2, yet develop from traditional pre-cDC precursors (Scott et al. 2015; Cerovic et al. 2013).These findings question the role of moDCs in antigen transport and subsequent induction of adaptive immune responses in lymph nodes but emphasize the need for suitable markers that can be used to distinguish moDCs from *bona fide* cDCs. This became even more clear when it was shown that, in lung inflammation, *bona fide* cDC2s can express CD64, a marker that is generally used to distinguish monocytes and macrophages from cDCs (Bosteels et al. 2020a). Until reliable markers are found that can distinguish moDCs from cDCs, irrespective of tissue or inflammatory stimulus, precursor transfers as well as fate tracking experiments remain

necessary. Finally, at least some of the cells traditionally described as moDCs may actually belong to the newly characterized DC3 subset of DCs, which develop from unique MDP progenitors, distinct from both pre-cDCs and monocytes (Liu et al. 2023).

The best characterized example of monocyte-derived cells with DC-like functionality are the so-called “lysoDCs” which are prevalent in Peyer’s patches (PPs), inductive sites located along the small intestine. LysoDCs excel at taking up particulate luminal antigen including intestinal bacteria. Upon stimulation, LysoDCs migrate into the interfollicular region of the Peyers patch in a CCR7 dependent manner where they are capable of priming naïve T cells (Wagner et al. 2020).

Langerhans cells (LCs) are APCs that reside in the epidermis, the outermost layer of the skin. LCs share many properties with cDCs including antigen acquisition and processing, CCR7-dependent migration into skin draining lymph nodes as well as antigen presentation and induction of adaptive immune responses (Forster et al. 1999). The main difference to cDCs is their ontogeny. Indeed, LCs share the ontogeny with many tissue macrophages as they derive from prenatal precursors, are independent of Flt3 signaling and self-sustain in tissue independently of blood precursor replenishment (Merad et al. 2008; Liu et al. 2019). Other examples of prenatally derived macrophages include microglia in the brain, Kupffer cells in the liver or Tim4+ macrophages in the intestine and other tissues (Mass et al. 2023; Bain et al. 2016). Therefore, LCs seem to represent a unique adaptation whereby a macrophage-like population takes on some DC-like functions. A notable difference between LCs and cDCs is their migration kinetics. While half of the dermal cDCs in the skin draining LNs are replenished after 20 hours, the LN LC compartment replenishes with significantly reduced migration kinetics (Tomura et al. 2014).

1.3. Ontogeny of cDCs

Like most immune cells, cDCs originate from hematopoietic stem cells (HSCs) in the bone marrow. DCs develop from the myeloid lineage and share their developmental origin with monocytes with a common ancestor named monocyte-DC progenitors (MDP) (Liu et al. 2009). The earliest dedicated precursors of cDCs in the bone marrow are the common DC progenitors (CDPs) which can give rise to pre-cDCs and pDCs (Naik et al. 2007; Guilliams et al. 2014). Pre-cDCs already show commitment to cDC1s (pre-cDC1) and cDC2s (pre-cDC2) in the bone marrow and at that stage are able to seed many different lymphoid and non-lymphoid tissues to give rise to immature cDCs (Schlitzer et al. 2015). A common feature of the cDC lineage is their dependence on the cytokine Flt3L, as Flt3L deficient mice show severely reduced numbers in CDPs, pre-cDCs and cDCs (McKenna et al. 2000; Kingston et al. 2009). Therefore, expression of Flt3, the Flt3L receptor can be used to distinguish cells from a *bona fide* cDC lineage from other myeloid cells such as monocytes, which express CSF1R and are dependent

on the macrophage colony-stimulating factor CSF1 (Rojo et al. 2019). Together with the recently described RORγt-expressing APCs in the MLN, expression of the transcription factor Zbtb46 has been shown to be highly expressed by cells of the cDC lineage (Akagbosu et al. 2022; Kedmi et al. 2022; Zhou et al. 2022; Satpathy et al. 2012). While Zbtb46 deficient mice did not show any defects in the cDC compartment, it serves as a suitable marker for *bona fide* cDCs (Satpathy et al. 2012).

1.4. Life-cycle of conventional dendritic cells

1.4.1. Definition of cDC nomenclature

Since cDCs can drastically change their phenotype along their lifecycle, different researchers introduced different nomenclatures to refer to the cDC differentiation states. The inconsistent use of these terminologies occasionally led to confusion in the field of cDC biology (Cabeza-Cabrerizo et al. 2021a) The terms and definitions used in this thesis are as follows (also see Figure 1):

The term **differentiation** is used to describe transitions between distinct states of cDCs. First, differentiation describes the developmental transition from a pre-cDC to an immature cDC as it occurs when pre-cDCs enter lymphoid and non-lymphoid tissues. Secondly, differentiation is also used to describe the transition of tissue resident cDCs towards a distinct environmentally conditioned subset as it occurs for Esam+ cDC2s in the spleen or CD103+ cDC2s in the small intestine.

Maturation of cDCs describes the gradual phenotypic changes of tissue residing cDCs. Maturation is associated with an increase in surface expression of MHCI and MHCII and the so-called maturation markers CD40, CD80 and CD86.

Activation of cDCs describes the functional switch of cDCs from an antigen acquisition mode towards an antigen processing and presentation mode. The activation of cDCs occurs in both the steady state and in inflammation and is accompanied by the expression of CCR7. Since activated cDCs also express high levels of MHC and maturation markers this state is often referred to as fully mature cDCs.

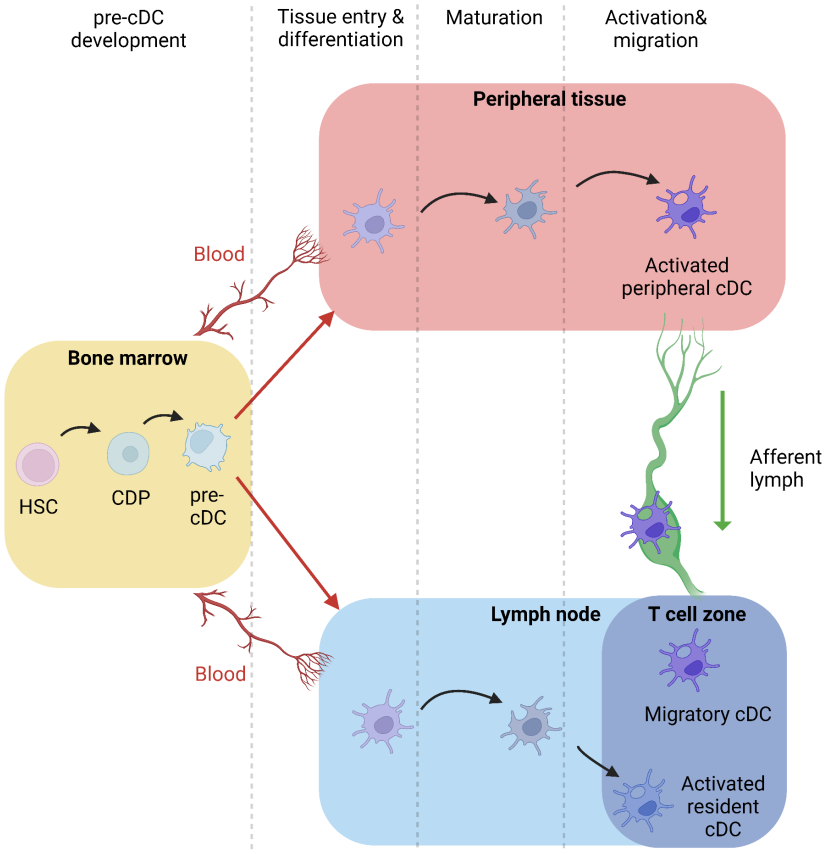


Figure 1 Life cycle of cDCs. Bone marrow-derived cDC precursors (pre-cDCs) are able to enter the blood and seed both non-lymphoid and lymphoid tissues where they differentiate into immature cDCs. In the tissue, cDCs mature until they are activated which is accompanied by upregulation of CCR7 and subsequent migration. While activated lymph node resident cDCs migrate into the T cell zone, peripherally activated cDCs migrate via afferent lymph into draining lymph nodes before they also enter the T cell zones to present acquired antigen to naïve T cells. **HSC**: hematopoietic stem cell; **CDP**: common dendritic cell progenitor; **cDC**: conventional dendritic cell; Created with BioRender.com.

1.4.2. Tissue seeding

The receptors and signals that regulate pre-cDC egress out of the bone marrow and seeding of different tissues remain largely unknown. Experiments using mixed bone marrow chimeras showed that CXCR4 expressed on pre-cDCs is needed for their retention in the bone marrow (Nakano et al. 2017). Experiments using CCR2 knockouts showed that upon inflammation caused by Influenza A virus, pre-cDC BM egress is accelerated in a CCR2-dependent manner (Cabeza-Cabrerizo et al. 2021b). Additionally, expression of CX3CR1 was shown to be partially required for pre-cDC entry into the lung upon LPS inhalation, but not the spleen and only in this inflammatory setting (Nakano et al. 2017). Specific for the small intestine, retinoic acid (RA) was shown to act on pre-cDCs that express the integrin α4β7 and seed the SI LP to give rise to CD103+ cDCs (Zeng et al. 2013). Interestingly, pre-cDC entry into lymphoid tissues was assumed to occur via high endothelial venules (HEVs) in the paracortex via L-selectin (CD62L), analogously to pDCs and lymphocytes. This view has been challenged by a recent

publication by Ugur et al. that showed that, in a cDC1 depletion model, reseeding cDC1s enter the mesenteric lymph node via medullary LN HEVs and possibly also via medullary venules, rather than via HEVs in the paracortex (Ugur et al. 2023). Taken together, homing of pre-cDCs into tissues is dependent on a complex interplay of different chemokine receptors and ligands which may not only differ for certain tissues, but also change in response to inflammation.

1.4.3. Environmental sensing and maturation of cDCs

Immature cDCs in tissues are specialized in the sampling of both foreign and self-antigens. The uptake of antigen is mediated by receptor dependent endocytosis, macropinocytosis and phagocytosis (Liu et al. 2015). All of these mechanisms enable a transfer of exogenous antigen into endolysosomes where antigens are further processed by acidification and hydrolysis. Compared to macrophages, cDCs show lower acidification of phagolysosome in order to preserve the antigen for subsequent loading onto MHCII molecules (Savina et al. 2007). Additionally, cDCs are equipped with a variety of receptors that allow for immune sensing of the environment. PRRs are located on the cell surface or in endosomes such as TLRs, C-Type Lectins as well as in the cytoplasm such as the nucleic acid sensors MDA5 and RIG-I as well as the NOD-like receptors (NLRs) (Li et al. 2021). Tissue residing cDCs continually undergo maturation which is accompanied by phenotypical changes. For instance, maturing LN cDC1s gain surface expression of XCR1 and CD8α with increased residency time (Ugur et al. 2023). Similarly, splenic cDC1s show gradual increase in MHCII, CD40 and CD86 expression along their maturation (Bosteels et al. 2023). Eventually, maturation culminates in cDC activation which occurs in both inflammation as well as in homeostasis. This leads to drastic phenotypic changes that are accompanied by a shift from the antigen acquisition mode towards an antigen processing presentation mode. Besides the increased surface expression of MHCI and MHCII and maturation markers CD40, CD80 and CD86, which are all associated with T cell activation and priming, activated cDCs also express CCR7 which allows peripheral but also lymph node resident cDCs to migrate into the T cell zone of draining LNs (see below cDC migration). Maturation and ultimately activation are important steps for the function of cDCs as the stimulus of activation can dictate the signals which determine the ensuing adaptive immune response. While cDC activation by pathogen-associated molecular patterns (PAMPs) is thought to favor immunogenic immune responses, steady state activation of cDCs that occurs without any overt inflammatory stimuli is associated with tolerogenic immune responses (Lutz et al. 2002).

1.4.4. Lifespan of cDCs in tissues

For a long time, it had been unclear whether cDCs proliferate in tissues or whether they are solely maintained by BM derived pre-cDCs analogously to monocyte derived macrophages. This question was addressed using advanced experimental approaches including parabiotic

mice and BrdU incorporation. In parabiotic mice, an unequal distribution of cDCs between the parabioic partners indicated a short half-life of cDC precursors in the blood (Liu et al. 2007). After separation of parabionts, cDCs derived from the parabiotic partner could be detected for up 10-14 days in the LNs and spleen (Liu et al. 2007) and up to 30 days in the lung (Ginhoux et al. 2009), which validates that cDCs are dependent on blood precursor replenishment. Additionally, by transplanting spleens, Liu et al. showed that graft cDCs incorporate BrdU which confirms *in situ* proliferation which may also increase the total time of tissue residency (Liu et al. 2007). More recently, *in situ* proliferation was also indirectly shown for small intestinal cDCs using a multicolor fate mapping approach as cDCs exist in clonal clusters in the topologically separated villi (Cabeza-Cabrerizo et al. 2019).

cDC activation simultaneously marks the last step of the cDC lifecycle which is accompanied by a short remaining lifespan. This is supported by the fact that cDCs that migrate into the lymph node do not leave the lymph node again via efferent lymph (Haig et al. 1999). Additionally, cDCs are rapidly cleared after they have presented antigen in the lymph node (Ingulli et al. 1997). This is in accordance with more recent data using mice that express the photoconvertible protein KikGR. In this study, Tomura et al. showed that in skin draining lymph nodes, migratory LCs and cDC1s are replenished after 4-5 days while CD103- cDC2s are replenished after a maximum of 2 days. In addition, cDC2s displayed higher caspase activity than LCs and cDC1s which is consistent with their increased replenishment rate (Tomura et al. 2014).

1.5. cDC migration

The ability to migrate through the body is a fundamental property of almost all leukocytes, encompassing egress from the bone marrow, circulation in the blood, extravasation and tissue entry. Unique for cDCs, and crucial for their function, is the ability to exit peripheral tissues via lymphatics in order to present acquired antigen to naïve cells of the adaptive immune system that are restricted to lymphoid organs and the blood. This migration process ultimately depends on the expression of the G protein-coupled receptor (GPCR) CCR7 which recognizes the CC-chemokine ligands 21 (CCL21) and CCL19 (Förster et al. 2008). CCR7-expressing cDCs are able to migrate into highly fenestrated lymphatic capillaries by haptotaxis, guided by CCL21, which is expressed and bound on the surface of lymphatic endothelial cells (LECs) (Weber et al. 2013; Schwarz et al. 2017). Compared to chemotaxis, which relies on gradients of soluble signaling molecules, haptotaxis relies on membrane bound ligands and their increasing concentration towards the lymphatic collecting vessels, thereby building a sustained “road” that cDCs use for their migration (Schwarz et al. 2017). After entry into lymphatic capillaries and the active migration along the LECs, cDCs reach lymphatic collecting vessels with a larger

diameter where they are further carried by passive lymph flow. This leads to ~200-fold increased velocity of migration with up to 1200 µm/min (Tal et al. 2011). Lymph transports the cDCs into the subcapsular sinus of the LN where again CCL21 gradients guide the cDCs into the LN parenchyma (Braun et al. 2011). In the LN itself, both CCL21 and CCL19 expressed by stromal cells guide the CCR7+ activated cDCs into the T cell zones where antigen presentation takes place (Luther et al. 2000) (migration process summarized in Figure 2).

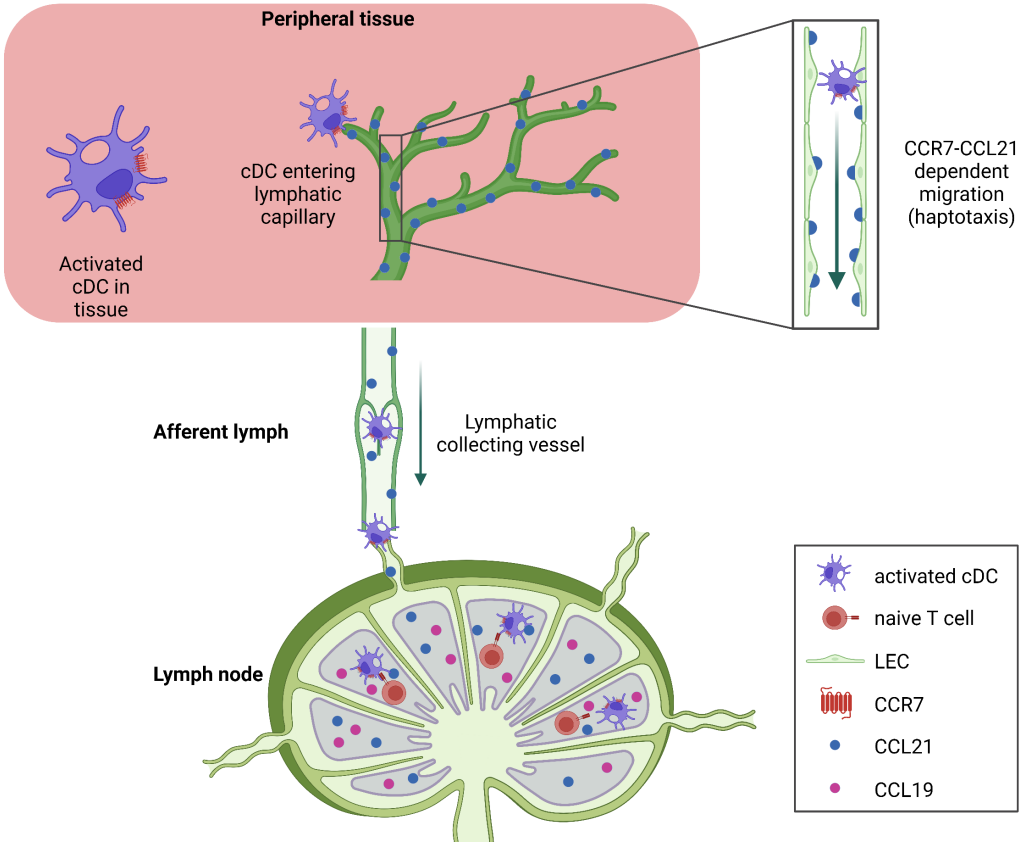


Figure 2 cDC migration from peripheral tissues to lymph nodes. Activated CCR7-expressing cDCs are able to enter lymphatic capillaries which are the terminal lymph vessels in peripheral tissues. After entry, cDCs crawl along increasing gradients of CCL21 that is expressed and bound on the cell surface membrane of lymphatic endothelial cells (LECs). Once cDCs reach the bigger lymphatic collecting vessels they are further transported to lymph nodes by passive lymph flow. In the lymph node, both CCL19 and CCL21 guide the activated cDCs into T cell zones where they can present acquired and processed antigen to naïve T cells. Created with BioRender.com.

Activation and subsequent CCR7-dependent migration of peripheral cDCs into the draining lymph nodes is crucial for the induction of *de novo* adaptive immune responses (Itano et al. 2003; Ohl et al. 2004). The migration of cDCs is often associated with a response to an inflammation. However, there is also constant CCR7-dependent migration of cDCs in the steady-state without any overt inflammation which is evidenced by the consistent presence of cDCs in afferent lymph as well lymph nodes as migratory cDCs at any given time (Cerovic et al. 2013; Ohl et al. 2004; Pugh et al. 1983). This steady-state migration of cDCs is crucial for the induction of peripheral tolerance against self but also harmless foreign antigens and therefore plays a major role in the prevention of autoimmune- and allergic reactions. The

maintenance of this peripheral tolerance is mediated by different mechanisms. Homeostatic migration of autoantigen-bearing cDCs favors the inactivation of self-reactive T cells that escaped thymic selection via mechanisms that include T cell deletion, induction of anergy or conversion to Tregs (Hasegawa et al. 2018). This was mainly shown using transgenic T cells that are specific for specific model antigens such as ovalbumin (OVA). For instance, in experiments where OVA is expressed by keratinocytes of the skin or intestinal epithelial cells, steady-state migrating cDCs transport the antigen to the draining lymph nodes and that mediate the deletion of OVA-specific CD8+ T cells or generate OVA-specific Tregs (Waithman et al. 2007; Azukizawa et al. 2011). Similarly, in a model of autoimmune encephalomyelitis, the generation of myelin oligodendrocyte glycoprotein-specific Tregs is mediated by migrating cDCs after s.c. immunization and is abolished in CCR7-deficient mice (Idoyaga et al. 2013). Similarly, the maintenance of prostate-specific Tregs is dependent on the homeostatic migration of cDCs from the prostate into its draining lymph node thereby promoting tissue-specific tolerance (Leventhal et al. 2016).

Due to their tolerogenic properties, steady-state migrating cDCs are a suitable target for the development of novel drugs and vaccines that aim to prevent or treat autoimmune diseases and allergies (Van Brussel et al. 2014). However, the signals and mechanisms that control CCR7 expression and cDC migration in the steady-state remain elusive.

1.5.1. Induced migration of cDCs

Most of our knowledge of cDC migration comes from experiments that studied cDC migration under the influence of inflammatory stimuli. Different studies showed that administration of TLR agonists leads to an efflux of cDCs from peripheral tissues while the numbers of cDCs in the lymph nodes are increased. For instance, oral administration of R848, a potent TLR7/8 agonist, leads to a mobilization of intestinal cDCs that is evidenced by increased cDC numbers in the MLN and a strong reduction of cDCs in the lamina propria (Schulz et al. 2009; Garcias Lopez et al. 2020). Similar results were observed in mesenteric lymph-adenectomized rats that showed a more than 25-fold increase in DCs in pseudo-afferent lymph after R848 administration, highlighting the potency of this molecule in inducing cDC migration (Yrlid et al. 2006b). Administration of poly I:C, a molecule that mimics double stranded DNA and leads to activation of TLR3 also leads to the migration of both cDC subsets, suggesting that it is the TLR induced translocation of NF-κB that induces the upregulation of CCR7 in cDCs (Garcias Lopez et al. 2020). However, since TLR3 is only expressed by cDC1s but not by cDC2s, this raised the question whether the induced migration of cDCs is a result of direct TLR signaling on cDCs or whether other cell types are acting in *trans* to activate cDCs. Indeed, R848 induced migration of cDCs ultimately depends on tumor necrosis factor alpha (TNF-α) that is produced by pDCs (Yrlid et al. 2006b; Wendland et al. 2007). However, the cell type that expresses

TLR3 and induces cDC migration in response to poly I:C administration has not been described yet. Another example of subset specific TLR expression is TLR5 which is expressed by intestinal cDC2s but not by cDC1s. Interestingly, administration of the TLR5 agonist flagellin specifically and solely induces the migration of cDC2s which suggests that the migration is regulated by different mechanisms in response to different triggers (Flores-Langarica et al. 2012). Besides using TLR agonists, different studies also showed an impact of induced tissue damage on cDC migration in the skin. For instance, tape stripping leads to increased numbers of dermal-derived cDCs in the skin draining lymph node (Tomura et al. 2014). Similarly, migration of dermal cDCs can also be induced by skin painting using FITC dissolved in the sensitizing vehicle of acetone:dibutyl phthalate, emphasizing that cDC migration is not solely regulated by PAMPs that can act on receptors such as TLRs but may be more of a general response to external stressors (Shklovskaya et al. 2008).

1.5.2. Homeostatic migration of cDCs

The signals and mechanisms that drive the migration of cDCs in the steady-state are still largely an open question. The understanding of induced cDC migration quickly led to the idea that homeostatic cDC migration is also driven by inflammatory stimulation that differs in quantity but not in quality. This was further supported by the observation that the steady-state migration of cDCs is reduced in mice that lack the key TLR adaptor protein MyD88 (Hagerbrand et al. 2015). A similar pattern was observed in mice with disrupted NF-κB signaling, also indicating that tonic TLR signaling may be the driver of steady state DC migration (Baratin et al. 2015). However, cDCs that migrate in the steady-state differ in both their transcriptome and phenotype compared to cDCs that were induced by TLR agonists (Ardouin et al. 2016; Yrlid et al. 2006b). This is especially interesting with regards to early observations that show that disruption of E-cadherin and subsequent β-catenin activation induces cDC activation and CCR7 expression. Additionally, these β-catenin activated cDCs show tolerogenic properties and have a phenotype that is distinct of that from cDCs induced by TLR activation (Jiang et al. 2007). Furthermore, there is also neither reduction in the migration of intestinal cDCs of germ-free mice, nor in mice that have a genetic deletion of the TLR adapter protein TRIF, suggesting that the steady state migration of cDCs is regulated by mechanisms distinc from TLR-mediated PAMP recognition (Hagerbrand et al. 2015; Wilson et al. 2008). More recently, the uptake of apoptotic material has been suggested as a driver of homeostatic cDC activation. For instance, administration of lipid nanoparticles that mimic apoptotic material drives the homeostatic migration of cDC1s but not of cDC2s (Bosteels et al. 2023). A similar pattern was observed for both cDC subsets in tumors that showed increased migration associated phenotypes after uptake of apoptotic tumor cells (Maier et al. 2020). While many studies show that different signals and pathways play a certain role in the induction of homeostatic cDC migration, blockade of those pathways barely resemble the severe

reduction of migrating cDCs that is seen in CCR7 knockout mice (Hagerbrand et al. 2015; Baratin et al. 2015; Ohl et al. 2004). Thus, it remains an open question which signals and pathways regulate the homeostatic migration of antigen-bearing cDCs from periphery to the lymph nodes that is indispensable for maintaining immune tolerance and preventing deleterious inflammation.

1.5.3. Molecular mechanisms of cDC migration

1.5.3.1. Integrins and adhesion molecules in cDC migration

cDCs can express a variety of cell adhesion molecules (CAMs) such as cadherins and integrins which are involved in many different biological processes such as cell adhesion and migration. Integrins consist of heterodimers that are located on the cell membrane, enabling interaction with the extracellular matrix including fibronectin and collagen. Intracellularly integrins are connected to the actin cytoskeleton providing a direct linkage to extracellular proteins. Mechanical forces on integrin bindings can therefore be sensed by cells which can result in intracellular signaling (Sun et al. 2019).

Lämmermann et al. showed that integrins are important for the migration of DCs in a 2D environment as they are needed for the generation of traction forces. Interestingly, a complete loss of integrins did not affect migration into the lymph nodes nor the interstitial migration of cDCs *in vivo*. This is explained by an integrin independent way of migration that DCs use in 3D environments that relies on the sole force of actin dependent protrusions that is also referred to as flowing and squeezing (Lämmermann et al. 2008). However, other adhesion molecules have been shown to mediate cDC migration. For instance, a knockout of the adhesion molecule L1 in both endothelial cells and hematopoietic cells leads to reduced numbers of CD11c+ cells in the inguinal LN after FITC skin painting (Maddaluno et al. 2009). Others have shown that antibody mediated blockade of ICAM-1 and VCAM-1 leads to reduced numbers of migrating skin DCs after FITC skin painting, emphasizing the role for adhesion molecules for the migration process (Johnson et al. 2006). Consistentl with this, Arasa et al. showed that integrin β1 expressed on DCs interacts with VCAM-1 expressed on LECs and loss of these molecules leads to reduced migration of cDCs. This effect was most pronounced in inflammation which also leads to the upregulation of VCAM-1 on LECs (Arasa et al. 2021). Additionally, van Rijn et al. showed that Semaphorin 7A, a glycosylphosphatidylinositol (GPI) anchored protein, decreases the adhesive properties of DCs which eventually promotes the migration process (van Rijn et al. 2016). Overall, the experimental evidence for the role of adhesion molecules in cDC migration is somewhat contradictory and seems to depend on the experimental setup and inflammatory status. Therefore, the extent to which integrins and other adhesion molecules mediate cDC migration, especially in the steady-state, remains an open question and requires further studies.

1.5.3.2. ADAMs in cDC migration

The A disintegrin and metalloproteinase (ADAM) family of proteases comprises 21 and 24 different ADAMs in humans and mice, respectively, which are involved in many biological processes such as development, proliferation and migration of cells (Seals et al. 2003). ADAMs are expressed on the cell surface and contain different functional domains including a disintegrin domain and a metalloproteinase domain. ADAMs utilize their enzymatic domains to cleave many different substrates relevant for immune processes, such as the cleavage of TNF-α into its soluble form by ADAM17 and shedding of Notch by ADAM10 (Black et al. 1997; Hartmann et al. 2002). Both ADAM10 and 17 are expressed by cDCs and have been shown to play a role in cDC function. For instance, a CD11c-Cre driven knockout of ADAM10 leads to reduced numbers of Esam+ splenic DCs due to impaired Notch dependent differentiation (Lewis et al. 2011). Using the same mouse model, Damle et al. could show a deficiency in Th2 responses which could be rescued by Notch1 overexpression (Damle et al. 2018). ADAM10 has also been shown to be involved in E-Cadherin shedding, leading to β-catenin activation (Maretzky et al. 2005). Noably, disruption of E-cadherin adhesions and β-catenin activation have been described to induce the migration of cDCs (Jiang et al. 2007). Interestingly, experiments in skin explants showed an increase in the expression of metalloproteinases including ADAMs in LECs in response to inflammatory stimuli. This leads to degradation of collagen in the surrounding of the LECs which in turn enhances DC entry into lymphatic collecting vessels but not into lymphatic capillaries (Arasa et al. 2021).

1.5.3.3. S1PRs in cDC migration

Sphingosine-1-phosphate receptor (S1PR) signaling is well described to be involved in the migration process of leukocytes (Kumar et al. 2015). There are five S1PRs which are G-protein coupled receptors that all bind and subsequently internalize their ligand spingosine-1-phosphate (S1P) (Baeyens et al. 2020). A main source of S1P are red blood cells and endothelial cells which contain high amounts of S1P synthesizing enzymes. S1P concentrations are highest in blood and decrease towards the lymph whereas lymph nodes and peripheral tissues have the lowest concentrations of available S1P (Yanagida et al. 2017). These strictly regulated and tissue-dependent gradients of S1P allow this molecule to function as a guidance cue for cell migration across different tissues. The role of S1P in leukocyte migration has been best described in lymphocytes such as naïve B- and T cells. Naïve lymphocytes in LNs express S1PR1 and migrate along the S1P concentration gradient which is higher in efferent lymphatic vessels than the LN parenchyma therefore acting as guidance for lymphocytes to exit the lymph nodes. Blockade of S1PR signaling using the S1P analogue and functional antagonist Fingolimod (FTY720), that inhibits all S1PRs except for S1PR2, leads to lymphocyte retention in the lymph nodes and is therefore used as a drug in multiple sclerosis patients (Matloubian et al. 2004).

The role of S1PR signaling in cDC migration from the periphery to the lymph nodes is not entirely clear. cDCs express all five S1PRs and interestingly, upregulate S1PR1-3 upon stimulation with LPS indicating that activated cDCs may utilize S1PRs for their migration (Lan et al. 2005; Idzko et al. 2006). Both *in vitro* and *in vivo* experiments showed reduced numbers of migrating cDCs in the draining lymph nodes of the skin, lung and intestine of S1PR knockout mice compared to WT controls. For instance, it was shown that after FITC skin painting, skin-derived CD11c+ cells, consisting of cDCs and LCs, rely on S1PR1 but not S1PR3 signaling for the migration as quantified by reduced numbers of FITC+ cells in the skin draining lymph nodes in S1PR1 deficient mice or after using a S1PR1 specific inhibitor (Rathinasamy et al. 2010; Lamana et al. 2011). Intratracheal administration of FITC-labelled OVA in combination with FTY720 also led to reduced numbers of FITC+ DCs in the mediastinal lymph node while FITC+ DCs in the lung were increased, indicative of an impaired migration (Idzko et al. 2006). This was further demonstrated by competitive transfers of *in vitro* generated and LPS-matured bone marrow-derived DCs (BMDC) into the foot and trachea of mice, showing reduced migration of S1PR1- and S1PR3-deficient BMDCs compared to WT control BMDCs (Rathinasamy et al. 2010). Besides the effects on the migration of skin and lung DCs, it was also shown that small intestinal CD103+ DCs rely on S1PR signaling for their migration. Following either pharmacological inhibition of S1PR1 or in S1PR3 knockout mice, migration to the mesenteric lymph node (MLN) was reduced after LPS stimulation but not in the control group that received PBS, suggesting that S1PR signaling may be important for the migration of cDCs in inflammatory settings but not in the steady state (Rathinasamy et al. 2010). Although several studies show a clear effect of S1PR interference on the migration of DCs, no current hypothesis fully accounts for the effects of S1PR signaling on DC migration or where a possible S1P gradient might play a role. Furthermore, the experiments were mostly performed under inflammatory settings including FITC skin paintings or the administration of LPS, leaving the question open to what extent S1PR signaling is involved in the homeostatic migration of cDCs.

1.6. Conventional dendritic cells in the intestine

Both the small intestine (SI) and large intestine (LI) are central organs of the digestive system that contain the largest proportion of immune cells in the body. The SI is divided into the duodenum, the jejunum and the ileum and is specialized for the uptake of nutrients from diet. For this, the SI evolved villi, finger-like protrusions that reach into the gut lumen and drastically increase the surface area. The LI follows after the SI and is dedicated to the absorption of water, vitamins and electrolytes from the gut content. Both organs are populated by a vast number of mutualistic microorganisms called the microbiome. The number of microbes in the human intestine ascends from the SI towards the LI with up to 10^5 and 10^{11} microorganisms per ml of gut content, respectively (Sender et al. 2016). The vast amount of harmless dietary

antigen in the SI and bacterial antigen in both the small and large intestine require mechanisms that suppress an inappropriate activation of the immune system. Therefore, the intestine harbors many different immune cells that keep the delicate balance between immunity against pathogens but also tolerance to dietary proteins and commensal bacteria. Crucial for the generation and maintenance of this balance are intestinal cDCs and crucially their homeostatic migration into the mesenteric lymph node.

In the intestinal tracts, cDCs are located in gut-associated lymphoid tissues (GALTs) ((reviewed in (Luciani et al. 2022)) and throughout the lamina propria (LP), which is the connective tissue that is underneath the intestinal epithelial cell (IEC) layer (Figure 3) (Mowat et al. 2014). Both cDC1s and cDC2s can be found throughout the entire lamina propria of the SI and LI but the ratio of the cDC subsets changes along the different parts of the gut. The SI contains more cDC2s than cDC1s whereas this ratio is reversed in the LI, which itself is dominated by CD103+ cDC1s. Unique for the intestine is the occurrence of tissue-adapted CD103+ cDC2s. This cDC2 subset is most prevalent in the duodenum and jejunum whereas their proportion among all cDCs decreases towards the ileum, with only rare presence in the LI, where cDC2s are mainly CD103-. The phenotype of CD103+ cDC2s is dependent on Notch2 signaling and the interplay of transforming growth factor beta (TGFβ) and retinoic acid (RA), both of which are enriched in the SI environment and are associated with the induction of immune tolerance (Bain et al. 2017; Zeng et al. 2013; Satpathy et al. 2013). Uniquely, CD103+ cDC2s have been shown to colonize the epithelium by RA-induced migration, which further imprints a tolerogenic phenotype (Farache et al. 2013; Rivera et al. 2021). Developmentally, CD103+ cDC2s likely arise from CD103- cDC2s as a result of tissue conditioning. This is supported by a reduction of CD103+ cDC2s in TGFβR1 knockout mice with a concomitant increase in CD103- cDC2s (Bain et al. 2017). Similarly, a CD11c-Cre driven knockout of the receptor Notch2 showed a strong reduction in CD103+ cDC2s while CD103- cDC2s increased, indicating their dependency and suggesting that the CD103- cDC2s are a less differentiated intermediate (Satpathy et al. 2013).

In general, all cDC subsets of the intestinal LP are able to migrate via lymph into the tissue draining lymph node called mesenteric lymph node (MLN) which is located in the mesenteric fat (Cerovic et al. 2013). The MLN consists of several lymph nodes which each drain different parts of the intestine (Houston et al. 2016; Esterhazy et al. 2019). The migration of cDCs from the intestinal LP to the MLN is crucial for the induction of both inflammatory but also tolerogenic immune responses. For instance, intestinal cDC1s are important Th1 inducers in response to *Toxoplasma gondii* infection due to their ability to produce high amounts of IL-12 (Mashayekhi et al. 2011). Intestinal cDC2s on the other hand are an important source of IL-23 and are potent inducers of Th17 responses as evidenced by reduced numbers of IL17+ T cells in the MLN of

mice that have strongly reduced numbers of cDC2s using a conditional deletion of IRF4 (Persson et al. 2013; Scott et al. 2015). More importantly, the small intestine contains a huge amount of foreign harmless dietary antigens which require mechanisms of tolerance in order to prevent the generation of food allergies or inflammatory bowel diseases (IBD). The induction of systemic tolerance against orally fed antigen, a process called oral tolerance, is dependent on the steady state migration of intestinal cDCs which mediate the uptake, processing and transport of antigen from the intestine into the MLN (Worbs et al. 2006). For instance, it was shown that mice that lack MLNs fail to induce oral tolerance (Spahn et al. 2002). Abrogation of oral tolerance was later also shown in a model of DC depletion using conditional expression of the diphtheria toxin receptor driven by Zbtb46 and CD11c, further highlighting the non-redundant role for cDCs in this process (Esterházy et al. 2016). More importantly, CCR7-deficient mice in which the migration of cDCs is abrogated also failed to induce tolerance against orally fed antigen, emphasizing the importance of steady state migration of cDCs in the induction of oral tolerance (Worbs et al. 2006). Intestinal cDCs are also equipped with a variety of molecular mechanisms that favor the generation of Foxp3+ Tregs, which are generally associated with the induction of tolerance. For instance, intestinal cDCs in both the intestine and the MLN express high levels of aldehyde dehydrogenases which convert the Vitamin A (retinol)-derived retinal into retinoic acid (RA) (Sun et al. 2007). Additionally, intestinal cDCs also express the integrin αVβ8 which catalyzes the conversion of pro-TGF-β into active TGF-β (Worthington et al. 2011). Both RA and TGF-β favor the induction of Tregs which also directly links the generation of tolerogenic adaptive immune responses with steady state migration of cDCs (Sun et al. 2007; Worthington et al. 2011). Due to their crucial role in maintaining systemic tolerance, a better understanding of how the life cycle and migration of intestinal cDCs is regulated will be an important step in the development of new therapeutic strategies aimed at enhancing the tolerogenic properties of migrating cDCs.

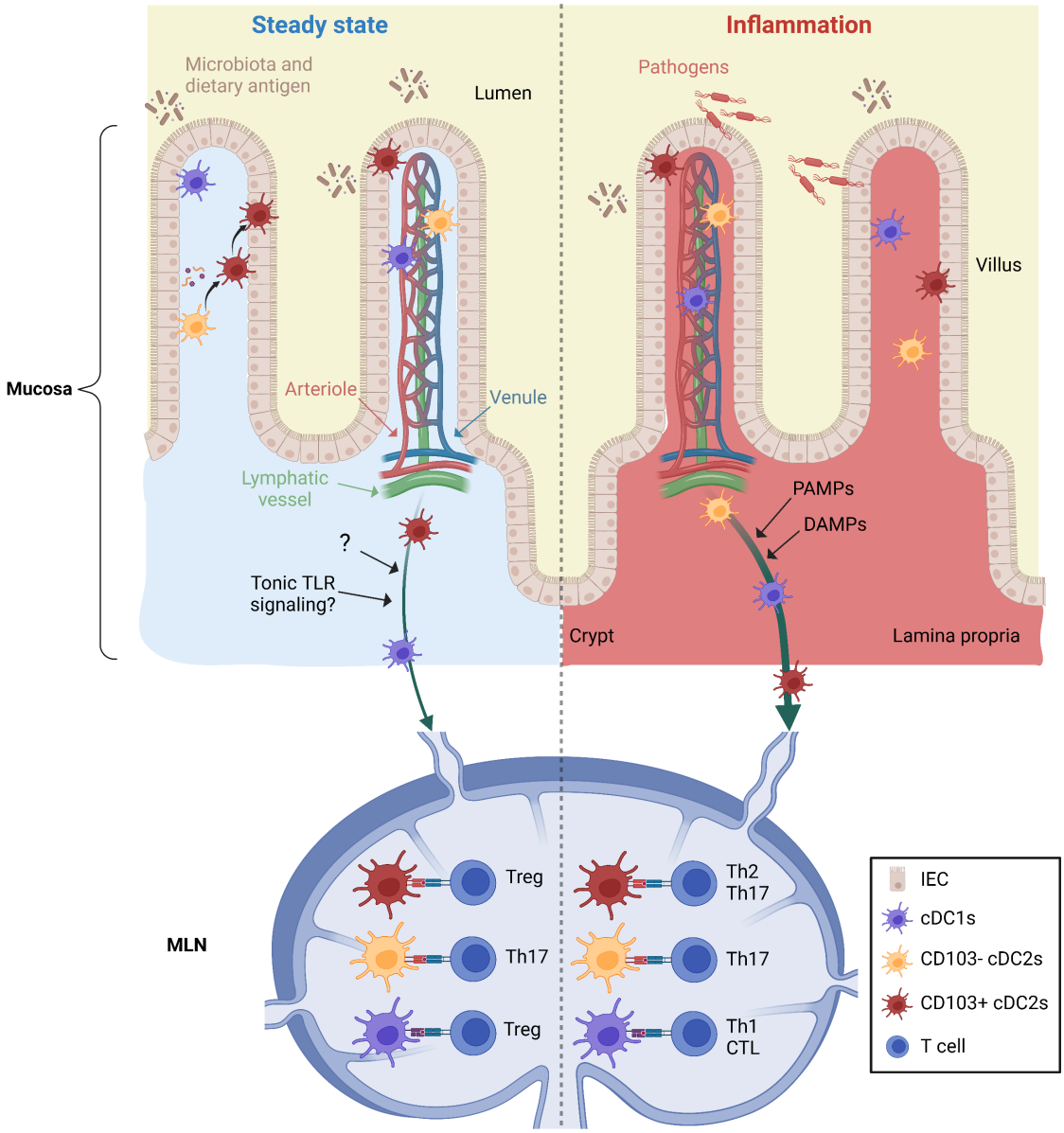


Figure 3 cDCs in the small intestinal lamina propria in steady-state and inflammation. In the steady-state, the three distinct subsets of the small intestinal lamina propria (SI LP) cDCs are constantly sampling their environment which contains self-, dietary and microbiota-derived antigens. After homeostatic activation by unknown signals, cDCs migrate to the mesenteric lymph node (MLN) where they maintain tolerance to harmless antigen by the deletion of food-specific T cells and the induction of regulatory T cells. In inflammation, cDC migration is accelerated by pathogen- as well as damage associated molecular patterns (PAMPs and DAMPs). Depending on the pathogen, this favors the induction of immunogenic Th1, Th2, as well as Th17 and cytotoxic T lymphocyte (CTL) responses. Created with BioRender.com.

1.7. Aims

To date, many studies of cDCs have been performed under the influence of inflammatory stimuli, which induce cDC activation and migration. With increasing incidences of autoimmune diseases, food allergies and inflammatory bowel diseases, understanding the regulatory mechanisms of the immune system has become even more important. A crucial step in the induction and maintenance of tolerance against self and harmless foreign antigen are cDCs that are continuously migrating from peripheral tissues into lymph nodes. While the tolerogenic property of steady state migrating cDCs has been best studied in the context of the gut where it is essential for the induction of oral tolerance, the signals and mechanisms that drive the migration are still not understood. The aim of this thesis is to characterize the cDC compartment of the small intestine, the molecular mechanisms controlling homeostatic maturation and ultimately, steady state migration. Here, we undertake a detailed quantification of cDC migration kinetics and analyze the transcriptomic, phenotypic and functional changes in the cDCs throughout the maturation process in order to develop a holistic model of these mechanisms central to cDC biology. Understanding the principles of steady state cDC maturation and migration is a fundamental prerequisite for the development of future therapeutic treatments that target cDCs and aim to treat deleterious inflammation and maintain tolerance to harmless antigens.

2. Material and Methods

2.1. Mice

Wild-type (WT) C57BL/6 (CD45.2), C57BL/6.SJL (CD45.1), C57B1/6.SJL (CD45.1/CD45.2), Vav-Cre-ADAM10$^{flox/flox}$ (a gift from Andreas Ludwig, RWTH Aachen (Pruessmeyer et al. 2014)), CD11c-Cre-ADAM10$^{flox/flox}$, Vav-H2B-Dendra2 (Dendra) (Ugur et al. 2018) and CCR7$^{gfp/gfp}$ (a gift from Reinhold Förster, Medizinische Hochschule Hannover) were bred and reared under SPF conditions at the animal facility of the Uniklinik RWTH Aachen. For generation of ADAM10$^{flox/flox}$-CD11c-Cre mice, ADAM10^{floxflox} mice were bred with B6.Cg-Tg(Itgax-cre)1-1Reiz/J (#008068) mice. ADAM10$^{flox/flox}$ mice were used as negative controls for both Vav-Cre and CD11c-Cre expressing mice. CCR7$^{gfp/+}$ mice were generated by crossing CCR7$^{gfp/gfp}$ mice with WT mice. All mice were socially housed in individually ventilated cages at 21°C with a conventional light cycle of 12 hours. Standard bedding was changed weekly. Acidified water (pH 3) and standard chow was accessible *ad libitum*. The experiments were approved by the North Rhine-Westphalia State agency for nature, environment and consumer protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV). All experiments were performed in accordance with the local guidelines and ethical regulations (Tierschutzgesetz).

2.2. Photoconversion of organs and tissues

Before surgical procedure, mice received 5 mg/kg Carprofen (Zoetis) analgesic by intraperitoneal injection. For photoconversion of the SI or the MLN, Dendra mice were anesthetized by inhalation of Isoflurane (Piramal). For access to the peritoneal cavity an approx. 1,5 cm long medial incision of the skin was made below the sternum. This was followed by an approx. 1 cm long incision in the muscle along the *linea alba* after the surrounding skin had been detached from the abdominal muscle wall. For photoconversion we used the BlueWave 75 light curing system (Dymax) with an installed 390/40 nm band pass filter at an intensity of 120 mW/cm^2. For photoconversion of SI segments, the gut was carefully moved out of the peritoneal cavity on wet (PBS-soaked) cotton sheets using sterile cotton swabs. Afterwards, two distinct sections (approx. 2 cm long) were photoconverted four times for 15 seconds while keeping the tissue wet with PBS. For photoconversion of the MLN chain, the SI and the mesentery harboring the MLN was taken out of the peritoneal cavity and exposed on wet (PBS-soaked) cotton sheets. After covering the SI parts surrounding the MLN with aluminium foil, the lymph nodes were photoconverted three times for 15 seconds while keeping the tissue wet with PBS. After photoconversion the organs were carefully placed back into the peritoneal cavity. Abdominal muscle wall was sutured by continuous stitching using surgical suture (Marlin violet, HR17, catgut) and the skin was closed with metal clips (Fine Science

Tools, 9 mm). After surgery mice were given Novalgine (Ratiopharm) *ad libitum* (0,9 ml per 500 ml of drinking water) until mice were sacrificed for analysis.

2.3. FTY720 administration

In order to test whether the sphingosine-1-phosphate receptor (S1PR) signaling is important for homeostatic DC migration we administered the inhibitor FTY720 (Fingolimod, Sigma Aldrich). Mice received 1 mg/kg of FTY720 diluted in PBS for 3 consecutive days by intraperitoneal injection (i.p.). Last injection was given directly after photoconversion of the MLN, 16 h before end of experiment.

2.4. Tissue isolation

Mice were sacrificed by CO_2 inhalation, followed by cervical dislocation. After the peritoneal cavity was opened by a medial incision, organs of interest were excised and kept on ice in PBS supplemented with 3% fetal calf serum (FCS) (PBS/FCS). Cell isolation protocols differed according to the tissue and cell type to be isolated.

2.4.1. Intestinal cell isolation

For isolation of cells of the small intestinal (SI) and large intestinal (LI) lamina propria (LP), intestines were excised and put in PBS/FCS on ice. After removal of remaining fat and of Peyer´s patches from the SI, the intestines were cut longitudinally and rinsed with PBS to remove intestinal content. Guts were then cut into small pieces (0,5 cm) and transferred into Hank´s balanced salt solution (HBSS) with 2 mM ethylenediaminetetraacetic acid (EDTA). The mixture was shaken for 20 min at 37°C at 225 rpm. Afterwards, the tissue was collected by filtering the mixture through a 50 µm nylon nitex gaze (Sephar) and transferred into fresh HBSS with 2mM EDTA for 20 min at 37°C at 225 rpm. After incubation, the tissues were collected and rinsed with HBSS. For digestion of colonic tissue, pieces were incubated at 37°C at 225 rpm in an enzyme mix consisting of 10 ml RPMI supplemented with 10% FCS, containing 30 µg/ml DNAse (Roche), 1 mg/ml Dispase (Gibco), 1,25 mg/ml Collagenase D (Roche) and 0.85 mg/ml Collagenase V (Sigma Aldrich). For digestion of SI tissue, pieces were incubated at 37 °C at 225 rpm in an enzyme mix consisting of 15 ml RPMI supplemented with 10% FCS, containing 1 mg/ml collagenase VIII (Sigma Aldrich). Tissues were digested for about 30 min or until no large pieces remained. Intermittently during the incubation, samples were vigorously shaken manually. Afterwards, cell suspensions were filled up to 40 ml with PBS/FCS and

filtered through a 100 µm cell strainer and centrifuged at 400 g for 5 min at 4°C. The pellet was resuspended in PBS/FCS for further counting and staining of cells.

2.4.2. Lymph node cell isolation

For isolation of lymph node (LN) cells, LNs were excised, cleaned from remaining fat and put into serum-free RPMI containing 2 mg/ml collagenase D (Roche). After cutting the LN into small pieces, the mixture was incubated for 45 min at 37 °C at 225 rpm. After digestion, the remaining pieces were mashed and filtered through a 50 µm nylon nitex gaze (Sephar). The suspension was centrifuged at 400 g for 5 min at 4 °C and pellets were taken up in PBS/FCS for further counting and staining of cells.

2.4.3. Bone marrow cell isolation

For isolation of bone marrow (BM) cells, humeri, femurs and tibiae were carefully removed from surrounding muscle and connective tissue. Afterwards, bones were cut at one end and put opened-end downwards into swab extraction tubes (Roche) with a hole at the bottom. Tubes were put into 2 ml collection tubes and centrifuged for 30 sec at 2400 x g. Afterwards, red blood cell lysis was performed by resuspending the pellet in 10 ml of erythrocyte lysis buffer (0.17 M NH_4CL, 10 mM $KHCO_3$, 0.1 mM EDTA) for 8 min at RT. Then, the tube was filled with PBS/FCS and cells were centrifuged at 400 x g for 5 min at 4°C. Afterwards, cells were taken up in PBS/FCS for further counting and staining.

2.5. Enrichment of dendritic cells

For enrichment of DCs in a mixed single-cell suspension we used Magnisort™ Streptavidin Negative Selection Beads (Thermo Fisher).

Briefly, after cell isolation, cells were counted and resuspended at a maximum concentration of $1x10^7$ cells / 100 µl (minimal volume 300 µl) in the biotinylated antibody staining mix (see below). After incubation for 20 min at 4°C, samples were washed in cell separation buffer (PBS+ 3% FCS and 1 mM EDTA) and spun at 400xg for 5 min. For depletion of cells, the pellet was resuspended at 10^7 cells/100 µl and 5 µl of Magnisort™ streptavidin beads per 10^7 cells were added. After incubating the cells for 10 min at RT on a tube rotator (VWR™), the tubes were filled up to 1 ml with cell separation buffer and placed on a DynaMag™ 2 magnet for 5 min. The supernatant, containing the enriched DC fraction was transferred into new tubes. Cells were washed in PBS/FCS for further staining (see 2.8).

Table 1 Antibodies used for DC enrichment

Antibody specificity	Conjugate	Clone	Company	Concentration
B220	Biotin	RA3-6B2	BioLegend	1:200
CD19	Biotin	6D5	BioLegend	1:200
CD3	Biotin	17A2	BioLegend	1:200
CD64	Biotin	X54-5/7.1	BioLegend	1:200
IgA	Biotin	RMA-1	BioLegend	1:50
Ly6G	Biotin	1A8	BioLegend	1:200

2.6. Cell counting via hemocytometer

Cells were counted using the improved Neubauer hemocytometer (Marienfeld GmbH). An aliquot of the cell suspension was mixed in a 1:1 ratio with 0.4 % Trypan Blue (Gibco) in PBS. Pre-dilutions were set that the live cells to be counted were approx. 100. For calculating the total amount of cells in the original cell suspension we used following formula:

Total cell number = mean of counted live cells * dilution factor * 10^4 * volume of cell suspension (in ml)

2.7. Generation of bone marrow chimaeras

Recipient C57Bl/6.SJL (CD45.1/CD45.2) were lethally irradiated with 10 gray (Gy) in a Faxitron CP-160 (Faxitron Bioptics). Five hours later, bone marrow (BM) was reconstituted by intravenous (i.v.) injection of a BM mixture consisting of $5x10^6$ WT (CD45.1)-derived and $5x10^6$ Vav-Cre-ADAM10$^{flox/flox}$ (CD45.2)-derived donor cells. Mice received 1.25 mg/ml of the antibiotic cotrimoxazole (Ratiopharm) in the drinking water for two weeks. Mice were sacrificed 8 weeks later for analyses.

2.8. Staining for flow cytometry and cell sorting

2.8.1. Surface staining

Before surface staining, single cell suspensions were blocked, using PBS/FCS containing 5% of rat serum for at least 10 min at 4°C. When Zombie NIR (BioLegend) was used as live-dead dye, cells were stained with the PBS/NIR mix (1:1000) for 10 min at 4°C in the dark before staining with surface antibodies. After washing cells with PBS/FCS, cells were resuspended in the antibody mixture for at least 20 min on ice. All antibodies and concentrations used for the experiments are listed in Table 2. When 7-Aminoactinomycin D (7AAD) (BioLegend) was used as live-dead dye it was added for the last 5 min into the antibody staining mixture at a dilution

of 1:20. After staining, cells were washed, taken up in PBS/FCS and analyzed on the LSR Fortessa flow cytometer (BD) using the BD FACSDiva software (BD).

Table 2 Antibodies and proteins used in flow cytometry

Antibody specificity	Conjugate	Clone	Company	Concentration
B220	BV421	RA3-6B2	BioLegend	1:200
B220	BV510	RA3-6B2	BioLegend	1:200
B220	BV711	RA3-6B2	BioLegend	1:200
CD101	PE	Moushi101	eBioscience	1:200
CD103	AF488	2E7	BioLegend	1:200
CD103	AF647	2E7	BioLegend	1:200
CD103	PE-Cy7	2E7	BioLegend	1:200
CD11b	BV786	M1/70	BioLegend	1:200
CD11b	BV421	M1/70	BioLegend	1:200
CD11b	BV786	M1/70	BioLegend	1:200
CD11c	APC-Cy7	N418	BioLegend	1:200
CD11c	APC-F750	N418	BioLegend	1:200
CD11c	PerCP	N418	BioLegend	1:200
CD11c	Biotin	HL3	BD	1:200
CD172α	APC	P84	BioLegend	1:200
CD172α	PE	P84	BD	1:200
CD19	PE-Cy7	6D5	BioLegend	1:200
CD4	BV650	RM4-5	BioLegend	1:200
CD40	PE	3/23	BioLegend	1:200
CD45	PE-Cy7	30-F11	BioLegend	1:200
CD45	BV510	30-F11	BioLegend	1:200
CD45.1	FITC	A20	BioLegend	1:200
CD45.2	AF488	104	BioLegend	1:200
CD45.2	BV650	104	BioLegend	1:200
CD45.2	PE	104	BioLegend	1:200
CD62L	BV711	MEL-14	BioLegend	1:200
CD64	PE	X54-5/7.1	BioLegend	1:200
CD64	PE-Cy7	X54-5/7.1	BioLegend	1:200
CD64	Biotin	X54-5/7.1	BioLegend	1:200
CD80	PE	16-10A1	BioLegend	1:200
CD81	PE	Eat-2	BioLegend	1:200
CD86	PE	GL-1	BioLegend	1:200
F4/80	BV421	BM8	BioLegend	1:200
F4/80	PE	BM8	BioLegend	1:200
IgA	PE	mA-6E1	eBioscience	1:200
Ki67	eF660	SolA15	eBioscience	1:200
Ly6C	BV711	HK1.4	BioLegend	1:200
MHCII	BV510	M5/114.15.2	BioLegend	1:800
MHCII	BV711	M5/114.15.2	BD	1:800
MHCII	AF700	M5/114.15.2	BioLegend	1:800
Streptavidin	BV650	-	BioLegend	1:200
Streptavidin	BV711	-	BioLegend	1:200
Tim4	PE-Cy7	RMT4-54	BioLegend	1:200
Trem1	PE	174031	R&D	1:200
XCR1	BV421	ZET	BioLegend	1:200
XCR1	PE	ZET	BioLegend	1:200

2.8.2. Intracellular staining

For intracellular staining, cells were first stained with the Zombie NIR live-dead dye followed by surface antibody staining. After washing, stained cells were fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher) according to the manufacturer´s protocol. Briefly, pelleted cells were thoroughly resuspended in 200 µl of the fixation buffer and incubated in the dark for 20 min at RT. After spinning down the cells at 400 g for 5 min, they were resuspended in 1x permeabilization buffer for 20 min at RT. Afterwards, cells were spun down and resuspended in the antibody mix, using permeabilization buffer as diluent for the antibodies. After 1 h of incubation cells were washed twice with PBS/FCS and analyzed by flow cytometry.

2.9. BrdU proliferation assay

For quantification of cell proliferation, mice were administered 2 mg of BrdU (Sigma Aldrich) in 100 µl PBS by i.p. injection. 24 h later SI and MLNs were excised and cells were isolated according to the aforementioned protocols (see 2.4.1 and 2.4.2). Afterwards, cells were stained with a live/dead dye, followed by surface staining and fixation/permeabilization according to described protocols (see 2.8.1 and 2.8.2). In order to stain cells with the anti-BrdU antibody, cells were washed in PBS/FCS and resuspended in PBS/FCS containing 300 µg/ml of DNAse I (Roche) for 1 h at 37°C. Afterwards, cells were washed in permeabilization buffer and taken up in permeabilization buffer containing anti-BrdU- and anti-GFP antibody and stained for 1 h at RT (see Table 3). After incubation, cells were washed with PBS/FCS and analyzed by flow cytometry.

Table 3 Antibodies used after DNAse treatment

Antibody specificity	Conjugate	Clone	Company	Concentration
GFP	AF647	FM264G	BioLegend	1:100
BrdU	PE	Bu20a	BioLegend	1:100

2.10. EdU proliferation assay

For quantification of cell proliferation in Dendra mice, 1 mg of EdU (Base Click) was administered in 100 µl PBS by i.p. injection, 24 h before sacrifice. For analysis, SI and MLNs were excised and cells were isolated according to the aforementioned protocols (see 2.4.1 and 2.4.2). Afterwards, cells were stained with a live/dead dye, followed by surface staining and fixation/permeabilization according to described protocols (see 2.8.1 and 2.8.2). In order to detect EdU that was incorporated into the DNA we utilized a chemical click-reaction using the ClickTech EdU Cell Proliferation Kit (BaseClick) using a modified manufacturer's protocol.

Briefly, cells were washed once with PBS/FCS after permeabilization. EdU-Click reaction was performed by incubating cells for 30 min at RT in 50 µl of the click reaction buffer containing 0.25 µM of Eterneon-Red 645 Azide. Afterwards, cells were washed twice with PBS/FCS and analyzed by flow cytometry.

2.11. Library preparation and Single-cell RNA sequencing

For single cell sequencing, 36000 GFP^- and 12000 GFP^{int-hi} SI LP DCs pooled from two $CCR7^{gfp/+}$ mice were sorted into sterile filtered PBS containing 0,04 % BSA (Miltenyi). Cells were centrifuged at 300 x g for 10 min at 4°C supernatant was removed. Afterwards, cells were resuspended in 30 µl of 0,04 % BSA in PBS to set the cell concentration to 1400 cells/µl. We aimed to sequence 5000 single cells. For GEM generation we used the Next GEM Chip K Kit according to the Chromium Next GEM Single Cell 5-v2 User guide (10x genomics; RevD). Library was prepared according to User guide Step 5. We used a total amount of 50 ng DNA with 14 total cycles in Step 5.5. Quality control (QC), quantification and sequencing steps were done by the "Interdisziplinäres Zentrum für Klinische Forschung" (IZKF) of the University Hospital RWTH Aachen. QC and quantification of RNA was done on a high sensitivity D1000 ScreenTape Chip for Tape Station 4200 (Agilent) following the user guide. DNA concentration was determined using a Quantus (Promega). Sequencing was done on a NextSeq 500 sequencing system (Illumina). GEM generation and library generation for single-cell sequencing were performed by Dr. Lydia Kopplin.

2.12. Bioinformatics

Raw single-cell sequencing data were processed by CellRanger pipeline with default parameters. We modified the Mus Musculus reference genome (mm10) to add an additional sequence of the gene encoding *EGFP*. We used Seurat (https://satijalab.org/seurat/) to perform downstream analysis: Cells having the percentage of mitochondrial higher than 10 % and percentage of contaminated ambient RNA higher than 50 % were excluded from the analysis as those are indicating dead cells. Doublets were excluded by using DoubletFinder (https://github.com/chris-mcginnis-ucsf/DoubletFinder). For dimensionality reduction, we applied a principal component analysis to transform top 2000 most highly variable genes to 25 principal components. A Uniform Manifold Approximation and Projection (UMAP) transformation followed, which transformed the gene expression profiles of all cells to two dimensions. Clustering was done at cluster resolution of 1. For RNA velocity inference, velocyto (http://velocyto.org/) and scVelo (https://scvelo.readthedocs.io/en/stable/) were used. Bioinformatics analyses were performed in cooperation with Dr. Hieu Trong Nguyen.

2.13. Software and statistics

FACS data was analyzed using FlowJo (BD Life Sciences, version 10.7.2). Statistical analyses were performed as indicated in the figure legends using GraphPad Prism Software (GraphPad Prism 10). P-values ≤ 0.05 were considered significant. Graphical elements (intestines and mice) were adapted from Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 4.0 Unported License. Figures were created using www.BioRender.com or Adobe Illustrator (Adobe, version 27.8.1).

3. Results

3.1. Life cycle of tissue cDCs

In order to characterize the life cycle of tissue cDCs, we focused on cDCs of the small intestinal lamina propria (SI LP). By using a combination of flow cytometry and transcriptomic based analyses, we wanted to get a better understanding of how the phenotypic and functional properties of cDCs changes in the timeframe they stay in the tissue. In the thesis we mainly focused on homeostatic small intestinal cDCs. Steady-state migration of SI LP DCs is of particular interest as it is a necessary step for the initiation of oral tolerance, the systemic unresponsiveness to orally fed antigen (Worbs et al. 2006). As a break of oral tolerance can lead to the induction of inflammatory bowel diseases and food allergies it is of great interest to better understand how this delicate balance between tolerance and immunity is regulated by migrating cDCs (Pabst et al. 2012).

3.1.1. Transcriptome of steady state cDCs in the small intestinal lamina propria

To study the transcriptome of cDCs in the SI LP in the steady-state, we performed single-cell RNA sequencing (scRNAseq), using the 10x Genomics platform. cDCs were identified by FACS, gating on live, leukocytes, $MHCII^+CD11c^+$, single cells and macrophages were excluded using CD64. The three major cDC subsets in the intestine were identified by differential expression of integrins CD103 and CD11b. While cDC1s are $CD103^+CD11b^-$, the cDC2 compartment ($CD11b^+$) can be split into $CD103^-CD11b^+$ and $CD103^+CD11b^+$ cDC2s (Figure 4 A). As the main focus of the thesis was the characterization of the cDC migration, we utilized $CCR7^{gfp/+}$ reporter mice in which one allele of the CCR7 gene is disrupted by a knock-in of the green fluorescent protein (GFP) gene. In the steady-state ~1% of cDCs in the SI LP are GFP^+, meaning they express CCR7 and are about to migrate out of the tissue (Figure 4 B). We refer to those cDCs as “pre-migratory” as they are already expressing CCR7, but have not yet actively migrated out via lymphatics. In the homozygous $CCR7^{gfp/gfp}$ mice, where both alleles of CCR7 are disrupted by the GPF gene, there is an increase of GFP^+ cDCs to ~10% as they lack a functioning CCR7, which is obligatory for cDC emigration from tissues (Ohl et al. 2004)(Figure 4 B).

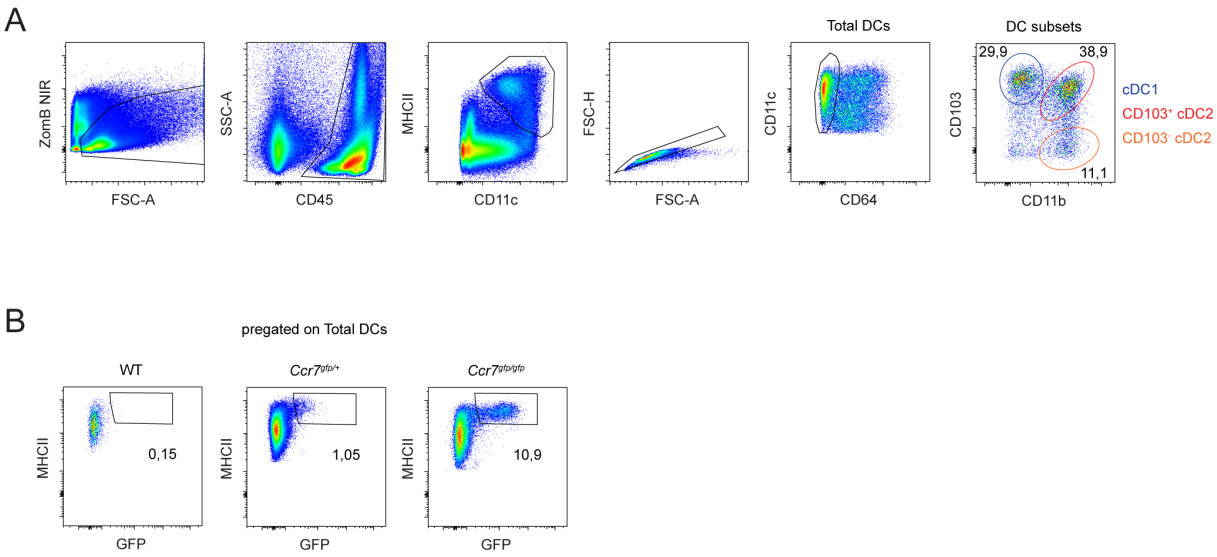


Figure 4 Gating strategy for small intestinal dendritic cell subsets and characterization of the utilized CCR7-reporter mouse model. A: Gating strategy to identify DCs in the SI LP by FACS. After depletion of plasma cells, macrophages, neutrophils and T-cells, cDCs were gated as live, leukocytes (CD45$^+$), MHCII$^+$ CD11c$^+$, single cells, CD64$^-$ (further macrophage exclusion). cDC subsets were identified by differential expression of integrins CD103 and CD11b, where cDC1s are CD103$^+$CD11b$^-$ and the cDC2 compartment (CD11b$^+$) can be split into a CD103$^+$ CD11b$^+$ and CD103$^-$ CD11b$^+$ population. **B:** Comparison of GFP expression amongst total SI LP cDCs between a WT (CCR7$^{+/+}$), CCR7$^{gfp/+}$ and a CCR7$^{gfp/gfp}$ mouse.

For scRNA sequencing we pooled SI LP cells of two CCR7$^{gfp/+}$ mice and sorted the total cDC fraction. As we were most interested in the transcriptomic changes that occur in the interface between CCR7$^-$ and CCR7$^+$ cDCs, we sorted the GFP$^-$ and the GFP$^{int\text{-}hi}$ population separately (Figure 5 A). The sorted populations were mixed at a ratio of 75 % (GFP$^-$) to 25 % (GFP$^{int\text{-}hi}$) to enrich for CCR7$^+$ cDCs. After library generation, sequencing and running the Seurat package pipeline, the unsupervised clustering generated a UMAP consisting of 1576 cells falling into 12 distinct clusters (Figure 5 B). The UMAP can be subcategorized into 3 main clusters: cDC1s on the left (clusters 7, 8, 0) as seen by the ubiquitous expression of *Xcr1* and *Irf8*, cDC2s on the right (clusters 2, 5, 3, 1, 10, 4, 6) as seen by the expression of *Sirpa* and *Irf4* and pre-migratory cDCs at the bottom (cluster 9) as seen by the expression of *Ccr7, GFP* and the migration associated gene *Fscn1*, which encodes for an actin-binding protein (Figure 5 B, C and D).

To get a better understanding of the sub clusters of the cDC1 and cDC2 compartments we generated a heatmap displaying the top 5 differentially expressed genes (DEGs) for each cluster. Except for a small number of contaminating cells (cluster 11, probably macrophages due to high expression of *Lyz1* and complement factors *C1qa, C1qb* and *C1qc*), the UMAP resembled the sorted cDC compartment identified by flow cytometry (Figure 4 A and Figure 5 E). For analysis of changes associated with cDC maturation and life-cycle we mainly focused on cDC1s. Compared to cDC2s, cDC1s represent an ontogenically, phenotypically and

functionally homogenous subset of cDCs. Where possible, we still applied similar methods for cDC2s to also emphasize the similarities as well as the differences for both cDC subsets. While clusters 7, 8, 0 were all characterized by a cDC1 phenotype, they most likely reflect different maturation states. Cluster 7 had the highest expression of cell cycle associated genes like *Hist1h2ae* and *Top2a*, suggesting actively proliferating cells. Interestingly, expression of cDC1 defining marker genes like *Xcr1* and *Cd8a* was lowest in these proliferating cells, possibly due to a more immature stage (Figure 5 D and E). Neighboring cluster 8 was characterized by high expression of genes of the *Mcm* family which had been shown to be necessary for the induction of DNA replication, suggesting that those cells are preparing for proliferation (Figure 5 E)(You et al. 1999). Cluster 0 most likely consists of the majority of cDC1s in G0 phase found in tissues, characterized by highest expression of *Xcr1*, while cell-cycle associated genes are downregulated (Figure 5 D and E). Interestingly, some cells in cluster 0 already expressed *Ccr7* (Figure 5 C and E). This suggests that *Ccr7* expression is an early marker of cells that prepare for migration, while the majority of genes associated with pre-migratory cDCs are not expressed yet. Conversely, we also observed cells that expressed a cDC1 signature within the more separate migratory cDC cluster 9. All cells in this cluster were characterized by high expression of migration associated genes (e.g. *Ccr7* and *Fscn1)*, marking (pre-)migratory cDCs in the SI LP. However, they also exhibited heterogeneity, so the left side of the cluster showed expression of cDC1 defining markers *Irf8* and *Xcr1,* while cells on the right of cluster 9 have residual expression of *Sirpa* and *Irf4*, which are characteristic of cDC2s. This indicates that transcriptional changes that occur upon the induction of the migrating cDC program are shared by both cDC1s and cDC2s, making them cluster together, separately from their more immature progenitors (Figure 5 C and D). Taken together the data show that cDC1s in the SI LP exist in different maturation states, marked by gene expressions associated with proliferation and migration.

To characterize the different cDC2 sub clusters, we first tried to classify them into the different cell states according to the expression of key markers that we had previously defined from the analysis of cDC1s. Similar to cDC1 cluster 7, cluster 2 represented cDC2s in the cell cycle as evidenced by high expression of *Hist1h2ae* and *Top2a* (Figure 5 B, C and E). Also, the neighboring cluster 5 was characterized by the expression of genes of the *Mcm* family, again suggesting that those cells might be preparing for proliferation (Figure 5 B and E). The other cDC2 sub clusters were more difficult to define which is partly due to the fact that the ontogenetic and developmental relationships between different subpopulations of cDC2s in the SI LP are not completely clear. The SI LP contains at least two functionally distinct subsets of cDC2s, distinguished by surface expression of CD103 (see Figure 4 B) (Cerovic et al. 2013; Scott et al. 2015; Rivera et al. 2021). A difficulty in assigning the cDC2 clusters to defined cDC2 SI LP subsets is that it had been shown that $CD103^-$ cDC2s can give rise to $CD103^+$

cDC2s in a TGF-β-dependent manner (Bain et al. 2017). Additionally, at least some of the CD103- cDC2s may represent a developmentally separate lineage, possibly equivalent to the monocyte-dendritic cell progenitor (MDP) derived DC3 which were recently identified in spleen (Liu et al. 2023). In order to characterize the other cDC2 sub clusters, the first approach was to examine the expression pattern of *Itgae* (encoding for CD103) and *Itgb7* which together make up the E-cadherin binding integrin αEβ7 (Kilshaw 1999). The expression pattern of both integrins revealed that *Itgae* expression was lowest in clusters 10 and 6 compared to the other cDC2 clusters whereas *Itgb7* expression was lowest in clusters 4 and 6 (Figure 5 D). As both integrins need to be expressed simultaneously to build the integrin αEβ7, which can be stained on the surface by flow cytometry (using anti-CD103), this suggested that clusters 10, 6 and 4 are most unlikely to be the main cDC2 population of $CD103^+$ cDC2s found in the SI LP. *Vice versa*, this suggested that clusters 1 and 3 may represent $CD103^+$ cDC2s. Additionally, since the two cDC2 subsets of the SI LP defined by surface expression of CD103 have different functions, we utilized markers described in the literature to further characterize the different clusters. It was shown for example, that $CD103^+$ cDC2s can migrate interstitially utilizing *Plet1* (Karrich et al. 2019). Others have shown that $CD103^+$ cDC2s express *Pilra*, which helps them to exit the lamina propria towards the epithelium, granting them a more tolerogenic phenotype (Rivera et al. 2021). As *Itgae, Plet1* and *Pilra* expression was also expressed by cells in clusters 3 and 1, these cluster consisted of *bona fide* $CD103^+$ cDC2s (Figure 5 D and E). Interestingly, gene expression pattern of cluster 6 was quite similar to cluster 3 but was enriched for *Gm42418*, a long non-coding RNA associated with ribosomal contamination (Liu et al. 2020). Therefore, cluster 6 probably represented dying cells. Since cluster 10 was enriched in *Cd7* and *CD209d* and *Ly6c2* expression, which all have recently been associated with progenitors of cDC2s, these cells are *bona fide* pre-cDC2s (Liu et al. 2023). Lastly, cluster 4 expressed significantly more *Il22ra2* which has been associated with $CD103^-$ cDC2s in the SI LP (Bain et al. 2017; Martin et al. 2014). Interestingly, there was also expression of Lyz2 and Csf1r, both of which have recently been described to be expressed by DC3s found in spleen. While the transcriptome of *bona fide* $CD103^-$ cDC2s in the SI LP shared some overlap with splenic DC3s, further experiments like fate-tracking are needed to unambiguously differentiate cDC2s found in different tissues.

From this basic characterization we hypothesized that the clustering recapitulates different states of cDC differentiation and maturation of both cDC1s and cDC2s, culminating in upregulation of CCR7 and the subsequent migration of cDCs in the steady-state.

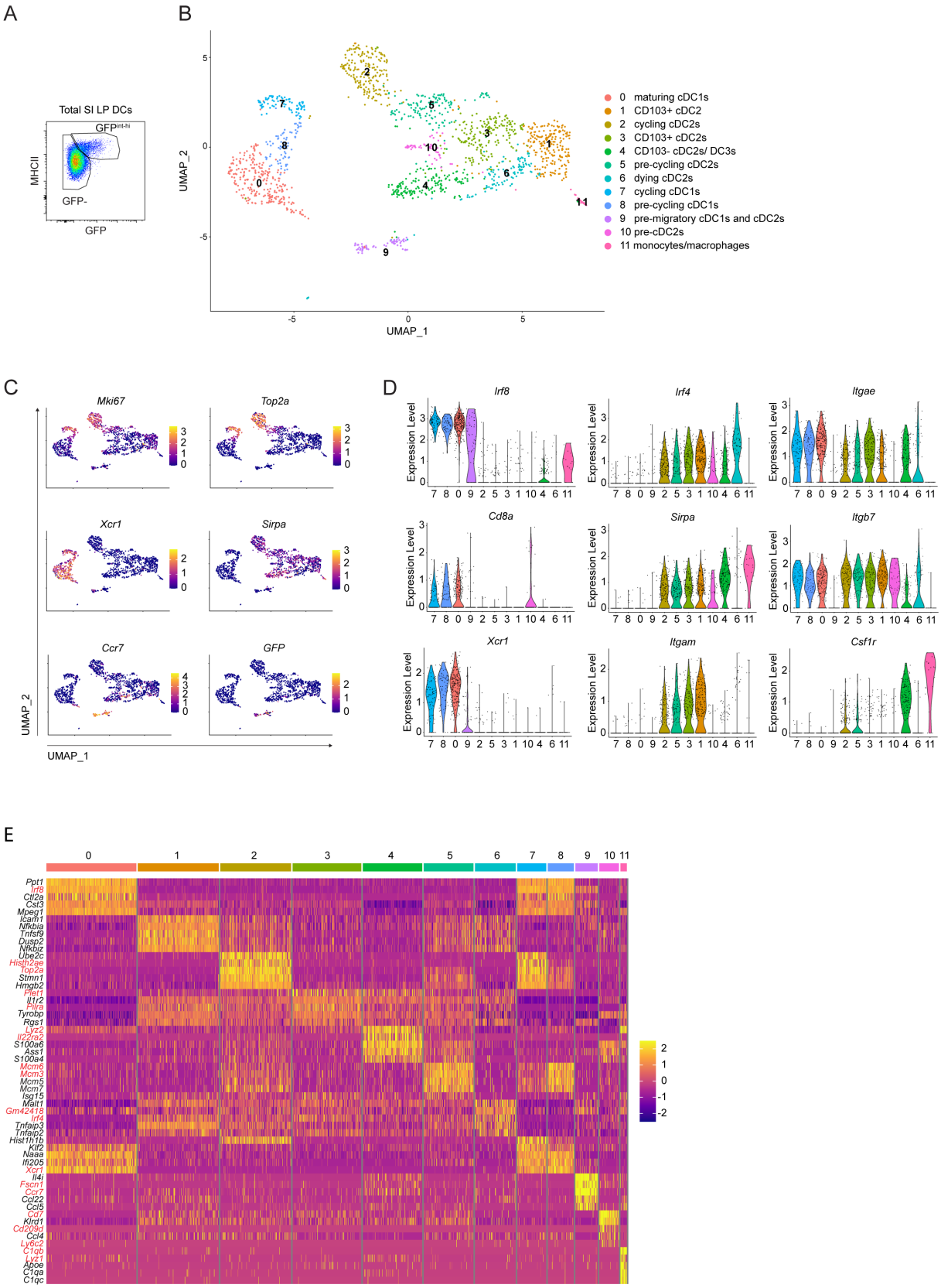


Figure 5 Single cell RNA-seq reveals the transcriptomic phenotype of cDC subsets in the steady-state small intestinal lamina propria. A: Gating strategy for sorted GFP^{-} and GFP$^{int\text{-}hi}$ cDCs from the SI LP of two pooled (12-week-old) CCR7$^{gfp/+}$ mice. **B:** UMAP projections of scRNA-seq data of sorted SI LP cDCs. **C:** Heatmaps represent

the expression of selected genes in single cells overlayed on the UMAP analysis from B. **D:** Violin plots showing the relative expression of selected cDC subset marker genes in single cells. Numbers along the x-axis indicate the clusters identified in B. **E:** Heatmap of the top 5 DE genes for each cluster of the UMAP in B.

3.1.2. Dendritic cell proliferation declines with maturation

To better understand the relationship between clusters of the single-cell dataset, we utilized RNA velocity which is based on the idea that cells can be put along a differentiation trajectory by comparing the amount of nascent mRNA of selected genes between cells (Figure 6 A). A decrease in nascent and thus un-spliced transcripts is an indicator of a more differentiated cell state when compared with a cell that still has more nascent transcripts (Gorin et al. 2022). The data show a general trend of maturation from the top to the bottom of the UMAP (Figure 6 A). Trajectory vectors for both cDC1s and cDC2s originate in the clusters that are associated with proliferation (clusters 7 and 2 (Figure 6 A and B)). Since these clusters represent a substantial proportion of SI LP cDCs we sought to further assess intestinal cDC proliferation. For this, CCR7$^{gfp/+}$ mice were administered 1 mg of bromodeoxyuridine (BrdU) i.p. which is incorporated into the newly synthesized DNA of proliferating cells and can be detected by antibody staining. 24 hours later CCR7^{-} and CCR7^{+} cDCs were analyzed for BrdU incorporation. Approximately 20 % of the main SI LP cDC population (CCR7^{-}) incorporated BrdU at the time of administration, while only 10 % of the pre-migratory CCR7+ cDCs had proliferated. This suggests that cDCs lose their proliferative capacity as they acquire a migratory phenotype (Figure 6 C). To further investigate the relationship between cDC maturation and their proliferative capacity, we divided the cDC compartment into *bona fide* maturation states, using the level of surface MHCII expression as a marker as suggested previously (Bosteels et al. 2023). Therefore, we divided the main cDC compartment (CCR7^{-}) into four equally sized “bins” according to their MHCII expression. We propose that the lowest MHCII expression resembles more immature cDCs (bins M1 and M2), while higher expression of MHCII resembles more mature cDCs (bins M3 and M4). High expression of MHCII and CCR7 marks the latest maturation stage of peripheral tissue cDCs. BrdU incorporation amongst the defined maturation stages revealed a gradual decrease of proliferative capacity along the maturation stages of cDCs (Figure 6 D). Since it was previously shown that cDCs form clonal clusters in the SI LP and the degree of proliferating cDCs, as seen by BrdU incorporation, is relatively high compared to other myeloid cells like macrophages, we sought to determine the extent to which cDCs proliferate *in situ* (Cabeza-Cabrerizo et al. 2019).

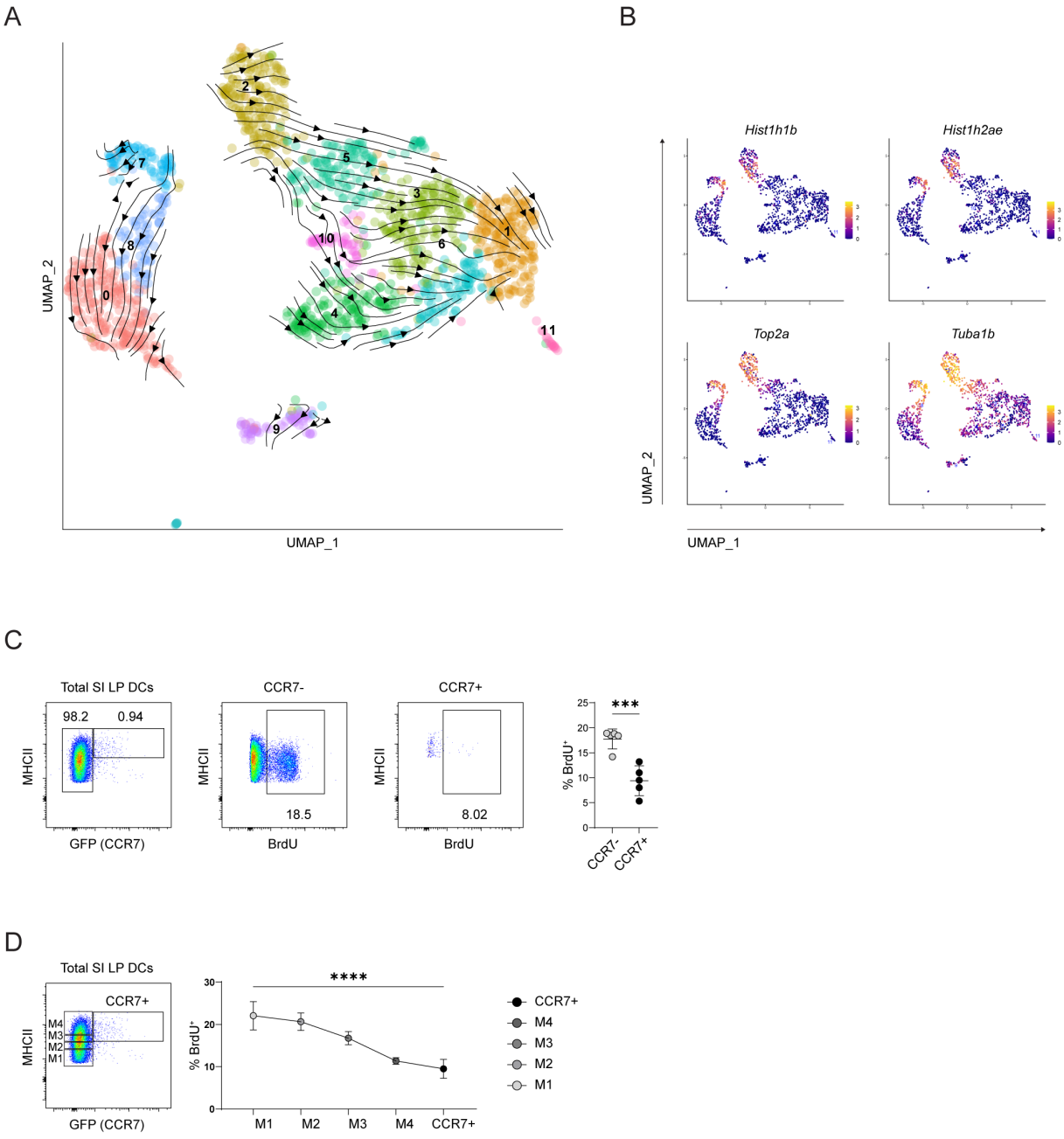


Figure 6 The proliferative capacity of cDCs decreases with maturation. A: RNA velocity of the scRNA seq dataset of total SI LP cDCs (see Figure 5 B). **B:** Heatmaps of selected proliferation and cell-cycle related genes. **C:** Gating strategy and BrdU incorporation amongst CCR7$^-$ and CCR7$^+$ SI LP cDCs, 24h after i.p. administration of 1 mg BrdU. The chart on the right shows the percentage of BrdU$^+$ cells among CCR7$^-$ (grey) and CCR7$^+$ (black) SI LP cDCs. Each dot represents an individual mouse. Statistical comparison was performed using a Student´s t-test (*** P≤0,001). **D:** Gating strategy for cDC maturation states according to surface MHCII expression and quantification of BrdU incorporation amongst defined groups. CCR7$^-$ cDCs were split into four equally sized populations (bins) according to surface MHCII expression. Statistical comparison was performed using a one-way ANOVA with Bonferroni correction (**** P≤0,0001). Data are shown as mean ± SD. n=5 mice in two independent experiments.

3.1.3. Dendritic cells proliferate in peripheral tissues

To directly test the extent to which cDCs proliferate in the SI LP and to what extent the BrdU labeling was due to the influx of BrdU+ precursors, we utilized an *in vivo* photolabeling method based on the Dendra transgenic mouse model, in which all hematopoietic cells express the histone-fused photoconvertible Dendra-2-protein (Ugur et al. 2018). Upon exposure to violet light, the Dendra-2-protein irreversibly changes conformation, resulting in a shift in fluorescence emission from green (Dgreen) to red (Dred), which can be detected by flow cytometry. For spatiotemporal labeling of cDCs, we performed laparotomy and photoconverted defined sections of the SI (~2 cm in length). Simultaneously, we administered 1 mg of ethynyldeoxyuridine (EdU), which, similarly to BrdU, is incorporated into the DNA of proliferating cells around the time of administration and can be detected by click chemistry. 24 h after photoconversion, we analyzed the EdU incorporation in newly arrived ($Dred^-$) cells and in cells that were already present in the tissue at the time of photoconversion ($Dred^+$) (Figure 7 A). As an internal technical control, photoconversion efficacy was verified by quantifying $Dred^+$ Tim4-expressing, locally maintained macrophages, as these cells are characterized by long-term residence and slow turnover (Shaw et al. 2018). In all analyzed tissue samples, $Tim4^+$ macrophage exhibited $Dred^+$ frequencies of over 95 %, ensuring that the initial photoconversion was highly efficient. To validate the setup, we first examined the EdU incorporation amongst $Dred^+$ macrophages. They show negligible amounts of EdU incorporation which is consistent with published data (Bain et al. 2013). However, about 5% of $Dred^-$ macrophages are EdU^+, most likely representing newly differentiated macrophages from incoming blood monocytes (Figure 7 B). In comparison, cDCs show much higher EdU incorporation than macrophages. Among $Dred^+$ cDCs 20 % were EdU^+, which was similar to the overall cDC population indicating that that this represented genuine *in situ* proliferation (Figure 7 B and Figure 6 C). Surprisingly, newly arriving cDCs that entered the gut tissue in the timeframe of 24 h after photoconversion ($Dred^-$) showed significantly lower proliferation than the $Dred^+$ compartment (Figure 7 B). These data suggest that the majority of BrdU/EdU labelling in the SI LP compartment was due to local proliferation and moreover, that there appears to be an induction of proliferation upon tissue entry. As both cDC1s and cDC2s show a similar degree of EdU incorporation amongst both the $Dred^-$ and $Dred^+$ compartment, it seems probable that they depend on the same signals that drive their proliferation *in situ*.

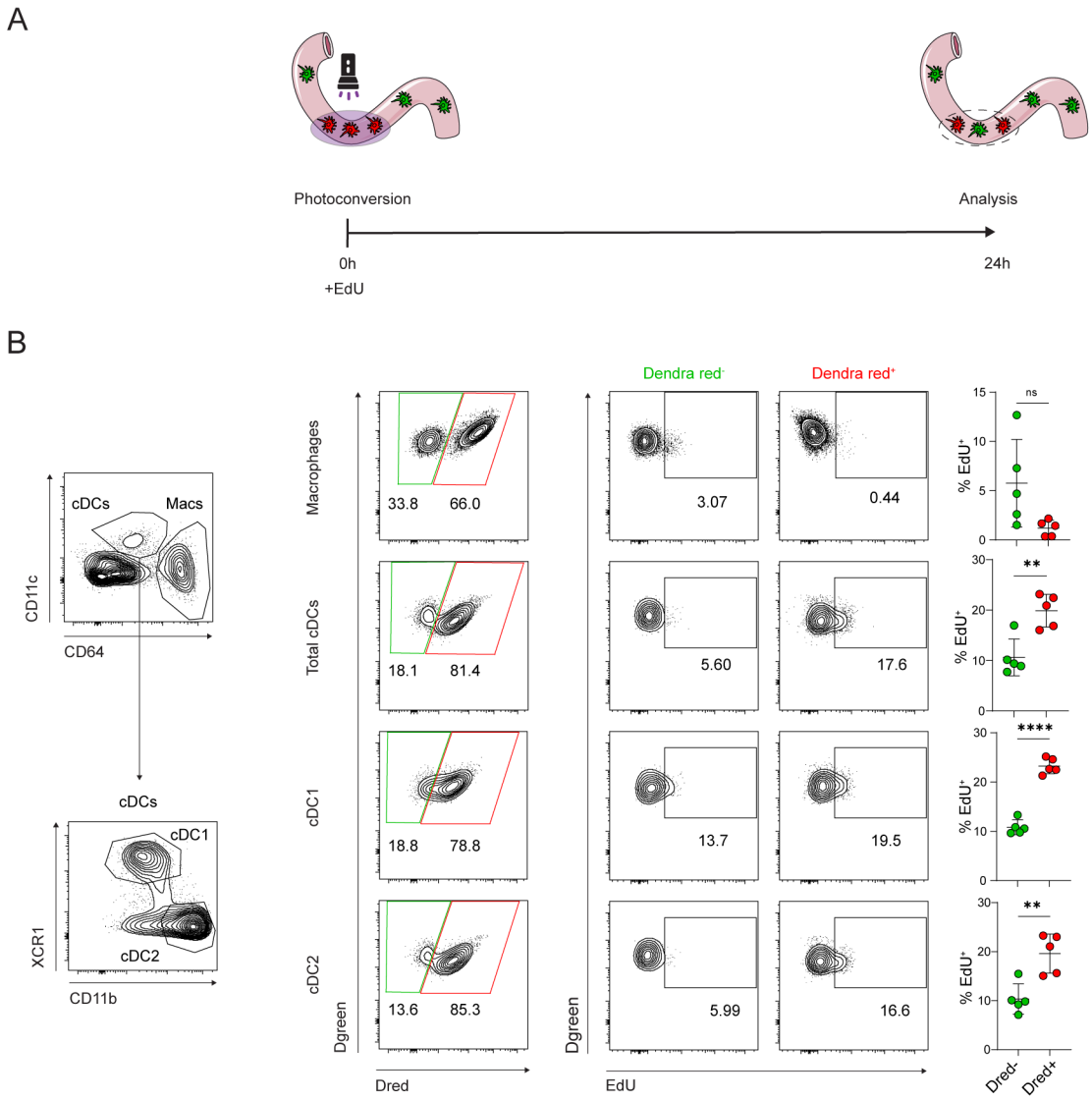


Figure 7 Dendritic cells proliferate in the small intestinal lamina propria. A: Schematic illustration of the experimental setup. After laparotomy, 2 cm segments of the SI of Dendra mice were photoconverted by exposure to violet light. At the same time, mice were administered with 1 mg of EdU i.p. After 24 h, mice were sacrificed and the photoconverted parts were analyzed for EdU incorporation by flow cytometry analysis of Dred$^-$ and Dred$^+$ cells. **B:** Gating strategy and quantification of EdU incorporation amongst Dred$^-$ and Dred$^+$ macrophages and cDCs. Cells were gated as live, leukocytes, single cells, MHCII$^+$ CD11c$^+$, while macrophages were identified as CD64$^+$ and cDCs as CD64$^-$. cDC subsets were further analyzed by differential expression of XCR1 (cDC1s) and CD11b (cDC2s). n=5, each dot represents an individual biological replicate, calculated as the mean of the same population in two different segments of the same intestine. Data are presented as mean ± SD, Statistical comparison was performed using a Student´s t-test. Asterisks indicate statistical significance (ns= not significant; ** P≤0.01; **** P≤0.0001).

3.1.4. Dendritic cells continuously mature towards a migratory phenotype

Once we established that cDCs can proliferate *in situ* and lose their proliferative capacity as they mature, we sought to look for other phenotypic changes that occur along the cDC maturation. Since the phenotypic changes may be different for cDC1s and cDC2s, we analyzed both subsets separately.

To better understand cDC1 maturation, we again utilized our scRNA seq dataset of SI LP cDCs, focusing on cDC1 clusters 7, 8, 0 and the (pre)migratory cluster 9 (see Figure 5 B). After re-clustering, the cDC1 only UMAP consisted of 6 clusters. While cluster 2 consisted of proliferating cDC1s (old cluster 7), cluster 1 consisted of cDC1s preparing for proliferation (old cluster 8 (Figure 5 B, Figure 8 A and B). Also unchanged was cluster 4 that consisted of pre-migratory cDCs (old cluster 9). Old cluster 0 was now split into the new clusters 0, 5, 3 and 6. While clusters 0, 3 and 6 most likely refer to different maturation states of cDC1s in G0 phase, cluster 5 was enriched for mitochondrial transcripts, indicating a break in membrane integrity. For an unbiased approach of maturation analysis, we again performed RNA velocity. The velocity trajectories suggest a maturation program starting at cluster 2, pointing towards clusters 1, 0, 5, 3, 6 into cluster 4 (Figure 8 A). As the merge of the cDC subsets suggested that subset defining markers are downregulated in fully matured (pre-migratory) cDCs (cluster 9 in Figure 5 D and E) we focused to look into the expression pattern of the typical cDC1 subset marker genes *Xcr1* and *Cd8a*. We saw that both markers show increased expression from cluster 2 towards cluster 3, while it drops again in cluster 6. Cluster 4, which consists of $CCR7^{+}$ pre-migratory cDCs showed lowest subset marker expression (Figure 8 B). Another set of genes associated with matured cDCs in general are the costimulatory molecules CD40, CD80 and CD86 and the tetraspanin CD81 (Arabpour et al. 2022; Lutz et al. 2002). Interestingly, while the expression of those genes was highest in the more mature clusters 6 and 4, there was already detectable expression of *Cd81* and *Cd86* in the other clusters. This expression also increased along the proposed maturation clusters, supporting the hypothesis that cDC maturation is a continuous process while the induction of cDC migration is rather associated with a spontaneous event. To validate these findings, we also measured the expression of the afore mentioned markers on a protein level using flow cytometry-based analyses. *Bona fide* cDC1 maturation was defined according to the increasing expression of surface MHCII and CCR7 (Figure 8 C). In general, we observed the same trend with a decrease in subset marker CD8a and XCR1 expression and an increase in costimulatory marker expression (CD80, CD86, CD40) with maturation (Figure 8 D).

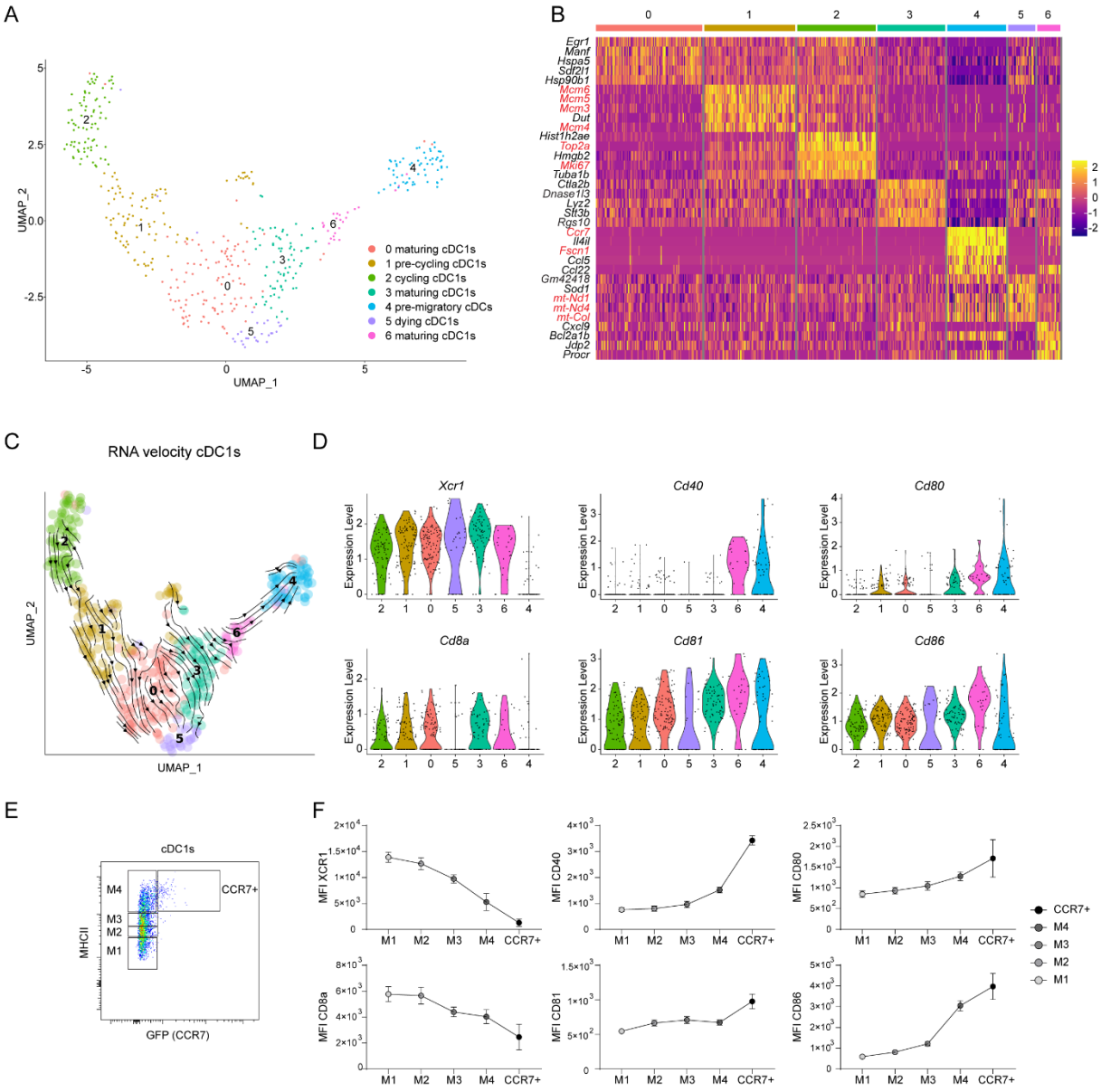


Figure 8 cDC1 maturation is accompanied by a decrease of subset defining markers and an increase of costimulatory molecules.A: UMAP projections of scRNA-seq data of selected cDC1 clusters 7, 8, 0 and 9 from UMAP in Figure 5 B. **B:** Heatmap with the top 5 DE genes per cluster of scRNA-seq UMAP of panel A. **C:** RNA velocity plot of selected SI LP cDC1s from UMAP in panel A. **D:** Violin plots showing relative expression of selected cDC1 subset and maturation marker genes. Numbers along the x-axis indicate the clusters identified in A and C. **E:** Gating strategy for cDC1 maturation states according to surface MHCII and CCR7 expression. **F:** MFI of selected cDC1 subset and maturation marker genes along cDC1 maturation states as gated in E.

To see if the same changes occur in cDC2s, we applied the same methodology to cDC2 clusters analysis. Re-clustering resulted in 11 clusters (Figure 9 A). By displaying the top 5 DE genes amongst the clusters, we first sought to exclude clusters that were not clearly related to a cDC2 phenotype. Cluster 4 likely consists of dying or apoptotic cells (old cluster 6) as identified by high expression of mitochondrial genes such as *mt-Nd3* and *mt-Nd4*. We also excluded cluster 9, which shows a clear macrophage phenotype as evidenced by the

expression of *Lyz1, Apoe* and complement system genes from the *C1q* family. Since cluster 10 which is characterized by high expression of interferon induced genes, did not express typical cDC2 markers, we excluded it as well for further analysis of cDC2 maturation. In order to rank the remaining clusters according to a maturation gradient we also performed RNA velocity for the cDC2 clusters (Figure 9 C). Similar to what we observed for cDC1s, velocity trajectories start in the proliferation associated cluster 1 and point towards clusters 3 and 2 to cluster 0. Another set of trajectories starts in cluster 6 and points towards cluster 5, both of which could both be different states of $CD103^-$ cDC2s/DC3s as seen by the expression of *IL22ra2*, *Lyz2* and *S100a4*. Interestingly, none of the trajectories point to the most mature pre-migratory cluster 7, characterized by the expression of *Ccr7* and *Fscn1,* which was the case for cDC1s (Figure 9 C and Figure 8 C). Nevertheless, based on the RNA velocity data we ranked the clusters accordingly starting from cluster 1 towards clusters 3, 2, 0 and a second trajectory starting from cluster 6 towards 5, both with cluster 7 as the terminal tissue maturation state. As we knew from cDC1s that subset defining markers decline with maturation we sought to analyze whether general cDC2 marker genes like *Sirpa* and *Itgam*, but also $CD103^+$ cDC2 marker genes like *Itgae* and *Trem1* showed first an increase and then a drop of expression along the maturation. Indeed, all 4 marker genes showed increased expression along clusters 1, 3, 2. While *Itgam* expression continued to increase in cluster 0, the other genes start to decreased from cluster 0 on, with all of the subset markers drastically downregulated in most mature cDC cluster 7 (Figure 9 C and D). It is important to note that cluster 7 consisted of both pre-migratory cDC1s and cDC2s which *a priori* led to reduced cDC2 marker expression. However, since the residual expression was close to zero, this indicated that there was a cessation of transcription of typical cDC subset marker genes. Interestingly, Sirpα, CD11b and CD103 protein levels increased along the early maturation bins which was different to what we observed for cDC1s (Figure 9 F and H). Similarly to cDC1s, costimulatory markers *Cd40*, *Cd80* and *Cd86* increased along the maturation continuum, while expression of the tetraspanin *Cd81* was only seen in pre-migratory cluster 7. When comparing the expression of those markers on protein level we could observe the same pattern.

Taken together the data showed that both cDC1s and cDC2s in the SI LP show a similar maturation pattern which is accompanied a gradual increase of costimulatory molecules such as CD80 and CD86.

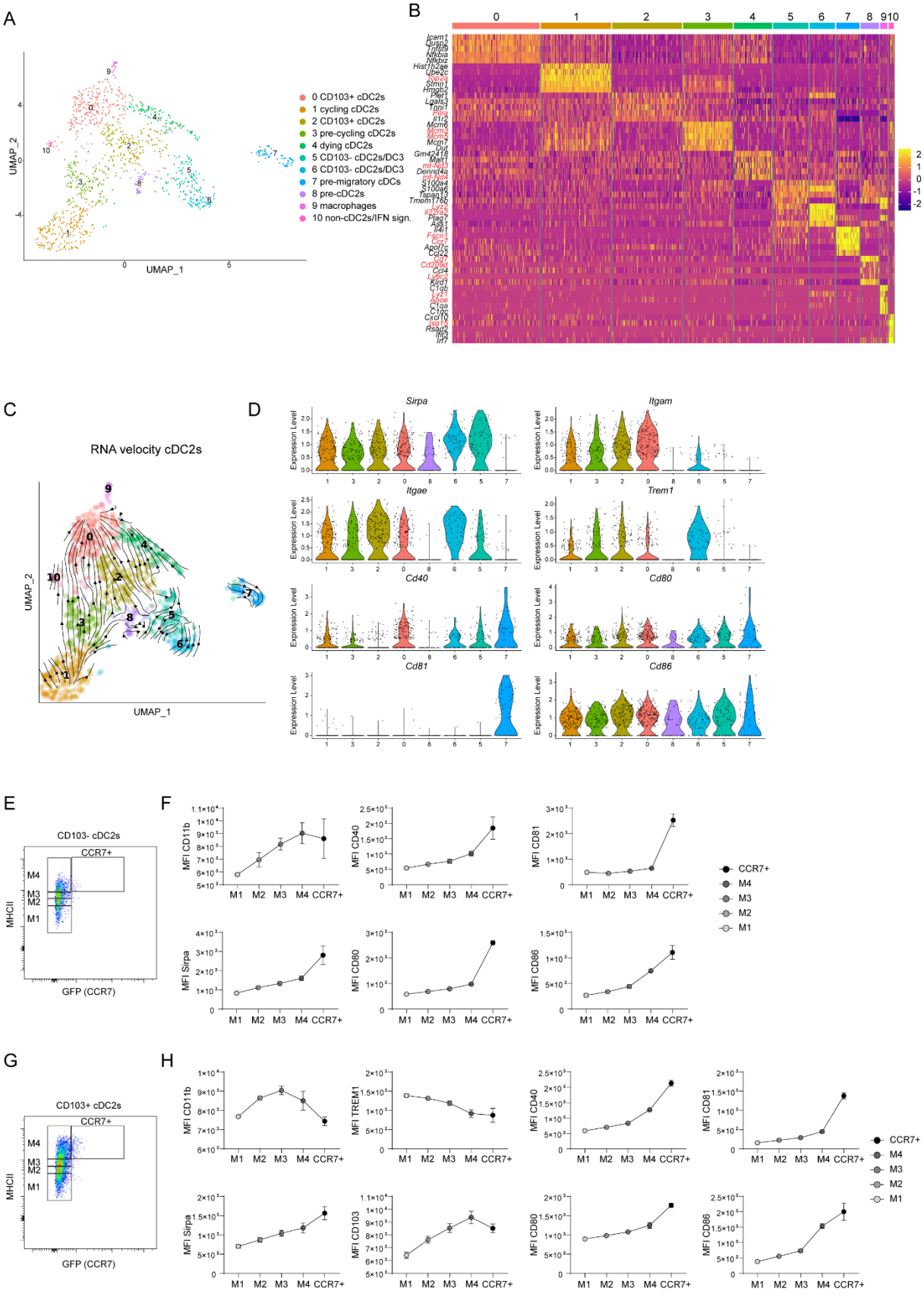


Figure 9 cDC2 maturation is accompanied by an increase of subset defining markers and costimulatory molecules. A: UMAP projections of scRNA-seq data of selected cDC2 clusters 2, 5, 3, 1, 10, 4, 6 and 9 from UMAP in Figure 5 B. **B:** Heatmap with the top 5 DE genes per cluster of scRNA-seq UMAP of panel A. **C:** RNA velocity plot of selected SI LP cDC2s from UMAP in panel A. **D:** Violin plots showing relative expression of selected cDC2

subset and maturation marker genes. Numbers along the x-axis indicate the clusters identified in A and C. **E:** Gating strategy for CD103$^-$ cDC2 maturation states according to surface MHCII and CCR7 expression. **F:** MFI of selected cDC2 subset and maturation marker genes along the CD103$^-$ cDC2 maturation states as gated in E. **G:** Gating strategy for CD103$^+$ cDC2 maturation states according to surface MHCII and CCR7 expression. **H:** MFI of selected CD103$^+$ cDC2 subset and maturation markers along the CD103$^+$ cDC2 maturation states as gated in G.

3.1.5. Conclusion Part 1

The data presented so far reveal the transcriptomic and phenotypic changes during the life cycle, maturation and early stages of migration of the SI LP cDC compartment in the steady state. Both cDC1s and cDC2s are present in different maturation and differentiation states culminating in acquisition of a pre-migratory phenotype which is characterized by CCR7 expression. In addition, we provide direct evidence that cDCs are not only capable of proliferating *in situ* but that the proliferative capacity is induced upon initial entry into the tissue and subsequently decreases along the maturation program. This maturation program is defined by a gradual increase of costimulatory molecules such as CD80 and CD86, both on the transcriptional and the protein levels. Additionally, the induction of migration which is associated with a drastic change of transcriptome, including loss of subset marker expression, seems to be a switch, rather than a gradual change, supporting the hypothesis that homeostatic migration of cDCs is triggered by dedicated signals and pathways.

3.2. Kinetics of cDC migration

The general analysis of the cDC life cycle in the SI LP revealed the stages of cDC development, proliferation and ultimately maturation and migration. We next sought to investigate the kinetics of these processes. For this we made following assumptions. Since the number of cDCs in the intestine remains constant in homeostasis, there must be an equilibrium between cDC input and cDC output (Figure 10 A). The input, that is the factors contributing to an increase in cDC numbers depend either on pre-cDCs entering the tissue or to local proliferation of cDCs. The output, on the other hand, comprises active cDC migration out of the tissue via lymphatic vessels and cDCs that die while present in the tissue. For simplicity and due to the difficulty of quantifying apoptosis in an *in vivo* system, we assumed that the contribution of cDCs dying in the peripheral tissue is a relatively minor contributor to the output of cDCs from the system. Therefore, we postulate that the egress of cDCs through lymphatic migration to be approximately in balance with the cDC input, which is a sum of new recruitment of cDC/cDC progenitors and local cDC proliferation *in situ*.

3.2.1. Kinetics of cDC replenishment of the small intestinal lamina propria

To assess the kinetics of total cDC input (and consequently also output) in the SI LP, we performed photoconversion experiments using the Dendra mouse model. Similar to the setup for quantification of EdU incorporation of SI LP cDCs (see Figure 7), we photoconverted ~2 cm parts of the SI which irreversibly shifts the emission of the Dendra protein from green to red. To quantify the kinetics of cDC turnover in the tissue over time, we analyzed the illuminated areas of the SI 1, 2 and 3 days after photoconversion (Figure 10 B). cDCs that were Dred^{+} at the time of the analysis had already been present in the LP at the timepoint of photoconversion. On the other hand, any Dred^{-} cDCs must have entered the tissue from the blood in the intervening period. It should be noted that, since proliferating cells lose Dred signal due to newly synthesized Dgreen by daughter cells, there is a possibility that some of the Dred^{-} cDCs may therefore not represent newly arriving cDCs, but cells that had undergone several rounds of proliferation. However, it had been shown, that the Dred signal remains detectable by flow cytometry even after 3 proliferation cycles of T-cells (Ugur et al. 2018). Therefore, we limited the cDC kinetics analysis to a maximum of 3 days after photoconversion, to ensure that the amount of Dred^{-} cDCs primarily reflect the ingress of cDCs/cDC precursors into the SI LP. As an internal control for the photoconversion efficacy, we again used Tim4-expressing macrophages which have a slow turnover in the gut (Figure 10 C). In all tissue samples analyzed, Tim4^{+} macrophage exhibited Dred^{+} frequencies of over 90 %, ensuring that the photoconversion procedure was highly efficient. Since the frequencies and numbers of CD103^{-} cDC2s were too low for proper analysis, and were sometimes difficult to unambiguously differentiate from CD11b^{+} monocytes, we focused on cDC1s and CD103^{+} cDC2s (Figure 10 C). While both subsets showed a linear decay of Dred^{+} cDCs over three days, cDC1s had an approximately two times faster turnover rate than the CD103^{+} cDC2s (Figure 10 D). Extrapolation of the kinetics showed that the cDC1 compartment would completely turn over in 4.2 days while the CD103^{+} cDC2s would take 8.2 days. Interestingly, as the amount of proliferation was the same for both cDC subsets (Figure 7 B), this suggests that cDC1s mature faster towards a migratory phenotype than cDC2s and spend less time in the SI LP. It is important to note that the experimental setup is dependent on laparotomy, which itself could affect the turnover rates of the cDC subsets. A similar experimental approach performed by Tomura et.al. in ear skin did not involve surgery and showed a much slower turnover for both CD103^{+} and CD103^{-} dermal cDCs compared to our data in the intestine (Tomura et al. 2014). The CD103^{+} dermal cDCs, likely representing cDC1s, showed approx. 20 % replenishment 5 days after photoconversion, while CD103^{-} dermal cDC2s showed approx. 40 % replenishment at 5 days. Whether the faster replenishment kinetics in the SI LP are due to possible effects of the surgery or whether those are genuine differences between the skin and the intestine itself requires further investigation. Taken together, the data shows that 25 % of SI LP cDC1s and

15 % of CD103$^+$ cDC2s turn over each day. Since we assume that cDC input is equal to the cDC output this means that 15-25 % of the SI LP migrate towards the draining lymph node every day.

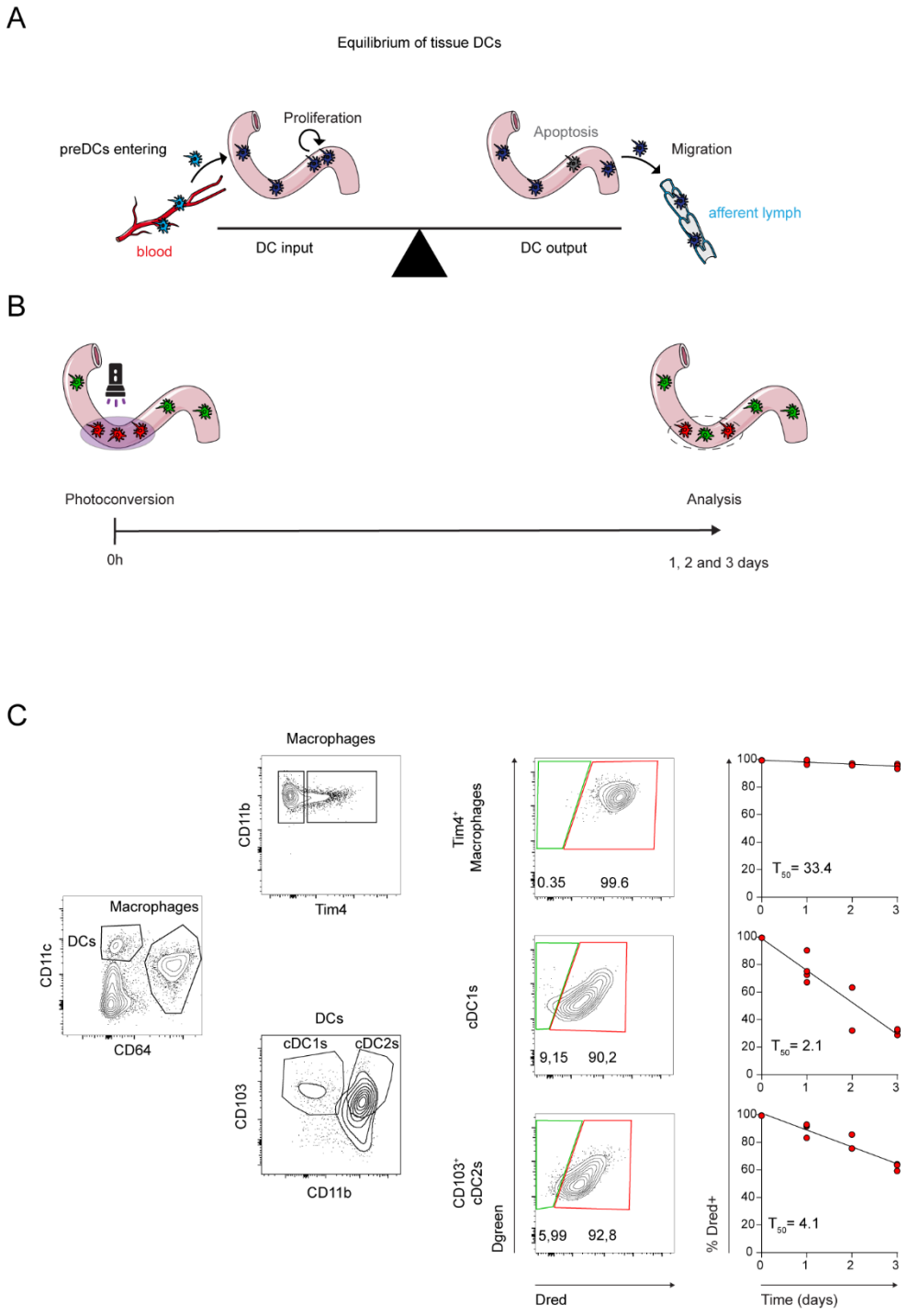


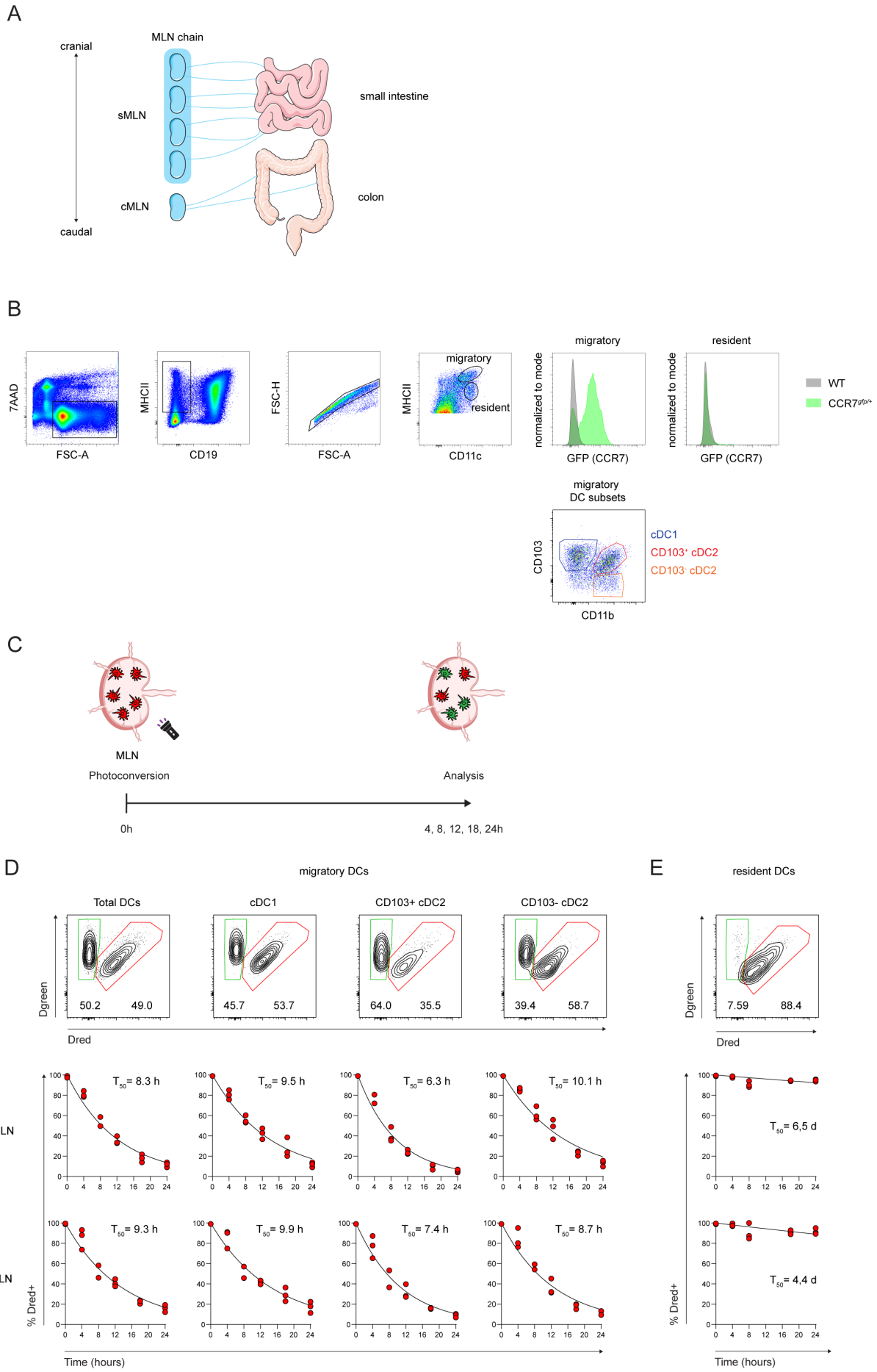
Figure 10 SI LP cDCs are replenished at a rate of 15-25 % per day. A: Schematic illustration of the equilibrium of tissue cDCs. **B:** Schematic illustration of the experimental setup. After laparotomy, 2 cm segments of the SI of Dendra mice were photoconverted by exposure to violet light. 1, 2 or 3 days later, mice were sacrificed and the photoconverted parts were analyzed. **C:** Gating strategy and quantification of Dred$^+$ SI LP cDCs and Tim4$^+$ macrophages. Cells were gated as live, leukocytes, single cells, MHCII$^+$CD11c$^+$, macrophages were identified as

$CD64^+Ly6C^-$ while cDCs were identified as $CD64^-$. cDC subsets were further analyzed by differential expression of integrins CD103 and CD11b with cDC1s being $CD103^+CD11b^-$ and the analyzed cDC2 subset being $CD103^+CD11b^+$. Each dot represents a biological replicate with n=3 for timepoints d0 and d3, n=4 for timepoint d1 and n=2 for timepoint d2 from a total of 3 independent experiments. T_{50}= time after which 50 % of the cells were replenished by $Dred^-$ cells.

3.2.2. Kinetics of cDC entry into lymph nodes

Having defined the turnover rates of SI LP cDCs, we went on to establish how this highly dynamic process is reflected in the draining mesenteric lymph node (MLN). The MLN chain consists of discrete lymph nodes which drain the small intestine (sMLN) or the colon (cMLN)(Houston et al. 2016; Esterhazy et al. 2019)(Figure 11 A). In lymph nodes, cDCs can be identified by flow cytometry, gating on live, $MHCII^+$, $CD19^-$ to exclude B-cells. From here we can distinguish cDCs that entered via lymphatics (migratory) and cDCs that entered directly via blood (resident) by differential expression of MHCII and CD11c. While migratory cDCs express high levels of MHCII and intermediate to high levels of CD11c, resident cDCs express an intermediate level of MHCII and a high level of CD11c. This gating strategy for migratory and resident cDCs was validated by differential expression of CCR7 (Figure 11 B).

To measure cDC migration kinetics in the MLN, we utilized the aforementioned *in vivo* photolabeling method based on the Dendra mouse model. After laparotomy, the entire MLN chain was exposed to violet light, photoconverting all leukocytes in the tissue. At different time points after photoconversion, we quantified the frequency of $Dred^+$ cDCs, which represented the cDCs which had been present in the MLN at the time point of photoconversion (Figure 11 C). For both the sMLN and the cMLN, the three cDC subsets showed comparable kinetics with half of the migratory cDCs being replenished every 6-10 h. Unexpectedly, $CD103^+$ cDC2s had fastest kinetics amongst the migrating intestinal cDC subsets which is not consistent with the increased replenishment rates of cDC1s in the SI LP as shown previously (Figure 10 D and Figure 11 D). After 24 h, almost all migratory cDCs were replenished by newly immigrating cDCs. These data not only indicate very high steady-state migration rates, but they also suggest that cDCs have a short life span once they migrate and enter the lymph nodes. In contrast, resident cDCs have a much slower turnover than migratory cDCs, similar to that of peripheral tissue cDCs (Figure 10 D and Figure 11 E). Taken together, the data show that steady-state migration of intestinal cDCs is a highly kinetic process that requires a tremendous amount of resources, underscoring the importance of this process.


A
cranial
caudal
MLN chain
sMLN
cMLN
small intestine
colon
B
7AAD
FSC-A
MHCII
CD19
FSC-H
FSC-A
MHCII
migratory
resident
CD11c
migratory
normalized to mode
GFP (CCR7)
resident
normalized to mode
GFP (CCR7)
WT
CCR7gfp/+
migratory
DC subsets
CD103
CD11b
cDC1
CD103+ cDC2
CD103- cDC2
C
MLN
Photoconversion
Analysis
0h
4, 8, 12, 18, 24h
D
migratory DCs
Total DCs
cDC1
CD103+ cDC2
CD103- cDC2
Dgreen
Dred
50.2
49.0
45.7
53.7
64.0
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39.4
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sMLN
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T50= 8.3 h
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T50= 10.1 h
T50= 9.3 h
T50= 9.9 h
T50= 7.4 h
T50= 8.7 h
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Time (hours)
E
resident DCs
Dgreen
Dred
7.59
88.4
T50= 6,5 d
T50= 4,4 d
% Dred+
Time (hours)

Figure 11 Steady-state cDC migration is a highly kinetic process with 50 % replenishment of LN migratory cDCs every 6-10 hours. A: Schematic illustration of the distinct drainage pattern of the MLN (Houston et al. 2016). **B:** Gating strategy used for separation of migratory ($MHCII^{hi}CD11c^{int-hi}$) and resident ($MHCII^{int}CD11c^{hi}$) cDCs and cDC subsets in lymph nodes. **C:** Schematic illustration of the experimental setup used for D and E. MLN cDCs were analyzed at different timepoints after exposure to violet light (photoconversion). **D:** Gating and quantification of $Dred^{+}$ migratory cDC subsets of the sMLN and cMLN 4, 8, 12, 18 and 24h after photoconversion of the MLN. cDC subsets were identified by differential expression of integrins CD103 and CD11b, where cDC1s are $CD103^{+}CD11b^{-}$ and the cDC2 compartment ($CD11b^{+}$) were split into a $CD103^{+}$ $CD11b^{+}$ and $CD103^{-}$ $CD11b^{+}$ population. **E:** Gating and quantification of $Dred^{+}$ resident cDCs of the sMLN and cMLN 4, 8, 12, 18 and 24h after photoconversion of the MLN. Each dot represents a biological replicate with n=3 for each timepoint except of cMLN 8h with n=2 from 6 independent experiments. T_{50}= time after which 50 % of the cells were replenished by $Dred^{-}$ cells.

3.2.3. Conclusion Part 2

Taken together, the data show that 15-25 % of the SI LP cDCs are replenished every day by newly immigrating cDC precursors. This corresponds to the almost complete turnover of the migratory cDC compartment of the MLN every 24h. Therefore, SI cDC migration is a highly kinetic process that requires a huge amount of resources, highlighting the importance of this process.

3.3. Regulation of steady-state cDC migration

Our data shows that steady-state cDC migration is a continuous process accompanied by profound transcriptomic changes. However, the signals and pathways involved in the induction of homeostatic migration are still not fully understood. Accordingly, the last chapter of this thesis focuses on the transcriptome of migrating cDCs, which included to test the role of candidate pathways that may be involved in steady-state cDC migration.

3.3.1. cDC1 migration is accompanied by increased expression of genes associated with cytoskeletal rearrangement, apoptosis and T cell stimulation.

In order to understand the principle mechanisms and pathways that are involved in the steady-state migration of cDCs, the first aim was to define the migratory transcriptome which is shared by migrating cDCs. cDC1s can be readily identified across tissues by their unique and ubiquitous expression of XCR1 (Bachem et al. 2012). This allowed us to use published datasets of cDC1s to directly compare with our own scRNAseq dataset for cDC1s (Figure 8 5). As cDC2s show much greater heterogeneity and are known to have unique tissue adaptations (Sichien et al. 2017), we initially aimed to define a global migratory transcriptome for cDC1s and subsequently compare it with cDC2s. For information on early transcriptional changes of migrating cDC1s, we utilized the SI LP scRNAseq dataset described above (see

Figure 8). To find genes and pathways used by migrating cDC1s in general, we also utilized two publicly available datasets comparing the cDC1s from SI LP and MLN (Kastele et al. 2021) or lung and mediastinal LN (medLN), which is draining the lung (Ardouin et al. 2016). To identify common genes and pathways used by cDC1s during migration, we searched for genes that were significantly up- or down-regulated in migrating cDC1s in all three datasets. By overlaying the differentially expressed genes using Venn diagrams, we found 191 upregulated and 205 downregulated genes common to all analyzed datasets (Figure 12 A and B). For an unbiased classification of the in total 396 DE genes, we performed gene enrichment analysis using the web-based platform ShinyGO (Ge et al. 2019). For a general overview, we tested whether the gene set was enriched in pathways that were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) or the Gene Ontology (GO) database (Figure 12 B). While many of the pathways were associated with general terms associated with immune responses or diseases, there were also more specific pathways and biological processes that were associated with the gene set. These included terms such as Cell migration, Cellular senescence, Apoptosis, Antigen processing and presentation, and Cell adhesion molecules. Since this type of analysis only considered the DE genes without associated fold changes, we next sought to determine which of these pathways were up-, and which were downregulated. To do this, we generated a volcano plot of the cDC1s of the SI LP vs. MLN dataset (Figure 12 C). Amongst all the significant genes displayed are also the 396 DE genes that were found to be significantly expressed by migrating cDC1s in all three datasets. As expected, genes associated with cell migration were significantly upregulated in migrating cDC1s. Besides *Ccr7*, which was used as a defining property of the migrating cDC groups, there was also significant upregulation of genes involved in cytoskeletal regulation such as *Fscn1*, *Marcksl1*, *Swap70*, *Samsn1* (Figure 12 C). While these genes are involved in the polymerization of actin bundles, a necessary mechanism for cell migration (Insall et al. 2009), there was also increased expression of CC-chemokine ligands 5 (CCL5) and CCL22. This suggested, that cDC1s may also communicate with or attract other cells in the migration process. The gene set was also associated with the notion of cellular senescence, which is associated with a loss of the ability to proliferate, as evidenced by the silencing of cell cycle-associated genes encoding minichromosome maintenance proteins described to have helicase functions (*Mcm3*, *Mcm6*, *Mcm7*) (Bell et al. 2013), but also of *Ccnd1*, also known as cyclin D1. This is accompanied by upregulation of the cyclin-dependent kinase inhibitor *Cdkn2b*, also known as multiple tumor suppressor 2. This is consistent with our in vivo proliferation data (see Figure 6), emphasizing that cDCs gradually lose proliferative capacity as they mature, with $CCR7^+$ cDCs showing the lowest level of BrdU/EdU incorporation. Interestingly, apoptosis was one of the terms that was associated with the gene set expressed by migrating cDC1s. While all apoptosis-related genes, including *Birc2*, *Birc3*, *Gadd45b*, *Bcl2l14*, *Casp3*, *Fas* and *Cflar* were upregulated by

migrating cDC1s, the functions of these proteins are associated with both, pro- and anti-inflammatory functions (Figure 12 C). As these data included the corporate DEGs from early to late migrating cDCs, this supports the hypothesis that cDCs initiate an apoptotic program quite early in the process of migration. This is consistent with the observation that CCR7 expression peaks at approx. 10% of cDCs in CCR7$^{gfp/gfp}$ reporter mice, supporting the hypothesis that cDCs do not survive for a long time after upregulating CCR7 (Figure 4 B). Ultimately, after the migration into the lymph nodes, cDCs are well described to be able to present acquired antigen to T cells. Consistent with this, migrating cDC1s also showed increased expression of genes associated with antigen processing and presentation. Surprisingly, MHCII-related transcripts such as the positive regulator of MHCII expression *Ciita*, but also MHCII transcripts themselves such as *H2-Ab1* and *H2-DMa* were downregulated by migrating cDC1s (Figure 12 C). Despite the decrease in transcript levels, there is still an increased expression of MHCII on the cell surface of maturing/migrating cDCs (see Figure 8 and Figure 9), which is explained by decreased recycling and an increased transport of intracellularly stored MHCII molecules to the cell surface (van Niel et al. 2006). Other genes that fall under the term of antigen presentation and were upregulated by migrating cDC1s were the gene encoding for the co-stimulatory molecule CD40 and the gene encoding for the protein involved in lipid presentation CD1d, described to be involved in the interaction with natural killer T (NKT) cells (Girardi et al. 2012). With regards to T cell induction, there was a significant upregulation of genes associated with Treg generation and homeostasis. For example, cDC-derived CCL22 has been shown to promote DC-Treg interactions by acting on CCR4, which is expressed on Tregs (Rapp et al. 2019). In addition, there was a significant induction of the transcript encoding for the aldehyde dehydrogenase family 1-member A2 (*Aldh1a2*), which converts retinoic acid (RA) from retinaldehyde and has been described to be involved in Treg induction. Similarly, transcript levels of the gene encoding for integrin beta 8 (*Itgb8*), which dimerizes with integrin αV to convert pro- TGF-β to its active form that also supports Treg generation, were increased in migrating cDC1s (Coombes et al. 2007; Worthington et al. 2011). It is important to note that the expression of many typical cDC1 markers that are closely related to cDC1 functions were significantly downregulated upon migration which is consistent with the data shown previously (see Figure 8). These included transcripts of *Tlr3*, *Clec9a* and *Xcr1* which are associated with environmental sensing and uptake of apoptotic material, both functional characteristics of cDC1s in tissues. Another KEGG pathway that was associated with the DE genes of migrating cDC1s was cell adhesion molecules. CCR7^{+} cDC1s significantly downregulated integrins in all three datasets, with the sole exception of *Itgb8,* which was upregulated as mentioned above. These included *Itgb2*, *Itgb7* and *Itgae* (encoding for the αE subunit (CD103) of the integrin αEβ7). Since integrins are known to mediate cell

adhesion to the environment, e.g. by binding to the extracellular matrix (ECM), this led to the hypothesis that integrins might be detrimental to cDC migration.

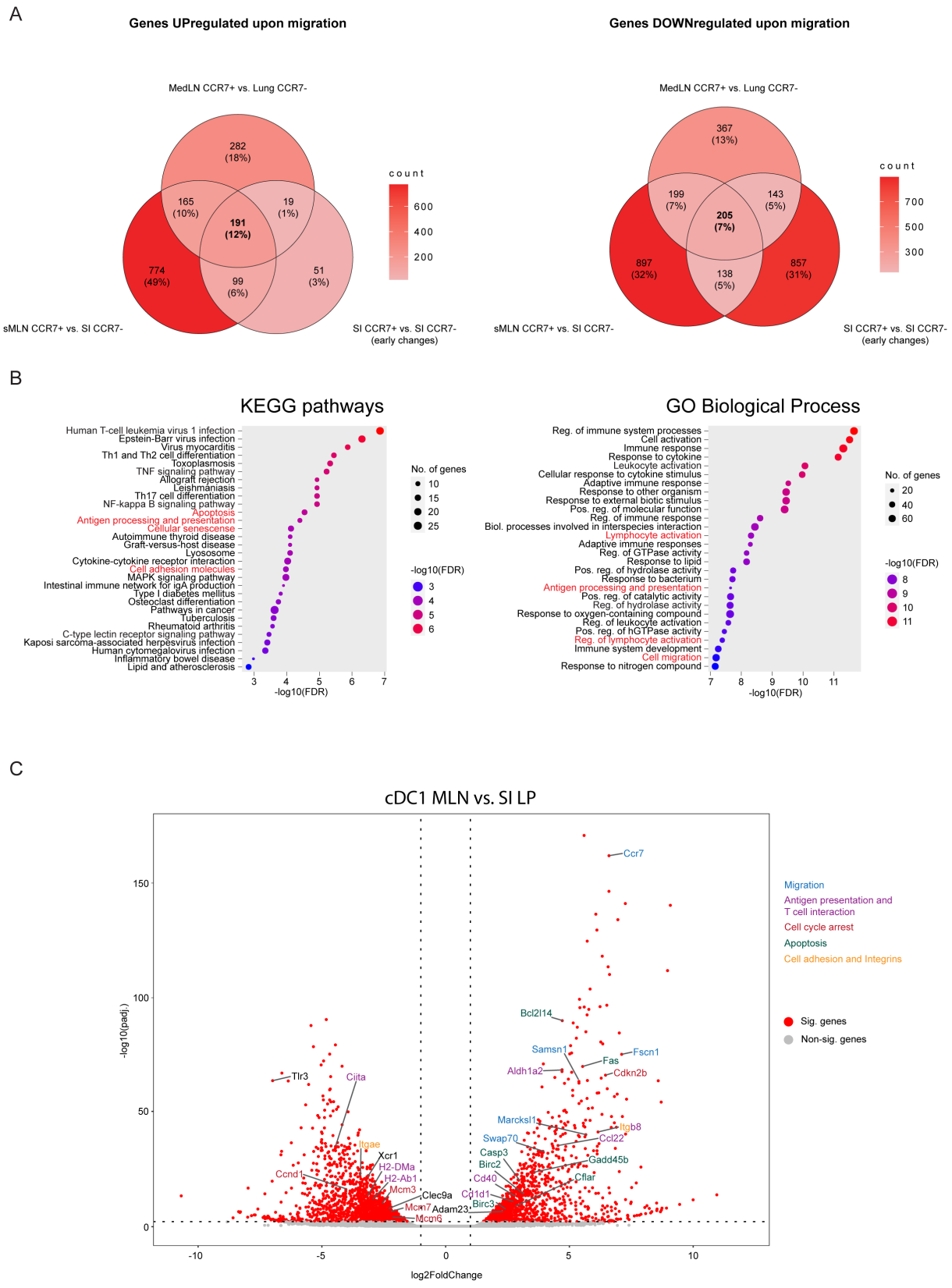

Figure 12 cDC1s of the small intestine and the lung utilize a congruent migration associated transcriptome. A: Venn diagrams displaying the number of genes that are significantly upregulated (left) or downregulated (right) in CCR7^{+} cDC1s compared to CCR7^{-} tissue resident cDC1s. SI CCR7^{+} vs. SI CCR7^{-} date is derived from the scRNA seq dataset comparing the pre-migratory cluster 4 vs. the other cDC1 clusters (see Figure 8 A) using an adjusted

p-value cutoff ≤ 0.05. The CCR7$^+$ sMLN vs. CCR7$^-$ SI bulk RNAseq dataset (GSE160156) was derived from (Kastele et al. 2021) using an adjusted p-value cutoff of ≤ 0.01 while microarray data of CCR7$^+$ medLN vs. CCR7$^-$ lung dataset (GSE71170) was derived from (Ardouin et al. 2016) using an adjusted p-value cutoff ≤ 0.05. **B:** Top 30 pathways and biological processes associated with the 396 genes that were differentially expressed by migrating cDC1s in all three datasets as shown in panel A. Analysis was done using the web-based platform ShinyGO with an FDR cutoff of 0.05 (Ge et al. 2019). **C:** Volcano plot highlighting selected genes associated with pathways that were found to be enriched in migrating cDC1s (see panel B). The plot was generated based on the cDC1 CCR7$^+$ sMLN vs. CCR7$^-$ SI dataset using an adjusted p-value cutoff of ≤ 0.01. Selected genes were differentially expressed in all three datasets.

Taken together the data show that both small intestinal and lung cDC1s utilize a congruent migratory transcriptome which is characterized by upregulation of genes associated with cytoskeletal rearrangement, apoptosis, and Treg inducing co-stimulatory molecules, while integrins and proliferation associated genes are significantly downregulated.

3.3.2. cDC1s already express low levels of CCR7 and apoptosis related genes prior to the full onset of the migratory transcriptome

Based on this general analysis of transcriptomic changes, we next sought to determine whether some of the described pathways could be detected very early or even before CCR7 expression, indicating that they might be involved in the induction of CCR7 expression and the migration itself. Therefore, we used the scRNAseq dataset of the steady-state SI LP cDC1s and compared selected DE genes between the three most mature clusters 3 and 6 and the pre-migratory cluster 4 (see Figure 8 A). Interestingly, genes associated with migration and actin polymerization (such as *Ccr7*, *Marcksl1* and *Swap70*) already showed increased expression in cluster 6, which is before the major transcriptional changes that are associated with migration occur. A similar pattern was also observed for apoptosis-related genes (such as *Gadd45b*, *Fas* and *Cflar*) which also showed increased expression in cluster 6 compared to earlier cluster 3. This suggests, that upregulation of these genes could be an early hallmark of migration and that the signaling of these pathways themselves may further accelerate the transition towards a migration associated phenotype in a positive feedback mechanism. Genes associated with antigen presentation (*H2-DMa* and *H2-Ab1*) or cDC1 function (*Clec9a* and *Xcr1)* showed a clear downregulation in cluster 4 compared to clusters 3 and 6, indicating that cessation of these genes occurs after induction of the migration process.

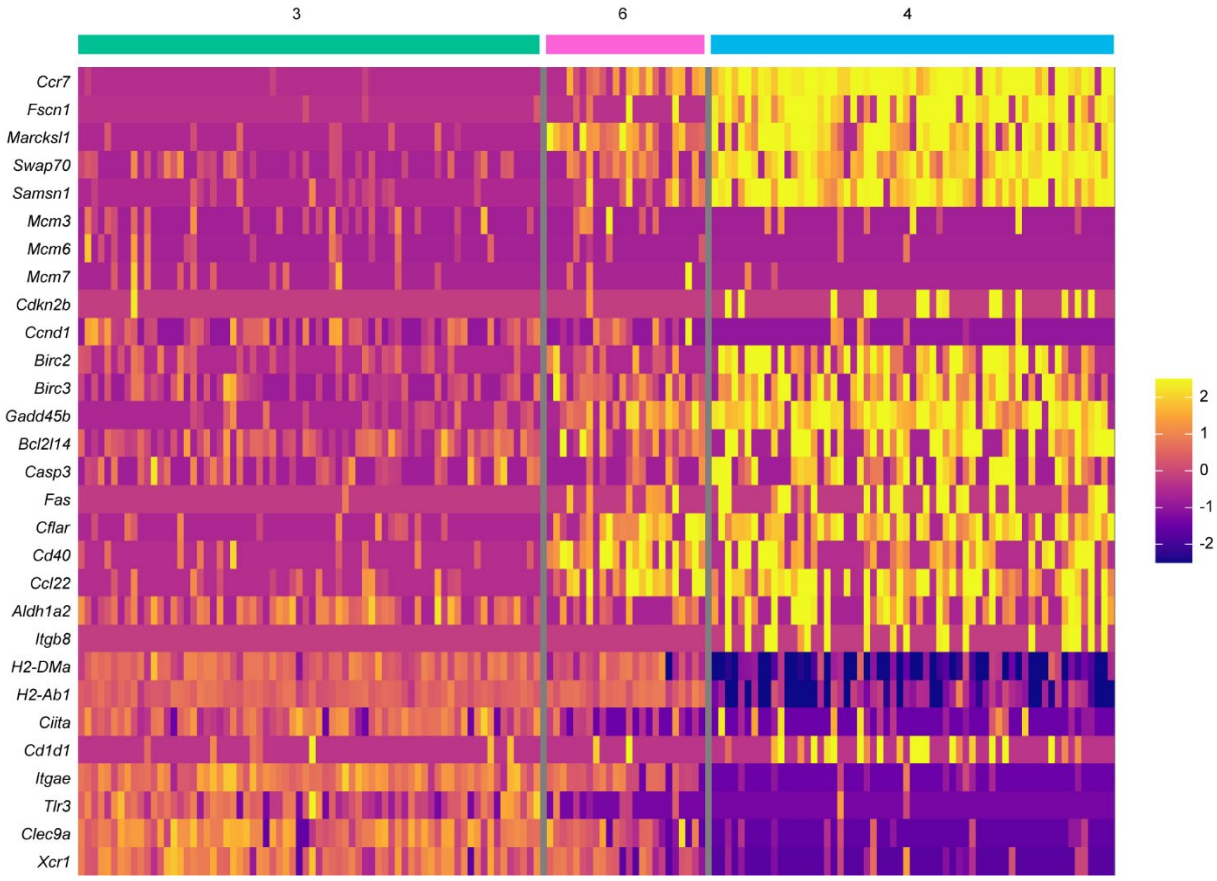

Figure 13 Cytoskeletal remodeling- and apoptosis-associated genes are already upregulated before the major transition to the broad migratory phenotype. Heatmap displaying the expression of selected genes (described in Figure 12) among the maturing cDC1 clusters 3, 6 and the pre-migratory cluster 4 from the scRNAseq dataset (see Figure 8 A).

3.3.3. cDC2s utilize a similar migratory transcriptome as cDC1s

Next, we wanted to analyze whether the key features of the migratory transcriptome of cDC1s are also associated with migration in cDC2s. For this, we used the published dataset comparing the transcriptome of MLN and SI LP cDCs (Kastele et al. 2021). The major advantage of this dataset is that both CD103^{+} and CD103^{-} cDC2s were sorted and analyzed separately. To test whether both cDC2 subsets of the SI LP use similar pathways as cDC1s for their steady-state migration, we overlaid key genes that were observed to be differentially expressed by migrating cDC1s. In general, both CCR7^{+} cDC2 subsets showed increased expression of the identical migration-associated genes as migrating cDC1s which included *Ccr7*, *Fscn1*, *Marcksl1*, *Samsn1*, and *Swap70* (Figure 14 A and B). This showed, that all intestinal cDC subsets, and at least also migrating lung cDC1s, utilize the same conserved migration machinery upon activation in the steady-state. Similarly, both subsets also showed increased expression of apoptosis-related genes upon migration, which is consistent with the high replenishment rates migratory cDC subsets in the MLNs as shown previously (Figure 14 A and B and Figure 11 D). The same pattern was seen for integrins, which were collectively

downregulated upon migration, again with the exception of integrin β8, which was upregulated. Interestingly, while it was known that all migratory cDC subsets in the MLN are able to produce RA using the enzyme encoded by *Aldh1a2* (Cerovic et al. 2013), expression of the integrin β8 was less associated with cDC2s (Boucard-Jourdin et al. 2016). In particular, the expression of both genes in migrating $CD103^-$ cDC2s was surprising, as this subset has been shown to favor T cell differentiation towards Th1 and Th17, rather than a Treg phenotype (Cerovic et al. 2013). In addition, $CD103^+$ cDC2s did not show a cessation of cell cycle related genes upon migration as observed for migrating cDC1s and $CD103^-$ cDC2s. Unfortunately, the experiments analyzing the proliferative capacity of the cDC sub sets lacked markers to differentiate between the cDC2 subsets (see Figure 6 and Figure 7). Therefore, whether or not there is a subset-specific regulation of proliferation needs to be further investigated. Finally, there was also downregulation of subset-associated genes in migrating cDC2s including the integrins CD11c (*Itgax)* and CD11b (*Itgam*) for both subsets and the $CD103^+$ cDC2-specific transcripts Glycoprotein 2 (*Gp2*) (Luda et al. 2022) and Triggering Receptor Expressed On Myeloid Cells (*Trem1*) (Bain et al. 2017), which is a similar pattern to the downregulation of *Clec9a* and *Tlr3* we observed in cDC1s.

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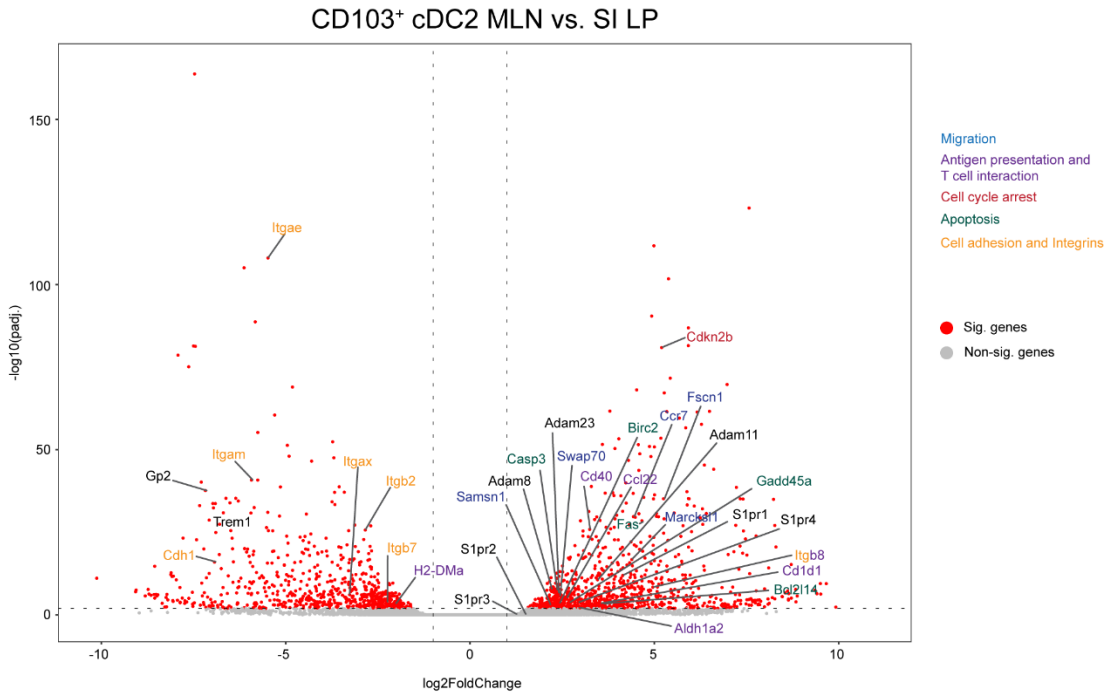

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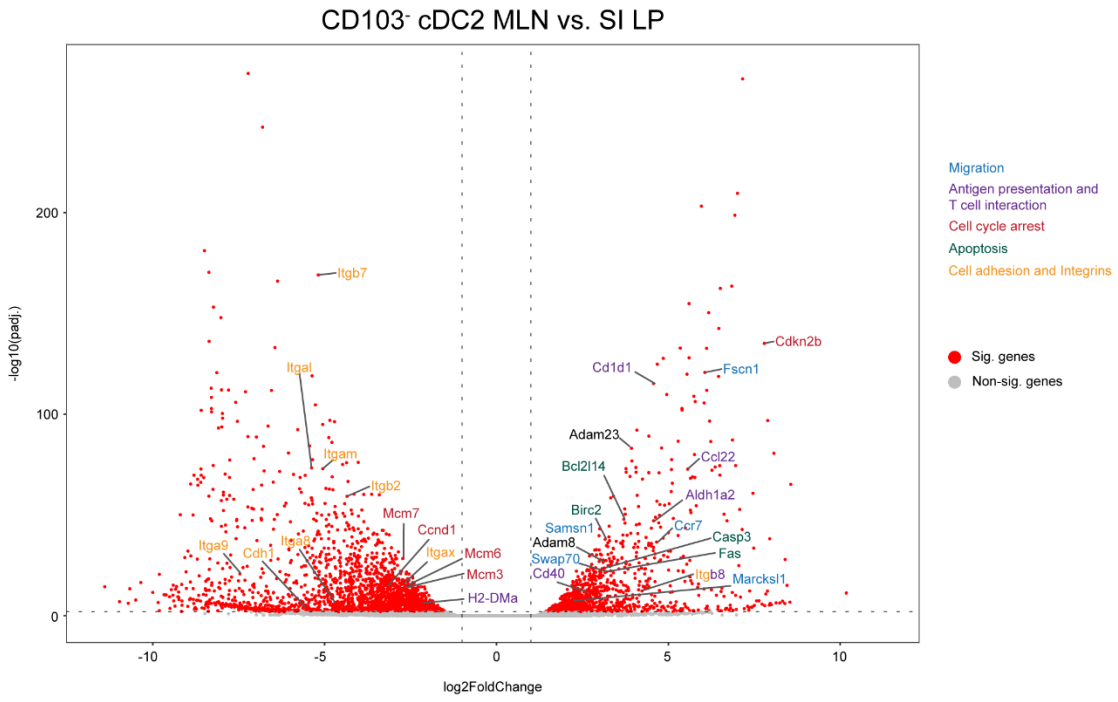

Figure 14 The cDC2 subsets of the small intestinal lamina propria utilize a similar migration associated transcriptome as cDC1s. A: Volcano plot highlighting selected genes associated with pathways that were found to be enriched in migrating cDC1s (see Figure 12 B). The plot displays the DE genes between CCR7^{+} CD103^{+} cDC2 in the sMLN and. CCR7^{-} CD103^{+} cDC2 of the SI LP with an adjusted p value cutoff ≤ 0.01. **B:** Volcano plot highlighting selected genes associated with pathways that were found to be enriched in migrating cDC1s (see Figure 12 B). The plot displays the DE genes between CCR7^{+} CD103^{-} cDC2 in the sMLN and. CCR7^{-} CD103^{-} cDC2 of the SI LP with an adjusted p value cutoff ≤ 0.01.

3.3.4. ADAM10 expression supports steady-state migration of SI cDC1s and CD103$^+$ cDC2s

Transcriptomic analysis of migrating cDCs revealed that the migration process is accompanied by a general downregulation of integrins (see Figure 12 C and Figure 14). As this indicated that downregulation of integrins may be an important step in the migration process, we hypothesized that the cessation of integrin expression may be paralleled by a mechanism to reduce integrin function or expression levels on the cell surface. Interestingly, we also found increased expression of transcripts encoding for ADAM23, ADAM8 and ADAM11 in the migrating cDC subsets (see Figure 12 C and Figure 14). Enzymes of the A disintegrin and metalloprotease (ADAM) family have been shown to have the ability to bind integrins to block their function (White 2003). Among the various known ADAMs, ADAM10 has been described to play a role in leukocyte migration (Pruessmeyer et al. 2014). To test whether ADAM10 plays a role in the steady-state migration of cDCs, we compared the cDC compartment of the SI and the MLN of mice lacking ADAM10 on all hematopoietic cells (Vav-Cre$^+$-ADAM10$^{p/p}$) with littermate control mice that were able to express ADAM10 (Vav-Cre$^-$-ADAM10$^{p/p}$). We hypothesized that, if ADAM10 is required for proper cDC migration, Vav-Cre$^+$-ADAM10$^{p/p}$ mice would have reduced frequencies and numbers of migratory cDCs in the MLN, while they would remain unchanged or increase in the SI. Indeed, there was a significant reduction of migratory cDCs in the MLNs of ADAM10-deficient mice compared to controls (Figure 15 A and B). Importantly, there was no reduction in the numbers or proportion of SI LP cDCs, or blood-derived, resident MLN cDCs, indicating that cDC development was unaffected. In fact, we observed a modest (but non-significant) increase of cDCs in the SI LP, which was consistent with a migration defect in ADAM10 deficient mice. To see if the phenotype was cDC subset specific, we analyzed the intestinal cDC subsets separately. Interestingly, cDC1s showed the strongest reduction in the frequency of migratory cDCs, followed by CD103+ cDC2s, while CD103- cDC2s were not affected by the loss of ADAM10 (Figure 15 C and D). However, the total numbers were only significantly decreased in migratory CD103$^+$ cDC2s that lacked ADAM10. Taken together, these data support the hypothesis that ADAM10 expressed on hematopoietic cells is involved in the migration of small intestinal cDC1s and CD103$^+$ cDC2s.

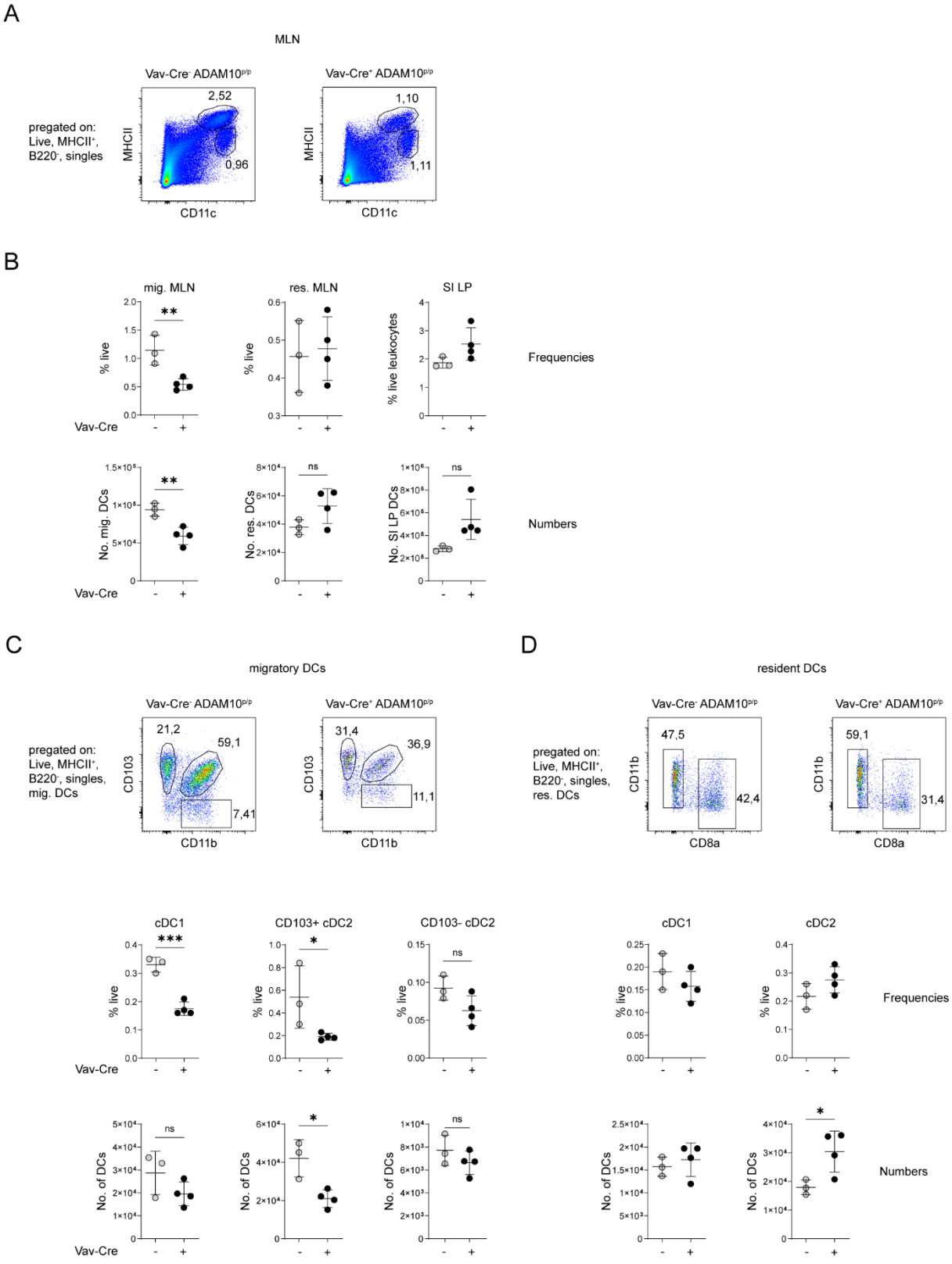

Figure 15 Lack of ADAM10 on hematopoietic cells leads to reduced migration of cDC1s and CD103⁺ cDC2s. A: Exemplary gating of migratory (MHCII^{hi}CD11c^{int}) and resident (MHCII^{int}CD11c^{hi}) cDCs of the mesenteric lymph node (MLN) of mice lacking ADAM10 on all hematopoietic cells (Vav-Cre⁺-ADAM10$^{p/p}$) and mice expressing ADAM10 on all hematopoietic cells (Vav-Cre⁻-ADAM10$^{p/p}$). **B:** Quantification of the frequencies of either total live cells (for MLN samples) or of live leukocytes (CD45⁺, for small intestinal (SI) samples) and total numbers of the indicated cDC populations. **C:** Exemplary gating and quantification of the migratory (mig.) cDC subsets which were identified by differential expression of integrins CD103 and CD11b, where cDC1s are CD103⁺CD11b⁻ and the

cDC2 compartment (CD11b$^+$) was split into a CD103$^+$ CD11b$^+$ and CD103$^-$ CD11b$^+$ population. **D:** Exemplary gating and quantification of the resident (res.) cDC subsets where cDC1s are CD8α$^+$ and cDC2s are CD11b$^+$CD8a-. Each dot represents an individual mouse with Vav-Cre$^-$ (n=3) and Vav-Cre$^+$ (n=4) from 1 experiment. Data are shown as mean ± SD. Statistical comparison was performed using a Student´s t-test (ns = not significant; * P≤0,05; ** P≤0,01; *** P≤0,001).

Next, we wanted to address whether ADAM10 needs to be expressed on cDCs themselves or whether expression in *trans* is sufficient to support the migration process. To this end, we generated competitive bone marrow (BM) chimeras. After irradiation of CD45.1/CD45.2 WT recipients, they were reconstituted with a 50/50 mixture of WT (CD45.1) and Vav-Cre$^+$-ADAM10$^{p/p}$ (CD45.2) BM. After 8 weeks, the cDC compartments of the SI, large intestine (LI) and the draining lymph nodes were analyzed for their donor origin (Figure 16 A). To do this, we excluded any remaining host cells (CD45.1.2) and pre-gated on donor-derived cells (CD45.1+CD45.2). If the reduced migration of cDCs is dependent on a cell-intrinsic ADAM10 expression on cDCs, then we would expect to see a skew towards ADAM10 KO-derived cDCs in the intestine, as these cDCs would be at a disadvantage in migrating out of the intestine. Conversely, we would expect to see a skew toward WT-derived cDCs in the mig. cDC compartment of the draining LNs, as these cDCs would have a competitive advantage at migrating compared to cDCs lacking ADAM10. On the other hand, if the effect of ADAM10 on cDC migration was cell extrinsic we would expect no difference between the two donor-derived cDCs as surrounding hematopoietic cells from the WT could provide the ADAM10. Analysis of the frequency of ADAM10-deficient cDCs, directly comparing this compartment in both the SI and the sMLN as well as the LI and the cMLN, showed a significant decrease in the relative proportion of ADAM10 KO migratory cDCs in LNs compared to the intestinal tissues. This skew was mainly driven by a highly significant decrease of migratory cDC1s in the ADAM10 deficient cDCs. These results suggest that ADAM10 needs to be expressed by the cDCs themselves to support the migration process. Interestingly, while the migration of CD103$^-$ cDC2s was generally not affected, there was a decrease in the frequency of ADAM10-deficient CD103$^+$ cDC2s in the sMLN compared to the SI. Taken together, these data suggest that intrinsic ADAM10 expression on cDCs aids their migration from peripheral tissues to the draining LNs. However, ADAM10 deficiency does not abolish cDC migration but rather has subtle and subset-specific effects. In addition, the data also does not exclude the possibility, that other cell types may still act in *trans* to aid cDC migration.

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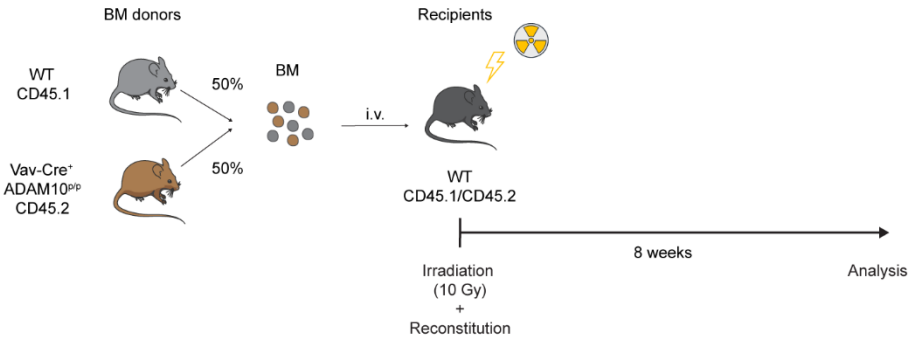

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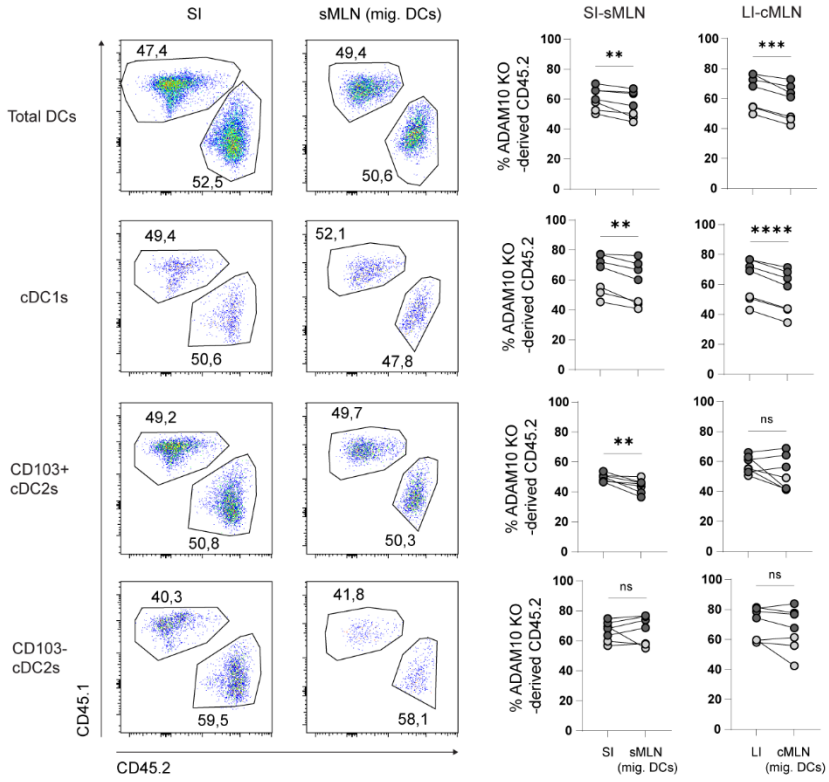
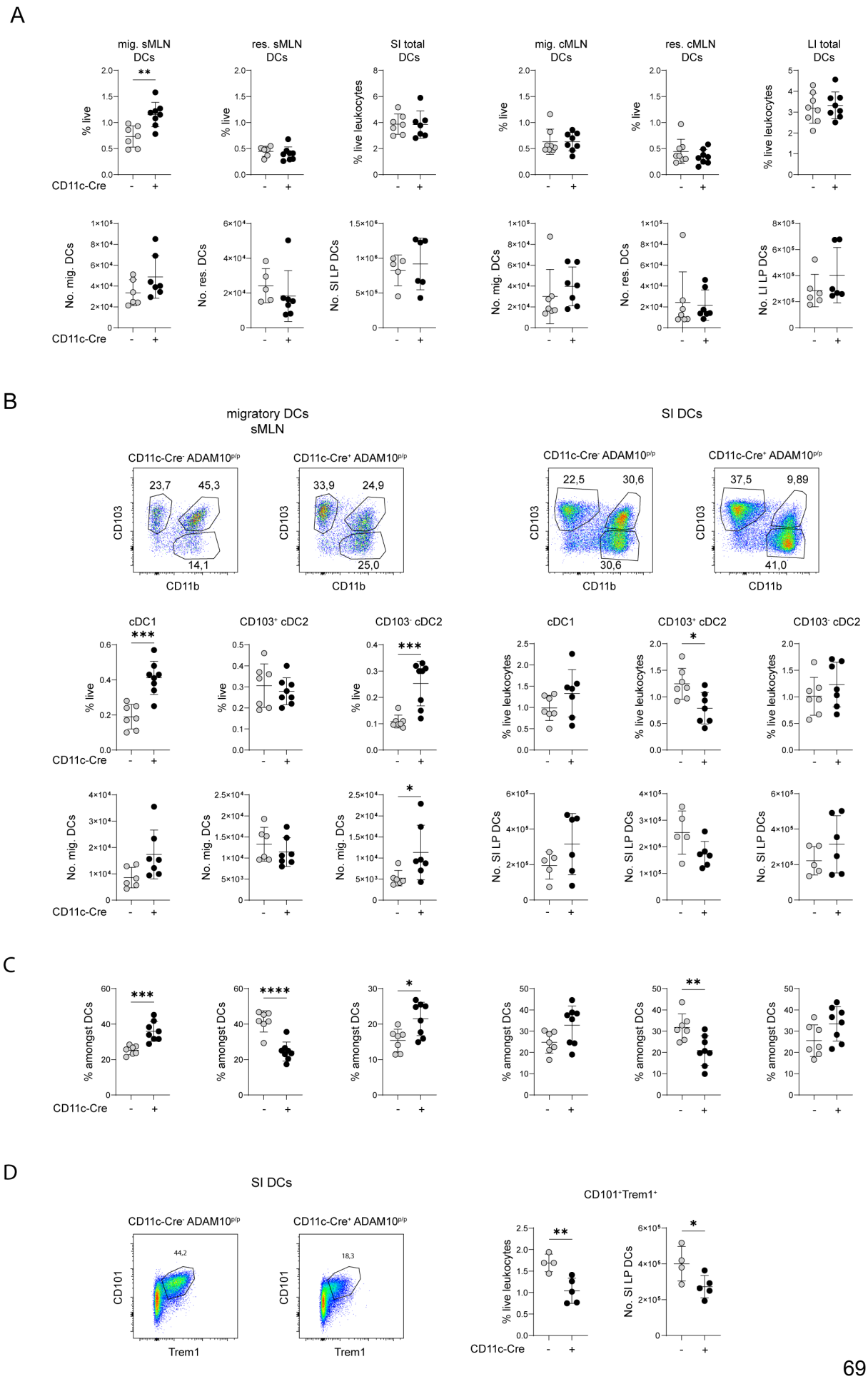


Figure 16 ADAM10 deficiency suppresses cDC1 migration in a cell intrinsic manner. A: Schematic illustration of the generation of competitive bone marrow (BM) chimeras. After irradiation, WT CD45.1/CD45.2 recipients were reconstituted with a 50/50 mix of WT (CD45.1)- or ADAM10 deficient (CD45.2)-derived BM. **B:** Gating strategy and quantification of ADAM10 KO derived (CD45.2$^+$) cDCs and cDC subsets amongst the SI and LI and the migratory cDCs of the sMLN and the cMLN. Any remaining host cells (CD45.1.2) were excluded before quantification of ADAM10 knockout (KO)-derived (CD45.2) cDCs. Each connected pair of dots represents an individual mouse. Dot colors indicate the two independent experiments (dark grey n=4) and (light grey n=3). Statistical comparison was performed using a paired t-test (ns = not significant; ** P≤0,01; *** P≤0,001; **** P≤0,0001).

In order to confirm our conclusion that ADAM10 mainly acts intrinsically to support steady-state cDC migration, we made use of another system using the CD11c-Cre driven ADAM10 knockout model. Although CD11c is expressed on several different cell types, especially macrophages, it still narrows down the pool of cells that could be involved (Abram et al. 2014). Similar to the analysis of the Vav-Cre driven ADAM10 knockout mice (see Figure 15), we compared the frequency and number of the cDCs in CD11c-Cre^{+}ADAM10$^{p/p}$ mice and compared them to ADAM10 sufficient CD11c-Cre^{-} ADAM10$^{p/p}$ mice. Surprisingly, the only difference was seen in the mig. cDC compartment of the sMLN, with an increase in the frequency of the Cre-expressing mice lacking ADAM10 on CD11c^{+} cells (Figure 17 A). This is in contrast to our previous data, which showed an increase in migrating cDCs rather than a decrease when cDCs lacked ADAM10. To test whether this increase was driven by a specific cDC subset, we analyzed the cDC subsets of the sMLN and the SI separately (Figure 17 B). The increase in the frequency of migrating cDCs in the sMLN was mainly driven by an increase in cDC1s and CD103^{-} cDC2s, while the main cDC subset in this compartment, the CD103^{+} cDC2s, was not affected. In the SI the overall trend was similar, although only the reduction in the frequency of CD103^{+} cDC2s was significant. While the data do not allow to further interpretation of the potential effect of ADAM10 expression on cDC migration, we observed a possible effect of ADAM10 deficiency on the cDC subset composition. The frequencies of CD103^{+} cDC2s in the SI and sMLN of CD11c-Cre^{+}ADAM10$^{p/p}$ mice were significantly lower compared to CD11c-Cre^{-}ADAM10$^{p/p}$ mice (Figure 17 C). In order to test whether the reduced proportion of CD103-expressing cDC2s was due to a loss of CD103 expression or a genuine reduction in the subset of CD103^{+} cDC2s, we stained for CD101 and Trem1, both markers that are specifically expressed by CD103^{+} but not CD103^{-} SI cDC2s (Bain et al. 2017). Since there was also a reduction in CD101^{+}Trem1^{+} cDCs, this suggests that lack of ADAM10 on CD11c expressing cells leads to an impaired differentiation of the intestinal CD103^{+} cDC2 subset (Figure 17 D). This observation could be explained by the fact that one of the factors involved in the differentiation of intestinal CD103^{+} cDC2s is Notch-2 signaling (Lewis et al. 2011), which has been reported to be downstream of ADAM10 (Gibb et al. 2010).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mig. sMLN DCs
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res. cMLN DCs
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No. SI LP DCs
No. LI LP DCs
CD11c-Cre
B
migratory DCs sMLN
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CD11c-Cre- ADAM10p/p
CD11c-Cre+ ADAM10p/p
CD103
CD11b
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Figure 17 CD11c-Cre driven knockout of ADAM10 does not decrease the steady-state migration of cDCs. A: frequencies of cDCs among total live cells (for MLN samples) or live leukocytes ($CD45^+$, for small intestinal (SI) and large intestinal (LI) samples) and total numbers of the indicated cDC populations. **B:** Exemplary gating, frequencies and numbers of the cDC subsets of the sMLN and the SI that were identified by differential expression of integrins CD103 and CD11b, where cDC1s are $CD103^+CD11b^-$ and the cDC2 compartment ($CD11b^+$) was split into a $CD103^+CD11b^+$ and $CD103^-CD11b^+$ population. **C:** Proportion of cDCs within the MLN (mig.) cDC subsets (left) or SI cDC subsets (right) in CD11c-$Cre^+ADAM10^{p/p}$ and CD11c-$Cre^-ADAM10^{p/p}$ mice. Each dot represents an individual mouse with n=8 except of CD11c-Cre- group of SI and sMLN with n=7 from a total of 6 independent experiments. **D:** Exemplary gating, frequencies and numbers of $CD101^+Trem1^+$ cDCs of the SI with n=4 for the CD11c-Cre^- group and n=5 for CD11c-Cre^+ group from 3 independent experiments. Data are shown as mean ± SD. Statistical comparison was performed using a Student´s t-test (* P≤0,05; ** P≤0,01; *** P≤0,001; **** P≤0,0001).

Taken together, the data provide somewhat conflicting results, depending on the model system used. While a Vav-driven knockout of ADAM10 resulted in reduced migration of cDC1 and $CD103^+$ cDC2s, this effect was not observed in the more cDC-specific driven knockout using CD11c-Cre^+ mice, indicating that the ADAM10 expressed by cells other than cDCs may be beneficial for the migration process of cDCs. Surprisingly, competitive bone marrow chimeras suggested rather a cDC-intrinsic effect of ADAM10 deficiency on cDC migration, which cannot be explained without further testing of the specificity of the used Cre-systems and validations of the affected cell types. In addition, there was a clear differentiation defect of $CD103^+$ cDC2s which was probably driven by a disturbed Notch2 signaling due to a cell intrinsic lack of ADAM10 in CD11c-Cre^+ mice. As the aim of this thesis was to investigate potential pathways that are involved in steady-state cDC migration we did not further investigate on the observed differentiation defect of $CD103^+$ cDC2s.

3.3.5. S1PR signaling selectively suppresses migration of $CD103^+$ cDC2s

Another set of genes that was significantly upregulated in migrating $CD103^+$ cDC2s were the Sphingosine-1-phosphate receptors (S1PRs) S1PR1 and S1PR4 with S1PR2 and S1PR3 also being upregulated, although not significantly (Figure 14 A). These receptors have been well described to be involved in the migration of immune cells, particularly in lymphocyte egress from lymphoid tissues (Matloubian et al. 2004). Several groups have suggested that cDCs utilize S1PR signaling for migration to the lymph nodes (Han et al. 2015; Reines et al. 2009). For instance, Rathinasamy et. al. described reduced numbers of $CD103^+$ cDCs in the MLN when S1PR1 or S1PR3 signaling was genetically disrupted or blocked by inhibitors (Rathinasamy et al. 2010). However, a limitation of these studies is, that they are often based solely on the quantification of cDC numbers and also in a setting of induced cDC migration using PRR agonists such as lipopolysaccharide (LPS).

In order to test whether S1PR signaling is required for the steady-state migration of cDCs, we used the afore established *in vivo* photolabeling in Dendra mice. By administration of the S1P functional antagonist FTY720, we were able to interfere with all S1PRs except of S1PR2. To quantify potential effects on the steady-state cDC migration, we administered FTY720 for 3 consecutive days. On the last day, the MLN chain was photoconverted and the frequency of $Dred^+$ cDCs was analyzed 16 h later (Figure 18 A). As positive control we quantified the frequency of $Dred^+$, $B220^+$ B cells as it is well described that their migration/circulation is drastically reduced by FTY720 (Chiba 2005). We saw a significant increase in $Dred^+$ B cells in FTY720 treated mice compared to the PBS control group. This confirmed that the inhibitor worked as described as the B cell circulation was drastically reduced and that the experimental setup is suitable for the measurement of cell migration (Figure 18 B). Compared to the effect seen in B cells, the effect on the cDC compartment was not as pronounced. Although not statistically significant, there was an increase in the proportion of $Dred^+$ mig. cDCs in the sMLN. This was driven by a selective increase in the proportion of $Dred^+CD103^+$ cDC2s. The same trend was observed among migratory $CD103^+$ cDC2s of the cMLN, suggesting that S1PR signaling selectively supports the migration of $CD103^+$ cDC2s while the migration of the other subsets remained unchanged.

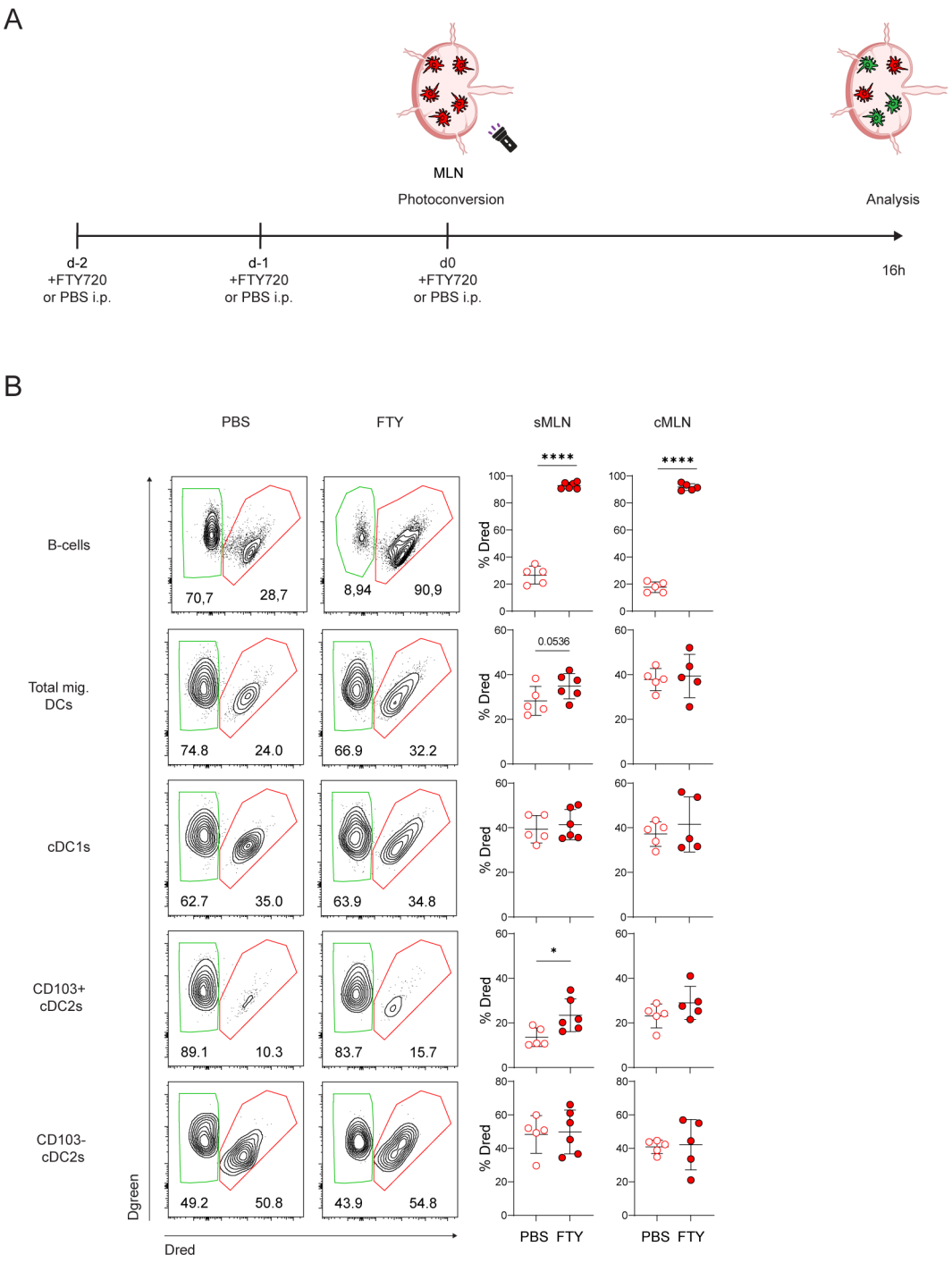

Figure 18 S1PR inhibition selectively suppresses migration of intestinal CD103⁺ cDC2s. A: Schematic illustration of the experimental setup. Dendra mice were administered 1 mg/kg S1PR functional antagonist FTY720 or PBS by i.p. injection for three consecutive days. On day 3 the MLN was photoconverted by exposure to violet light. 16 hours later, MLN were analyzed for the frequency of Dred⁺ B-cells and cDCs. **B:** Exemplary gating (sMLN) and quantification of Dred⁺ B-cells (B220⁺) and the migratory cDC subsets which were identified by differential expression of integrins CD103 and CD11b (see Figure 11 B). Data are shown as mean ± SD. Each dot represents an individual mouse with PBS control group (n=5) and FTY group (n=6) from two independent experiments. Statistical comparison was performed using a Student´s t-test (* P≤0,05; **** P≤0,0001).

3.3.6. Conclusion Part 3

The data presented here showed that all migrating cDCs, regardless of their tissue of origin, share a similar migration-associated transcriptome. This transcriptome is characterized by a general upregulation of genes associated with cytoskeletal rearrangement, apoptosis and antigen presentation while integrins and proliferation-associated genes are significantly downregulated. In addition, the data showed that ADAM10, as well as S1PR signaling is utilized by different cDC subsets during homeostatic migration, as the lack of these proteins led to a selective reduction of migrating cDCs.

4. Discussion

4.1. Summary

The homeostatic migration of cDCs is a fundamental process that maintains immunological tolerance to innocuous self- and foreign antigens. This process is particularly important in the context of the gut, where it induces and maintains immune tolerance to both dietary- as well as commensal-derived antigens, preventing exaggerated immune responses such as inflammatory bowel disease or food allergy. However, the mechanisms that drive the migration of cDCs in the steady state are still largely unknown. This thesis provides novel insights into the maturation as well as the migration process of intestinal cDCs. We showed that both intestinal cDC subsets proliferate *in situ* and that they share a similar transcriptional maturation program, which is accompanied by a gradual increase in classical maturation markers and a simultaneous decrease in proliferative capacity. Remarkably, the proliferative capacity of small intestinal cDCs increased upon tissue entry, suggesting that local signals can regulate cDC proliferation and maturation. Furthermore, migrating cDCs, regardless of tissue of origin or subset, use a common transcriptional program that is accompanied by the upregulation of an apoptosis-associated gene set. We further provided a comprehensive quantification of cDC migration kinetics and demonstrated an almost complete turnover of the migratory cDC compartment of the MLN every day. Finally, we showed that the migration of select subsets of small intestinal cDCs can be hindered by inhibition of S1PR or ADAM10 signaling. These findings also demonstrate that the photoconversion-based experimental systems used here allow for a dedicated system to further dissect the molecular mechanisms of cDC migration.

4.2. The homeostatic migration of small intestinal cDCs is a highly kinetic process

The quantification of cDC migration from peripheral tissues into lymph nodes has always been challenging. The majority of the studies that address the migration of cDCs are based on the use of fluorescent tracers or the transfer of *ex vivo* manipulated cDCs and rely on the enumeration of labelled cDCs in the lymph nodes. However, these methods harbor several disadvantages that impede an accurate quantification of cDC migration. For instance, the orthotopic transfer of labelled cDCs is dependent on prior enrichment of cDCs. However, both *in vitro* generated BMDCs as well as *ex vivo* enrichment of cDCs do not allow for the analysis of homeostatic cDC migration as they do not reflect the situation of endogenous cDCs in tissues. Similarly, while the application of fluorescent dyes is a suitable method to track migrating cDCs, FITC used together with a carrier solution induces the migration of dermal DCs and does not reflect the steady state (Ohl et al. 2004). Additionally the application of such

small molecules harbors the risk of labelling lymph node resident DCs, both by direct transport of the molecules via lymph or by transfer of antigen from migratory DCs, making it difficult to faithfully track the origin of labelled cDCs in the lymph node and to quantify their migration (Itano et al. 2003; Belz et al. 2004; Sixt et al. 2005). The use of transgenic photoconvertible proteins such as KikGR or Kaede mouse models, offer a quantitative approach to analyze cDC migration (Tomura et al. 2014; Houston et al. 2016).

By using the Dendra mouse model, we here presented for the first time a detailed quantification of the steady state migration kinetics of intestinal cDCs. We showed that the migration of intestinal cDC subsets is a highly kinetic process that is faster than that of dermal cDCs (Tomura et al. 2014). This observation further raises the question of how steady-state migration is induced in the steady state and how it is regulated in different tissues. Compared to the skin, the physical intestinal barrier only consists of a single layer of epithelial cells that is exposed to a huge amount of dietary and microbiota-derived antigens while also allowing for an exchange of nutrients. Therefore, the increased rate of migration of cDCs in the intestine may be a result of the enhanced antigen exposure of the intestinal environment that requires a quicker turnover to maintain the transfer of information to the adaptive immune system. However, it is important to note that compared to the quantification of dermal cDC migration, photoconversion of both the intestine as well as the mesenteric lymph node require laparotomy. While it was shown that the photoconversion procedure itself does not influence inflammatory parameters such as IL1-β or TNF-α, the surgical procedure however could potentially lead to an activation of intestinal cDCs, similar to an observation that tissue damage, induced by tape stripping, was shown to increase the migration rate of dermal cDCs (Tomura et al. 2010). Nonetheless, steady state migration is a very rapid process that necessitates a huge investment of resources, further illustrating the importance of steady state DC migration. With conservative estimates of 0,5-1x10^6 cDCs in the mouse small intestine our results suggest that up to 10^5 cDCs migrate to the MLN every day just from the small intestine, and subsequently undergo apoptosis (Pabst et al. 2010).

4.3. Regulation of homeostatic cDC migration

Despite the importance of homeostatic cDC migration in the induction of peripheral tolerance, the signals and mechanisms that drive it are still an enigma. Several studies have addressed the role of potential pathways that could be involved in the regulation of homeostatic cDC migration (Hagerbrand et al. 2015; Wilson et al. 2008; Bosteels et al. 2023). In a study by Baratin et al., IKKβ-induced NF-κB was shown to be a key regulator of homeostatic cDC migration, at least for dermal cDCs. However, the activated NF-κB network was independent of canonical upstream signals as deletions in IL-1β-, TLR- and TNF-α-signaling did not show

reduced numbers of migratory cDCs in draining lymph nodes. Furthermore, it was also independent of the IKKα-dependent non-canonical activation of NF-κB and thus raised the question whether steady state migration is even regulated by external stimuli or whether it may just be driven by an intrinsic stochastic process (Baratin et al. 2015). But what could the possible mechanisms that drive the homeostatic migration of cDCs be?

In a hypothetical model of stochastically regulated cDC migration, every cDC has the same chance of getting activated at any time, no matter when it entered the tissue and how long it has been there already. However, our results, as well as published data, do not align with this hypothesis. For instance, in a pre-cDC transfer experiment, the frequency of donor-derived migratory cDCs in the mesenteric lymph node increases with the days after transfer, peaking at day 7 (last day analyzed) (Scott et al. 2015). This suggests that cDCs stay in peripheral tissues for at least a few days before they become activated which does not align with the idea that the induction of cDC migration is a purely stochastic event. Since the main function of the steady state cDC migration is the continuous transfer of peripherally acquired antigen to inductive sites in order to maintain peripheral tolerance, time-based regulation would also maximize the amount of antigen acquired before the migration is induced. This is also in accordance with data presented here, which shows that the expression of CCR7 is tightly linked with maturation, which itself is a time-dependent gradual process. However, it still does not explain how the migration itself is induced.

In this thesis, we analyzed the transcriptional changes that occur in migrating cDCs and showed that cDC1s and cDC2s utilize a similar transcriptional program for their migration. Furthermore, we also found substantial overlap in the transcriptome of migrating cDCs of the lung and the small intestine while the common hallmarks of migrating cDCs can also be observed in published datasets that analyzed CCR7+ cDCs in the thymus and the skin draining lymph nodes (Ardouin et al. 2016). Taken together, these data indicate that the homeostatic migration of cDCs relies on a highly conserved program that is utilized by migrating cDCs, irrespective of tissue origin or subset and therefore suggests that the upstream induction may also rely on highly conserved signals and pathways. What could be possible intrinsic or extrinsic cues that regulate cDC maturation and subsequent migration, irrespective of the different tissues and microenvironments they reside in?

4.4. Growth factors as regulators of cDC maturation and migration

Whether tissue resident cDCs proliferate *in situ* or whether they solely depend on renewal from blood precursors, as it is the paradigm for monocyte derived tissue macrophages, has been an open question due to the lack of suitable *in vivo* methods to directly address it. First direct

evidence of local proliferation was provided by spleen transplantations in which graft cDCs incorporated BrdU, confirming *in situ* proliferation of splenic cDCs (Liu et al. 2007). However, this only provided information about the situation in the spleen and due to the experimental setup, did not allow a quantification to which extent entering precursors are contributing to the pool of proliferating cDCs. Using a photoconversion-based approach that allows for time- and tissue-stamping of cells, combined with EdU incorporation, we here showed that small intestinal cDCs are capable of proliferation *in situ*. This is in accordance with a publication that used multicolor fate mapping in Clec9a^{Confetti} mice revealing that lung as well as small intestinal cDCs are present in clonal clusters, which indicates local proliferation of peripheral tissue cDCs (Cabeza-Cabrerizo et al. 2019). More interestingly, we here demonstrated that the proliferation rate of cDCs that reside in the small intestinal lamina propria is significantly higher than that of entering precursors. This observation suggests that small intestinal cDCs are provided with a signal that induces their proliferation upon tissue entry, a hitherto undescribed phenomenon. Furthermore, we showed that the proliferative capacity of cDCs drops with their maturation, ultimately resulting in the cessation of cell-cycle associated gene expression in CCR7+ cDCs, which represent the most mature stage of tissue cDCs. Taken together, these results not only suggest that the maturation, proliferation and the migration are tightly linked processes, but further raise the question how this inherent and continuous process may be regulated.

Growth factors such as the granulocyte-macrophage colony-stimulating factor (GM-CSF), Lymphotoxin-β (LTβ) and fms-like tyrosine kinase 3 ligand (Flt3L) have all been shown to be involved in the maintenance of cDC homeostasis. For instance, lymphotoxin-α1β2 produced by B cells was shown to induce proliferation of splenic cDC, which was further enhanced by overexpression of the cytokine. Accordingly, splenic cDC numbers are strongly reduced when there is a genetic deletion of the lymphotoxin-β receptor, supporting its role in cDC homeostasis, at least in spleen (Kabashima et al. 2005). However, a later study that analyzed mice with conditional deletions of LTβ on T and B cells showed that while there is a decrease in splenic and lymph node DCs, there is a simultaneous increase of blood DCs suggesting that LTβ may mainly be involved in the process of tissue entry (Junt et al. 2006). However, the effects of LTβ on cDC homeostasis have not been examined in peripheral tissues. The effects of GM-CSF and Flt3L on cDC homeostasis have been better characterized. Experiments in mice with genetically deleted GM-CSF or Flt3L demonstrated that both cytokines are involved in maintaining cDC numbers in spleen and skin (Kingston et al. 2009). In particular, Flt3L has a profound effect on cDC numbers and development in both lymphoid and non-lymphoid tissues (Wilson et al. 2021; Ginhoux et al. 2009; Waskow et al. 2008). It was further shown to be a central regulator of splenic cDC proliferation as demonstrated by reduced BrdU incorporation in Flt3-deficient cDCs compared to WT control cDCs in a parabiotic setup (Waskow et al. 2008). In addition, Flt3L has recently been described to be involved in the

maturation of splenic cDC1s. Following depletion of splenic cDC1s, there is an increase in local Flt3L levels which in return leads to an accelerated maturation process as evidenced by increased expression of MHCII, CD11c and XCR1 on the repopulating cDC1s. The maturation process was further enhanced when Flt3L was given systemically in line with the hypothesis that it acts as a major regulator of the cDC maturation process (Ugur et al. 2023). Notably, since Flt3L is internalized and degraded upon receptor binding, it is scavenged from the local environment. Therefore, a possible mechanism by which Flt3L could regulate the homeostatic cDC migration is by local and population-based sensing of Flt3L levels. In the context of the small intestine, villi that contain none or few cDCs would have higher levels of local Flt3L and thereby potentially favor both the attraction of blood precursors into an empty niche as well as the proliferation and maturation of newly entering cDCs. This hypothetical model is also in accordance with the observation that in Clec9a^{Confetti} mice, the chance of cDC clusters in villi to be of a single-color is around 80%, suggesting that when a niche is occupied, it is more likely to be filled by local proliferation of cDCs rather than by newly entering precursors (Cabeza-Cabrerizo et al. 2019). At a certain population size, local Flt3L concentrations could reach a critical level that is below a threshold to further induce proliferation which may act as a signal for cell cycle cessation and subsequent migration. This self-regulated feedback mechanism could explain how local cDC populations intrinsically regulate their migration, irrespective of cDC subset or tissue. Furthermore, this model would allow for a certain time of tissue residence which would guarantee for an efficient uptake of antigens. Interestingly, such a maturation and proliferation linked induction of migration may also explain why Langerhans cells (LCs) show far slower migration kinetics than cDCs, which would be explained by the slow turnover of LCs in the steady state as demonstrated by minimal BrdU incorporation after several days of BrdU administration (Tomura et al. 2014; Merad et al. 2002). Furthermore, since MHC-peptide complexes could be spread among dividing sister cells, this would distribute the acquired antigens, increasing the chance of antigen presentation in the lymph node, particularly if all cDCs in a particular microenvironment receive a signal to migrate simultaneously. Both intestinal epithelial cells (IECs), as well as T cells have been shown to produce Flt3L, and could therefore act as a stable source of Flt3L in the intestinal lamina propria (Saito et al. 2013; Sikder et al. 2023). Moreover, it was shown that CD4 T cells increase Flt3L expression upon proliferation (Saito et al. 2013). This mechanism could further explain how cDC maturation and migration speed could be accelerated on-demand in response to local inflammation. However, future studies are needed to further elucidate the exact roles of growth factors such as Flt3L in cDC maturation, proliferation and migration. Expanding our experimental setup to other tissues and organs will address whether the observed induced proliferation of cDCs after tissue entry is a gut specific phenomenon or whether it is a tissue independent mechanism that applies to cDCs in all tissues. Furthermore, precursor transfers

and fate tracking models of cDCs will be of great value in further addressing how cDC maturation and migration are regulated. The recently published, tamoxifen-inducible, *Prtn3*-driven precursor fate-tracking would allow the assessment of cDC maturation and proliferative capacity over a period of time and provide additional information on the kinetics of cDC maturation in different tissues (Ugur et al. 2023). Furthermore, this model would also allow to indirectly test whether cDC proliferation may be regulated locally by clonal clusters of cDCs. If this hypothesis is true, histological analysis of small intestinal villi should show a proportional increase in cluster sizes with time after precursor labelling. If cDCs within the same microenvironment migrate in a coordinated fashion we expect to observe a reduction in the number of bigger cDC clusters, supporting a local regulatory mechanism of cDC migration. Furthermore, while it has been shown that administration or genetic overexpression of Flt3L increases overall cDC numbers, the direct effects on the migration of cDCs had not been addressed yet. The generation of dedicated mouse models that allow for a specific disruption of Flt3 signaling in cDCs could be a valuable tool in studying the potential role of Flt3L in the induction of homeostatic cDC migration. For instance, conditional Flt3 (CD135) knockout strains such as XCR1-CreERT or huLangerin-CreERT driven knockouts could answer whether Flt3L signaling inhibition leads to changes in cDC migration without affecting total numbers of cDCs. Crossed to Dendra mice this model would allow for direct quantification of the cDC migration and answer whether deprivation of Flt3 signaling may be a key driver of steady state DC migration.

4.5. Apoptosis as a regulator of the homeostatic migration of cDCs

A highly conserved biological program that is associated with migrating cDCs is apoptosis (Ingulli et al. 1997; Tomura et al. 2014; Leverkus et al. 2000). Apoptosis is responsible for the short half-life of cDCs upon migration to the LN and it mediates the high replenishment rate of migratory cDCs in lymph nodes, as quantified in this thesis. Notably, we showed that certain apoptosis related transcripts are upregulated together with CCR7, before other major migration-associated transcriptional changes occur. This leads to the hypothesis that apoptosis may not just be induced alongside with migration but that it may also be a potential driver of the migration program itself. What evidence exists that speaks for or against the idea of apoptosis acting as a regulator of homeostatic cDC migration? Apoptosis can be induced by intrinsic or extrinsic stress signals such as DNA damage or FAS signaling, respectively. The chance of apoptosis induction usually increases with the lifetime of a cell and therefore could serve as a suitable mechanism that controls the steady state migration of cDCs while allowing for a certain time of tissue residency. Furthermore, apoptosis is a highly conserved biological program and could therefore explain how steady state migration could be regulated,

irrespective of the cDC subset and the tissue the cDCs reside in. However, when cells undergo apoptosis they usually express certain “eat-me” signals which mediate a quick clearance by surrounding phagocytes, such as macrophages. In the case of cDCs, this process would be highly disadvantageous since their main function is to present acquired antigen to T cell in the lymph nodes which would be prevented by early efferocytosis of cDCs in peripheral tissues. Interestingly, cDCs may have evolved mechanisms to overcome this problem. For instance, it was shown that CCR7 signaling in DCs induces anti-apoptotic signals via AKT and NF-κB pathways (Sanchez-Sanchez et al. 2004). Furthermore, compared to maturing cDCs, activated cDCs show reduced sensitivity to apoptotic signals induced by death receptors or CTLs which demonstrates a delicate balance of pro- and anti-apoptotic mechanisms in migrating cDCs (Leverkus et al. 2000; Medema et al. 2001). However, addressing a potential link of apoptosis in inducing the migration of cDCs *in* vivo is restricted due to limitations in available models as interfering with this process can have a profound effect on the overall homeostatic cellular balance. Still, it was shown that ionizing- as well as low dose radiation induces the expression of CCR7 and augments the migration of cDCs both *in vitro* and *in vivo* (Yu et al. 2018; Cummings et al. 2012). Additionally, FAS ligation was shown to induce the maturation of bone marrow-derived DCs as shown by increased expression of MHCII, CD86 and CD40 while it did not induce apoptotic cell death in these cells (Rescigno et al. 2000). However, these studies do not provide information regarding possible effects on the migration of cDCs. Furthermore, full body irradiation does not allow for any conclusions regarding a DC intrinsic or extrinsic effect of apoptosis in the activation process. This is especially important since the uptake of apoptotic material itself had also been shown to induce the migration of cDCs (Penteado et al. 2017; Bosteels et al. 2023).

Further studies will be needed to test whether cDC intrinsic apoptosis drives the induction of homeostatic cDC migration. However, future *in vivo* studies will require a cDC-specific and controlled interference with the apoptosis program. The Dendra mouse model offers an elegant way to quantify the migration of dermal and intestinal cDCs. Crossed with mice that allow for controlled apoptosis induction, such as a DC-specific CreERT-driven deletion of the antiapoptotic protein Bcl-2, could be a promising approach to investigate the role of apoptosis in cDC migration. However, even if apoptosis turns out to be a key regulator of homeostatic cDC migration, there would still be the question as to which signals induce the apoptosis in the first place which brings us back to the initial question.

Ultimately, the factors that induce the homeostatic migration of cDCs remain an open question. While the data presented here suggest that it may be regulated by a highly conserved mechanism that is shared between cDCs and possibly even Langerhans cells, it is still possible that the activation and subsequent migration is just a result of cumulative stress on tissue

residing cDCs. Accordingly, this could explain the observation that physiologically independent pathways play a role in the induction of CCR7 in cDCs. For instance, physical stress that deforms the nucleus was shown to induce the migration of DCs *in vitro* (Alraies et al. 2023). Furthermore, the uptake of apoptotic material can induce the migration of cDCs which may also act as a measure of stress in the local environment (Bosteels et al. 2023; Maier et al. 2020). The idea of cumulative stress signals inducing homeostatic cDC migration would also explain why several studies only show limited effects on the homeostatic migration after interfering with potentially involved pathways (Baratin et al. 2015; Hagerbrand et al. 2015; Rathinasamy et al. 2010).

4.6. S1PR signaling in cDC migration

Several studies have suggested that DCs in the skin, lung and the small intestine utilize S1PR signaling for their migration from the periphery into lymph nodes (Rathinasamy et al. 2010; Czeloth et al. 2005; Idzko et al. 2006). In this thesis, we used a photoconversion-based approach to measure possible effects of S1PR inhibition on cDC migration. Quantification of the migratory cDC compartment of the mesenteric lymph node after FTY720 treatment validated the results shown by Rathinasamy et al. that reported that in mice with inhibited S1PR1 and S1PR3 signaling have reduced numbers of CD103+ cDCs in the MLN (Rathinasamy et al. 2010). Furthermore, our multiparametric flow cytometry-based approach revealed that the observed phenotype was due to selective inhibition of the migration of CD103+ cDC2s, which make up the majority of small intestinal CD103+ cDCs. In contrast, CD103+ cDC1s as well as CD103- cDC2s remained unaffected. Accordingly, transcriptomic analysis showed a selective upregulation of S1PR1 and S1PR4 in migrating CD103+ cDC2s but not in the other intestinal cDC subsets. These results demonstrate that, in addition to CCR7-signaling, further signals and pathways are selectively used by different cDC subsets for homeostatic migration. Furthermore, Rathinasamy et al. showed that the migration of skin derived APCs was reduced after S1PR1 blockade but not when S1PR3 was genetically deleted, while small intestinal DCs utilized both S1PR1 and S1PR3, suggesting that there are additional tissue-specific differences of S1P-dependent tissue DC migration (Rathinasamy et al. 2010). However, there is currently no data available that addresses how S1PR expression is transcriptionally regulated in cDC2s, nor why S1PR signaling is only used by CD103+ cDC2s, but not by the other cDC subsets in the intestine. To address this, experimental approaches such as ChIP- and ATAC-seq could offer valuable insight into this subset specific and migration-associated transcriptional regulation. Another cell type in which S1PR signaling was shown to be involved in the migration process are Langerhans cells. Topical application of FTY720 as well as S1P led to a significantly reduced migration of Langerhans cells from the

epidermis into the skin draining lymph node (Reines et al. 2009). A possible explanation as to why S1PR signaling is used by CD103+ cDC2s and Langerhans cells but not the other subsets may be the specific niches these cells occupy in the tissue. Unlike cDCs, Langerhans cells reside in the epidermis and, upon activation, require an initial migration into the dermis to access the lymphatic capillaries. Similarly, CD103+ cDC2s in the gut egress the lamina propria to colonize and establish intimate contact with the intestinal epithelium which alters their transcriptional and functional profile (Rivera et al. 2021). These unique tissue niches occupied by Langerhans cells and gut CD103+ cDC2s may necessitate additional signals to home to the lymphatics, a role which may be fulfilled by S1P gradients. Even though natural S1P gradients are thought to be very low in peripheral tissues, S1P is produced by lymphatic endothelial cells, possibly generating a gradient towards lymphatic capillaries (Hla et al. 2008; Pham et al. 2010). Furthermore, it was demonstrated that such a S1P gradient is used by peripheral tissue T cells to enter afferent lymphatics and migrate to skin draining lymph nodes (Ledgerwood et al. 2008). Taken together, our data provide novel information about the selective use of S1PR signaling in the migration of small intestinal CD103+ cDC2s which may offer a way to therapeutically target this unique intestinal cDC subset. For instance, topical application of FTY was shown to reduce allergic reaction due to inhibiting the migration of Langerhans cells (Reines et al. 2009). Additionally, local administration of FTY to the lung attenuated experimental asthma at least in part by inhibiting cDC migration to the mediastinal lymph node (Idzko et al. 2006). Similarly, drugs that target S1PR signaling in the intestine may offer a promising way to selectively target the migration of small intestinal CD103+ cDC2s. Here, finding mechanisms that boost the S1P mediated migration of CD103+ cDC2s may be of great value, making use of the tolerogenicity of this unique cDC subset to treat exaggerated immune reactions in the gut.

4.7. The role of ADAM10 in cDC migration

The metalloprotease ADAM10 is involved in a variety of immune cell functions across different cell types (Seals et al. 2003). Here, using competitive bone marrow chimeras, we revealed a yet undescribed, cell-intrinsic role of ADAM10 in the migration of intestinal cDC1s. However, the exact molecular mechanism by which ADAM10 selectively supports the migration of intestinal cDC1s remains unknown. One of the best described function of ADAM10 is the cleavage of the extracellular domain of the Notch receptor upon ligand binding, inducing subsequent intracellular signaling (Hartmann et al. 2002; Tian et al. 2008). Interestingly, Notch signaling has been described in both the differentiation as well as the maturation of cDC1s and cDC2s. For instance, in an *in vitro* setting, adding Notch to bone marrow derived DC (BMDC) cultures increases the differentiation and maturation of cDC1s while also enhancing their

expression of CCR7 and boosting their ability cross-prime T cells *in vivo* (Kirkling et al. 2018). Furthermore, a CD11c-cre driven deletion of ADAM10 was shown to inhibit both the terminal differentiation of splenic Langerin+ cDC1s as well as their maturation, as seen by reduced numbers of Langerin+ cDC1s and a decreased expression of CD8α (Diener et al. 2021). In accordance with our previous observations of a strong link between the maturation and the migration process of cDCs in steady state, an ADAM10-deficiency induced reduction of Notch signaling could therefore explain the reduced migration capability of cDC1s in the intestine. Here, follow up experiments that analyze the maturation of intestinal cDC1s in mice with genetic deletions of ADAM10 will provide further insight into the role of ADAM10 and Notch in the maturation of cDCs. Accordingly, we also found a strong reduction in the proportion of intestinal CD103+ cDC2s in mice with a CD11c-cre driven deletion of ADAM10 compared to littermate controls, with a concomitant increase in CD103- cDC2s. Since these results are almost identical to observations in mice with a CD11c-cre driven knockout of Notch2, it is likely that the defects in cDC2 differentiation are due to the downstream effect of an ADAM10 deletion on Notch signaling (Lewis et al. 2011). Taken together, our data provide novel insights into the role of ADAM10 in the migration process of intestinal cDC1s. However, whether the ADAM10-Notch axis reduces the migration of intestinal cDC1s due to possible effects on the maturation and whether the migration of cDC1s in other organs are also affected remain an open question and require further analysis.

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Appendix

List of Abbreviations

°C	degree Celsius
7AAD	7-Aminoactinomycin D
ADAM	A disintegrin and metalloproteinase
AF	Alexa Fluor
ANOVA	Analysis of variance
APC	antigen presenting cell
approx.	approximately
Batf3	Basic Leucine Zipper ATF-like Transcription Factor 3
BM	bone marrow
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BV	Brilliant Violet
CCL	CC-type motif chemokine ligand
CCR	CC-type motif chemokine receptor
CD	Cluster of differentiation
cDC	Conventional or classical dendritic cell
CLEC	C-type lectin
cMLN	colon-draining MLN
CSF1R	Colony stimulating factor 1 receptor
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular pattern
DC	dendritic cell
dd	double distilled
DEGs	differentially expressed genes
e.g.	exempli gratia
EDTA	ethylenediaminetetraacetic acid
EdU	ethynyldeoxyuridine
FCS	fetal calf serum
Fig.	figure
FITC	Fluorescein isothiocyanate
Flt3	Fms-related tyrosine kinase 3
Flt3L	Fms-related tyrosine kinase 3 ligand
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMDP	granulocyte-monocyte and DC progenitor
GO	Gene Ontology
GPCR	G protein-coupled receptor
Gy	gray
hi	high
i.e.	id est
i.p.	intra peritoneal
i.v.	intra venous
IBD	inflammatory bowel disease
Id2	Inhibitor of DNA binding 2
IEC	Intestinal epithelial cell
IL	Interleukin
iLN	inguinal lymph node
int	intermediate
IRF	Interferon regulatory factor
KEGG	Kyoto Encyclopedia of Genes and Genomes
Klf4	Krüppel-like factor 4
KO	knockout

LANUV	Landesamt für Natur-, Umwelt- und Verbraucherschutz
LC	Langerhans cell
LI	large intestine
LN	lymph node
LP	lamina propria
M	Molar
MDP	monocyte-dendritic cell progenitor
medLN	mediastinal lymph node
MHC	major histocompatibility complex
MLN	mesenteric lymph node
moDC	monocyte-derived dendritic cell
mRNA	messenger ribonucleic acid
mW	milliwatt
MyD88	Myeloid differentiation primary response 88
n.s.	not significant
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLR	NOD-like receptor
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PE	Phycoerythrin
PP	Peyer's patch
PRR	Pattern recognition receptor
RNA	ribonucleic acid
RT	room temperature
S1PR	sphingosine-1-phosphate receptor
scRNA-seq	single cell RNA sequencing
seq	sequencing
SI	small intestine
sMLN	small intestinal-draining MLN
TAP	Transporter associated with antigen processing
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
Treg	regulatory T cell
TRIF	TIR domain-containing adaptor protein inducing interferon beta
UMAP	Uniform Manifold Approximation and Projection
WT	wild-type
XC-Motif Chemokine Receptor 1	XCR1

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Acknowledgements

First of all, I would like to thank Prof. Dr. Oliver Pabst and Prof. Dr. Martin Zenke for supervising me and this thesis.

Thank you, Oliver, for the opportunity to be a part of the institute. I really appreciated all the direct and indirect input you gave me along the way and I hold you in high regard for your vast knowledge and experience in all kinds of topics and the way you manage so many tasks and people while being a great scientist. Thank you for providing such a nice and open working environment and also for giving us students the freedom to approach our own ideas about how things could work. I think that this cannot be taken for granted and I really think it has helped me also in how I approach experiments and I am sure it has also contributed in making me a better scientist.

I would also like to thank Prof. Zenke for all the great scientific input and for taking the time to listen to my project updates over the past few years.

Next, I would like to thank Vuk Cerović, my unofficial but most direct supervisor. I still remember when you offered me the PhD position after my job interview and wrote: “Take some time to think about if you really want to do a PhD, it will be a long and certainly not always easy journey”. And today I am more than happy that I decided to do it and to do it together with you! You became not only my supervisor, but also my mentor, teacher and a friend. Thank you for all the time you invested into listening to all of my questions, for boosting my interest in immunology even more and all the hours of discussions with and without a beerchen. Thank you for all the patience you had to bring when I took a bit longer sometimes (e.g. in teaching me how one can analyze cell-intrisic and cell-extrinsic effects using competitive chimeras or going through one of the Bainer papers). Thanks for all the white board sessions and your way of explaining complex topics in a simple and understandable way. I hope I get as good as you at that someday. Of course, I want to thank you for all the emotional support when things didn’t go that well and all the times you kept me company or brought breakfast when I mis-planned experiments and had to come in on the weekend. Thank you for showing me squash, something I now wish we could have started much earlier. By the way, are you up for squash next week? I also appreciated all the chats about cooking, music, movies and also sometimes books, even though I could not add much there (and no matter how well you sold reading books as a fun hobby, you didn’t manage to make me a passionate reader; unless its science, that’s always fun to read). Also, thanks for all the time you put into reading and correcting this thesis and my germalish at supersonic speed and being available whenever I struggled. Let’s see where future takes us but who knows, maybe Andrea will find a good job and we can follow her? I would be up for another period of working in AG Cerović! :)

Next, I would like to thank you, Ana, for all the chats and discussions, the coffee breaks and the help in writing TVAs. I really appreciate all the effort you put into the journal clubs and for teaching me how to critically think about papers and the way to setup experiments the right way. But also thank you for many other things such as the trouble of picking up parcels for me in Belgium that ended up at a crossroads in the middle of nowhere!

I would like to thank Prof. Simon Milling for the several meetings we had over the past years and for all your scientific input and support. I really appreciated to have you as an external supervisor and I really enjoyed all the online as well as personal meetings we had.

Chronologically I now would like to thank you, Andrea, for helping me with basically everything when I started this PhD. I am still a bit sad/angry that you left me alone with Vuk (wasn't that bad eventually, though) and I wish our time in the institute had overlapped a bit more, but I am more than grateful we met. I learned a lot from you, especially how to organize myself, but I also copied the salad you used to eat for example. It's always been fun to work with you and it's good to know who to call if I ever need some help in calculating how many kg of chow I would need for my planned setup :)

I would also like to thank Kadda for all the coffee and lunch breaks. Thanks for cheering me up when things were not going well, for all the random chats about basically everything, all the memes and your tries to make me a bit more of a "Macher". It really felt like something was missing since you left. Fingers crossed for your viva in a few days and greetings to Blue and Nala! *Insert cat meme here*

Next, I would like to thank Britta for being the best target of Asmae's and my jokes. Thanks for making me a plasma cell expert just by sitting next to you. I hope I was helpful in all your questions you introduced with "Ich bräuchte mal etwas Schwarmintelligenz..". Thanks for correcting my official german letters where I sometimes felt I don't know how to german. It has always been fun to work with you and I am more than happy we even managed to submit our thesis on the same day.

I would also like to thank you, Sraddha, for organizing all the events after work, all your help in the lab and all the chats we had in between. Sorry for losing the best (I guess not so secret) secret santa present ever! And thank you even more for all the effort you put into crafting it again. I really enjoy working with you and I am sure you will make it big in the future!

Thank you Asmae for all your help and in teaching me "How to Histo" for beginners and experts. It's been a lot of fun to work with you and I appreciate the way you approach things, teach yourself all the bioinformatics/AI and the way you present your data and ideas. I still have to laugh when I think about that morning when you popped up behind me in the lab with a ladder shouting WWE! Keep going, you are a great scientist!

I would also like to thank you, Lydia, for all the funny chats and the time we spent together in the office, for your help on the single-cell experiments and also for teaching me a bit more how to pipette working in cell culture or with RNA. Sometimes I still hear your voice in my head saying “Lieber einmal zu vorsichtig als andersherum” and I am sure that way I worked even more “sterile” and it helped me keeping my cell cultures clean.

Thank you Hieu for all the magic and effort you put in, turning complex data into nice looking and understandable plots. Sorry for bombarding you with questions in the middle of the night asking about if it is possible to do this and that. I am still amazed by how you always understood what I wanted to do and also being able to address all my questions after I just came by with a piece of paper trying to draw and explain my data. And also, thank you for always staying till the end at Christmas parties together with Sraddha and me! Btw, are you up for squash next week? :)

I would also like to thank you, Johanna, for all you direct but also indirect input along the way. I also want to thank you for your suggestions for my conference posters that made them look nicer and more authentic! (didn’t get a poster prize, though :/)

Thank you, Nathalie and Silke, for always keeping the flow cytometers in good shape! Thank you for making my not so well planned „spontaneous“ cell sorts possible and for shutting down a machine after a long day in the lab, allowing me to take a rest half an hour earlier. Also thank you for hotfixing the Fortessa (btw at this point I would also like to thank the Fortessa for only very rarely letting me a down!; it’s been a pleasure working with you) when I panicked and thought my 8-week experiment is ruined and I couldn’t acquire the data. It’s always been fun to chat to you about “God and the world”.

Thanks to Marius, Maria and all the Alumni/Alumnae? I met along the way who contributed to the great working environment and also made me feel like a teacher sometimes (I hope I was helpful).

I would also like to thank Christina and Kristina for all your help and support during my experiments. Thanks for keeping the lab running and well equipped while best I could offer in response was making it all dirty again.

Next, I would like to thank Heike, Necla and Betül for keeping everything organized in the background and making sure everything official goes smoothly.

An dieser Stelle möchte ich mich natürlich bei meiner Familie bedanken. Danke Mama, danke Oma für all eure Unterstützung über die Jahre, dass ihr immer an mich geglaubt habt und ihr keine Sekunde an mir gezweifelt habt. Ich möchte mich auch bei denjenigen bedanken, die mich auf meinem Weg begleitet haben und die diesen Moment leider nicht mehr mit mir

zusammen erleben durften. Natürlich möchte auch meinen Geschwistern Max, Philipp und Marie danken und auf eure immer wiederkehrende Frage „Wann bistn endlich Doktor?“ vage ich es nun zu sagen „Es dauert nicht mehr allzu lange!“ :)

Zu guter Letzt möchte ich noch all meinen Freunden danken die mich auf diesem Weg begleitet haben. Besonders möchte mich bei den „Wittgensteinerhustlern“ für all die witzigen Konversationen und Erlebnisse bedanken und dafür, dass ihr dafür gesorgt habt, dass ich nach einem stressigen Labortag gut abschalten konnte. Ihr seid wie eine zweite Familie und ich wüsste nicht ob ich das ganze ohne euch so gut durchgezogen hätte.

Eidesstattliche Erklärung / Declaration of academic honesty

Ich, Fabian Tobias Hager

erkläre hiermit, dass diese Dissertation und die darin dargelegten Inhalte die eigenen sind und selbstständig, als Ergebnis der eigenen originären Forschung, generiert wurden.

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Weitere Erklärung / Further declaration

GEM generation and library construction for single-cell sequencing were performed by Dr. Lydia Kopplin. Bioinformatics analyses were performed in collaboration with Dr. Hieu Trong Nguyen.