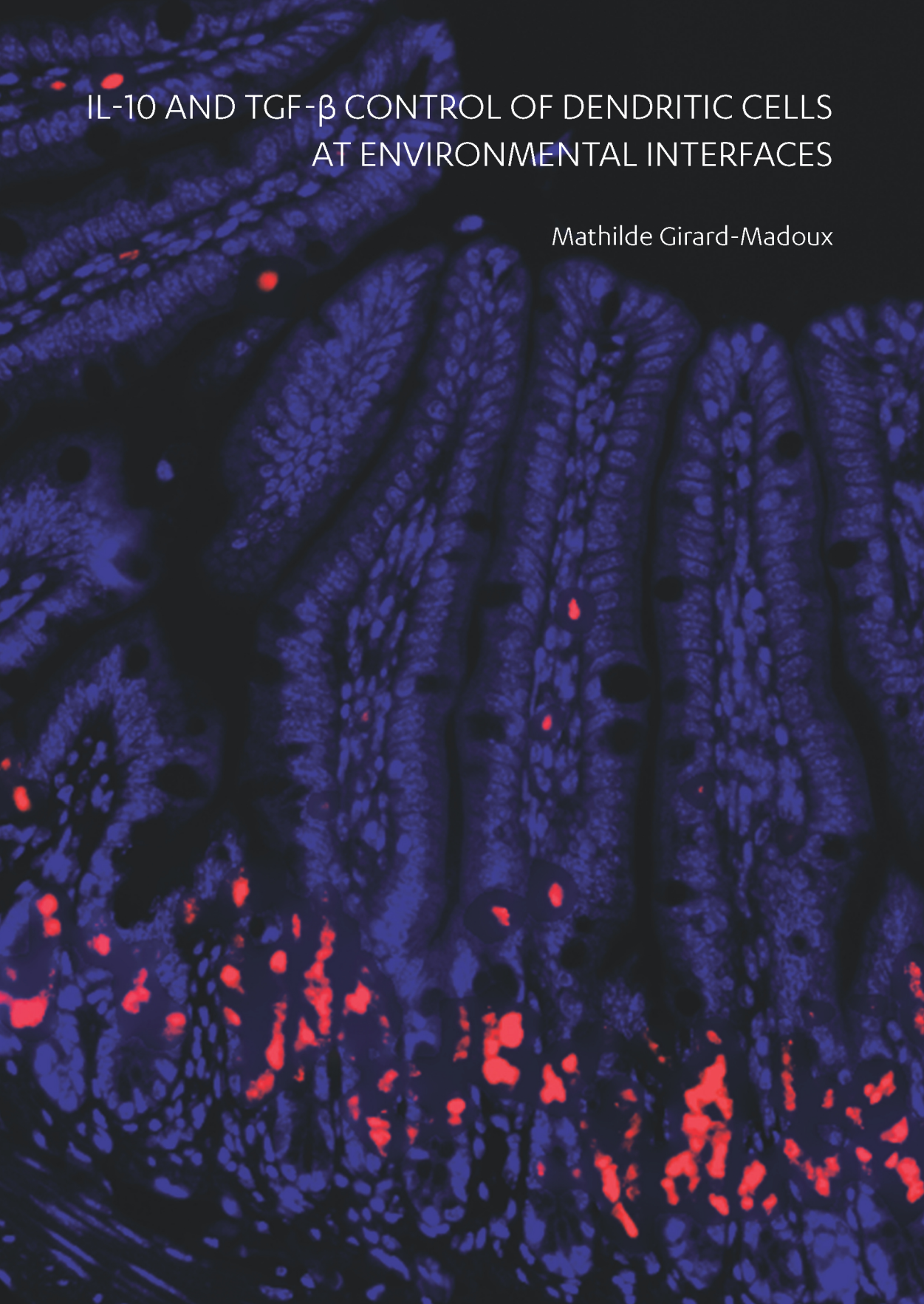
IL-10 AND TGF-β CONTROL OF DENDRITIC CELLS AT ENVIRONMENTAL INTERFACES

Mathilde Girard-Madoux



IL-10 and TGF-β Control of Dendritic Cells at Environmental Interfaces

The studies described in this thesis were mainly performed at the Department of Immunology, Erasmus MC, University Medical Center Rotterdam, the Netherlands, and partly performed at the Department of Cell Biology and Histology, Academic Medical Center of the University of Amsterdam, the Netherlands, and at the Department of Dermatology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany.

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IL-10 and TGF-β Control of Dendritic Cells at Environmental Interfaces

IL-10 en TGF-β controle van dendritische cellen op het grensvlak met de omgeving

Thesis

To obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the rector magnificus

Prof.dr. H.A.P Pols

and in accordance with the decision of the Doctorate Board
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Promoters:	Prof.dr. Jon D. Laman
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Charly Nelson, you were right!
This is for you

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CHAPTER 1

GENERAL INTRODUCTION

Every day we are exposed to a multitude of environmental agents which can reach the interior of our body through epithelial interfaces of the skin, the lungs and the gastrointestinal tract. Dendritic cells (DC) are immune cells which reside at such barriers and indiscriminately pick up antigens (Ag), irrespectively of whether these are harmful or innocuous. DC subsequently migrate to local lymph nodes (LN) and initiate immune or tolerogenic responses. DC present pernicious Ag derived from viruses or pathogenic bacteria to naïve T cells to induce Ag-specific pro-inflammatory effector T cell responses. On the other hand, DC also promote tolerance to harmless Ag such as commensal bacteria and food proteins through induction of T cell anergy and regulatory T cells (Treg). DC are therefore crucial in the balance between immunity and tolerance. Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) are potent anti-inflammatory cytokines which act on many (immune) cells including DC. Both cytokines have the potential to inhibit DC maturation in vitro. In this thesis, we dissected the role of IL-10 and TGF-β in controlling DC function in vivo, particularly at environmental borders, notably the skin, lungs and gastrointestinal tract. To this aim, we generated mice with a DC-specific IL-10 receptor or TGF-β receptor deficiency to study the lack of IL-10 or TGF-β signaling in DC in the steady state but also in several in vivo models of immune responses. In this first chapter, the origin, role and tissue-specific subsets of DC are introduced, as well as the role of IL-10 and TGF-β in vitro and on selected immune cell types in vivo.

INNATE AND ADAPTIVE IMMUNE RESPONSES

Our immune system has evolved to protect against pathogens such as viruses and bacteria but also to ensure efficient elimination of infected and damaged host cells. Moreover, both tasks are accomplished by two distinct branches of the immune system. Innate immunity comprises several components. The first lines of defense are anatomical barriers such as the multi-layered skin and the mucosal epithelium in the lung and gut. Defensins, lysozymes, the complement system and the presence of commensal microbiota prevent the invasion of and eliminate pathogens. Granulocytes, macrophages, DC, natural killer (NK) cells and mast cells form the cellular components of the innate immune system. They express pattern recognition receptors (PRR) which recognize pathogen-associated molecular patterns (PAMP). PAMP are common components shared by a family of pathogens such as lipopolysaccharides (LPS) in walls of gram^{neg} bacteria or nuclear RNA of viruses. Upon interaction with pathogens or detection of infected/damaged cells, innate immune cells respond quickly by secreting pro-inflammatory molecules such as proteases, chemokines and cytokines and by phagocytosing invading agents.[1] Innate immunity is activated very rapidly upon PRR signaling but does not promote immunological memory.

The second, adaptive arm of the immune system is Ag-specific and responds after a latent activation phase which creates a lag time between Ag exposure and the specific response. T and B cells constitute the adaptive effector cells. In particular, the T cell receptor (TCR) complex on the surface of T cells recognizes Ag broken-down into peptides bound to major histocompatibility complex (MHC) molecules which are

present at the surface of Ag-presenting cells (APC) such as DC. Each T cell is unique by expressing a distinct TCR and therefore can only respond to one specific peptide/MHC complex, which results in the induction of a highly specific response. One other feature of the adaptive immune system is its capacity to induce immunological memory. After first encounter and response against a given pathogen, a few T and B cells become memory cells which recirculate and stay quiescent. Upon second encounter with the same Ag, memory cells become activated extremely quickly, proliferate and mediate the adaptive response against the Ag. This allows faster and more efficient secondary responses upon recurring infections and provides life-long protection against individual pathogens.

DENDRITIC CELLS

Specialized APC linking innate and adaptive immune responses

In 1973, Steinman and Cohn identified a novel cell type which they called dendritic cells (DC) due to their "extended cytoplasmic processes".[2] DC were localized mainly in the spleen and presented several unique morphological and functional characteristics, notably their high efficiency in presenting Ag to T cells in a mixed leucocyte reaction.[3] Since then DC have been studied extensively and have emerged as the crucial link between innate and adaptive immunity. DC are now recognized as specialized APC. DC are present at all environmental interfaces such as the skin, the airways and gut mucosa where they form a dense network by extending their dendrites to capture Ag.[4] Besides these tissue-resident DC, subsets of DC are also found in the spleen, LN and thymus.[4] These lymphoid organ-resident DC do not migrate but present peptides locally to T cells. On the other hand, tissue-resident DC are the classical migratory DC, characterized by three main functions.[5,6] (1) As innate immune cells present at epithelial barriers, immature DC are very efficient in Ag capture. To this aim they use several mechanisms such as phagocytosis, micropinocytosis and receptor-mediated endocytosis.[6] (2) DC are equipped with highly efficient machineries to process Ag and generate peptides embedded in MHC molecules, forming a peptide/MHC complex at the cell surface.[6] (3) DC migrate from peripheral tissues to draining LN where, as specialized APC, they present their peptide/MHC complexes to naïve T cells, thereby linking innate and adaptive responses.[6] Recognition of PAMP via their PRR (such as Toll-like receptors) and/or cytokine activation initiates DC migration but also their maturation, characterized by phenotypic and functional changes such as upregulation of MHC-II and costimulatory molecules and secretion of cytokines.[6] These characteristics of immature versus mature DC are summarized in Table 1. By the time DC reach the LN, they are fully competent to induce T cell responses and are no longer Ag-capturing cells but specialized APC.

DC development

DC develop from hematopoietic stem cells (HSC) present in the bone marrow (BM) (Figure 1).[7-9] There, HSC differentiate into common lymphoid precursors (CLP) and common myeloid precursors (CMP). CMP give rise to macrophage-DC progenitors (MDP),

Table 1. Main characteristics of immature and mature DC.

	Immature DC	Mature DC
Main function	Antigen capture	Antigen presentation
Antigen uptake	High phagocytic and endocytic receptors	Low phagocytic and endocytic receptors
Morphology	Expression of adhesion molecules, low motility	Loss of adhesion molecules, cytoskeleton remodeling, high motility
Lysosomal compartment	High CD68	Low CD68 High DC-LAMP
MHC-II expression	High intracellular MHC-II	High surface MHC-II
Tissue-tropic chemokine receptors	High CCR1, CCR5, CCR6	Low CCR1, CCR5, CCR6
LN-tropic chemokine receptor	Low CCR7	High CCR7
Co-stimulatory molecules	Low CD80, CD86, CD40, PD-L1, PD-L2	High, CD80, CD86, CD40, PD-L1, PD-L2
Cytokine production	Low	High production of polarizing cytokines such as IL-12p70, IL-4, IL-6, IL-10 and pro-inflammatory molecules like TNF-α and IL-6

which subsequently differentiate into common DC progenitor cells (CDP), monocytes and some types of macrophages. CDP diverge into plasmacytoid DC (pDC) and a precursor 'classical' DC population (pre-DC). Pre-DC and monocytes migrate in the blood to reach lymphoid organs and peripheral tissues where they undergo differentiation, proliferation and reside as lymphoid organ-resident DC or tissue-resident DC, respectively.[7-9] In contrast to CMP, CLP contribute little to DC and this pathway remains unclear. Moreover, circulating monocytes can differentiate into DC mostly during inflammation but also in the steady state, for example giving rise to CX_3CR1^+ cells in the lamina propria (LP).[7-9] Notably, epidermal Langerhans cells (LC) are not derived from HSC but differentiate from an embryonic precursor that colonizes the epidermis before birth.[10] Therefore, LC are not dependent on BM for their renewal in the steady state. Instead, LC self-renew in situ from proliferative units composed of dividing LC and their terminally differentiated daughter cells.[11] Additionally, LC can undergo massive proliferation under inflammatory conditions.[10] After UV radiation, the LC network is replenished by a first wave of short-lived LC which are derived from $Gr1^{hi}$ monocytic precursors followed by a second wave of long-lived 'classically-derived' LC.[12]

DC and mononuclear phagocytes subsets at epithelial borders

The DC compartment is very heterogeneous with DC present in various organs and expressing particular surface marker profiles. It is believed that each DC subset has a specialized function although plasticity between DC populations exists under certain conditions.[13]

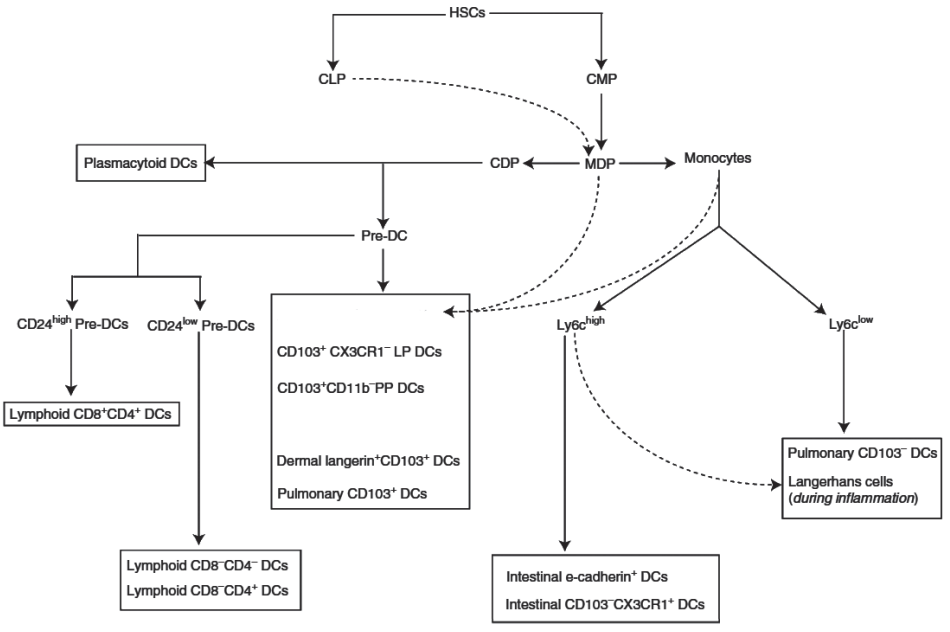

Figure 1. Differentiation of DC from hematopoietic stem cells (HSC). HSC differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP); CMP subsequently differentiate into monocytes and pre-DC in the bone marrow (BM). Then, monocytes and pre-DC enter the blood and migrate to lymphoid organs and peripheral tissues, where they give rise to lymphoid organ-resident DC and tissue-resident DC (only selected subsets of skin, lung and gastrointestinal tract DC are depicted here). In addition to CMP, CLP also have the potential to give rise to DC, but their contribution is not well understood. Adapted from Kushwah and Hu.[8]

In the skin, several subsets of DC have been identified (Table 2). The epidermis harbors Langerhans cells (LC) which express Langerin (CD207) and represent the classical type of migratory DC.[14] LC have numerous dendrites sampling their environment, continuously picking up Ag and migrate towards the draining LN where they present Ag to naïve T cells. LC display both tolerogenic and immunogenic functions depending on the conditions.[15] In addition to trans-migrating LC, the dermis contains several distinct subsets of DC: Langerin^{neg}CD11b^{+} cells constitute the major dermal DC population, whereas Langerin^{neg}CD11b^{neg}, Langerin^{+}CD103^{+}, Langerin^{+}CD103^{neg} DC are present in lower numbers.[14,16] All subsets of dermal DC are able to migrate to skin-draining LN (sdLN) and initiate immune responses but particular functions are performed by distinct subsets. For example, Langerin^{+}CD103^{+} dermal DC are very efficient at cross-presenting self-peptides,[17] Langerin^{neg}CD11b^{+} cells produce retinoic acid (RA) which is capable of inducing Treg,[18] and all dermal DC populations are able to present viral Ag to naïve T cells.[16]

Table 2. Skin DC subsets in steady state.

Subset	Phenotype	Major function
Langerhans cells	MHC-II^{+} Langerin^{+} CD103^{neg} EpCam^{+}	Sample the environment and pick up antigens Migrate to sdLN Present viral antigens to CD4^{+} cells
Langerin^{+} CD103^{+} dDC	MHC-II^{+} CD11c^{+} CD11b$^{neg/low}$ Epcam^{neg}	Sample the environment and pick up antigens Migrate to sdLN Prime CD4^{+} T cells Cross-present self-derived or viral antigens to CD8^{+} T cells
Langerin^{+} CD103^{neg} dDC	MHC-II^{+} CD11c^{+} CD11b$^{neg/low}$ Epcam^{neg}	Sample the environment and pick up antigens Migrate to sdLN Function not well characterized
CD11b^{+} dDC	MHC-II^{+} Langerin^{neg} CD103^{neg} Epcam?	Sample the environment and pick up antigens Migrate to sdLN Prime CD4^{+} T cells Can express RALDH and induce Treg
CD11b^{neg} dDC	MHC-II^{+} Langerin^{neg} CD103^{neg} Epcam?	Sample the environment and pick up antigens Migrate to sdLN Function not well characterized

According to Romani et al.[14], Guilliams et al.[16] and Henri et al.[17]

In the lung, three major populations of DC are present in the steady state (Table 3).[19] The DC compartment can be grossly divided into two subsets: conventional DC (cDC) expressing high levels of CD11c and pDC which express Ly6C, B220, Siglec-H and low levels of CD11c. pDC are essential in the maintenance of tolerance through the induction of Treg development in response to inhaled Ag. pDC most likely perform their task through IFN-α secretion which inhibits Th2 development.[20] cDC can be further divided into CD103^{+} and CD11b^{+} DC populations. CD103^{+} DC, also known as intraepithelial DC, express Langerin and project their dendrites through the epithelial cell layer to sample the airway lumen. Their main function is therefore to survey the luminal surface, pick up Ag and migrate to the draining LN where they transfer Ag to lymphoid organ-resident DC.[20,21] CD103^{+} DC are particularly efficient at cross-presenting Ag to CD8^{+} T cells for example during influenza viral infection.[21] CD11b^{+} DC reside in the LP and express SIRPα, involved in DC migration. CD11b^{+} DC are a major source of pro-inflammatory chemokines and cytokines such as CCL17, CCL22 and TNF-α which attract Th2 and CD8^{+} T cells to the lung and contribute to the development of asthma.[20] The alveolar space also contains MHC-II^{+}CD11c^{+}CD103^{+} DC and highly autofluorescent macrophages which express CD11c but lack CD11b.[20] Alveolar macrophages are phagocytic cells also called 'dust cells' which remove airway debris and necrotic and dead cells from the airways.[19]

Table 3. DC and macrophage subsets in the healthy lung.

Subset	Phenotype	Function
Intraepithelial CD103$^+$ DC	MHC-II^{hi} CD11c^{hi} CD11b^{neg} Langerin$^+$	Extend dendrites into the airway lumen and pick up antigens Migrate to mediastinal lymph node but poorly induce CD4$^+$ T cells Efficient in cross-presenting Ag to CD8$^+$ T cells
Lamina propria CD11b$^+$ DC	MHC-II^{hi} CD11c^{hi} CD103^{neg} SIRPα$^+$ CX3CR1$^+$	Secrete pro-inflammatory chemokines and cytokines which recruit T cells to the lung during allergen challenge Efficient at priming naïve CD4$^+$ T cells and reactivating effector T cells
Plasmacytoid DC	MHC-II$^+$ CD11c^{dim} CD11b$^+$ SiglecH$^+$ Ly6c$^+$	Induce tolerance to inhaled antigens Promote Treg development Secrete IFN-α upon viral infection
Alveolar DC	MHC-II^{hi} CD11c^{hi} CD103$^+$ CD11b$^{neg/+}$	Actively scan the alveolar airspace and pick up antigens Function not clearly defined : migrate slowly but also accumulate in the airway-adjacent region and could boost effector responses
Alveolar macrophage (dust cell)	CD11c^{hi} F4/80$^+$ CD11b^{neg} autofluorescent	Phagocytosis of airway particles, necrotic and dead cells

Adapted from Gill.[20]

The gastrointestinal tract contains four major subsets of DC/mononuclear phagocytes (Table 4).[22] CD11c$^+$CX$_3$CR1$^+$ mononuclear phagocytes and CD11c$^+$CD11b$^+$CD103$^+$ DC compose the DC compartment in the LP. CX$_3$CR1$^+$ cells are specialized in picking up Ag from the gut lumen by extending their dendrites in between epithelial cells.[23] They do not migrate and are poor inducers of T cell responses.[24] Conversely, CD11b$^+$CD103$^+$ DC migrate from the LP to the gut draining LN and are potent inducers of Treg and oral tolerance to food Ag.[25] They also are crucial in imprinting T cells with gut-tropic markers[26] and can play a pro-inflammatory role by inducing Th17 responses.[27] CD11c$^+$CD11b^{neg}CD103$^+$ DC are present in the Peyer's patches and isolated lymphoid follicles and have characteristics of lymphoid organ-resident DC.[28] Finally, CD11c$^{neg/low}$F4/80$^+$ phagocytes reside in the LP and spontaneously secrete IL-10 which mediates Foxp3$^+$ T cell differentiation.[29]

Tailoring T cell responses

Depending on the microenvironment and the type of Ag encountered in the periphery, DC will induce different type of responses, best fitted to the individual pathogenic/innocuous Ag. Three signals are required for the differentiation of naïve T cells into activated effector cells[30,31] (Figure 2). First, the MHC/peptide complex binds to the TCR complex on the surface of T cells. On one hand, intracellular Ag are presented by MHC class I (MHC-I)

Table 4. Intestinal lamina propria DC and mononuclear phagocyte subsets in the steady state.

Subset	Phenotype	Function in the SI	Functions in the colon
CX_3CR1^+ mononuclear phagocytes	$CD11c^+$ $CD103^{neg}$ $MHC\text{-}II^{hi}$ $F4/80^+$ $CD11b^+$	Sample lumen with transepithelial dendrites Do not migrate to mLN Poorly stimulate T cells Regulate T cell-independent IgA class-switching Restrict commensals translocation	Maintains $Foxp3^+$ T cell pool Induce Th17 cell upon activation by commensal-derived ATP Promote TNF-dependent colitis Inhibit Th17 cells during DSS-colitis
$CD103^+CD11b^+$ DC	$CD11c^{hi}$ $CX3CR1^{neg}$ $MHC\text{-}II^{hi}$ $F4/80^{neg}$	Carry antigens to mLN in a CCR7-dependent manner Promote generation of Treg Induce tolerance to orally administered antigens Imprint gut-tropism to T cells Induce activation of Th17 cells and IL-22 secretion by ILC upon flagellin administration Promote T cell-independent IgA class-switching	Not described
CD103+$CD11b^{neg}$ DC	$CD11c^{hi}$ $CX3CR1^{neg}$ $MHC\text{-}II^{hi}$ $F4/80^{neg}$ $CD8^+$	Show phenotypic and ontogenic characteristics of lymphoid organ 'classic' DC Most prevalent in the peyer's patches and isolated lymphoid follicles	Show phenotypic and ontogenic characteristics of lymphoid organ 'classic' DC
$CD11b^+$ $F4/80^+$ macrophages	$CD11c^{neg/low}$ $CX3CR1^{neg/+}$ $MHC\text{-}II^+$ $CD103^{neg}$	Spontaneously secrete IL-10 and promote differentiation of $Foxp3^+$ Treg	Phagocytose and eradicate bacteria but are refractory to TLR stimulation Promote colonic wound healing

ILC, innate lymphoid cells. Adapted from Varol et al.[22]

to $CD8^+$ T cells. On the other hand, peptides from extracellular Ag are embedded in MHC class II (MHC-II) and recognized by specific $CD4^+$ T cells. Only APC are able to process and present extracellular Ag in the context of MHC-II. The second signal consists of the interaction between costimulatory molecules on DC and their ligands on T cells. For example, positive costimulatory molecules CD80 and CD86 on DC interact with CD28 on T cells to induce differentiation of effector T cells. These activated effector T cells then start to express CTLA-4 which limits their excessive activation and inflammation-associated tissue damage, as demonstrated by the severe lymphoproliferative disorder in CTLA-4-deficient mice that die at age of 2 weeks of multi-organ lymphocytic infiltrations.[32] Similarly, binding of negative costimulatory PD-L1 or PD-L2 on DC with PD-1 on the surface of T cells inhibits their activation and proliferation.[33] The levels of MHC-II and the fine balance between positive and negative co-stimulatory signals on DC

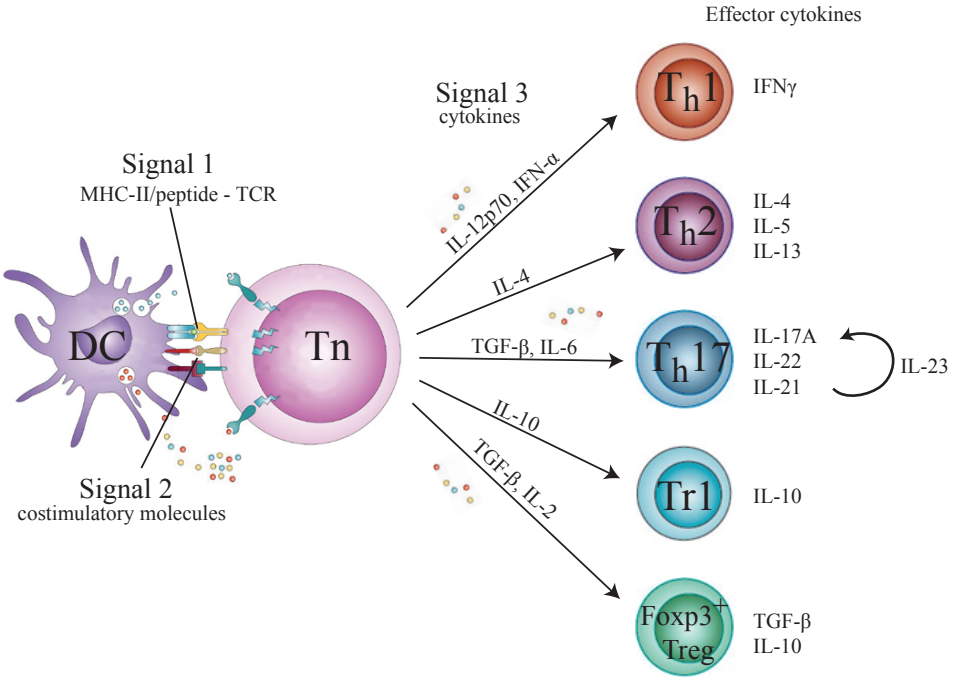

Figure 2. Priming of T helper cells by DC. The differentiation of naïve CD4$^+$ T cells into effector T helper (Th) cells is orchestrated by DC and driven by three signals. The Ag-specific signal 1 consists of the recognition of peptides embedded in major histocompatibility class II (MHC-II) at the surface of DC by the matching T cell receptor (TCR) on T cells. Signal 2 corresponds to costimulatory signals. The interaction between CD86 on DCs and CD28 on the surface of T cells is an example of positive costimulation. Binding of PD-L1 present on DCs and PD-1 on T cells acts as a negative costimulatory signal inhibiting T cell proliferation. The polarizing signal 3 is mediated through the production of distinct cytokine profiles by DC dependent on the pathogen type and local microenvironment, which promote skewing of CD4$^+$ T cell differentiation towards a specific Th lineage, characterized by their effector cytokine production.

modulate the strength of the immune response.[34] The third signal consists of cytokines secreted by DC in order to skew naïve CD4$^+$ T cells towards the most appropriate effector T helper (Th) type. Depending on the type of Ag encountered and signals from the environment, DC will secrete different cytokines (Figure 2). IL-12p70 production by DC will induce the differentiation of Th type-1 (Th1) cells, which are characterized by their IFN-γ production. Th1 cells are particularly effective against intracellular pathogens such as viruses and certain types of bacteria. Their main function is to regulate the activation of CD8$^+$ T cells and macrophages.[35] DC can also secrete IL-4 which promote the differentiation of naïve T cells into Th2 cells. Th2 cells secrete IL-4, IL-5 and IL-13 and are critical for immunity against extracellular pathogens such as helminthes. Th2 cells control the recruitment and activation of eosinophils and stimulate the production

of antibodies by B cells.[35] DC producing IL-6 in combination with TGF-β induce Th17 cells, which are characterized by their production of IL-17, IL-22 and IL-21. Th17 cells are crucial in responses against microbes such as bacteria and fungi at epithelial borders. These cells recruit neutrophils and play a major role in barrier function and repair.[36] Besides their role in inducing pro-inflammatory Th cell responses, DC can also induce regulatory T cells (Treg).[37] Unlike Th1, Th2 and Th17 cells, Treg have an inhibitory role and actively suppress Th cell functions. DC induce the differentiation of $Foxp3^+$ Treg by secreting TGF-β and of regulatory type 1 T cells (Tr1) through IL-10 production.[38] Other factors such as retinoic acid are important in combination with TGF-β to induce Treg at mucosal interfaces.[25] $Foxp3^+$ Treg secrete TGF-β and/or IL-10 and can also exert their regulating function by cell-to-cell contact.[39] Tr1 cells produce IL-10 and are particularly important at mucosal interfaces like the gut.[40]

Only after receiving these three signals at appropriate levels, activated T cells start proliferating through clonal expansion and polarized cells migrate to the site of infection/inflammation to exert their effector or regulatory role.

Balance between tolerance and immunity

To maintain immune homeostasis, a tight balance between tolerance and immunity is fundamental. Tolerance must be induced towards non-pathogenic Ag such as allergens and commensals in the gut as well as self-Ag. DC are crucial in initiating and maintaining tolerance, as mice devoid of DC develop spontaneous fatal autoimmune disease.[41] Central self-tolerance is generated in the thymus and assures that autoreactive T cells bearing high affinity TCR for self-Ag are deleted by negative selection.[42] Thymic DC, by presenting self-Ag, play an essential role in controlling negative selection.[43] In the thymus, DC also induce the differentiation of naturally occurring $Foxp3^+$ Treg.[38,43] However, a few self-reactive T cells escape this tight regulation and are found in the circulation even in healthy individuals and can cause autoimmunity.[44] Moreover, the body is continuously exposed to non-pathogenic Ag present in the environment. Thus, mechanisms of tolerance are also operational in the periphery.[45] DC contribute to peripheral tolerance through several mechanisms, such as deletion of auto-reactive T cells, induction of T cell anergy or the differentiation of inducible TGF-β and/or IL-10 producing Treg.[43,46] Naturally occurring Treg instructed in the thymus as well as inducible Treg generated in the periphery are able to inhibit pathogenic $CD4^+$ and $CD8^+$ T cells by cytokine secretion or cell-to-cell contact.[39] Mice devoid of $Foxp3^+$ Treg develop a severe systemic autoimmune phenotype.[47] Accordingly, humans suffering from a deficiency in Treg development (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, or IPEX syndrome) develop multi-organ autoimmune pathologies such as enteropathy, dermatitis and endocrinopathy.[48]

In addition to tolerance to non-harmful Ag, immune responses need to be mounted against pathogens and infected host cells. These are characterized by the induction of effector $CD8^+$ or $CD4^+$ T cells, and each pathogenic Ag induces a selective type of response

(Th1, Th2 and/or Th17). The overactivation and/or inappropriate activation of T cells can be harmful to the host. Th1 and Th17 cells are responsible for tissue damage during chronic inflammation and promote autoimmune diseases such as inflammatory bowel disease (IBD), multiple sclerosis, arthritis and psoriasis.[35,49,50] Th2 cells can be deleterious during the initiation and persistence of asthma and other allergic diseases.[35] Therefore, a controlled balance between tolerance promoted in part by Treg and immunity ensured by effector T cells is necessary to maintain immune homeostasis. A dysregulation of this balance induces chronic inflammatory disease and autoimmunity.[35] DC, by their unique capacity to induce both Treg and effector T cells are essential to equilibrate immunity and tolerance. DC therefore play a central role in autoimmune diseases[51] such as IBD and celiac disease[52], autoimmune pancreatitis[53] and rheumatoid arthritis[54].

The type of Ag is crucial in the conditioning of DC. In addition, the cytokine and chemokine environment appears to be decisive in promoting either tolerogenic or pro-inflammatory DC.[34] Particularly at epithelial interfaces, the constantly changing microenvironment will modulate DC function and promote immune reponses,[55] such as in the gut,[56-58] lung[59] and skin.[60] IL-10 and TGF-β are two anti-inflammatory cytokines that are present at environmental barriers, which are essential in the balance between tolerance and immunity and are able to control DC function.

INTERLEUKIN-10

Receptor and signaling

IL-10 has a broad anti-inflammatory role and is crucial to maintain tolerance. IL-10 signals via the IL-10 receptor, which is a member of the interferon receptor family and is composed of two chains (Figure 3). The β chain is the signal transducing subunit and is present constitutively and ubiquitously on cell surfaces.[61-63] This chain is not IL-10 specific but shared by other cytokines such as IL-22.[64] The α chain however exclusively binds IL-10 and is expressed only on hematopoietic cells, including T cells and DC.[65-67] Since IL-10 mainly acts to downregulate ongoing immune responses, the expression of the α chain is often upregulated on activated cells.[68,69] In order for IL-10 to transduce its signal to the nucleus and thus mediate its biological effect, two α and two β chains must combine to form a tetramer.[70] In target cells, this association activates the kinases Janus tyrosine kinase-1 (JAK1) paired with the α chain and tyrosine kinase-2 (Tyk2) coupled with the β chain,[71] mediating the phosphorylation of the intracellular domain of the α chain. These phosphorylated residues are then recognized by the transcription factor STAT3 which is in turn phosphorylated and can form homodimers that translocate into the nucleus and bind with high affinity to promoters of IL-10 target genes.[72,73]

IL-10 sources

IL-10 was first described as a Th2-derived cytokine able to inhibit Th1 cell activation and cytokine production[74]. Later it became evident that IL-10 can be secreted by a plethora of cells, including $CD4^+$ T cells,[75] $CD8^+$ T cells,[76] B cells,[77] activated monocytes,[78,79]

macrophages,[80] DC,[79,81-83] mast cells[84] but also by non-immune cells such as keratinocytes in the skin[85] and epithelial cells in the lungs.[86] In general, activation of cells will trigger their IL-10 production.[87] For example, TCR activation will induce IL-10 production by several subsets of T cells.[87] Overall, regulatory Tr1 cells appear to be the prominent source of IL-10 in the periphery.[40,88] Interestingly, in the gastrointestinal tract Foxp3^{+} Treg emerge as main IL-10 producers in the colon whereas Tr1 cell-derived IL-10 predominates in the small intestine (SI).[89] Furthermore, in a tolerance model consisting of repeated injections

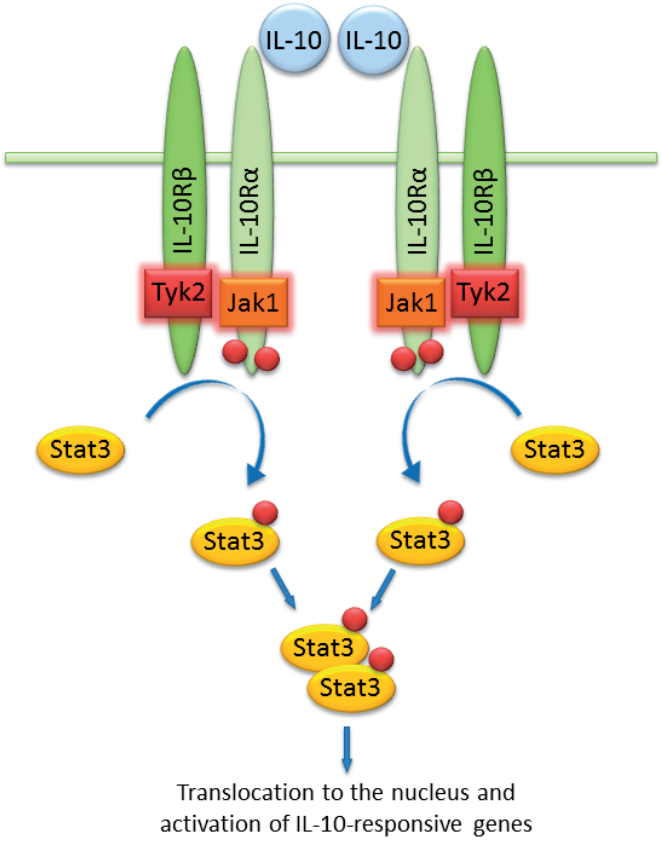

Figure 3. IL-10R signaling. Functional IL-10 Receptor (IL-10R) complexes are tetramers composed of two ligand-binding subunits (IL-10Rα) and two accessory signaling subunits (IL-10Rβ). Binding of IL-10 to the extracellular domain of IL-10Rα activates phosphorylation of janus kinase-1 (JAK1) and tyrosine kinase-2 (Tyk2) which are constitutively associated with IL-10Rα and IL-10Rβ respectively. These kinases subsequently phosphorylate the intracellular domain of the IL-10Rα chain. Phosphorylated residues then serve as temporary docking sites for the transcription factor signal transducer and activator of transcription-3 (STAT3). STAT3 binds to these sites and is phosphorylated by the receptor associated JAK1. Phosphorylated STAT3 then homodimerizes and translocates into the nucleus where it binds with high affinity to various IL-10-responsive gene promoters.

of anti-CD3 agonist antibody, the main source of IL-10 were intraepithelial lymphocytes and Peyer's patch T cells in the SI.[79] IL-10 production by DC and macrophages is triggered by their activation through specific PRR and particularly the strength of intracellular TLR signaling will determine the amount of IL-10 produced by these cells.[87] IL-10 secretion by DC is important to generate tolerance in vivo. IL-10-producing DC induce Tr1 cells and Ag-specific tolerance. In IL-10 transgenic mice this population of $CD11c^{low}$ DC is increased and expresses low levels of MHC-II, CD80 and CD86 on their surface even after LPS stimulation.[90] Pulmonary DC which produce IL-10 are necessary to induce allergen-tolerance and prevent asthma.[81] Finally, genetically engineered IL-10-producing DC induce allergen-specific Treg and tolerance in a model of asthma.[91]

IL-10 control of DC in vitro

IL-10 is not only produced by many cell types, but also plays an essential role in regulating several cell functions. IL-10's major task is to limit excessive inflammatory reactions.[92-94] The first described role of IL-10 was the inhibition of cytokine production by T cells and NK cells but this appeared to be an indirect effect via monocytes/macrophages.[95-97] Early in vitro studies on APC revealed that IL-10 inhibits the expression of MHC-II and co-stimulatory molecules on the surface of macrophages, monocytes and DC[95,98-100] and their production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α.[78,100-102] In contrast, addition of IL-10 neutralizing antibody to LPS-maturing DC enhances surface expression of MHC-II and co-stimulatory molecules and augments their secretion of IL-12 and TNF-α.[103] IL-10 treated DC induce Ag-specific $CD4^+$ and $CD8^+$ T cell anergy in vitro.[99,104,105] DC cultured in the presence of IL-10 inhibit T cell proliferation/activation during both priming and reactivation after delayed type hypersensitivity (DTH) challenge.[106] In a mouse model of multiple sclerosis, IL-10-treated DC secreted lower levels of IL-12, failed to induce T cell proliferation and limited central nervous system autoimmune disease in vivo.[107] In asthma models, IL-10-differentiated DC induce effective and prolonged allergen-specific tolerance through upregulation of Treg activities both in prophylactic and therapeutic settings.[108-110] On the other hand, in a melanoma tumor model, incubation of DC with IL-10 rendered both naïve and primed $CD8^+$ T cells unresponsive and unable to mount an anti-tumor immune response.[111]

Of note, besides its prominent anti-inflammatory role on APC and T cells, there are occasional reports that IL-10 can also have a stimulatory role on cells. For example, IL-10 induces the proliferation and differentiation of B cells into antibody-producing plasma cells[112] and promotes the proliferation and lytic activity of $CD8^+$ T cells.[93]

IL-10 as a downregulator of immune responses in vivo

In vivo, the crucial immuno-suppressive role of IL-10 is demonstrated in IL-10 deficient ($IL\text{-}10^{-/-}$) mice, which fail to control inflammation.[113] IL-10 deficient mice exhibit normal maturation and differentiation of T and B cells but spontaneously develop severe enteropathy affecting both the small and large intestine. The mice do not succumb to autoimmunity but mount an aberrant Th1 response to common non-pathogenic gut

microbiota. In fact, enhanced levels of IFN-γ, TNF-α, IL-6, IL-1 and nitric oxide were detected in colon explant cultures of diseased IL-10$^{-/-}$ animals.[114,115] Treatment of IL10$^{-/-}$ mice with anti-IL-12 or anti-IFN-γ antibodies prevented young mice from acquiring colitis confirming that the pathology is Th1-driven.[114,116] The inflammation observed in IL-10$^{-/-}$ mice is strictly enteric-Ag driven since IL-10$^{-/-}$ mice do not develop disease in germ-free conditions.[117] IL-10$^{-/-}$ mice initiate enhanced innate and adaptive responses in the skin[118] and succumb to LPS-induced endotoxic shock.[119] Further, IL-10$^{-/-}$ animals are unable to promote proper Th2 immunity and induce a Th1 response against nematodes[113] and fail to control lung inflammation after endobronchial infection.[120] Overall, IL-10$^{-/-}$ mice mount increased Th1 responses, also to non-harmful Ag.

Accordingly, IL-10Rα$^{-/-}$ mice demonstrate an increased susceptibility to dextran sodium sulfate (DSS) induced colitis and fail to initiate a proper Th2 response against the worm *Trichuris muris*, which results in enhanced intestinal inflammation, comparable to IL-10$^{-/-}$ animals.[121] Similarly, IL-10Rβ$^{-/-}$ mice develop splenomegaly and enterocolitis although the phenotype of these mice might not be solely IL-10 mediated since the IL-10Rβ chain is shared with other cytokines including IL-22.[62,64]

In contrast, mice harboring an MHC-II-driven overexpression of IL-10 show a loss of DC/macrophage function associated with lower IL-12p40 and TNF-α production and are unable to mount efficient Th1 or Th2 responses. These mice therefore demonstrate an increased susceptibility to bacterial and parasitic infections, which can result in death.[122-124]

Cell type-specific IL-10 and IL-10R-deficient animals

The Cre/*loxP* system[125] allowed for cell type specific IL-10 or IL-10R deletion, which provided tools to investigate the particular sources and targets of IL-10 in specific disease models. These studies highlight the fact that IL-10 secretion by several cell types, especially by T cells, is important at epithelial interfaces. In the skin, mast cell-specific IL-10$^{-/-}$ animals revealed the unexpected role of mast cells during a contact hypersensitivity reaction (CHS) and UVB irradiation. Mast cell-derived IL-10 is critical to prevent leukocyte infiltration, inflammation and resulting tissue damage during CHS.[84] LC-derived IL-10 is necessary to control the priming of naïve T cells during CHS, as LC-specific IL10$^{-/-}$ mice develop increased ear swelling.[83] T cell- and Foxp3^{+} Treg-derived IL-10 is also crucial during CHS as both conditional IL-10$^{-/-}$ mice show enhanced inflammation in the ears.[126,127] At the gut mucosal interface, T cell-derived IL-10 is crucial to maintain homeostasis since T cell- and Foxp3^{+} Treg-specific IL10$^{-/-}$ mice (Foxp3-IL10$^{-/-}$) develop enterocolitis similar to IL10$^{-/-}$ animals.[126,127] In the lung, Foxp3-IL10$^{-/-}$ mice develop increased OVA-induced lung inflammation.[127] These studies emphasize the essential role of Treg-derived IL-10 at environmental barriers since Foxp3-IL10$^{-/-}$ animals develop enhanced inflammation in the skin, lungs and colon.[127]

On the other hand, cell type-specific IL-10R knock-out mice identified the targets of IL-10 in vivo. During LPS induced septic shock, IL-10 signaling in monocytes/macrophages and/or neutrophils is critical to downregulate the immune response whereas

IL-10 signaling in T or B cells is not required.[121] However, during *Trichuris muris* infection, IL-10 signaling in either monocyte/macrophages or T cells dispensable for the expulsion of the nematode.[121]

T cells are a major target of IL-10 in vivo, especially in the gastrointestinal tract. A dominant negative IL-10R expressed specifically on T cells demonstrated that memory/effector T cells require to be controlled by IL-10 to avoid IL-22 mediated colitis.[128] Colitis transfer models showed that Th17 cells themselves express the IL-10R and need to be controlled directly by Treg in an IL-10-dependent manner to be maintained at homeostatic levels.[129] Foxp3 expression is dependent on IL-10 signaling in Treg and necessary to maintain their suppressive function and prevent inflammation in the colon.[130] When IL-10 signaling is disrupted in Foxp3$^+$ Treg, they fail to control Th17 cells which selectively proliferate and induce colonic inflammation.[131] Also, upon bacterial infection CD8$^+$ T cells transiently upregulate the expression of the IL-10R and are directly regulated by IL-10 which modulates the memory T cell response.[68] In conclusion, IL-10 balances T cell homeostasis by promoting and maintaining Treg and inhibiting effector T cell function.

IL-10 not only acts in a paracrine manner but cells such as T cells,[75] DC[103,132] and macrophages[124] use IL-10 also in an autocrine mode to control their own state of activation and downregulate their function.

Finally, some viruses[133,134] and tumor cells[135,136] secrete IL-10 in order to maintain immune cells in a non-responsive state and thereby escape the immune system. Other pathogens induce IL-10 production by host cells in order to inhibit Ag-specific responses and rather induce tolerance.[92] Persistence of *Leishmania major* parasites in healed hosts correlates with the presence of IL-10 at the lesion site.[137,138] IL-10$^{-/-}$ animals, through elevated secretion of IFN-γ, induce a potent anti-*leishmania* response and completely clear the parasites,[137] which results in the loss of T cell memory. Therefore maintenance of residual parasites sustains Ag-specific memory T cells conferring immunity against reinfection. However T cell memory comes with the risk of severe disease reactivation at times of immune suppression.

IL-10 and IL-10 signaling deficiencies in humans

In humans, defective IL-10 production or IL-10 signaling result in development of immune responses against harmless Ag at epithelial interfaces. Patients suffer from gastrointestinal diseases, airway hypersensibility and skin pathologies.[139] A study on celiac disease patients revealed that polymorphisms in the IL-10 gene resulting in lower IL-10 production are strongly correlated to severity of disease and age of onset.[140] Moreover, a defect in IL-10 production by DC induces a severe pathology in patients with Crohn's disease.[141] A subset of patients with early-onset inflammatory bowel disease (IBD) harbored mutations in the IL-10Rα or IL-10Rβ which abrogated IL-10 signaling. Peripheral blood mononuclear cells from these patients secreted elevated levels of pro-inflammatory cytokines inducing aberrant inflammation in the intestine.[142] Recent studies identified patients with loss of function mutations in the IL-10R (α or β chain) or IL-10 genes who all suffered from very early-onset IBD associated with folliculitis and/or

arthritis in some patients.[143-145] Restoration of IL-10 signaling by allogenic hematopoietic stem cell transplantation induced clinical remission.[144-146]

In conclusion, these studies in mice and humans demonstrate that IL-10 is important in downregulating immune responses rather than preventing systemic autoimmunity. T cells and in particular Treg are the predominant sources and targets of IL-10 but studies in vitro demonstrate that IL-10 modulates DC function. Some of the goals of this thesis was to elucidate the role of IL-10 signaling in DC in vivo.

TRANSFORMING GROWTH FACTOR-β

TGF-β signaling

TGF-β, similarly to IL-10, is a pleiotropic cytokine with multiple functions in several cell types. TGF-β has diverse roles in cell differentiation, proliferation, apoptosis, as well as during development and embryogenesis. In mammals, there are three isoforms: TGF-β1, TGF-β2, TGF-β3 which share similar functions but are differentially expressed in tissues and during development.[147] TGF-β1 (referred to as TGF-β) is the major isoform expressed in the immune system. It is secreted in an inactive form, as a dimer associated with latency-associated protein (LAP) and can also be bound to latent TGF-β binding protein (LTBP) which induces its deposition in the extracellular matrix. TGF-β activating protein induces LAP degradation or change of conformation which releases the TGF-β dimer which becomes active.[148] Active TGF-β can then bind to its receptor (Figure 4), a tetrameric complex composed of two TGF-βR1 chains and two TGF-βR2 chains.[149,150] The TGF-βR1 chain (also known as Alk5) is not required for binding of TGF-β but is necessary for intracellular signaling. Both chains are serine/threonine kinases which can activate several downstream signaling molecules primarily through phosphorylation of Smad transcription factors.[151] Specifically, Alk5 phosphorylates receptor-associated Smad 2 and 3 which subsequently associate with the common-mediator Smad 4. This complex translocates into the nucleus and recognizes Smad-binding elements on DNA. Further association of the Smad trimer with cofactors induces the transcription of several TGF-β target genes.[150] On the other hand, TGF-β signaling is negatively regulated through inhibitory Smad 7 which competes with receptor-associated Smads for the binding to Alk5.[151]

TGF-β: a key regulator of tolerance

TGF-β is secreted by a variety of cells and tissues and TGF-β1 is the predominant isoform expressed by leukocytes. The majority of immune cells are able to secrete TGF-β, such as activated T cells,[152] B cells,[153] NK cells,[154] mast cells,[155] granulocytes,[156] macrophages[157] and DC.[158,159]

TGF-β is essential to maintain immune homeostasis. TGF-β acts as a negative regulator of all immune cells and prevents autoimmunity. TGF-β knock-out (TGF-$\beta^{-/-}$) mice rapidly develop a severe wasting syndrome and die at 3 weeks of age from multifocal inflammation.[160,161] Their disease is characterized by increased levels of auto-antibodies, massive infiltration of lymphocytes and macrophages in tissues which resemble human

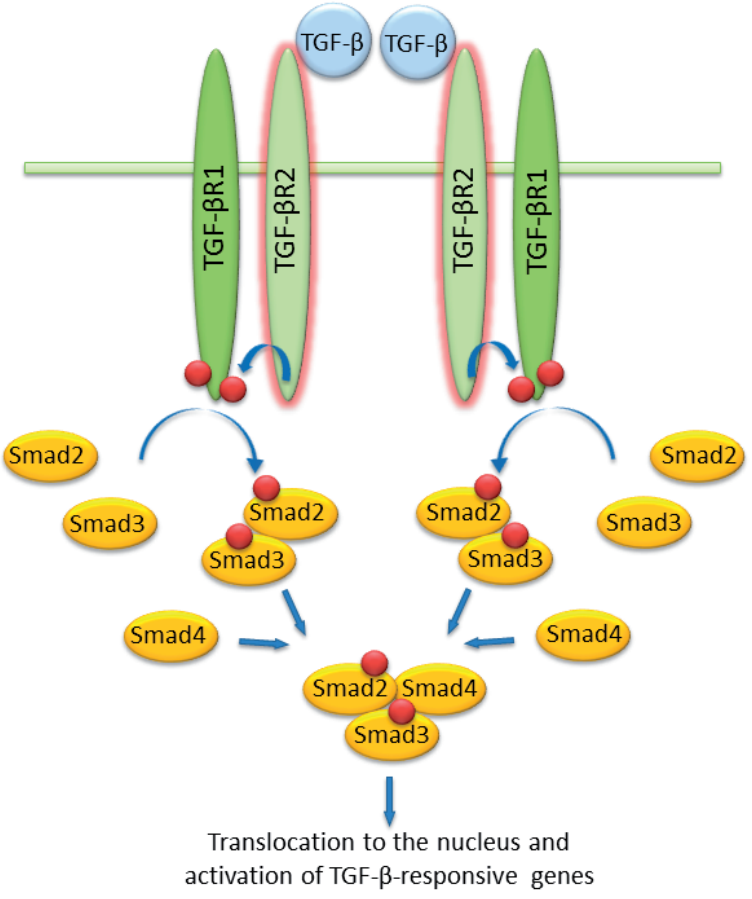

Figure 4. TGF-βR signaling. The TGF-β receptor is composed of two chains. The TGF-βR2 chain is the binding subunit while the TGF-βR1, also referred to as Alk5, represents the signaling subunit. Both are serine/threonine kinases. Binding of the active TGF-β1 dimer to the TGF-βR2 induces the formation of a tetramer composed of two TGF-βR1 and two TGF-βR2 units. This allows TGF-βR2 to phosphorylate TGF-βR1 sites. TGF-βR1 then propagates the signal through phosphorylation of SMAD2 and SMAD3. Receptor-activated SMAD2 and SMAD3 proteins form hetero-oligomeric complexes with SMAD4. Activated SMAD complexes translocate to the nucleus where they regulate the transcription of TGF-β1 target genes.

autoimmune diseases.[162] This was confirmed by the fact that TGF-$\beta^{-/-}$ mice still develop autoimmunity under germfree conditions,[163] unlike the colitis phenotype of IL10$^{-/-}$ animals which is microbiota-induced.[117]

T cells are simultaneously the major source of TGF-β and its most important target. T cell-specific TGF-β deficient mice develop an autoimmune phenotype resembling that of total TGF-$\beta^{-/-}$ animals.[164] In fact, production of TGF-β by T cells is required to inhibit CD4^{+} and CD8^{+} T cell proliferation and activation but is dispensable to maintain Treg in the periphery. Furthermore, T cell-derived TGF-β inhibits Th1 and Th2 cytokine

production.[164] As a consequence, T cell- and Treg-derived TGF-β inhibits Th1-mediated colitis.[164-166] However, the function of Treg-produced TGF-β remains controversial and might not participate in the suppressive function of Treg.[167,168]

TGF-β exerts its primary function on T cells by inhibiting their proliferation, differentiation and survival. T cells themselves produce TGF-β which acts in an autocrine loop to inhibit IL-2 signaling.[152] Importantly, Ag stimulation of T cells in the presence of TGF-β induces Foxp3 expression and Treg cell development in vitro.[169] However, if combined with IL-6, TGF-β induces the development of Th17 cells which drive autoimmune diseases like experimental autoimmune encephalomyelitis (EAE).[170,171] Both TGF-β secretion and signaling in T cells is important for Th17 induction in vivo. T cell-specific TGF-$\beta^{-/-}$ animals despite developing auto-immunity are resistant to EAE induction because of lower Th17 differentiation.[164] Furthermore, mice expressing a dominant negative form of the TGF-βRII on T cells (T-DN-TβRII$^{-/-}$) develop severe fatal autoimmune disease induced by the spontaneous activation of T cells.[172] However these mice, similarly to TGF-$\beta^{-/-}$ animals, do not develop Th17 cells and are thus resistant to EAE induction despite the increased systemic levels of Th1 cytokines.[173] Accordingly, mice with a T cell-specific TGF-βRII deletion exhibit a similar phenotype to that of TGF-$\beta^{-/-}$ mice. These mice develop a fast wasting syndrome and severe autoimmunity caused by an elevated number and activation of Th1 cells and reduced numbers of Treg.[174]

Moreover, TGF-β has an immuno-modulatory role on other immune cells including DC (see below). TGF-β inhibits the proliferation and immunoglobulin secretion of B cells[175] and promotes the differentiation of IgA-producing plasma cells.[151] Additionally, TGF-β maintains NK cell homeostasis and inhibits their cytolitic activity and IFN-γ production.[176,177] Also, TGF-β inhibits granulocyte and mast cell activation.[151]

TGF-β regulation of APC

TGF-β has an inhibitory role on APC through downmodulating their immunogenic function. TGF-β restrains macrophage activation and their production of IL-6 and IL-12.[178,179] In vitro, TGF-β induces a tolerogenic phenotype of DC by preventing their maturation.[180] TGF-β added to cultures of LPS-stimulated DC represses their Ag presenting function by blocking the upregulation of MHC-II, CD1d, CD80, CD86 and inhibiting IL-12 production.[181,182]

Besides its tolerogenic function on DC, TGF-β is necessary for the differentiation of LC from hematopoietic precursors or monocytes.[183-185] Both paracrine and autocrine TGF-β signaling are crucial for LC development in the skin in vivo[186-188] and TGF-β null mice are devoid of LC in the skin.[189] Beyond its role in LC development, TGF-β suppresses hapten-induced DC maturation, cytokine secretion and apoptosis which are suggested as mechanisms for LC not to become overactivated in the presence of harmless Ag.[190] Also, TGF-β reduces trafficking of LC to the LN by inhibiting their expression of the chemokine receptor CCR7.[191]

IL-10 and TGF-β-differentiated tolerogenic DC secrete lower levels of IL-12p70 and IL-23, induce anergic T cells and promote Treg development.[192] Accordingly, in a transplant

model, host DC incubated with donor Ag in combination with TGF-β induce alloAg-specific hypo-responsive T cells which prolongs graft survival.[193,194] In human autoimmune disease, TGF-β differentiated DC have been used in therapeutic and prophylactic approaches. Treatment of Ag-specific DC with TGF-β and IL-10 induced Ag-specific tolerance of $CD4^+$ T cells in diabetic patients.[195] However, in vivo, the expression of a dominant negative TGF-βRII on DC does not affect their homeostasis or IL-12 production.[177] In contrast with TGF-$\beta^{-/-}$ or T cell specific DN-TβRII mice, animals bearing a dominant negative TGF-βRII on DC did not develop autoimmunity. However, they develop enhanced EAE associated with high levels of Th1 and Th17 cytokines but similar Treg frequency and no differences in T cell proliferation compared to wild type animals.[196] On the other hand, mice with a DC-specific TGF-βRII deficiency develop autoimmunity with spontaneous T and B cell activation, associated with lower Treg activation.[197] However, whether this phenotype might be driven by residual deletion of the receptor in T cells remains elusive.[198]

TGF-β in human diseases

No deficiencies of TGF-β or TGF-βR signaling have been reported in humans, most likely because TGF-β signaling is crucial during embryogenesis. However, polymorphisms in the TGF-β promoter were found in patients with asthma[199] and osteoporosis.[200] TGF-β is essential in regulating (autoimmune) diseases in humans.[151] A correlation between disease severity and TGF-β serum levels was found in patients with asthma,[201] where TGF-β played a pro-inflammatory role as a potent neutrophil chemoattractant. Also, TGF-β has a prominent function in cancers. Defects in TGF-β signaling pathways,[202,203] in the TGF-β allele[204] and higher serum levels of TGF-β[205] have been causally linked to several cancers in humans.

In summary, TGF-β is crucial to maintain immune homeostasis and, similarly to IL-10, T cells are suggested to be the most important sources and targets of TGF-β. In vitro studies demonstrate that TGF-β inhibits DC function and is crucial for LC homeostasis, which we sought to investigate in vivo in this thesis.

AIM OF THIS THESIS

Since their first characterization by Steinman and Cohn in 1973, DC have emerged as crucial regulators of immune responses. Through their potent Ag presenting abilities and capacity to efficiently induce both Ag-specific effector and regulatory T cells, DC are pivotal players in the balance between immunity and tolerance. However, the in vivo role of cytokines to control the tolerogenic/regulatory function of DC remains poorly understood. IL-10 and TGF-β are two tolerogenic cytokines crucial to respectively downregulate immune responses and to prevent autoimmunity. Both IL-10 and TGF-β are secreted by and act on many immune and stromal cell types and are crucial in regulating diverse cell functions. In vitro, DC matured in the presence of IL-10 and/or TGF-β develop a tolerogenic phenotype, inducing T-cell anergy and/or Ag-specific tolerance. In this thesis we investigated the role of IL-10 and TGF-β signaling in DC in vivo, taking

advantage of mice with a DC-specific knock out of the IL-10Rα or TGF-βRI (DC-IL10R$^{-/-}$ and DC-TβRI$^{-/-}$ animals, respectively).

It was previously demonstrated that IL-10 modulates the expression of MHCII and co-stimulatory molecules on the surface of DC in vitro. Notably, the presence of anti-IL10R antibody in the medium of maturing DC induces the enhanced expression of maturation markers. In **Chapter 2**, we therefore investigated whether the constitutive lack of IL-10 signaling in DC affects the maturation of spleen, LN and skin DC in the steady state. Next, we examined to what extent DC are targets of IL-10 during contact hypersensitivity (CHS), a model of atopic contact dermatitis. While IL-10 secreted by LC and T cells is necessary to prevent an excessive CHS reaction, its role in controlling DC remained elusive.

In **Chapter 3** we further studied skin immune responses using a model of cutaneous leishmaniasis. IL-10-deficient animals mount an enhanced anti-parasite Th1/Tc1 reaction and totally clear the parasite, rendering them susceptible to reinfection. Also, T cell-derived IL-10 was shown to be essential in maintaining the presence of parasites in the chronic phase of the disease. Here, we evaluated the role of IL-10 in controlling DC in the course of the anti-*Leishmania major* response, during both the acute and chronic phases of the disease.

IL-10 was first described as an essential cytokine to maintain immune homeostasis in the gastrointestinal tract as IL-10-deficient animals develop severe inflammation both in the small intestine and the colon. Particularly, T cell and Treg-derived IL-10 is critical to prevent excessive Th1/Th17 activation in the colon. In **Chapter 4** we therefore investigated the particular role of IL-10 signaling in DC both in the colon and small intestine of DC-IL10R$^{-/-}$ mice.

Chapter 5 moves on to the third epithelial barrier, i.e. the lungs, and describes preliminary data on the role of IL-10 in governing DC function during OVA-induced asthma. In allergy and asthma, IL-10 is protective and the disruption of IL-10 signaling promotes the development of these pathologies. Also, IL-10-treated tolerogenic DC inhibit disease development. However, the role of IL-10 in modulating DC during the inflammatory response of asthma remained to be investigated.

In the second part of this thesis, we focus on the TGF-β regulation of DC. TGF-β is necessary for the development of LC but its role in regulating other DC subsets and functions remained elusive. In **Chapter 6**, we examined the role of TGF-β control of LC and dermal DC in the steady state and during CHS.

In **Chapter 7** we first assess the lethal autoimmune phenotype that spontaneously develops in DC-TβRI$^{-/-}$ animals. Since in the colon TGF-β is secreted predominantly by epithelial cells to create a tolerogenic microenvironment at this epithelial barrier, we next analyzed the specific role of TGF-β signaling in colonic DC using Rag-deficient DC-TβRI$^{-/-}$ mice.

In **Chapter 8** the findings of our work are summarized and discussed with regard to current knowledge in the field. Finally, I consider future perspectives and implications of our findings.

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CHAPTER 2

IL-10 CONTROLS DENDRITIC CELL-INDUCED T-CELL REACTIVATION IN THE SKIN TO LIMIT CONTACT HYPERSENSITIVITY

Mathilde J.H. Girard-Madoux[1,2], Junda M. Kel[1], Boris Reizis[3] and Björn E. Clausen[1]

[1]Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands
[2]Department of Cell Biology & Histology, AMC, University of Amsterdam, Amsterdam, the Netherlands
[3]Department of Microbiology, Columbia University Medical Center, New York, NY, USA

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ABSTRACT

IL-10 is a pleiotropic cytokine and potent negative regulator of both innate and adaptive immune responses. Consequently, IL-10-deficient mice develop enhanced contact hypersensitivity (CHS) to topical hapten. Although the importance of IL-10 production by (regulatory) T cells and Langerhans cells in regulating CHS has been established by cell type-specific *il-10* gene targeting, it remains elusive to what extent IL-10 controls dendritic cell (DC) function *in vivo*. To this aim, we generated mice with a DC-specific deletion of the IL-10 receptor. Despite the ability of IL-10 to inhibit DC maturation *in vitro*, DC of resting DC-IL10R$^{-/-}$ mice retained their immature phenotype *in vivo*. In contrast, IL-10R-deficient DC produced elevated levels of proinflammatory cytokines and IL-10 following *in vitro* stimulation. Induction of CHS was indistinguishable from control mice at 24h post hapten challenge, but resulted in increased ear swelling at 48h and delayed resolution of the inflammatory reaction. Adoptive T-cell transfer experiments revealed that only T-cell reactivation and not sensitization by IL-10R-deficient DC leads to enhanced CHS. Accordingly, the expression of proinflammatory cytokines and IL-10 was augmented in the skin of DC-IL10R$^{-/-}$ mice after hapten challenge. Our data demonstrate that IL-10 signaling in DC is dispensable during naïve T-cell priming, but is critical to prevent an exaggerated effector T cell response in the skin.

Keywords: Conditional gene targeting, dendritic cells, immune regulation, IL-10 receptor signaling, Langerhans cells, skin.

Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell(s); KO, knockout; LC, Langerhans cell(s); LN, lymph node(s); mLN, mesenteric LN; sdLN, skin-draining LN; Treg, regulatory T cell(s).

INTRODUCTION

IL-10 is a key regulatory cytokine with pleiotropic effects on multiple cell types. The primary function of IL-10 is to limit exuberant T cell responses to microbial pathogens to prevent chronic inflammation and tissue damage.[1,2] IL-10 signals via a heterodimeric receptor (IL-10R) consisting of a ligand-binding IL-10Rα subunit, expressed at high levels on macrophages and DC, and an IL-10Rβ chain ubiquitously and constitutively expressed.[3] IL-10 is pivotal to control innate immune responses by inhibiting monocyte/macrophage function through down regulation of their proinflammatory cytokine production.[3,4] By acting on dendritic cells (DC) and T cells, IL-10 modulates adaptive immune responses. On one hand, IL-10 suppresses DC maturation and proinflammatory cytokine secretion. On the other hand, IL-10 inhibits T helper type-1 (Th1) cell differentiation, restrains effector T cell function and promotes the development and function of regulatory T cells (Treg).[3-5] In addition to its inhibitory capacity, IL-10 is also suggested to have a stimulatory role in $CD8^+$ T-cell homeostasis.[6] IL-10 not only acts on many different immune cells, but is also produced by a variety of leukocytes including activated macrophages, DC and, in particular, Treg cells.[7] As a gatekeeper of excessive immune responses to foreign antigens, IL-10 is expressed at epithelial borders to the environment. In the skin, IL-10 is produced by keratinocytes,[3] macrophages and DC upon exposure to contact sensitizer or ultraviolet radiation.[8,9] Consequently, IL-10-deficient ($IL\text{-}10^{-/-}$) mice mount exaggerated Th1 responses and exhibit increased irritant and contact hypersensitivity (CHS) reactions in the skin.[10,11]

CHS is a relevant mouse model for allergic contact dermatitis, one of the most common occupational diseases in man.[12] In mice, topical application of a contact sensitizer (hapten) onto the skin initiates the production of proinflammatory cytokines that activate skin DC to prime hapten-specific T cells in draining lymph nodess (LN). Subsequent painting of the same hapten onto the ear initiates a transient ear swelling reaction that is mediated by IFN-γ-producing $CD8^+$ T cells and controlled by $CD4^+$ cells secreting IL-10.[12,13] Epidermal Langerhans cells (LC) have been critically linked to the initiation of contact sensitivity responses; however, recent experiments suggest functional redundancy of the different skin DC subsets in mediating CHS.[14,15]

DC are key players in maintaining immune homeostasis.[16] They continuously probe their environment for invading microorganisms and induce robust T cell responses to pathogen-derived antigens. DC uptake and presentation of innocuous foreign and self-antigens mediates T cell tolerance,[17] and IL-10 can skew DC function towards a tolerogenic rather than an immunogenic phenotype.[2] Despite ample *in vitro* data suggesting a role for IL-10 in regulating DC function, it is still unknown to what extent DC are targets of IL-10 *in vivo*. To this aim, we generated mice with a DC-specific deletion of the *il-10rα* gene ($DC\text{-}IL10R^{-/-}$ mice). In this study, we have focused on dissecting the role of IL-10 control of DC to govern innate and adaptive immune responses in the skin.

RESULTS

The IL-10R is efficiently and specifically deleted on DC

DC-IL10R$^{-/-}$ animals appeared healthy, however, the mice demonstrated splenomegaly (Fig. E1A), with a 40% expansion in total cell number (Fig. E1B). In addition, splenic DC frequency was significantly increased (Fig. E1C), which was not the result of reduced DC apoptosis (Fig. E1D and E). The CD4^{+} and CD8^{+} T cell ratio was similar to controls (Fig. E1F). Skin-draining LN (sdLN) from DC-IL10R$^{-/-}$ and non-TG mice were similar in size. Furthermore, total cell number and DC subset frequencies were comparable in whole ear, epidermis, dermis and sdLN (Fig. E2). In sdLN, dermis and epidermis, surface expression of the IL-10R on DC was determined by flow cytometry. The IL-10R was expressed on DC in all of these tissues, and efficiently deleted on cells prepared from DC-IL10R$^{-/-}$ animals (Fig. 1A). The same was found for DC in other LN, spleen and thymus (Fig. E3). In the steady state, IL-10R expression on T and B cells was undetectable. To demonstrate that the IL-10R can be expressed on activated T cells of DC-IL10R$^{-/-}$ mice, splenic T cells were stimulated *in vitro* and IL-10R expression was assessed. Three days after culture on anti-CD3 coated plates, 20-25% of the cells had upregulated the IL-10R, at a level comparable to activated non-TG T cells (Fig. 1B). These data prove specific and robust deletion of the IL-10R in DC.

IL-10 signaling is dispensable to maintain the immature DC phenotype in the steady state

In the presence of neutralizing anti-IL-10 antibody, DC undergo spontaneous maturation *in vitro*[18] and mediate a stronger immune response *in vivo*. [19] To determine to what extent

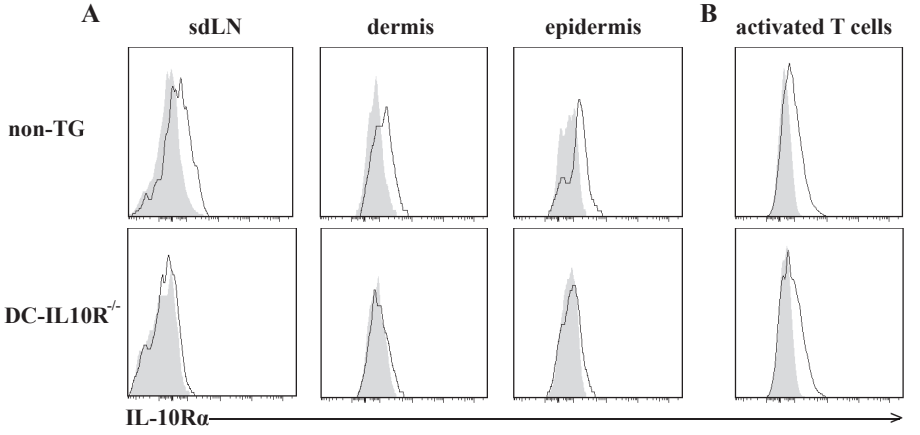

Figure 1. Efficient DC-specific deletion of the IL-10R in DC-IL10R$^{-/-}$ mice. **(A)** Skin-draining LN, epidermal and dermal cell suspensions were analyzed for IL-10Rα expression after gating on CD11c^{+}MHCII^{+} DC or MHCII^{+} LC, respectively. **(B)** IL-10Rα expression on activated splenic T cells. Black lines represent the IL-10Rα staining and filled grey diagrams the isotype control.

the lack of IL-10 signaling affects the maturation of DC *in vivo* in the steady state, we assessed the expression of MHCII and costimulatory molecules on IL-10R-deficient DC. In sdLN and spleen of DC-IL10R$^{-/-}$ animals, IL-10R-deficient DC displayed comparable levels of MHCII, CD86 and CD40 as control cells (Fig. E4A). During the cross-talk between DC and T cells, the PD-1:PD-L1/PD-L2 pathway is critical to prevent excessive immune reactions.[20] In steady state, the expression of the inhibitory costimulatory molecules PD-L1 and PD-L2 was not altered on the surface of IL-10R-deficient DC in sdLN and spleen (Fig. E4A).

To determine whether IL-10 deficiency in DC affects the T cell compartment, we assessed helper and cytotoxic T cell activation status as well as Treg levels in the spleen of resting DC-IL10R$^{-/-}$ animals. The expression levels of the activation markers CD69, CD62L, and CD44 on CD4^{+} and CD8^{+} T cells (Fig. E4B) and Treg frequencies were comparable between non-TG and DC-IL10R$^{-/-}$ animals, both in the CD4^{+} and in the CD8^{+} T cell population (Fig. E4C). In conclusion, in steady state DC-IL10R$^{-/-}$ mice, IL-10R-deficient DC are immature, the T cell compartment is non-activated, and the lack of IL-10 signaling in DC does not alter Treg frequencies.

IL-10 control of DC is dispensable during an innate immune response but necessary to prevent enhanced CHS

Irritant response to croton oil is an innate immune reaction of the skin, mainly driven by neutrophils and monocytes.[21] Total IL-10 KO mice display an increased irritant response, whereas mice with a T cell-specific deletion of the *il-10* gene exhibit a similar reaction as compared to non-TG.[11] Topical application of croton oil onto the ears of DC-IL10R$^{-/-}$ and non-TG animals resulted in comparable ear swelling at 24h, which was resolved at 72h (Fig. E5). These data determine that IL-10 mediated control of DC does not contribute to contain a local innate immune reaction in the skin.

CHS is a transient skin immune response driven by hapten-specific Th1/Tc1 cells and controlled by CD4^{+} T cells secreting IL-10.[12,13] To determine the role of IL-10 control of DC in regulating CHS, DC-IL10R$^{-/-}$ and non-TG control mice were sensitized and 5 days later challenged onto the ear with oxazolone. In contrast to previous findings in total and cell type-specific IL-10 knockouts,[15] DC-IL10R$^{-/-}$ and non-TG animals developed a similar ear swelling reaction 24h after hapten challenge (Fig. 2A). Intriguingly, while non-TG started to resolve the inflammation, ear swelling in DC-IL10R$^{-/-}$ mice further increased, peaked at 48h and was still significantly increased at 72 and 96h. These results establish that IL-10-mediated control of DC is crucial to contain the T cell-dependent immune response during CHS.

IL-10 controls DC function during the effector phase of CHS

To dissect whether IL-10 signaling in DC is required during T cell priming or during the effector phase of CHS, we performed adoptive T cell transfer experiments. Specifically, sdLN T cells of hapten sensitized donor mice were injected intravenously into naïve recipient animals that were subsequently challenged with oxazolone onto the ears.

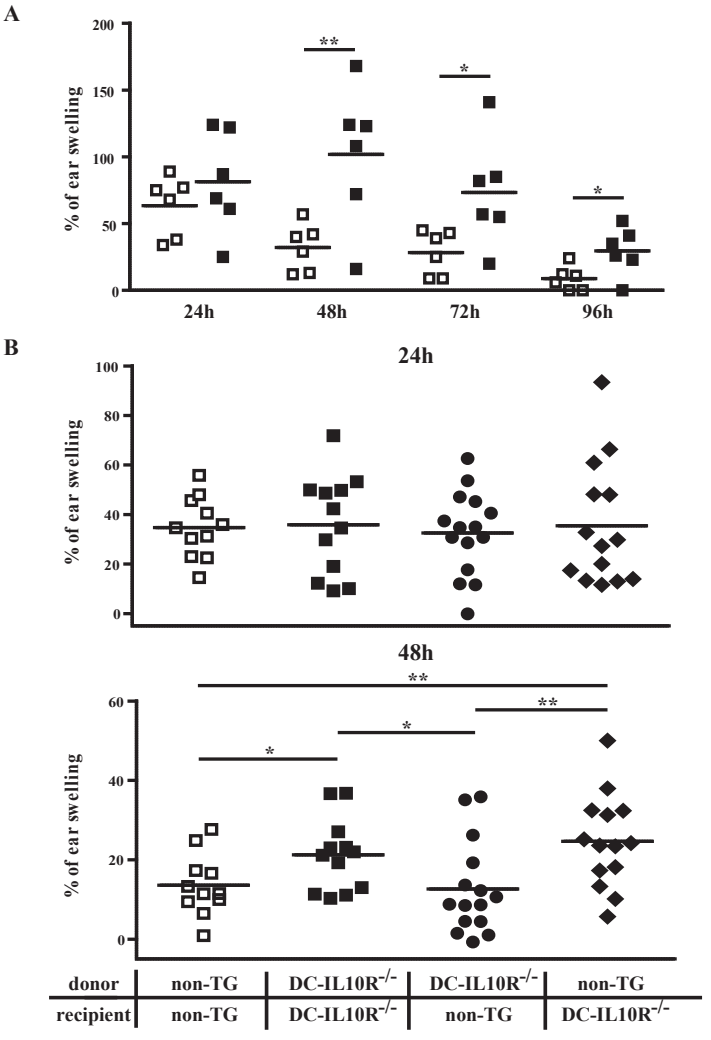

Figure 2. IL-10 control of DC is critical during the effector phase of CHS. **(A)** Ear thickness after oxazolone hapten challenge. □ : non-TG and ■: DC-IL10R$^{-/-}$ mice. One representative experiment out of 3 is shown. **(B)** Purified T cells from sensitized mice were injected i.v. into recipient animals as indicated. One day later the recipients were challenged with oxazolone onto the ears. Ear swelling was measured at 24h and 48h after hapten challenge. Data of 2 combined experiments are presented.

T cells primed in non-TG or DC-IL10R$^{-/-}$ mice were injected into both non-TG and DC-IL10R$^{-/-}$ animals. As expected from the previous experiment (Fig. 2A), similar ear swelling developed at 24h post hapten-challenge in all experimental groups (Fig. 2B, upper panel). In contrast, at 48h the ear swelling reaction was significantly stronger in DC-IL10R$^{-/-}$ recipients, irrespective of the genotype of the donor mice in which the T cells were sensitized (Fig. 2B, lower panel). That is, T cells primed by non-TG DC and

reactivated by IL-10R-deficient DC mounted enhanced CHS, while T cells primed by IL-10R-deficient DC and reactivated by non-TG DC mediated similar CHS as compared to non-TG controls. These findings reveal that IL-10 control of DC is dispensable during naïve T-cell priming in sdLN, but essential to prevent exaggerated reactivation of effector T cells in the skin.

IL-10R-deficient DC produce increased levels of cytokines after in vitro stimulation

To investigate DC maturation and cytokine secretion after activation, we stimulated splenic DC from non-TG and DC-IL10R$^{-/-}$ mice. *In vitro* activation with LPS induced similar phenotypic maturation of IL10R$^{-/-}$ and control DC (Fig. E6). In contrast, LPS-stimulated IL10R$^{-/-}$ DC produced significantly increased amounts of TNF-α, IL-6 and IL-10 as compared to non-TG DC (Fig. 3A). To mimic T-cell encounter, LPS-stimulated DC were cultured with soluble CD40L (sCD40L) for 24h. The secretion of IL-6 and IL-10 by DC was further enhanced as compared to LPS treatment alone and significantly higher in IL-10R$^{-/-}$ DC cultures (Fig. 3A). No TNF-α could be detected after culture with sCD40L. Addition of recombinant mouse IL-10 (rmIL-10) significantly blocked the production

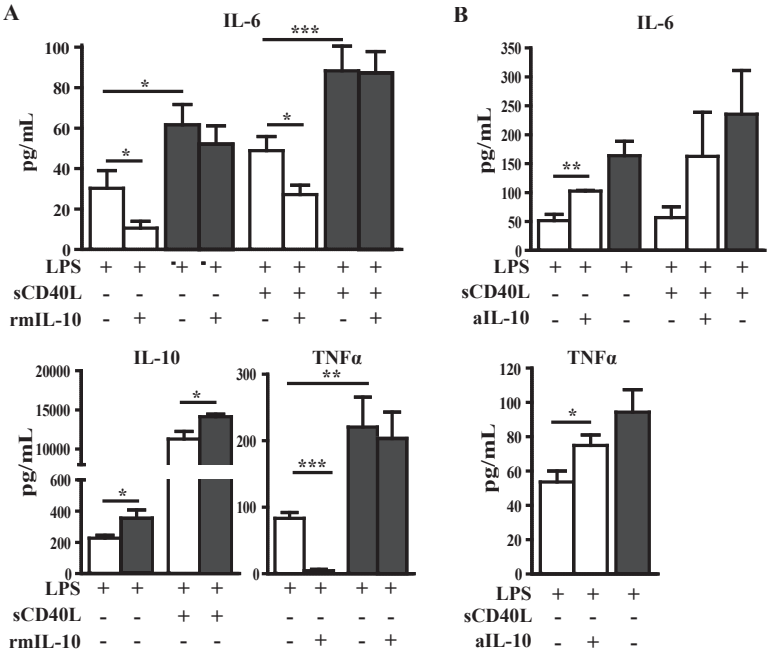

Figure 3. Elevated cytokine production of *in vitro* activated IL-10R$^{-/-}$ DC. **(A)** TNF-α, IL-6 and IL-10 levels were measured in supernatants of DC cultures after LPS ± sCD40L ± rmIL-10 or **(B)** ± aIL-10 stimulation as indicated. Open and shaded columns represent non-TG and DC-IL10R$^{-/-}$ animals respectively.

of TNF-α and IL-6 by control DC, but not IL-10R$^{-/-}$ DC (Fig. 3A). Corroborating this observation, non-TG DC cultured in the presence of neutralizing anti-IL-10 antibody (aIL-10) produced significantly higher levels of both cytokines (Fig. 3B). These findings confirm that enhanced cytokine secretion by IL-10R$^{-/-}$ DC is specifically mediated by defective IL-10 signaling in the mutant DC. The levels of IL-12p70, IL-23 and TGF-β were below the detection limit in all DC cultures. These data strongly suggest that activated IL-10R-deficient DC drive enhanced adaptive immunity by elevated secretion of proinflammatory cytokines.

Enhanced cytokine production and recruitment of innate immune cells in DC-IL10R$^{-/-}$ skin during elicitation of CHS

Under steady state conditions, immune homeostasis was not affected in the ears of DC-IL10R$^{-/-}$ and control animals. In particular, the occurrence of T cells (CD3^{+}), antigen presenting cells (MHCII^{+}) and granulocytes (Gr1^{+}) as well as the expression of IL-1β, TNF-α, IFN-γ and TGF-β was similar, while IL-6 and IL-10 were not detectable (Fig. E7). Next, we isolated ear tissue from mice at 24h and 48h post hapten-challenge and assessed the presence of cytokines by quantitative RT-PCR. Already at 24h we observed elevated mRNA expression of the proinflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ in DC-IL10R$^{-/-}$ mice as compared to controls (Fig. 4A). IL-10 mRNA levels were increased as well, whereas no differences could be observed for TGFβ. After peaking at 24h, all cytokine mRNA levels were similar or lowered at 48h both in DC-IL10R$^{-/-}$ and controls, although IL-1β and TNF-α mRNA were still enhanced in DC-IL10R$^{-/-}$ ears. Corroborating our mRNA data, augmented TNF-α protein expression was detected in cryosections of DC-IL10R$^{-/-}$ ears at both 24h and 48h (Fig. E8A). H&E staining revealed numerous infiltrating cells in both DC-IL10R$^{-/-}$ and control mice 24h post-challenge and the foci were more abundant in DC-IL10R$^{-/-}$ mice (Fig. E8B), although ear thickness was not yet enhanced at this time point (Fig. 3A). MHCII and Gr1 staining suggested that a greater number of antigen presenting cells and granulocytes accumulated in the ears of DC-IL10R$^{-/-}$ mice (Fig. E8B). At 48h, when ear swelling was increased (Fig. 3A), a massive influx of MHCII^{+} and Gr1^{+} cells was observed in DC-IL10R$^{-/-}$ animals, while fewer infiltrates were detectable in controls (Fig. 4B). In contrast, a similar number of NK cells was present in the ears of DC-IL10R$^{-/-}$ and non-TG mice both at 24h and 48h post-challenge (Fig. E8C).

To determine the magnitude and the nature of the T cell response during CHS in DC-IL10R$^{-/-}$ versus control animals, we analyzed CD3 T cell infiltration and the mRNA levels of CD3 and the transcription factors Tbet and Foxp3. While there was no difference between DC-IL10R$^{-/-}$ and non-TG both at the protein and mRNA level (Fig. 4C and E8D), we found an increase in CD3 expression at 48h as compared to 24h post hapten challenge, which was largely due to an enhanced influx of Foxp3^{+} Treg (Fig. 4C).

Taken together, these data demonstrate that the increased ear swelling reaction at 48h in DC-IL10R$^{-/-}$ animals is associated with enhanced *in situ* production of inflammatory cytokines and accumulation of innate immune cells starting already at 24h post hapten challenge.

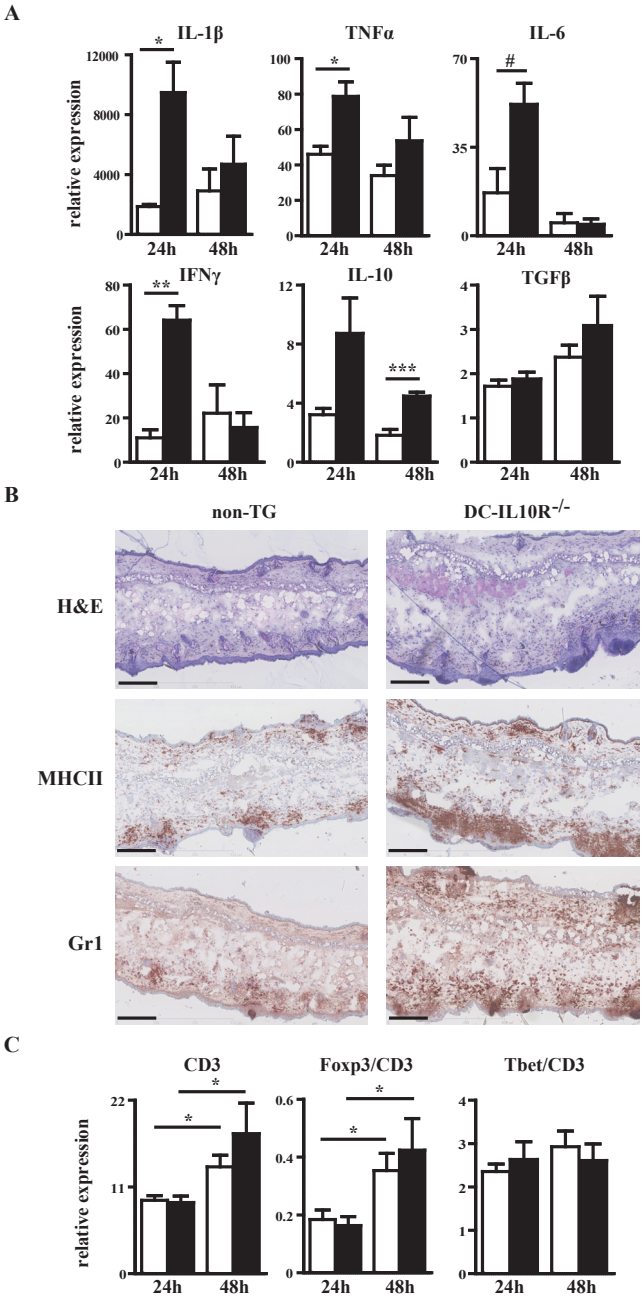

Figure 4. Increased cytokine mRNA expression and cellular influx in the ears of DC-IL10R$^{-/-}$ mice during CHS.Ears were isolated 24 and/or 48h post oxazolone challenge. **(A)** Cytokine mRNA expression. **(B)** H&E (top), anti-MHCII (middle) or anti-Gr1 (lower panels) staining at 48h. Size bars represent 200µm. **(C)** mRNA levels of CD3, Foxp3 and Tbet. In **(A)** and **(C)** □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals.

DISCUSSION

We generated mice with a DC-specific IL-10R deficiency, which enables for the first time the assessment of IL-10-mediated control of DC homeostasis and function *in vivo*. In this study, we focus on skin immune responses and reveal that IL-10 signaling in DC is dispensable during T cell priming but is critical to prevent exaggerated T cell-mediated inflammation.

DC-IL10R$^{-/-}$ mice did not develop spontaneous auto-immunity, but displayed splenomegaly and an increase in splenic DC frequency. The latter suggests that IL-10 plays a role in DC homeostasis, although it has not been reported that IL-10 or IL-10R KO mice have an altered DC compartment. Recently, IL-10 was found to induce apoptosis of mature human DC,[22] however, we could not detect any changes in DC apoptosis in the spleen of DC-IL10R$^{-/-}$ mice. Against our expectation based on *in vitro* studies,[4,17,18] IL-10R-deficient DC displayed no signs of spontaneous maturation in the steady state. In agreement with this immature DC phenotype, T cell activation and (Treg) homeostasis was unaffected in DC-IL10R$^{-/-}$ mice.

We next analyzed DC-IL10R$^{-/-}$ mice in innate and adaptive immune activation. DC have an essential role in innate immunity by inducing proliferation and activation of NK cells and macrophages.[23] Mice with a specific deletion of the *il-10* gene in neutrophils and macrophages show increased local inflammation following subcutaneous injection of LPS.[24] While the secretion of IL-10 controls the innate immune cells in an autocrine way as recently demonstrated in mice with a neutrophil/monocyte-specific IL-10R deficiency,[25] IL-10 may also act in a paracrine fashion via DC. While IL-10 KO mice mount an increased irritant response to croton oil,[10] as expected T cell-specific IL-10 deletion does not affect the innate ear swelling reaction.[11] Here we show that the lack of IL-10 signaling in DC also does not alter irritant responses in the skin. Taken together, although local IL-10 production is crucial to limit innate skin inflammation, IL-10 mediated regulation of DC is not required in this process. Rather, IL-10 directly acts on the innate immune cells themselves.[25]

IL-10 has a crucial role in controlling adaptive immune responses in the skin,[26,27] as established by increased CHS in IL-10 KO mice.[10] IL-10 can exert its immune regulatory function during both the induction and the effector phase of CHS: Intradermal injection of IL-10 into the ears of mice shortly before sensitization[28] and intraperitoneal application of IL-10 before hapten challenge both suppress ear swelling.[26,27] During the sensitization phase of CHS, cognate interaction of LC with CD4^{+} T cells and LC-derived IL-10 are required to limit ear swelling, since mice with a LC-specific deletion of MHCII or IL-10 exhibit increased CHS responses.[29] Both T cell-specific and Treg-specific IL-10 KO mice display enhanced CHS,[11,30] demonstrating that T-cell derived IL-10 plays an essential role in limiting the reaction. Treg derived IL-10 appears to be of particular importance since transfer of naive CD4^{+}CD25^{+} Treg prior to hapten challenge suppresses CHS via IL-10.[31]

Despite its important contribution to limit CHS, the cellular targets of IL-10 and, in particular, the role of IL-10 signaling in DC during a CHS response remain

unknown. Unlike any other (cell type-specific) IL-10 KO mouse model, DC-IL10R$^{-/-}$ mice developed similar ear swelling as non-TG controls 24h after elicitation of CHS. Ear swelling further increased at 48h resulting in a delayed resolution of the reaction in the absence of IL-10 signals in DC. To determine whether DC are targets of IL-10 at the time of hapten sensitization and/or challenge, we performed adoptive T cell transfer experiments after sensitization. Irrespective of whether T cells were primed by non-TG or IL-10R-deficient DC, reactivation of the cells by IL-10R$^{-/-}$ DC resulted in enhanced ear swelling reactions at 48h. Conversely, T cells from either DC-IL10R$^{-/-}$ or non-TG donor mice reactivated in non-TG recipients mediated reduced CHS as compared to DC-IL10R$^{-/-}$ controls. These data strongly support the conclusion that IL-10 control of LC/DC is not required during T cell priming, but is crucial to contain the effector response of the CHS reaction.

IL-10R-deficient DC produce elevated amounts of proinflammatory cytokines (TNF-α, IL-6) upon LPS stimulation, which are further augmented by sCD40L treatment mimicking activation by T cells *in situ*. TNF-α and IL-6 play pivotal roles in CHS, since KO mice of either cytokine mount decreased ear swelling responses.[32] Already 24h after hapten challenge, expression of IL-1β, TNF-α and IL-6 mRNA is significantly increased in the ears of DC-IL10R$^{-/-}$ mice as compared to non-TG. These proinflammatory cytokines promote increased activation of hapten-specific effector T cells as suggested by amplified expression of IFN-γ in DC-IL10R$^{-/-}$ ears. The fact that ear swelling is still similar between both groups at this time point may be explained by enhanced production of IL-10 by IL-10R deficient DC *in vitro* and in the ears of DC-IL10R$^{-/-}$ mice. Eventually, the elevated levels of proinflammatory cytokines secreted by both DC and T cells lead to an exaggerated influx of innate immune cells into the skin, which triggers enhanced edema and ear swelling in DC-IL10R$^{-/-}$ mice at 48h. Although cytokine levels are downregulated at 48h as compared to 24h after hapten challenge, the sustained presence of higher levels of proinflammatory cytokines causes delayed resolution of the CHS reaction in DC-IL10R$^{-/-}$ mice. Intriguingly, mRNA levels of CD3 and the transcription factors Tbet (Th1) and Foxp3 (Treg) were similar in the ears of DC-IL10R$^{-/-}$ and controls demonstrating that lack of IL-10 signaling in DC does not affect the number of infiltrating T cells or the Th1/Treg balance. Therefore, the increased expression of IFN-γ in the ears of DC-IL10R$^{-/-}$ mice strongly suggests enhanced activation of hapten-specific Th1 cells by IL-10R deficient DC.[12] In addition, the ratio of Tbet expressing Th1 cells stays the same during the first two days of the effector response, whereas the number of Foxp3^{+} Treg has doubled by 48h after hapten challenge. Since Treg exert their regulatory/suppressive function largely via secretion of IL-10, this finding implies that Treg control of effector T cells is insufficient to limit disease. Rather, Treg-derived IL-10 is essential to prevent excessive DC activation in CHS and cannot be compensated for by adenosine production.[33] This finding also elucidates why DC-IL10R$^{-/-}$ mice develop an exacerbated hapten-specific adaptive immune response, whereas an innate irritant reaction to croton oil is similar that seen in non-TG.

In conclusion, it is becoming increasingly clear that the collaboration of DC, Treg and effector T cells is essential to prevent excessive immune activation and that IL-10 is a central messenger within this regulatory network. Our analysis of DC-specific IL-10 *receptor* deficient animals establishes that IL-10 signaling in DC is dispensable during T cell priming, but critically required to limit the magnitude and duration of the CHS reaction. This may have considerable implications for human immunotherapy since releasing DC from IL-10 control may increase their immunogenicity *in situ*, for example against tumors, without the risk of emerging autoimmunity due to enhanced DC priming of naïve T cells.

MATERIAL AND METHODS

Mice

CD11c-Cre mice[34] were crossed with floxed IL-10Rα mice[25] to generate DC-IL10R$^{-/-}$ mice. Non-transgenic (non-TG) littermates were used as controls. The mice were housed at the animal facilities of the AMC in Amsterdam and the Erasmus MC in Rotterdam. All animal experimentation was in compliance with EU as well as national laws and approved by the local ethical committees.

Cell preparation

Spleens, LN and ears of mice were collected and single-cell suspensions were prepared as previously described.[35] Briefly, spleens and LN were mechanically disrupted and digested with 4000 U/mL collagenase IV (Worthington) for 30 min at 37°C. Dorsal and ventral sides of ears were floated on HBSS supplemented with 1,25mg/mL dispase (Roche) for 1h at 37°C. Epidermis was further incubated with 0.5% trypsin (Invitrogen), while dermis was digested with collagenase IV for 30 min at 37°C. Cells were then mashed through a 70 μm nylon cell strainer (BD Falcon) to obtain a single-cell suspension for flow cytometry or MACS-sorting (Miltenyi Biotec).

Flow cytometry

Cells were surface stained at 4°C for 45-60 min with the following monoclonal antibodies: CD11c-A488 (N418), MHCII-A647 (M5/114.15.2) and IL-10Rα (CD210)-biotin (1B1.3a) from BioLegend. PE-Cy7-conjugated streptavidin was used to visualize the biotin-conjugated antibody (BD Pharmingen). Appropriate isotype controls were utilized when necessary. Cells were analyzed on a FACS Canto (BD Biosciences) using FlowJo software.

In vitro T cell activation

Splenic T cells were enriched by MACS using pan-T cell microbeads (Miltenyi Biotec), plated on anti-CD3-coated plates and cultured for 3 days in complete RPMI (cRPMI: supplemented with 10% FCS, 2mM L-glutamine, 100μg/mL streptomycin, 100U/mL penicillin, 50μM β-mercaptoethanol), before harvesting and staining for flow cytometry.

CHS

CHS was induced by painting the shaved abdomen of mice with 50µL of 2% oxazolone (Sigma-Aldrich) in a 4:1 mixture of acetone:olive oil (AOO) for sensitization. The mice were challenged 5 days later with 25µL of 0.5% oxazolone in AOO onto the right ear. Percentage ear swelling was calculated by comparing ear thickness before and up to 96h after hapten challenge using a micrometer (Mitutoyo).

Adoptive T cell transfer

Mice were painted with 2% oxazolone as described above and inguinal LN cells were isolated 5 days after sensitization. T cells were MACS enriched (pan-T cell kit, Miltenyi Biotec), resuspended in sterile PBS and $4x10^6$ cells were injected i.v. into recipient mice. One day later, the animals were challenged with 0.5% oxazolone on the ear and swelling was measured 24 and 48h later.

DC culture

Splenocytes were prepared as described above and DC were purified by MACS with CD11c-PE labeling and anti-PE microbeads (Miltenyi Biotec). Cells were resuspended in cRPMI with 125ng/mL of LPS (*E. coli* 026.B6, Sigma) and 5ng/mL recombinant mouse IL-10 (R&D) or 5µg/mL anti-IL-10 neutralizing antibody (kindly provided by E. Lubberts) where mentioned, and plated onto non-adherent plates (Corning) at a concentration of $0.5x10^6$ cells/mL for O/N culture. The supernatants were then collected, DC washed with PBS and plated with 400ng/mL sCD40L (PreproTech). After 24h, supernatants were stored at -20°C and DC were analyzed by flow cytometry.

Cytokine Bead Array

Supernatants from LPS- and LPS+sCD40L-stimulated DC were stored at -20°C. Levels of TNF-α, IL-10, IL-6, IL-12p70, IL-23 and TGF-β were determined by cytometric bead assay (Bender MedSystems) according to the manufacturer's instructions. Samples were analyzed on a FACS Canto-II (BD Bioscience) and cytokine concentrations were calculated using the FCAP ArrayTM software (BD Bioscience).

RT-qPCR

mRNA was extracted from total ears using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). cDNA was synthesized from mRNA with SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. TaqMan real-time quantitative PCR assays were designed to determine transcript levels of several markers (primer sequences can be found in Table E1). The expression levels were normalized to control gene *ABL*. For Foxp3 and Tbet, relative expression to CD3 is depicted. All reactions were run on a 7900HT Fast Real Time PCR machine (Applied Biosystems)

Histology

Ears were cut longitudinally into 6 µm-thick sections on a CM305 0S microtome (Leica). Tissue sections were stained with haematoxylin-eosin to study their microarchitecture.

Immunohistochemistry was performed as follows: sections were fixed in acetone containing 0.02% hydrogen peroxide and incubated O/N at 4°C with primary rat antibodies (Pharmingen) against MHCII (M5/114.15.2), Gr1 (RB6-8C5) or TNF-α (MP6-XT22) in PBS/0.1% BSA. The next day, rabbit anti-rat secondary antibody (Dako) was applied in PBS/0.1% BSA supplemented with 2% normal mouse serum for 1h at RT. Enzyme activity was revealed using the vectastain ABC kit (Vector Laboratories) and aminoethylcarbazole was used as a chromogen for horseradish peroxidase activity. Sections were mounted with glycerol-gelatin. Slides were scanned with the NanoZoomer 2.0HT scanner (Hamamatsu) and analyzed with the NanoZoomer Digital Pathology viewer software (Hamamatsu) to obtain pictures at 10X magnification.

Statistical analysis

All experiments were repeated at least 3 times, with 4 to 8 mice per group. ANOVA and Student's *t*-test were used to analyze the data. Statistics: #p=0.05, *p<0.05; **p<0.01; ***p<0.001 compared with non-TG.

ACKNOWLEDGMENTS

We thank Julia Ober-Blöbaum and Jon Laman for critical reading of the manuscript, Cerithsa Martina, Inge Brouwers-Haspels and Sabina Onderwater for expert technical assistance and Robert Jack for the kind gift of floxed IL-10Rα mice. This work was supported by grants from the Netherlands Organization for Scientific Research (NWO, VIDI 917-76-365) and the Landsteiner Foundation for Blood Transfusion Research (LSBR, 0414F) to B.E.C.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Flow cytometry

Cells were surface stained at 4°C for 45-60 min with the following monoclonal anti-mouse antibodies: CD11c-A488 (N418), MHCII-A647 (M5/114.15.2), IL-10Rα (CD210) -biotin (1B1.3a), EpCam-PerCP-Cy5.5 (G8.8) from BioLegend, CD45-PE-Cy7 (30-F11), NK-FITC (Dx5), SIRPα-APC (P84), CD103-PE (2E7), CD86-PE (GL1), CD40-biotin (MR1), CD3-APC (145-2C11), CD4-PerCP-Cy5.5 (RM4-5), CD8-APC-Cy7 (53-6.7), CD69-biotin (H1.2F3), CD25-APC (3C7) and CD62L-FITC (MEL-14) from BD Pharmingen, PD-L1-biotin (MIH5) and PD-L2-PE (TY25) from eBioscience. A commercially available kit was employed for FoxP3 staining (FJK-165, eBioscience). PE-Cy7-conjugated streptavidin was used to visualize biotin-conjugated antibodies (BD Pharmingen). Apoptosis staining was performed using the Annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturer's instruction. Intracellular staining with Langerin-A488 or -A647 (929.F3, Dendritics) or Bcl2-PE (3F11, BD Pharmingen) were performed in 0.5% saponinebuffer (Sigma) after fixing the cells with 4% PFA for 15min at room temperature. Appropriate isotype controls were utilized when necessary. Cells were analyzed on a FACS Canto (BD Biosciences) using FlowJo analysis software.

Irritant response

The irritant response was elicited by applying 25μL of 1% (vol/vol) croton oil (Sigma-Aldrich) in olive oil onto the dorsal side of the right ear. Ear thickness was determined before and up to 72h after irritant application using a micrometer (Mitutoyo).

Histology

CD3 staining was performed as described in the material and methods with primary antibody rat anti-mouse CD3 (KT3, Abcam) and rabbit anti-rat secondary antibody (Dako).

SUPPLEMENTARY FIGURES AND TABLE

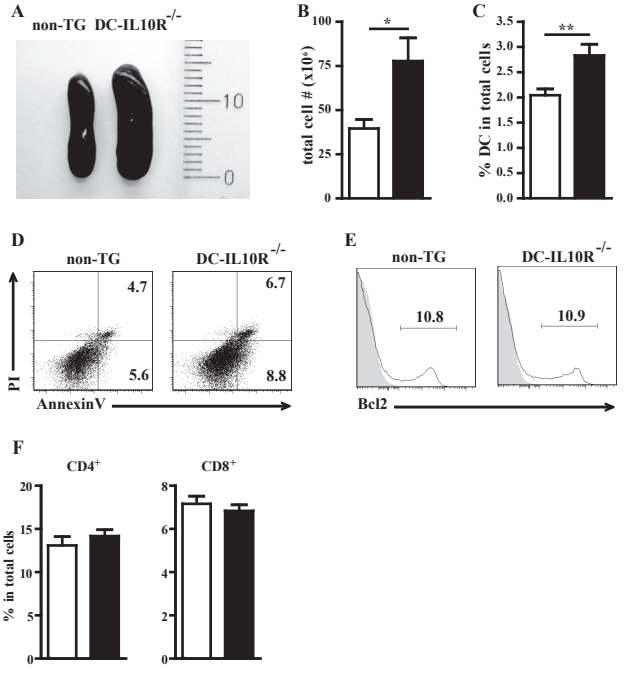

Figure E1. Splenomegaly with increased DC frequency in DC-IL10R$^{-/-}$ mice. **(A)** Enlarged spleen in DC-IL10R$^{-/-}$ as compared to non-TG control. **(B)** Spleen total cell number. **(C)** CD11c^{+}MHCII^{+} DC frequency. **(D)** DC from spleen were stained for AnnexinV and incubated with propidium iodide (PI) to determine apoptosis. Cells were gated on CD11c^{+}MHCII^{+}. **(E)** Bcl2 expression was assessed in DC activated with LPS overnight. Cells were gated on CD11c^{+}MHCII^{+}. Black lines represent Bcl2 expression and grey diagrams the isotype control. **(F)** Splenic CD3^{+}CD4^{+} and CD3^{+}CD8^{+} T cell frequencies as determined by flow cytometry. □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals.

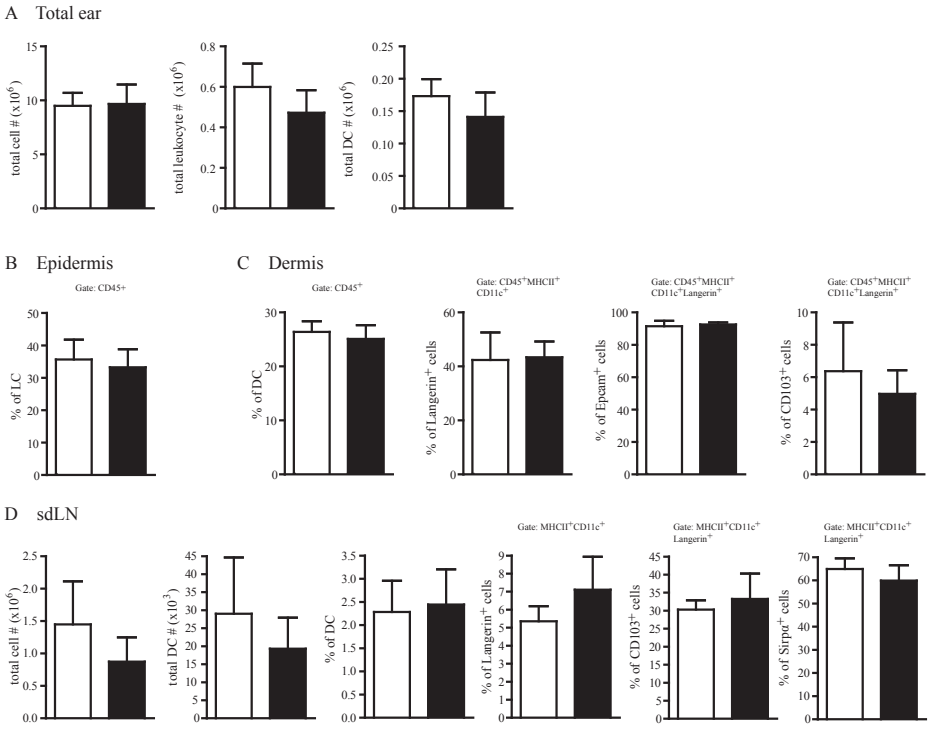

Figure E2. Similar total cell and DC numbers and frequencies in ears and sdLN of DC-IL10R$^{-/-}$ mice. **(A)** Total cell, leukocyte and DC numbers in whole ear. Cell suspensions were stained with CD45, MHCII and CD11c. **(B)** CD45^{+}MHCII^{+}Langerin^{+} LC frequency in the epidermis. **(C)** Total DC and DC subset frequencies in dermis. Dermal cell suspensions were gated on CD45^{+}MHCII^{+}CD11c^{+} cells and analyzed for Langerin expression. To discriminate between migratory LC and Langerin^{+} dermal DC, expression of EpCam and CD103 was assessed. **(D)** Total cell and DC number and frequencies of DC subsets in sdLN. MHCII^{+}CD11c^{+} DC were stained for Langerin, CD103 and Sirpα to identify all Langerin^{+} LN DC and to resolve LC and Langerin^{+}CD103^{neg} from Langerin^{+}CD103^{+} dermal DC. □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals.

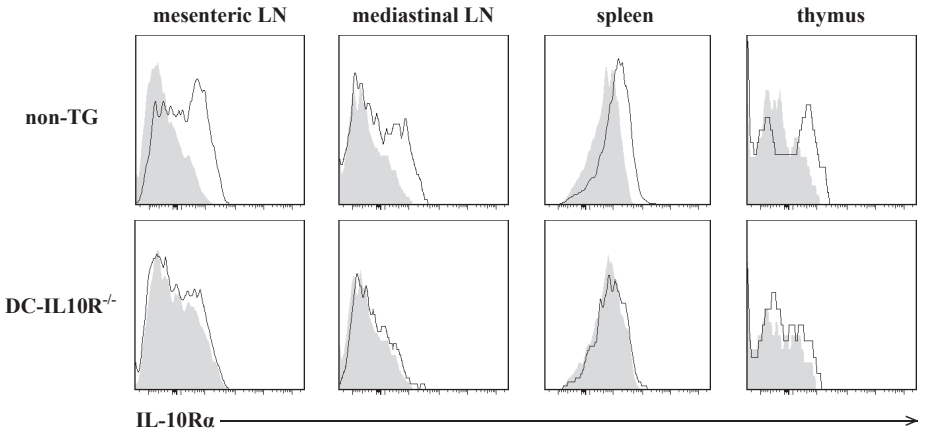

Figure E3. The IL-10R**α** is selectively and efficiently deleted on DC in DC-IL10R-deficient mice. Mesenteric LN, mediastinal LN, spleen and thymus single cell suspensions were stained for IL-10Rα expression and gated on $MHCII^{+}CD11c^{+}$ DC. Black diagrams represent the IL-10Rα staining and filled grey diagrams the isotype control.

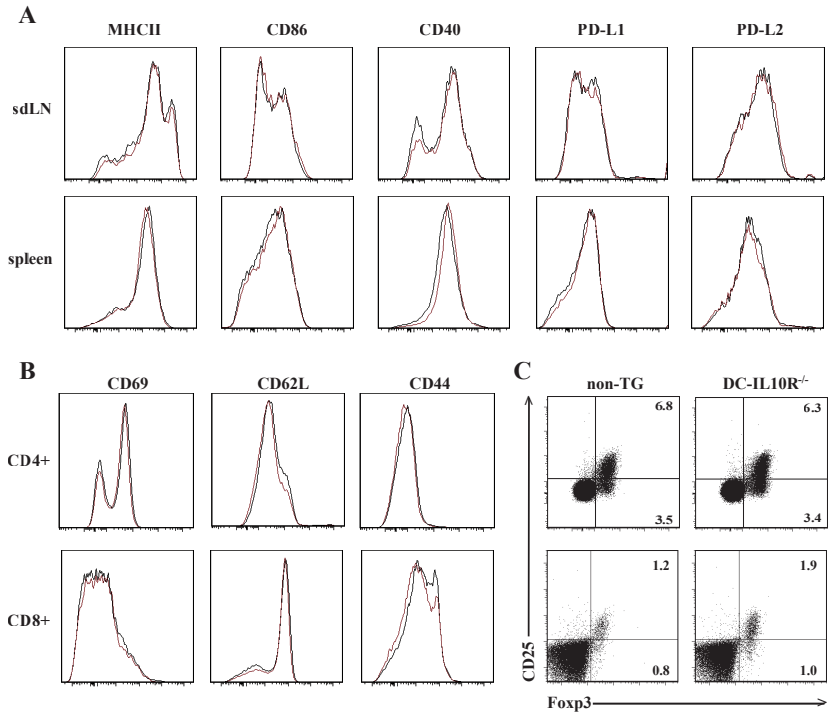

Figure E4. Lack of spontaneous DC maturation and T cell activation in DC-IL10R$^{-/-}$ mice as compared to non-TG in the steady state. **(A)** sdLN and spleen cells were stained for CD11c, MHCII, CD86, CD40, PD-L1 and PD-L2. Diagrams shown are gated on MHCII^{+}CD11c^{+} cells. **(B)** Activation of CD3^{+}CD4^{+} and CD3^{+}CD8^{+} T cells from spleen was evaluated by staining for CD69, CD62L and CD44 and **(C)** CD25 and Foxp3. In **(A)** and **(B)** black and red diagrams represent respectively non-TG and DC-IL10R$^{-/-}$ mice.

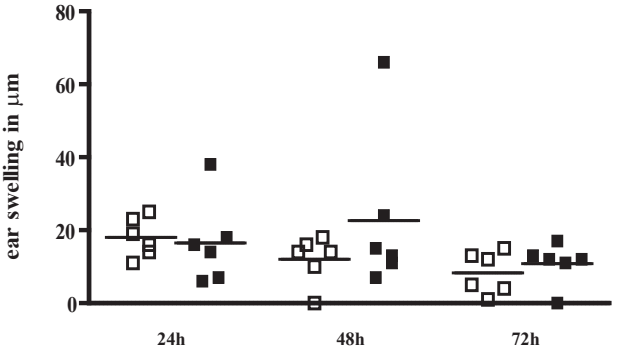

Figure E5. IL-10 control of DC is dispensable during an innate immune response. Irritant responses in non-TG and DC-IL10R$^{-/-}$ mice were elicited by topical application of 1% croton oil onto the ears. Ear swelling was measured after 24, 48 and 72h. □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals.

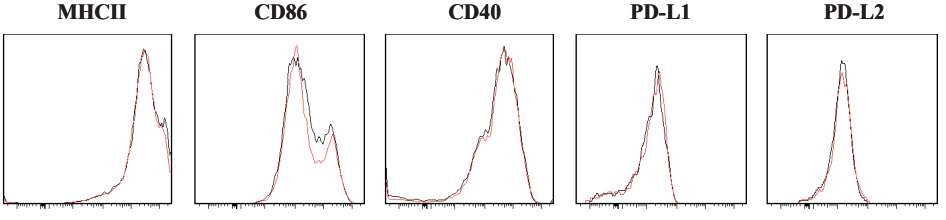

Figure E6. Similar maturation marker expression on *in vitro* activated IL10R-deficient DC. Splenic DC were isolated from DC-IL10R$^{-/-}$ and non-TG control mice and matured with LPS overnight. DC were stained with MHCII, CD86, CD40, PD-L1 and PD-L2 for flow cytometry. Black lines represent non-TG control and red lines represent DC-IL10R$^{-/-}$ mice.

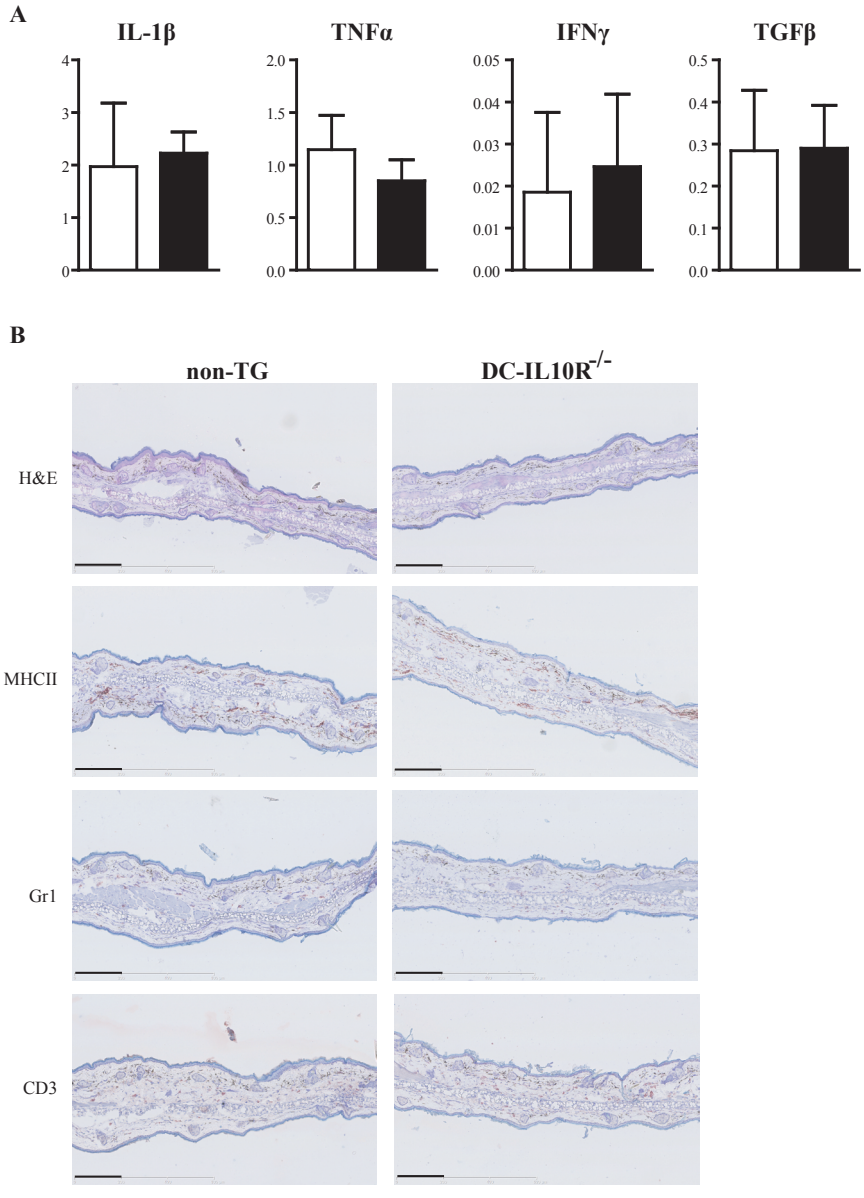

Figure E7. Similar cell composition and cytokine levels in steady state ears of DC-IL10R$^{-/-}$ as compared to non-TG mice. **(A)** Cytokine mRNA expression. □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals. **(B)** H&E, anti-MHCII, anti-Gr1 and anti-CD3 staining on cryosections. Size bars represent 200µm.

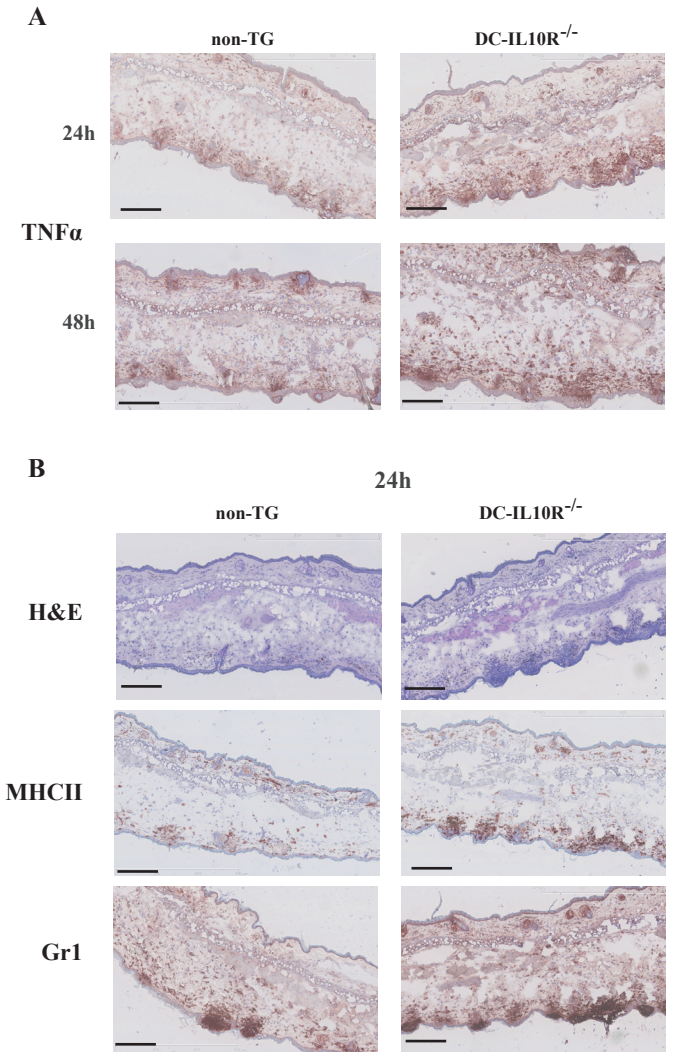

Figure E8. Increased inflammation and recruitment of innate cells in ears of DC-IL10R$^{-/-}$ mice as compared to non-TG. Cryosections of ears from non-TG and DC-IL10R$^{-/-}$ animals prepared at 24 or 48h after hapten challenge were stained **(A)** with anti-TNF-α antibody, **(B)** with H&E, anti-MHCII or anti-Gr1 as indicated and **(D)** with anti-CD3 antibody. Size bars represent 200µm. **(C)** Total CD45^{+}MHCII^{neg}CD3^{neg}NK^{+} NK cell number and percentage were determined in ears 24 or 48h after hapten challenge by flow cytometry. □ : non-TG and ■ : DC-IL10R$^{-/-}$ animals.

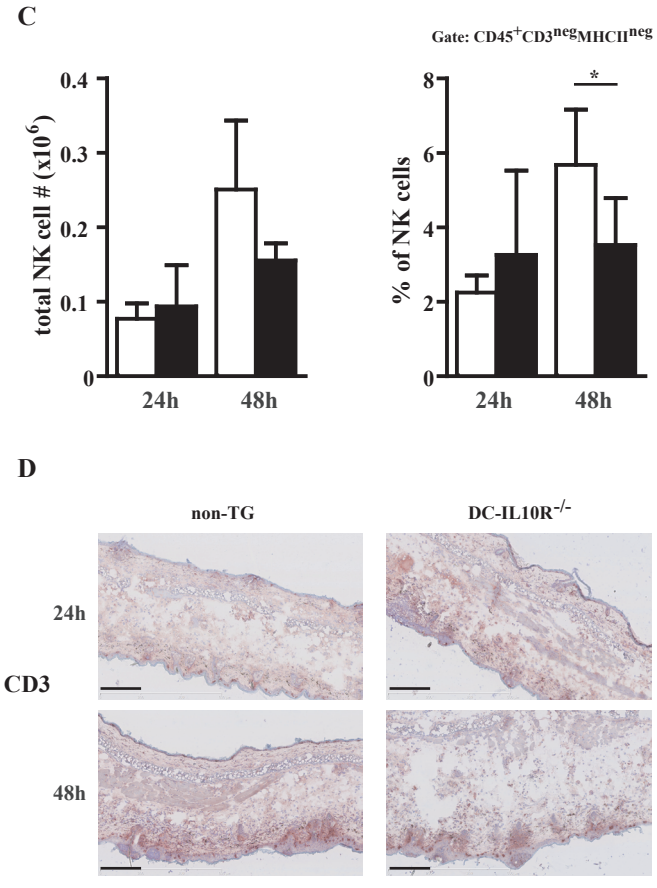

Figure E8. (*Continued*).

Table E1. RT-qPCR primers.

Gene	Forward primer sequence	Reverse primer sequence
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
TNF-α	CCACGTCGTAGCAAACCAC	TTTGAGATCCATGCCGTTG
IL-10	CAGAGCCACATGCTCCTAGA	GTCCAGCTGGTCCTTTGTTT
IFN-γ	ATCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGGTA
CD3e	AACACGTACTTGTACCTGAAAGCTC	GATGATTATGGCTACTGCTGTCA
Tbet	TCAACCAGCACCAGACAGAG	AAACATCCTGTAATGGCTTGTG
Foxp3	AGAAGCTGGGAGCTATGCAG	GCTACGATGCAGCAAGAGC

CHAPTER 3

LACK OF IL-10 SIGNALING IN DENDRITIC CELLS ENHANCES ANTI-*LEISHMANIA MAJOR* IMMUNITY

Mathilde J.H. Girard-Madoux[1,4], Kordula Kautz-Neu[2,4], Esther von Stebut[2,5], and Björn E. Clausen[1,3,5]

[1]Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands, [2]Department of Dermatology and [3]Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University, 55131 Mainz, Germany

[4,5]Both first and senior authors contributed equally to this work

Submitted

CHAPTER 4

IL-10 CONTROL OF DENDRITIC CELLS IS ESSENTIAL TO MAINTAIN IMMUNE HOMEOSTASIS IN THE SMALL INTESTINE

Mathilde J.H. Girard-Madoux[1], Julia L. Ober-Blöbaum[1,2], Junda M. Kel[1], Dicky J. Lindenbergh-Kortleve[3], Janneke N. Samsom[3,4], and Björn E. Clausen[1,2,4]

[1]Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands, [2]Institute for Molecular Medicine, Johannes Gutenberg-University, 55131 Mainz, Germany and [3]Department of Pediatric Gastroenterology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands

[4]Both senior authors contributed equally to this work

Submitted

CHAPTER 5

REDUCED AIRWAY HYPERREACTIVITY IN THE PRESENCE OF A SIMILAR TH2 RESPONSE DURING ASTHMA IN MICE LACKING IL-10 SIGNALING IN DENDRITIC CELLS

Mathilde J.H. Girard-Madoux[1], Julia L. Ober-Blöbaum[1,2], Mirjam Kool[3], Rudi W. Hendriks[3], and Björn E. Clausen[1,2]

[1]Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands, [2]Institute for Molecular Medicine, Johannes Gutenberg-University, 55131 Mainz, Germany and [3]Department of Pulmonary Medecine, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands

Manuscript in preparation

CHAPTER 6

TGF-β IS REQUIRED TO MAINTAIN THE POOL OF IMMATURE LANGERHANS CELLS IN THE EPIDERMIS

Junda M. Kel[1], Mathilde J.H. Girard-Madoux[1,2], Boris Reizis[3] and Björn E. Clausen[2]

[1] Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands,
[2] Department of Cell Biology and Histology, AMC, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands,
and [3] Department of Microbiology and Immunology, Columbia University Medical Center, New York, NY 10032, USA

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ABSTRACT

The pivotal role of TGF-β in Langerhans cell (LC) development has been previously established in TGF-β deficient mice, which lack epidermal LC. As to whether TGF-β also governs LC homeostasis and function remains elusive. To assess the role of TGF-β mediated control of cutaneous dendritic cells (DC) *in vivo*, we generated mice with a conditional knockout of the TGF-β receptor 1 (TβR1) under a DC-specific promotor (DC-TβR1^{del} mice). While initial LC seeding occurred in DC-TβR1^{del} mice, the cells disappeared from the epidermis during the first week of life. TβR1-deficient LC demonstrated spontaneous maturation and gained migratory potential, based on increased surface expression of MHC class II, co-stimulatory molecules and CCR7, and down-regulation of E-cadherin. In parallel to their early loss from the epidermis, migrating LC were reduced in the dermis and skin-draining lymph nodes of adult DC-TβR1^{del} mice, while the number of Langerin^{+} dermal DC was similar to wild type. In the absence of LC, low dose contact hypersensitivity in DC-TβR1^{del} mice was significantly diminished. On the other hand, ear swelling was restored to wild type levels when a higher hapten dose was applied to efficiently target TβR1-deficient dermal DC. In conclusion, TGF-β inhibits *in vivo* LC maturation and migratory phenotype, identifying TGF-β as a critical factor controlling LC homeostasis in the steady state.

Key words: Dendritic cells, Langerhans cells, Skin, Transforming growth factor β, Transgenic/Knockout mice.

Abbreviations: DC, dendritic cells; LC, Langerhans cells; TGF-β, transforming growth factor beta-1; TβR1, TGF-β receptor 1; LN, lymph node; CHS, contact hypersensitivity.

INTRODUCTION

The skin forms a first protective barrier between the host and its external environment and is equipped with a sophisticated system of immune surveillance to combat invading pathogens and control peripheral tolerance. Skin immunity relies on a network of sentinel dendritic cells (DC) in both the epidermis and dermis.[1,2] The DC populating the epidermis, known as Langerhans cells (LC), can be identified by the expression of Langerin, a C-type lectin receptor associated with formation of the characteristic Birbeck granules in LC.[3] Dermal DC belong to a broader subset of interstitial DC characterized by the expression of CD11c and MHC class II (MHCII) in the absence of macrophage markers. The DC population in the dermis is heterogeneous and comprises Langerin^{+} and Langerin^{neg} cells.[4] Bone marrow (BM) chimera models and the different repopulation kinetics of LC and dermal DC demonstrated that a fraction of the Langerin^{+} DC in the dermis are not transmigrating LC but represent a distinct subset of DC residing in the dermis itself.[5-7] The two Langerin^{+} skin DC populations can be distinguished by the differential expression of the adhesion molecule EpCam and the integrin CD103. Both LC and (Langerin^{+}) dermal DC are able to migrate to the skin draining lymph node (LN) in the steady state as well as under inflammatory conditions.[8]

In the steady state, LC in the epidermis have a slow turnover and are sessile but active, continuously probing their environment for invading pathogens by extending and retracting their dendrites.[9,10] During inflammation the local production of pro-inflammatory cytokines, such as IL-1β and TNF-α by keratinocytes, drives LC maturation and mobilization towards the draining LN within 3 to 4 days.[11,12] LC down-regulate E-cadherin to dissociate from neighboring cells and up-regulate α6-integrin to enable passage through the basal membrane in order to access the dermal layer of the skin. LC then enter the dermal lymphatics, and finally the LN T cell area, in a CCR7-dependent way.[13-15] DC are present in the dermis as individual cells as well as in clusters and, in contrast to the more static LC network, can freely move around. Dermal DC are replaced every 10-15 days and mobilize quickly upon local stimulation; emigrated dermal DC can be detected in the draining LN already 1 to 2 days after activation.[12] As for LC, the migration of (Langerin^{+}) dermal DC towards cutaneous LN is CCR7-dependen.[7] Although it is largely unknown what triggers the emigration of LC and dermal DC from the skin in the steady state, the concept that a continuous flow of immature DC from the skin to the draining LN contributes to the maintenance of peripheral tolerance has been generally accepted.[16] Indeed, Hemmi *et al* were the first to demonstrate that a cutaneous self-antigen is transported from the skin towards the draining LN by transforming growth factor-beta1 (TGF-β1) dependent cells under steady state conditions.[17]

TGF-β1 is an essential factor in LC development, because the epidermis of TGF-β1 deficient mice is devoid of LC.[18] Recently, it was demonstrated that autocrine production of TGF-β1 is responsible for LC development in the murine epidermis.[19] The importance of TGF-β signaling in LC lineage decisions has been further highlighted in a knockout model for Id2, a signaling molecule downstream of the TGF-β receptor, in which LC

development was abolished.[20] TGF-β1 signals exclusively through a tetrameric complex of two transmembrane receptors, namely, TGF-β receptor 1 (TGF-βR1) and TGF-β receptor 2 (TGF-βRII). Initial complex formation of TGF-β1 with TGF-βRII leads to phosphorylation of TGF-βR1 on serine-threonine residues and propagation of the intracellular signal into the nucleus via Smad proteins.[21,22]

Ample *in vitro* evidence has been collected with respect to the inhibitory effect of TGF-β on DC activation and maturation. Monocyte-derived DC expanded in the presence of IL-10 and TGF-β demonstrated reduced IL-12 and IL-23 production and favored T cell anergy and the induction of FoxP3$^+$ regulatory T cells.[23] In a similar way tolerogenic DC can be gerenated from mouse bone marrow (BM) that increased graft survival after adoptive transfer into transplanted hosts.[24] In addition, hapten-induced monocyte-derived DC maturation and apoptosis were suppressed by TGF-β, unless signals of tissue damage were mimicked by addition of ATP.[25]

Although these *in vitro* experiments suggest an important role for TGF-β in controlling DC maturation, the contribution of TGF-β signaling to govern LC and dermal DC homeostasis and function *in vivo* is largely unknown. To this aim, we have generated mice with a conditional knockout of TGF-βR1 under a DC-specific promotor, which results in deletion of TGF-β receptor signaling in all CD11c$^+$ (skin) DC (DC-TβR1^{del} mice). Initial seeding of LC was observed in the epidermis of DC-TβR1^{del} mice, but in the absence of TβR1 expression the cells spontaneously matured, gained increased migratory capacity and gradually disappeared from the epidermis. In the absence of LC, low dose contact hypersensitivity (CHS) responses were reduced. In contrast, the phenotype and function of (Langerin$^+$) dermal DC were unaffected. Taken together, beyond the important role of TGF-β in LC development, our findings provide the first evidence that TGF-β signals are critical to control LC maturation and emigration from the epidermis in the immunological steady state.

RESULTS

Delayed CD11c-Cre mediated target gene recombination in cutaneous DC

To determine CD11c-Cre transgene activity in cutaneous DC, the CD11c-Cre mutation[26] was crossed to Rosa26-tdRFP Cre reporter mice.[27] From day 0 to 3 after birth 65.6 ± 10.1% of the MHCII$^+$ LC that seeded the epidermis expressed red fluorescent protein (RFP), although at low levels. In comparison, RFP intensity in adult mice was enhanced about 4 times and the frequency of RFP-positive LC increased to 78 ± 4.5% (Figure 1A and Table SI). Analysis of the MHCII$^+$ cells in the dermis of neonatal animals revealed 46.5 ± 6.7% RFP$^+$ cells, while in adult mice 80.1 ± 6.7% of CD11c$^+$MHCII$^+$ dermal DC expressed RFP with doubled intensity (Figure 1B). In conclusion, the CD11c-Cre transgene becomes gradually more active in cutaneous DC, including LC, but Cre-mediated RFP expression was not yet detectable in about 35% of LC and 55% of MHCII$^+$ dermal DC immediately after birth.

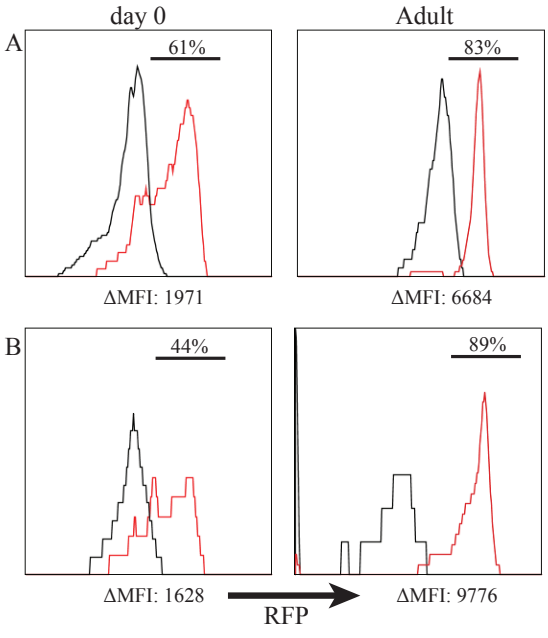

Figure 1. Delayed CD11c-Cre transgene activity in cutaneous dendritic cells. To determine CD11c-Cre transgene activity in skin DC, we crossed CD11c-Cre to Rosa26-tdRFP Cre reporter mice. **(A)** Part of the MHCII^{+} LC expressed RFP already on day 0; although RFP intensity (ΔMFI) and frequency were diminished as compared to adult animals. **(B)** Similarly, RFP expression was reduced in MHCII^{+}CD11c^{low} cells in the dermis of neonatal animals as compared to MHCII^{+}CD11c^{+} cells in adult mice. The epidermis **(A)** and dermis **(B)** of n=9 neonatal (days 0-3 after birth) and n=4 adult mice were analyzed. Representative histograms are depicted (black line: CD11c-Cre/Rosa26-tdRFP mice, grey line: negative controls) and detailed ΔMFI data per time point are summarized in Supplementary Table SI.

Gradual loss of TGF-βR1-deficient LC from the epidermis in the first week of life

The assessment of TGF-β mediated control of LC homeostasis *in vivo* has been hampered by the critical role of TGF-β in LC ontogeny. Thus, when we crossed CD11c-Cre mice with animals carrying a floxed TGF-βR1 allele, thereby generating mice harboring DC that cannot respond to TGF-β (DC-TβR1^{del} mice), we expected to find an empty LC compartment. However, in the epidermis of neonatal DC-TβR1^{del} mice MHCII^{+} cells were present, although LC numbers were reduced about 50% as compared to non-transgenic littermates (Figure 2A). Recently, it was reported that a single wave of precursors populates the epidermis during embryonic life, which subsequently differentiate into LC. These cells then undergo a massive proliferative burst during the first week of life to form the typical LC network.[28,29] Indeed, we observed a 4-fold expansion of LC in non-transgenic controls from 0,54 ± 0,14% on day 0 to 2,42 ± 0,12% on day 3. Although a reduced number

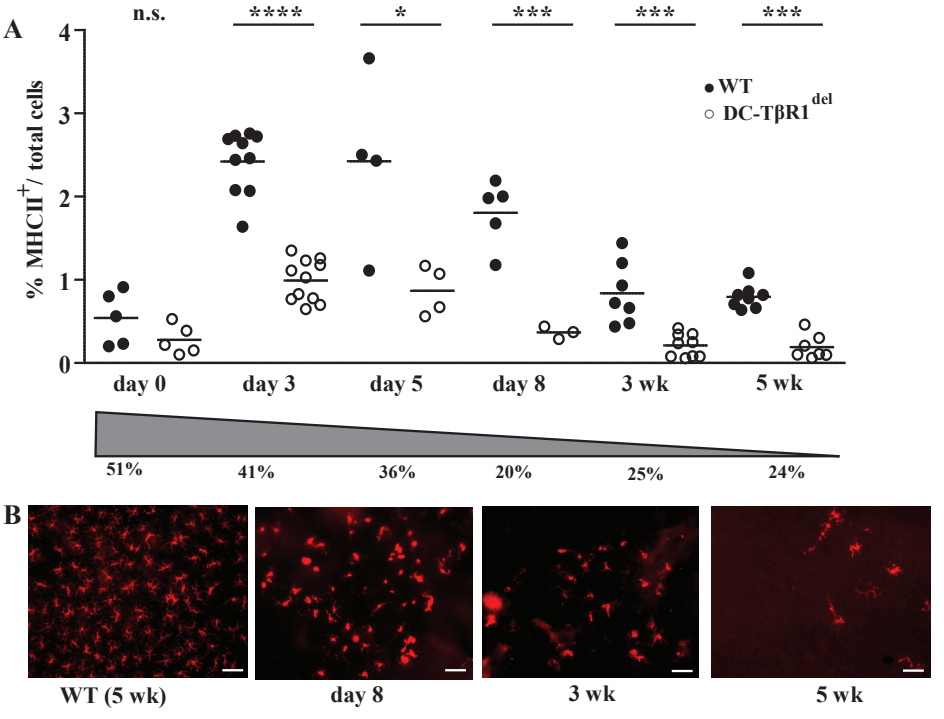

Figure 2. Gradual loss of TβR1-deficient LC from the epidermis. The number of MHCII$^+$ cells in the epidermis of DC-TβR1^{del} mice and non-transgenic littermates was assessed. **(A)** Gradual loss of LC from the epidermis of DC-TβR1^{del} mice as compared to non-transgenic controls during the first week of life as demonstrated by flow cytometry. At each time point n=4-11 animals were analyzed. **(B)** MHCII staining of epidermal sheets at the indicated time points after birth. One representative epidermal ear sheet is shown from n=5 mice analyzed at each time point (scale bar: 50 mm).

of LC was present in the epidermis at day 0 (0,28 ± 0,08), a comparable 4-fold increase in the number of cells was detected in DC-TβR1^{del} mice, resulting in 0,99 ± 0,08% of LC on day 3. Intriguingly, afterwards the LC population gradually disappeared and only an occasional patch of LC remained visible in the epidermis of 5 week-old DC-TβR1^{del} mice (Figure 2B). These findings suggest that TGF-β signals are not only critical during LC ontogeny, but are also required to maintain the cells in the epidermis.

TβR1-deficient LC spontaneously mature in situ and gain emigration potential

To elucidate the fate of TβR1-deficient LC after seeding of the epidermis in DC-TβR1^{del} mice, the cells were analyzed directly (day 0) and on day 3 after birth by flow cytometry. On day 0 Langerin expression was undetectable in MHCII$^+$ LC in the epidermis, as has been previously reported[30] (Figure 3A). Moreover, while up-regulation of Langerin expression could be detected in LC of non-transgenic littermates by day 3, this was significantly

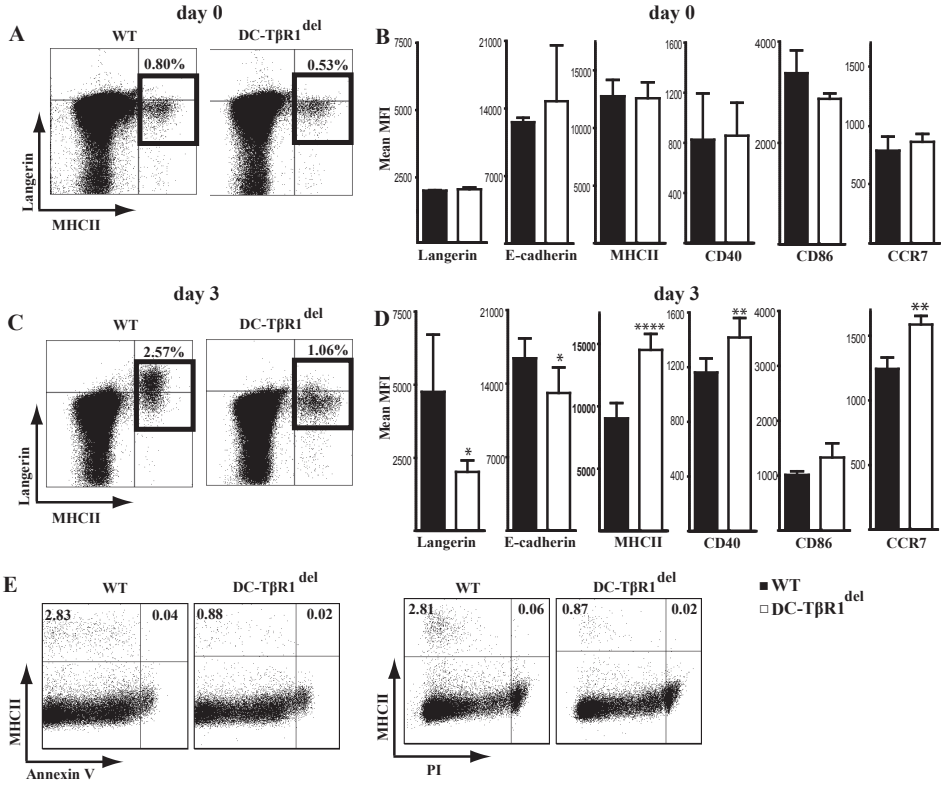

Figure 3. Lack of TGF-β signaling results in spontaneous LC maturation. Single cell suspensions from the epidermis of DC-TβR1^{del} mice and non-transgenic littermates were prepared directly after birth or 3 days later and LC were identified as MHCII^{+} cells, as indicated by the square gates in A and C (one representative out of 2-8 animals is depicted). **(A)** LC isolated from the epidermis of DC-TβR1^{del} mice and non-transgenic littermates immediately after birth do not express Langerin. **(B)** While upregulation of Langerin expression was detectable in LC isolated from non-transgenic controls 3 days after birth, this was not observed in TβR1-deficient LC. **(C)** On day 0 no differences were observed in the expression (average MFI ± SD) of the indicated maturation markers. **(D)** In contrast, on day 3 LC in DC-TβR1^{del} mice expressed increased levels of CD86, CD40 and CCR7 and reduced levels of Langerin and E-cadherin, as compared to controls. N=2-8 mice were analyzed at each time point and detailed MFI data are summarized in Table SII. **(E)** Analysis of Annexin V and PI staining on day 3 did not reveal any signs of LC apoptosis. One representative of n=2-5 animals is shown.

diminished in DC-TβR1^{del} mice (Figure 3C). Immediately after birth TβR1-deficient LC displayed an immature phenotype similar to wild type (Figure 3B), but on day 3 surface expression of MHCII, CD86 and CD40 was enhanced as compared to control LC (Figure 3D and Table SII). Concomitant with the augmented expression of maturation markers, the cells significantly down-regulated E-cadherin and up-regulated CCR7 expression, suggesting that LC migration is triggered in the absence of TGF-β signaling

(Figure 3D and Table SII). Although the number of LC in the epidermis of DC-TβR1^{del} animals was considerably diminished seven days after birth, the remaining cells showed significantly increased expression of CD86 and CCR7 (Table SII). Corroborating these data, LC survival in the epidermis was not affected in the absence of TGF-β signals, since we failed to detect any signs of LC apoptosis in 3 day-old DC-TβR1^{del} mice (Figure 3E). Taken together, our observation of spontaneous maturation and increased migratory potential of TβR1-deficient LC establishes TGF-β as an essential factor to control the immature state of LC and to promote their persistence in the steady state epidermis.

TGF-β signals do not control dermal DC homeostasis and maturation in the steady state

Our knowledge on dermal DC development and homeostasis in neonatal mice is currently limited. Therefore, we isolated the dermis immediately and on days 3 and 5 after birth. In wild type neonatal dermis, MHCII^{+}CD11c^{low} cells were present and increased in number with age (Figure 4A and S1A). Surface expression of CD11c by dermal DC was detectable starting at 1 week of age (Figure 4B and S1B). To evaluate a possible role for TGF-β to

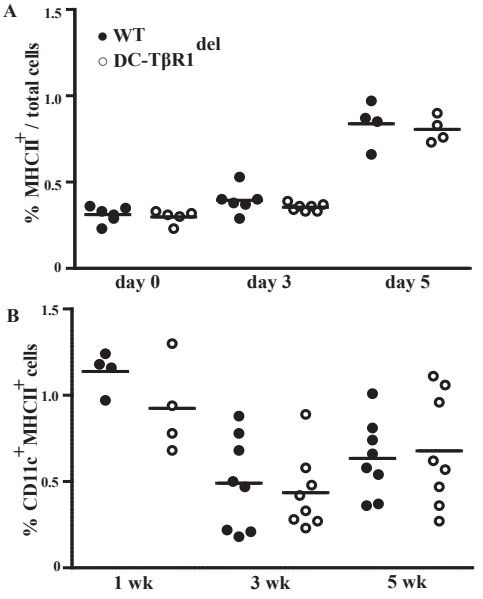

Figure 4. Dermal DC homeostasis is TGF-β independent. The frequency of DC in dermal cell suspensions from DC-TβR1^{del} mice (open symbols) and controls (filled symbols) was determined by flow cytometry. **(A)** On days 0, 3 and 5 increasing numbers of MHCII^{+}CD11c^{low} cells were collected from the dermis and no difference between DC-TβR1^{del} and non-transgenic littermates was observed (gating strategy as indicated in Figure S1A). **(B)** CD11c^{+} DC were first detectable in one week-old animals and equal numbers of MHCII^{+}CD11c^{+} DC were present in the dermis of DC-TβR1^{del} mice and controls at all time points analyzed (gating strategy as in Figure S1B). N=4-6 mice were analyzed at each time point.

govern DC homeostasis in the dermis, DC-TβR1^{del} mice and controls were compared, but DC frequencies and surface expression of MHCII and CD86 were similar in both groups (Figure 4 and Table SIII).

To distinguish Langerin^{+}EpCam^{neg} dermal DC and Langerin^{+}EpCam^{+} LC,[5,6] we analyzed the expression of Langerin as well as EpCam by MHCII^{+} dermal cells. In accordance with the loss of LC from the epidermis during the first week of life, the number of transmigrating LC was significantly decreased in the dermis of adult DC-TβR1^{del} mice (Figure 5A and B). In contrast, similar numbers of Langerin^{+} dermal DC were present in DC-TβR1^{del} animals and controls, confirming that Langerin^{+} DC development in the

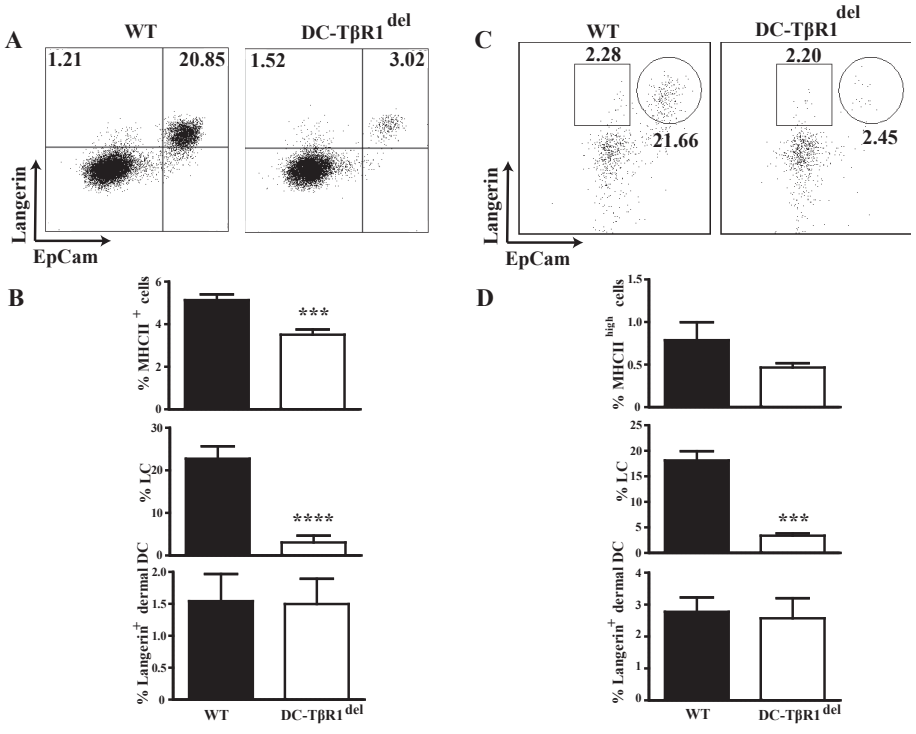

Figure 5. Steady state migration of cutaneous DC in DC-TβR1^{del} mice. **(A)** Langerin and EpCam expression were evaluated after gating on MHCII^{+} dermal cells to define transmigrating LC (upper right quadrant) and Langerin^{+} dermal DC (upper left quadrant). **(B)** The frequency of MHCII^{+} cells was significantly reduced in adult DC-TβR1^{del} mice as compared to controls due to the absence of transmigrating LC, while Langerin^{+} dermal DC were not affected. **(C)** Langerin and EpCam expression on gated MHCII^{high} skin immigrants in the draining LN to resolve migrating LC and Langerin^{+} dermal DC. **(D)** Skin draining LN cells were isolated from adult DC-TβR1^{del} and wild type animals and although the frequency of total MHCII^{high} cells was not significantly different, migration of Langerin^{+}EpCam^{+} LC into the LN of adult DC-TβR1^{del} mice was diminished as compared to controls. In contrast, Langerin^{+}EpCam^{neg} dermal DC immigrants reached the LN similarly to wild type mice. One out of two independent experiments is presented, 8-12 week-old mice (n=3-5 per group) were tested and average cell frequencies (±SD) are depicted.

dermis is TGF-β independent, as has recently been demonstrated in TGF-β1 deficient mice.[31] Moreover, in line with the profound LC deficiency, we observed a reduced accumulation of LC descendants within the population of MHCII^{high} skin immigrants in the LN of adult DC-TβR1^{del} mice as compared to controls, while Langerin^{+} DC from the dermis efficiently reached the nodes (Figure 5C and D). Taken together, the frequency, state of maturation and steady state migration of (Langerin^{+}) dermal DC was similar to wild type, suggesting that the lack of TβR1 signaling does not affect the homeostasis of this cutaneous DC population.

Dermal DC function in contact hypersensitivity is not regulated by TGF-β

To determine whether the lack of TGF-β mediated control of dermal DC impairs their ability to contain a T-cell dependent immune response, CHS to topical hapten was induced in DC-TβR1^{del} mice. To control for the profound reduction in LC numbers in adult DC-TβR1^{del} animals, we included Langerin-DTR mice treated with DT on day -14 to selectively deplete LC, but not Langerin^{+} dermal DC during the sensitization phase.[5,31,32] In response to a low dose of oxazolone, DC-TβR1^{del} as well as LC depleted Langerin-

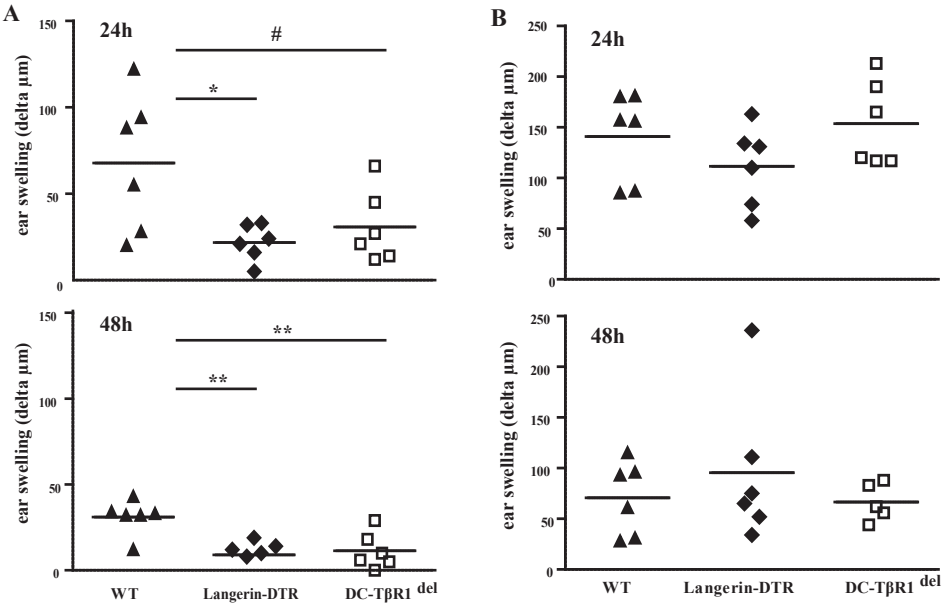

Figure 6. TGF-β signaling does not control dermal DC function in CHS. CHS responses elicited by sensitization and challenge with a low dose **(A)** or a high dose **(B)** of Oxazolone were compared between wild type controls, LC-depleted Langerin-DTR and DC-TβR1^{del} mice. Ear swelling was evaluated 24h and 48h after hapten challenge. **(A)** At a low dose of Oxazolone the CHS response was significantly diminished in both Langerin-DTR and DC-TβR1^{del} mice as compared to wild type. **(B)** Comparable ear swelling was observed in all groups using a high dose of Oxazolone. For both low dose **(A)** and high dose **(B)** CHS responses at least 5 mice per group were tested and one out of two independent experiments is presented.

DTR mice showed significantly diminished CHS responses as compared to wild type (Figure 6A), which corroborates our previous work that LC are required for efficient initiation of CHS [33,34]. At the same time, dermal DC can contribute to traffic hapten to the draining LN, although LC and dermal DC may migrate with differential kinetics [12,34,35]. In addition, Shkloskaya *et al* demonstrated that Langerin^{+} dermal DC participate in the transport of contact sensitizer [36]. To investigate whether TGF-β signals control the function of DC in the dermis during CHS, a higher hapten dose was topically applied to efficiently target dermal DC. This elicited similar ear swelling in wild type, LC depleted Langerin-DTR and, in particular, DC-TβR1^{del} animals (Figure 6B). Neither the amount nor the duration of ear swelling were enhanced by TβR1-deficient dermal DC as compared to appropriate controls, demonstrating that TGF-β signaling does not govern (dermal) DC function in CHS.

DISCUSSION

For a long time, TGF-β has been well known for its vital role in LC development.[16] Here we describe that once the cells have seeded the epidermis, TGF-β is also a critical factor to maintain the pool of immature LC in the epidermis. We crossed CD11c-Cre transgenic to floxed TβR1 mice to generate DC-TβR1^{del} mice, in which the DC compartment is insensitive to TGF-β signals. Considering the importance of TGF-β in LC ontogeny, we anticipated that the epidermis would be devoid of LC. Indeed, the epidermal compartment of adult DC-TβR1^{del} animals was empty, except for an occasional patch of residual LC. In contrast, and quite unexpected, we detected significant numbers of LC (about 50% of wild type) in neonatal mice and during the first days of life (Figure 2). These findings corroborate work by others demonstrating a lack of LC in the epidermis of adult Langerin-Cre/TGF-βRII^{del} mice, however, the presence of LC in young (<1 wk of age) mice with LC-specific TGF-βRII deficiency was not addressed in this study.[19] LC seeding of the epidermis in DC-TβR1^{del} mice correlates with partial promotor activity of the CD11c-Cre transgene in newborn animals as we could demonstrate in crosses to Rosa26-tdRFP Cre reporter mice (Figure 1). In neonatal skin, only about half of the LC and dermal DC are RFP(Cre)-positive. This is paralleled by moderate RFP expression in only a fraction of splenic DC right after birth, which increases approximately four times in intensity in the >95% RFP-positive DC detectable in adult spleen[26] (Figure S2). The increasing expression of Cre during the first week of life created the opportunity to assess TGF-β mediated control of LC that had seeded the epidermis in DC-TβR1^{del} mice. The contiguous network of epidermal LC develops from a single wave of LC precursors that enter the epidermis and subsequently undergo a massive burst of proliferation.[28,29] Indeed, a 4-time increase in LC frequency could be detected in non-transgenic controls between day 0 and 3 after birth (Figure 2). Interestingly, a similar 4-fold expansion was observed in DC-TβR1^{del} animals, suggesting that TβR1 deficiency does not affect the proliferative capacities of LC. Alternatively, the cells seeding and expanding in the epidermis may not yet have lost their TβR1 as suggested by the data shown in figure 1. Intriguingly, the

population of LC in 3 day-old DC-TβR1^{del} mice was gradually lost from the epidermis. Upon investigating the fate of the cells, we failed to detect any signs of LC apoptosis, suggesting that TβR1-deficient LC do not die *in situ*. Instead, the LC that seeded the epidermis displayed an immature phenotype immediately after birth, which was converted into a mature state in the absence of TGF-β signaling by day 3 (Figure 3). Notably, the subtle but significant changes in surface expression levels of MHCII and co-stimulatory molecules three days after epidermal seeding likely reflect physiologic LC maturation under steady state conditions *in vivo*, and are not represented by the large differences in the expression of these molecules observed during artificial LC/DC maturation *in vitro*. Furthermore, the spontaneous *in situ* maturation of TβR1-deficient LC was associated with down-regulation of E-cadherin and increased expression of CCR7, pointing towards an enhanced (e)migratory potential of the cells. Indeed, CCR7 gene activity in DC can be inhibited by TGF-β signaling *in vitro*[37] and lower levels of E-cadherin as well as *de novo* expression of CCR7 are linked to LC dissociation from keratinocytes and migration towards local LN.[15,38]

It has been well established that murine LC emigrate from the epidermis towards the skin-draining LN in the steady state and that the cells can transport self-antigens,[17,39] prompting the hypothesis that this route of continuous LC migration contributes to the maintenance of tolerance.[16,40] Homeostatic LC migration seems to involve partial maturation, since CD86 and CD40 expression are enhanced upon LC arrival in the draining nodes.[41] This observation is in agreement with the spontaneous phenotypic maturation of TβR1-deficient LC *in situ*. In addition, disruption of E-cadherin binding and enhanced β-catenin signaling drive *in vitro* DC maturation towards a regulatory phenotype, including up-regulation of CCR7, but without production of pro-inflammatory cytokines.[42] To what extent these pathways control LC maturation and mobilization *in vivo* awaits further investigation. However, the spontaneous maturation of TβR1-deficient LC was associated with reduced E-cadherin levels and elevated expression of CCR7, suggesting that the cells gain the capacity to migrate out of the epidermis. In conclusion, our data establish TGF-β as an essential factor to control the persistence of immature LC in the epidermis and suggest that TGF-β signaling might be involved in governing steady state LC (e)migration for the maintenance of self-tolerance.

Langerin expression was absent in wild type MHCII^{+} LC at the time of seeding of the epidermis and became up-regulated by day 3 after birth (Figure 3), i.e. once the confluent LC network had been established.[30] In TβR1-deficient LC this up-regulation of Langerin was diminished. Notably, this by itself does not alter LC function or differentiation as has been demonstrated in Langerin knockout animals.[43] Rather it supports the crucial role of TGF-β in the induction of Langerin expression by LC.[3] In contrast to LC, the number of (Langerin^{+}) dermal DC in DC-TβR1^{del} animals were similar to wild type, indicating that Langerin expression and (Langerin^{+}) DC development in the dermis is TGF-β independent, as has been demonstrated in TGF-β1 deficient mice.[31] Furthermore, the immature phenotype of TβR1-deficient dermal DC *in situ* suggests that the lack of

TGF-β signaling does not affect the maturation and homeostasis of this cutaneous DC population.Therefore, it was important to assess the role (if any) of TGF-β signals to govern dermal DC function. To this aim, we elicited CHS responses to topical hapten in DC-TβR1^{del} mice and included LC-depleted Langerin-DTR mice to control for the profound absence of epidermal LC (Figure 5). On one hand, ear swelling was significantly reduced in both mouse models as compared to wild type mice, after skin painting of a low hapten dose. This is in agreement with our previous work demonstrating that LC are required for efficient induction of CHS,[33,34] most likely due to ineffective transport of topically applied antigen to the draining LN by reduced numbers of skin DC in DC-TβR1^{del} and LC-depleted Langerin-DTR mice.[34] On the other hand, ear swelling was restored to wild type, but not enhanced, in DC-TβR1^{del} mice upon topical application of a higher dose of oxazolone. This supports independent results that dermal DC can drive CHS responses in the absence of LC[33] and that TGF-β control of (dermal) DC function is dispensable to regulate the magnitude and duration of the reaction, although a role for TGF-β/SMAD3 signals to control inflammation during CHS has been suggested.[44] Moreover, attenuated ear swelling in LC-deficient DC-TβR1^{del} mice argues against a regulatory role of LC in CHS.[45]

Accumulating evidence indicates that tissue-derived signals control the maturation and function of DC and LC. TGF-β produced by splenic stromal cells drives the differentiation of regulatory DC[46] and Rescigno *et al.* demonstrated that the induction of regulatory T cells by interstitial DC is promoted via TGF-β secreted by gut epithelial cells.[47] In the skin, over-expression of RANK-L in the epidermis drives LC-mediated induction of regulatory T cells,[48], while over-expression of CD40L in keratinocytes leads to LC maturation and emigration, accompanied by chronic skin inflammation.[49] Here, we provide the first evidence that TGF-β is also an essential mediator governing LC maturation and mobilization in the immunological steady state, while TGF-β does not contribute to dermal DC homeostasis and function. Beyond its well-established and vital role in LC development our data establish that TGF-β is also a critical factor to maintain the pool of immature LC in the epidermis. Consequently, LC intrinsic impairment of TGF-β signals may account for their slow steady state turnover and migration to local LN for the induction of peripheral tolerance.

MATERIALS AND METHODS

Mice

Bacterial artificial chromosome (BAC) transgenic CD11c-Cre mice[26] were crossed to floxed TGF-βR1 (ALK5) mice, kindly made available by S. Karlsson, Lund University Hospital, Sweden,[50,51] to generate CD11c-Cre/ALK5$^{fl/fl}$ (DC-TβR1^{del}) mice and backcrossed to C57BL/6 for 10 generations. CD11c-Cre mice crossed to Rosa26-tdRFP Cre reporter mice, a generous gift from H.J. Fehling, University Clinics Ulm, Germany,[27] were kindly provided by T. Brocker (University of Munich, Germany). Mice were bred and housed under SPF conditions and animal experimentation was in compliance with EU as well

as national laws and approved by the local ethical committee. We used non-transgenic littermates as wild type experimental controls. Adult mice were used between eight and twelve weeks of age.

Epidermal sheets

Preparation, fixation and staining of epidermal sheets from ear skin was performed as previously described.[33] From 8 day-old mice whole body skin was isolated and analyzed. Epidermal sheets were stained with the following mAb: MHCII-FITC (M5/114, Pharmingen), CD3-PE (HL3, Pharmingen) and Langerin (929.F3, Dendritics) in combination with anti-rat-Cy3 (Jackson ImmunoResearch Europe Ltd.). After washing, the sheets were mounted on slides with Vector shield mounting medium (Vector Laboratories). Images were generated with a DMRA fluorescent microscope (objectives 10x or 25x; Leica) and a Kx14 camera (Apogee Instruments Inc.) at RT and analyzed with Image-Pro Plus software.

Preparation of single cell suspensions

Whole body skin from 1 to 8 day old pups and ears from older mice were floated on HBSS/dispase (1.25 mg/ml) for 1h at 37˚C. Skin layers were separated, the epidermis was further digested with 0.5% trypsin and the dermis with 4000 U/ml collagenase type IV (Worthington) for 1h at 37˚C. For E-cadherin staining HBSS/0.5% trypsin was applied to isolate the epidermis and HBSS/0.25% trypsin for preparation of the single cell suspension, all in the presence of 10 mM Hepes and 1 mM $CaCl_2$. Skin-draining axillary and inguinal LN were excised from resting adult mice and incubated with collagenase type IV. Cells were passed through a 70 μm cell strainer (BD Falcon) to obtain single cell suspensions.

Flow cytometry

Cells were incubated with Fc-block (2.4G2, Pharmingen) and appropriate combinations of the following antibodies obtained from Pharmingen, unless stated otherwise: MHCII-FITC and MHCII-PerCPCy5.5 (M5/114), CD11c-Alexa647 and CD11c-PE (N418), CD86-PE (GL-1), CD40-biotin (3/23), CCR7-Alexa647 (4B12) and EpCam-Alexa647 (G8.8). Intracellular staining for Langerin-Alexa647, Langerin-Alexa488 (929.F3, Dendritics) or E-cadherin-FITC (36/E-cadherin) was performed in the presence of 0.5% saponine (Sigma) after fixation with 3% paraformaldehyde. Annexin V-FITC and PI staining was carried out according to the instructions of Pharmingen. Appropriate mAb isotype controls were used. Data were acquired on a FACS LSR and analyzed with FlowJo software (BD Biosciences).

Induction of contact hypersensitivity

Contact hypersensitivity (CHS) with a low (sensitization 0.5%, challenge 0.25%) and high (sensitization 2%, challenge 0.5%) dose of Oxazolone was induced as previously described [34]. Ear swelling responses were measured as the difference in ear thickness of

the right ear before challenge and 24h and 48h later. To achieve selective depletion of LC, Langerin-DTR mice were injected i.p. with 400 ng diphtheria toxin (DT) in PBS 2 weeks before sensitization.

Statistics

Statistical analysis was performed with GraphPad Prism, using ANOVA or a two-tailed Student's t-test. Levels of significance: ****, $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, # $P = 0.07$.

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SUPPLEMENTARY MATERIAL

Table SI. Enhanced RFP expression in adult as compared to neonatal CD11c-Cre/Rosa26-tdRFP mice.

Age (days)	ΔMFI in LC	ΔMFI in dermal DC
0	1971 (1)	1628 (1)
1	1400 (1)	1242 (1)
2	2670 ± 279 (3)	1812 ± 494 (3)
3	2077 ± 104 (4)	956 ± 221 (4)
Adult	7935 ± 3372 (4)	4114 ± 4019 (4)

RFP expression in LC and dermal DC of neonatal and adult CD11c-Cre/Rosa26-tdRFP mice was evaluated by flow cytometry. The relative RFP expression level (ΔMFI) of each individual animal was calculated by subtracting the average background fluorescent signal of age-matched control animals. LC were identified as $MHCII^+$ cells, dermal DC as $MHCII^+$ cells in neonatal mice and $CD11c^+MHCII^+$ cells in adult mice (see figure S1 for the gating strategy of dermal DC). Mean RFP expression ± SD is indicated for each time point and the number of mice analyzed is annotated in between brackets.

Table SII. Spontaneous maturation of TβR1-deficient LC in the epidermis on day 3 after birth.

Age (days)		Langerin	E-cadherin	MHCII	CD40	CD86	CCR7
0	WT	1933 ± 20 (3)	12596 ± 446 (3)	12760 ± 1405 (3)	826 ± 369 (3)	3379 ± 448 (3)	779 ± 118 (3)
	DC-TβR1^{del}	1985 ± 71 (2)	14760 ± 5796 (2)	12572 ± 1399 (2)	859 ± 262 (2)	2872 ± 107 (2)	852 ± 66 (2)
3	WT	4754 ± 691 (8)	16425 ± 669 (8)	9028 ± 436 (8)	1163 ± 36 (8)	1016 ± 46.00 (2)	1244 ± 44 (4)
	DC-TβR1^{del}	2017 ± 175 (5)	13116 ± 1085 (5)	14523 ± 573 (5)	1418 ± 64 (5)	1330 ± 130.8 (4)	1585 ± 39 (3)
	P-value	0.0112	0.0185	< 0.0001	0.003	0,1882	0.0025
7	WT	n.d	n.d	n.d	n.d	117 ± 13 (4)	3141 ± 132 (2)
	DC-TβR1^{del}	n.d	n.d	n.d	n.d	245 ± 41 (4)	5842 ± 486 (2)
	P-value					0.0249	0.033

$MHCII^+$ LC were gated as indicated in figure 3a and 3c. The average expression levels of the indicated markers on LC are presented (MFI ± SEM) and the number of analyzed mice is annotated in between brackets.

Table SIII. Expression of MHCII and CD86 on dermal DC.

Age		MHCII	CD86
3 wk	WT	18975 ± 3635 (8)	1204 ± 658 (8)
	DC-TβR1^{del}	19301 ± 3378 (8)	843 ± 265 (8)
5 wk	WT	14293 ± 2790 (8)	740 ± 260 (8)
	DC-TβR1^{del}	13183 ± 1809 (7)	1542 ± 617 (7)

Dermal DC in adult mice were identified as CD11c^{+}MHCII^{+} cells. The average expression levels of the indicated markers are presented (MFI ± SD) and the number of mice analyzed is annotated in between brackets.

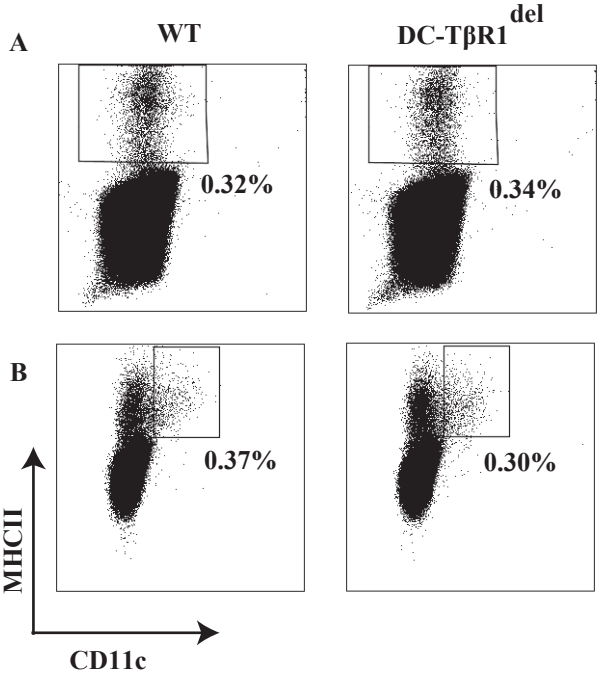
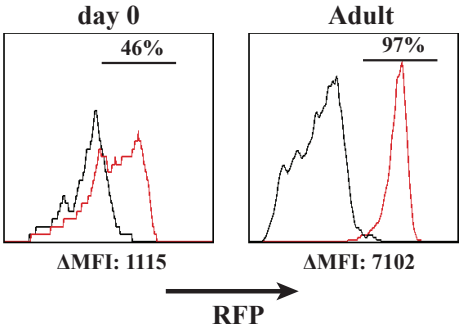


Figure S1. Gating strategy to identify dermal DC in neonatal and adult mice. Dermal cell suspensions were stained for CD11c and MHCII. **(A)** In neonatal animals MHCII^{+} cells expressed low levels of CD11c, while **(B)** CD11c^{+}MHCII^{+} dermal DC could be visualized in adult mice. One representative mouse of n=4-6 mice is depicted.



Age (days)	ΔMFI in splenic DC
0	1115 (1)
1	638 (1)
2	746 ± 121 (3)
3	457± 84 (4)
Adult	4887± 1512 (4)

Figure S2. Delayed CD11c-Cre transgene activity in splenic DC. CD11c-Cre transgene activity was assessed in splenic DC from CD11c-Cre/Rosa26-tdRFP mice. Part of the splenic DC expressed RFP directly after birth and RFP intensity (ΔMFI) and frequency were diminished as compared to adult animals.

CHAPTER 7

TGF-β SIGNALING IN DENDRITIC CELLS IS PIVOTAL TO PREVENT T-CELL MEDIATED COLITIS

Junda M. Kel[1], Mathilde J.H. Girard-Madoux[1], Julia Ober-Blöbaum[1,3], Dicky Lindenberg-Kortleve[2], Janneke N. Samsom[2], and Björn E. Clausen[1,3]

Departments of [1]Immunology and [2]Pediatric Gastroenterology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, The Netherlands, [3]Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany

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CHAPTER 8

GENERAL DISCUSSION

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In addition to the generation of protective immune responses, effective immune defense strategies depend on intricate negative regulation to restrict tissue damage due to inflammation. IL-10 and TGF-β are immunosuppressive cytokines produced by and acting on many different leukocyte subsets, including DC and T cells.[1,2] In particular, Treg exert their immunosuppressive function largely via the secretion of IL-10 and/or TGF-β.[3] However, IL-10 and TGF-β do not assume redundant roles since IL-10$^{-/-}$ and TGF-β$^{-/-}$ mice display distinct phenotypes. IL10$^{-/-}$ develop an enteric flora-mediated IBD which therefore is absent when mice are housed in germ-free conditions.[4,5] IL-10$^{-/-}$ mice also exhibit enhanced T cell responses upon induction of an immune reaction.[4] In contrast, TGF-β$^{-/-}$ animals develop severe autoimmunity and die at 3 weeks of age, which is not dependent on external Ag.[6-8]

DC have the unique capacity to balance immunity and tolerance and hence are critical in the maintenance of immune homeostasis during infection/inflammation as well as in steady-state conditions.[9] DC continuously probe their environment and induce robust, protective T-cell responses to pathogen-derived Ag as well as T-cell tolerance to innocuous foreign and self-Ag, for instance via the induction of Treg.[9] DC cultured in the presence of IL-10 and/or TGF-β exhibit a tolerogenic phenotype and inhibit T cell activation and proliferation.[10-13]

The particular role of IL-10 and TGF-β signaling in DC in vivo and to what extent DC contribute to the phenotypes of total IL-10 and TGF-β knock-out mice remains unclear. This unresolved question has been the subject of this thesis and our findings, which will be discussed in this chapter, are summarized in Box 1 and 2 and Figures 1 and 2.

IL-10 CONTROL OF DC

IL-10 signaling in resting DC

In vitro studies demonstrate that IL-10 acts on APC and suppresses the expression of MHC-II and costimulatory molecules on their surface. Monocytes and macrophages cultured in the presence of IL-10 exhibit attenuated Ag-presenting capacities associated with reduced MHC-II expression.[14,15] Accordingly, addition of anti-IL10 to maturing DC induces the upregulation of surface MHC-II and costimulatory molecule expression.[16] However, a recent study establishes that IL-10 treatment does not influence the expression of MHC-II and costimulatory molecules on DC in vitro.[17] Therefore we investigated whether DC constitutively lacking the IL-10R would undergo spontaneous maturation. We demonstrate in this thesis that, in vivo, IL-10R-deficient DC express similar amounts of MHC-II and costimulatory molecules such as CD86, CD40, PD-L1 and PD-L2. This is true for DC present in LN (Chapter 2), spleen (Chapter 2) and LP (Chapter 4). Notably, after LPS and soluble CD40L activation, IL10R$^{-/-}$ DC do not express higher levels of MHC-II or costimulatory molecules than WT cells (Chapter 2). Therefore the addition of IL-10 or IL-10R blocking antibodies to in vitro generated DC (from BM or peripheral blood monocytes) alters their maturation, whereas DC

Box 1. Main findings on the role of IL-10 in controlling DC function in vivo.

- Lack of IL-10 signaling in DC does not alter the expression of MHC-II and co-stimulatory molecules
- IL-10 controls DC cytokine production, notably IL-6, TNF-α, IL-23 and IL-10
- IL-10 regulation of DC is dispensable during priming of naïve T cells in lymph nodes
- In the lungs, IL-10 signaling in DC might be dispensable to govern Th2 cells during asthma
- In the absence of IL-10 control, skin and small intestinal lamina propria DC promote enhanced effector/memory Th1 and Th17 responses

constitutively lacking IL-10 signaling in vivo retain a WT phenotype with respect to surface expression of MHC-II and costimulatory molecules.

In contrast, IL-10 signaling in DC plays an essential role in their cytokine release. Monocytes and macrophages treated with IL-10 secrete lower levels of IL-1β, IL-6 and TNF-α.[14,18] IL-10 suppresses the capacity of peritoneal macrophages to secrete TNF-α [19] and inhibits DC to produce IL-12, TNF-α, IL-6 and IL-1β.[11] Accordingly, in the presence of anti-IL10 antibody, DC secrete elevated levels of TNF-α and IL-12.[16] We demonstrate that IL10R$^{-/-}$ DC secrete enhanced levels of IL-6 and TNF-α when stimulated with LPS and soluble CD40L in vitro (Chapter 2). Corroborating previous findings, culture with anti-IL10 mAb enhances WT DC production of pro-inflammatory cytokines while additional IL-10 in the medium inhibits their cytokine secretion (Chapter 2).

IL-10 plays an important autocrine role to modulate DC function and thereby T cell activation.[16,20] DC cultured in the presence of anti-IL10 neutralizing Ab express lower levels of *il-10* mRNA in one[16] but secrete more IL-10 in another study.[17] We show here that DC constitutively lacking the IL-10R produce higher levels of IL-10 when stimulated in vitro (Chapters 2 and 4). These results suggest that DC lacking IL-10 signaling secrete elevated IL-10 in an attempt to control their own activation status.

In accordance with the immature phenotype of DC, T cells in DC-IL10R$^{-/-}$ animals are not activated and the frequency of Treg is similar to WT mice. Therefore IL-10 signaling in DC is dispensable to regulate DC-induction of Treg, both in the thymus during central tolerance, and in the tissues/secondary lymphoid organs for the establishment of peripheral tolerance.

Furthermore, subsets of DC in DC-IL10R$^{-/-}$ mice are comparable to WT, demonstrating that IL-10 signaling in DC is not required for their development and differentiation. In the skin, LC, Langerin^{+} dermal DC and Langerin^{neg} dermal DC are present at normal frequencies (Chapter 2). In the small intestinal LP, the proportion of CD103^{neg} and CD103^{+} DC is also similar to WT (Chapter 4). None of the studies on IL-10$^{-/-}$ or IL10Rβ$^{-/-}$ mice show data on DC development and subset homeostasis. Wakkach et al. demonstrated that

Tr1 are induced in the periphery by IL-10-producing pDC, which are elevated in IL-10 transgenic mice. However, pDC are still present in IL-10 deficient mice showing that IL-10 signaling in DC is not required for their differentiation.[21] This study and our work attest that IL-10 control of DC is dispensable for their development, but rather is crucial in modulating their cytokine secretion.

IL-10 control of DC homeostasis in situ

Several studies demonstrate that IL-10 treated DC gain a tolerogenic phenotype and secrete lower amounts of pro-inflammatory cytokines which impairs T cell proliferation and activation resulting in T cell anergy[12,14,22-24] or the induction of Treg.[25,26] These studies suggest that IL-10 control of DC is necessary at the time of first encounter of DC with naïve T cells during the initiation of the immune response in LN. It is established that IL-10 secretion by DC regulates the induction of effector T cells and in vivo, lack of IL-10 production by LC during priming of T cells induces an enhanced CHS reaction.[27] IL-10 can act in an autocrine manner,[16] therefore DC- but also T cell-derived IL-10 could regulate DC function during priming of T cells in the LN. However, in this thesis we show that lack of IL-10 signaling in DC does not affect T cell priming but rather controls effector/memory T cell function.

IL-10 control of DC in the skin during contact hypersensitivity

As a gatekeeper of excessive immune responses against foreign Ag, IL-10 is expressed at epithelial interfaces to the environment, including the skin. Consequently, IL-10$^{-/-}$ mice develop increased contact hypersensitivity (CHS),[28] a relevant mouse model for allergic contact dermatitis.[29] Following the topical application of a contact sensitizer (hapten), DC prime hapten-specific T cells in sdLN. The subsequent painting of the same hapten onto the ears triggers a transient ear-swelling reaction, driven by IFN-γ-secreting Th1 cells and regulated by CD4^{+} T cells producing IL-10.[29] Mice bearing a T cell- or Treg-specific deletion of IL-10 exhibit aggravated CHS, demonstrating the essential role of T cell-derived IL-10 to curtail the inflammatory skin reaction.[30,31]

In addition to LC in the epidermis, the skin harbors distinct dermal DC populations, and our group recently established the functional redundancy of distinct cutaneous DC subsets in mediating CHS.[32] During hapten sensitization, the production of IL-10 by LC – and presumably also by dermal DC – is required to limit ear swelling, as mice bearing a LC-specific IL-10 knockout mount aggravated CHS responses.[27] In Chapter 2 we demonstrate that in contrast to total, T cell-, Treg- and LC-specific IL10$^{-/-}$ mice,[27,28,30,31] DC-IL10R$^{-/-}$ and WT mice develop a similar ear-swelling reaction 24 h after hapten challenge. Intriguingly, at 48 h ear swelling further increases in DC-IL10R$^{-/-}$ mice, while the inflammation begins to resolve in WT animals. The adoptive transfer of T cells primed in WT or DC-IL10R$^{-/-}$ donor mice into either type of recipient animals, confirm similar ear swelling 24 h after hapten challenge. In contrast, at 48 h the ear-swelling reaction is significantly stronger in DC-IL10R$^{-/-}$recipients, whereas T cells that were primed by IL-10R-deficient and reactivated by WT DC fail to elicit exaggerated ear swelling. These

results demonstrate that IL-10 controls DC function during the effector phase of CHS but not during priming of T cells in the sdLN. Further, enhanced cytokine expression and cellular influx occur in the skin of DC-IL10R$^{-/-}$ animals during the elicitation of CHS. As soon as 24 h after hapten challenge, the expression of pro-inflammatory cytokines and IL-10 is increased in the skin of these animals as compared with their WT counterparts, causing a massive infiltration of innate, MHC-II^{+} and T cells at 48 h. Whereas the global extent of infiltration is lower in WT mice, the number of skin-infiltrating T cells is similar in both strains. Intriguingly, the increase in T cells observed from 24 to 48 h is mainly caused by the recruitment of Foxp3^{+} Treg into the skin.

Our data show two major points. (1) The lack of IL-10 signaling in DC does not alter T-cell priming in sdLN during hapten sensitization. Most likely, the balanced, albeit elevated secretion of pro-inflammatory cytokines and inhibitory IL-10 by IL-10R-deficient skin-infiltrating DC drives a similar differentiation of effector cells and Treg as in WT mice. Therefore, ear swelling in DC-IL10R$^{-/-}$ mice is not augmented 24 h after a hapten challenge. Accordingly, the production of IL-10 by LC, which is essential to prevent exaggerated T-cell priming and ear swelling at 24 h,[27] exclusively acts on naïve T cells, rather than in an autocrine fashion on LC/DC. Moreover, the potential paracrine regulation of mature DC function by IL-10 is dispensable during T-cell priming. (2) Rather, DC are the target of IL-10 to limit excessive T-cell reactivation during the elicitation of CHS, as demonstrated by the exaggerated ear swelling observed 48 h after hapten challenge in DC-IL10R$^{-/-}$ mice. In contrast to priming, the IL-10-mediated autocrine/paracrine negative feedback on DC in situ is required to curtail inflammation and tissue damage. This highlights a critical role of skin DC in the maintenance of immune homeostasis that goes beyond their classical function as migratory APC. Whether such DC are resident and/or inflammatory remains to be determined.

In conclusion, IL-10 control of DC in the skin is necessary to prevent the hyperactivation of effector/memory T cells in a Th1-dependent CHS model (Figure 1A).

IL-10 control of DC in the skin upon *Leishmania major* infection

The ability of IL-10 to dampen immune responses and inhibit the activation of immune cells can be exploited by harmful microbes.[33] In fact, IL-10 is expressed by certain pathogens to trick the immune system and induce pathogen-specific tolerance. Some viruses secrete a viral form of IL-10 with high homology to human IL-10 that can bind to the human IL-10R.[34,35] As a result, treatment with anti-IL-10R induces an efficient and rapid resolution of a persistant viral infection without causing immunopathology.[36] Other pathogens induce IL-10 production by various host cells which inhibits effector T cells and promotes the induction of Ag-specific Treg.[33] For example, the immune response against *Lysteria monocytogenes* is enhanced in the presence of anti-IL10.[37] The timing of IL-10 secretion, the source and targets of IL-10 all form an intricate network which can induce overwhelming infection and/or severe tissue damage if deregulated. Presence of IL-10 is also correlated with long-term maintenance of certain pathogens, for instance

Leishmania major parasites, at the site of infection. On one hand this maintains Ag-specific memory T cells which confer immunity against reinfection, but on the other hand persistence of the parasites can induce severe disease reactivation. Notably, *L. major* resistant mouse strains such as C57BL/6 (DC-IL10R$^{-/-}$ are bred on a C57BL/6 background) develop a protective Th1/T cytotoxic type 1 (Tc1) response upon infection and manifest self-healing lesions similar to humans. During infection with *L. major*, IL-10 controls the acute phase of infection and also enables parasite persistence in the infected tissue, which is required for *Leishmania*-specific T cell memory. IL-10$^{-/-}$ animals produce increased levels of IFN-γ which mediates total clearance of the parasite.[38] Consequently, immunological memory is lost and IL-10$^{-/-}$ mice develop bigger lesions than WT upon reinfection.[38] Accordingly, WT mice treated with anti-IL10R during the chronic stage of infection achieve sterile immunity and are no longer at risk of disease reactivation. In contrast, mice overexpressing IL-10 have an increase susceptibility to *L. major* and fail to control the infection.[39] At the peak of the anti-*Leishmania* response, IL-10 is abundantly produced at the site of infection by innate immune cells and CD4^{+} T cells, both of which could contribute to govern DC.[40,41] Persistence of a small number of parasites at the site of infection correlates with the presence of IL-10 producing Treg cells.[42,43] Belkaid et al. suggested that APC could be modulated by IL-10 during both the acute and latent phases of infection.[42] In Chapter 3 we demonstrate that IL-10 control of DC is important during the acute phase but not the latent phase of infection. IL-10R-deficient DC induce an increased Th1/Tc1 to Th2 ratio resulting in enhanced parasite clearance (Figure 1A). Similarly to hapten challenge during CHS (Chapter 2), IL-10R$^{-/-}$ DC secrete elevated pro-inflammatory cytokines such as IL-6, TNF-α and IL-12 which favors skewing toward Th1/Tc1. In both models, the enhanced T cell response does not induce a chronic state of inflammation, and there is resolution of infection and inflammation. During the anti-*Leishmania* response, IL-17A was significantly enhanced in sdLN of DC-IL10R$^{-/-}$ mice but its role in the course of infection is still unclear. In susceptible mice IL-17A induces parasite persistence.[44] In resistant mice inoculated with a high parasite dose, elevated levels of IL-17A provoke the increased recruitment of neutrophils to the lesions.[45] In DC-IL10R$^{-/-}$ animals inoculated with a low dose of parasites, we do not observe elevated numbers of neutrophils in the lesions despite elevated levels of IL-17A in LN. Thus, IL-17A might not be a relevant pathogenic cytokine in this infectious model and its role remains to be investigated.

In contrast to IL-10$^{-/-}$ mice, DC-IL10R$^{-/-}$ mice do not totally eliminate parasites and develop minimal to no lesions upon reinfection, similar to WT. These results imply that even though parasite clearance is more efficient in DC-IL10R$^{-/-}$ animals, the lack of IL-10 signaling in DC does not induce a Th1/Tc1 reaction potent enough to eliminate all parasites during the acute phase of infection. Furthermore, DC do not appear to be the target of Treg-derived IL-10 which is necessary in the latent phase for persistence of parasites. Hence, IL-10 signaling in T cells and Treg appears to be sufficient to generate and maintain anti-*Leishmania* T-cell memory.

IL-10 control of DC in the gastrointestinal tract

Only a single layer of epithelial cells separates the interior of our body from the external environment. Thus, the balance between tolerance and immunity must be tightly regulated. The importance of IL-10 in the gastrointestinal tract is underscored by the fact that IL10$^{-/-}$ animals develop colitis due to an aberrant Th1 response to non-pathogenic commensal bacteria.[4,5,46] In Chapter 4 we demonstrate that DC-IL10R$^{-/-}$ animals acquire small intestinal inflammation equivalent to that of IL-10$^{-/-}$ mice, but no colitis under SPF conditions. Once bacterial exposure increases (in open cages), DC-IL10R$^{-/-}$ mice develop a prolapse, which is a sign of colonic inflammation. Like IL-10$^{-/-}$ mice,[4] DC-IL10R$^{-/-}$ animals harbor increased numbers of T cells and IgA^{+} plasmablasts in the LP. Similar to what we observed during CHS in the skin (Chapter 2), priming of naïve T cells in mLN is comparable to WT. Using an adoptive OT-II transfer followed by OVA gavage, we demonstrate that mLN T cells instructed by IL-10R$^{-/-}$ DC proliferate and secrete equivalent amounts of cytokines as compared to controls. These data further confirm that IL-10 control of DC does not play a role during priming of naïve T cells in LN.

In this thesis we establish that IL-10 is essential to control LP DC and maintain immune homeostasis in situ. In DC-IL10R$^{-/-}$ mice the Th17 compartment is particularly disturbed with higher number of LP Th17 cells and IL-17A-producing iEL. Th1 cells are present at the same frequency as in controls but secrete elevated levels of IFN-γ in the LP of DC-IL10R$^{-/-}$ mice. Thus, IL-10 control of LP DC is essential to maintain T cell homeostasis and to contain Th1/Th17 activation (Figure 1A).

We demonstrate that IL10R-deficient LP DC express WT levels of MHC-II and costimulatory molecules on their surface. However, they secrete elevated levels of pro-inflammatory cytokines such as IL-23, IL-12p70 and IL-6 which can directly act on memory/effector LP T cells and iELs. All three cytokines are involved in the maintenance and reactivation of memory T cells.[47-50] IL-12p70 and IL-23 are mainly produced by DC and can therefore be considered DC-derived cytokines. Moreover, IL-12p70 and IL-23 are both crucial in maintaining gastro-intestinal homeostasis. IL-10 therapy of IL10$^{-/-}$ mice suppresses their phenotype presumably through the inhibitory effect of IL-10 on IL-12(p40) production by APC.[46] In fact, anti-IL-12 administration (neutralizing both IL-12p70 and IL-23) reverses established colitis in IL-10$^{-/-}$ mice.[51] Accordingly, in a model of TNBS-induced colitis, treatment of WT mice with anti-IL-12 improves and often completely abrogates established disease.[52] More recent studies have demonstrated that IL-23 is crucial for the maintenance and reactivation of effector/memory Th17 cells both in the colon and SI.[47] In the colitis transfer model, IL-23 directly acts on T cells to promote inflammation.[53] In a model of anti-CD40-induced colitis, colonic LP DC-derived IL-23 induces T-cell independent IL-17A production resulting in mucosal inflammation.[54] In the SI, CD8^{neg}CD11b^{neg} DC from the ileum constitutively secrete IL-23 in response to enteric microflora.[55] Also, upon activation by bacterial Ag, LP CD103^{+}CD11b^{+} DC rapidly secrete IL-23 to stimulate IL-22 release from innate lymphocytes.[56] Notably, IL-23 may act on macrophages and DC in an

autocrine amplification loop which promotes tissue inflammation.[54,57,58] In conclusion, we establish that in the LP, IL-10 is necessary to control DC to inhibit their secretion of IL-23, IL-12p70, IL-6 and TNF-α which all promote effector/memory Th1 and Th17 cell maintenance and/or reactivation.

We further demonstrate the enhanced recruitment, proliferation and survival of lymphocytes in the LP of DC-IL10R$^{-/-}$ mice, most likely through the elevated secretion of pro-inflammatory cytokines and chemokines by IL-10R-deficient DC. CCL5 and CCL28 are two chemokines which recruit respectively T cells and IgA-producing plasmablasts to the LP and are enhanced in IBD patients.[59-61] In DC-IL10R$^{-/-}$ animals both chemokine levels are increased in the SI resulting in elevated numbers of T cells and plasmablasts in the LP of these mice. Whether IL10R-deficient LP DC directly secrete these two chemokines remains to be established. However, we demonstrate that IL10R-deficient DC secrete augmented levels of IL-6, which promotes IgA secretion in a T cell-independent manner.[62] Therefore the increased IgA-producing plasmablasts observed in the LP could be directly induced by the elevated levels of IL10R-deficient DC-derived IL-6. In the SI, secreted IgA is the first line of defense in preventing pathogen invasion and the augmented number of IgA^{+} cells could be a compensatory mechanism to counteract the enhanced T cell activation and inflammation in the LP.[62]The phenotype of DC-IL10R$^{-/-}$ animals evolves with age, strongly suggesting that exposure to environmental Ag is crucial in developing the SI inflammation. Both bacterial and/or food Ag could promote IL10R-deficient LP DC to secrete elevated levels of pro-inflammatory cytokines. LP DC express several TLR[63] and the colitis observed in IL10$^{-/-}$ mice is TLR dependent[64] and therefore absent in germ-free animals.[5] LP DC constitutively secrete IL-10 which is enhanced by TLR stimulation, but they fail to secrete IL-12p40 in the steady state or after TLR ligation.[63] Addition of anti-IL10R antibody to freshly isolated LP DC cultures results in enhanced IL-12p40 production, suggesting that IL-10 signaling maintains LP DC unresponsive to TLR ligation.[63] Accordingly, depletion of IL-10 from human colonic explant cultures induces enhanced IFN-γ, IL-17 and TNF-α production upon commensal bacteria exposure.[65] These studies suggest that the phenotype of DC-IL10R$^{-/-}$ animals could be induced by an exaggerated pro-inflammatory response to TLR signaling. However TLR are mostly expressed by distal SI LP DC[63] and DC-IL10R$^{-/-}$ mice develop the most severe inflammation in the proximal part of the SI. These results suggest that other Ag, such as food Ag which are predominantly present in the proximal SI could drive the inflammatory phenotype in our mice. Alternatively, the type of bacteria present in the animal facility could also influence the phenotype of DC-IL10R$^{-/-}$ animals. IL10$^{-/-}$ mice kept in a germ-free environment and reconstituted with two different strains of commensal bacteria develop enterocolitis that is variable in the location, onset and severity.[66] Moreover, DC-IL10R$^{-/-}$ mice develop a Th17 phenotype which might be triggered by the presence of segmented filamentous bacteria (SFB). SFB is prone to induce IL-17A production by T cells in the SI, however these Th17 cells have been proposed to play a protective role against

pathogenic microorganisms.[67] The presence of SFB in DC-IL10R$^{-/-}$ mice remains to be determined. In addition, further studies with gluten-free diet or recolonization of germ-free DC-IL10R$^{-/-}$ mice with distinct types of bacteria will help to address remaining questions. Yet, we can safely conclude that when IL-10 signaling is disrupted, LP DC continuously exposed to non-pathogenic foreign Ag become constitutively active and induce inflammation. By constantly secreting elevated levels of pro-inflammatory cytokines, IL-10R-deficient DC maintain and recruit Th17/Th1 cells to the LP leading to chronic inflammation.

In conclusion, through enhanced cytokine and chemokine secretion in the SI, LP IL10R-deficient DC induce enhanced recruitment, proliferation and activation of Th1/Th17 cells in situ, while T-cell priming in LN is not affected. Whether IL-10 controls both LP CD11c^{+}CD103^{+} or CD11c^{+}CX$_3$CR1^{+} cells remains to be determined.

IL-10 control of DC in the lung during OVA-induced asthma

While it is established that IL-10 is essential in the induction of Th2 responses, it is still unclear how IL-10 controls the persistence of the asthmatic reaction. Studies with IL-10$^{-/-}$ mice give conflicting results making it difficult to hypothesize on the influence of IL-10 on Th2 cytokine production or typical asthma features such as airway inflammation, eosinophilia, mucus production and AHR.[68-70] Discrepancies also exist on the role of IL-10 in controlling DC during an asthmatic reaction. IL-10 treated DC were shown to prevent allergen sensitization and reverse the inflammatory phenotype of asthma.[26,71] Another study suggests that DC differentiated in the presence of IL-10 express lower MHC-II and costimulatory molecules and inhibit proliferation of Ag-specific T cells. No differences in cytokine production and airway inflammation are noticed compared to controls.[72] Our preliminary data presented in Chapter 5 indicate that in an OVA-induced asthma model, DC-IL10R$^{-/-}$ mice develop less AHR but similar Th2 differentiation/maintenance and mucus production. In light of our previous work in skin (Chapter 2) and gut (Chapter 4), it would not be surprising if IL-10 control of DC does not play a role during the differentiation of naïve T cells into effector Th2 cells in the LN, but this remains to be investigated. Surprisingly, IL10R$^{-/-}$ DC do not seem to play an essential role in the reactivation of Th2 cells in situ, as no overt inflammation in the lungs was observed. IL-4, IL-5 and IL-13 levels in BALF were similar to WT, as well as *il-4* mRNA in the lungs. After their differentiation, Th2 cells induce the production of mucus from goblet cells and promote chemotaxis of eosinophils.[73] Mucus production and expression levels of *ccl11* (eotaxin-1), a chemokine specifically recruiting eosinophils, are comparable in the lungs to WT, suggesting that Th2 cells are not hyperactivated in situ. Importantly, we also do not observe enhanced activation of Th1 or Th17 cells. IFN-γ in BALF and *ifn-γ* mRNA in lungs are similar to WT, and levels of IL-17A remain undetectable both in WT and DC-IL10R$^{-/-}$ mice. These results demonstrate that DC-IL10R$^{-/-}$ animals do not mount an inappropriate Th1 or Th17 response in reaction to allergen as one would have expected if the Th1-Th17/Th2 ratio had been disbalanced.

Therefore, IL-10 control of DC is essential during reactivation of Th1 and Th17 cells but might not be crucial for Th2 cells. Conversely, during leishmaniasis, significantly lower IL-4 is present in the sdLN of DC-IL10R$^{-/-}$ animals which shifted the Th1/Th2 balance towards a protective Th1 response (Chapter 3). However, unlike asthma, infection with *L. major* induces a preferential Th1 response, and Th1 cells have a potent capacity to inhibit Th2 cells.[74] In conclusion, in contrast to Th1 and Th17 responses, in the context of a strict Th2 response IL-10 signaling in DC does not seem to play a crucial role in controlling Th2 cells in situ.

The levels of *foxp3* are elevated whereas *gata3* is comparable to WT in the lungs of DC-IL10R$^{-/-}$ mice. These data suggest that the number of Foxp3^{+} Treg is increased but that total Th2 cells are similar to WT. The balance between Treg and Th2 cells is particularly important in controlling allergic asthma. Depletion of Treg induces enhanced Th2 responses and elevated AHR in mice.[75] Moreover, asthmatic patients have a severly reduced Treg/Th2 ratio.[76] In DC-IL10R$^{-/-}$ animals, the *foxp3/gata3* ratio (reflecting Treg/Th2) is enhanced in the lungs implying a favorable balance towards tolerance. This increased Treg/Th2 ratio could explain the reduced AHR observed in DC-IL10R$^{-/-}$ mice.

Treg secrete IL-10 which is essential to downregulate the asthmatic reaction.[77,78] In fact, Foxp3-specific IL-10$^{-/-}$ mice develop increased OVA-induced lung inflammation during asthma.[31] Accordingly, injection of in vitro generated Foxp3^{+} IL-10-secreting cells protect from asthma.[79] In the lungs of DC-IL10R$^{-/-}$ animals, levels of *il-10* are significantly elevated. Even though the source of IL-10 remains to be identified, and may be several cell types, the increased *il-10* correlated with the increased levels of *foxp3* mRNA. Therefore we hypothesize that increased levels of Treg produce elevated levels of IL-10 and mediate reduced AHR in the presence of an only mildly attenuated Th2 response.

Lung Treg homeostasis is controlled in part by DC. Pulmonary DC produce IL-10 which promotes the differentiation of Treg that mediate tolerance in response to allergen exposure.[80] Accordingly, injection of DC constitutively producing IL-10 results in increased Foxp3^{+}IL10^{+} Treg which protect from allergic asthma.[81] We establish that splenic and LP-derived IL10R$^{-/-}$ DC produce elevated levels of IL-10 (Chapter 2 and 4), therefore it is very likely that IL10R$^{-/-}$ lung DC secrete enhanced levels of IL-10, which would promote augmented Treg homeostasis. Whether the increased DC-derived IL-10 induces elevated differentiation and/or proliferation/maintenance of Treg remains to be elucidated. However, extrapolating from previous results described in Chapter 2 and 4, we suggest that IL-10 control of DC is particularly important in situ in the lungs rather than in the draining LN during an asthmatic reaction.

In summary, IL10R$^{-/-}$ DC initiate a normal or slightly reduced Th2 response to allergen in a model of asthma. However, these DC might produce elevated amounts of IL-10 which promotes enhanced Treg reactivation/proliferation in situ (Figure 1B). IL-10-producing Treg (Foxp3^{+} and possibly Tr1) are then responsible for the decreased AHR observed in DC-IL10R$^{-/-}$ animals.

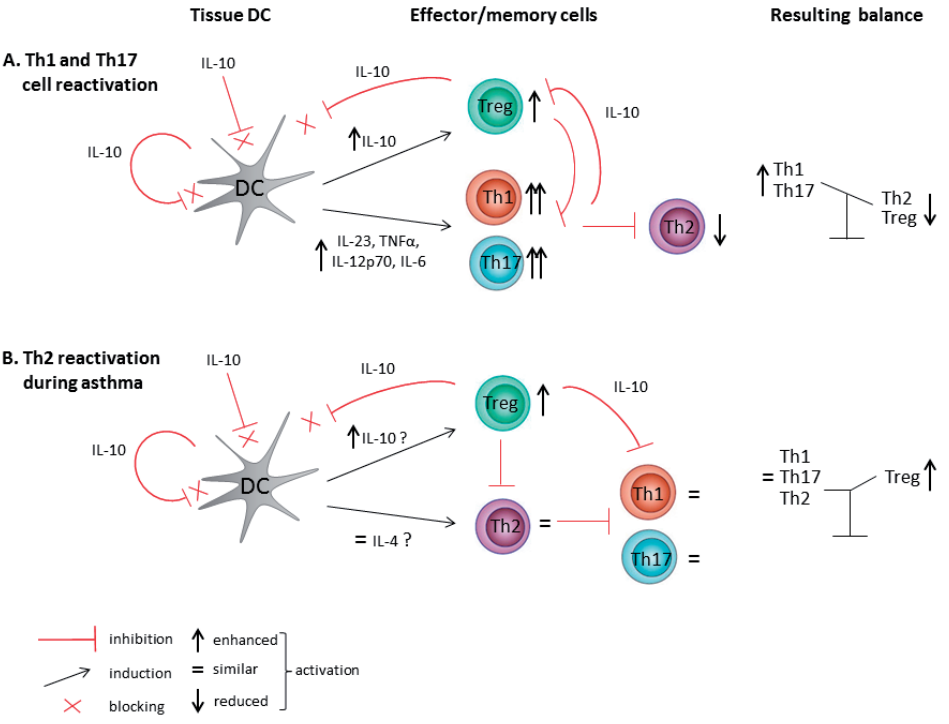

Figure 1. Proposed model on how lack of IL-10 signaling in DC modulates effector/memory T cells in situ. **(A)** Skin and lamina propria IL-10R-deficient DCs secrete elevated levels of pro-inflammatory cytokines and IL-10, which enhance Th1 and Th17 reactivation in situ. Treg activity might be elevated and Th2 activation reduced. The net balance results in augmented Th1 and Th17 reactivation while Th2 and Treg activities are diminished. **(B)** During asthma, IL10R-deficient lung DC might secrete WT levels of cytokines to maintain Th2 cells, and augmented levels of IL-10 which promotes Treg (re)activation. Th1 and Th17 cells remain unaffected. The resulting balance is an enhanced Treg function while Th activation is similar to WT. Adapted and expanded from Clausen and Girard-Madoux, OncoImmunology 2013; 2:e23186.

DC, helper T cells, regulatory T cells and IL-10 in between: an intricate ménage à quatre

Our data establish that IL-10 control of DC is dispensable during priming of T cells. However, IL-10 signaling in DC is crucial to modulate DC-reactivation of T cells and attenuate DC function during inflammation in situ (Figure 1). Little is known about the role of DC as innate immune cells able to maintain effector T cells and/or reactivate memory T cells in situ. LN-resident $CD8^+$ DC reactivate Ag-specific memory T cells during influenza infection.[82] In a model of herpes simplex virus infection, tissue-resident T cells are reactivated by DC demonstrating that memory T cell responses can be initiated within peripheral tissues.[83] This thesis emphasizes the role of DC as tissue-resident modulators of homeostasis and regulators of immune responses. IL-10 control of DC is

necessary to prevent excessive activation of Th1/Th17 effector/memory T cells in the skin (Chapter 2 and 3) and the SI (Chapter 4), but does not play a crucial role in controlling Th2 cells in the lung during asthma (Chapter 5).

A fine balance between DC, effector T cells and Treg must be kept where IL-10 signaling takes center stage.[84] First, DC promote both effector T cells and Treg differentiation and control their reactivation in situ. In this thesis we demonstrate that priming of naïve T cells is similar in DC-IL10R$^{-/-}$ mice as in WT and that frequencies of Foxp3^{+} and Foxp3^{neg} Treg are not affected (Chapters 2 and 4). Splenic and LP IL-10R$^{-/-}$ DC secrete elevated levels of pro-inflammatory cytokines if stimulated with LPS/sCD40L, which enhances the reactivation of effector Th1 in the skin and Th1/Th17 in the SI. However, IL-10R$^{-/-}$ DC also produce elevated levels of IL-10 suggesting that Treg maintenance and/or reactivation is also enhanced. In Chapter 5 we strongly suggest that IL10R-deficient lung DC secrete enhanced levels of IL-10 which results in the elevated *foxp3* expression in situ. In fact, Foxp3^{+} cells are more numerous in the SI of DC-IL10R$^{-/-}$ (Chapter 4) which is also observed in colitis mice and patients with IBD.[85] However, since effector T cells are also enhanced in number, the net balance effector T cells/Treg remains untouched in DC-IL10R$^{-/-}$ animals.

Second, Treg are crucial to suppress effector T cell functions and have an essential role in controlling DC activation. In fact, Treg keep DC in a tolerogenic state creating a regulatory loop through mutual interactions.[86] Treg exert control over DC maturation and cytokine secretion in an IL-10-dependent manner[87] and we suggest that Treg are the main source of IL-10 which controls DC. Treg enable DC suppressive activity and stimulate DC IL-10 secretion which can act in an autocrine loop to induce the expression of the inhibitory costimulatory molecule B7-H4 on DC.[88] Treg rather than naïve CD4^{+} or memory/effector CD4^{+} T cells induce an immunosuppressive phenotype of DC even in the presence of LPS.[89] In vivo, depletion of Treg from asthma susceptible mice provokes an inflammatory DC phenotype which induces enhanced T cell activation[75] and in asthmatic patients, Treg displaying reduced IL-10 secretion are unable to maintain DC in an unresponsive state.[90] Likewise, in a model of diabetes, Treg maintain DC in a tolerogenic state, with low levels of MHC-II and costimulatory molecules.[91] Lastly, Foxp3^{+} Treg induce tolerogenic DC which in turn promote Tr1 differentiation in vitro and in vivo.[92]

Finally, effector T cells inhibit Treg and control DC homeostasis through their secretion of pro-inflammatory cytokines. In addition, all effector CD4^{+} and CD8^{+} T cells can produce IL-10, which mainly acts in an autocrine feedback loop to downregulate their own function.[93] However, effector T cell-derived IL-10 could also participate in controlling DC function in situ.

In conclusion, signaling between effector T cells, Treg and DC, notably through IL-10, must be maintained in balance in order to sustain homeostasis. Especially, IL-10 signaling in DC is essential to further regulate effector T cell and possibly Treg in situ. Certainly, T cells are not the only source of IL-10 regulating DC function. At environmental barriers, epithelial cells are important producers of IL-10 such as in the

skin[94], lung[95] and gastrointestinal tract,[65] which regulate the local microenvironment and can contribute to modulate DC function.

IL-10 signaling in T cells is not always sufficient to maintain homeostasis

Our data also establish that IL-10 signaling in T cells is not sufficient to dampen immune responses (Chapters 2 and 4). In the skin during CHS, Treg-specific IL10$^{-/-}$ mice develop increased ear swelling.[31] The exaggerated ear swelling observed at 48 h in DC-IL10R$^{-/-}$ animals (Chapter 2), despite the elevated expression of IL-10 in the skin, indicates that the regulation of T cells by IL-10 alone is inadequate to restrict adaptive immune responses in inflamed tissues. Therefore, Foxp3^{+} Treg preferentially recruited into the skin 48 h after elicitation of CHS exert their regulatory function largely by IL-10 control of DC and to a lesser extent of effector T cells.

Similarly, IL-10 control of T cells is not sufficient to control small intestinal homeostasis, but rather DC need to be controlled by (Treg-derived) IL-10 in order to keep Th1/Th7 cell homeostasis (Chapter 4). Treg-derived IL-10 is crucial to maintain immune homeostasis as Treg-specific IL-10 deficient mice develop colitis.[31] Likewise, in the T cell transfer model of colitis, Treg exert their immunosuppressive function through IL-10.[85,96] In DC-IL10R$^{-/-}$ animals, the levels of *il-10* throughout the SI is enhanced, and restimulated LP and iEL secrete elevated amounts of IL-10, available to control T cells. Furthermore LPS-activated DC themselves produc enhanced IL-10. However, IL-10 is not sufficient to inhibit Th17 and Th1 cells and/or to promote Treg capable of counteracting this overt inflammation.

In a model of leishmaniasis (Chapter 3) we demonstrate that parasites disappear much more quickly from DC-IL10R$^{-/-}$ than controls. However, IL-10 control of cells other than DC (presumably T cells) is sufficient to prevent eradication of parasites during the acute phase of infection and to maintain small numbers of the parasites in the lesions during the latent phase. Indeed, lack of IL-10 signaling in DC was not sufficient to eliminate the anti-*Leishmania* T cell memory response.

In contrast, in the context of a Th2 response, IL-10 signaling in DC does not seem to be essential in controlling Th2 cells but rather affects lung Treg homeostasis (Chapter 5). Lung IL-10R$^{-/-}$ DC most likely produce elevated levels of IL-10 which promotes Treg maintenance and/or reactivation in situ. IL-10R$^{-/-}$ DC might not secrete elevated levels of IL-4 which would enhance effector Th2 cell responses. In this Th2 model, the relevance of Treg-derived IL-10 feedback on DC remains to be determined but seems dispensable as far as our data show. Thus, in a context of Th2-driven asthma, IL-10-dependent T cell regulatory circuits seem sufficient to maintain immune homeostasis.

In conclusion, our findings are threefold. First, IL-10 signaling in DC is dispensable during T cell priming in LN. Second, IL-10 control of DC is necessary to dampen effector/memory Th1/Th17 responses but not Th2 cells in situ, and might have a role in maintaining Treg. Finally, IL-10 signaling in skin and SI Th1 and Th17 cells is not sufficient to prevent excessive effector T cell reactivation.

TGF-β CONTROL OF DC

TGF-β signaling in Langerhans cells

In vitro, TGF-β is crucial for the differentiation of monocytes or hematopoietic precursors into LC.[10,97,98] TGF-β$^{-/-}$ mice are devoid of LC[99] and autocrine/paracrine TGF-β signaling is necessary for LC development.[100-102] Our group generated Langerin-Cre mice and demonstrated that Langerin-TβR1$^{-/-}$ mice are nearly devoid of LC in the epidermis with only an occasional patch of LC present in adult mice.[103] These data show that TGF-β is essential for the differentiation of LC. Here we establish that TGF-β is not solely required for LC development but also necessary for their maintenance in the epidermis (Figure 2A). In DC-TβR1$^{-/-}$ mice, due to delayed CD11c-Cre mediated deletion of the TβR1, a large fraction (approximately 50%) of developing LC can still integrate TGF-β signals, migrate to and seed the epidermis as in WT animals. At this stage (day 0 after birth), LC are still immature, with low levels of MHC-II and costimulatory molecules on their surface. A gradual loss of LC occurs during the first week of life, correlating with the efficient deletion of the TβR1 on LC as visualized by CD11c-Cre driven GFP expression. Therefore, TGF-β is also essential for the maintenance of LC in the epidermis.

It was previously established that migrating LC express elevated MHC-II and CD86 in the steady state, forming a continuous flow of LC that have sampled Ag in the epidermis.[104] Migrating LC also upregulate CCR7 which mediates DC migration to LN,[105] and downregulate E-Cadherin, a molecule necessary to anchor LC to keratinocytes.[106] TGF-β signaling in differentiating human LC inhibits the expression of CD1d, an Ag presenting molecule, in vitro. Accordingly, LC recovered from human skin are devoid of CD1d on their surface and are in an immature phenotype.[107] Here we demonstrate that LC that are not under the control of TGF-β upregulate MHC-II, CD40 and slightly CD86, corresponding to a phenotypically mature state. At the same time, TβR1$^{-/-}$ LC express higher CCR7 and lower levels of E-Cadherin, associated with their dissociation from the neighboring keratinocytes. TGF-β signaling is also required for Langerin expression, as TβRI-deficient LC express diminished levels of Langerin. Our data were confirmed by other groups in models of inducible TβR2 or TGF-β depletion specifically in LC.

Box 2. Main findings on the role of TGF-β in controlling DC function in vivo.

- TGF-β signaling in Langerhans cells is necessary for their maintenance in the healthy epidermis
- TGF-β control of dermal DC is dispensable both for their development and homeostasis in steady state and during CHS
- TGF-β is required for expression of CD103 and CD11b on migratory gut DC
- Lack of TGF-β signaling in DC promotes spontaneous colonic inflammation

Both TβR2 and TGF-β excision in LC induces their migration to the draining LN.[108] TGF-$\beta^{-/-}$ LC have a mature phenotype with higher levels of CD86 and CCR7 and lower E-cadherin on their surface, in agreement with an autocrine loop for TGF-β in regulating LC homeostasis.[108] Notably, the migration of LC is MyD88 independent, showing that TGF-β signaling in LC does not render LC unsensitive to TLR agonists.[108]

These results combined with ours demonstrate that TGF-β is not only crucial for the development of LC but is also required to maintain these cells in the epidermis in an immature state. Consequently, LC which lack TGF-β signaling undergo spontaneous maturation, inducing their migration to the draining LN.

TGF-β control of other DC populations

In 2007 a new Langerin^{+} dermal DC subset was discovered[109-111] and we asked wether their development and/or maturation is TGF-β dependent similar to LC. In Chapter 6 we establish that Langerin^{+} dermal DC homeostasis is comparable to WT and these cells are present in normal frequencies in DC-TβR1$^{-/-}$ mice. These results demonstrate that TGF-β signaling is not required for the development and maintenance of dermal Langerin^{+} DC, as previously shown in TGF-$\beta^{-/-}$ mice and confirmed and extended by our group in LC-TβR1$^{-/-}$ animals.[103,112] In Langerin^{+} dermal DC, the expression of Langerin was also not dependent on TGF-β, in contrast to LC, further emphasizing the distinct nature and origins of LC and Langerin^{+} dermal DC.

Studies on the role of TGF-β mediated phenotypic maturation of DC give contrasting results. IL-10/TGF-β differentiated DC express similar levels of MHC-II, CD80 and CD86 as WT DC.[113] In another study, TGF-β treatment augments E-cadherin, reduces CD86 surface expression and inhibits IL-1β and TNF-α production.[114] The homeostasis of DC expressing a dominant-negative TGF-βR2 (DC-DNTβR2) is not perturbed, and during *L. major* infection these DC secrete WT levels of IL-12.[115] However, in an atherosclerosis model, DC from DC-DNTβR2 mice secrete elevated levels of TNF-α and IL-12, which induce enhanced effector T cell activation while costimulatory surface markers did not differ from controls.[116] Here, we find that TβR1-deleted dermal DC (including both Langerin^{+} and Langerin^{neg} subsets) express WT levels of MHC-II and CD86, attesting that TGF-β does not influence dermal DC homeostasis. These discrepancies might be due to the source of DC, the efficiency of TGF-β signaling inhibition and whether the body endures chronic inflammation.

In Chapter 6, we demonstrate that splenic TβR1$^{-/-}$ DC display a WT phenotype in contrast to what was expected from in vitro studies.[10,13,107] These results establish that, except for LC, DC unable to integrate TGF-β signals do not spontaneously mature and do not induce systemic autoimmunity.

Role of skin DC subsets during contact hypersensitivity

The role of distinct DC populations during CHS has been controversial.[117] We performed two distinct experiments to decipher the role of LC and the importance of TGF-β signaling in dermal DC.

First, in a low dose CHS model in which the hapten preferentially reaches cells present in the epidermis, the CHS reaction is similar between LC-deficient DC-TβR1$^{-/-}$ animals and LC-depleted Langerin-DTR animals. Notably, both mice have significantly reduced ear swelling compared to WT. This corroborates our previous findings that LC are necessary to efficiently initiate the hapten-specific T cell response in a low dose model and that the reduced CHS reaction in the absence of LC is most likely due to inefficient hapten transport to the sdLN.[103,118,119] While these observations are supported by work from other groups,[120,121] they are in contrast to findings using human Langerin (hLangerin) transgenic mice. Both hLangerin-DTA mice, which constitutively lack LC, and hLangerin-DTR animals, following inducible LC ablation, develop an increased T cell reaction to a high dose of contact sensitizer.[122,123] The authors argue that hLangerin-DTA mice lack LC from birth which could influence the development of peripheral tolerance mechanisms during ontogeny of the immune system. Moreover, using adoptive T-cell transfer experiments, they demonstrate that enhanced CHS in the absence of LC is due to elevated priming of T cells rather than reactivation upon challenge. Consequently, the authors suggest that LC play a tolerogenic role, inhibiting the CHS reaction[122]. However, in DC-TβR1$^{-/-}$ animals, which constitutively lack LC (Chapter 6), as well as in Langerin-DTR mice after inducible LC ablation, we (and others) find a lower, and never observed an enhanced CHS reaction. The reason for this discrepancy is not clear, and why hLangerin-DTA and hLangerin-DTR mice mount exaggerated reactions remain elusive.

Second, since both langerin^{+} and langerin^{neg} dermal DC are able to carry Ag/contact sensitizer and induce the differentiation of naïve T cells in the sdLN,[124] we assessed the role of TGF-β in controlling dermal DC during CHS. For this purpose, we used a high dose model, where the hapten also efficiently reaches dermal DC. DC-TβR1$^{-/-}$, diphtheria toxin-treated Langerin-DTR and WT animals all develop similar ear swelling, demonstrating that TGF-β signaling in dermal DC is not necessary to control the CHS reaction. When only Langerin^{+} cells (LC and Langerin^{+} dermal DC) lack TGF-β signaling, CHS is also reduced.[103] These results are in accordance with other studies from our group showing that LC and Langerin^{+} dermal DC exert a redundant role rather than distinct functions during CHS.[32] Hence, the ear swelling reaction is dependent on the number of LC/DC (both in the epidermis and dermis) that transfer the hapten to sdLN for naïve T cell priming.[32]

In conclusion, LC are necessary for efficient initiation of the CHS reaction and TGF-β does not control dermal DC function to limit ear swelling in CHS.

TGF-β control of DC in the gastrointestinal tract

TGF-β signaling is crucial to maintain homeostasis in the gut. Paradoxically, patients with IBD often express elevated levels of TGF-β, but their T cells are refractory to TGF-β signals.[125,126] This is due to the fact that these patients also express elevated levels of Smad7 which inhibits TGF-β intracellular signal transduction. In colonic mucosa explants from patients, restoration of TGF-β signaling through the inhibition of Smad7 results

in decreased levels of pro-inflammatory cytokines.[125,126] Likewise, in mice, blockade of TGF-β signaling in T cells induces colonic inflammation.[127] In the colitis transfer model, Treg could not exert their inhibitory function in the presence of anti-TGF-β antibody.[128] The source of TGF-β in the colon during IBD remains controversial, but it is suggested that Treg might be the main, albeit not the only source.[129,130]

Since DC-TβR1$^{-/-}$ mice die at 10 weeks of age because of the uncontrolled proliferation of activated T cells that have deleted the TβR1, we crossed DC-TβR1$^{-/-}$ mice onto a Rag-deficient (Rag$^{-/-}$) background which allowed us to study TGF-β signaling specifically in DC and in the absence of inflammation. As expected, Rag$^{-/-}$DC-TβR1$^{-/-}$ mice remain healthy. We demonstrate that in steady state, TGF-β signals in DC are necessary for CD103 expression. In Rag$^{-/-}$DC-TβR1$^{-/-}$ mice, lack of TGF-β signaling results in significantly lower expression of CD103 on mLN DC (Chapter 7). This observation is supported by an in vitro study in which CD103 expression on CD8^{+} T cells is TGF-β mediated.[131] Accordingly, CD8^{+} T cells expressing a DN-TRβ2 are highly deficient in CD103 in vivo.[132] CD103 binds to E-cadherin,[133] and has a crucial role in maintaining T lymphocytes in situ since CD103-deficient mice harbor fewer T cells in the LP.[134] Also, DN-TRβ2 CD8^{+} T cells which express low levels of CD103, migrate to the LP but are retained there much less efficiently.[135] In the gastrointestinal tract, epithelial cells (EC) express E-cadherin and are important anchors for CD103^{+} DC.[136,137] Therefore CD103^{+} DC are found predominantly close to the epithelium.[138] TβR1-deficient DC express lower levels of CD103 and we hypothesize that they are therefore less retained in the LP and consequently migrate to the mLN. In fact, MHC-II^{hi} cells from Rag$^{-/-}$DC-TβR1$^{-/-}$ mLN express elevated levels of CCR7, a chemokine receptor necessary for migration of DC to the mLN.[139] Besides their anchoring properties, intestinal EC are essential in modulating DC function in situ.[137] Through their production of TGF-β and retinoic acid (RA), EC condition CD103^{+} DC to become immunosuppressive. Tolerogenic CD103^{+} DC then promote the differentiation of Treg.[137,140] Therefore our data suggest that lack of TGF-β signaling in DC firstly inhibits their retention in the LP and secondly prevents their tolerogenic conditioning (Figure 2B). When T cells are adoptively transferred into Rag$^{-/-}$DC-TβR1$^{-/-}$ mice, these animals develop significantly enhanced colonic inflammation compared to Rag$^{-/-}$ recipients. Colitis is caused by CD4-driven elevated levels of IFN-γ and IL-17A in situ. Corroborating our results, human colon mucosa explants secrete enhanced levels of IFN-γ in the presence of anti-TGF-β antibody.[65] CD103^{+} DC are necessary to maintain homeostasis and prevent colitis.[141] Through their secretion of TGF-β and RA, CD103^{+} DC promote Treg induction and inhibit Th1/Th17 effector T cells.[140,141] In light of these data, our results imply that TβR1-deficient DC, which are not properly conditioned by EC in the LP, induce enhanced Th1/Th17 activation and/or reduced Treg stimulation. LP cell isolations will further characterize LP TβR1-deficient DC in their expression of surface markers and cytokine production. However, we suggest that TβR1-deficient CD103^{+} DC secrete lower amounts of TGF-β and/or express lower levels of avβ8, which is crucial to activate TGF-β.[142] Both would result in reduced Treg

differentiation, which would promote colitis. TβR1-deficient LP DC could also secrete elevated levels of pro-inflammatory cytokines. In fact, DC expressing a DNTβR2 secrete elevated levels of IL-12 and TNF-α which induce enhanced activation of Th1, Th2 and Th17 cells.[116] Similarly, MOG-immunized DC-DNTβR2 mice develop EAE with increased infiltrates of $CD4^+$ T cells in situ and elevated levels of Th1 and Th17 in the periphery.[143] In conclusion, we demonstrate that TGF-β signaling in DC is necessary for CD103 expression, and suggest that lack of CD103 expression does not affect DC homing to the colon but regulates their retention in the LP and their conditioning towards tolerogenic DC (Figure 2B).

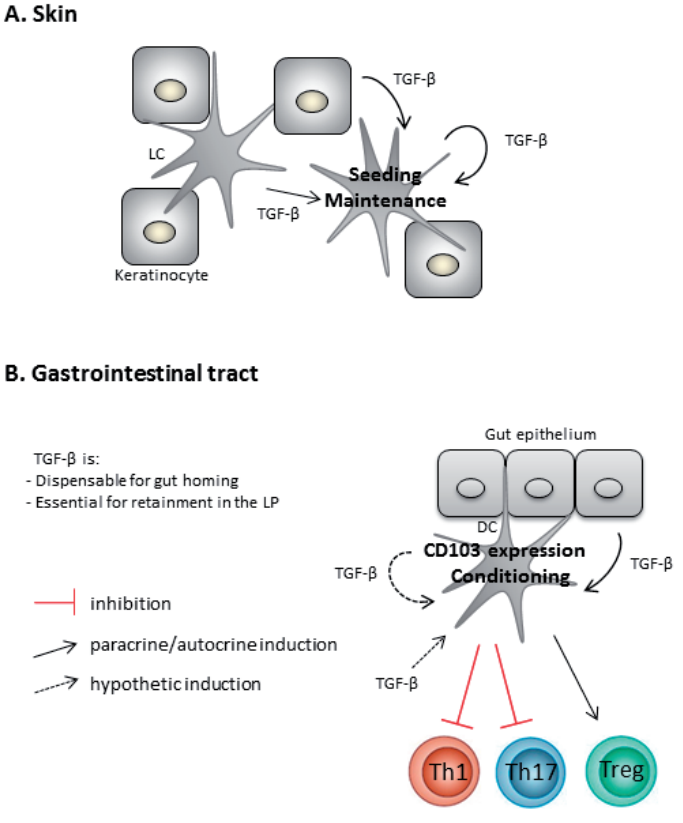

Figure 2. Proposed model on the importance of TGF-β signaling in two different subsets of DC. **(A)** In the skin, autocrine/paracrine TGF-β is necessary for seeding as well as the maintenance of LC in the epidermis.TGF-β has not obvious role in dermal skin DC populations. **(B)** TGF-β promotes CD103 expression on DC, which does not influence homing but regulates their retainment in the lamina propria. Moreover, TGF-β control of $CD103^+$ DC promotes their tolerogenic function. In the absence of TβR1, $CD103^+$ DC cannot be conditioned by gut epithelial cells and mediate a pro-inflammatory Th1/Th17 response instead of promoting Treg.

Difficulties of studying TGF-β signaling in vivo

The inconsistent results from DC-DNTβR2[115], DC-TβR2$^{-/-}$[144] and our DC-TβR1$^{-/-}$ mice (Chapters 6 and 7) illustrate the difficulties of studying TGF-β signaling in DC in vivo. The first major challenge is that not all target cells are affected by the mutation. DC-DNTβR2 animals only disrupt 50-80 % of TGF-β signaling in DC.[145] In transgenic mice resulting of the use of Cre recombinase, the efficiency of floxed target gene excision is known to vary between genes.[145] Further, the deletion efficiency may vary depending on the turnover of the target cells, which is low in the case of LC and high in other DC populations. Since LC ontogeny is dependent on TGF-β, a correlation exists between the presence of LC and the disruption of TGF-β signaling in DC allowing us to estimate the efficiency of TβR1 excision on LC. We demonstrate in Chapter 6 that DC-TβR1$^{-/-}$ animals are devoid of LC with only a few patches in adult mice, which certifies that TGF-β signaling is efficiently disrupted in LC.

Another difficulty resides in targeting DC and DC *only*. The CD11c-Cre transgene is expressed primarily by DC but also by a small fraction of other immune cell types such as T cells and NK cells.[146] Since TGF-β exerts a potent and crucial role in the regulation of cell function, deletion of TGF-β signaling in a fraction and/or subset of cells other than DC can induce a strong phenotype. Therefore it has been a challenge to study TGF-β signaling only in DC in vivo. In DC-DNTβR2 mice, TGF-β signaling is abrogated in DC but also in NK cells which proliferate, secrete elevated amounts of IFN-γ and induce enhanced Th1 responses.[115] We show in Chapter 7 that in DC-TβR1$^{-/-}$ mice, a small fraction of T cells delete the TβR1 at the genomic level and expand massively. When adoptively transferred into WT animals, these T cells induce severe autoimmunity, recapitulating the phenotype of T cell-specific TβR2$^{-/-}$ and DNTβR2 mice.[147,148] Therefore the phenotype observed in DC-TβR1$^{-/-}$ mice is not solely DC-driven, and a small fraction of T cells actually induce the inflammatory phenotype. Therefore, we next made use of Rag$^{-/-}$DC-TβR1$^{-/-}$ reconstituted with WT T cells to study the DC-specific phenotype. These mice develop colitis and slight inflammation in the liver and lungs, a much milder phenotype than the one of DC-TβR1$^{-/-}$ animals. Indeed, to study the biology of TGF-β Sanjabi and Flavell recommend to cross animals onto a Rag$^{-/-}$ background or the use of transfer models to be certain to target one cell population and to eliminate the severe T-cell driven autoimmune phenotype.[145] In light of our results, we speculate that the severe autoimmune phenotype observed in DC-TβR2$^{-/-}$ animals may be largely driven by a proportion of T cells that have deleted the receptor.[144]

In conclusion, it is still a challenge to study the role of TGF-β signaling in DC in vivo in the absence of specific Cre mice. We generated Rag$^{-/-}$DC-TβR1$^{-/-}$ which reconstituted with WT T and B cells provide a tool to investigate TGF-β signaling selectively in DC. In the future, the use of promoters more specific to DC (subsets) will allow more stringent mutations and will overcome the leaky phenotype of mice generated with target genes using the CD11c promoter. For example, the newly discovered transcription factor Zbtb46[149-151] and the C-type lectin receptor DNGR-1[152] seem to be expressed

exclusively by conventional DC and will facilitate conditional gene targeting in this DC population.

IL-10 and TGF-β: an old couple with complementary roles

IL-10 and TGF-β both control immune homeostasis and their role is complementary rather than redundant.[153] In several cell types, IL-10 and TGF-β cooperate to modulate cell function such as in B cells where IL-10 and TGF-β have a synergistic role in promoting IgA secretion.[154] IL-10 and TGF-β can also influence each other's secretion. IL-10 regulates TGF-β signaling in human bronchial epithelial cells by reducing Smad7 expression,[155] and in Th1 cells, TGF-β activates IL-10 promoter transcription through Smad4.[156]

Early studies suggest a differential role of IL-10 and TGF-β on macrophages in modulating their cytokine production.[157] In vitro, the effect of IL-10 on cytokine release is detectable 4 times faster than that of TGF-β, IL-10 inhibits TNF-α secretion 25-fold more than TGF-β and completely abrogates IL-1β release. At the molecular level, TGF-β inhibits translation whereas IL-10 mediates the degradation of cytokine mRNA.[158]. Several studies make use of IL-10 and TGF-β treated DC to induce tolerance, and DC treated with either one of the two cytokines are not as efficient in inducing tolerance as when treated with both, demonstrating their synergetic roles in DC.[113] These IL-10/TGF-β treated DC induce tolerance against diabetes[159] and during transplantation to induce allograft tolerance.[160]

TGF-β controls development of cells and prevents autoimmunity. On the other hand IL-10 inhibits reaction to nonpathogenic enteric microbiota and controls the downregulation of immune responses. Accordingly, in this thesis we demonstrate that the roles of IL-10 and TGF-β in controlling DC function in vivo are different but complementary. In the skin, TGF-β signaling is crucial for LC development, their immature phenotype and maintenance in the epidermis, but is dispensable for the homeostasis of other DC subsets. IL-10 control of DC does not play a role in the homeostasis of skin DC. However, during a CHS reaction, IL-10 signaling in DC is crucial to control the reactivation of T cells in situ whereas TGF-β control of dermal DC is not necessary either during priming or to control the reaction upon challenge. In contrast with DC-IL10R$^{-/-}$ mice which develop inflammation predominantly in the SI upon antigen exposure, Rag$^{-/-}$DC-TβR1$^{-/-}$ animals reconstituted with WT T cells develop inflammation only in the colon. Both IL-10 and TGF-β control of DC seem to have a similar function in controlling Th1 and Th17 responses as has also been suggested by a study on human colon explants.[65] However, IL-10R-deficient LP DC induce enhanced effector/memory T cell reactivation through elevated secretion of pro-inflammatory cytokines, whereas TGF-β seems important to control the maintenance and conditioning of LP CD103^{+} DC.

In conclusion, IL-10 signaling in DC is crucial to regulate the magnitude and phenotype of immune responses to (non-)harmful Ag, whereas TGF-β signals to maintain DC in situ, and control their immature state and conditioning.

FUTURE PERSPECTIVES

Since 1973 and the discovery of DC, the knowledge on these cells has evolved considerably. As initiators of adaptive immune responses, DC are now considered to have a great potential to be used as therapeutic tools.[9,161] Further understanding the role of IL-10 and TGF-β, the two most potent anti-inflammatory cytokines, in the control of DC will help to further decipher disease mechanisms and eventually will enable us to create new prophylactic and curative DC therapies.

We aim to further elucidate the role of IL-10 and TGF-β in gut homeostasis. DC-IL10R$^{-/-}$ develop spontaneous small intestinal inflammation and phenocopy most features of celiac disease. DC-IL10R$^{-/-}$ animals therefore represent a model of spontaneous murine celiac disease and can be used to investigate the complex immuno-pathogenesis of this pathology affecting millions of people worldwide. In vitro, recombinant human IL-10 suppresses gliadin-specific activation of T cells which suggests that IL-10 could be used as a therapy for this disease.[162] It is therefore important to understand the effects that injected IL-10 could have on other cells than T cells, notably on DC in vivo. Moreover, mutations in the IL-10 gene or the IL-10 signaling pathway are associated with severe IBD and lung and skin diseases in patients.[163-165] In mice, disruption of TGF-β signaling also induces IBD,[166] and in humans, a polymorphism in the TGF-β codon is associated with Crohn's disease.[167] To unravel which part of these phenotypes are induced by the lack of IL-10 or TGF-β signaling in DC will provide crucial information to further elucidate the mechanisms of IBD and celiac disease. It will also reveal whether DC are potential therapeutic targets. For example, targeting TGF-β to LP DC could promote their tolerogenic conditioning.

Moreover, DC-IL10R$^{-/-}$ and DC-TβR1$^{-/-}$ mice may be valuable tools to dissect the role of IL-10 and TGF-β in DC in cancer and viral infection models. Both IL-10 and TGF-β are secreted by tumor cells or tumor-associated cells (i.e.: macrophages, T cells)[168,169] as well as pathogens such as viruses[33] in order to bypass the immune system via induction of Ag-specific tolerance and by modulating DC function.[170] IL-10 promotes tumor growth by inducing Treg which inhibit DC function through IL-10 and TGF-β.[171-173] Therefore, targeting anti-IL-10R and TβR1 antibodies specifically to DC could inhibit the conditioning of DC by tumor/pathogen-derived IL-10 and TGF-β and thus restore normal responses to these harmful antigens. In vivo, mice vaccinated with DC modified with small interfering RNA against IL-10R or IL-10 develop enhanced CD8^{+} T cell responses against the human papilloma virus and tumors.[174] Particularly, our studies establish that IL-10 signaling in DC is dispensable during T-cell priming but essential to limit the magnitude and duration of the effector response both in the skin and gastrointestinal tract. Thus, the release of DC from the control mediated by IL-10 might promote their immunogenicity without increasing the risk for autoimmunity due to enhanced T-cell priming. It is therefore likely that blocking IL-10 signaling in DC would induce a strong Th1/Tc1 Ag-specific response that could result in enhanced clearance of tumors/pathogens, without affecting memory responses. DC also represent tools that can be used as vaccines and several clinical studies

are in trial.[175] In the skin, targeting of DC or a subset of DC could increase the efficacy of vaccines.[176] Especially, targeting CD205 on the surface of LC and dermal DC induces a potent Ag-specific immune reaction.[177]

In summary, DC represent great potential tools for immunotherapy. Further understanding the role of IL-10 and TGF in controlling DC function in vivo will hopefully enable to rationally further develop such therapies.

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SUMMARY

Dendritic cells (DC) are necessary to maintain homeostasis and are essential in regulating immune responses. DC induce effector T cell responses to invading pathogens and promote regulatory T cell (Treg) differentiation to harmless antigens. Interleukin-10 (IL-10) and transforming growth factor β (TGF-β) are anti-inflammatory cytokines displaying potent tolerogenic abilities. IL-10-deficient animals spontaneously develop colitis and mount enhanced Th1 responses to (innocuous) antigens. Mice lacking TGF-β1 die early in life due to severe multi-organ autoimmunity. Both cytokines are secreted by and act on a plethora of (non-) immune cells including T cells and DC. In particular, T cells are a prominent source and crucial targets of these cytokines in vivo. In vitro studies demonstrated that both IL-10 and TGF-β attenuate DC maturation and function by inhibiting their expression of MHC-II and co-stimulatory molecules and preventing cytokine secretion. In this thesis, we investigated the role of IL-10 and TGF-β signaling in DC in vivo, using mice with a DC-specific deletion of the IL-10Rα and TGF-βR1, respectively (DC-IL10R$^{-/-}$ and DC-TβR1$^{-/-}$ mice).

Chapter 1 provides a general introduction summarizing the current knowledge on the pivotal role of DC in balancing immunity and tolerance, as well as the essential contribution of IL-10 and TGF-β in maintaining immune homeostasis.

In **Chapter 2** we first analyzed DC-IL10R$^{-/-}$ mice in the steady state. It was previously demonstrated that IL-10 suppresses DC maturation in vitro and that culture in the presence of anti-IL10 neutralizing antibody induces a pro-inflammatory DC phenotype. In contrast, IL10R-deficient DC in spleen, skin-draining lymph nodes (LN) and skin did not express enhanced surface MHC-II or co-stimulatory molecules in vivo. However, upon in vitro stimulation, IL10R-deficient DC secreted elevated amounts of TNF-α, IL-6 and IL-10. We next analyzed the consequences of deficient IL-10 signaling in skin DC using a model of contact dermatitis. During contact hypersensitivity (CHS), DC-IL10R$^{-/-}$ mice displayed enhanced ear swelling starting at 48h after hapten challenge. Using an adoptive T cell transfer model, we established that IL-10 control of DC is dispensable during T cell priming in skin-draining LN, but crucial in regulating the reactivation of effector/memory T cells in the skin. Our results also strongly suggest that IL-10 signaling in T cells is not sufficient to control the ear swelling reaction.

C**hapter 3** continues to focus on the IL-10 regulation of skin DC, using a cutaneous infection model of *Leishmania major* (*L. major*) parasites. Lack of IL-10 signaling in DC promoted an enhanced Th1/Tc1 to Th2 balance which enabled more efficient parasite killing in the skin lesions. However, this response was not potent enough to achieve complete parasite clearance. Residual parasites were still present in DC-IL10R$^{-/-}$ mice after resolution of the inflammation and immunological memory was still intact. Therefore, DC-IL10R$^{-/-}$ mice were protected against reinfection with *L. major*, comparable to wild types (WT). Our results suggest that IL-10 control of DC is important during the acute phase but not the latent phase of leishmaniasis.

IL-10 is essential to maintain homeostasis in the gastrointestinal tract, as evidenced by severe colitis in IL-10-deficient animals. In **Chapter 4**, we demonstrate that IL-10 signaling in DC is crucial to maintain immune homeostasis in the small intestine. DC-IL10R$^{-/-}$ mice spontaneously developed enteropathy characterized by lamina propria (LP) T cell and IgA^{+} plasmablast infiltrates, crypt hyperplasia and increased numbers of intraepithelial lymphocytes. IL-10R-deficient LP DC expressed elevated levels of IL-23, IL-6, TNF-α and IL-12p70 that induced hyperactivation of T cells, particularly in the Th17 compartment. Moreover, IL-10R-deficient DC did not instigate augmented differentiation and activation of naïve T cells in the mesenteric LN, but rather promoted the enhanced recruitment, proliferation and survival of T cells in the LP. Therefore, IL-10 control of DC is necessary to dampen effector/memory Th1/Th17 cell reactivation and maintain immune homeostasis in the small intestine.

In **Chapter 5**, we examined the relevance of IL-10 signaling in DC at the environmental barrier of the lung, using an OVA-induced asthma model. DC-IL10R$^{-/-}$ mice developed less airway hyperresponsiveness (AHR) compared to controls. However, the levels of Th2-derived IL-4, IL-5 and IL-13 in broncho-alveolar lavage fluid and *il-4* and *gata3* mRNA in the lung were similar to WT, suggesting that IL-10 control of DC is dispensable to regulate Th2 cell function during asthma. Nonetheless, the expression of *foxp3* and *il-10* was enhanced in the lungs of DC-IL10R$^{-/-}$ animals. We hypothesize that IL-10R-deficient DC secrete elevated levels of IL-10, as observed in the spleen and small intestinal LP, which could enhance Treg activity and regulate AHR in situ.

The role of TGF-β in regulating DC homeostasis and function in vivo is investigated in chapter 6 and 7. It was previously shown that autocrine/paracrine TGF-β signaling is essential for Langerhans cell (LC) seeding of the skin. In **Chapter 6** we demonstrate that TGF-β is necessary for the maintenance of LC in the epidermis as DC-TβR1$^{-/-}$ mice gradually lost LC from the skin. Lack of TGF-β signaling in LC induced the downregulation of Langerin and E-cadherin and the upregulation of MHC-II, CD40 and CCR7, which promoted their migration to skin-draining LN. We also establish that TGF-β-signaling is dispensable for the homeostasis of other skin DC subsets, notably Langerin^{+} dermal DC. In a low hapten dose CHS model, the ear swelling reaction was diminished in the absence of LC whereas a high hapten dose restored the response to WT, emphasizing the redundant role of skin DC in inducing CHS reactions. Our results also demonstrate that TGF-β control of dermal DC is dispensable during CHS.

In **Chapter 7** we first describe the lethal multi-focal inflammation in DC-TβR1$^{-/-}$ mice. Unexpectedly, this phenotype was partially caused by T cells that have deleted the TβR1 and are activated and proliferate in vivo. We therefore crossed DC-TβR1$^{-/-}$ mice onto a Rag-deficient (Rag$^{-/-}$) background to study the role of TGF-β signaling specifically in DC. Intriguingly, mesenteric LN DC expressed lower levels of CD103 and CD11b and higher levels of CCR7, surface markers important in the maintenance and migration of DC in the LP. When WT T cells were infused into Rag$^{-/-}$/DC-TβR1$^{-/-}$ mice, the animals developed colitis characterized by enhanced Th1 and Th17 cell activation, crypt hyperplasia and

loss of goblet cells. We hypothesize that in DC-TβR1$^{-/-}$ animals, CD103^{+} cells cannot be conditioned by epithelial cell-derived TGF-β to become tolerogenic since they lack the TβR1. In addition, TβR1-deficient CD103^{+} cells populate the LP but are retained less effectively in the gut due to their low CD103 expression and thus are inefficiently conditioned by other tolerizing mediators. Consequently, TβR1-deficient CD103^{+} DC induce the elevated activation of pro-inflammatory T cells, which provoke colitis.

In **Chapter 8** the findings of this thesis are discussed in relation to the current literature and I address future perspectives emerging from the work presented in this thesis.

SAMENVATTING

Dendritische cellen (DC) zijn nodig om homeostase te handhaven en zijn essentieel in het reguleren van immuunresponsen. DC induceren effector T cel responsen tegen pathogenen en stimuleren differentiatie van regulatoire T cellen (Treg) tegen onschadelijke antigenen. Interleukine 10 (IL-10) en transforming growth factor-β (TGF-β) zijn anti-inflammatoire cytokines met potente tolerogene eigenschappen. IL-10-deficiente dieren ontwikkelen spontaan colitis en hebben verhoogde Th1-responsen tegen (onschadelijke) antigenen. Muizen zonder TGF-β1 sterven jong ten gevolge van multi-orgaan autoimmuniteit. Beide cytokines worden gesecreteerd door en werken op een breed palet van (non) immuuncellen inclusief T cellen en DC. Met name T cellen zijn een prominente bron zowel een cruciaal doelwit van deze cytokines in vivo. In vitro studies toonden aan dat zowel IL-10 als TGF-β de maturatie en functie van DC beperken door remming van expressie van MHC-II en costimulatoire moleculen en het remmen van cytokine secretie. In dit proefschrift onderzochten we de rol van IL-10 en TGF-β signalering in DC in vivo, gebruikmakend van muizen met een DC-specifieke deletie van de IL-10Rα en TGF-βR1, respectievelijk DC-IL10R$^{-/-}$ en DC-TβR1$^{-/-}$ muizen.

Hoofdstuk 1 biedt een algemene introductie die de huidige kennis samenvat over de beslissende rol van DC in de balans van immuniteit en tolerantie, en over de essentiele bijdrage van IL-10 en TGF-β in het handhaven van immuunhomeostase.

In **Hoofdstuk 2** analyseerden we allereerst DC-IL10R$^{-/-}$ muizen in de steady state. Het was eerder aangetoond dat IL-10 de maturatie van DC onderdrukt en dat kweek in de aanwezigheid van IL-10 neutraliserende antistof een pro-inflammatoir DC fenotype induceert. In contrast hiermee brachten IL-10R-deficiente DC in milt, huid-drainerende lymfeklieren en de huid, MHC-II of costimulatoire moleculen niet verhoogd tot expressie in vivo. Echter, na in vitro stimulatie secreteerden IL-10-deficiente DC verhoogde hoeveelheden TNF-α, IL-6 en IL-10. Vervolgens analyseerden we de consequenties van deficiente IL-10 signalering in huid DC gebruikmakend van een model voor contact dermatitis. Tijdens contactovergevoeligheid (CHS) vertoonden DC-IL10R$^{-/-}$ muizen versterkte oorzwelling vanaf 8 uur na hapteen challenge. Middels het overbrengen van T cellen stelden we vast dat IL-10 controle van DC niet noodzakelijk is tijdens de priming van T cellen in huid-drainerende lymfeklieren, maar wel cruciaal is in het reguleren van de reactivatie van effector/memory T cellen in de huid. Onze resultaten suggereren ook sterk dat IL-10 signalering in T cellen niet voldoende is om de reactie van oorzwelling te controleren.

Hoofdstuk 3 zet de focus door op IL-10 regulatie van huid DC, gebruikmakend van een huidinfectie model met *Leishmania major* (*L. major*) parasieten. Het ontbreken van IL-10 signalering in DC bevorderde een versterkte Th1/Tc1 over Th2 balans, die meer efficiente doding van de parasiet in huidlesies mogelijk maakte. Echter, deze respons was niet voldoende potent om complete eliminatie van de parasieten te bewerkstelligen. Residuele parasieten bleven aanwezig in de DC-IL10R$^{-/-}$ muizen na het oplossen van de

ontsteking, en het immunologisch geheugen was intact. Daarom waren DC-IL-10R$^{-/-}$ muizen beschermd tegen herinfectie met *L. major*, vergelijkbaar met wild type (WT).

IL-10 is essentieel om homeostase in het darmstelsel in stand te houden, mede blijkend door het optreden van ernstige colitis in IL-10-deficiente dieren. In **Hoofdstuk 4** tonen we aan dat IL-10 signalering in DC cruciaal is voor het behouden van immuun homeostase in de dunne darm. DC-IL10R$^{-/-}$ muizen ontwikkelden spontane enteropathie gekarakteriseerd door infiltraten van lamina propria (LP) T cellen en IgA^{+} plasmablasten, crypte hyperplasie en toegenomen aantallen van intraepitheliale lymfocyten. IL-10R-deficiente LP DC brachten verhoogde niveaus van IL-23, IL-6, TNF-α en IL-12p70 tot expressie die hyperactivatie van T cellen induceerden, met name in het Th17 compartiment. Bovendien bevorderden IL-10R-deficiente DC geen versterkte differentiatie en activatie van naieve T cellen in de mesenteriale lymfeklieren, maar leidden juist tot verhoogde recrutering, proliferatie en overleving van T cellen in de LP. Hieruit blijkt dat IL-10 controle van DC nodig is voor reactivatie van effector/memory Th1/Th17 cellen te dempen en immuunhomeostase in de dunne darm te handhaven.

In **Hoofdstuk 5** onderzochten we het belang van IL-10 signalering in DC op de barriere van de long met de omgeving, middels een OVA-geinduceerd astma model. DC-IL10R$^{-/-}$ muizen ontwikkelden minder luchtweg hyperresponsiviteit (AHR) vergeleken met controles. Echter, de niveaus van Th2-geproduceerd IL-4, IL-5 en IL-13 in bronchoalveolaire lavage en *il-4* en *gata3* mRNA in de long waren vergelijkbaar met WT. Dit suggereert dat IL-10 controle van DC niet vereist is voor het reguleren van de Th2 cel functie tijdens astma. Niettemin was de expressie van *foxp3* en *il-10* versterkt in de longen van DC-IL10R$^{-/-}$ dieren. We hypothetiseren dat IL-10R-deficiente DC verhoogde niveaus van IL-10 secreteren, zoals vastgesteld in de milt en de LP van de dunne darm, die activiteit van Treg zouden kunnen verhogen en de AHR in situ reguleren.

De rol van TGF-β in de regulatie van DC homeostase en functie in vivo wordt onderzocht in Hoofdstuk 6 en 7. Het was eerder aangetoond dat autocriene/paracriene TGF-β signalering essentieel is voor het koloniseren van de huid door Langerhans cellen (LC). In **Hoofdstuk 6** tonen we aan dat TGF-β nodig is voor het in stand houden van het LC netwerk in de epidermis, omdat DC-TβR1$^{-/-}$ muizen de LC uit de huid geleidelijk verliezen. Het ontbreken van TGF-β signalering in LC leidde tot verminderde expressie van Langerine en E-cadherine en de opregulering van MHC-II, CD40 en CCR7, wat hun migratie naar huid-drainerende lymfeklieren bevorderde. We toonden tevens aan dat TGF-β signalering niet nodig is voor de homeostase van andere DC subsets in de huid, met name Langerin^{+} DC in de dermis. In een CHS model met lage hapteen dosis was de oorzwellingsreactie verminderd in de afwezigheid van LC, terwijl een hoge hapteen dosis de respons tot op het niveau van WT terugbracht. Dit benadrukt de redundante rol van huid DC bij de inductie van CHS reacties. Onze resultaten tonen ook aan dat TGF-β controle van dermale DC niet van belang is.

In **Hoofdstuk 7** beschrijven we eerst de lethale multi-focale ontsteking in DC-TβR1$^{-/-}$ muizen. Onverwachterwijs werd dit fenotype gedeeltelijk veroorzaakt door T cellen

die de TβR1 deleteren en die in vivo worden geactiveerd en prolifereren. We kruisten daarom DC-TβR1$^{-/-}$ muizen terug op een Rag-deficiente achtergrond (Rag$^{-/-}$) om de rol van TGF-β signaling specifiek in DC vast te stellen. Intrigerenderwijs brachten DC uit de mesenteriale lymfeklieren lagere niveaus van CD103 en CD11b tot expressie, en hogere niveaus van CCR7, membraanmoleculen die belangrijk zijn voor instandhouding en migratie van DC in de LP. Wanneer WT T cellen werden toegediend aan Rag$^{-/-}$DC-TβR1$^{-/-}$ muizen, ontwikkelden die colitis gekarakteriseerd door verhoogde Th1 en Th17 activatie, crypte hyperplasie en verlies van goblet cellen. We hypothetiseren dat in DC-TβR1$^{-/-}$ dieren CD103^{+} cellen niet worden geconditioneerd door TGF-β van epitheelcellen om tolerogeen te worden omdat ze de TβR1 mankeren. Bovendien kunnen TβR1-deficiente CD103^{+} cellen de LP wel populeren, maar worden minder effectief vastgehouden in de darm door hun lage CD103 expressie, waardoor zij inefficient door andere tolerogene factoren worden geconditioneerd. Om die reden induceren TβR1-deficiente CD103^{+} DC verhoogde activatie van pro-inflammatoire T cellen, die colitis veroorzaken.

In **Hoofdstuk 8** worden de bevindingen van dit proefschrift geinterpreteerd in het licht van de bestaande literatuur, en ik bespreek perspectieven voor de toekomst, voortkomend uit werk beschreven in dit proefschrift.

LIST OF PUBLICATIONS

- **Girard-Madoux MJ**, Ober-Blöbaum JL, Kel JM, Lindenbergh-Kortleve DJ, Samsom JN[*], Clausen BE[*]. IL-10 control of dendritic cells is crucial to maintain immune homeostasis in the small intestine. *Submitted.* [*]Authors contributed equally.

- **Girard-Madoux MJ**[*], Kautz-Neu K[*], von Stebut E, Clausen BE. Lack of IL-10 signaling in dendritic cells enhances anti-Leishmania major immunity. *Submitted.* [*]Authors contributed equally.

- Clausen BE, **Girard-Madoux MJ**. IL-10 control of dendritic cells in the skin. *OncoImmunology 2013; 2:e23186*

- Mandaric S, Walton SM, Rülicke T, Richter K, **Girard-Madoux MJ**, Clausen BE, Zurunic A, Kamanaka M, Flavell RA, Jonjic S, Oxenius A. IL-10 suppression of NK/DC crosstalk leads to poor priming of MCMV-specific CD4 T cells and prolonged MCMV persistence. *PLoS Pathog. 2012 Aug;8(8):e1002846*

- **Girard-Madoux MJ**, Kel JM, Reizis B, Clausen BE. IL-10 controls dendritic cell-induced T-cell reactivation in the skin to limit contact hypersensitivity. *J Allergy Clin Immunol. 2012 Jan;129(1):143-50.*

- Kel JM, **Girard-Madoux MJ**, Reizis B, Clausen BE. TGF-β is required to maintain the pool of immature Langerhans cells in the epidermis. *J Immunol. 2010 Sep 15;185(6):3248-55.*