In vivo functions of intestinal dendritic cells

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA BY

Nathan Edward Welty

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Adviser: Daniel H. Kaplan, M.D., Ph.D.

September 2014

Acknowledgements

I owe a considerable debt of gratitude to many people who made this work possible over the last four years and beyond.

First and foremost, I would like to thank my advisor Dan Kaplan, who took a risk giving a second year med student with limited immunology experience an independent project after the most minimal of rotations. I will always be grateful for your faith in me and for your ongoing scientific and professional mentorship.

Thanks to all the present and past members of the Kaplan lab, especially Krystal Haley, Beth Jarrett, and Brian Chicoine who have prevented us from descending into utter chaos. To Zach Zelickson for assisting with endless gut preps. Many thanks also to Botond Igyártó for the initial observations that started this project.

I thank the MSTP and MICaB administrators who have made my time at Minnesota possible. To Tucker LeBien, Yoji Shimizu, Chris Pennell, and Steve Jameson for excellent leadership and support. To Susan Shurson, Nick Berg, and Louise Shand for always knowing or finding out the answers to all of my questions.

Thanks to my past and present committee members Steve Jameson, Marc Jenkins, Brian Fife, Alex Khoruts, and Dave Masopust for their valued input and guidance.

I would like to thank all those who have collaborated on this research. I especially thank the members of Marc Jenkins' lab, in particular Ryan Nelson, Jon Linehan, and T. Dileepan, for generously providing numerous reagents and valuable advice. To the Jenkins, Mueller and Fife labs for their feedback in joint lab meetings. To Chris Staley and Mike Sadowsky for providing the data that put our paper over the finish line. And to recent collaborators in the Littman and Reizis labs, thank you.

Thank you to my past scientific mentors, particularly Karen Iles and John Hartman IV.

I am forever indebted to all those who have invested themselves in teaching and took a special interest in my success. In particular, I thank Jane Mahon for making high school chemistry unforgettable, Paul Delaney for supporting me in any and every way he could, and Eileen McMahon McQuade for first introducing me to immunology.

A great fortune of a pursuing a dual-degree is the chance to make a new set of friends at every stage. I'm especially thankful for the support and friendship of Cori Russell, Katherine Janssen, Chika Nwulu, Minelva Nanton, Caitlin Heiderscheidt, Lokesh Kalekar, Shirdi Schmiel, Michelle Smith, Sam Dunmire, Joe Belanto, and Sarah Parker during my graduate career. For all the other MICaB, MSTP, and medical students who have been helpful colleagues and friends. And I am especially thankful for those friends who have been there for me since college, especially Katie Rea and Jennifer Lorden.

Lastly I thank the very most important people in my life. In my grandparents Willis, Catherine, Hilda, and Jacob, I am fortunate to have cherished models of life and love. I would not have gotten far without the loving support of my mother, Connie, my father, Tim, and my brother and sister, Luke and Elisabeth. And finally, to Josh, my constant companion, best friend, and true love thank you.

Dedication

For Josh and Griffin

Abstract

Dendritic cells (DCs) in the intestinal lamina propria (LP) are composed of two $CD103^+$ subsets that differ in CD11b expression. We report here that Langerin is expressed by human LP DCs and that transgenic human langerin drives expression in $CD103^+$ $CD11b^+$ LP DCs in mice. This subset was ablated in huLangerin-DTA mice, resulting in reduced LP Th17 cells without affecting Th1 or T reg cells. Notably, cognate DC–T cell interactions were not required for Th17 development, as this response was intact in huLangerin-Cre I-$A\beta^{fl}$ mice. In contrast, responses to intestinal infection or flagellin administration were unaffected by the absence of $CD103^+$ $CD11b^+$ DCs. huLangerin-DTA x $BatF3^{-/-}$ mice lacked both $CD103^+$ LP DC subsets, resulting in defective gut homing and fewer LP T reg cells. Despite these defects in LP DCs and resident T cells, we did not observe alterations of intestinal microbial communities. Thus, $CD103^+$ LP DC subsets control T cell homeostasis through both non-redundant and overlapping mechanisms.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

PRR: pattern recognition receptor
PAMP: pathogen associated molecular pattern
TLR: toll-like receptor
TCR: T cell receptor
IL: interleukin
MHCII: major histocompatibility complex class II
APC: antigen presenting cell
DC: dendritic cell
LC: Langerhans cell
cDC: conventional dendritic cell
MLN: mesenteric lymph node
LP: lamina propria (small intestine unless specified otherwise)
RA: retinoic acid
ILC: innate lymphoid cell
BATF: basic leucine zipper, atf-like transcription factor
Ova: ovalbumin
SFB: segmented filamentous bacteria
WT: wild type
huLangerin: human langerin

Chapter 1: An overview of dendritic cell functions

Introduction

A basic function of immune surveillance is to discriminate between self and nonself particles and to link these distinctions to the generation of an appropriate immune response (Janeway, 1989). In higher-order vertebrates like mammals, carrying out this function requires the coordinated actions of both the innate and adaptive arms of the immune system. The innate immune system encodes pattern recognition receptors (PRRs), which detect conserved microbial products, known as pathogen associated molecular patterns (PAMPs). Due to their conserved nature, PAMPs reliably signify the presence of foreign microbes to the host. Activation of PRRs, which include the families of Toll-like receptors, NOD-like receptors, and lectin receptors, by PAMPs stimulates rapid responses in innate immune cells. These responses may include the enhancement of phagocytic activity, release of anti-microbial molecules, or the production of pro-inflammatory cytokines that recruit other cells to the site of infection.

Unlike innate immune cells, cells of the adaptive immune system, including B cells and CD4 and CD8 T cells, express receptors with essentially limitless potential specificities. These receptors, known as the B cell receptor (BCR) and T cell receptor (TCR) respectively, are generated randomly through recombination of germline DNA segments within these cells during development. As a result, the frequency of cells expressing any given BCR or TCR is extremely low. T cell receptors recognize peptide fragments, or antigens, processed from

self or foreign proteins and displayed on major histocompatibility complex (MHC) molecules on the surface of other cells. CD8 T cells recognize peptides presented in the context of MHC class I molecules, which are present on most cells of the body, while CD4 T cells recognize peptides displayed on MHC class II, which is expressed only by certain immune cells known as antigen presenting cells (APCs). In addition to stimulation through the TCR by peptide-MHC, T cells require several signals for their full stimulation and acquisition of effector functions. These include costimulatory signals, the most prominent of which is delivered through CD28, which is activated by CD80 or CD86 on the surface of the APC.

APCs also deliver additional signals, some of which are in the form of secreted cytokines that bind receptors present on the T cell and can cause responding T cells to acquire distinct fates. Thus, naïve CD4 T cells can become helper T cells that express distinct combinations of cytokines and transcription factors that provide protection from different types of pathogens (Figure 1-1). For instance, T helper type 1 (Th1) cells produce the canonical cytokine interferon-γ (IFNγ), which activates macrophages to clear intracellular infections, while Th17 cells produce interleukin-17 (IL-17), which recruits neutrophils to clear extracellular infections. Thus, the cellular components of the innate immune system determine the character and effectiveness of the adaptive immune response, particularly those innate cells that express MHC molecules. One such cell is the dendritic cell.

The dendritic cell paradigm

Ralph Steinman and Zanvil Cohn first described dendritic cells (DCs) in the early 1970s. These “large stellate cells” were present in the peripheral lymphoid organs of mice and named for their distinctive dendritic morphology (Steinman & Cohn, 1973; 1974; Steinman, Lustig, & Cohn, 1974). Subsequent studies revealed that DCs, and related epidermal Langerhans cells (LCs), were potent activators of T cell proliferation *in vitro* (Steinman & Witmer, 1978), which eventually led to their designation as APCs. However, DCs were not constitutively mitogenic (Romani et al., 1989; Schuler & Steinman, 1985), leading to the idea that dendritic cells existed in two functional states and had to under go “maturation” in order to stimulate a T cell response. Further studies revealed that functional DC maturation coincided with marked alterations in DC behavior, including reduced phagocytosis, cytoplasmic reorganization, enhanced migration, and upregulation of MHC molecules and the costimulatory molecules CD80 and 86 (Larsen, Ritchie, Pearson, Linsley, & Lowry, 1992; Larsen et al., 1990; Reis e Sousa, Stahl, & Austyn, 1993). These activities were found to be induced in DCs by microbial stimuli like LPS (Roake et al., 1995; Sallusto, Cella, Danieli, & Lanzavecchia, 1995), as was their upregulation of the chemokine receptor CCR7 (Dieu et al., 1998), which allowed trafficking of DCs to draining lymph nodes (LNs) along CCL19/21 gradients (Förster et al., 1999).

Thus, these nearly two decades of research generated a basic paradigm of DC function in the immune system, namely that innate detection and

acquisition of microbes by “immature” DC in peripheral tissues led to both their upregulation of MHC and costimulatory molecules as well as their migration to draining LNs. Here, they could encounter rare naïve T cells with receptors specific for antigens acquired in the periphery and stimulate their proliferation and acquisition of effector functions (Reis e Sousa, 2006). Dendritic cells thus represented a physical link between innate detection of microbial products and generation of adaptive immune responses that had been hypothesized by Janeway (Janeway, 1989). There have been important revisions to this concept, most notably the observation that steady-state migration of dendritic cells occurs even in the absence of overt microbial stimuli and is an important mechanism for maintaining immune tolerance (Steinman, Hawiger, & Nussenzweig, 2003). Nonetheless this basic model still underlies our understanding of DC functions today.

Dendritic cell ontogeny

Dendritic cells are bone marrow derived cells that are thought to arise from hematopoietic stem cells (HSCs) through several intermediate stages (Fig. 1-2). The first step thought to represent commitment to the mononuclear phagocyte lineage; a term that collectively refers to monocytes, macrophages, and dendritic cells; is the so-called macrophage/DC progenitor (MDP). MDPs are CX_3CR1^{int} cells that differentiate from a common myeloid progenitor (CMP) and retain the capacity to reconstitute macrophage and DC compartments but not other myeloid lineages (Fogg et al., 2006). MDPs can subsequently differentiate

into common DC progenitors (CDP), which express lower levels of CD117 (c-kit) and have the potential to reconstitute both the plasmacytoid (pDC) and conventional (cDC) compartments, but do not give rise to macrophages. Alternatively, MDPs can also differentiate into Ly6C^{hi} monocytes, which can give rise to circulating monocytes and some tissue resident macrophages (Bogunovic et al., 2009; Ginhoux et al., 2006; S. H. Naik et al., 2007; Onai et al., 2007b; Tamoutounour et al., 2013). Finally, CDPs can further differentiate into pre-cDCs, which are CD11c^{+} MHCII^{-} cells present in both the bone marrow and in the circulation, from which they seed peripheral tissues, including secondary lymphoid organs, to give rise to terminally differentiated DC subsets (Liu et al., 2009). From the MDP stage onward, DC progenitor populations express Fms-like tyrosine kinase 3 (Flt3), making Flt3L dependence a common strategy to used to identify “true” DCs as opposed to tissue resident monocytes or inflammatory macrophages (Waskow et al., 2008).

There are some exceptions to these developmental mechanisms, most notably with respect to Langerhans cells in the epidermis, which develop from embryonic precursors prior to birth and locally self-renew within the skin (Hoeffel et al., 2012; Merad, Ginhoux, & Collin, 2008). Recently, reporter mice for the transcription factor Zbtb46 and the c-type lectin DNGR1 have been used to define the DC lineage more restrictively in both the bone marrow and the periphery (Meredith et al., 2012; Satpathy, KC, Albring, Edelson, Kretzer, et al., 2012a; Schraml et al., 2013). Both these strategies as well as single cell analysis

of HSC progenitors have suggested that pDC precursors may in fact be distinct from those that give rise to cDCs. Additionally, analysis of HSC progenitors at the single cell level reveals some propensity of even early “multipotent” HSCs to form only certain subsets (S. H. Naik et al., 2013), suggesting that DC lineage specification does not occur in a rigid step-wise fashion but instead takes place through graded commitment and may begin earlier than previously suspected.

Dendritic cell subsets

Conventional DCs in peripheral tissues have a short half-life and are continually replaced by pre-cDCs circulating in the blood. After entering peripheral tissues, these cells undergo further terminal differentiation steps to generate the full complement of cDC subsets present in both lymphoid and nonlymphoid organs. Although the markers used to identify these subsets can vary between tissues, there are presently two broad DC subsets present in most peripheral lymphoid and nonlymphoid tissues that can be defined by phenotypic markers, ontogeny, transcription factor expression profiles, and function (Satpathy, Wu, Albring, & Murphy, 2012b).

$CD8\alpha^+$ and $CD103^+$ DCs

CD8α has long been used to identify heterogeneous populations of DCs resident in secondary lymphoid organs (Vremec et al., 1992), but this subset has recently been broadened to include DCs expressing the integrin CD103 that are $CD8\alpha^-$ and are present in most nonlymphoid tissues. These subsets share expression of several phenotypic markers, including XCR1 and Clec9a (DNGR1),

but lack expression of CD11b (Crozat et al., 2010; 2011; Poulin et al., 2012; 2010). More importantly, these subsets share a common requirement for the transcription factors BatF3, IRF8, and Id2 during development (Edelson et al., 2010; Hacker et al., 2003; Hildner et al., 2008; Schiavoni et al., 2002; Tussiwand et al., 2012), which initiate unique developmental and functional programming in these cells and identify them as a unified and distinct DC lineage. Additionally these cells have been aligned with human $CD141^+$ DCs present in tissues, which also express DNGR1, and have a similar transcriptional profile and functional properties (Haniffa et al., 2012; Jongbloed et al., 2010; Poulin et al., 2010).

CD8α lymph node resident DCs and $CD103^+$ nonlymphoid tissue DCs have likewise been shown to share a number of important immunological functions, most notably the capacity to take up exogenous antigens and display them as peptide-MHC class I complexes instead of peptide-MHC class II complexes (Bedoui et al., 2009; Hildner et al., 2008), a process known as cross-presentation. Cross presentation thus allows antigens acquired from other infected cells to be presented to MHCI restricted, cytotoxic CD8 T cells. Hence, CD8α and $CD103^+$ DCs are of critical importance for the generation of antiviral immunity and for tumor rejection, and are also likely important detectors of "danger" signals through their ability to cross-present necrotic cell derived antigens (Bedoui et al., 2009; Hildner et al., 2008; Sancho et al., 2009). This subset of DCs has also been implicated in development to of type 1 immunity, a term that refers to both Th1 cells as well as innate sources of type 1 cytokines,

such as group 1 innate lymphoid cells (ILCs) and natural killer cells, during infection (Briseño, Murphy, & Murphy, 2014; Igyarto et al., 2011; Mashayekhi et al., 2011). This requirement is due at least in part to the ability of CD8α/CD103$^+$ DCs to produce IL-12, which is required for Th1 responses (Hsieh et al., 1993).

CD11b$^+$ DCs

The identification of CD11b$^+$ DC as a distinct DC lineage has been complicated by the difficulty of distinguishing these cells from tissue resident macrophages, which also express high levels of CD11b, as well as other phenotypic markers of this subset. Recently, some cell surface markers have been identified that are capable of distinguishing these subsets, although they may not be faithful in every tissue (Gautier et al., 2012; Schraml et al., 2013; Tamoutounour et al., 2012). CD11b$^+$ DCs were originally identified in secondary lymphoid organs by their modest expression of CD4 and lack of CD8α expression. The first transcription factor identified to be required for development of this subset was RelB (L. Wu et al., 1998), followed by TRAF6 and interferon regulatory factor-2 (IRF2) (Ichikawa et al., 2004; Kobayashi et al., 2003), although the basis of these requirements is still unclear. It is also not known whether these transcription factors are required for differentiation of nonlymphoid tissue CD11b$^+$ DCs. Mice with a DC-restricted TRAF6 deficiency, for instance, show a normal distribution of DC subsets in the gut (Han et al., 2013). More recently, the transcription factor IRF4 has been identified as a common requirement for both lymphoid and nonlymphoid tissue CD11b$^+$ DCs (Persson et

al., 2013; Schlitzer et al., 2013). However, loss of this transcription factor in nonlymphoid tissue CD11b$^+$ DCs has a relatively modest effect, and IRF4 appears to also affect this subset by inhibiting migration to draining lymph nodes (Bajana, Roach, Turner, Paul, & Kovats, 2012). Finally, signaling through the Notch2 pathway is required for development of both a subset of CD11b$^+$ ESAM$^+$ DCs in the spleen as well as CD11b$^+$ DCs in the gut but does not appear to affect the development of other CD11b$^+$ subsets (K. L. Lewis et al., 2011; Satpathy et al., 2013).

Importantly, many of the transcription factors involved in CD11b$^+$ DC development appear to also affect the development or maturation of other DC subsets (Bajana et al., 2012; Kobayashi et al., 2003; Satpathy et al., 2013), complicating functional analyses of these cells. Nonetheless, these DCs appear to have complementary functions to the CD8α/CD103 DC lineage. Several studies have shown that, while they are not particularly effective at cross presentation to CD8 T cells, CD11b$^+$ DCs are much more effective than CD8α DCs in stimulating MHCII dependent CD4 T cell responses (Dudziak et al., 2007; K. L. Lewis et al., 2011; Vander Lugt et al., 2013). Additionally, we and others have shown that these cells are important in the generation of type 3 immunity, which refers to both Th17 cells and ILC3s that produce type 3 cytokines like IL-17 and IL-22 (K. L. Lewis et al., 2011; Persson et al., 2013; Satpathy et al., 2013; Schlitzer et al., 2013; Welty et al., 2013). The mechanisms by which CD11b$^+$ DCs mediate these responses are less clear, and are discussed at length in later

chapters. Finally, recent evidence has also begun to accumulate that $CD11b^+$ DCs may regulate type 2 immunity (Gao et al., 2013; Kumamoto et al., 2013; Plantinga et al., 2013; J. W. Williams et al., 2013).

Dendritic cells and the intestinal immune system

The intestine represents a unique barrier tissue because it is colonized by a dense and diverse cohort of commensal microbes in numbers greater than there are cells in the human body (Maynard, Elson, Hatton, & Weaver, 2012). Despite the fact that these microbes express the same PAMPs as pathogenic organisms, and are themselves capable of causing disease in certain settings (Hepworth et al., 2013; Sonnenberg et al., 2012), the immune system remains tolerant of these organisms. In part this state of tolerance is maintained by mechanisms that limit contact with commensal organisms (Macpherson, Slack, Geuking, & McCoy, 2009). However, commensals can and do shape the immune development of the host (H. Chung et al., 2012). Additionally, the gut represents a common portal for many pathogens that must be discriminated from commensals and eliminated. Understanding the mechanisms that promote intestinal immune homeostasis and responses to gut pathogens is thus of great importance.

A large and diverse cohort of dendritic cells in the intestinal lamina propria resides immediately below the single-cell intestinal epithelial layer (Fig. 1-3). DC subsets present in the gut consist of both $CD103^+$ $CD11b^-$ DCs that are similar to CD8α/CD103 DCs in other tissues, and $CD103^+$ $CD11b^+$ DCs, which, despite

their unique expression of CD103, share a common transcriptome and ontogeny with $CD11b^+$ DCs at other sites (Bogunovic et al., 2009; K. L. Lewis et al., 2011; Miller et al., 2012). Additionally, $CD103^-$ CX_3CR1^+ intestinal macrophages represent a large proportion of the antigen presenting cells in the gut and derive from a $Ly6C^{hi}$ monocyte precursor (Bogunovic et al., 2009; Varol et al., 2009). Distribution of these three major subsets varies along the length of the intestine, with $CD103^+$ DCs being more predominant in the small intestine (Denning et al., 2011; Jaensson-Gyllenbäck et al., 2011). In addition to the lamina propria, the gut also contains subanatomical lymphoid structures including isolated lymphoid follicles (ILFs) and Peyer's patches, in which $CD103^+$ $CD11b^-$ DCs are thought to predominate (Bogunovic et al., 2009).

While the development and phenotype of these diverse APC subsets have been well characterized, their *in vivo* functions are less understood. CX_3CR1^+ macrophages extend transepithelial dendrites into the lumen, where they can sample antigens under both steady-state and inflammatory conditions (Niess et al., 2005; Rescigno et al., 2001). While they appear to migrate to mesenteric lymph nodes (MLNs) only poorly in the steady-state, loss of commensal signaling can induce migration of these cells and transport of bacteria acquired from the lumen (Diehl et al., 2013; Schulz et al., 2009). In contrast, $CD103^+$ cDC express CCR7 and migrate to MLNs under steady-state and inflammatory conditions (Schulz et al., 2009). An important function of these cells appears to be their ability to produce retinoic acid (RA) metabolizing enzymes, which is thought to be

important in maintaining immune homeostasis and tolerance toward intestinal commensals (Coombes & Powrie, 2008; Hall, Grainger, Spencer, & Belkaid, 2011). However, CX_3CR1^+ macrophages are also important for maintaining tolerance (Hadis et al., 2011; Zigmond et al., 2014), and the individual functions of the two distinct $CD103^+$ has been poorly defined.

In the following chapters, the specific functions of these distinct intestinal dendritic cell subsets are explored. Chapter 2 will describe the development of *in vivo* mouse models of DC subset deletion. Chapter 3 investigates the functional requirement for these subsets in several relevant models of infection and inflammation. Chapter 4 describes the role of intestinal dendritic cells in maintaining immune and commensal homeostasis in the gut. Chapter 5 examines the mechanism whereby a particular DC subset contributes to Th17 responses induced by specific commensals. Finally, Chapter 6 offers brief concluding remarks on dendritic cells in disease pathogenesis and the potential for DC based therapeutics.

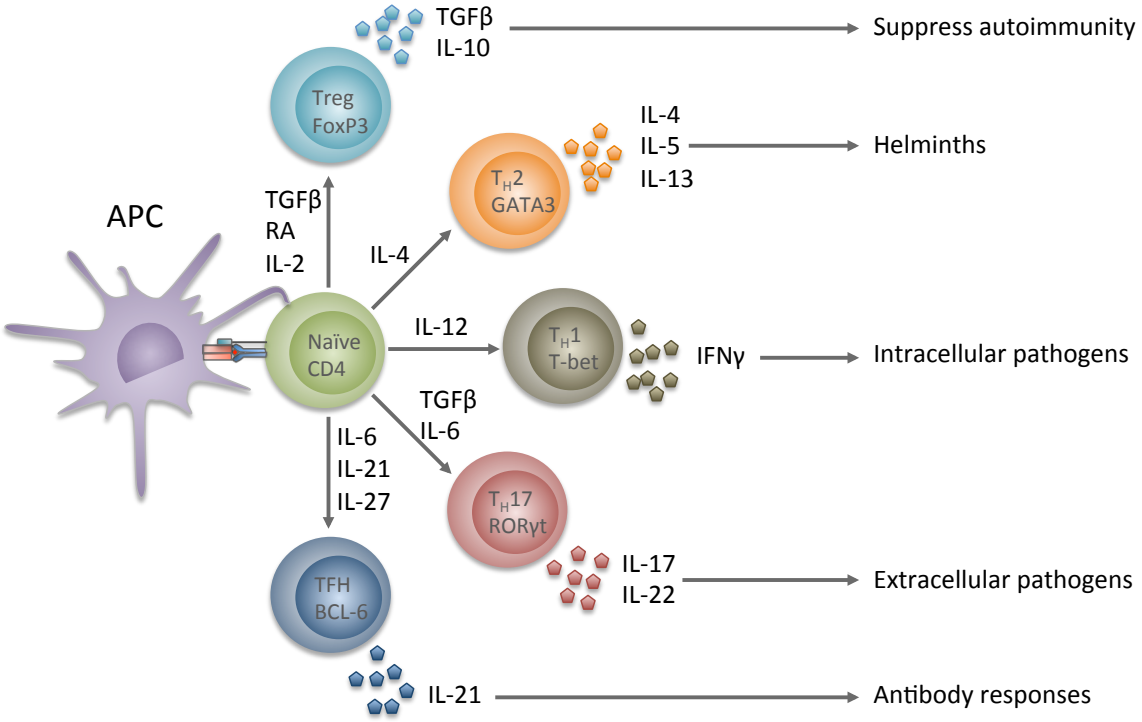


Figure 1-1: Three signal model of CD4 T cell differentiation

Naïve helper CD4 T cells encountering cognate peptide/MHCII complexes presented by APCs (signal 1) and costimulatory molecules (signal 2) in the context of the indicated cytokines (signal 3) undergo differentiation to different T helper lineages, marked by expression of canonical transcription factors and effector cytokines. These differentiated effector cells then carry out distinct functions during an immune response

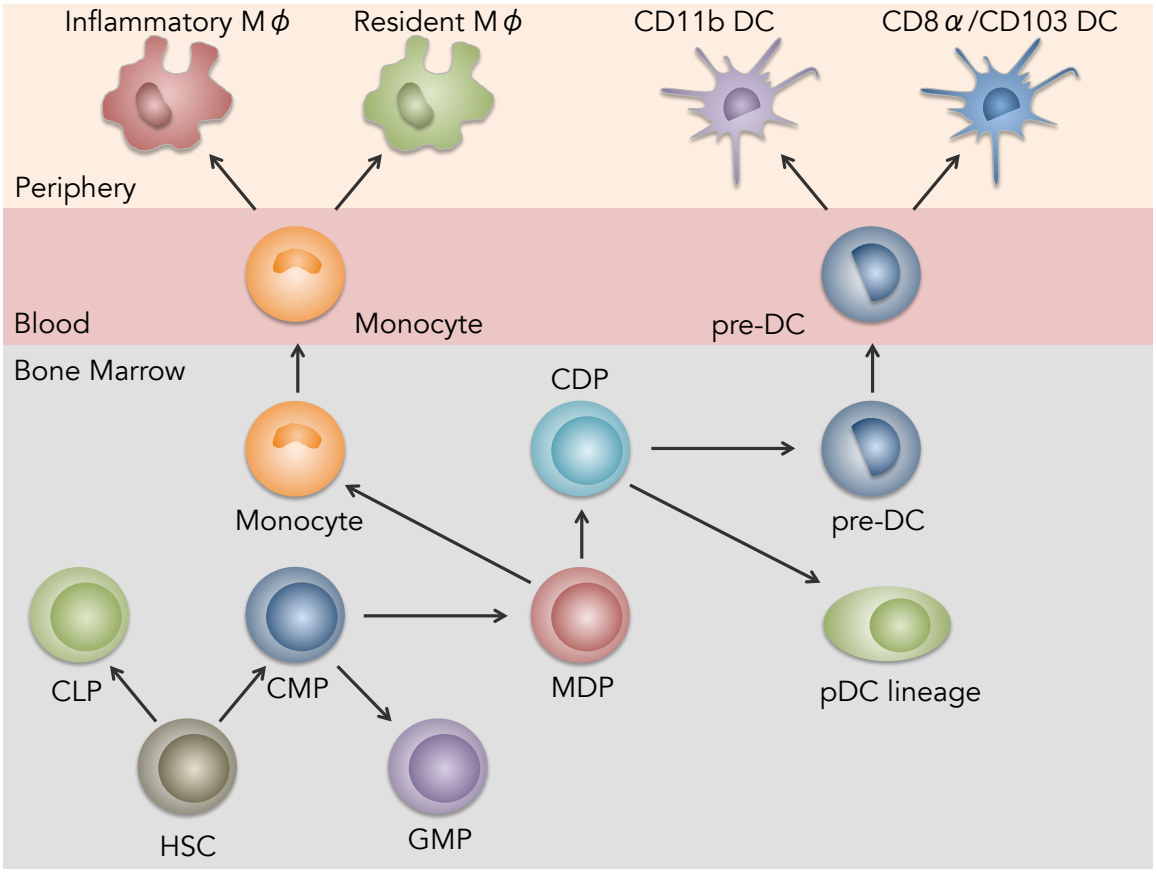


Figure 1-2 Dendritic cell ontogeny

Dendritic cells develop from hematopoietic stem cells (HSC) that differentiate through a series of intermediate states (CMP, MDP, CDP) in the bone marrow before entering the circulation as pre-DCs. Pre-DCs enter peripheral tissues like the lymph nodes, spleen, and nonlymphoid organs and undergo terminal differentiation to either the CD8α/CD103 or CD11b cDC lineages. In contrast, tissue resident macrophages are derived from circulating monocytes that split off from the macrophage/DC precursor (MDP) stage.

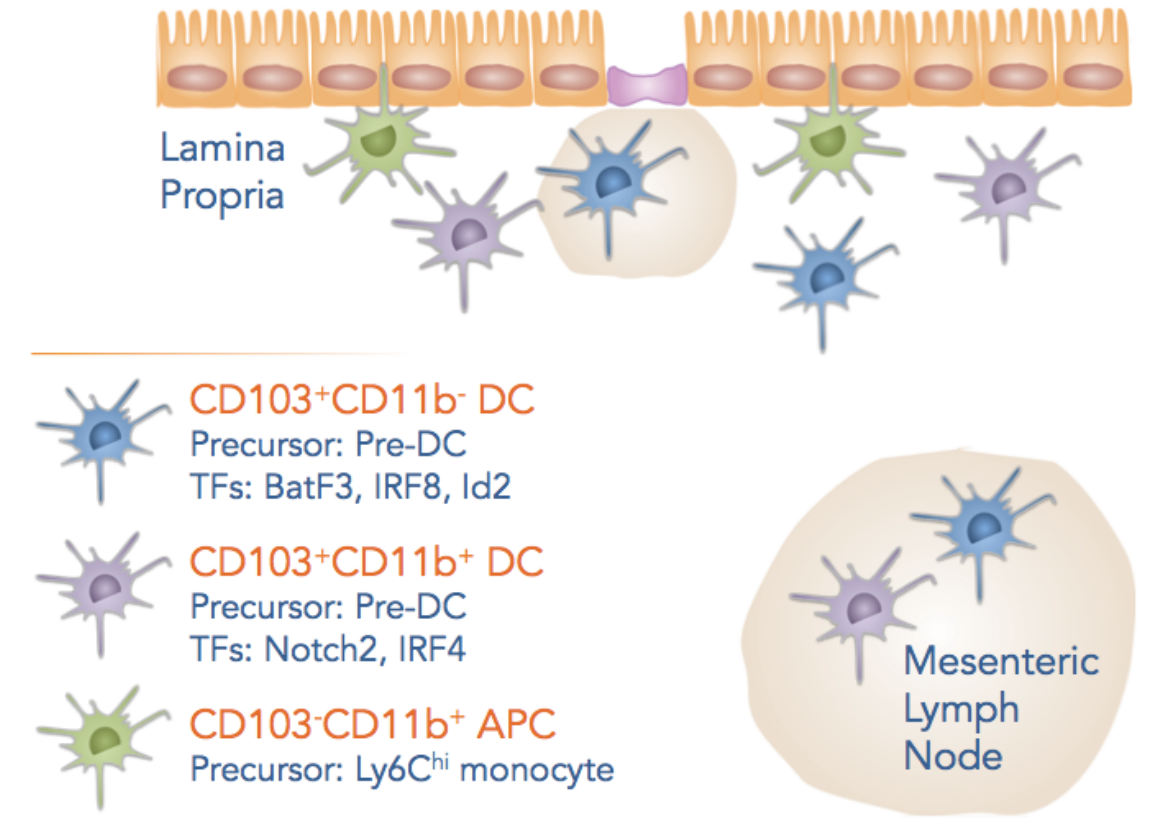


Figure 1-3 Dendritic cell diversity in the gut

Terminally differentiated DCs and macrophages derived from indicated precursors populate the steady-state intestine. In the gut, unlike other peripheral tissues, both DC subsets express CD103 despite dependence on distinct canonical transcription factors. DCs are the predominant cell capable of migrating to the mesenteric lymph node in the steady state. $CD103^-$ $CD11b^+$ macrophage APCs, in contrast, express CX_3CR1 and extend transepithelial dendrites into the lumen to sample antigens locally.

Chapter 2: Mouse models for investigating intestinal dendritic cell function

Introduction

Here we describe a novel set of mouse reagents designed to investigate the specific functions of intestinal DC subsets. Several strategies have been previously employed to investigate the functions of dendritic cell subpopulations *in vivo*. One approach relies on the deletion of transcription factors required for DC subset development. Fms-like tyrosine kinase 3 is a receptor required for the development of conventional dendritic cells, and $Flt3^{-/-}$ mice have a prominent deletion of conventional DC lineages (Bogunovic et al., 2009; Onai, Obata-Onai, Schmid, & Manz, 2007a). The transcription factor BatF3 is required for the development of certain DC subsets, and $BatF3^{-/-}$ mice lack $CD8\alpha^{+}$ splenic DCs, as well as $CD103^{+}$ $CD11b^{-}$ peripheral DC subsets (Edelson et al., 2010; Hildner et al., 2008). In addition to these whole-mouse knockout models, other transcription factors and signaling pathways required for DC subset development have been deleted only in cells expressing the α_x integrin CD11c through the use of Cre-lox technology (Caton, Smith-Raska, & Reizis, 2007; K. L. Lewis et al., 2011; Persson et al., 2013; Satpathy et al., 2013; Schlitzer et al., 2013).

A second approach is the development of transgenic mouse strains to delete dendritic cell subsets based on transgenic expression of diphtheria toxin or diphtheria toxin receptor. For instance, mice that express the human receptor for diphtheria toxin, heparin-binding EGF-like growth factor (HB-EGF), under control of the CD11c promoter (CD11c-DTR mice) are a commonly used model

to investigate the function of dendritic cells (Jung et al., 2002). This strategy has also been employed in a more restricted approach using the conventional DC specific transcription factor Zbtb46 and in several instances to investigate the functions of langerin expressing cells in the skin (Kaplan, Kissenpfennig, & Clausen, 2008; Meredith et al., 2012). Such mice allow for inducible ablation of transgene-expressing cells upon injection of active diphtheria toxin, for which the transgenically expressed human receptor has ~10^3-10^5 greater sensitivity than the endogenous murine receptor (Saito et al., 2001).

In summary, current models that allow for *in vivo* deletion of DC subsets are valuable tools to study DC function (Chow, Brown, & Merad, 2011). However, multiple DC subsets are often affected by whole-body or CD11c promoter based strategies, preventing the attribution of particular functions to an individual subset. For instance, in the small intestine, $Flt3^{-/-}$ mice have greatly reduced numbers of $CD103^+$ $CD11b^+$ DC in the lamina propria, but approximately 40% of $CD103^+$ $CD11b^-$ DC, as well as a statistically significant number of $CD103^-$ $CD11b^+$ cells, are also absent (Bogunovic et al., 2009). Likewise, CD11c-Cre $Notch2^{fl/fl}$ mice lack $CD103^+$ $CD11b^+$ DC, but have a concomitant increase in $CD103^+$ $CD11b^-$ LP DC, along with a loss of splenic $CD11b^+$ $Esam^{hi}$ DC (K. L. Lewis et al., 2011).

To investigate the function of DC subsets in the skin, we previously generated mice that ablate epidermal Langerhans cells (LCs) based on transgenic expression of human Langerin (huLangerin-DTA mice) (Kaplan,

Jenison, Saeland, Shlomchik, & Shlomchik, 2005). In this study, we report that, in addition to LCs, $CD103^+$ $CD11b^+$ LP DCs selectively express human Langerin (huLangerin) and are absent in these mice. Because all other DCs in the LP and MLN are intact, we use huLangerin-DTA mice, as well as $BatF3^{-/-}$ mice that lack $CD103^+$ $CD11b^-$ DC, to dissect the in vivo roles of specific $CD103^+$ DC subsets in establishing innate and adaptive immune responses.

Materials and Methods

Mice

All strains were backcrossed for at least eight generations onto the C57BL/6 genetic background. huLangerin-Cre *Rosa26*-$Stop^{fl}$-YFP (Kaplan et al., 2007), muLangerin-EGFP (Kissenpfennig et al., 2005), huLangerin-$CreER^{T2}$ *Rosa26*-$Stop^{fl}$-YFP (Bobr et al., 2012), and huLangerin-DTA (Kaplan et al., 2005) mice have all been previously described. huLangerin-DTR mice have been previously described (Bobr et al., 2010), and the F618 and F605 lines were derived from separate insertions of the BAC transgene into distinct founders. $BatF3^{-/-}$ mice (Hildner et al., 2008) were a kind gift from K. Murphy (Washington University, St. Louis, MO). DTAxYFP mice were generated by intercrossing huLangerin DTA and huLangerin-Cre *Rosa26*-$Stop^{fl}$-YFP mice. DTAx$BatF3^{-/-}$ mice were generated by intercrossing C57BL/6 $BatF3^{-/-}$ mice and huLangerin-DTA mice and backcrossing the resulting offspring to obtain $BatF3^{-/-}$ homozygous breeding pairs. Experiments were performed with 6–12-week-old age- and sex-matched, cohoused littermates. Mice were housed in specific pathogen-free

microisolator cages and fed irradiated food and acidified water. The University of Minnesota Institutional Animal Care and Use Committee approved all animal protocols.

Immunohistochemistry

Tissues were washed in PBS and fixed in 10 volumes of 1% phosphate-buffered paraformaldehyde containing 75 mM l-lysine (Sigma-Aldrich) and 10 mM $NaIO_4$ (Sigma-Aldrich). Tissues were then washed and dehydrated in 15% sucrose to preserve architecture. Fixed, dehydrated samples were embedded in OCT compound (Tissue-Tek) before freezing, cutting, and mounting. Sections were then blocked and stained with fluorochrome-conjugated and biotinylated antibodies and DAPI. Fluorochrome-conjugated streptavidin secondary was used to detect biotinylated antibodies. Stained tissues were imaged using a DM5500B epi-fluorescent microscope (Leica).

Cell Isolation

Lymph nodes and spleens were isolated and mechanically disrupted before digestion for 1 hour at 37°C with 400 U/mL collagenase D (Roche) in RPMI-1640 containing 10% FBS to isolate dendritic cells. Cell suspensions were passed through 40 μm filters and washed with 3% PBS containing 5 mM EDTA and 0.04% NaN_3 (FACS buffer) in preparation for staining and flow cytometry. To isolate lamina propria cells, small intestines were dissected and cleaned in situ of mesenteric fat and connective tissue. Peyer's patches were removed with scissors and the entire small intestine was cut into 1 cm pieces for digestion.

These pieces were first washed in HBSS before incubation at 37°C for 20 min in 3% RPMI containing 1 mM DTT, and 5 mM EDTA. The supernatant, containing the intraepithelial lymphocyte fraction, was discarded. The remaining lamina propria fraction was then washed twice in RPMI with 2 mM EDTA before digestion with 0.1 mg/ml Liberase TL (Roche) and 0.05 mg/ml DNase (Sigma-Aldrich) for 30 min at 37°C. LP suspensions were passed through a 70-μm filter, washed, and resuspended in 5 ml Histopaque-1077 (Sigma-Aldrich) to enrich for lymphocytes. Samples were overlaid with 2 ml RPMI before room-temperature centrifugation at 2,000 g for 30 min with no break. The interface was collected and cells were washed in FACS buffer before staining and analysis by flow cytometry.

Flow cytometry

Single-cell suspensions from lymph nodes or small intestine LP were blocked in 24G2 for 10 minutes before staining or stained directly in 24G2 with fluorochrome-conjugated antibodies to CD8α, CD11c, CD64, B220, I-A/I-E (MHCII), CD103, and CD11b purchased from BioLegend. Anti-huLangerin clone DCGM4 and anti-hu/muLangerin clone 929F3 were purchased from Dendritics. Unlabeled anti-huLangerin clone 2G3 was a kind gift from Sandra and Gerard Zurawski (Baylor University, Houston, TX) and was conjugated to Alexa Fluor-647 using an Alexa Fluor-647 conjugation kit (Molecular Probes). Intracellular GFP was detected by fixing and permeablizing cells with a Cytofix/Cytoperm kit (BD Biosciences) and performing intracellular staining with FITC conjugated anti-

GFP antibody (Rockland). Fixable viability dye was purchased from eBioscience.

Stained samples were analyzed on an LSRII or LSRFortessa flow cytometer (BD), and data were processed using FlowJo software (Tree Star). Cell numbers were calculated from flow cytometry frequencies of viable single cells multiplied by hemocytometer counts of Trypan blue–excluding cells.

Statistics

All statistical analysis was performed using GraphPad Prism software 5.0c (La Jolla, CA). Significance was assessed by unpaired two-tailed student's T tests with a confidence interval of 95% except where noted.

Results

$CD103^+$ $CD11b^+$ lamina propria DCs express human Langerin

LP DCs in C57BL/6 mice do not express Langerin under steady-state conditions (Chang & Kweon, 2010). In contrast, Langerin expressing DCs have been reported in human LP (Chikwava & Jaffe, 2004; Kaser et al., 2004). To confirm Langerin expression in human LP, we examined fresh samples of human intestine by immunofluorescence. We observed numerous cells expressing Langerin that co-expressed MHC-II, identifying them as LP APCs (Fig. 2-1)[1].

In mice, endogenous Langerin is expressed primarily by LCs and $CD103^+$ dermal DCs in the skin. In contrast, human LCs express Langerin, but DCs in the dermis do not (Haniffa et al., 2012). We previously reported that the human promoter of the gene *langerin* drives expression in murine LCs, but not $CD103^+$

dermal DCs (Bursch et al., 2007). Because human Langerin transgenic mice appear to recapitulate the human pattern of Langerin expression in the skin, we considered the possibility that LP DCs might express human Langerin in these transgenic mice.

We previously generated huLangerin-Cre x *Rosa26*-Stop^{fl}-YFP mice (henceforth referred to as huLang-Cre YFP) that faithfully report the expression of transgenic human Langerin (Kaplan et al., 2007). Immunofluorescent microscopy of the small intestine of huLang-Cre YFP mice revealed numerous cells co-expressing MHC-II and huLangerin in the LP that were not present in control littermates (Fig. 2-2A). huLangerin- expressing cells could also be detected in the colon, but at a reduced frequency (Fig. 2-2B). YFP^{+} MHC-II^{+} cells could be easily detected by flow cytometry of collagenase-digested small intestinal LP (Fig. 2-2C). Importantly, all YFP^{+} cells were CD11c^{hi} and MHC-II^{+}, identifying them as LP APCs (Fig. 2-2D). In contrast, reporter mice for murine Langerin (muLangerin-eGFP) (Kissenpfennig et al., 2005) confirmed that murine langerin was not expressed in MHCII^{+} cells of the LP, although it was easily detected in epidermal LCs (Fig. 2-2E). Thus, the human BAC containing the gene for *langerin* that we have previously used to generate several lines of transgenic mice recapitulates the human pattern of Langerin expression and drives expression in intestinal LP DCs.

To determine which DC subsets express huLangerin, single-cell suspensions were generated from the small intestinal LP of huLang-Cre YFP and

control mice. To identify LP DCs, we gated on CD11c^{hi} MHC-II^{+} cells, further dividing them into three subsets (I–III) based on their expression of CD103 and CD11b (Fig. 2-2F) (Bogunovic et al., 2009). We did not observe YFP expression in CD103^{+} CD11b^{-} DCs, CD103^{-} CD11b^{+} macrophages, or control littermates. In contrast, we observed robust YFP expression in CD103^{+} CD11b^{+} DCs isolated from huLang-Cre YFP mice (Fig. 2-2G). These data suggest that huLangerin expression marks a phenotypically and developmentally distinct subset of intestinal DCs.

YFP expression in these constitutive Cre reporter mice could potentially result from huLangerin transgene expression in a DC precursor population. Moreover, surface expressed huLangerin cannot be detected in these mice because the insertion of Cre into the first exon of the transgene disrupts recognition by most commercial antibodies. We therefore stained DCs from huLangerin-DTR F618 mice in which the DTR was inserted into the 3' UTR of human *langerin* (Bobr et al., 2010). We also examined huLangerin-CreER^{T2} *Rosa26*-Stop^{fl}-YFP mice in which YFP expression is induced by tamoxifen administration (Bobr et al., 2012). Gating on the three subsets of LP APCs as before (Fig. 2-3A), we observed huLangerin protein expression in CD103^{+} CD11b^{+} DC of huLangerin-DTR F618 mice, but not in other DC/macrophage subsets or transgene-negative controls (Fig. 2-3B). Likewise, in tamoxifen injected huLangerin-CreER^{T2} YFP mice we observed inducible expression of YFP only in mature CD103^{+} CD11b^{+} DC but not in CD103^{+} CD11b^{-} DC that also

arise from a conventional DC precursor or in monocyte-derived CD103$^-$ cells (Fig. 2-3C) (Bogunovic et al., 2009; Varol et al., 2009). Thus, we conclude that the gene for human *langerin* drives YFP and huLangerin expression selectively in CD103$^+$ CD11b$^+$ LP DCs of BAC transgenic mice. Because we observed few huLangerin$^+$ DCs in the colon, which agrees with previous data showing that CD103$^+$ CD11b$^+$ DCs are predominantly present in the small bowel (Denning et al., 2011), we focused on the small intestinal LP for the remainder of our studies.

huLangerin-DTA mice lack CD103$^+$ CD11b$^+$ DCs

huLangerin-DTA BAC transgenic mice were engineered to express an attenuated form of diphtheria toxin driven by the same human Langerin promoter used by huLangerin-Cre mice. In the skin, these mice have a selective ablation of epidermal LCs (Kaplan et al., 2005). To determine whether LP DCs are also ablated in these mice, we compared DC populations in the small intestinal LP of huLangerin-DTA and littermate control mice. Using the same gating strategy shown in Fig. 2-2F, we noted a near-complete absence of CD11c^{hi} MHC-II$^+$ CD103$^+$ CD11b$^+$ DCs (Fig. 2-4 A and B, subset II). Importantly, the numbers of CD103$^+$ CD11b$^-$ DCs (subset I) and CD103$^-$ CD11b$^+$ macrophages (subset III) in the LP were unaffected. We noted a similar absence of CD103$^+$ CD11b$^+$ DCs in the draining MLNs of huLangerin- DTA mice (Fig. 2-4C).

To confirm ablation of CD103$^+$ CD11b$^+$ DCs in huLangerin-DTA mice, we bred huLangerin-DTA mice with YFP reporter mice. As expected, a population of CD11c$^+$ cells expressing YFP could be detected in the LP of huLang-Cre YFP

but not control mice. These cells were absent from the LP of huLang-Cre YFP x huLangerin-DTA mice (Fig. 2-4D). The same was observed in the MLNs (Fig. 2-4E). We next investigated huLangerin-DTR mice, which allow for inducible deletion of huLangerin expressing cells by administration of diphtheria toxin (Bobr et al., 2010). Surprisingly, unlike mice derived from the huLangerin-DTR F618 founder (Fig. 2-3), a line of these mice derived from a distinct founder (hereafter termed) showed robust huLangerin expression in skin-draining lymph nodes (pooled axillary, brachial, and inguinal nodes) (Fig. 2-5A) but not in LP DCs (Fig. 2-5B). Moreover, two days after injection of diphtheria toxin, we observed profound deletion of LC in the SDLN (Fig. 2-5C), but only a slight reduction in the frequency of $CD103^+$ $CD11b^+$ DC in the LP (Fig. 2-5D). This was in contrast to huLangerin-DTR F618 mice, which showed a near-complete ablation of $CD103^+$ $CD11b^+$ LP DC after DT injection (Fig. 2-5E). Nonetheless, despite the founder effects observed in huLangerin-DTR F605 mice, our findings in multiple other independently derived transgenic lines support our conclusion that huLangerin is specifically expressed in terminally differentiated $CD103^+$ $CD11b^+$ DCs in the LP and MLN. This DC subset is selectively ablated in huLangerin-DTA and DTR F618 transgenic mice, providing a novel and powerful tool to study the function of $CD103^+$ $CD11b^+$ DCs *in vivo*.

DTAxBatF3$^{-/-}$ mice lack all CD103$^+$ DCs

$Batf3^{-/-}$ mice lack $CD103^+$ $CD11b^-$ DCs in the LP, as well as other peripheral tissues. In order to account for possible redundancies between

$CD103^+$ DC subsets, we generated huLangerin-DTA x $BatF3^{-/-}$ animals (henceforth referred to as DTAxBatF3$^{-/-}$). As expected, in DTAxBatF3$^{-/-}$ mice, both subsets of $CD103^+$ DCs were constitutively deleted in the LP (Fig. 2-6A) and MLN (Fig. 2-6B).

Although originally reported to be macrophages, $CD103^-$ $CD11b^+$ APCs in the gut are heterogeneous for conventional DC specific markers, such as Zbtb46, and show some dependence on Flt3L for their development (Bogunovic et al., 2009; Satpathy et al., 2013; Satpathy, KC, Albring, Edelson, Kretzer, et al., 2012a). Recent reports have identified CD64 (FcγRI) as a marker capable of distinguishing CX_3CR1^+ intestinal macrophages from DCs (Gautier et al., 2012; Tamoutounour et al., 2012). Therefore we used CD64, as well as the monocyte marker Ly6C to distinguish additional APC populations in our transgenic mice. $MHCII^+$ cells could easily be divided into $CD64^+$ and $CD64^-$ fractions. Within the $CD64^+$ macrophage gate, we identified $Ly6C^-$ cells that were either $CD11c^+$ or $CD11c^{lo/-}$, which identifies lamina propria and serosal/muscularis macrophages, respectively (Bogunovic et al., 2009; Muller et al., 2014). $CD11c^+$ LP macrophages were uniformly $CD103^-$ and $CD11b^+$ (Fig. 2-7A, top right panel). In contrast, $MHCII^+$ $CD64^-$ $CD11c^+$ $Ly6C^-$ DCs were heterogeneous for CD103 and CD11b expression, forming 3 identifiable populations (Fig. 2-7A, bottom right panel). We next investigated the expression of huLangerin in each of these subsets by using the same gating strategy in huLangerin-Cre YFP mice. As before, we found that LP macrophages (subset I) and $CD103^+$ $CD11b^-$ DCs

(subset II) did not express huLangerin, while nearly all $CD103^+$ $CD11b^+$ DCs (subset III) were huLangerin$^+$ (Fig. 2-7B). Interestingly, in $CD103^-$ $CD11b^+$ DCs (subset IV), we observed a small population of huLangerin$^+$ cells (Fig. 2-7B).

Finally, we looked at the number of cells in these populations in our transgenic mice. While $CD64^+$ macrophages were by far the most abundant cells isolated from the LP, we did not observe any significant differences their numbers for any genotype (Fig. 2-7C). In contrast, as previously shown (Fig. 2-4, 2-6), BatF3$^{-/-}$ mice and DTAxBatF3$^{-/-}$ mice lacked $CD103^+$ $CD11b^-$ DCs, while huLangerin-DTA and DTAxBatF3$^{-/-}$ mice had a near-complete ablation of $CD103^+$ $CD11b^+$ DCs (Fig. 2-7D). While the $CD103^-$ $CD11b^+$ DC fraction was much smaller than the other two LP DC subsets, we observed a significant reduction of this population in huLangerin-DTA and DTAxBatF3$^{-/-}$ mice compared to their respective littermates. Interestingly, this reduction was more pronounced in DTAxBatF3$^{-/-}$ mice due to increased numbers of $CD103^-$ $CD11b^+$ DCs in BatF3$^{-/-}$ mice (Fig. 2-7D, far right panel). Together, these data demonstrate that huLangerin-DTA and DTAxBatF3$^{-/-}$ mice represent novel tools to dissect the contributions of developmentally distinct DC subsets in intestinal immune responses.

Discussion

Here, we report that huLangerin transgenic mice that were developed to target LCs in the skin also have robust and selective transgene expression in $CD103^+$ $CD11b^+$ LP DCs. While isolated previous reports had described rare

langerin$^+$ cells in the human gut (Chikwava & Jaffe, 2004; Kaser et al., 2004), it was unclear whether these cells were a part a defined DC subset present in normal individuals. Excitingly, recent studies of DCs isolated from the human intestine have corroborated our discoveries in huLangerin transgenic mice (Longman et al., 2014; Watchmaker et al., 2014). In one of these reports, the human counterparts of murine intestinal DC subsets were defined, and the pattern of langerin expression among these subsets precisely matched that of huLangerin transgenic mice (Watchmaker et al., 2014). While the factors responsible for driving different tissue expression patterns of human versus murine langerin remain unclear, these findings strongly support the use of huLangerin transgenic animals as a faithful humanized model to investigate the *in vivo* functions of relevant DC subsets in a preclinical setting.

We subsequently developed and described several specific DC deletion models for use in functional studies. HuLangerin-DTA mice have a constitutive deletion of CD103$^+$ CD11b$^+$ DC, while huLangerin-DTR F618 mice ablate CD103$^+$ CD11b$^+$ DCs after injection of diphtheria toxin. Interestingly, an independently generated huLangerin-DTR F605 line showed transgene expression and deletion only in LC but not CD103$^+$ CD11b$^+$ DC. We believe that this may be due to the integration of fewer copies of the huLangerin BAC into this founder, which can affect gene expression levels (Feng et al., 2001). In line with this hypothesis, we observed a modest reduction in the frequency of CD103$^+$ CD11b$^+$ DC after DT injection, which could potentially be consistent with low-

level transgene expression below the limit of detection by our antibody. We have also previously observed that the level of Langerin expression is ~1 log lower in LP DC than in epidermal LC.

We also successfully generated huLangerin-DTA mice crossed to previously described BatF3$^{-/-}$ animals in order to delete both CD103^{+} DC subsets. Interestingly, using more recent gating strategies that allow for the distinction of CD64^{+} macrophages from intestinal DCs, we found that a fraction of CD103^{-} CD11b^{+} cells also expressed huLangerin and were deleted in huLangerin-DTA mice. The identity of these cells is unclear. While CD103^{-} DCs have been reported to be Flt3 responsive, various fractions of these cells have been reported to express the DC transcription factor Zbtb46 and intermediate levels of CX_3CR1. Formal developmental studies have not been performed, and it is not clear whether these cells represent an ontogenetically distinct subset or are an immature precursor population (Briseño et al., 2014; Cerovic et al., 2013; Satpathy et al., 2013). Our own finding that this population is increased in BatF3$^{-/-}$ mice could support the latter hypothesis, although it is also possible that differences in the microbiome could explain this effect. Alternatively, it is tempting to speculate that CD103^{-} DCs might be localized in a distinct subanatomical compartment, e.g. innate lymphoid follicles, since the ligand for CD103 is expressed by intestinal epithelial cells (DeNucci, Mitchell, & Shimizu, 2009).

Footnotes

Portions of this work have been previously published. Reprinted from *The Journal of Experimental Medicine*, Volume 210. Nathan E Welty, Christopher Staley, Nico Ghilardi, Michael J. Sadowsky, Botond Z. Igyártó, and Daniel H. Kaplan. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism, pp. 2011-2024. Copyright 2013, Welty et al.

[1] Immunohistochemistry in Figures 2-1A and 2-2A performed by B. Igyártó.

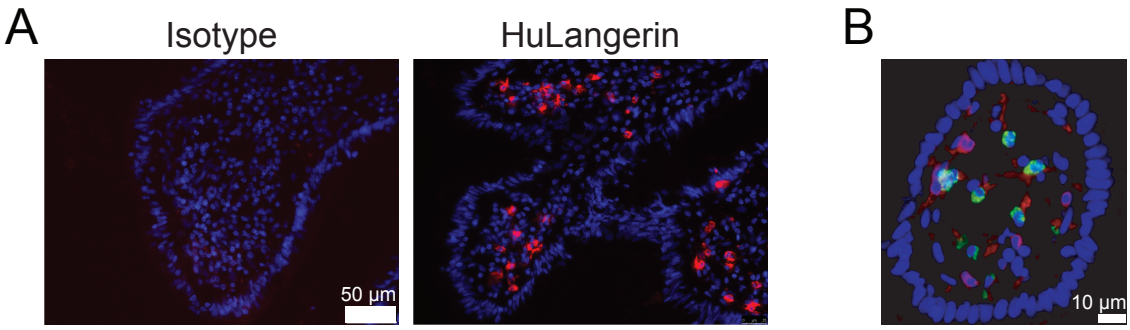


Figure 2-1: Human intestinal APCs express langerin

(A) Human ileal biopsies were stained with DAPI (blue) and isotype control (left) or anti-huLangerin antibody (right, red). (B) Human colonic villus stained with DAPI (blue) and antibodies against HLA-DR (red) and Langerin (green).

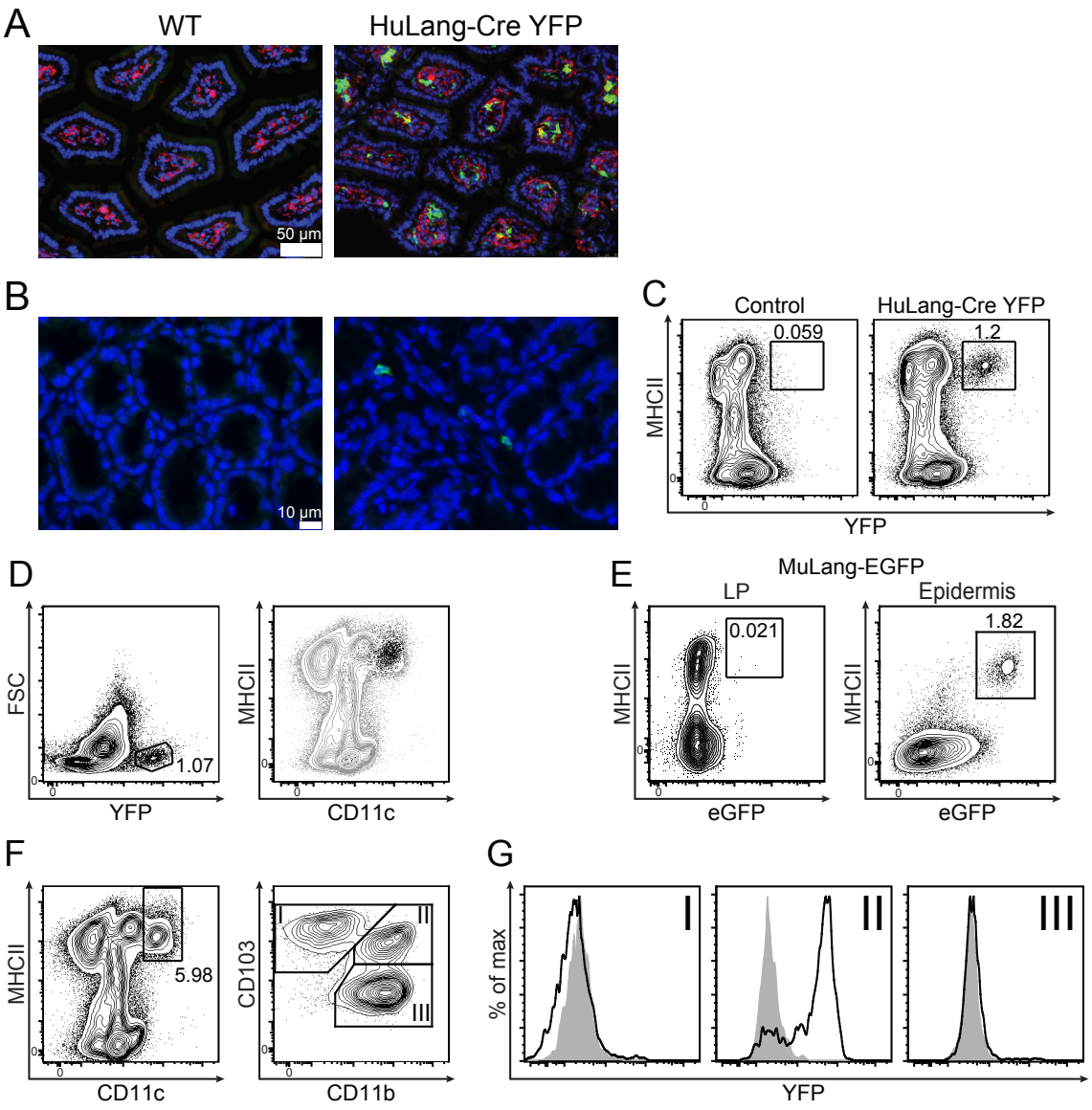


Figure 2-2: CD103⁺ CD11b⁺ lamina propria DC express human but not murine langerin

(A) Ileum stained with anti-YFP (green), anti-MHCII (red), and DAPI (blue). (B) Colon stained with anti-YFP (green) and DAPI (blue). (C) LP cells isolated from mice of the indicated genotypes. (D) Small intestinal LP suspensions from HuLang-Cre YFP mice gated on live single cells before gating for total YFP+ cells (left). YFP+ cells (black dots) were compared for expression of MHCII and CD11c with live-gated cells (gray contour, right). (E) Cell suspensions from the indicated tissues of murine Langerin-EGFP mice. (F) Gating strategy for LP DC subsets (G) YFP expression in the indicated subsets from HuLang-Cre YFP (black line) or littermate control (shaded gray) mice.

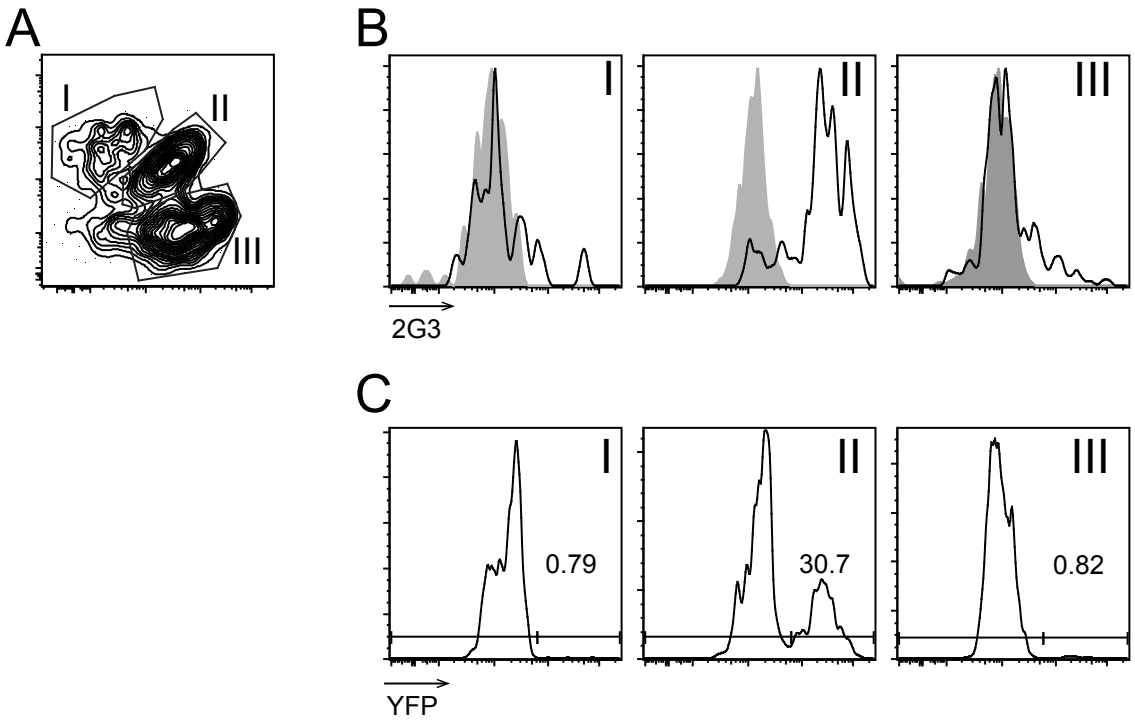


Figure 2-3: HuLangerin is constitutively expressed by lamina propria DCs

(A) CD11c^{hi} MHCII^{+} cells gated for CD103 and CD11b. (B) huLangerin expression in the indicated DC subsets isolated from huLangerin-DTR F618 (black line) or littermate control (shaded gray) mice. (C) huLangerin-CreER^{T2} YFP mice injected with Tamoxifen once daily for 5 days, harvested 4 days later, and stained for YFP expression in the indicated subsets. All data are representative of at least 2 independent experiments.

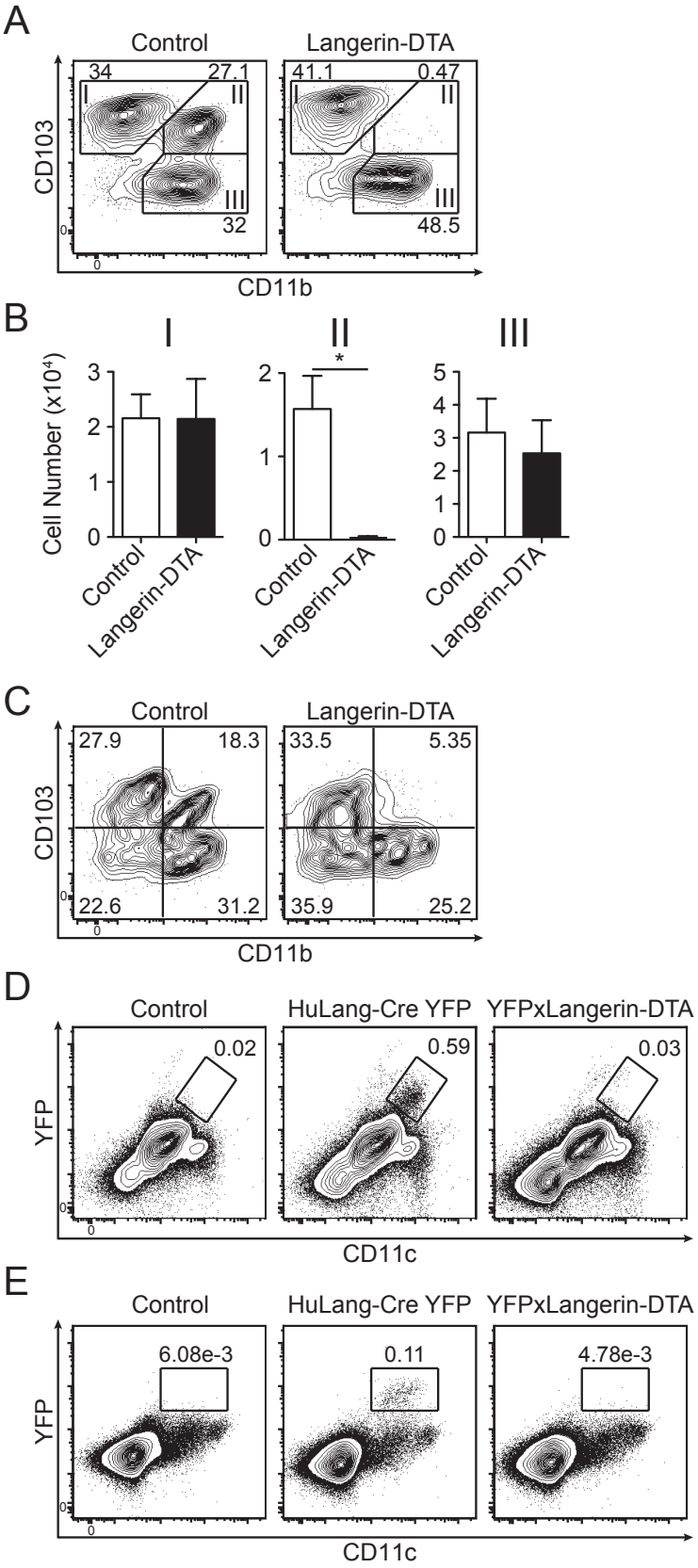


Figure 2-4: CD103^{+} CD11b^{+} LP DCs are ablated in huLangerin-DTA mice

A) CD11c^{hi} MHCII^{+} LP cells analyzed for CD103 and CD11b expression. (B) The number of cells for each subset in A is shown (*n* = 6–8 mice per genotype). (C) MLN cells analyzed as in A. (D–E) Flow cytometry of live single cells from LP (D) or MLN (E) isolated from littermates of the indicated genotypes. Data are representative of at least three experiments.

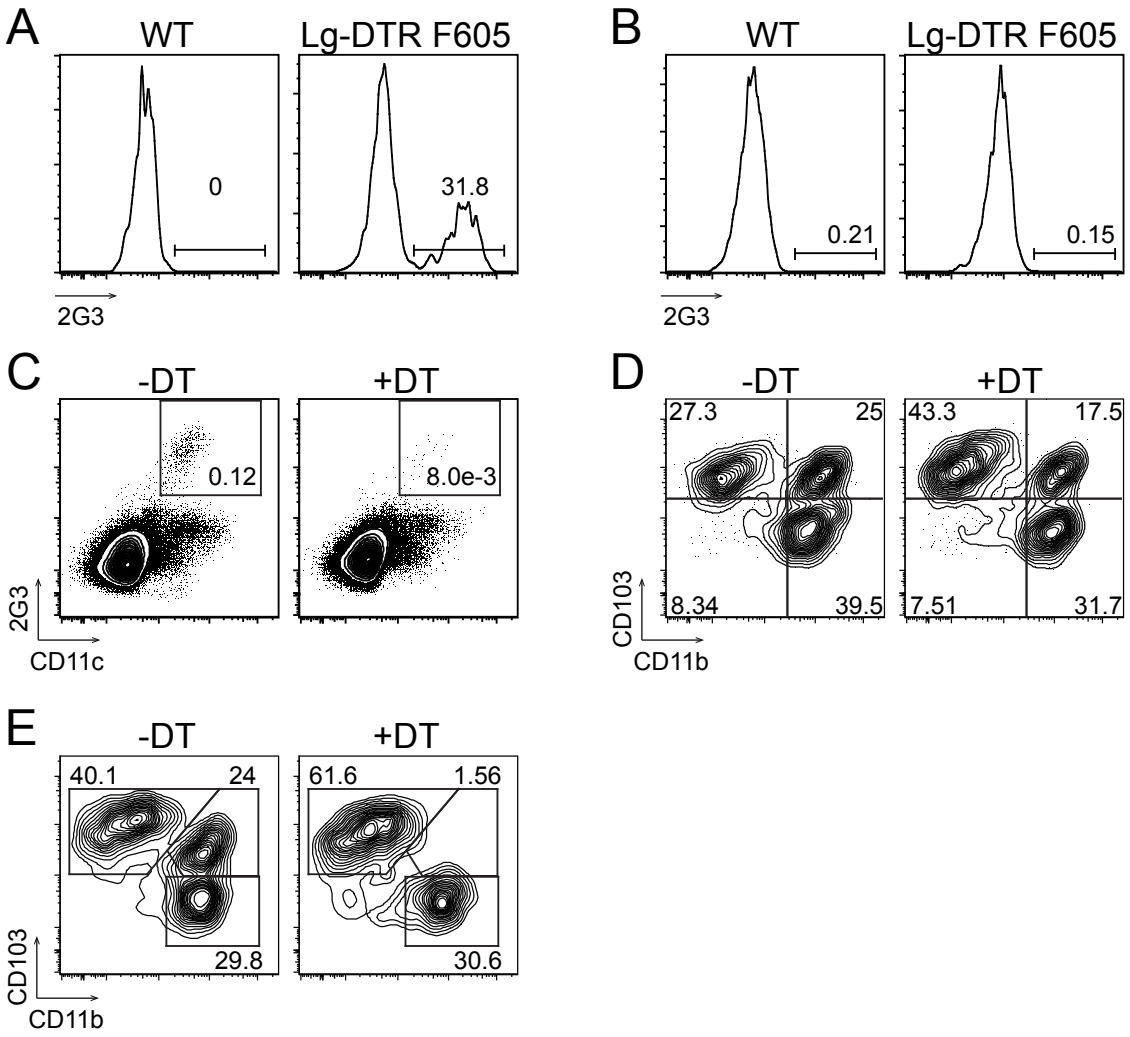


Figure 2-5: Human langerin is not expressed in CD103$^+$ CD11b$^+$ DC in huLangerin-DTR F605 mice

Cells from (A) skin-draining lymph nodes or (B) SI LP of huLangerin-DTR F605 gated on CD11c^{hi} MHCII$^+$ cells and stained with a huLangerin-specific antibody (2G3). (C) Cells from SDLN of huLangerin-DTR F605 mice left untreated (-DT) or injected with 1 µg diphtheria toxin 2 days prior to harvest. (D and E) CD11c$^+$ MHCII$^+$ SI LP cells from (D) huLangerin-DTR F605 or (E) huLangerin-DTR F618 mice treated with diphtheria toxin as in (C).

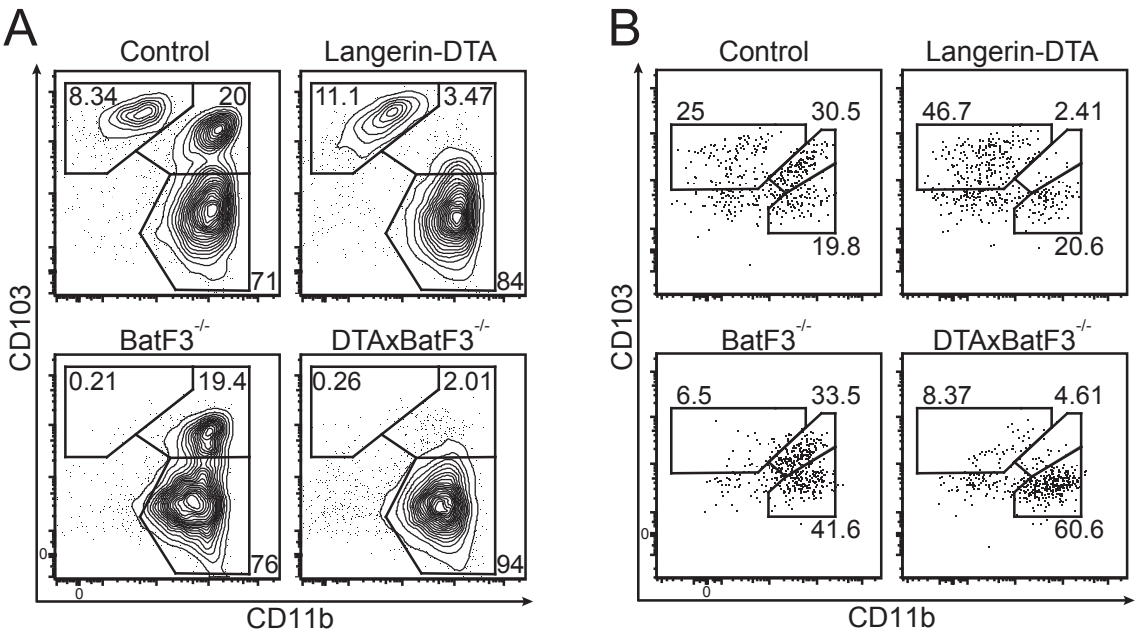


Figure 2-6: HuLangerin-DTA x BatF3$^{-/-}$ mice lack all CD103^{+} LP DC

Flow cytometry of CD11c^{hi} MHCII^{+} cells from (A) SI LP or (B) MLN of the indicated genotypes. Data represent 3 experiments (*n* = 3–5 per group).

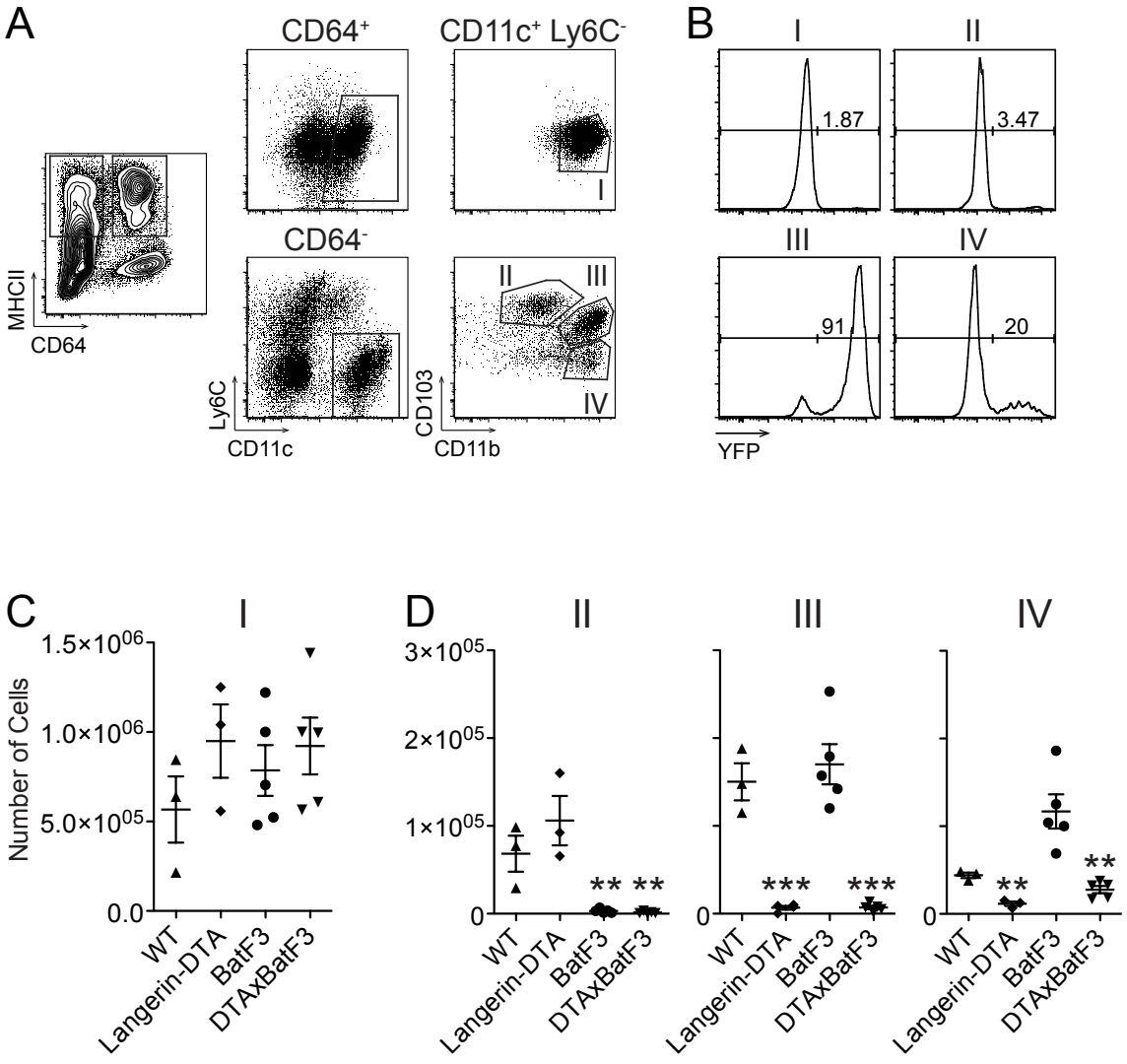


Figure 2-7: Investigation of CD103$^-$ DCs in transgenic mice

(A) Gating strategy for SI LP cells using CD64. (B) SI LP cells from huLangerin-Cre YFP mice gated as in (A). (C) Number of CD64$^+$ LP macrophages gated as in (A) in SI LP of transgenic mice. (D) Number of cells in indicated populations from designated transgenic mice. **p<0.01, ***p<0.001 compared to wild type or littermate controls as appropriate

Chapter 3: Functional characterization of dendritic cell deficient mice

Introduction

Previous studies have suggested numerous functional roles for $CD103^+$ LP DC during inflammation and infection but lacked specific deletion models to define the particular requirements for individual subsets. One such model involves oral administration of dextran-sodium sulfate (DSS), which generates an acute hemorrhagic enterocolitis and is a standard assay of the intestinal inflammatory response (Wirtz et al., 2007). LP DC- and macrophage-depleted mice reconstituted only with CX_3CR1^+ macrophages by adoptive transfer of $Ly6C^{hi}$ monocyte precursors developed enhanced DSS colitis dependent on TNF-α production by this subset (Varol et al., 2009). This suggested that CX_3CR1^+ macrophages drive DSS colitis, while $CD103^+$ DCs may play a protective role. Likewise, mice with a CD11c-restricted loss of β-catenin signaling also showed enhanced DSS colitis that was proposed to be related to a reduction in anti-inflammatory mediators from $CD103^+$ LP DCs (Manicassamy et al., 2010). However, due to the lack of specificity of the CD11c promoter, it remains possible that this effect was instead mediated by loss of β-catenin signaling in macrophages (K. M. Murphy, 2011). Finally, $BatF3^{-/-}$ mice, which have a relatively restricted deficiency of $CD103^+$ $CD11b^-$ DC showed no alteration in susceptibility to DSS (Edelson et al., 2010).

In addition to their potential functional role in innate inflammation, LP DCs

and macrophages have also been proposed to play a number of functions during infection. *S. enterica* is a common gut pathogen that can cause both an acute, self-limited gastroenteritis as well as chronic infection. While *S. enterica* has classical been thought to invade primarily through microfold cells overlying small intestinal Peyer's patches, from which it disseminates through the host, a number of studies have implicated LP APCs in the spread of this pathogen (Jantsch, Chikkaballi, & Hensel, 2011). In a models using noninvasive strains of the bacteria that have a defective type III secretion system, several studies have shown that LP macrophages acquire the bacteria and are important for its transportation to the MLNs (Diehl et al., 2013; Varol et al., 2009). In contrast, other studies using virulent or attenuated *S.* Typhimurium have shown instead that Flt3-dependent LP DCs are required for its transport to MLNs (Bogunovic et al., 2009; Voedisch et al., 2009). However, in these studies the particular DC subset(s) required are unclear. Moreover, the requirement for particular intestinal APC subsets in the development of an adaptive immune response against the pathogen has not been investigated.

Lastly, LP DCs have been proposed to play a significant role in the maintenance of mucosal barrier integrity. In particular, $CD103^+$ $CD11b^+$ DCs are thought to exert innate immune functions through their ability to detect flagellin via Toll-like receptor 5 (Fujimoto et al., 2011; Uematsu et al., 2006). Flagellin administration induces IL-22 from innate lymphoid cells in the LP and is thought to enhance innate resistance to intestinal pathogens (Kinnebrew et al., 2010; Van

Maele et al., 2010). Elaboration of IL-22 depends on TLR5 and DC-derived IL-23. Reduced IL-22 production in $Flt3^{-/-}$ mice and the expression of TLR5 by $CD103^{+}$ $CD11b^{+}$ DCs has suggested that this DC subset is required for IL-22 production (Kinnebrew et al., 2012). Additionally, IL-23–dependent IL-22 is required for innate resistance to *Citrobacter rodentium*, a mouse model for enteropathogenic *Escherichia coli* infection (Zheng et al., 2008) and may also be important in resistance to *S.* Typhimurium gastroenteritis (Godinez, Keestra, Spees, & Baumler, 2011). Lastly, a recent report showed that CD11c-Cre $Notch2^{fl}$ mice, which have a robust depletion of $CD103^{+}$ $CD11b^{+}$ DC, are profoundly susceptible to *C. rodentium* through an IL-23 dependent mechanism (Satpathy et al., 2013).

In this study, we use mice with specific LP DC deficiencies to define the functional requirements for these subsets in a variety of inflammatory and infectious models. We first show that Langerin-DTR F618 mice are not a viable model to investigate intestinal DC function due to ectopic transgene expression in this founder line. Using huLangerin-DTA and $BatF3^{-/-}$ mice, we then show that $CD103^{+}$ $CD11b^{+}$ DC are dispensable for protection from DSS colitis and adaptive immune responses to *S.* Typhimurium, but that BatF3-dependent DC are required to generate a *Salmonella*-specific CD4 T cell response. Finally, we provide evidence that $CD103^{+}$ DC are not strictly required for innate IL-22 production or resistance to IL-23 dependent infections, but that additional DC or macrophage subsets may cooperate in these responses.

Materials and Methods

Mice

C57BL/6 IL-23 p19$^{-/-}$ mice have been previously described (Ghilardi et al., 2004) and were a gift of N. Ghilardi (Genentech, South San Francisco, CA). FoxP3-DTR mice (J. M. Kim, Rasmussen, & Rudensky, 2006) were a kind gift of D. Mueller (University of Minnesota, Minneapolis, MN). Mice were housed as described above, and the University of Minnesota Institutional Animal Care and Use Committee approved all animal protocols.

DSS colitis

Mice were treated with 2 or 5% wt/vol dextran sodium sulfate (MP Biomedicals) in the drinking water and monitored daily for morbidity and weight loss, as previously described (Wirtz, Neufert, Weigmann, & Neurath, 2007). Mice that were moribund or lost 20% or more of their initial body weight were sacrificed.

Flagellin administration and LP mRNA extraction

1 µg ultrapure flagellin (InvivoGen) in sterile PBS was injected intravenously through the tail vein before LP isolation and mRNA extraction as follows. A 1.5-cm segment of proximal small intestine 2 cm distal to the pylorus was dissected and opened longitudinally with scissors. Peyer's patches were removed from this segment before incubation at 37°C for 15 min in HBSS containing 25 mM Hepes, 1 mM DTT, and 10 mM EDTA to remove the

epithelium. Segments were then pulse-vortexed eight times and washed in sterile PBS before stabilization in RNAlater (QIAGEN) for mRNA extraction and analysis. To deplete LP DCs, CD11c-DTR mice were injected i.p. with 1 μg diphtheria toxin 18 h before flagellin injection. To deplete Tregs, FoxP3-DTR mice were injected i.p. with 1 μg diphtheria toxin every other day for 6 days (3 injections total) prior to flagellin injection.

Quantitative real-time PCR

RNA was extracted from RNAlater-stabilized tissues using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA was reverse-transcribed using a high-capacity cDNA RT kit and analyzed via qPCR with TaqMan Gene Expression Assays and an ABI 7900HT (Applied Biosystems) as previously described (Haley et al., 2012). Data were normalized to Hprt expression, and relative expression values calculated using the comparative Ct method. Data are presented as $2^{-\Delta\Delta Ct}$.

Infections

C. rodentium strain DBS100 was purchased from the American Type Culture Collection. Bacteria were subcloned and cultured for 12 h overnight in Luria-Bertani media, harvested by centrifugation, and suspended in sterile PBS. Bacterial concentration was estimated by measuring optical density at 600 nm (OD 600) and confirmed by plating serial dilutions. Mice were deprived of food for 12 h before orogastric inoculation with 2×10^9 bacteria in 200 μl PBS by gavage.

For *S. enterica* infection, attenuated AroA$^-$ *S. enterica* serovar Typhimurium expressing the 2W1S epitope (AroA$^-$ ST-2W) was a gift from M. Jenkins (University of Minnesota, Minneapolis, MN) and K. Smith (University of Washington, Seattle,WA). Either this strain or the naturally streptomycin resistant wild-type *S. enterica* serovar Typhimurium strain SL1344 were subcloned and cultured as above in the presence of 0.1 mg/ml streptomycin. Bacteria were harvested and animals infected as above with $5x10^7$–10^8 *S. enterica*. To induce *S. enterica*-dependent enterocolitis, animals were treated as previously described (Barthel et al., 2003). In brief, mice were pretreated by gavage with 20 mg streptomycin sulfate in 100 µl sterile PBS. 24 hours later, mice were gavaged with 100 µl 5% $NaHCO_3$, pH 9.0, before orogastric inoculation with $5x10^7$ *S. enterica* strain SL1344. Live bacterial loads were determined at indicated time points after infection by homogenizing tissues in 0.1% Triton X-100 buffer and plating serial dilutions on MacConkey agar. Detection of 2W1S-specific CD4 T cells after infection was performed as previously described (Moon et al., 2007) with an I-A^b-2W1S tetramer, a kind gift from M. Jenkins.

Statistics

All statistical analysis was performed using GraphPad Prism software 5.0c (La Jolla, CA). Significance was assessed by unpaired two-tailed student's T tests with a confidence interval of 95% except where noted.

Results

huLangerin-DTR F618 mice have ectopic expression of huLangerin

Although huLangerin-DTA mice have a robust and selective ablation of intestinal $CD103^+$ $CD11b^+$ DC (Fig. 2-4), we also wanted to assess a mouse model of inducible, rather than constitutive, ablation of this subset. As we have previously shown, huLangerin-DTR F618 mice, unlike a separate F605 founder, robustly express a huLangerin BAC transgene genetically engineered to contain the high-affinity diphtheria toxin receptor *HB-EGF* in $CD103^+$ $CD11b^+$ DC (Fig. 2-3B, Fig. 2-5). We had previously observed that, unlike F605 mice that do not express huLangerin in the gut, these mice became moribund and succumbed 5-7 days after diphtheria toxin injection (data not shown and Fig. 3-1B). However, we also found huLangerin expression at ectopic sites in F618 mice that were not observed in our other huLangerin transgenic mice, including the thymus, spleen, liver, and lungs (data not shown and Fig. 3-1A)[1]. In order to assess whether the rapid death observed in these animals was due to inducible ablation of $CD103^+$ $CD11b^+$ LP DC or to ablation of ectopic transgene-expressing cells, we crossed huLangerin-DTR F618 mice to huLangerin-DTA mice that constitutively lack $CD103^+$ $CD11b^+$ LP DC. Unlike wild type or huLangerin-DTA only littermates, DTAx618DTR double transgenic mice rapidly succumbed to diphtheria toxin injection at a similar rate to mice transgenic for huLangerin-DTR F618 alone (Fig. 3-1B). Thus, we conclude that the susceptibility of the huLangerin-DTR F618 founder line to diphtheria toxin is independent of $CD103^+$ $CD11b^+$ LP DC and that

these animals do not represent a viable tool for investigating the function of this DC subset.

CD103$^+$ DC are dispensable for protection from DSS colitis

huLangerin-DTA mice have been well characterized (Igyarto et al., 2009; Kaplan et al., 2005). They are long-lived and do not develop spontaneous autoimmunity. Because the ablation of CD103$^+$ CD11b$^+$ LP DCs in these mice was previously unappreciated, we tested their response to an intestinal inflammatory challenge with DSS. We administered DSS in the drinking water of huLangerin-DTA and littermates for 10 d and monitored their weight loss. As expected, treated groups lost a significant amount of weight. However, huLangerin-DTA mice lost weight at the same rate as littermate controls when treated with either 5% (Fig. 3-2A) or 2% (Fig. 3-2E) DSS. Treated control and huLangerin- DTA animals also exhibited an identical degree of colonic shortening, a measure of colitis severity (Fig. 3-2B). In order to test for potential redundancy between CD103$^+$ DC subsets, we also examined DSS treated DTAxBatF3$^{-/-}$ mice compared to BatF3$^{-/-}$ littermate controls. Although colitis induction was slightly delayed in BatF3$^{-/-}$ compared to wild-type litters, we observed no difference in weight loss or colon length in mice that lacked one (BatF3$^{-/-}$) or both (DTAxBatF3$^{-/-}$) CD103$^+$ LP DCs (Fig. 3-2C-D). Thus, CD103$^+$ LP DCs are not required for the development of, or protection from, DSS enterocolitis.

CD103$^+$ CD11b$^+$ are not required for adaptive immunity to *S.* Typhimurium

To investigate whether adaptive immunity to *S.* Typhimurium requires CD103$^+$ CD11b$^+$ DCs, we orally infected mice with an attenuated strain of *Salmonella* that expresses a previously characterized 2W1S CD4 T cell epitope, allowing us to track a pathogen-specific adaptive immune response during infection (Moon et al., 2007; Nelson, McLachlan, Kurtz, & Jenkins, 2013). Ten days after inoculation, near the peak of the response, huLangerin-DTA mice bore a bacterial burden equivalent to that of littermate controls in both the spleen (Fig. 3-3A) and MLN (Fig. 3-3B). As expected, *Samonella*-2W1S infection led to an ~20-fold increase in the number of 2W1S- specific endogenous CD4 T cells, as well as their upregulation of the activation marker CD44. Notably, the lack of CD103$^+$ CD11b$^+$ LP DCs in huLangerin-DTA mice did not impair the development of a pathogen-specific CD4 T cell response. However, BatF3$^{-/-}$ mice had a significantly impaired expansion of *Salmonella*-specific 2W1S$^+$ CD4 T cells (Fig. 3-3C). Thus, CD103$^+$ CD11b$^+$ LP DCs are unnecessary for the generation of CD4 T cell responses to a flagellated gut pathogen, while BatF3-dependent cells are required.

CD103$^+$ DC are dispensable for IL-23 dependent innate immunity

CD103$^+$ CD11b$^+$ DCs express TLR5 (Fujimoto et al., 2011; Uematsu et al., 2006) and are reportedly required for IL-23– dependent amplification of IL-22 production by gut innate lymphoid cells in response to intravenously administered flagellin (Kinnebrew et al., 2012). Because these studies were performed in mice

with defects in multiple gut DC lineages (e.g., CD11c-DTR and Flt3$^{-/-}$ mice), we tested the response to intravenous flagellin in huLangerin-DTA mice. As expected, we observed a dramatic increase in LP IL-22 mRNA expression in the small intestine of control mice 2 h after treatment with flagellin. This increase was absent in IL-23 p19$^{-/-}$ mice and significantly reduced in CD11c-DTR mice treated with diphtheria toxin prior to flagellin injection. However, IL-22 production was unaffected in huLangerin- DTA mice, indicating that CD103^{+} CD11b^{+} DCs are not required for innate immune responses to i.v. flagellin (Fig. 3-4A). Surprisingly, when we repeated the experiment in huLangerin-Cre MyD88^{fl} mice in which CD103^{+} CD11b^{+} DC are present but lack the ability to signal through the TLR and IL-1R family adapter protein MyD88, we observed a significant decrease in LP IL-22 expression compared to Cre^{-} littermate controls (Fig. 3-4B), suggesting that there may be compensatory mechanisms promoting innate IL-22 production in the absence of CD103^{+} CD11b^{+} DC.

We next investigated innate IL-22 production in the absence of both CD103^{+} DC subsets. Two hours after i.v. flagellin, BatF3$^{-/-}$ mice lacking CD103^{+} CD11b^{-} DC had a similar upregulation of IL-22 production as that observed in wild-type controls, suggesting that this subset is not required for innate IL-22. Surprisingly, in DTAxBatF3$^{-/-}$ mice, which have an additional deletion of CD103^{+} CD11b^{+} DC, IL-22 mRNA expression was enhanced approximately two-fold over wild-type controls or BatF3$^{-/-}$ littermates (Fig. 3-5A). As innate ILC3 IL-22 production in this model is dependent on earlier upregulation of IL-23 (Kinnebrew

et al., 2012), we next investigated whether these mice also have enhanced IL-23 production. One hour after i.v. flagellin administration, we detected a significant upregulation of IL-23 p19 mRNA in wild type and BatF3$^{-/-}$ animals. DTAxBatF3$^{-/-}$ mice had a further increase in IL-23 p19 expression that was similar in magnitude to the augmented IL-22 observed 2 hours after flagellin injection (Fig. 3-5B).

CD103^{+} DC have been implicated in FoxP3^{+} regulatory T cell development *in vitro* through their ability to produce retinoic acid and TGF-β (Coombes et al., 2007; Denning et al., 2011; Sun et al., 2007). To test whether innate IL-23/22 upregulation in the absence of CD103^{+} DC might be due to a defect in the immune suppressive Treg compartment, we injected FoxP3-DTR mice (J. M. Kim et al., 2006) with diphtheria toxin to delete Treg prior to flagellin administration. We observed no difference in IL-22 upregulation in Treg-depleted mice versus littermate controls, suggesting that IL-22 augmentation in CD103^{+} DC deficient mice occurs through a Treg independent mechanism.

CD103^{+} CD11b^{+} DC are not required for resistance to IL-23 dependent infections

We next sought to determine whether CD103^{+} CD11b^{+} DCs are required for IL-23/IL-22-dependent immune responses during infection. We orogastrically inoculated mice with 2x10^{9} *C. rodentium*. As previously reported (Basu et al., 2012; Mangan et al., 2006), IL-23 p19$^{-/-}$ mice lost a significant amount of weight beginning at day 4 after infection (Fig. 3-6A) and succumbed in 8–12 d (Fig. 3-6B). One very old >50 g mouse did not succumb to infection in this time frame

but did lose ~20% of its body weight. However, huLangerin-DTA mice and their wild-type littermates did not lose weight or succumb to *C. rodentium* infection (Fig. 3-6A-B). We considered that, though they may be an important source of IL-23, $CD103^+$ $CD11b^+$ DCs might not be required for protection from *Citrobacter* because of the lack of TLR5 ligation by this aflagellate pathogen (Khan et al., 2008). We therefore infected mice with virulent *S.* Typhimurium strain SL1344, using an antibiotic pretreatment model of acute *Salmonella*–induced gastroenteritis (Barthel et al., 2003). We observed that CFU of *S.* Typhimurium in the MLN was significantly increased in IL-23 $p19^{-/-}$ mice 48 h after infection, but was unaffected in huLangerin-DTA mice. We also observed a similar trend in the spleen (Fig. 3C-D). Thus, $CD103^+$ $CD11b^+$ DCs do not appear to be required for resistance to IL-23/22-dependent infections.

In contrast to our results, a recent report using CD11c-restricted Notch2 deletion, which also depletes most $CD103^+$ $CD11b^+$ LP DC, suggested that this subset was required for IL-23/22 dependent resistance to *C. rodentium* (Satpathy et al., 2013). In order to test the hypothesis that Notch2 might mediate resistance to *Citrobacter* through other $CD11c^+$ cells, we pretreated mice with a Notch2 selective blocking antibody to inhibit signaling through this receptor prior to infection (Y. Wu et al., 2011). Such treatment recapitulates the phenotype of mice that have a genetic ablation of Notch2 (B. Reizis, personal communication). We found that both wild type and huLangerin-DTA mice treated with Notch2 blocking antibody lost weight after infection with *C. rodentium* compared to

isotype-treated control littermates (Fig. 3-7B). Importantly huLangerin-DTA mice did not exhibit increased susceptibility compared to treated controls. Thus, we propose that sensitivity to *C. rodentium* observed in CD11c-Cre Notch2^{fl} mice is due to Notch2 ablation in other CD11c^{+} subsets.

Discussion

In this study we have used targeted dendritic cell ablation strategies to probe the functional requirement for individual DC subsets during inflammation and infection. We first assessed the utility of a previously developed huLangerin transgenic founder that expressed a huLangerin-DTR BAC construct in CD103^{+} CD11b^{+} LP DC. We had previously observed that, unlike a founder that did not express huLangerin in this subset, these mice rapidly died after the injection of diphtheria toxin. However, we found that this was due to ectopic expression of the transgene in other tissue sites. The reason for ectopic expression in this founder is unclear, although previous studies have shown that both the tissue expression pattern and degree of expression of BAC transgenes mice can vary between founders, presumably due to the site of transgene integration and the number of copies of the transgene that integrate (Feng et al., 2001; Gong et al., 2003). Whatever the cause, this particular transgenic founder does not provide a useful model for interrogating the functions of intestinal dendritic cells.

Using an DSS-induced innate model of toxic enterocolitis, we found that CD103^{+} CD11b^{+} DC were dispensable for the development or protection from colitis both when deleted alone and when ablated in combination with CD103^{+}

CD11b$^-$ DC. These data are somewhat different from those reported in a previous study, in which mice were first depleted of all LP DCs and macrophages and then reconstituted with monocyte precursors that give rise to CX_3CR1^+ macrophages but not CD103$^+$ DC were more susceptible to DSS (Varol et al., 2009). These experiments was performed using bone marrow chimeras and long-term DT injection to maintain CD11c$^+$ cell depletion, and the inflammatory and microbial cues driving colitis in this setting might differ considerably from our steady-state genetic ablation model. Moreover, the cell populations mediating colitis in this model were not specifically identified. Alternatively, it is possible that differences in the method for colitis detection explain discrepancies the results.

Earlier reports have suggested that intestinal dendritic cells, and CD103$^+$ CD11b$^+$ DC in particular, are required to transport *S.* Typhimurium from the LP to the MLN during infection (Bogunovic et al., 2009; Voedisch et al., 2009). In contrast to these studies, we found no defect in dissemination to either draining lymph nodes or systemically in the absence of CD103$^+$ CD11b$^+$ DC. Likewise, mice that lacked this DC subset mounted appropriate pathogen-specific CD4 T cell responses. However, BatF3$^{-/-}$ mice, which lack CD103$^+$ CD11b$^-$ DC, exhibited a markedly impaired *Salmonella*-specific adaptive immune response. The mechanism behind this deficiency remains unclear. In addition to CD103$^+$ CD11b$^-$ DC, BatF3$^{-/-}$ mice lack resident CD8α$^+$ DC in the spleen (Hildner et al., 2008), but CD8α$^+$ DC resident in the lymph node remain relatively intact on the C57BL/6 genetic background (Edelson et al., 2011). Thus, impaired immunity to

S. Typhimurium may be due to the loss of splenic $CD8\alpha^+$ DC rather than $CD103^+$ $CD11b^-$ LP DC. Indeed, $BatF3^{-/-}$ mice are highly susceptible to *Toxoplasma gondii* infection due to a lack of IL-12 production by splenic $CD8\alpha^+$ DC. Given that CD4 T cell responses to *Salmonella* are dominated by Th1 effectors (Griffin & McSorley, 2011), and that IL-12 is critical for Th1 generation (Hsieh et al., 1993), it is possible that a similar mechanism is at play here. Investigating the kinetics of the anti-*Salmonella* response at earlier timepoints after infection may shed light onto the specific DC subset(s) involved.

Recent reports using CD11c-DTR and CD11c-Cre $Notch2^{fl}$ mice have proposed that $CD103^+$ $CD11b^+$ DCs are required for flagellin-induced expression of IL-22 and for resistance to infection with the attaching and effacing pathogen *C. rodentium* through an IL-23 dependent mechanism (Kinnebrew et al., 2012; Satpathy et al., 2013). However, the CD11c promoter affects multiple DC and macrophage subsets in the gut, and the transcriptional profile even of cells that are not deleted is affected in CD11c-Cre $Notch2^{fl}$ mice (K. M. Murphy, 2011; Satpathy et al., 2013). In contrast to these studies, huLangerin-DTA mice, which have a much more restricted deletion of $CD103^+$ $CD11b^+$ DC, showed no defects in flagellin-induced IL-22 production or resistance to IL-23 dependent models of *S.* Typhimurium gastroenteritis and *C. rodentium* infection. While this could be due to inefficient deletion of DC, huLangerin-DTA mice have a more efficient ablation of this subset than CD11c-Cre $Notch2^{fl}$ mice. Moreover, we found that huLangerin-DTA mice could be rendered susceptible to *C. rodentium* by

blockade with a Notch2 selective antibody, suggesting that Notch2 signaling in other cells may be important for defense against this pathogen. The specific cell affected remains unclear, but could potentially be a Notch2-dependent recruited cell, resident $CD103^-$ macrophages, or Flt-3 dependent $CD103^-$ $CD11b^+$ DCs (Briseño et al., 2014; Cerovic et al., 2013). Two recent studies have proposed that resident $CD103^-$ macrophages may be responsible for innate, Notch2-dependent IL-23 production. The first study combined CD11c-restricted Notch deletion with CX_3CR1 reporter mice to show that CX_3CR1^+ intestinal macrophage development is impaired in the absence of Notch signaling (Ishifune et al., 2014). The second found that CX_3CR1^+ cells are required for IL-22 dependent resistance to *C. rodentium* infection using a novel CX_3CR1 and CD11c dual promoter-based deletion strategy (Longman et al., 2014). While these data are somewhat difficult to reconcile with prior studies that excluded a role for macrophages using on CCR2 promoter-based deletion strategies, it is possible that the effects observed in these mice are mediated by a small population of $CD103^-$ $CD11b^+$ DC that have been reported to be CX_3CR1^{int} (Cerovic et al., 2013).

Intriguingly, in our own studies using a model of i.v. flagellin administration, we found a defect in IL-22 production when $CD103^+$ $CD11b^+$ DCs were present but rendered unable to respond to MyD88 dependent signals. Furthermore, we noted a Treg-independent upregulation of IL-23/22 when both $CD103^+$ DC subsets were deleted. At face value, these data appear difficult to

reconcile with normal innate IL-22 production in mice that lack $CD103^+$ $CD11b^+$ DC alone. However, Notch signaling pathways were originally identified during neural development because of their role in cell-cell communication and fate patterning between neighboring cells. This occurs through the process of lateral inhibition, such that activation of Notch in one cell promotes its inhibition in neighboring cells, thus amplifying developmental distinctions between the two (Axelrod, 2010; Chitnis, 1995; J. Lewis, 1998). $CD11b^+$ DC show evidence of ongoing Notch2 signaling even in the mature state, although the source of Notch ligands remains unclear (K. L. Lewis et al., 2011). Thus, our data could be consistent with a model in which Notch2 is required, not only for the development of $CD103^+$ $CD11b^+$ LP DC, but also for functional patterning between mature intestinal DC and macrophage subsets. In such a model, deletion of $CD103^+$ $CD11b^+$, as in huLangerin-DTA mice, would relieve Notch2 suppression in other myeloid subsets, allowing these cells to carry out Notch2 dependent functions. However, when $CD103^+$ $CD11b^+$ DC are present but rendered functionally defective, as in Langerin-Cre $MyD88^{fl}$ mice, Notch2 expression in other myeloid cells would remain suppressed, allowing a defect in innate IL-22 production to emerge. Such a model would also be consistent with our observations of huLangerin-DTA mice treated with Notch2 blocking antibody.

Alternatively, although maintenance of innate mucosal barrier immunity in other circumstances is tightly integrated with tonic signaling from the commensal microbiome (Brandl et al., 2008), the role of the microbiome remains relatively

unexplored with respect to innate IL-22 production. Thus, there may be as yet undefined microbial signals that are required to generate the IL-23/22 cytokine amplification loop. It is likewise possible that Notch2 signaling itself causes alterations in commensal microbial communities that are responsible for the defects observed in CD11c-Notch2^{fl} mice. Future studies that employ the use of germ free animals or those reconstituted with a defined commensal flora may be required.

Footnotes

Portions of this work have been previously published. Reprinted from *The Journal of Experimental Medicine* volume 210, Nathan E Welty, Christopher Staley, Nico Ghilardi, Michael J. Sadowsky, Botond Z. Igyártó, and Daniel H. Kaplan. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism, pp. 2011-2024. Copyright 2013, Welty et al.

[1] HuLangerin-DTR F618 IHC was performed by E. Ampudia Mesias

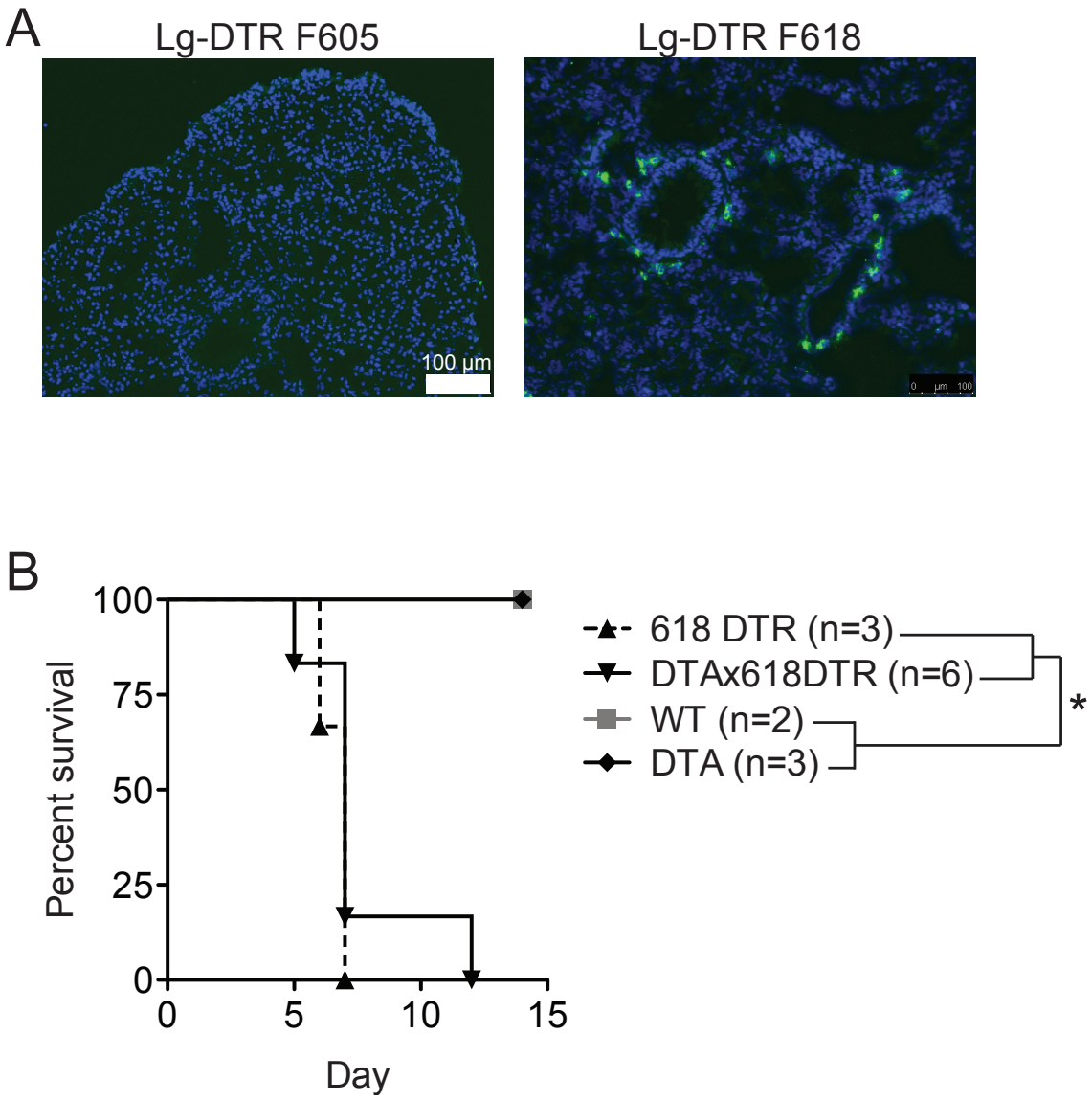


Figure 3-1: Langerin-DTR F618 mice die after DT injection due to ectopic transgene expression

(A) Lung tissue from mice of indicated genotypes stained with anti-huLangerin specific antibody (green) and DAPI (blue). (B) Survival of mice after i.p. injection of 1 µg diphtheria toxin. *p<0.05 by Log-Rank (Mantel-Cox) test.

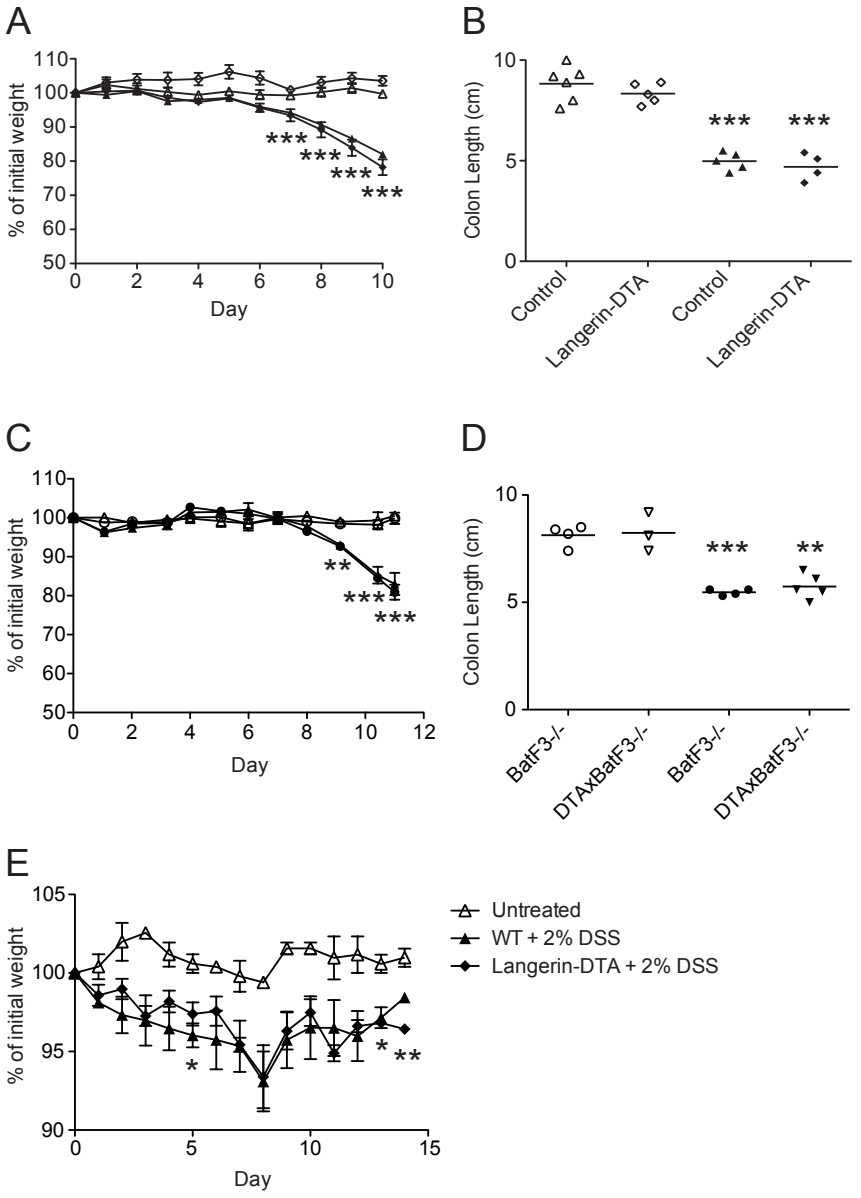


Figure 3-2: CD103^{+} DC are not required for protection from DSS colitis

(A) Wild-type (triangles) and huLangerin-DTA (diamonds) mice were given water (open symbols) or 5% DSS (filled symbols) *ad libitum* and monitored for weight loss. Data shown as mean ± SEM. (B) Colon length upon sacrifice at day 10. (C) BatF3$^{-/-}$ (circles) and DTAxBatF3$^{-/-}$ (triangles) littermate mice were given water (open symbols) or 5% DSS (filled symbols). Data shown as mean ± SEM (D) Colon length upon sacrifice at day 10. Symbols represent individual mice and lines represent the mean. A-D represent one of two experiments. (E) Animals were treated with 2% DSS and monitored for weight loss. *p<0.05, **p<0.01, ***p<0.001

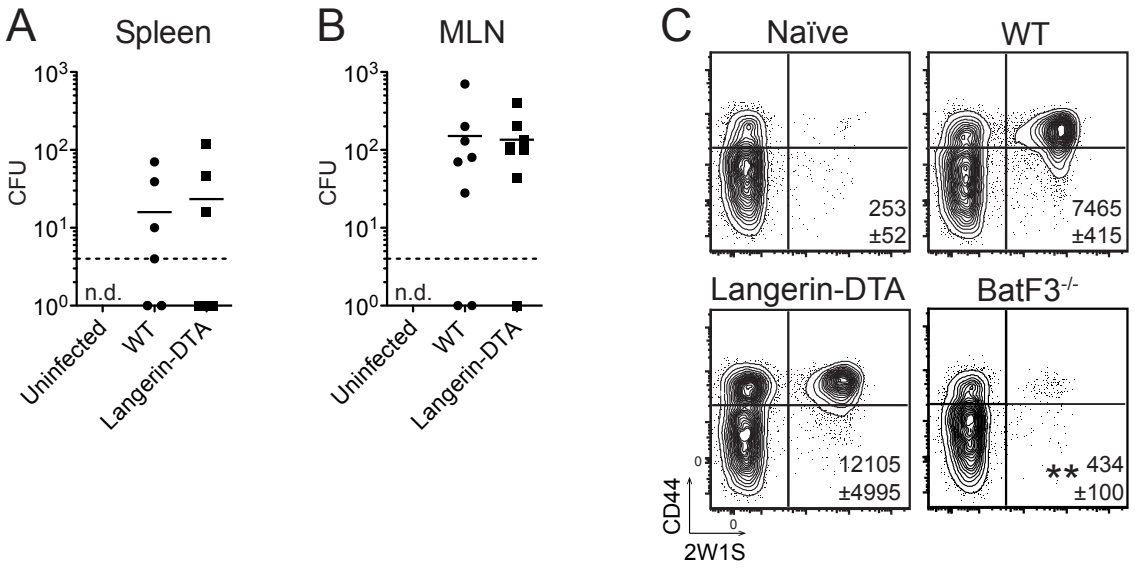


Figure 3-3: CD103^{+} CD11b^{+} DC are dispensable for immunity to* S. *Typhimurium while BatF3-dependent DC are required

CFU from (A) spleen or (B) MLN of mice infected with attenuated AroA^{-} *S.* Typhimurium expressing 2W1S 10 days after infection. Dotted lines represent the limit of detection. (C) Spleens and MLN were pooled from uninfected (naïve) and AroA^{-} *Salmonella*-2W1S infected mice after 10 d and magnetically enriched for 2W1S-specific cells. Flow plots represent the binding of 2W1S-I-A^{b} tetramer by CD4 T cells. Numbers represent the total number of tetramer-specific cells±SEM. n.d. not detected, **p<0.01 compared to wild type

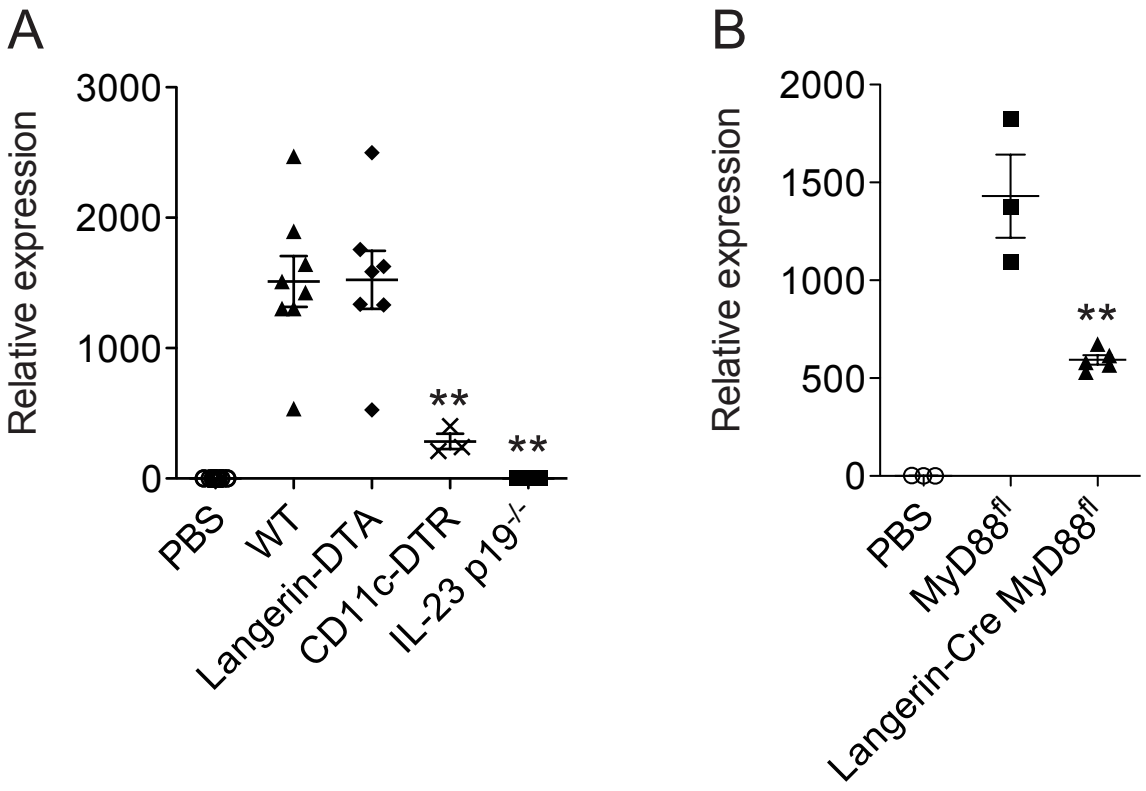


Figure 3-4: CD103+ CD11b+ DC are not required for innate IL-22 production

(A and B) IL-22 expression in animals treated intravenously with PBS (open symbols) or 1 μg flagellin (closed symbols) and euthanized 2 h later for LP mRNA extraction and qPCR analysis. CD11c-DTR mice were treated with 1 μg diphtheria toxin 18 h before flagellin injection. MyD88^{fl} mice are Cre^{-} control littermates of Langerin-Cre MyD88^{fl} mice. Data are normalized to HPRT and displayed as fold change over PBS-treated mice. **p<0.01 compared to wild type or Cre^{-} controls

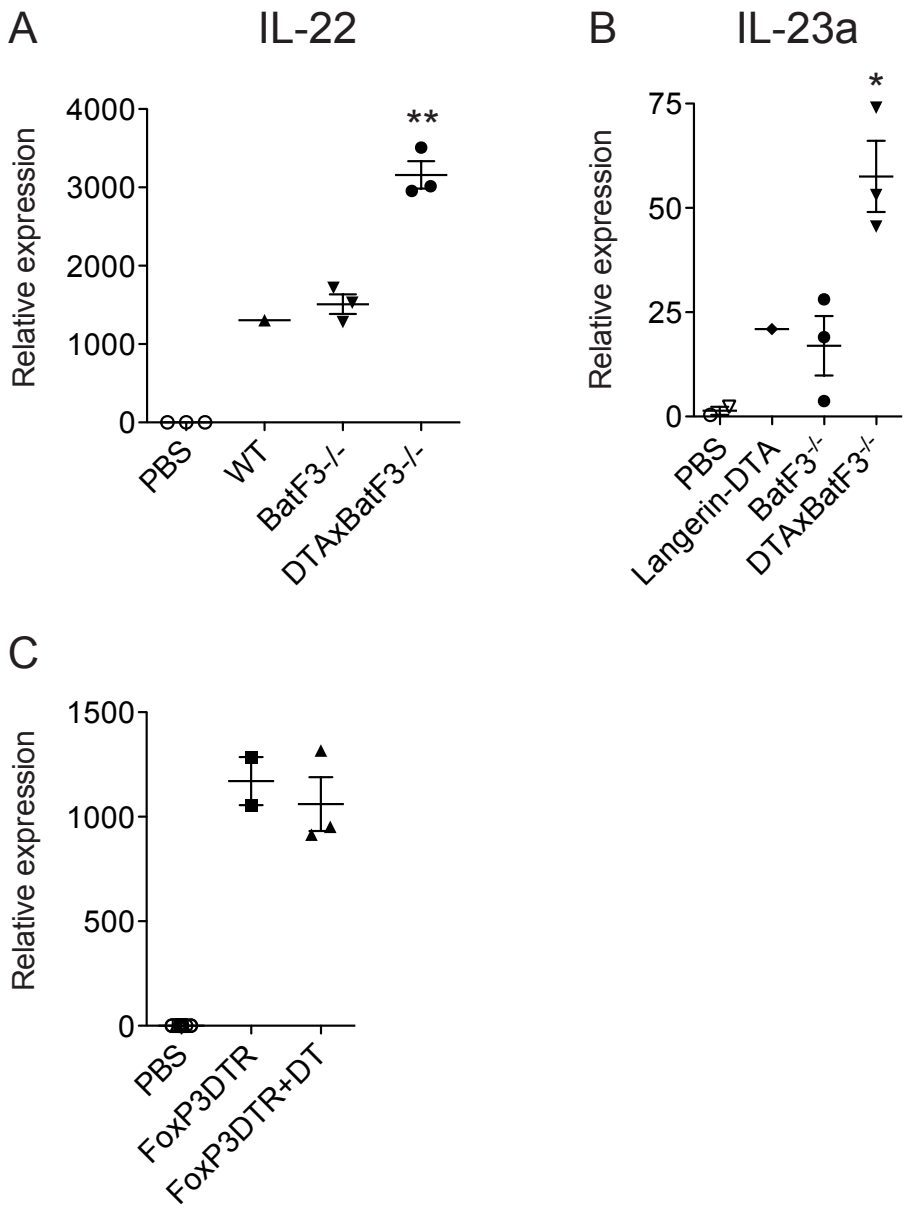


Figure 3-5: CD103^{+} DC deficient mice have enhanced innate IL-23/22 responses through a Treg independent mechanism

(A,C) IL-22 or (B) IL-23a mRNA expression in mice treated i.v. with flagellin and euthanized (A,C) 2 or (B) 1 hr later for LP mRNA extraction and qPCR analysis. FoxP3DTR+DT mice were treated with 1µg diphtheria toxin every other day for a total of 3 injections. Data are normalized to HPRT and displayed as fold change over PBS-treated mice. *p<0.05, **p<0.01

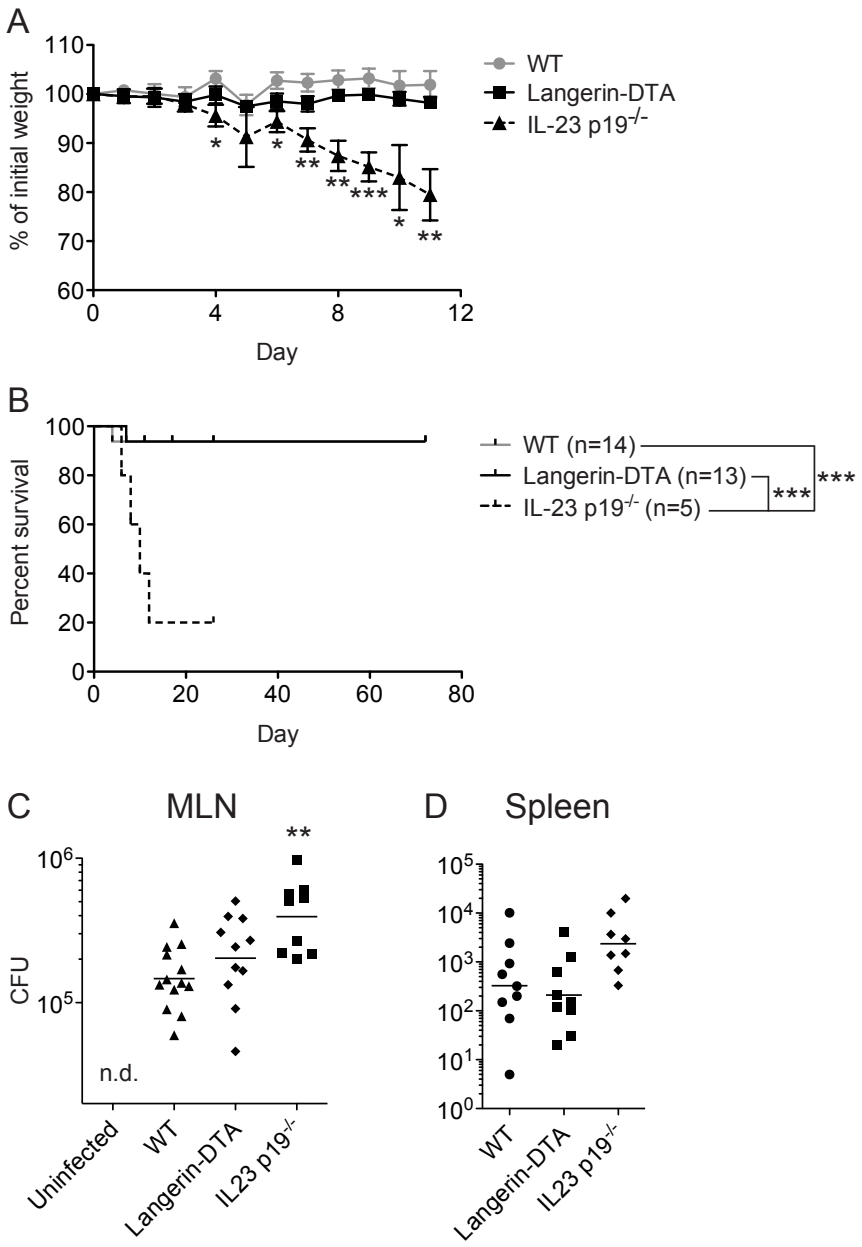


Figure 3-6: CD103$^+$ CD11b$^+$ DC are not required for IL-23 dependent resistance to intestinal infection

(A and B) Mice were infected with 2x10^9 *C. rodentium* by gavage and monitored for (A) weight loss and (B) survival. Weight loss is presented as the mean ± SEM. Survival curves were compared by Log-Rank (Mantel-Cox) Test. Data are pooled from 3 independent experiments. (C and D) Mice were pretreated with 20 mg streptomycin sulfate 24 h before infection with 5x10^7 *S.* Typhimurium strain SL1344 by gavage. (C) MLNs and (D) spleens were harvested 48 h later and analyzed for CFU. n.d. not detected, *p<0.05, **p<0.01, ***p<0.001

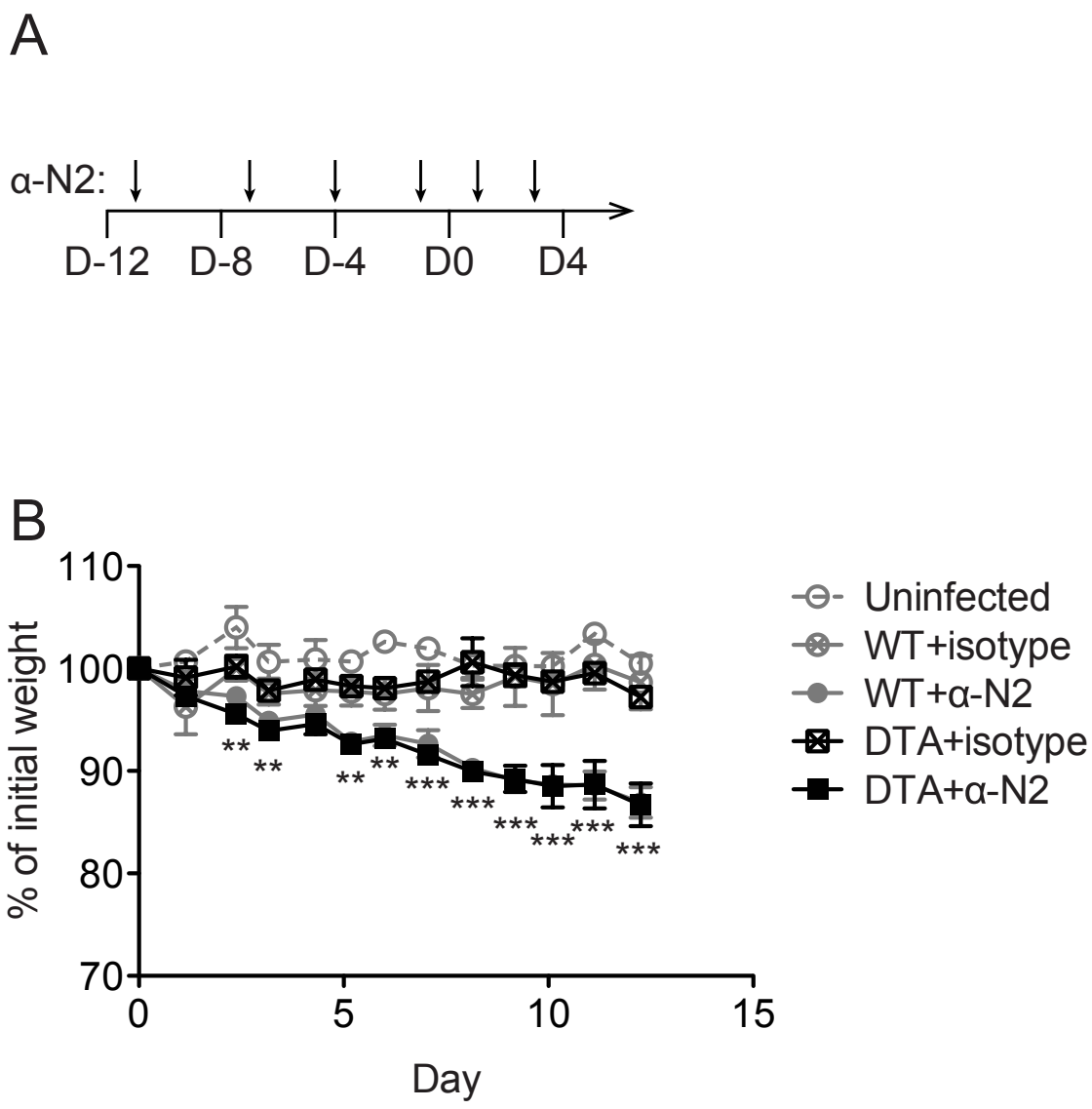


Figure 3-7: Notch2 inhibition sensitizes CD103^{+} CD11b^{+} DC deficient mice to IL-23 dependent infection

(A) Mice were treated with i.p injection of ~5 μg/g Notch2 blocking antibody (α-N2) or control Ig (isotype) prior to and after infection on day 0. Arrows represent antibody injections. (B) Weight loss of mice infected with 2x10^{9} *C. rodentium* by gavage. Data are displayed as mean ± SEM with at least 3 mice per group and represent 1 of 2 experiments. **p<0.01, ***p<0.001

Chapter 4: Homeostatic functions of intestinal dendritic cell subsets

Introduction

While there are multiple mechanisms whereby the immune system prevents deleterious activation against commensal microbial antigens, maintenance of steady-state adaptive immune homeostasis depends in part on the establishment of immunological tolerance to normal intestinal contents (Lathrop et al., 2011; Macpherson et al., 2009). $CD103^+$ DCs have been proposed to be required for this process, yet the participation of specific subsets remains unclear. Moreover, the capacity of intestinal DC to shape the makeup of the intestinal microbiome has not been investigated.

$CD103^+$ intestinal dendritic cells are thought to be crucial to the development of tolerance (Coombes & Powrie, 2008). These cells express high levels of CCR7 and migrate to MLNs under steady-state and inflammatory conditions (Schulz et al., 2009). They also produce retinoic acid (RA), which, in combination with transforming growth factor β (TGF-β) is capable of inducing differentiation of $FoxP3^+$ Treg cells *in vitro* that express gut-homing molecules like the chemokine receptor CCR9 (Coombes et al., 2007; Jaensson et al., 2008; Sun et al., 2007). However, it remains unclear whether these functions are the property of only one $CD103^+$ DC subset or whether there is functional redundancy between subsets. In line with the latter hypothesis, recent studies have shown that all intestinal macrophage and DC populations express RA metabolizing enzymes and are capable of inducing Treg development *in vitro*,

raising the possibility that there might be considerable functional redundancy between these subsets *in vivo* (Denning et al., 2011; Guilliams et al., 2010).

It has long been appreciated that oral administration of protein antigens induces a state unresponsiveness to those same antigens when re-administered systemically (Wells & Osborne, 1911). The mechanisms underlying oral tolerance have been proposed to be similar to those that render immunological tolerance to the commensal flora, namely that antigen presentation by $CD103^+$ DC in the MLN induces the formation of antigen-specific regulatory T cells (Pabst & Mowat, 2012; Weiner, da Cunha, Quintana, & Wu, 2011). Interestingly, Treg homing back to the gut after initial differentiation appears to be a critical step, as animals deficient for gut homing receptors, like CCR9, and chemokines fail to develop oral tolerance (Cassani et al., 2011; Hadis et al., 2011). One study has suggested that this might be the case because Treg undergo significant expansion within the intestinal lamina propria under the influence of IL-10 produced by resident CX_3CR1^+ macrophages (Hadis et al., 2011). The steps mediating egress of the proliferated Treg to provide systemic suppression remain unclear. Moreover, the evidence for the functional role of $CD103^+$ DC is largely based on *in vitro* data. Indeed, *in vivo* data from a model that used directed antigen targeting to $CD103^+$ DC actually found that these DC were quite ineffective at generating $FoxP3^+$ Treg in the MLN, although responding cells were efficiently imprinted with gut-homing receptors (Semmrich et al., 2011).

To test the requirements for specific $CD103^+$ DC in the maintenance of host-commensal homeostasis, we use huLangerin DTA, $BatF3^{-/-}$, and $DTAxBatF3^{-/-}$ animals that lack these subsets to determine their functional roles in the steady state. We provide evidence, that, though they are redundant for Treg development, oral tolerance, and maintenance of the steady-state microbiome, $CD103^+$ $CD11b^+$ LP DC cooperate with $CD103^+$ $CD11b^-$ DC to promote LP Treg homeostasis. Surprisingly, we also uncover a potential novel role for $CD103^+$ $CD11b^+$ DC in the maintenance of naïve CD4 T cell homeostasis in the MLN.

Materials and Methods

Mice

OT-II (Barnden, Allison, Heath, & Carbone, 1998), TEα (Grubin, Kovats, deRoos, & Rudensky, 1997), and OT-I (Hogquist et al., 1994), transgenic mice that contain T cells specific for $Ova_{323-339}$, H2-$E\alpha_{52-68}$, and $Ova_{257–264}$, respectively, have all been previously described. OT-II and TEα mice were maintained on a $RAG1^{-/-}$ background and were a kind gift of M. Jenkins. huLangerin-Cre I-$A\beta^{fl}$ mice have been previously described (Igyarto et al., 2009). All mice were housed as previously stated. The University of Minnesota Institutional Animal Care and Use Committee approved all animal protocols.

Oral tolerance induction and delayed-type hypersensitivity

Oral tolerance was assessed using a previously described assay (Hadis et al., 2011). Briefly, animals were tolerized by two oral gavages 24 hours apart of 50 mg either grade V ovalbumin or bovine serum albumin (Sigma-Aldrich) as a negative control. Four days later, mice were immunized subcutaneously with 300 µg ova dissolved at 1 mg/mL in PBS and emulsified 1:1 in complete Freund's adjuvant (Sigma-Aldrich). For IL-12 administration, mice were immunized with Ova/CFA and given 3 daily s.c. injections thereafter of 0.5 µg recombinant murine IL-12 (PeproTech) in 200 µL sterile PBS. For other forms of DTH, mice were immunized s.c. with 200 µg Ova/alhydrogel (Sigma-Aldrich) or 400 µg Ova+10^7 heat-killed *Candida albicans* (HK Ca). Two weeks later mice were anesthetized with ketamine/xylazine and baseline footpad measurements were recorded using a micrometer. Mice were then challenged with 40 µg ova at 1 mg/mL in PBS in the right footpad or PBS alone in the left footpad. Footpad swelling was measured in a blinded fashion daily thereafter and calculated as [right footpad thickness – right footpad thickness$_{d0}$] – [left footpad thickness – left footpad thickness$_{d0}$].

Adoptive transfers and *in vivo* Treg conversion assay

Treg development *in vivo* was assessed using a modification of a previously described assay (Sun et al., 2007). Briefly, spleens and lymph nodes were harvested from CD90.1 OT-II T cell receptor (TCR) transgenic mice. Cells were labeled with CFSE and resuspended in sterile PBS. The frequency of OT-II

T cells were calculated based on flow cytometric analysis of a small aliquot of the sample and the cell concentration was adjusted such that $1x10^5$ OT-II in 200 µL PBS were adoptively transferred to receptor mice via i.v. tail vein injection. One day later, mice were either left untreated or given 1.5% wt/vol ovalbumin in their drinking water, replaced every 2 days. Mice were euthanized after five days and the number and phenotype of OT-II cells in organs of recipient mice was determined by flow cytometry.

For adoptive transfer of multiple TCR transgenic cells into the same mice, cells were harvested and processed separately and combined prior to injection. $3x10^5$ CFSE labeled cells ($1x10^5$ of each congenically marked TCR transgenic cell type) in 200 µL sterile PBS were then injected into recipient mice through the tail vein. Five days after transfer, cells were detected in recipient animals by staining with antibodies against CD45.1 (OT-I), CD90.1 (Teα and OT-II), and TCR Vβ6 (Teα) in addition to standard T cell markers.

Fecal sample collection and bacterial DNA extraction

Immediately after euthanization, fresh fecal pellets were obtained from the distal colon. Ceca were then opened with sterile scissors and cecal contents were scraped out with forceps. All samples were immediately frozen and stored in sterile microfuge tubes at -80°C. Genomic DNA was extracted from samples using a Power-Soil DNA extraction kit (MoBio) in accordance with the manufacturer's instructions. Approximately 0.3 g of each sample was used for extraction.

16S rDNA amplification and pyrosequencing

DNA extracts were normalized to a concentration of 2.5 ng μl^{-1} by dilution in nuclease-free water and used as template for a PCR assay targeting the hypervariable V6 region of the 16S rDNA using the 967F/1046R primer set (Sogin et al., 2006). Each sample was amplified with a reverse primer containing a unique 6-bp nucleotide sequence on the 5' terminus to allow samples to be pooled for sequencing and separated later (Sogin et al., 2006). Library construction and paired-end sequencing (2 x 100 nt) of amplicons was performed by the BioMedical Genomics Center at the University of Minnesota (Saint Paul, MN) using the Illumina HiSeq platform.

16S sequence analysis and statistics

Raw sequence reads were pair-end aligned, separated by sample, and trimmed for quality as described previously using mothur software v. 1.27.0 (Schloss et al., 2009). Sequences of abundance <2 over the entire dataset were excluded from analysis. Sequences were aligned to the RDP taxonomic database (Cole et al., 2009), and analysis of OTUs was performed at a sequence cutoff of 0.03. The number of sequence reads associated with each group was subsampled to that of the smallest group (containing 217,483 sequences) for comparisons of β diversity and relative taxonomic abundance. Alpha diversity indices (Shannon, non-parametric Shannon, and Simpson indices) were calculated using mothur. The Bray-Curtis measure of dissimilarity was used to construct distance matrices among sites (Bray & Curtis, 1957). Unweighted

UniFrac analyses (Lozupone & Knight, 2005) and analysis of molecular variance (Excoffier, Smouse, & Quattro, 1992) were also calculated using mothur. Diversity indices and relative taxon abundance were compared via two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test at $\alpha=0.05$ using SPSS Statistics software v. 19.0 (IBM).

Results

Steady-state characterization of DC-deficient mice

We first characterized the number and phenotype of cells in the secondary lymphoid organs of mice that lacked $CD103^+$ DC subsets. While we found that the total number of cells in the spleen and in the pooled brachial, axillary, and inguinal skin-draining lymph nodes (collectively SDLN) did not differ when one or both LP DC subsets were deleted (Fig. 4-1A-B). This was despite the absence of other peripheral $CD103^+$ DCs as well as splenic $CD8\alpha^+$ DCs in $BatF3^{-/-}$ mice and epidermal Langerhans cells in huLangerin-DTA mice. However, we noted a modest ~30-40% decrease in total mesenteric lymph node cellularity in mice that lacked $CD103^+$ $CD11b^+$ DC, but not in $BatF3^{-/-}$ mice that lacked $CD103^+$ $CD11b^-$ DC (Fig. 4-1C). Dendritic cells make up a small fraction of the total cell number in secondary lymphoid organs, particularly when organs are not collagenase digested, which is required to release significant numbers of dendritic cells (Inaba et al., 2009). Therefore, it is unlikely the numerical reduction in migratory DC in huLangerin-DTA mice can account for the overall change in MLN cellularity. Likewise, the difference in total MLN cellularity was not due to a reduction in a

specific lymphocyte compartment, as the frequency of CD4 and CD8 T cells as well as $B220^+$ B cells was not altered by the absence of $CD103^+$ $CD11b^+$ DC (Fig. 4-1D).

Colonization of germ-free mice with a host-specific commensal microbiome has been reported to cause host immune maturation and enhanced T cell activation in mucosal secondary lymphoid organs (H. Chung et al., 2012). We therefore investigated markers of antigen experience on CD4 T cells in the secondary lymphoid organs of DC-deficient mice. huLangerin-DTA mice that lacked only $CD103^+$ $CD11b^+$ DC had a normal frequency of $CD44^{hi}$ $CD62L^-$ antigen experienced MLN CD4 T cells compared to their wild-type littermates. In contrast, DTAxBatF3$^{-/-}$ mice lacking both $CD103^+$ subsets had a reduced frequency of $CD44^{hi}$ $CD62L^-$ cells compared to BatF3$^{-/-}$ littermates that lacked only $CD103^+$ $CD11b^-$ DC (Fig. 4-2A, C). This difference appeared to be due in part to a trend toward increased activated T cells in BatF3$^{-/-}$ litters. Whether this increase in activation was due to genotype or to vertical transmission of microbial flora cannot be determined as BatF3$^{-/-}$ litters were maintained separately from their wild type and huLangerin-DTA only counterparts. There were no changes in CD44 or CD62L expression on CD4 T cells in the SDLNs for any genotype (Fig. 4-2B, D). Together, these data suggest that $CD103^+$ DC are critical for microbe induced host immune development in gut-associated lymphoid tissues, although this dependence may require the presence of specific commensal species.

CD103^{+} DC are not required for steady-state intestinal microbial homeostasis

Numerous studies have demonstrated that the presence of specific commensal microorganisms in the intestine direct host immune development (H. Chung et al., 2012; Honda & Littman, 2012). In contrast, the capacity of the host immune system to shape the intestinal microbiome has been less robustly examined. We have now characterized two genetic mouse models in which intestinal DC subsets have been ablated. Because huLangerin-DTA and DTAxBatF3$^{-/-}$ mice do not develop inflammation, we used these mice to test whether DCs regulate steady-state intestinal commensal communities. We obtained fresh fecal pellets and scraped cecal contents from age- and sex-matched huLangerin-DTA and wild-type littermate controls. We also sampled DTAxBatF3$^{-/-}$ mice and BatF3$^{-/-}$ littermate controls. We then performed high-throughput sequencing on 16S bacterial rDNA amplified from these samples[1]. A total of 1.11×10^{8} paired-end sequence reads (2 x 100 nt) were generated, with 2.17×10^{5} sequence reads per sample meeting our quality criteria. From these reads, 19,592 operational taxonomic units (OTUs) were identified among all samples, with a mean of 2,338 ± 261 OTUs present in individual samples. Sequence analyses identified a total of 28 phyla, which were dominated by Firmicutes (49 ± 7%), Bacteroidetes (21 ± 8%), and Proteobacteria (13 ± 5%; Fig. 4-3A-B).

Nonmetric multidimensional scaling (NMDS) on the Bray-Curtis distance matrix was used to visualize microbial community structure along axes of maximal variance (Bray & Curtis, 1957). Unweighted UniFrac analysis revealed that the loss of $CD103^+$ $CD11b^+$ DCs in huLangerin- DTA mice did not significantly affect the microbial composition in colonic (Fig. 4-3C) or cecal (Fig. 4-3D) contents (P = 0.962 and P = 0.662, respectively). There were also no significant differences in Shannon and Simpson diversity indices between groups (unpublished data). Of note, we did observe clustering of different litters, most evident in the cecum, demonstrating the importance of parental transmission in shaping steady-state gut microbial ecology. Similarly, deletion of all CD103+ DCs in DTAxBatF3$^{-/-}$ animals also did not significantly alter composition of the colonic (Fig. 4-3E) or cecal (Fig. 4-3F) microbiota (P = 0.683 and P = 0.959, respectively). Thus, the absence of $CD103^+$ $CD11b^+$ LP DCs, either alone or on the BatF3$^{-/-}$ background, does not significantly affect the composition of the steady-state enteric microflora.

Although we did not observe differences in the composition of the microbiome between mice from the same litter, there were alterations in commensal microbial communities between BatF3 sufficient and deficient litters that were derived from different breeding pairs. Differences between non-littermates were significant for all comparisons regardless of the presence or absence of the huLangerin-DTA transgene (Fig. 4-4). We therefore further compared microbial composition between BatF3 sufficient and deficient litters to

understand which taxa might account for this difference. Because of the short sequence reads used for identification, we could only identify commensals with a high degree of reliability at the taxonomic class level. We observed a significant increase in the number of sequence reads assigned to the class *Clostridia* in both the cecum (Fig. 4-5A) and colon (Fig. 4-5B) of BatF3$^{-/-}$ litters compared to BatF3 sufficient litters. Within the eight most abundant classes of bacteria present in our samples, which on average accounted for approximately 88.7% of the total sequence reads obtained per sample, a change in the abundance of *Clostridia* was the only significant difference observed (Fig. 4-5, only the 3 most abundant classes are shown for ease of display). Because of the importance of parental transmission in determining the offspring's microbial constituents (Ubeda et al., 2012), we consider it likely that an increase in *Clostridia* abundance is due to vertical transmission within the BatF3$^{-/-}$ line rather than the loss of the *BATF3* gene. However, this latter possibility cannot be excluded.

CD103^{+} DC are mutually required for regulatory T cell homeostasis

We next investigated whether loss of migratory dendritic cells affected the homeostasis of FoxP3^{+} regulatory T cells[2]. Contrary to our initial expectations, but in agreement with several subsequent models that target CD103^{+} CD11b^{+} DC (K. L. Lewis et al., 2011; Persson et al., 2013), we found that the frequency and number of FoxP3^{+} Treg in the MLN and small intestine LP was unaffected in huLangerin-DTA mice (Fig. 4-6). As T reg cell numbers have also reported to be unaffected in BatF3$^{-/-}$ mice (Edelson et al., 2010), we next examined T reg cell

development in DTAxBatF3$^{-/-}$ mice. The frequency and number of FoxP3^{+} T reg cells in the MLN was unaffected in DTAxBatF3$^{-/-}$ mice lacking both CD103^{+} DC subsets (Fig. 4-7A-B). However, there was a significant reduction in the number of FoxP3^{+} Treg in the LP of DTAxBatF3$^{-/-}$ mice compared with BatF3$^{-/-}$ littermates (Fig. 4-7C-D). We next investigated expression of gut-homing chemokine receptors on FoxP3^{+} Treg in the MLN. The frequency of T reg cells expressing the RA-inducible gut homing receptor CCR9 was greatly diminished in DTAxBatF3$^{-/-}$ mice compared with both wild type mice and mice lacking only one CD103^{+} DC subset (Fig. 4-7E-F). We therefore conclude that CD103^{+} CD11b^{+} and CD103^{+} CD11b^{-} DCs are individually redundant but are together required to imprint gut homing receptors on T reg cells in the MLN, thereby maintaining normal numbers of T reg cells in the LP.

Intestinal microbial communities dictate steady-state regulatory T cell phenotype

FoxP3^{+} regulatory T cells that co-express the transcription factor Helios (*IKZF2*) have been proposed to represent a population of thymically derived Tregs, while Helios^{-} cells may represent those that have arisen from encounter with antigen in the periphery, although this is a matter of some debate (Gottschalk, Corse, & Allison, 2012; Thornton et al., 2010). Gating on FoxP3^{+} cells present in the small intestinal lamina propria, we were able to identify defined populations of Helios^{+} and Helios^{-} Tregs in the steady state (Fig. 4-8A). Intriguingly, we found that the frequency of Helios^{-} Treg was markedly increased

in BatF3$^{-/-}$ animals compared to wild type mice. This increase did not depend on the presence of CD103^{+} CD11b^{+} DC as huLangerin-DTA and DTAxBatF3$^{-/-}$ mice had no significant difference in the frequency of Helios^{-} cells compared to their respective wild type or Batf3$^{-/-}$ littermates (Fig. 4-8B). We also did not observe any change in the frequency of Helios^{-} Treg in the MLN, suggesting that this phenomenon was not due to a defect in thymic Treg development (Fig. 4-8C).

To test whether the altered Treg phenotype in BatF3$^{-/-}$ animals was due to the loss of CD103^{+} CD11b^{-} LP DCs or to altered commensal microbial communities, we outcrossed BatF3$^{-/-}$ mice to obtain littermate BatF3$^{+/-}$ and BatF3$^{-/-}$ with the same parentally transmitted flora. In these outcrossed mice, we did not observe any difference in the frequency of Helios^{-} Treg in the MLN, small intestinal LP, or colonic LP (Fig. 4-9). Thus, we conclude that vertically transmitted commensal microflora exerts dominant effects on host LP Treg phenotypes irrespective of genotype at the *BATF3* locus.

CD103^{+} CD11b^{+} DC are not required to establish oral tolerance

To test the functional requirement for CD103^{+} DC during oral tolerance, we immunized mice that were previously tolerized by gavage of ovalbumin (Ova) or bovine serum albumin (BSA) with subcutaneous Ova emulsified in complete Freund's adjuvant (CFA) according to a previously published protocol (Hadis et al., 2011). Two weeks later, mice were challenged by footpad injection of Ova/PBS to assess a delayed-type hypersensitivity (DTH) response. Compared to unimmunized controls, we found a robust DTH response in wild type mice that

were immunized with Ova/CFA. This response was almost completely suppressed in mice that had been previously tolerized by oral administration of ovalbumin. We found that huLangerin-DTA mice had intact DTH responses that were robustly suppressed by prior administration of oral ovalbumin, demonstrating that $CD103^+$ $CD11b^+$ DC are dispensable for oral tolerance (Fig. 4-10).

We next sought to investigate oral tolerance in mice that lacked both subsets of $CD103^+$ LP DC. However, we found that, regardless of oral pretreatment regimen, $BatF3^{-/-}$ and DTAx$BatF3^{-/-}$ mice both failed to mount DTH responses to subcutaneous immunization with Ova/CFA (Fig. 4-11A). In addition to $CD103^+$ $CD11b^-$ DC in the LP, $BatF3^{-/-}$ mice also lack $CD103^+$ DC in the skin and skin draining lymph nodes, as well as $CD8\alpha^+$ DC in the spleen (Edelson et al., 2010; 2011). BatF3-dependent DCs have been shown to be a critical source of IL-12 (Mashayekhi et al., 2011), a cytokine that is necessary for the development of Th1 cells that are important for effective DTH responses. We therefore sought to rescue this response by immunizing mice with Ova/CFA and concomitant injection of recombinant IL-12, as well as the use of alternate adjuvants, including alum and heat-killed *C. albicans* (HK CA), but were unsuccessful (Fig. 4-11B). Thus, it remains unclear whether there is a functional requirement for $CD103^+$ DCs in oral tolerance.

CD103$^+$ CD11b$^+$ DC are not required for Treg generation to oral antigens

We next investigated the participation of CD103$^+$ DCs in Treg generation to oral antigen. We adoptively transferred 1x10^5 Ova-specific OT-II CD4 T cells (Sun et al., 2007) into either wild type or DC deficient mice. Compared to untreated controls, OT-II cells efficiently expanded in the mesenteric lymph nodes of wild-type hosts when animals were administered ovalbumin in the drinking water for five days (Fig. 4-12A). This expansion was due to proliferation of the transferred cells in response to Ova, as demonstrated by their dilution of CFSE (Fig. 4-12B). Surprisingly, we did not note a consistent reduction in CFSE dilution in treated mice that lacked one or both CD103$^+$ DC subsets (Fig. 4-12C), suggesting that CD103$^+$ DC were not required to present orally acquired ovalbumin.

Despite the fact we did not observe altered proliferation in CD103$^+$ CD11b$^+$ DC deficient mice, we did note that Ova-treated huLangerin-DTA and DTAxBatF3$^{-/-}$ mice had approximately ½-1 log reduction in the total number of OT-II in the MLNs compared to their respective wild-type or BatF3$^{-/-}$ littermates (Fig. 4-12D). Moreover, there was an inverse relationship between the number of proliferated OT-II and the percentage of these cells expressing the transcription factor FoxP3, such that mice lacking CD103$^+$ CD11b$^+$ DC actually had an increased frequency of FoxP3$^+$ regulatory T cells (Fig. 4-12E). We considered that these marked changes in cell numbers in the absence of apparent defects in proliferation might be due to differences in the number of naïve cells that were

capable of responding to Ova. We therefore looked at littermate animals within the same experiment that had received OT-II cells but not antigen. Surprisingly, we observed a similar pattern in both the frequency (not shown) and total number of OT-II cells (Fig. 4-12F) present in the MLN of mice that were not exposed to Ova as those that were. This difference was not due to variation in the number of cells initially transferred because the number of OT-II cells isolated from skin-draining lymph nodes of the same mice was highly consistent regardless of recipient genotype (Fig. 4-12G).

CD103$^+$ CD11b$^+$ DC maintain naïve T cell homeostasis

In order to avoid potential confounding due to differences in parental microflora, we next examined the homeostasis of naïve OT-II cells in BatF3$^{-/-}$ and DTAxBatF3$^{-/-}$ mice where one parent was heterozygous for the *BATF3* gene. We could then directly compare BatF3$^{+/-}$, DTAxBatF3$^{+/-}$, BatF3$^{-/-}$, and DTAxBatF3$^{-/-}$ mice from the same litter. After adoptive transfer of 1x10^5 OT-II cells into these animals, we again observed a significant reduction in both number (Fig. 4-13A) and frequency (Fig. 4-13D) of transferred cells in the MLNs of huLangerin-DTA and DTAxBatF3$^{-/-}$ mice after five days. There were no significant differences in the number or frequency of cells recovered from the SDLN (Fig. 4-13B,E), or Spleen (Fig. 4-13C,F) of recipient mice. Thus we conclude that CD103$^+$ CD11b$^+$ LP DC, but not CD103$^+$ CD11b$^-$ DC, are required for the homeostasis of OT-II cells in the MLN but not other secondary lymphoid tissues.

DCs have been reported to express lymphotoxin molecules and vascular endothelial growth factor (VEGF) that are required for maturation of high endothelial venules (HEVs). In CD11c-DTR mice chronically depleted of dendritic cells or in mice where DCs, but not T-cells, lack CCR7, impaired HEV development leads to markedly reduced entry of adoptively transferred T cells into lymph nodes that can be detected within 3-4 hours after i.v. transfer (Moussion & Girard, 2011; Wendland et al., 2011). Thus, to investigate whether a similar HEV-dependent mechanism might explain the reduced number of OT-II cells observed in the MLN of huLangerin-DTA mice, we looked at OT-II numbers at an earlier time point. Four hours after transfer we observed no change in the frequency of OT-II cells that were able to enter the MLN of $CD103^+$ $CD11b^+$ DC deficient mice compared to wild type controls (Fig. 4-13G), suggesting that this DC subset alters naïve T cell homeostasis through a different mechanism.

Maintenance of naïve T cells in the periphery requires interaction with self-peptide-MHC complexes and homeostatic cytokines. Intraclonal competition for maintenance MHC interactions can reduce the apparent half-life of adoptively transferred TCR transgenic cells, particularly in the case of non-physiologic high-transfer frequencies. However, at transfers closer to physiologic frequencies, modest proliferation of transferred cells can be observed (Hataye, 2006; Takada & Jameson, 2009). Assuming a typical parking efficiency of ~10-15% (Moon et al., 2009), transfer of $1x10^5$ cells should result in approximately $1x10^4$ surviving cells in the recipient mouse, which is close to the total number of OT-II cells we

were able to recover from the spleen, SDLNs, and MLNs in these experiments (8166±479 for BatF3$^{+/-}$ controls, note that this number does not include all secondary lymphoid organs, e.g. cervical LNs). While 1x10^4 cells is still many times the size of physiologic clonal populations (Nikolich-Zugich, Slifka, & Messaoudi, 2004), we nonetheless detected rare cells that had slightly diluted their CFSE in the MLNs of recipient mice (Fig. 4-13H). Such cells were detected at a similar frequency in a previous study (Hataye, 2006). Intriguingly, however, mice that lacked CD103$^+$ CD11b$^+$ DC had a complete absence of CFSE diluted cells in the MLN (Fig. 4-13I).

Finally, we sought to determine whether reduced maintenance of OT-II cells was dependent on T cell receptor interactions with MHCII on DCs. We adoptively transferred multiple transgenic clones with distinct TCR specificities into the same recipients. We detected MHC class I restricted CD8$^+$ OT-I cells with an antibody against the congenic surface marker CD45.1 (Fig. 4-14A) and MHC class II restricted CD4$^+$ OT-II and TEα cells with an antibody against CD90.1. TEα cells were distinguished from OT-II cells by staining for TCR Vβ6 (Fig. 4-14B). We then investigated whether maintenance of these distinct clonal populations was altered in the MLNs of huLangerin-DTA mice that lacked CD103$^+$ CD11b$^+$ DC or in huLangerin-Cre I-Aβ^{fl} mice in which CD103$^+$ CD11b$^+$ DC were present, but were unable to present peptides on MHCII. We found that MHCI restricted OT-I cells did not require CD103$^+$ CD11b$^+$ DC for their maintenance in the MLNs (Fig. 4-14C). In contrast, OT-II cells were highly

dependent on this subset, as previously observed. Interestingly, we also observed a similarly reduced frequency of OT-II cells in mice where $CD103^+$ $CD11b^+$ DCs were intact, but lacked expression of MHCII (Fig. 4-14D). Finally, we observed a much more modest, but significant, reduction in the frequency of TEα cells, although this was not as obviously dependent on MHCII expression by $CD103^+$ $CD11b^+$ DC (Fig. 4-14E). As before, the analysis of $CFSE^{lo}$ cells that had undergone slight proliferation corroborated these same trends (Fig. 4-14F-H). Taken together, these data suggest that TCR-MHCII interactions on $CD103^+$ $CD11b^+$ DC, but not $CD103^+$ $CD11b^-$ DC, are required for the maintenance of naïve CD4 T cells in the MLN and that the magnitude of this requirement may vary based on TCR.

Discussion

Here we have used targeted DC deletion strategies to determine their role in maintaining steady-state immune tolerance and commensal homeostasis. We found that $CD103^+$ $CD11b^+$ DC deficient mice had a modest reduction in overall mesenteric lymph node size, but that this was not due to a specific defect in B cells, CD4 T cells, or CD8 T cells. DTAxBatF3$^{-/-}$ mice additionally had a reduced frequency of effector/memory $CD44^{hi}$ CD4 T cells. While the latter results are consistent with a defect in the steady-state presentation of commensal antigens, it is interesting that there appears to be a specific requirement for $CD103^+$ $CD11b^+$ DC to maintain normal MLN cellularity. Although this effect is modest,

further studies investigating lymph node architecture and specific cytokines or other factors involved would be informative.

The significance of intestinal commensal microorganisms in establishing and maintaining normal gut immune maturation is very well appreciated (Honda & Littman, 2012). In contrast, the role played by the intestinal immune system in determining the constituents of the commensal microbial community has been less rigorously explored. Altered intestinal microbial communities that result from genetic defects have been reported for Nalp6$^{-/-}$ and Tbet$^{-/-}$ Rag2$^{-/-}$ mice (Elinav et al., 2011; Garrett et al., 2007). Altered commensalism in these mice occurs in the setting of spontaneous inflammatory bowel disease. Because huLangerin-DTA and DTAxBatF3$^{-/-}$ mice do not develop inflammation, they can be used to test whether DCs regulate steady-state intestinal commensal communities. Remarkably, the absence of CD103^{+} CD11b^{+} LP DCs did not alter intestinal microbiome of these mice. This is not the result of a technical limitation, as we were able to detect differences resulting from vertical transmission of maternal flora (Ubeda et al., 2012). Our data thus support a model in which the innate and adaptive immune functions of dendritic cells, with the possible exception of IgA production (Fagarasan et al., 2002), have a limited role in shaping the composition of the intestinal microbiome. Indeed, data from our lab and others has shown that, while there might be modest alterations in the steady-state intestinal flora of Rag$^{-/-}$ mice that lack both T and B cells, mice lacking innate toll like receptor signaling pathways have no significant alterations in intestinal

commensal communities (Scholz, Badgley, Sadowsky, & Kaplan, 2014; Ubeda et al., 2012). However, one recent study found that although genetic deletion of TLR5 did not alter the taxonomic composition of the commensal flora, metatranscriptomic analysis uncovered genotype-specific changes in global bacterial gene expression patterns (Cullender et al., 2013). Such analyses thus may uncover more subtle microbial alterations if applied to dendritic cell deficient mice.

Interestingly, BatF3$^{-/-}$ and DTAxBatF3$^{-/-}$ mice that had been maintained as separate litters had a distinct microbiota that was at least in part characterized by an increase in sequence reads assigned to the class *Clostridia*. While *Clostridia* represent a large and diverse set of microbes, certain clusters of the genus *Clostridium*, which are members of this class, have recently been implicated in the generation of FoxP3^{+} Treg cells in the colonic lamina propria (Atarashi et al., 2013; 2011). These induced Tregs were Helios^{-} and highly immunosuppressive, expressing high levels of IL-10 and CTLA4 (Atarashi et al., 2011). Notably, we also observed an increase of Helios^{-} Treg in the small intestine of BatF3$^{-/-}$ litters. This increase was not due to the BatF3 genotype, but rather could be vertically transmitted to BatF3$^{+/-}$ animals, suggesting a dominant role for the microbiota. It is thus tempting to speculate that the overrepresentation of *Clostridia* sequences and the altered Treg phenotype in BatF3$^{-/-}$ litters are causally related. Whatever the case may be, this line of inquiry supports a dominant role for the microbiome in determining the immune phenotype of the host.

CD103$^+$ DCs generate T reg cells *in vitro* through a retinoic acid-dependent mechanism (Coombes et al., 2007; Sun et al., 2007; Jaensson et al., 2008). The particular CD103$^+$ DC subset responsible for T reg cell development in vivo has not been identified because both CD103$^+$ LP DC subsets, as well as CX3CR1$^+$ macrophages, have the capacity to metabolize RA and to induce T reg cells in vitro (Guilliams et al., 2010; Denning et al., 2011). In agreement with others, we show that, in vivo, neither CD103$^+$ DC subset is individually required for T reg cell homeostasis (Edelson et al., 2010; Lewis et al., 2011). The reduction of T reg cells in DTAxBatF3$^{-/-}$ mice reconciles the in vitro and in vivo data by demonstrating that, though mutually redundant, CD103$^+$ DC subsets are jointly required to imprint CCR9$^+$ T reg cells in the MLN and thereby maintain normal T reg cell numbers in the LP. We also found that CD103$^+$ CD11b$^+$ DC are redundant in a model of oral tolerance, although we could not assess mice that lack both LP DC due to technical limitations. Nonetheless, these data suggest a joint dependence on CD103$^+$ DC subsets for normal Treg homeostasis.

Whether CD103$^+$ DCs were required for antigen presentation or were an obligate source of RA required could not be distinguished. Indeed, the mechanisms of steady-state antigen acquisition and presentation are currently highly controversial. Previous work suggested that LP DC can acquire steady-state antigen via the extension of transepithelial dendrites through tight junctions between intestinal epithelial cells and into the lumen, however this appears to be a property of CX_3CR1^+ macrophages and not CD103$^+$ DC (Niess et al., 2005;

Rescigno et al., 2001). $CD103^+$ DC may acquire antigen from CX_3CR1^+ cells via transfer through gap junction proteins (Mazzini, Massimiliano, Penna, & Rescigno, 2014). However, another recent study has proposed that $CD103^+$ DC instead directly acquire small soluble antigens via goblet cell antigen passages (McDole et al., 2012). Likewise, while early studies showed an obligate role for cell bound, CCR7-dependent antigen transport in initiating ova-specific immune responses in the MLN (Worbs et al., 2006), whether or not CX_3CR1^+ macrophages participate in this migration remains a debated subject (Diehl et al., 2013; Schulz et al., 2009). However, we found that there was still robust proliferation of Ova-specific cells in the MLNs of mice that lacked both $CD103^+$ DC subsets, strongly suggesting alternate means of antigen transport. Moreover, we found no reduction, and possibly an enhancement, of Treg conversion in $CD103^+$ DC deficient mice. This study bears repeating, and the conclusions may be compromised by the reduced number of OT-II precursors capable of responding to antigen in the MLN. However, at face value the data suggests that the defect in LP Treg homeostasis in DTAxBatF3$^{-/-}$ mice is not due a loss of Treg priming. Rather, the reduction in RA induced homing molecules like CCR9 may be the sole explanation for the phenotype.

Perhaps our most surprising finding was that the deletion of $CD103^+$ $CD11b^+$ DCs in huLangerin-DTA or DTAxBatF3$^{-/-}$ mice impaired maintenance of adoptively transferred CD4 T cells in the mesenteric lymph nodes. This effect did not occur in other secondary lymphoid organs and appeared to be mediated by

interactions between the T cell receptor and MHCII. Despite transfer at non-physiologic frequencies, CD4 T cells showed reduced CFSE dilution in huLangerin-DTA mice, suggesting a loss of homeostatic TCR signals. MHC class I restricted OT-I cells showed no phenotype after transfer, while clonal CD4 T cell populations with different TCRs showed a different degree of dependence on the presence of $CD103^+$ $CD11b^+$ DCs. Moreover, mice that lacked MHCII only on $CD103^+$ $CD11b^+$ DCs showed a similar phenotype. Importantly, altered T cell homeostasis did not appear to be due to impaired HEV development in the absence of migratory DCs, as has been reported in other systems (Moussion & Girard, 2011; Wendland et al., 2011), because of the data suggesting MHCII dependence and because the entry of cells into the MLN was not impaired early after transfer.

Our data suggest a dependence on a single DC subset for naïve T cell maintenance at a single secondary lymphoid tissue. The maintenance of naïve T cells in the periphery is thought to require low-level stimulation from self-peptide-MHC complexes as well as homeostatic signals through the IL-7 receptor (Takada & Jameson, 2009). Intriguingly, one recent study has found that TGF-β signaling can regulate IL-7Rα expression and that TGF-β is important for the peripheral maintenance of naïve CD4 T cells, particularly those with a low affinity for self (Ouyang et al., 2013). Thus, the unique requirement for $CD103^+$ $CD11b^+$ DCs, but not $CD103^+$ $CD11b^-$ DCs, could be due to their expression of integrin-β8, which is required for TGF-β activation (Fig. 5-7) (Païdassi et al., 2011;

Worthington, Czajkowska, Melton, & Travis, 2011; Z. Yang et al., 2007). Such a mechanism could also explain why OT-II cells, which have a low affinity for self-MHC molecules, appeared to be highly dependent on the presence of this subset. However, an important caveat to these experiments is that they were completed at non-physiologic frequencies. Transfers at lower numbers, at which proliferation of the cells is more easily observed due to reduced intraclonal competition (Hataye, 2006), would likely yield more convincing results. However, the utility of this technique might be limited by the need to obtain adequate cell numbers from individual SLOs. Therefore, these studies might also need to be supplemented with longitudinal kinetic studies of higher-frequency transfers. Another potential caveat is that the minimal proliferation observed could be due to low-level cross reactivity between OT-II cells and unknown antigens presented by $CD103^+$ $CD11b^+$ DCs. Thus, studies would need to be completed with multiple TCR transgenics and/or germ free mice. Nonetheless, this model would suggest that migratory intestinal DCs preferentially maintain low-affinity naïve T cells in mucosal lymph nodes. Such a mechanism could be important to prevent autoreactivity or to promote an optimally diverse set of potential TCRs to respond to infection.

Footnotes

Portions of this work have been previously published. Reprinted from *The Journal of Experimental Medicine* volume 210, Nathan E Welty, Christopher Staley, Nico Ghilardi, Michael J. Sadowsky, Botond Z. Igyártó, and Daniel H.

Kaplan. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism, pp. 2011-2024. Copyright 2013, Welty et al.

[1] C. Staley performed fecal DNA extraction and microbiome sequence analyses.

[2] Z. Zelickson assisted with cell isolation.

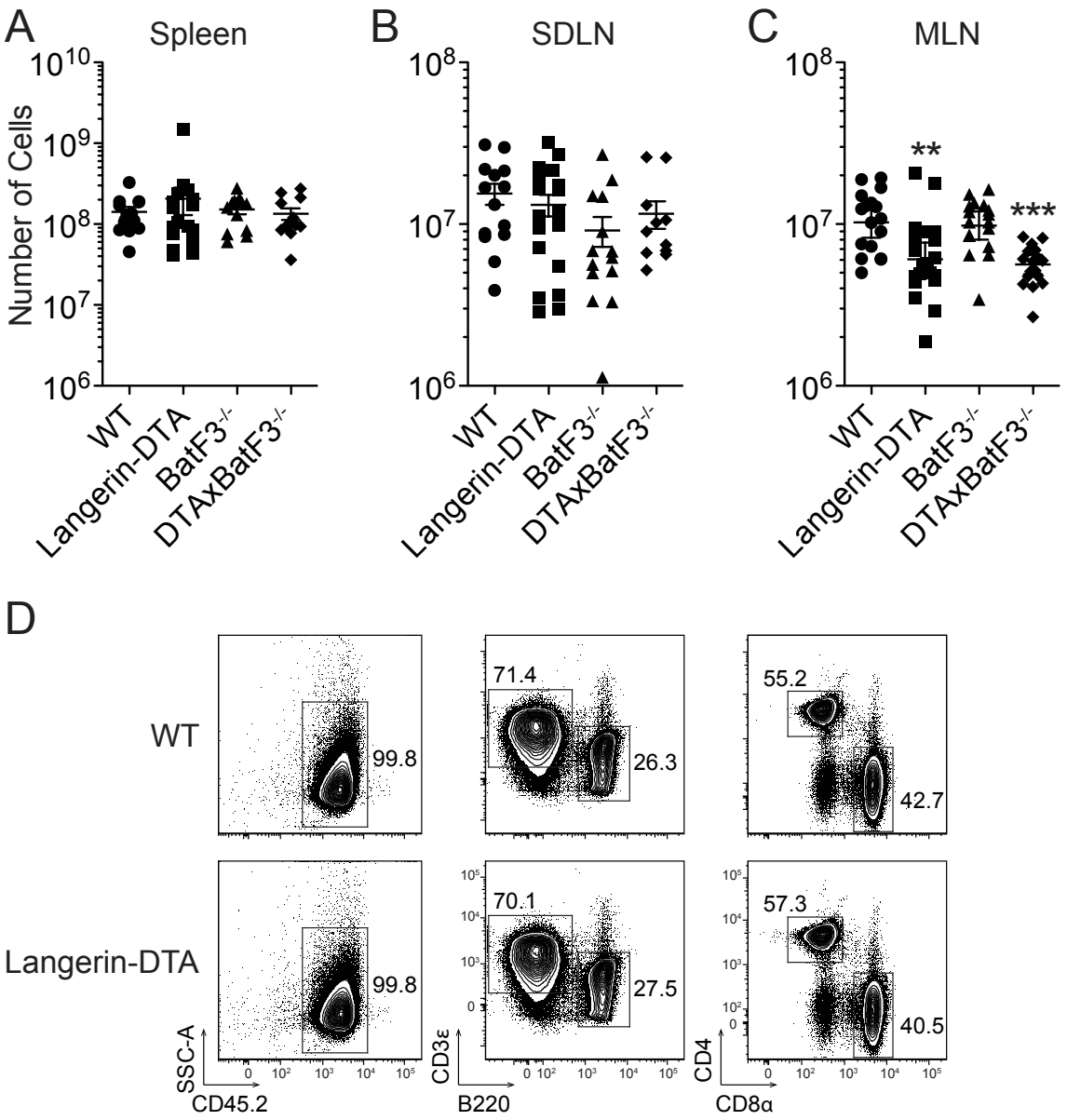


Figure 4-1: Langerin-DTA mice have reduced MLN cellularity but normal T and B cell compartments

(A-C) Total number of cells in (A) spleen, (B) skin-draining lymph nodes (SDLN), and (C) mesenteric lymph nodes (MLN) isolated from mice of the indicated genotypes. Symbols represent individual mice and lines represent the mean±SEM. (D) Flow cytometry plots of MLN cells stained for leukocyte (CD45.2), T cell (CD3ε, CD4, CD8), and B cell (B220) markers. Numbers represent the frequency of cells in each gate. **p≤0.01, ***p≤0.001 by unpaired student's T test

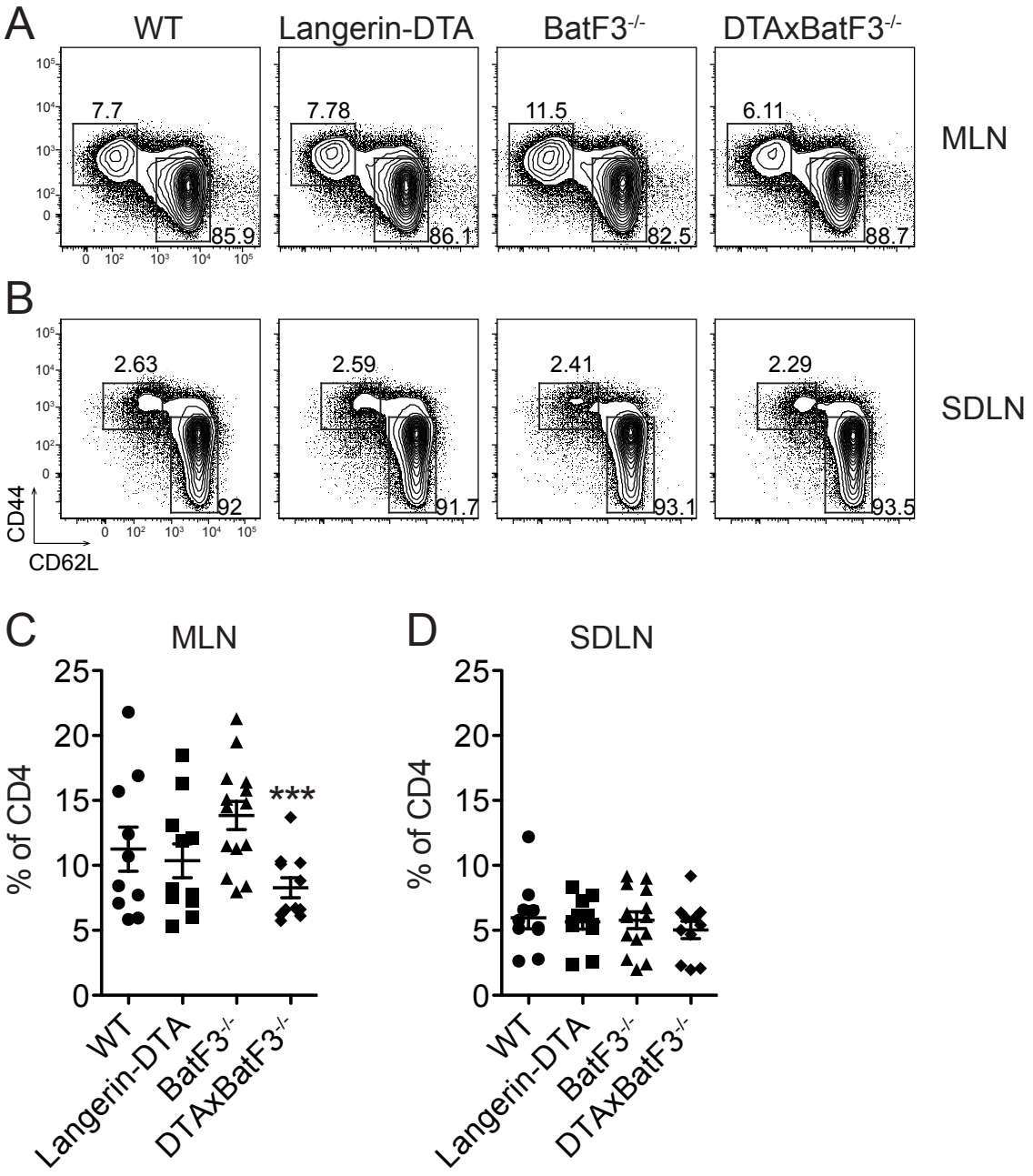


Figure 4-2: Mice lacking CD103+ DC have reduced steady-state CD4+ T cell activation

(A-B) Flow cytometric analysis of CD4 T cells isolated from (A) MLN or (B) SDLN of indicated mice. (C-D) The frequency of cells in each gate is plotted in (C) and (D) for each tissue type. Symbols represent individual mice and are pooled from four experiments. Unpaired student's T tests were performed for Langerin-DTA or DTAxBatF3$^{-/-}$ mice and their respective WT or BatF3$^{-/-}$ littermates. ***p≤0.001

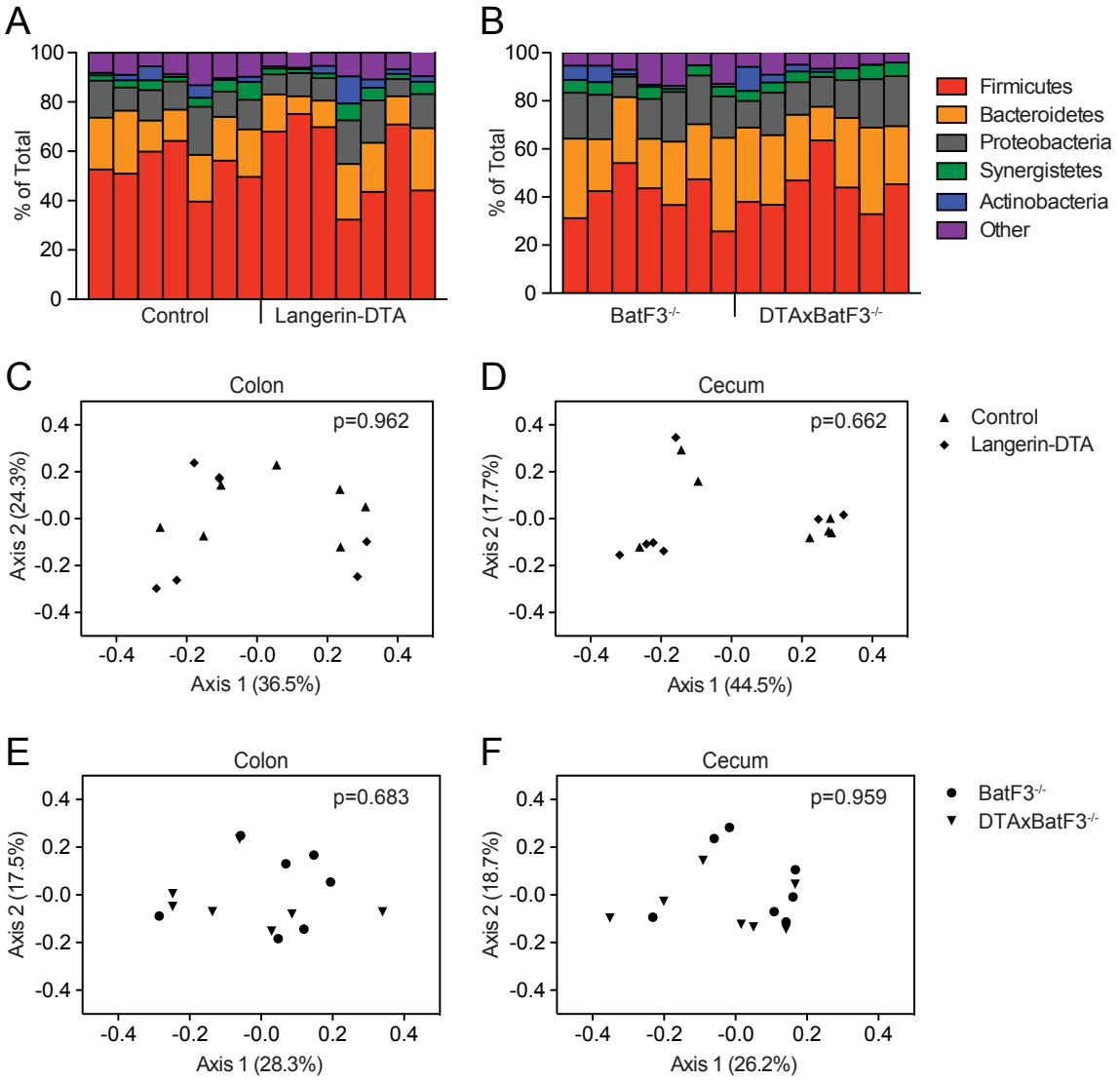


Figure 4-3: Loss of CD103^{+}CD11b^{+} LP DCs does not alter commensal microbial communities

(A and B) Phylogenetic classification of 16S rDNA sequence reads obtained from colonic fecal samples of wild-type/huLangerin-DTA littermates (A) or BatF3$^{-/-}$ /DTAxBatF3$^{-/-}$ littermates (B). Bars represent bacterial phyla present in individual mice (7 mice per genotype). (C–F) Principal component plots comparing overall bacterial composition in colonic (C and E) or cecal (D and F) stool samples. Symbols in C–F represent individual mice. P-values represent unweighted UniFrac comparisons of huLangerin-DTA or DTAxBatF3$^{-/-}$ animals to their respective wild type or BatF3$^{-/-}$ littermates at each anatomical site.

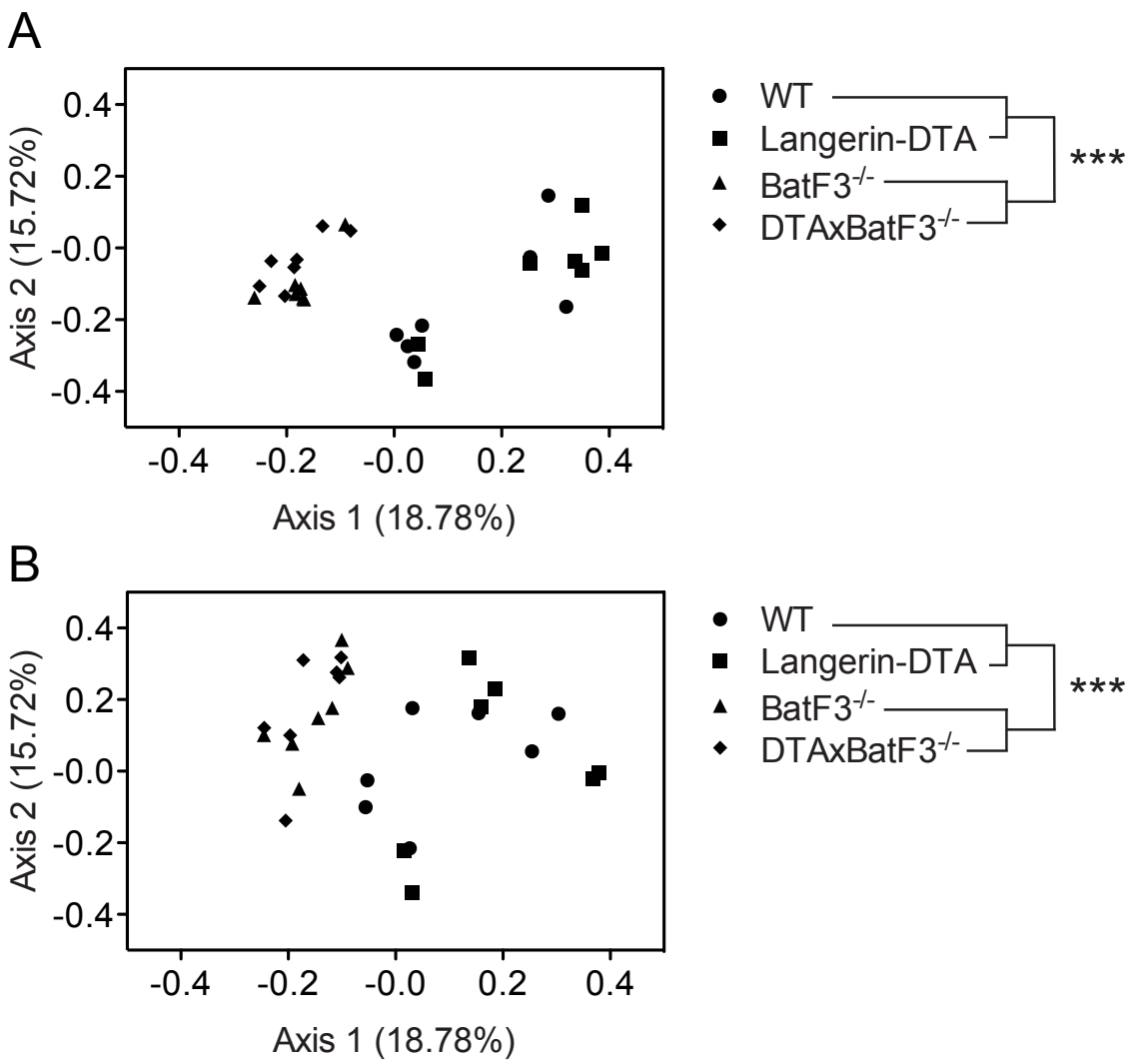


Figure 4-4: Altered commensal microbial homeostasis in BatF3$^{-/-}$ mice

Principal component analysis of 16S sequences from (A) cecal and (B) colonic samples of mice of all genotypes. P-values represent unweighted UniFrac comparisons between genotypes by AMOVA. ***p<0.001

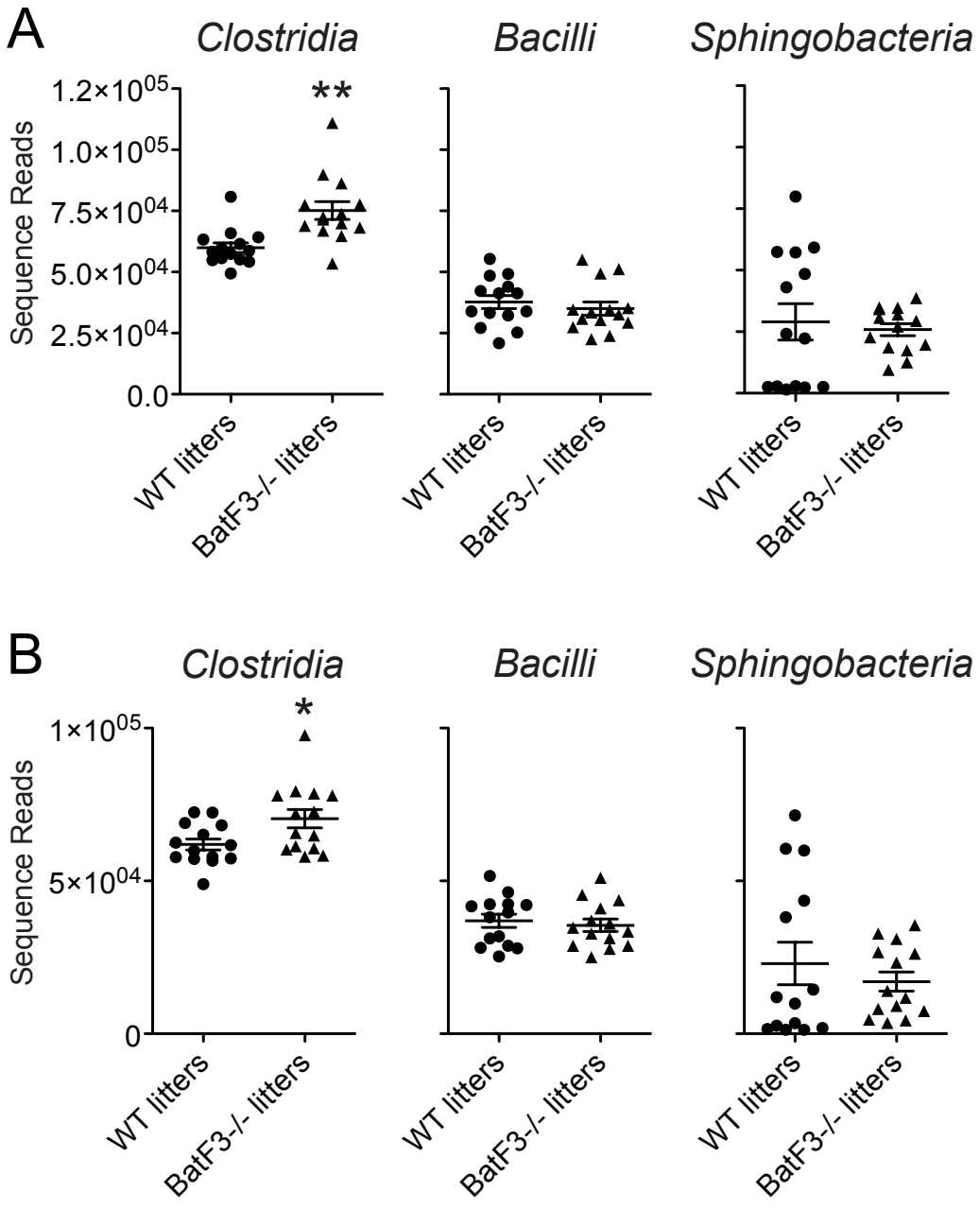


Figure 4-5: Increased* Clostridia *drive altered commensalism in BatF3$^{-/-}$ mice

The frequency of sequence reads from the 3 most abundant taxonomic classes obtained from (A) cecal and (B) colonic samples. For this analysis, wild type and huLangerin-DTA littermates were pooled (circles) and compared to BatF3$^{-/-}$ and DTAxBatF3$^{-/-}$ littermates (triangles).

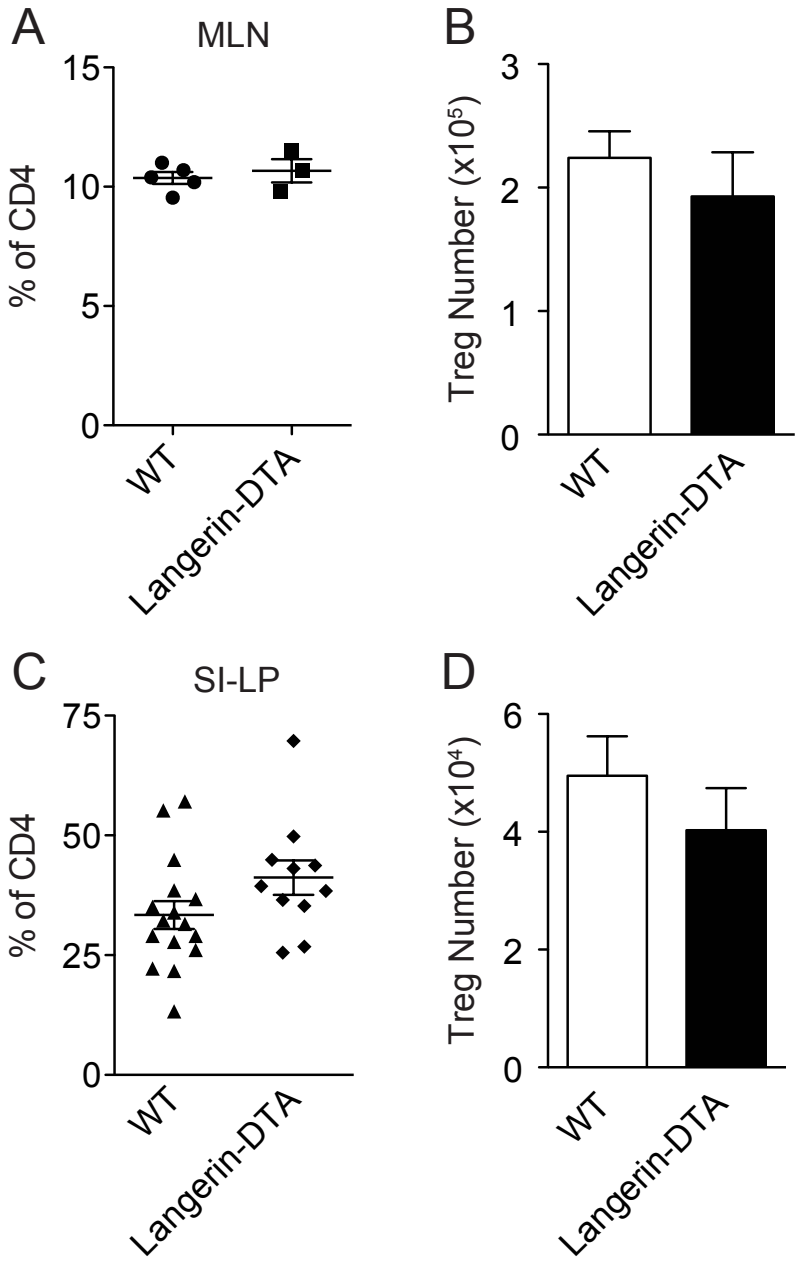


Figure 4-6: CD103⁺ CD11b⁺ DC are not required for FoxP3⁺ regulatory T cells

(A-D) Cells were isolated from (A,B) MLN or (C,D) small intestinal lamina propria and T reg cells were detected by intracellular staining for FoxP3 directly ex vivo. Data in (A) are representative data from one of >5 experiments. Data in (B-D) are presented as mean ± SEM and pooled from five independent experiments (n = 8–10 mice per group).

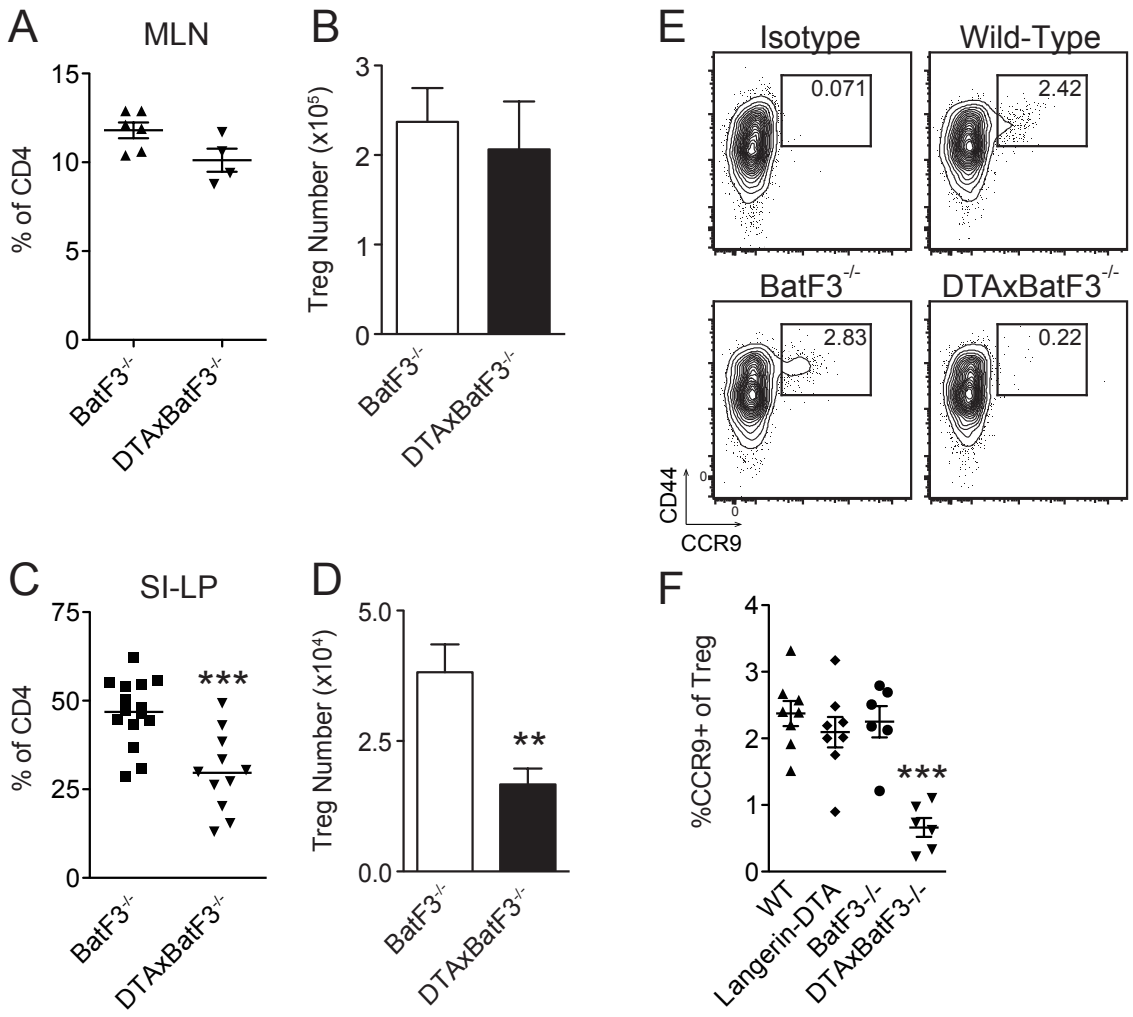


Figure 4-7: CD103^{+} DCs are required for LP Treg cells

(A-D) Cell suspensions from the indicated tissues were stained ex vivo for intracellular FoxP3 and enumerated by flow cytometry (n = 11–14 mice per group in B-D). (E) Representative flow plots of MLNs gated on CD3ε+ CD8α+ CD4+ FoxP3+ cells and stained with isotype control (top left, wild-type mouse) or anti-CCR9 antibody (indicated genotypes). (F) Frequency of CCR9+ MLN T reg cells in mice of indicated genotype. All data are presented as mean ± SEM and symbols represent individual mice. Animals are pooled from three different experiments. Statistics were calculated by unpaired Student's t tests, using Welch's correction in D. **p≤ 0.01; ***p≤ 0.001

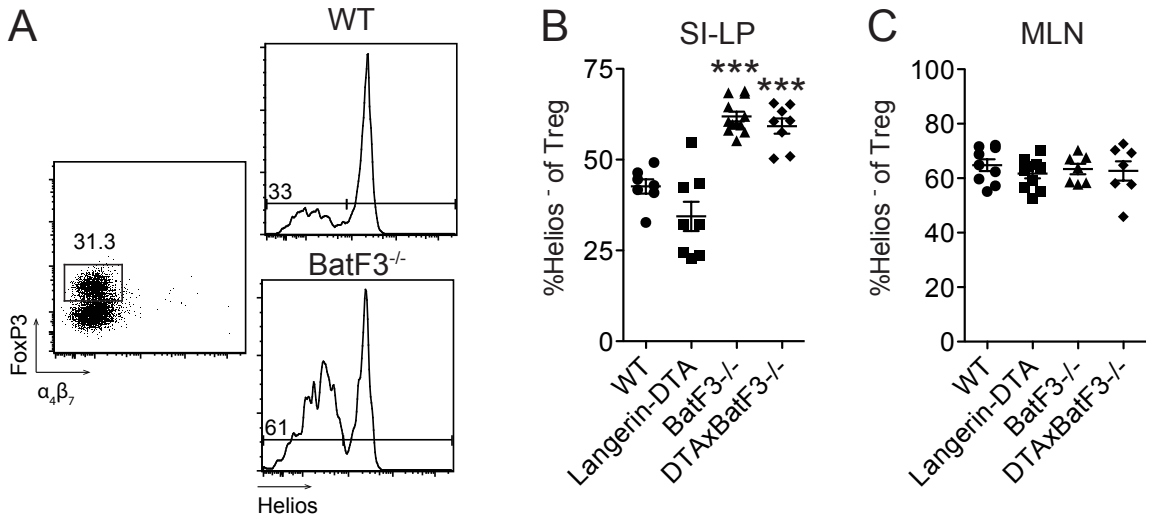


Figure 4-8: Altered regulatory T cell phenotype in BatF3$^{-/-}$ mice

(A) Gating strategy for FoxP3^{+} LP Tregs and Helios expression. (B and C) Frequency of Helios^{-} Treg in (B) small intestinal LP and (C) MLN of indicated genotypes. ***p<0.001

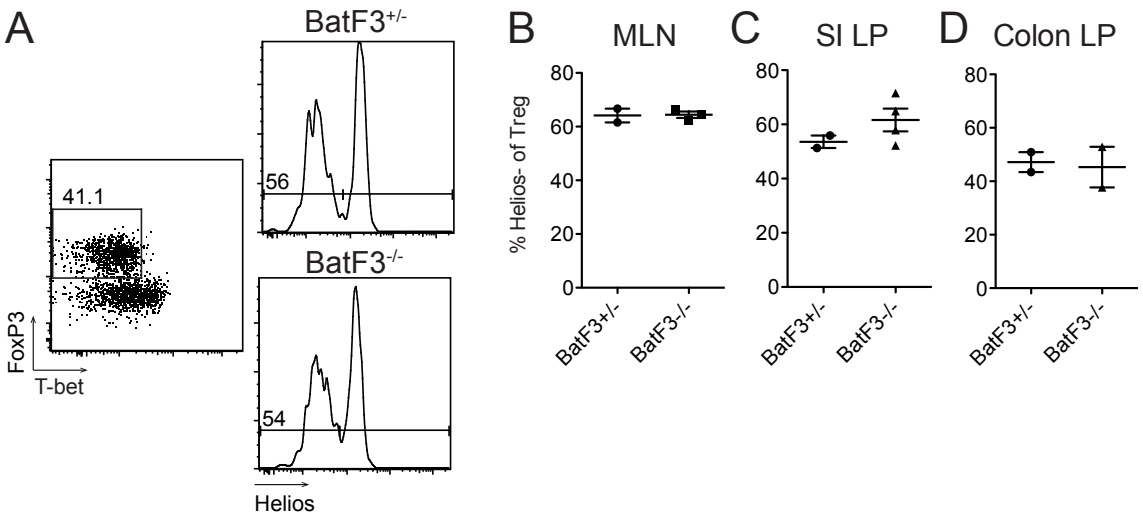


Figure 4-9: Altered regulatory T cell phenotype in BatF3$^{-/-}$ animals is due to vertical transmission of commensal microbial flora

(A) Gating strategy and frequency of Helios^{-} Treg in (B) MLN, (C), small intestinal LP, and (D) colonic LP of BatF3$^{+/-}$ and BatF3$^{-/-}$ littermates.

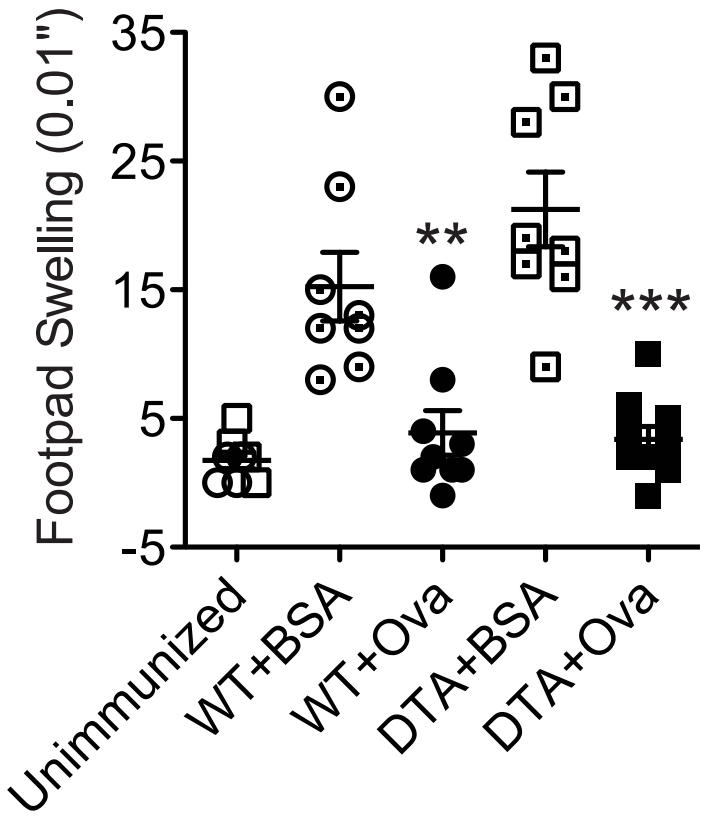


Figure 4-10: CD103^{+} CD11b^{+} DC are not required for oral tolerance

Footpad swelling of mice tolerized with either oral ovalbumin (Ova) or bovine serum albumin (BSA) as indicated prior to s.c. immunization with Ova/CFA. Footpad swelling was assessed in unmanipulated (unimmunized) or treated mice two days after injection of Ova/PBS in the right hind paw and PBS alone in the left hind paw. Background swelling in the left paw was subtracted from that in the right paw. P values represent unpaired student's T tests of mice of the same genotype tolerized with BSA versus Ova. **p≤ 0.01; ***p≤ 0.001

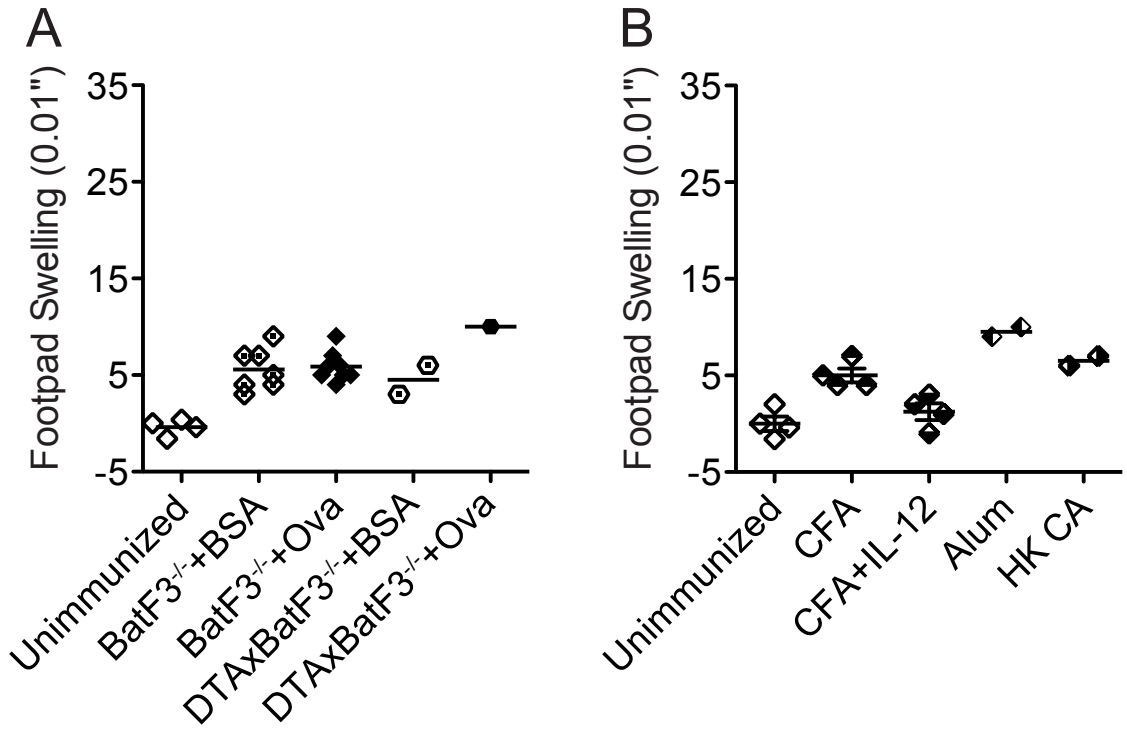


Figure 4-11: BatF3$^{-/-}$ mice do not develop delayed-type hypersensitivity responses

(A) Footpad swelling of mice tolerized with oral Ova or BSA as indicated prior to s.c. immunization with Ova/CFA and challenge with Ova/PBS as in Figure 4-5. (B) BatF3$^{-/-}$ mice immunized s.c. with Ova and the indicated adjuvant combinations.

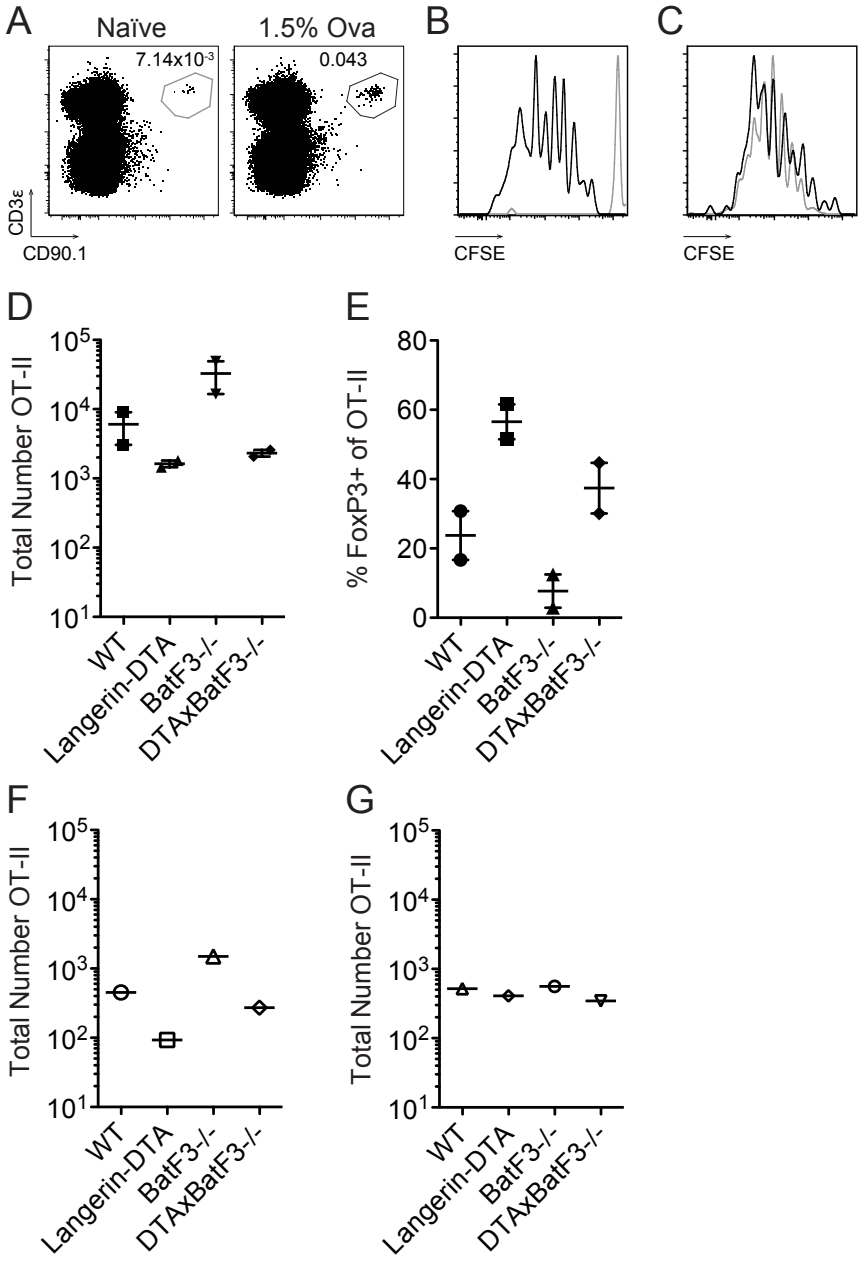
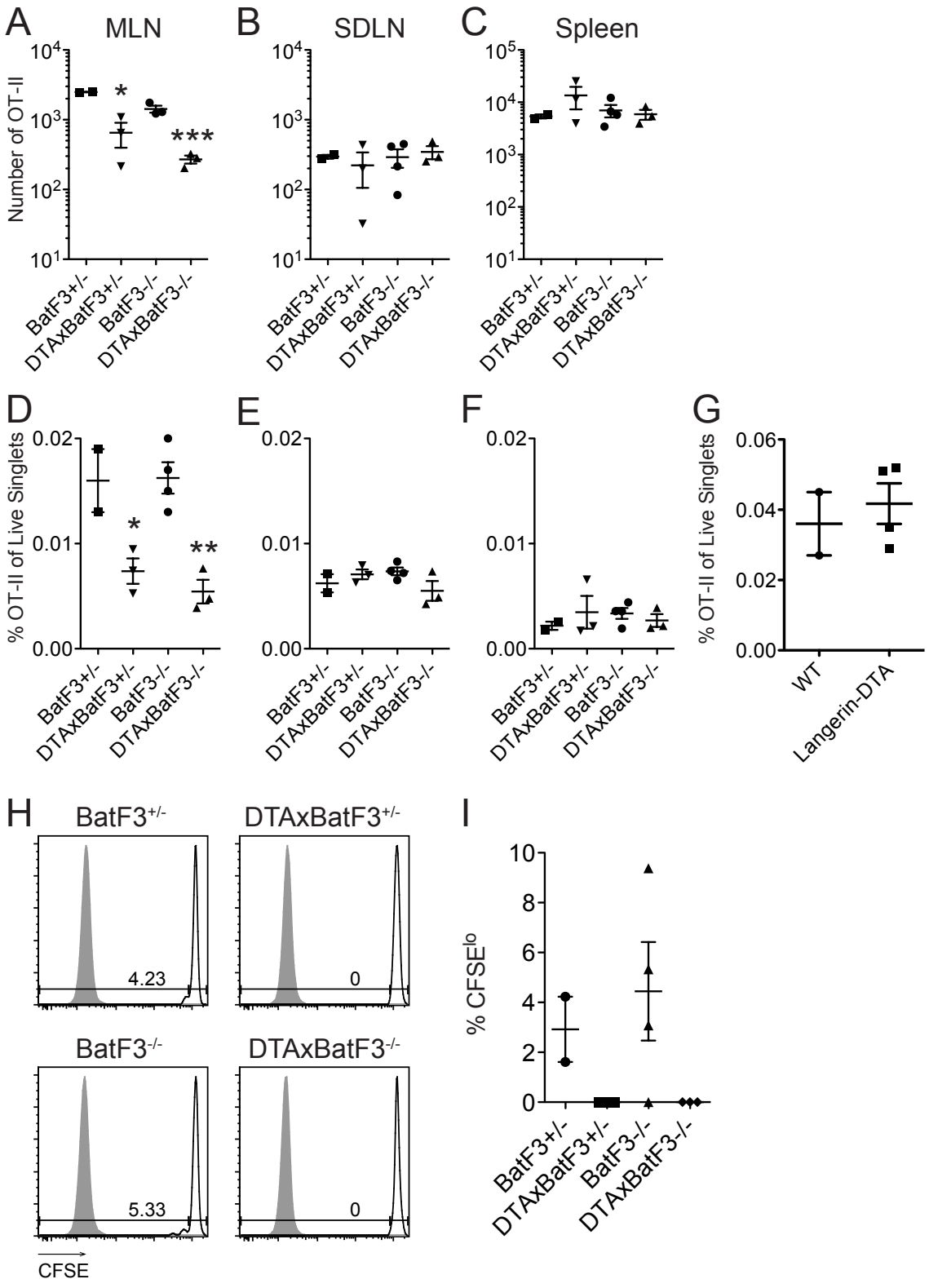


Figure 4-12: huLangerin-DTA mice have reduced responses to oral Ova

(A) Flow plots of MLNs from recipient mice left untreated (naïve) or given 1.5% Ova in the drinking water for 5 days after i.v. transfer of transgenic OT-II cells. (B) CFSE profile of OT-II cells in naïve (grey) or Ova treated (black) mice. (C) CFSE profile of ova treated BatF3$^{-/-}$ (grey) and DTAxBatF3$^{-/-}$ (black) mice. (D) Number of OT-II in treated mice of the indicated genotypes. (E) Frequency of FoxP3^{+} OT-II. (F-G) Number of OT-II in (F) MLNs or (G) SLDNs of untreated mice.


A
MLN
B
SDLN
C
Spleen
Number of OT-II
BatF3+/-
DTAxBatF3+/-
BatF3-/-
DTAxBatF3-/-
D
E
F
G
% OT-II of Live Singlets
WT
Langerin-DTA
H
BatF3+/-
DTAxBatF3+/-
BatF3-/-
DTAxBatF3-/-
4.23
0
5.33
0
CFSE
I
% CFSElo

(previous page)

Figure 4-13: CD103^{+} CD11b^{+} DC are required for transgenic T cell maintenance in MLNs in the absence of antigen

(A-C) Number and (D-F) frequency of OT-II cells isolated from indicated tissues five days after i.v. transfer of $1x10^{5}$ transgenic cells into mice of denoted genotypes. (G) Frequency of OT-II cells detected in the MLNs four hours after transfer. (H) Histograms showing the CFSE profile of transferred OT-II in the MLN of indicated mice five days after injection. (I) Percentage of OT-II designated CFSE^{lo} according to gating in (H). Data represent 1 of 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001

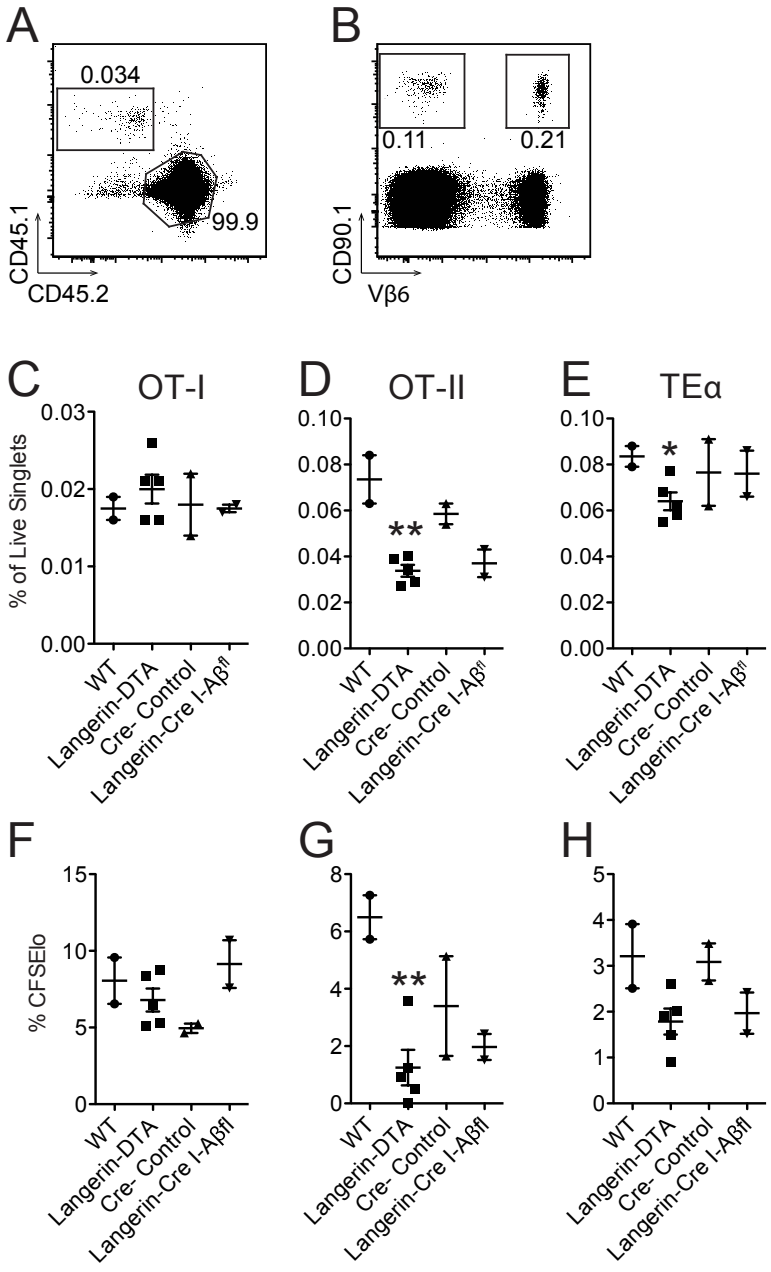


Figure 4-14: Reduced T cell maintenance varies based on TCR and is MHCII dependent

(A-B) Flow plots showing detection of adoptively transferred (A) OT-I or (B) OT-II ($CD90.1^+$ $V\beta6^-$) and TEα ($CD90.1^+$ $V\beta6^+$) transgenic cells in recipient mice. (C-E) Frequency of indicated transgenic T cell populations in the MLNs of recipients. (F-H) Percentage of designated transgenic T cells that were $CFSE^{lo}$ in the MLN. *p<0.05, **p<0.01, ***p<0.001

Chapter 5: Regulation of Th17 responses by CD103^{+} CD11b^{+} intestinal dendritic cells

Introduction

Th17 cells are a subset of CD4 helper T cells that are necessary for protection from extracellular bacterial and fungal infections but also play important pathogenic roles in autoiummune disease (Korn, Bettelli, Oukka, & Kuchroo, 2009). They produce the canonical cytokine IL-17A and require the master transcriptional regulator RORγt for their development, identifying them as a distinct lineage of T helper cells (Harrington et al., 2005; Ivanov et al., 2006; Langrish et al., 2005; Park et al., 2005). Although initially identified by their dependence on IL-23 (Cua et al., 2003; Langrish et al., 2005), Th17 cells were later shown to be induced by combinations of IL-6 and TGF-β1 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen, Hocking, Flavell, & Stockinger, 2006), which upregulate the IL-23 receptor to enforce the Th17 differentiation program (Zhou et al., 2007). TGF-β is secreted in an inactive form and requires activation by RGD binding integrins, including integrin $\alpha_v\beta8$ (Mu et al., 2002). Loss of these integrins also impairs Th17 development (Acharya et al., 2010; Melton et al., 2010). Th17 cells can also be generated by IL-21 in an IL-6 independent manner (Korn et al., 2007; Nurieva et al., 2007). Finally, IL-1β has also been shown to be critical for Th17 differentiation (Y. Chung et al., 2009; Hu, Troutman, Edukulla, & Pasare, 2011; Shaw, Kamada, Kim, & Nunez, 2012), although the requirement for IL-1 has been more controversial.

In the steady-state Th17 cells are rare in secondary lymphoid organs. However, a large population of Th17 cells can be found in intestinal lamina propria, most predominantly in the small bowel. Development of these cells requires the presence of certain commensal products and species in the lumen of the gut, in particular adenosine triphosphate (ATP) and segmented filamentous bacteria (SFB) (Atarashi et al., 2008; Ivanov et al., 2009; 2008). Understanding the mechanism whereby commensals promote LP Th17 responses is important because microbial colonization, and in particular colonization with SFB, can enhance autoimmune disease pathogenesis in several models (Chappert, Bouladoux, Naik, & Schwartz, 2013; Lee, Menezes, Umesaki, & Mazmanian, 2011; H.-J. Wu et al., 2010). ATP acts through P2X and P2Y receptors, which appear to be predominately expressed by CX_3CR1^+ APCs, to upregulate the production of several pro-Th17 mediators, including IL-6, IL-23, and integrins α_v and β8 (Atarashi et al., 2008). SFB induce production of serum amyloid A proteins in the gut, which can upregulate pro-Th17 cytokines like IL-6 and IL-23 on $CD11c^+$ cells in the lamina propria (Ivanov et al., 2009). Very recent studies have found that in fact the majority of LP Th17 cells are not only induced by the presence of SFB but are in fact specific for the bacteria (Goto et al., 2014; Y. Yang et al., 2014). However, although MHCII expression by $CD11c^+$ cells was required for SFB induced Th17 formation (Geem et al., 2014; Goto et al., 2014), the specific APC subset(s) required remain unclear.

Although initially thought to be required for regulatory T cell generation (Coombes et al., 2007; Sun et al., 2007), $CD103^+$ $CD11b^+$ intestinal DC have also been implicated in Th17 differentiation *in vitro* through their ability to produce IL-6 (Denning et al., 2011; Denning, Wang, Patel, Williams, & Pulendran, 2007; Fujimoto et al., 2011; Persson et al., 2013; Uematsu et al., 2008). Likewise, several models that target these cells *in vivo*, including CD11c-Cre $Notch2^{fl}$ mice and CD11c-Cre $IRF4^{fl}$ mice, show reduced frequencies of LP Th17 cells as well as impaired Th17 responses to systemic immunization and *Aspergillus fumigatus* infection in the lung (K. L. Lewis et al., 2011; Persson et al., 2013; Schlitzer et al., 2013). To investigate the *in vivo* requirements for this subset, we used DC-deficient mice, along with novel T cell transgenic and peptide-MHCII tetramer reagents specific for SFB. We found that $CD103^+$ $CD11b^+$ DCs were required for LP Th17 formation, but surprisingly they were not required to present SFB antigens. They were also not an obligate source of IL-6, IL-1β, or the TGF-β activating integrin β8. Together, our data suggested that LP DC subsets might have distinct functional roles in Th17 homeostasis, with $CD103^-$ $CD11b^+$ cells priming Th17 responses and $CD103^+$ $CD11b^+$ possibly controlling Th17 maintenance within the LP.

Materials and Methods

Mice

huLangerin-Cre $Itg\beta8^{fl}$ mice were generated by intercrossing huLangerin-Cre mice (Kaplan et al., 2007) with mice containing a floxed allele of the *ITGB8*

gene (Proctor, Zang, Wang, Wang, & Reichardt, 2005), a kind gift of Dean Sheppard (University of California-San Francisco, San Francisco, CA). IL-$6^{-/-}$ and IL-$1\beta^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed as described above, and the University of Minnesota Institutional Animal Care and Use Committee approved all animal protocols.

Segmented filamentous bacteria quantification and colonization

Fecal samples were collected as described above and genomic DNA was extracted using a Power-Soil DNA extraction kit (MoBio) in accordance with the manufacturer's instructions. Relative quantification of bacteria by real-time quantitative PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and primers targeting either mouse SFB or total 16S rDNA sequences as previously described (Barman et al., 2008). Specific primers used are listed in Table 5-1.

Colonization with SFB was performed either by cohousing with SFB^{+} donor mice (one donor mouse per cage of 4 recipients) for 3-4 weeks or by gavage of SFB^{+} fecal pellets. To ensure equal colonization by cohousing, donor mice were rotated between different cages and fecal material and bedding from each cage was distributed equally to others on a biweekly basis. For gavages, fresh fecal pellets were collected from live donors with sterile forceps. Pellets were weighed and then resuspended at 30 mg/mL in sterile PBS and passed through a 40 μm cell strainer. Mice were gavaged with 200 μL rehydrated fecal

material. SFB negative controls were gavaged with 200 µL autologous fecal material resuspended in PBS at 30 mg/mL.

In vitro restimulation and intracellular cytokine staining

For evaluating cytokine expression, cells were resuspended at $2.5x10^6$/mL in complete DMEM media with 10%FBS and stimulated for 3 h with PMA (50 ng/ml) and ionomycin (1.5 µM; Sigma-Aldrich) in the presence of brefeldin A for the final 2.5 h. Intracellular staining for IL-17A, IFNγ, IL-22, IL-10, and IL-13 was performed with Cytofix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer's instructions.

Cell sorting and quantitative PCR

LP cells from huLangerin-Cre YFP^{fl} were isolated and $CD11c^+$ $MHCII^+$ cells were sorted into $CD103^+$ $CD11b^-$, $CD103^+$ $CD11b^+$ YFP^+, and $CD103^-$ $CD11b^+$ subsets using a FACSAria cell sorter (BD). mRNA was extracted using an RNeasy mini kit (QIAGEN) according to the manufacturers instructions. RNA was reverse-transcribed using a high-capacity cDNA RT kit and analyzed via qPCR with TaqMan Gene Expression Assays and an ABI 7900HT (Applied Biosystems) as previously described (Haley et al., 2012). Data were normalized to Hprt expression using the comparative Ct method. Data are presented as $2^{-\Delta Ct}$.

T cell adoptive transfer

Three days after SFB colonization by gavage, congenically marked 7B8 transgenic T cells specific for SFB (Y. Yang et al., 2014) were sorted and CFSE

labeled. Cells were then counted and resuspended in sterile PBS. $5x10^4$ cells were transferred into recipients. Four days later, organs were harvested and 7B8 cells were identified by staining for CD45.1, CD45.2, and TCR Vβ14.

Tetramer pulldown and transcription factor staining

Previously described SFB-specific I-A^b T cell tetramers (Y. Yang et al., 2014) were a kind gift of M. Jenkins (University of Minnesota, Minneapolis, MN) and D. Littman (New York University, New York, NY). Detection of SFB-specific CD4 T cells was performed as previously described (Moon et al., 2009). In some experiments two different SFB tetramers (referred to as SFB2 and SFB3) were labeled with the same fluorochrome and used for detection together. In other experiments, single-cell suspensions isolated from secondary lymphoid tissues were simultaneously double-stained with phycoerythrin- and allophycocyanin-conjugated SFB3 tetramer and magnetically separated using a cocktail of anti-PE and anti-APC magnetic beads (Miltenyi Biotech). $CD3\varepsilon^+$ cells were gated away from a dump gate containing fixable viability dye, F4/80, CD11c, CD11b, and B220 antibodies conjugated to the same fluorochrome. Intracellular transcription factor staining was performed with a FoxP3 transcription factor staining buffer kit (eBioscience) and antibodies against RORγt (BD), T-bet, Gata-3 (BioLegend), and FoxP3 (eBioscience).

Mixed bone marrow chimeras

Six- to eight-week old sex-matched C57BL/6 or CD45.1 congenic mice were irradiated with a split-dose treatment of 1000 rads (500 rads per dose)

approximately 5 hours apart using an X irradiator. The next day, $5x10^6$ bone marrow cells isolated from specified transgenic donor mice were delivered intravenously. The mice were administered sulfamethoxazole in the drinking water for two weeks after irradiation and transplantation. Chimeric mice were placed into cohoused experimental cohorts with nonchimeric IL-$6^{-/-}$ controls during the antibiotic treatment period and were rested for at least one week after antibiotic withdrawal before any microbial colonization procedures were performed. Six to eight weeks after chimeras were generated, the efficiency of chimerism was assessed in PBMC by flow cytometry. Chimerism was also assessed at harvest in the DC subsets of interest by staining for CD45.1 and CD45.2.

Results

$CD103^+$ $CD11b^+$ DC are required for Th17 homeostasis

To determine whether the absence of $CD103^+$ $CD11b^+$ DCs affects Th17 development *in vivo*, we compared the numbers of CD4 T cell subsets in huLangerin-DTA and control mice. We found a nonsignificant trend toward fewer CD4 T cells in the small intestinal LP of huLangerin- DTA mice (Fig. 5-1A). To examine the requirement of $CD103^+$ $CD11b^+$ LP DCs for Th17 homeostasis, we compared the expression of IL-17A and IFNγ in PMA/ionomycin-stimulated cells isolated from the LP of huLangerin-DTA and littermate control mice. We observed that the frequency of CD4 T cells expressing IL-17 was greatly reduced

in huLangerin-DTA mice, whereas the expression of IFNγ was unaffected (Fig. 5-1B–D). As previously noted, CD4 T cells expressing IL-17 in the MLN were rare (not shown) (Atarashi et al., 2008).

We next examined the presence of Th17 and Th1 cells in the small intestinal LP of mice lacking both $CD103^+$ DC subsets. We compared $DTAxBatF3^{-/-}$ mice with littermate $BatF3^{-/-}$ controls to ensure that any observed changes were not caused by alterations in the gut microbiota. We observed reduced Th17 cells in $DTAxBatF3^{-/-}$ mice compared with $Batf3^{-/-}$ mice. However, Th1 cells in the LP were not affected (Fig. 5-1E-F). These data are quite similar to those obtained with huLangerin-DTA mice not bred to $Batf3^{-/-}$ animals. Because the additional deletion of $CD103^+$ $CD11b^-$ DCs did not alter the presence of Th17 or Th1 cells in the LP, these data demonstrate that $CD103^+$ $CD11b^+$ DCs are specifically required for steady-state Th17 homeostasis.

$CD103^+$ $CD11b^+$ affect Th17 homeostasis through an MHCII independent mechanism

Finally, we examined the LP of huLangerin-Cre I-$A\beta^{fl}$ mice. We first confirmed that $CD103^+$ $CD11b^+$ DCs from these mice lacked expression of MHCII (Fig. 5-2A). Surprisingly, despite the fact that >90% of $CD103^+$ $CD11b^+$ DCs lacked expression of MHC-II, these mice had a normal frequency of Th17 cells in the LP compared with control littermates (Fig. 5-2B-C). Thus, Th17 development in the LP depends on the presence of $CD103^+$ $CD11b^+$ DCs but does not require their cognate interaction with CD4 T cells.

Breeding within our mouse colony is tightly controlled, and animals are only intercrossed with other animals from the same line to regenerate breeding pairs. We noted that all animals maintained in-house in this manner had a relatively low frequency of LP Th17 cells. However, one line that had been generated by crossing in-house mice with a strain obtained from an outside institution had a high frequency of LP Th17 cells. Given the importance of intestinal commensals, particularly segmented filamentous bacteria (SFB), in generating LP Th17 cells (Atarashi et al., 2008; Ivanov et al., 2008; 2009), we tested these mice for the presence of SFB by qPCR. As expected, fecal pellets obtained from mice freshly shipped from Jackson Laboratories, which do not contain SFB in their flora (Ivanov et al., 2009), had a very low abundance of SFB 16S sequences relative to total eubacteria. As a positive control, we obtained fecal material from IL-23R$^{-/-}$ Rag2$^{-/-}$ mice, which have an overgrowth of SFB (Y. Yang et al., 2014); these mice had a high abundance of SFB by qPCR. We next tested mouse lines from our colony. As expected, we found that in-house lines in our colony had no detectable increase in SFB compared to mice obtained from Jackson Laboratories. In contrast, the line outcrossed with mice from another institution had a ~3 log increase in SFB abundance (Fig. 5-3A).

Horizontal transfer of SFB does not take place between SPF animals when housed separately but readily occurs upon cohousing SFB negative mice with SFB positive donors in the same cage (Ivanov et al., 2008; 2009). In order to investigate the requirement for CD103^{+} CD11b^{+} DC in the presence of SFB, we

cohoused wild type, huLangerin-DTA, huLangerin-Cre I-Aβ^{fl} mice with SFB positive donors in the same cage. We observed a marked increase in the frequency of LP Th17 cells of cohoused wild-type mice compared to animals normally maintained in our colony. However, as we previously observed, huLangerin-DTA mice that lacked CD103^{+} CD11b^{+} DCs had a reduced frequency of LP Th17 cells. This decrease was not dependent on MHCII expression as huLangerin-Cre I-Aβ^{fl} mice had a similar frequency of LP Th17 cells as wild-type controls (Fig. 5-3 B-C). We also investigated SFB-specific T cells in the MLN of these mice using recently described peptide-MHCII tetramers (Y. Yang et al., 2014). For this study, tetramers containing two different SFB epitopes but labeled with the same fluorochrome were used to identify SFB specific cells in the MLN. In parallel with our observations of bulk LP Th17 cells, we found a decrease in RORγt^{+} SFB-specific cells that was dependent on the presence of CD103^{+} CD11b^{+} DCs, but not on their expression of MHCII (Fig. 5-4).

CD103^{+} CD11b^{+} DC are not required to present SFB antigens

We next investigated the proliferation of SFB-specific cells to determine whether CD103^{+} CD11b^{+} DCs were required for antigen uptake or presentation. 7B8 cells are an SFB-specific TCR transgenic CD4 T cell recently generated by TCR hybridoma screening (Y. Yang et al., 2014). We adoptively transferred 5x10^{4} CFSE-labeled 7B8tg cells into mice colonized by gavage of autologous (SFB neg) or SFB^{+} donor material. Four days later, we observed no proliferation of transferred cells in the MLN of mice colonized by gavage of autologous fecal

material, confirming that our colony does not contain SFB. In contrast, there was robust proliferation of 7B8tg cells in mice gavaged with SFB^+ donor material, as demonstrated both by their dilution of CFSE and by expanded numbers of these cells in the MLN (Fig. 5-5A-B). We next examined mice lacking one or both $CD103^+$ DC subsets to determine if either of these was required to present SFB antigen. We found no difference either in the CFSE profile or expansion of 7B8tg cells in huLangerin-DTA, $BatF3^{-/-}$, or $DTAxBatF3^{-/-}$ mice, demonstrating that $CD103^+$ DCs are dispensable for SFB antigen presentation (Fig. 5-5A-D).

We also examined RORγt expression on adoptively transferred 7B8tg cells. Surprisingly, contrasting with our findings in endogenous SFB-specific cells, we found that expression of RORγt was not reduced on 7B8tg cells in the MLN of huLangerin-DTA (not shown) or $DTAxBatF3^{-/-}$ mice, although there was a significant reduction in Th17 differentiation in $IL\text{-}6^{-/-}$ hosts (Fig. 5-5E-F). Because 7B8tg experiments were performed in mice colonized by gavage of SFB^+ donor material rather than by cohousing, we next investigated endogenous SFB specific cells in animals that had been colonized by gavage. Two weeks after gavage of SFB^+ donor feces, we found that huLangerin-DTA mice had no reduction in the frequency of $ROR\gamma t^+$ SFB-specific cells in the MLN (Fig. 5-6A) or in the frequency of LP Th17 cells (Fig. 5-6B). We also confirmed that gavage of SFB^+ fecal material did not induce reconstitution of the $CD103^+$ $CD11b^+$ DC compartment by recruitment of other inflammatory myeloid cells (Fig. 5-6C).

Thus, colonization of mice by gavage of SFB^+ fecal material appears to override the requirement for $CD103^+$ $CD11b^+$ DCs.

$CD103^+$ $CD11b^+$ DC influence Th17 responses independently of integrin $\alpha_v\beta8$, IL-6, and IL-1β

To further investigate the mechanism whereby $CD103^+$ $CD11b^+$ DC control Th17 homeostasis, we examined the production of Th17 associated cytokines by these cells. We found that, compared to $CD103^+$ $CD11b^-$ DCs, $CD103^+$ $CD11b^+$ DCs expressed higher levels of a number of factors associated with Th17 differentiation, including IL-23 p19, IL-6, IL-1β, TGF-β1, and integrin β8, which activates latent TGF-β1 (Mu et al., 2002). Interestingly, this expression profile was largely shared by $CD103^-$ $CD11b^+$ APCs, with the exception of the low levels of integrin β8 detect in $CD103^+$ $CD11b^+$ DCs but not in $CD103^-$ APCs (Fig. 5-7).

Because gavage of SFB antigens overrode the requirement for $CD103^+$ $CD11b^+$ DCs, we tested the requirement for these cytokines in the setting of cohousing with SFB^+ donor mice. To test whether integrin β8 expression by $CD103^+$ $CD11b^+$ DCs was required for Th17 homeostasis, we generated huLangerin-Cre $Itg\beta8^{fl}$ mice in which $CD103^+$ $CD11b^+$ DCs are present, but lack expression of β8. Surprisingly, we found an increase in the number of endogenous SFB-specific cells we were able to detect in the MLN of these mice; however there was no defect in RORγt expression in this population (Fig. 5-8).

Thus, integrin β8 expression by $CD103^+$ $CD11b^+$ DCs is not required for Th17 generation to SFB antigens.

In order to test the requirements for other cytokines produced by $CD103^+$ $CD11b^+$ DCs, we used mixed bone marrow chimeras to generate animals with subset-specific cytokine knockouts[1]. Irradiated congenically marked wild type recipients received bone marrow from either wild type or huLangerin-DTA mice alone or mixed 1:1 with bone marrow from cytokine knockout mice. As expected, animals reconstituted with huLangerin-DTA bone marrow alone completely lacked $CD103^+$ $CD11b^+$ LP DCs, while those reconstituted with huLangerin-DTA bone marrow mixed with either IL-$6^{-/-}$ or IL-$1\beta^{-/-}$ bone marrow had a normal $CD103^+$ $CD11b^+$ DC compartment derived from the cytokine knockout source (Fig. 5-9A).

Because animals were cohoused for several weeks with SFB^+ donors in these experiments, we pooled spleen and MLN cells in order to detect those T cells that might have responded to SFB in the GALT but then entered into the circulation. To increase our sensitivity of detection, we also used a previously described strategy (Tubo et al., 2013) whereby cells were double-stained with a single SFB peptide-MHCII tetramer (hereafter referred to as SFB3) separately conjugated to two different fluorochromes. Thus, when cells were gated away from non-T cells and CD8 T cells as previously described (Fig. 5-9B) (Moon et al., 2009), an expanded population of SFB-specific cells positive for both

tetramer reagents could be identified in cohoused mice, along with a much smaller naïve population in non-cohoused controls (Fig. 5-9C).

Using this method, we examined the expansion of SFB-specific cells in response to cohousing. While non-cohoused, nonchimeric control (naïve) mice had a total number of SFB specific cells that was close to the limit of detection, we noted a significant ($p<0.05$ by student's T-test with Welch's correction for unequal variance) increase in the number of cells for each group over naïve animals. However, there were no significant differences in expansion between various cohoused groups. We also noted that the number of SFB specific cells varied markedly by ~1-1.5 logs within groups (Fig. 5-9D). This extreme variation did not appear to be a result of the cohousing strategy as even genetically identical nonchimeric littermates from the SFB^+ donor line that were colonized from birth showed striking differences in the number of SFB-specific cells (Fig. 5-9E).

We next investigated RORγt expression among SFB-specific cells. While we observed no RORγt expression in SFB-specific cells from naïve animals, we again observed a high degree of individual variation in cohoused mice. Surprisingly, this was even true of $IL\text{-}6^{-/-}$ controls cohoused in the same experiments, several of which had very high frequencies of RORγt expressing cells (Fig. 5-9F). To investigate whether differences in expansion might be related to the variability in RORγt induction, we pooled all groups and plotted the total number of SFB specific cells versus their expression of RORγt. We

observed a strong positive correlation between these variables (Fig. 5-9G). Thus, although loss of $CD103^+$ $CD11b^+$ DC or cytokines produced by this subset does not impact the average expansion of SFB-specific cells, in line with our previous findings, there is a high degree of inter-individual variability in the CD4 T cell response to this commensal epitope.

Finally we tested the role of $CD103^+$ $CD11b^+$ DC derived cytokines in LP Th17 homeostasis. Similar to the differences we observed in nonchimeric wild type and huLangerin-DTA animals, we found a significant, though not complete, reduction in the frequency of IL-$17A^+$ cells in cohoused huLangerin-DTA chimeras compared to controls (Fig. 5-10A and C). We observed a similar reduction in DTA+IL-$6^{-/-}$ chimeras. However, to control for the fact that half of all hematopoietic cells in these chimeras were also deficient for IL-6, we generated WT+IL-$6^{-/-}$ chimeras. In these control chimeras, although $CD103^+$ $CD11b^+$ DCs were intact, we observed a similarly reduced frequency of IL-$17A^+$ cells (Fig. 5-10B and D). In line with this observation IL-$6^{-/-}$ mice had a much more profound reduction in IL-17A production by LP CD4 T cells (Fig. 5-10D). Thus, a hematopoietic source of IL-6 is required for LP Th17 cells, but reduced production of this cytokine cannot explain the requirement for $CD103^+$ $CD11b^+$ LP DC in Th17 homeostasis.

In contrast to the reduced LP Th17 responses observed in DTA+IL-$6^{-/-}$ mice, DTA+IL-$1\beta^{-/-}$ chimeras did not show a significant decrease in IL-$17A^+$ CD4 T cells (Fig. 5-10D). To confirm that these findings were not simply an artifact

resulting from *in vitro* stimulation conditions, we also examined RORγt expression in LP CD4 T cells directly *ex vivo*. Although the frequency of RORγt expressing cells was higher in all instances than the frequency of CD4 T cells producing IL-17A, we observed the same pattern of transcription factor expression as we did for cytokine production (Fig. 5-10E-H). Thus, while $CD103^+$ $CD11b^+$ DCs are required for LP Th17 homeostasis in SFB colonized animals, they are not an obligate source of IL-6 or IL-1β.

$CD103^+$ $CD11b^+$ DC do not affect ILC MHCII expression

A recent report has suggested that, even in the absence of SFB colonization, expression of MHCII by group 3 innate lymphoid cells (ILC3) prevents LP Th17 generation (Goto et al., 2014). To determine if $CD103^+$ $CD11b^+$ DCs influence Th17 responses through such a mechanism, we gated on $CD11c^-$ $B220^{lo/-}$ $Thy1.2^+$ $CD3\varepsilon^-$ LP cells to identify ILC subsets by transcription factor staining (Fig. 5-11A). As previously reported (Hepworth et al., 2013), we observed MHCII expression most notably in T-bet^- ILC3 and to a much lesser extent in ILC2 and T-bet^+ ILC3 compared to $CD3\varepsilon^+$ $Thy1.2^+$ LP T cells. However, there were not marked differences in MHCII expression between wild type (Fig. 5-11B) and huLangerin-DTA (Fig. 5-11C) chimeric mice for any ILC subsets, suggesting that $CD103^+$ $CD11b^+$ LP DCs regulate Th17 responses independently of ILCs.

Discussion

LP Th17 cells contribute to autoimmune disease in humans and in animal models (Honda & Littman, 2012; Korn et al., 2009). Given this pathogenic potential, it is important to understand the mechanisms promoting LP Th17 differentiation. In agreement with other models that target $CD103^+$ $CD11b^+$ DCs (K. L. Lewis et al., 2011; Persson et al., 2013; Schlitzer et al., 2013), we observed that the frequency of Th17 cells in the LP was greatly reduced in the absence of $CD103^+$ $CD11b^+$ DCs. Other LP-resident T cell subsets were unaffected. Specific commensal species and bacterial products, namely segmented filamentous bacteria and luminal adenosine triphosphate (ATP) are sufficient to generate Th17 cells in the LP (Atarashi et al., 2008; Ivanov et al., 2009). Thus, the reduction in Th17 cells could potentially result from a decrease in the overall number of DCs presenting commensal antigens. Notably, Th17 development was not affected in $Batf3^{-/-}$ mice that lack $CD103^+$ $CD11b^-$ DCs, and $DTAxBatF3^{-/-}$ mice had a reduction of Th17 cells similar to that observed in huLangerin-DTA mice. Thus, $CD103^+$ $CD11b^+$ DCs have a nonredundant role in LP Th17 development that is independent of $CD103^+$ $CD11b^-$ DCs.

Segmented filamentous bacteria are the only known commensal organism to induce the development of LP Th17 cells (Ivanov et al., 2009). Very recent studies have shown that a majority of LP Th17 cells in SFB colonized hosts are specific for SFB derived antigens (Goto et al., 2014; Y. Yang et al., 2014). Moreover, MHCII expression by $CD11c^+$ cells is required for the induction of LP

Th17 cells by SFB (Geem et al., 2014; Goto et al., 2014). However, the specific DC subsets required to present antigen have not been defined. Remarkably, although $CD103^+$ $CD11b^+$ DCs were required for Th17 homeostasis even in SFB colonized mice, Th17 differentiation was intact in huLangerin-Cre I-$A\beta^{fl}$ mice regardless of SFB status. We further found using adoptive transfer of SFB-specific transgenic T cells that neither $CD103^+$ DC subset was required to present SFB antigens, suggesting that $CD103^-$ macrophages and/or DCs may be responsible for the initial priming and differentiation of SFB-specific cells. These data suggested that $CD103^+$ $CD11b^+$ DCs, though not required for cognate antigen presentation, might rather be required to establish the appropriate cytokine milieu for T cell differentiation, as has been reported to occur during infection (Perona-Wright, Mohrs, & Mohrs, 2010).

The cytokines required for Th17 differentiation in the LP have been somewhat controversial. SFB and luminal ATP both increase expression of a number of pro-Th17 mediators in the gut, including IL-6, IL-23, integrin α_v, and integrin β8 (Atarashi et al., 2008; Ivanov et al., 2009). Studies in cytokine knockout mice have established requirements for IL-6 (Ivanov et al., 2006), TGF-β1 (Ivanov et al., 2008), and integrin $\alpha_v\beta8$ (Melton et al., 2010), in LP Th17 differentiation, but not IL-21 or IL-23 (Ivanov et al., 2008) despite requirements for these cytokines in *in vitro* models of Th17 differentiation (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). The role of IL-1β is more controversial. $MyD88^{-/-}$ mice, which lack IL-1 receptor signaling, have a normal frequency of LP

Th17 cells in the steady-state in two studies (Atarashi et al., 2008; Ivanov et al., 2008). However, a third study found a that both IL-1R$^{-/-}$ and MyD88$^{-/-}$ mice, but not IL-6$^{-/-}$ mice, had reduced steady-state LP Th17 formation (Shaw et al., 2012). A fourth study of Th17 differentiation in an oral infection model found requirements for both IL-1 receptor signaling and IL-6 (Hu et al., 2011). The reasons for these discrepancies are not clear but could result from differences in the microbiota or the methods used to detect LP Th17 cells.

We found that CD103^{+} CD11b^{+} LP DCs expressed higher levels of many Th17 associated cytokines than CD103^{+} CD11b^{-} DCs. To test the requirement for production of these cytokines from CD103^{+} CD11b^{+} DCs, we used Cre-lox technology and mixed bone marrow chimeras to create subset-specific knockouts for integrin β8, IL-6, and IL-1β. We found no role for integrin β8 or IL-1β in Th17 homeostasis of SFB colonized animals. While we did observe a reduction in LP Th17 cells in huLangerin-DTA+IL-6$^{-/-}$ mixed bone marrow chimeras, this was not specific to the deletion of CD103^{+} CD11b^{+} but was rather consistent with an overall reduction in hematopoietic sources of IL-6. Thus, while we have confirmed previous studies showing the importance of IL-6 in Th17 development, we have not identified a cytokine-dependent requirement for CD103^{+} CD11b^{+} DCs. A recent report in CD11c-Cre IRF4^{fl} mice, which also have a reduced number of CD103^{+} CD11b^{+} DCs, found that this subset was required for Th17 priming in the MLN through an IL-6 dependent mechanism (Persson et al., 2013). However, IRF4 has been implicated not only in DC subset

development but also in the migration of DCs to draining lymph nodes (Bajana et al., 2012). There was evidence that loss of this transcription factor in $CD11c^+$ cells also reduced the numbers of $CD103^-$ cells in the MLN. This study also used a systemic immunization strategy with Ova, anti-CD40 antibody, and LPS, and demonstrated the role of IL-6 only *in vitro*. It is thus unclear to what extent these findings reflect the steady-state Th17 response to intestinal commensals.

Notably, we have not yet investigated the role of TGF-β1 production by $CD103^+$ $CD11b^+$ DCs. The LP Th17 defect in mice that express only an inactive form of TGF-β appears to be more profound than that observed in huLangerin-DTA mice (Ivanov et al., 2008). Additionally, Th17 cells have been reported to rely on an autocrine source of TGF-β1 in an EAE model (Gutcher et al., 2011). It is nonetheless possible that LP DCs represent an obligate steady-state source of TGF-β. A second cytokine we have not yet investigated is IL-23. While IL-23 is not absolutely required for LP Th17 cells, IL-23 $p19^{-/-}$ mice do have a modest reduction in LP Th17 frequency that is mostly due to the loss of IL-17A and IL-22 double-producing cells (Ivanov et al., 2008). This bears some similarity to the defect observed in huLangerin-DTA mice, which have a reduced frequency of LP Th17 cells but not a complete loss of this lineage. However, we did not observe a specific reduction in Th17 cells that also produce IL-22 in huLangerin-DTA mice (not shown).

Interestingly, the cytokine profile of $CD103^+$ $CD11b^+$ DCs was mirrored by that of $CD103^-$ $CD11b^+$ APCs. Given that $CD103^-$ cells appear to be sufficient to

present SFB antigens, it is possible that $CD103^+$ $CD11b^+$ DCs are instead required for the maintenance of LP Th17 cells rather than priming an SFB-specific Th17 response. Such a mechanism could be consistent with the fact that $CD103^+$ $CD11b^+$ DCs appear to be dispensable for LP Th17 cells after a bolus delivery of SFB by fecal gavage. Investigating the kinetics of the Th17 response to gavage versus natural acquisition of SFB through cohousing might yield key insights into the true function of $CD103^+$ $CD11b^+$ DCs. Likewise, the specificity of the responding cells in the LP should be better defined, as some studies have reported Th17 differentiation of bystander cells not specific for SFB in SFB colonized hosts (Geem et al., 2014; Lochner et al., 2011).

Finally, although it did not yield significant insight into the cytokine requirements for Th17 generation, we noted a high degree of inter-individual variability when investigating an epitope-specific response to SFB colonization using a sensitive tetramer-based detection method. This variability did not appear to merely be a result of the chimera or cohousing protocols used for these experiments as nonchimeric littermates colonized with SFB from birth also displayed marked differences in the magnitude of the SFB-specific response. Additionally, this variability occurred in marked contrast to the relative reproducibility of Th17 responses in the bulk LP CD4 T cell population of the same mice.

In parallel with the variability in the expansion of SFB specific cells, we also observed a high degree of variation in the differentiation of those cells to the

Th17 lineage. These two variables were strongly positively correlated. This data stands in contrast to data from SFB-specific transgenic T cells, which showed highly reproducible response patterns when transferred into SFB gavaged mice (Y. Yang et al., 2014). Together, these findings are strongly reminiscent of a recent report that individual CD4 T cell clones show markedly stochastic patterns of both expansion and differentiation in response to infection, yet reproducibly generate a characteristic immune response when these patterns are averaged together (Tubo et al., 2013). Indeed, the size of the SFB-specific population found in naïve animals was near the limit of detection. Thus, the SFB-specific response detected in these experiments might be due to the expansion of only one or possibly a few naïve CD4 T cell clones. The priming stimulus in these experiments is also arguably quite weak, which might contribute to the inter-individual variability observed. Intriguingly, even IL-$6^{-/-}$ mice, which should be relatively genetically resistant to generating Th17 cells, showed a high degree of SFB-specific Th17 differentiation in some individuals. This might suggest that individual TCRs have the capacity to override even quite strong environmental determinants of T helper lineage commitment. Such a finding would raise important implications for the generation of commensal-specific adaptive immunity, where similar stochastic responses might underlie individual variability in human susceptibility to diseases like Crohn's and ulcerative colitis.

Footnotes

Portions of this work have been previously published. Reprinted from *The Journal of Experimental Medicine* volume 210, Nathan E Welty, Christopher Staley, Nico Ghilardi, Michael J. Sadowsky, Botond Z. Igyártó, and Daniel H. Kaplan. Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism, pp. 2011-2024. Copyright 2013, Welty et al.

[1] Bone marrow chimeras were generated by S. Kashem.

Group	Primer name	Primer Sequence (5'-3')
Eubacteria (all bacteria)	UniF340	ACTCCTACGGGAGGCAGCAGT
	UniR514	ATTACCGCGGCTGCTGGC
Segmented filamentous bacteria	SFB736F	GACGCTGAGGCATGAGAGCAT
	SFB844R	GACGGCACGGATTGTTATTCA

Table 5-1: 16S primer sequences for detection of SFB

Sense and anti-sense primer sequences used to detect colonization with SFB by real-time qPCR (Barman et al., 2008).

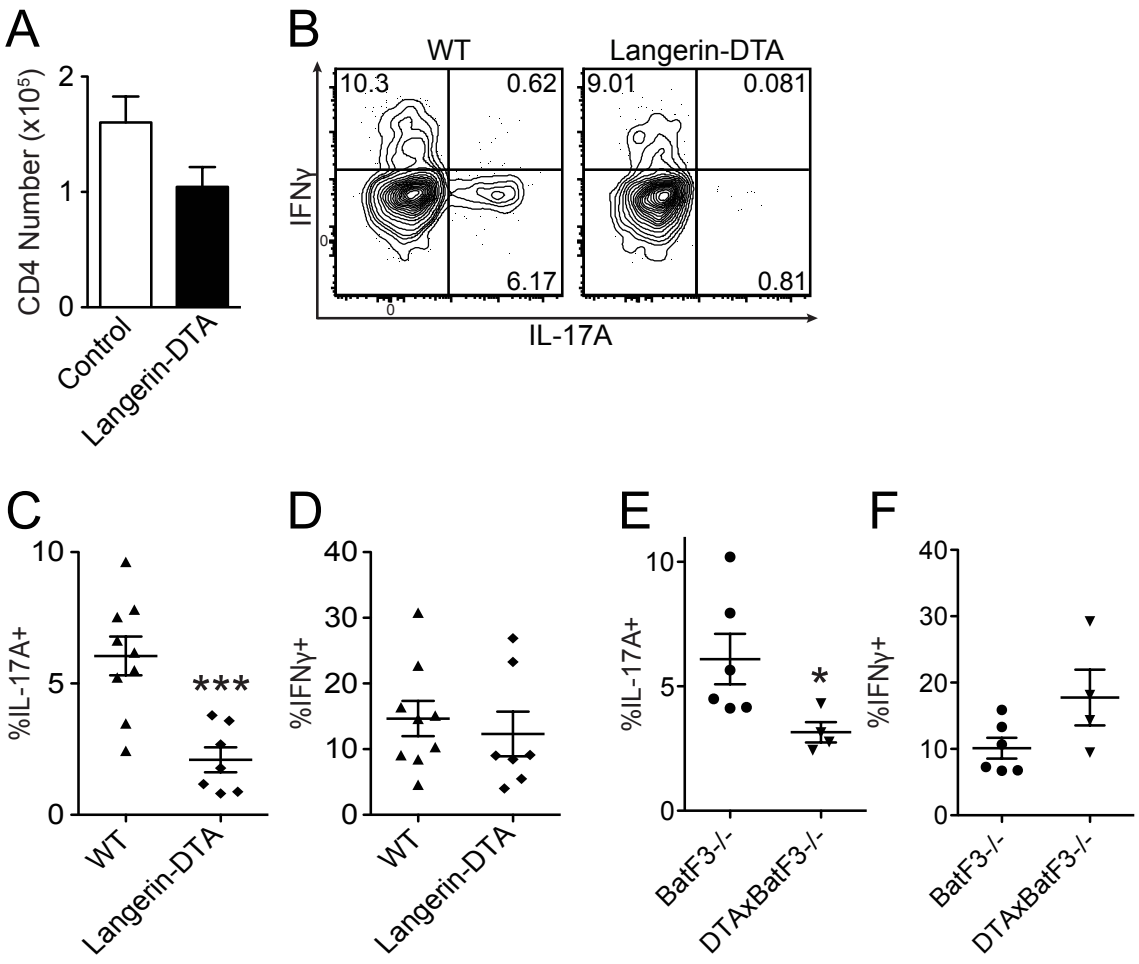


Figure 5-1: CD103$^+$ CD11b$^+$ DC are non-redundant for LP Th17 homeostasis

(A) CD4 cells gated as CD3ε+ TCRγδ- CD8α- CD4+ were isolated and enumerated from SI LP by flow cytometry. Data are presented as mean ± SEM and pooled from five independent experiments (*n* = 8–10 mice per group). (B) LP suspensions were restimulated ex vivo for 3 h with PMA/ionomycin and stained for intracellular cytokines. Representative flow plots gated on LP CD4 T cells as in A. (C–F) Frequency of restimulated CD4+ LP T cells expressing indicated cytokines. *p<0.05, ***p<0.001

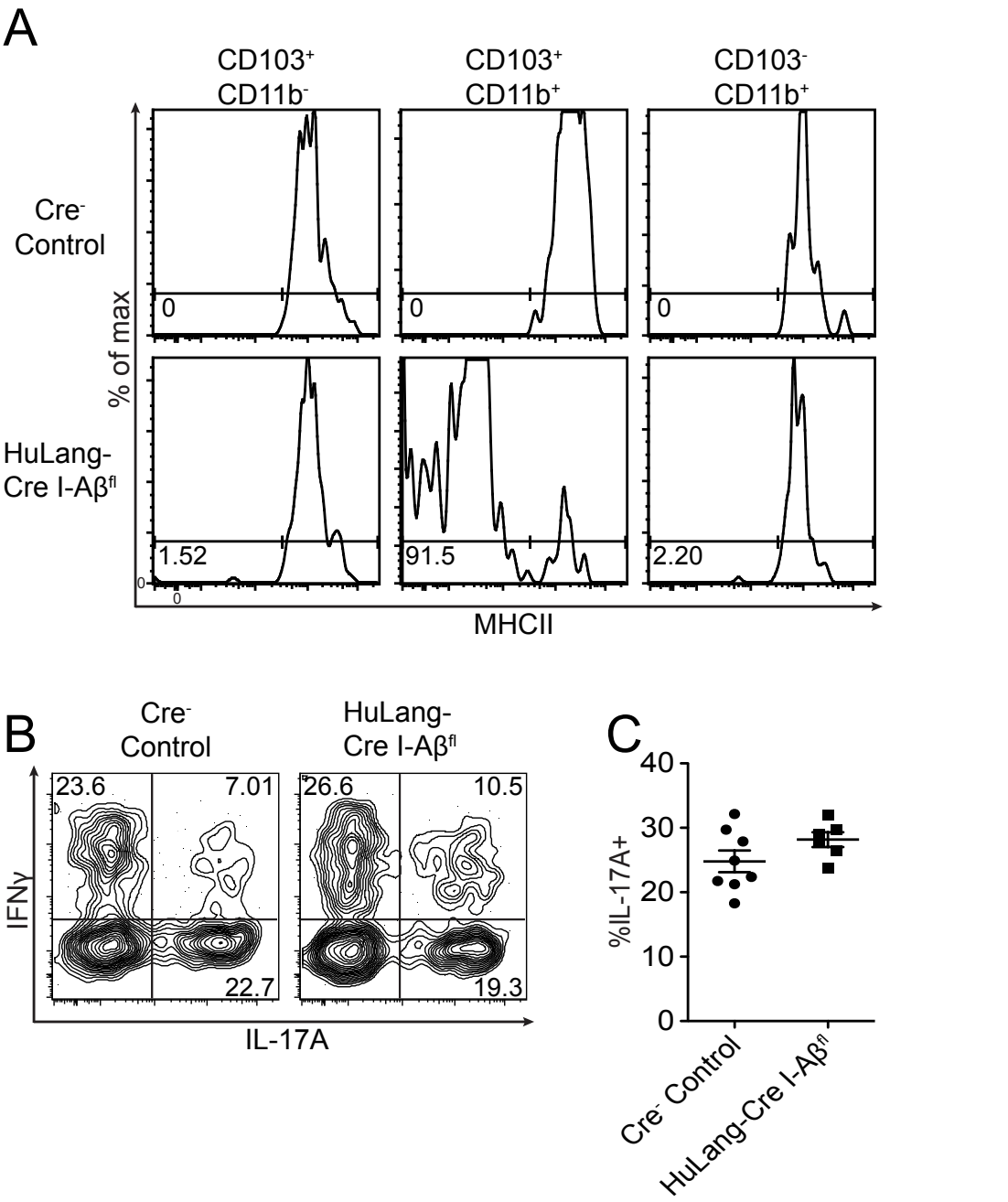


Figure 5-2: LP Th17 cells do not require MHCII expression by CD103+ CD11b+ DC

(A) Surface expression of MHCII on small intestinal LP DC subsets gated as CD3ε- B220- CD64- CD11c+ cells and subgated for CD103 and CD11b, as in Fig. 2-2F. Flow plots represent two independent experiments. (B-C) LP T cells restimulated with PMA/ionomycin and stained for cytokines as before

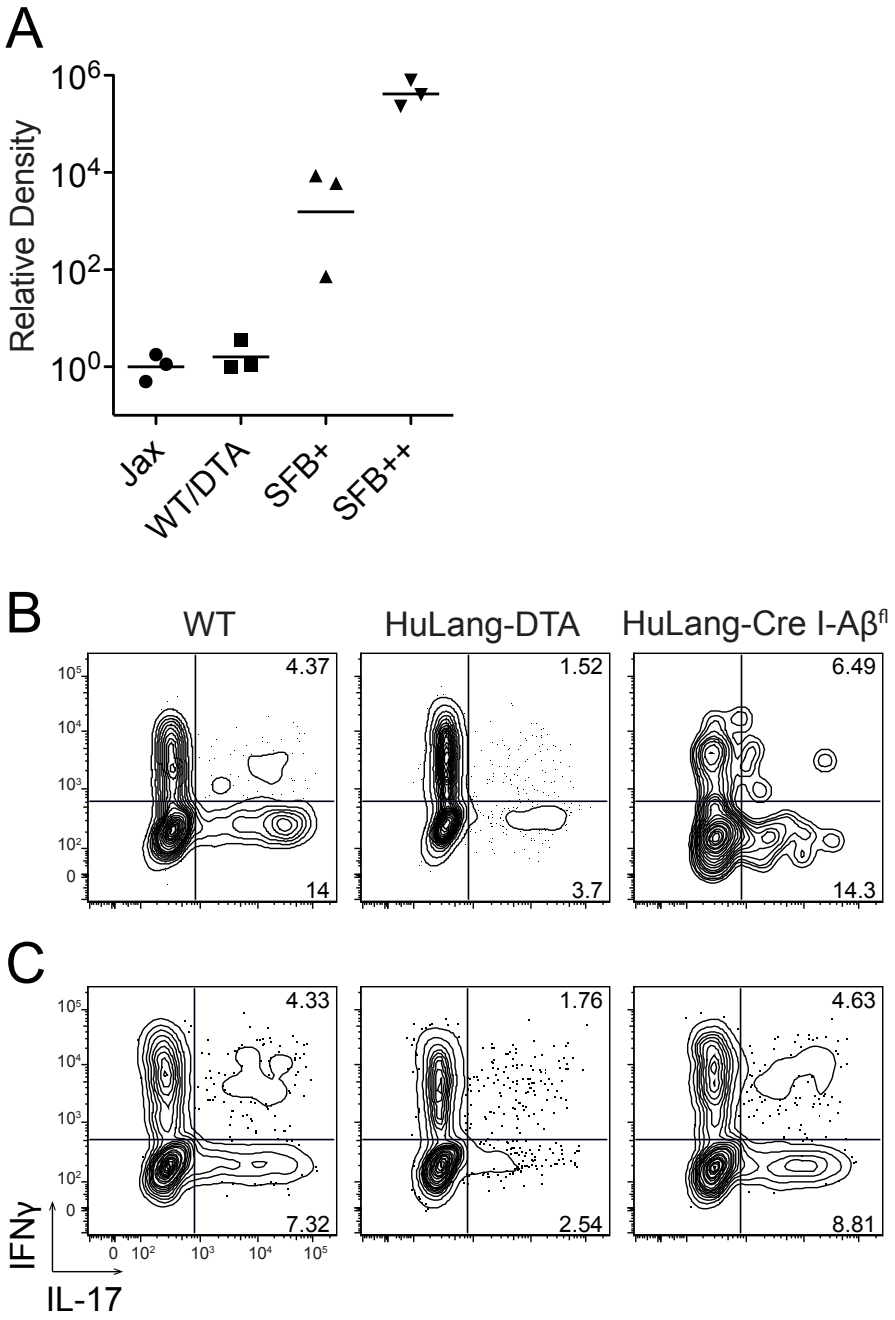


Figure 5-3: CD103$^+$ CD11b$^+$ DC are required for LP Th17 homeostasis in SFB colonized animals through a MHCII independent mechanism

(A) Abundance of SFB 16S rDNA relative to total eubacteria by real-time qPCR of fecal material obtained from Jackson Labs (Jax), our standard in-house mouse lines (WT/DTA), an SFB+ line identified in our facility (SFB+), or IL-23R$^{-/-}$ Rag2$^{-/-}$ mice (SFB++). (B and C) Mice were cohoused with SFB+ donors and LP cells were isolated, restimulated, and stained for cytokines as before

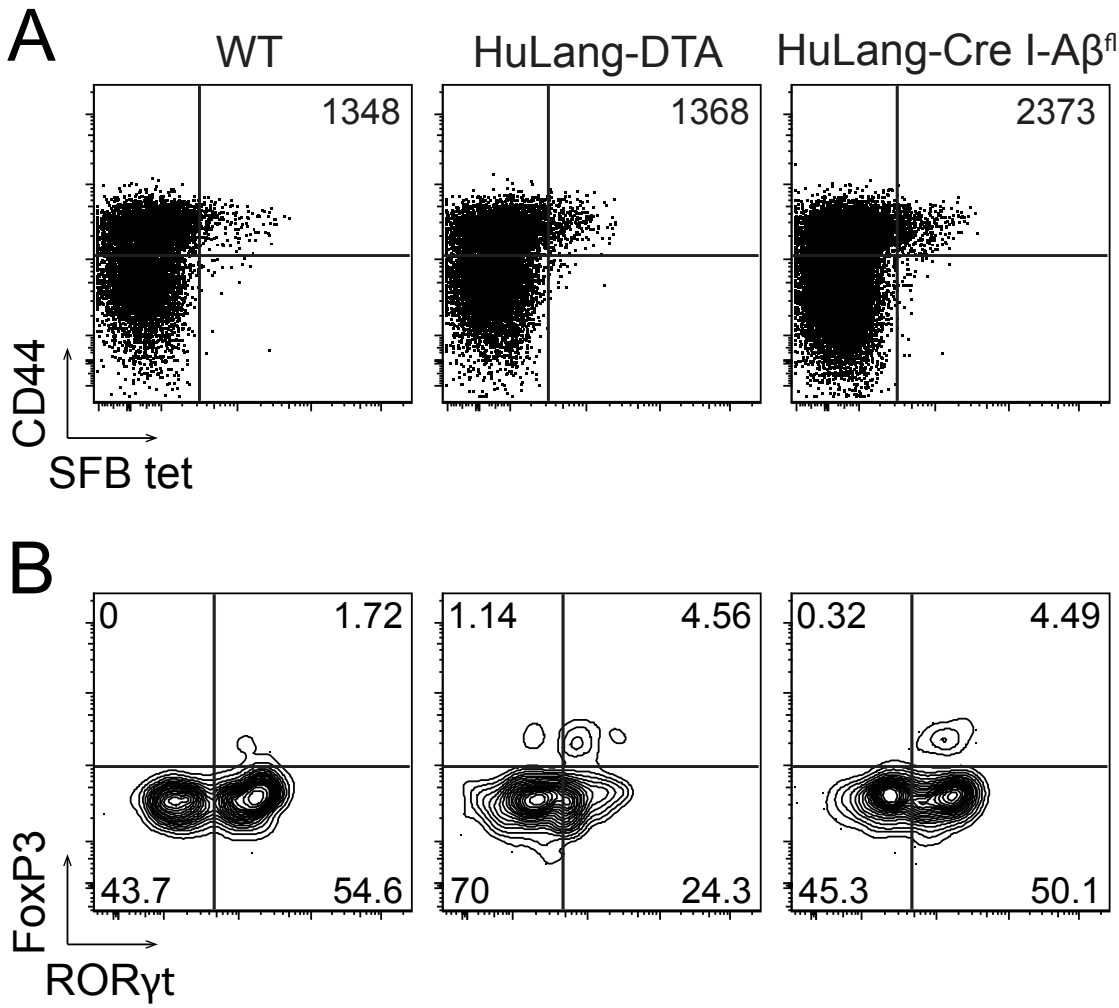


Figure 5-4: CD103^{+} CD11b^{+} DCs are required for RORγt induction in SFB-specific cells through a MHCII independent mechanism

(A) Live Dump- CD3ε+ CD4+ MLN cells gated stained with two distinct SFB specific tetramers both conjugated to APC. Numbers represent the total number of SFB specific cells in the indicated gate. (B) Intracellular staining for RORγt and FoxP3 on Tetramer+ cells.

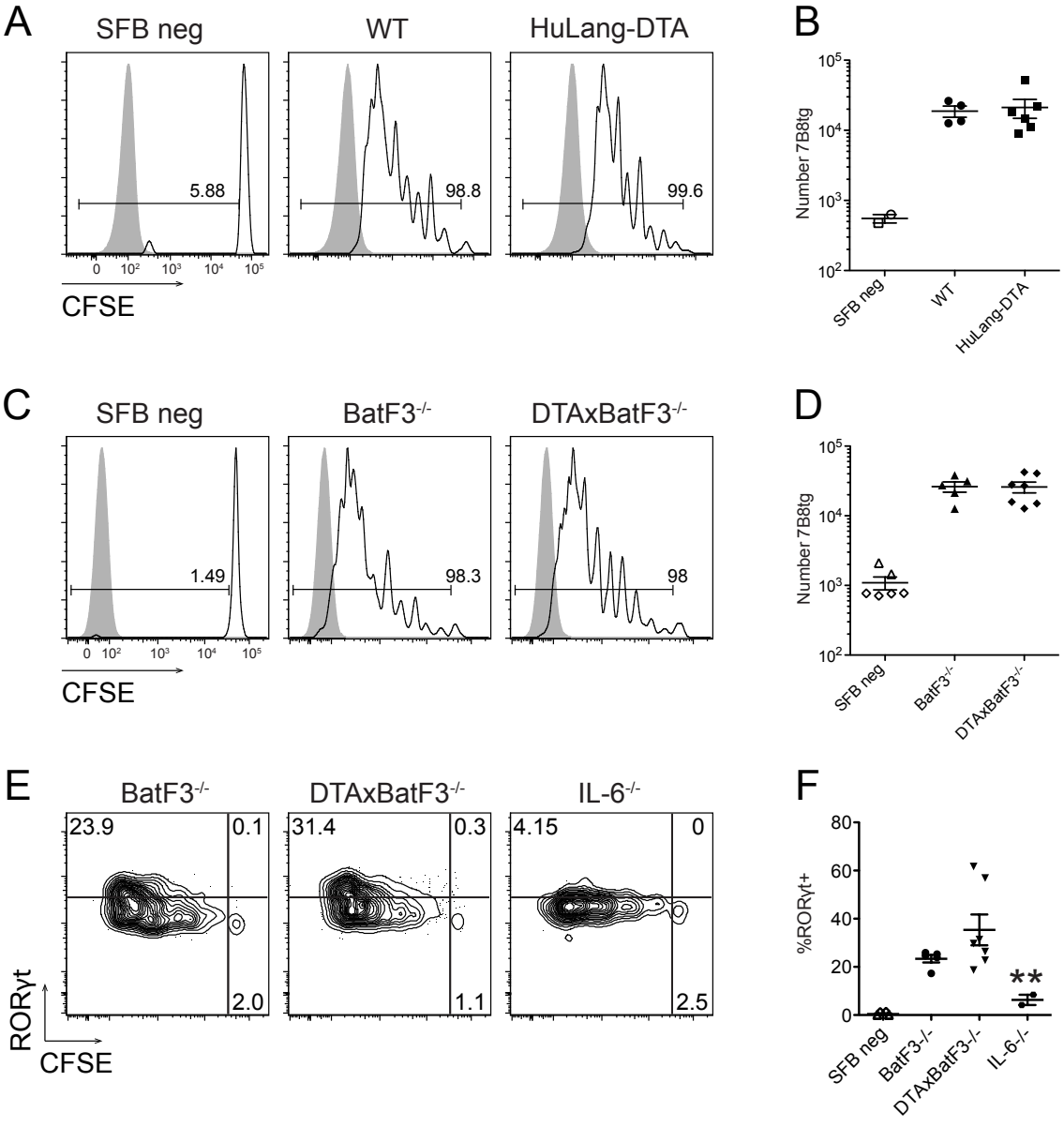


Figure 5-5: CD103+ DC are not required to present SFB antigens

(A,C) CFSE dilution and (B,D) total number of 7B8 transgenic cells isolated 4 days after transfer from the MLNs of mice previously colonized by gavage of autologous fecal material (SFB neg) or SFB+ donor material. (E,F) RORγt expression in 7B8 cells from mice of the indicated genotypes. **p<0.01

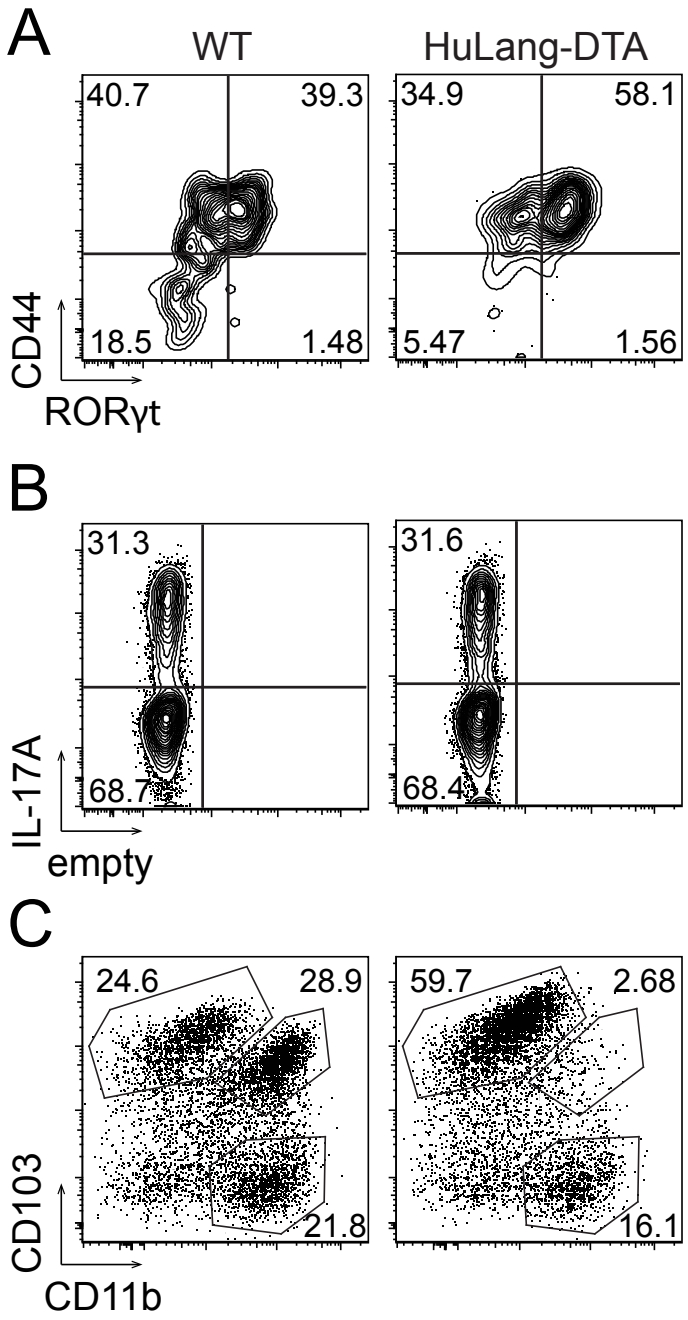


Figure 5-6: CD103^{+} CD11b^{+} DCs are not required for LP Th17 homeostasis after SFB gavage

(A) RORγt expression on SFB specific cells in the MLN two weeks after gavage of SFB+ donor material. (B) IL-17A staining in restimulated LP CD4 T cells of the same animals. (C) MHCII^{+} CD11c^{+} CD64^{-} LP DCs in the same animals.

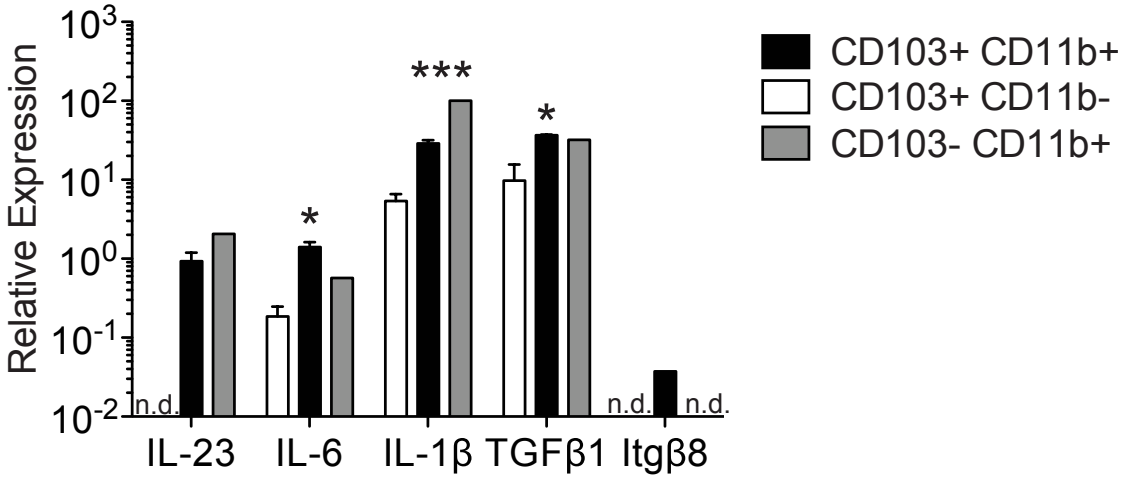


Figure 5-7: CD103$^+$ CD11b$^+$ DC produce Th17 associated cytokines

Relative mRNA expression of indicated cytokines in sorted DC subsets. *p<0.05, ***p<0.001 compared to CD103$^+$ CD11b$^-$ DCs.

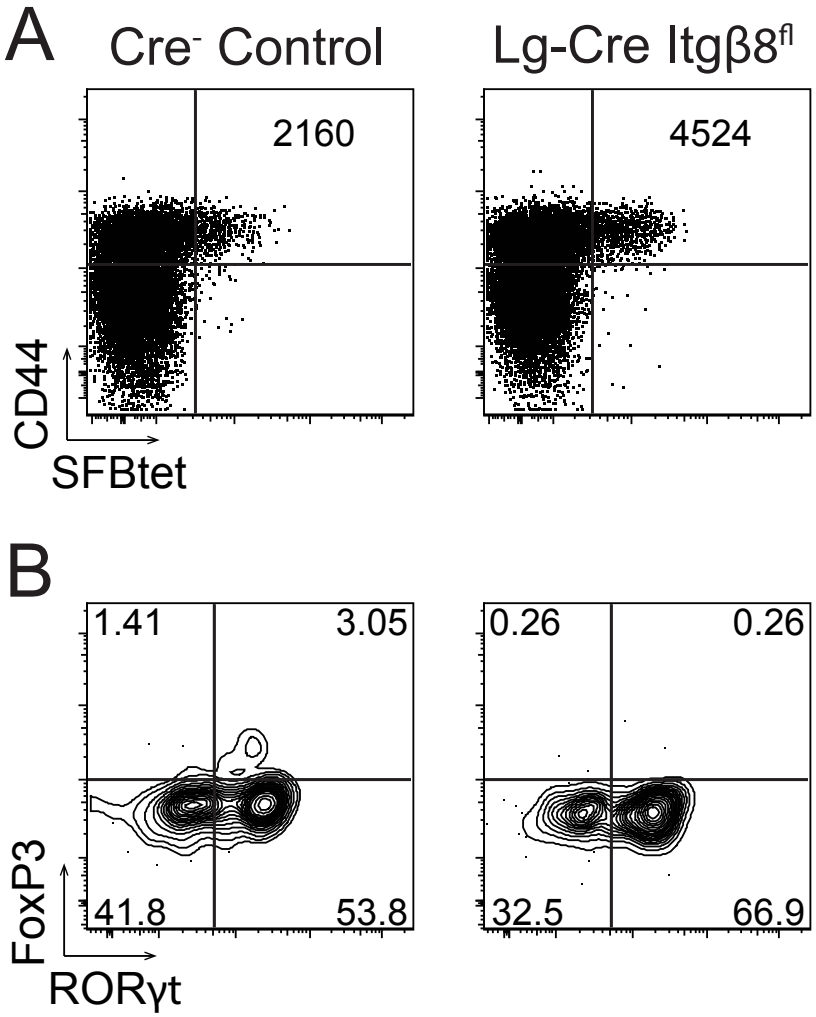
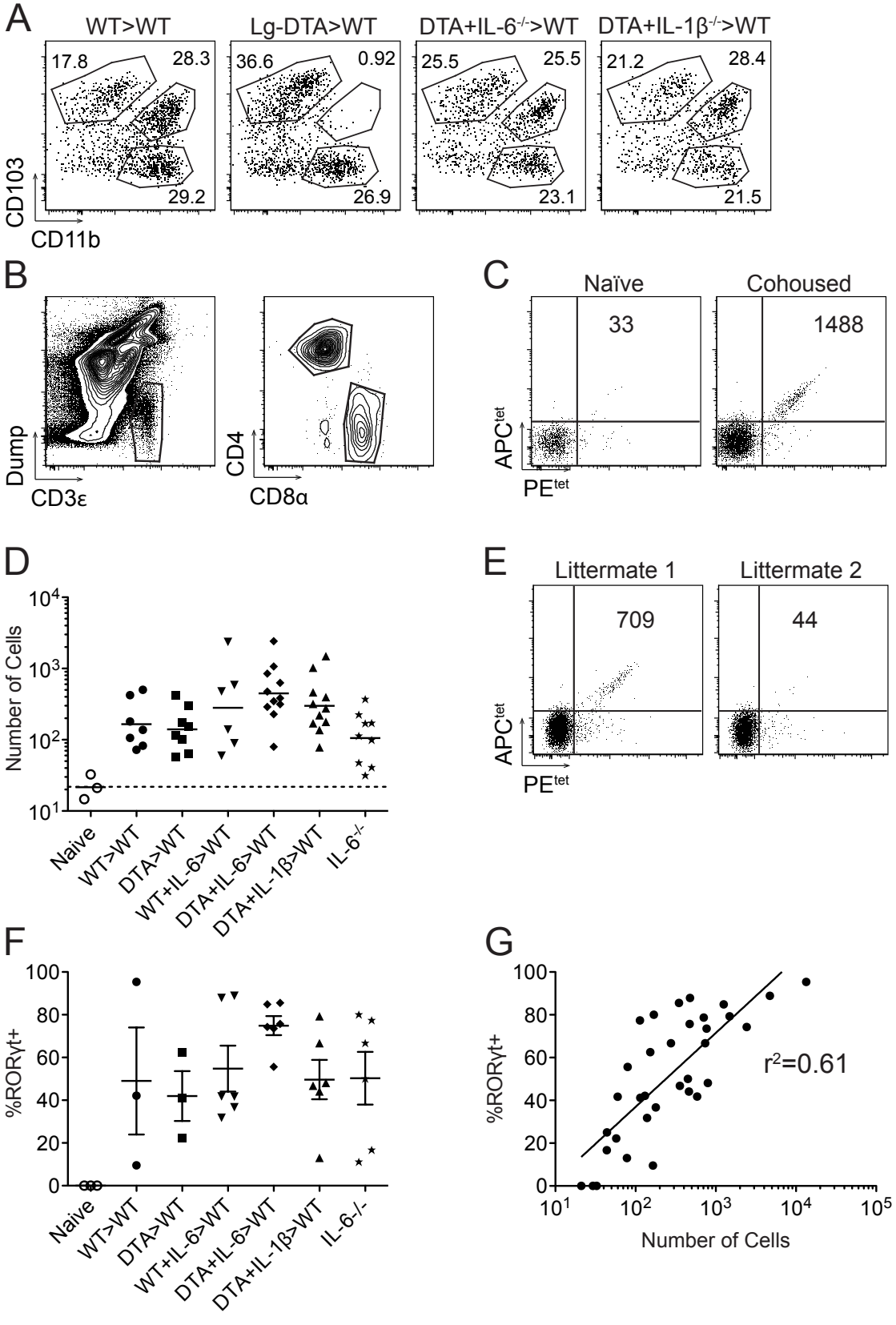


Figure 5-8: Expression of β8 integrin by CD103^{+} CD11b^{+} DCs is not required for Th17 homeostasis

(A) SFB specific cells stained and gated as in Fig. 5-4. Numbers represent total cell numbers in indicated gate. (B) RORγt staining of SFB^{+} cells.


A
WT>WT
Lg-DTA>WT
DTA+IL-6-/->WT
DTA+IL-1β-/->WT
17.8
28.3
29.2
36.6
0.92
26.9
25.5
25.5
23.1
21.2
28.4
21.5
CD103
CD11b
B
Dump
CD3ε
CD4
CD8α
C
Naïve
Cohoused
33
1488
APCtet
PEtet
D
Number of Cells
Naive
WT>WT
DTA>WT
WT+IL-6>WT
DTA+IL-6>WT
DTA+IL-1β>WT
IL-6-/-
E
Littermate 1
Littermate 2
709
44
APCtet
PEtet
F
%RORγt+
Naive
WT>WT
DTA>WT
WT+IL-6>WT
DTA+IL-6>WT
DTA+IL-1β>WT
IL-6-/-
G
%RORγt+
r2=0.61
Number of Cells

(previous page)

Figure 5-9: CD103$^+$ CD11b$^+$ DCs are not required for expansion of endogenous SFB specific cells in SLOs

(A) CD11c$^+$ MHCII$^+$ CD64$^-$ LP DCs isolated from indicated chimeric mice. (B) Tetramer gating strategy for pooled spleen and MLN pregated for single cells. (C) Detection of CD4 cells binding SFB3 tetramer in SFB negative (naïve) and cohoused mice. (D) Total number of SFB3-specific CD4 cells in pooled spleen and MLN of indicated cohoused chimeric mice. Dotted line represents the limit of detection based on SFB3 binding cells in the CD8α$^+$ CD4$^-$ gate. $p<0.05$ for all groups compared to naïve by student's T test with Welch's correction for unequal variance. (E) SFB3 tetramer binding CD4 cells in SFB$^+$ donor littermates. (F) Frequency of RORγt$^+$ cells among SFB3-specific CD4 cells. (G) Relationship between SFB3$^+$ cell number and RORγt expression in all chimeras. Line represents a semilog regression of the data.

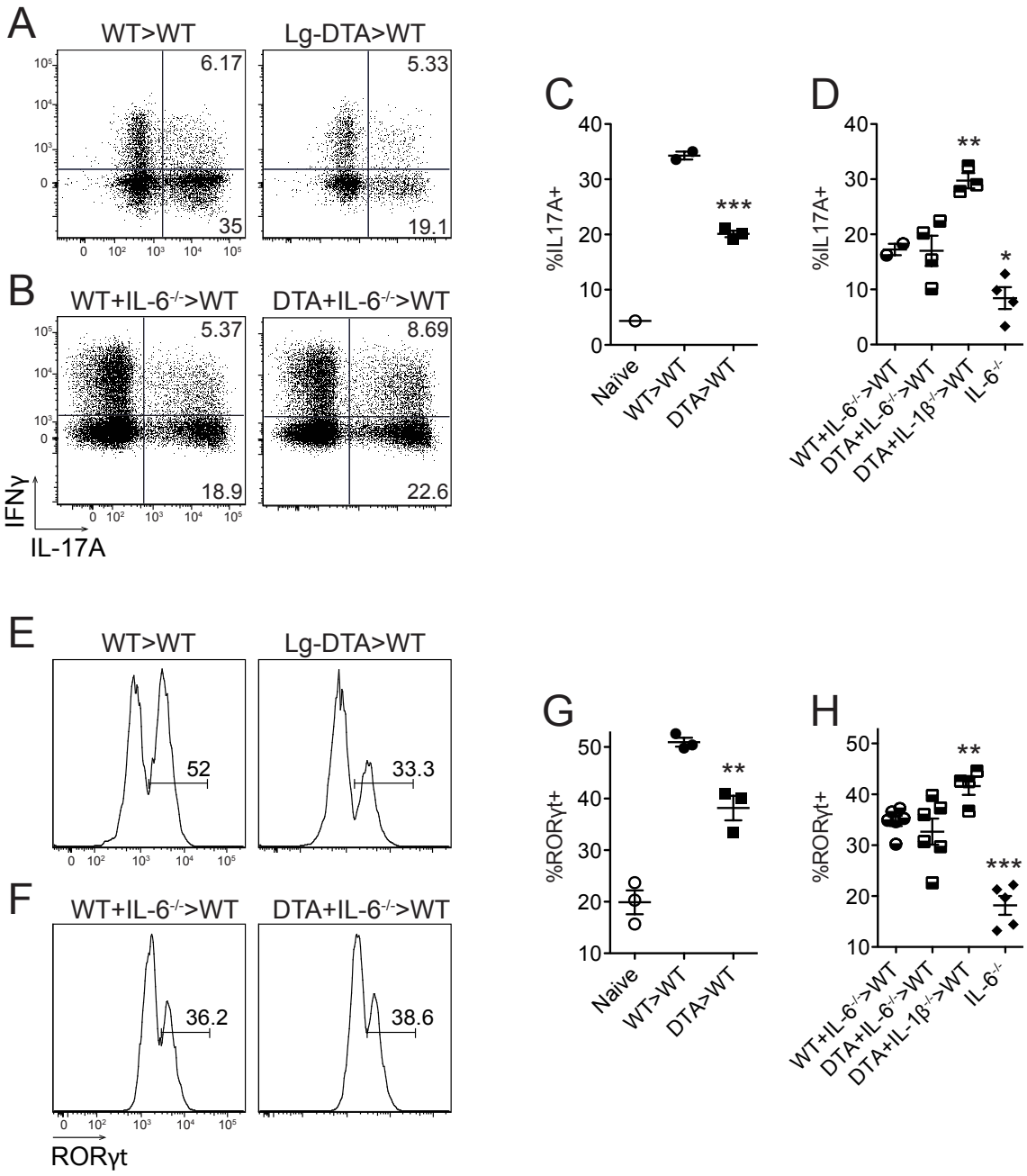


Figure 5-10: CD103$^+$ CD11b$^+$ DCs affect LP Th17 homeostasis independently of IL-6 and IL-1β

(A and B) LP CD4$^+$ T cells restimulated and stained for cytokines as before. (C and D) Frequency of IL-17A$^+$ cells in indicated chimeras. (D and E) LP CD4$^+$ T cells stained for RORγt. (F and G) Frequency of RORγt$^+$ cells in indicated chimeras. **p<0.01, ***p<0.001

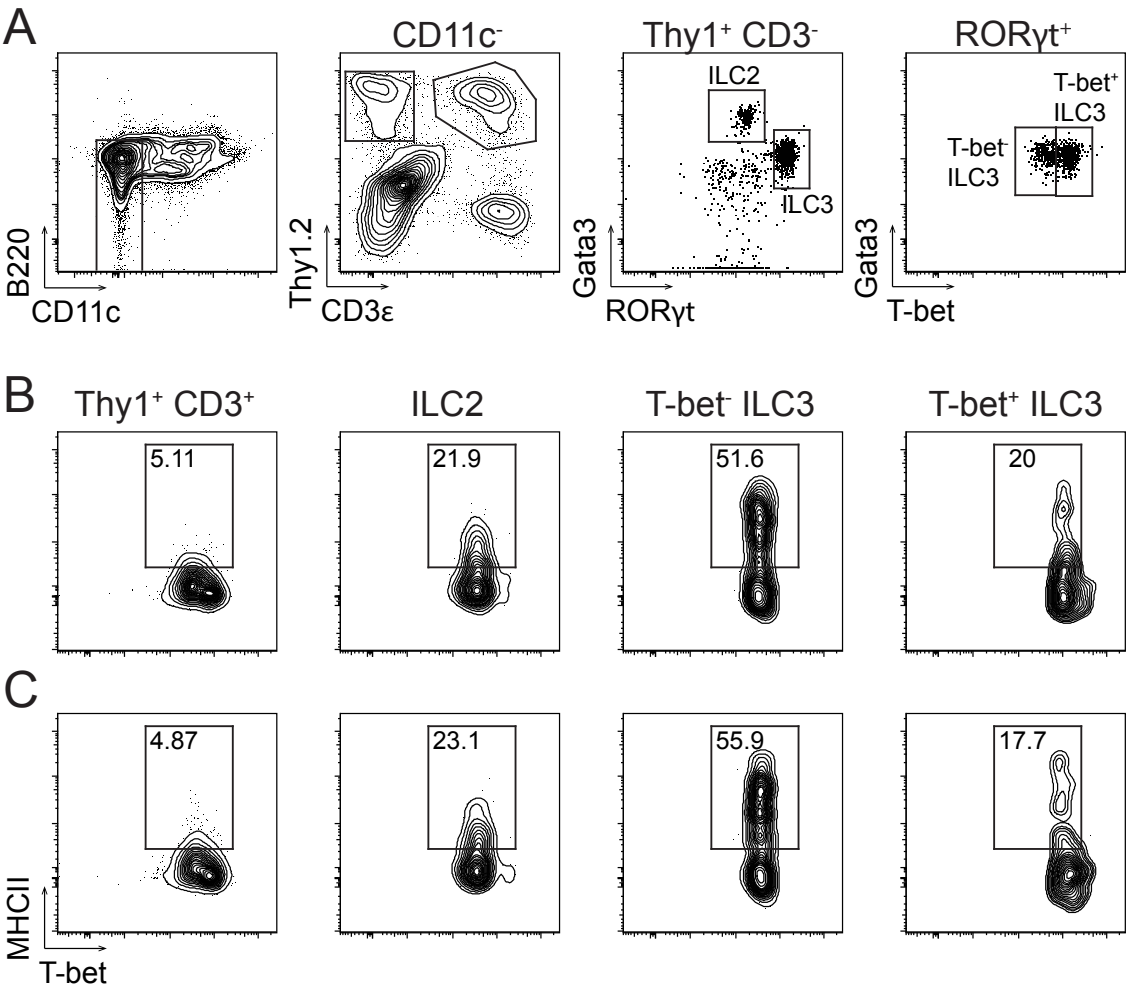


Figure 5-11: Deletion of CD103+ CD11b+ DCs does not affect ILC3 expression of MHCII

(A) Gating strategy for ILCs from small intestinal LP. (B and C) Expression of MHCII in indicated subsets from (A) WT→WT or (B) huLangerin-DTA→WT chimeric mice cohoused with SFB donor mice.

Chapter 6: Conclusion – Dendritic cell heterogeneity and immune based therapies

Although the importance of dendritic cells went unappreciated for many years, the centrality of this specialized cell type in the immune response is now undeniable. The past decade and a half has seen the development of numerous *in vivo* genetic and molecular tools that have underscored the significance of dendritic cells in a variety of both infectious and noninfectious settings. Indeed, even during the relatively short time period of the work described in these pages, our understanding of dendritic cell development and function has substantially evolved. These studies have led to an emerging paradigm of dendritic cell function: that functional heterogeneity within the DC lineage serves to orchestrate discrete innate and adaptive immune modules to provide optimal protection to the host (Briseño et al., 2014; Satpathy, Wu, Albring, & Murphy, 2012b). The exact cellular players and molecular mechanisms underlying these divisions of labor, as well as the degree to which DCs are rigidly committed to perform certain functions, remain to be fully described. Our own findings, discussed in the preceding chapters, would suggest that intestinal DC subsets retain a certain degree of functional plasticity, or at least that there is appreciable redundancy between distinct DC lineages in the gut.

While dendritic cells are by now well appreciated for their roles in the initiation of innate and adaptive responses, their therapeutic potential has yet to be fully realized. Given their role in the initiation phase of the immune response,

the most obvious application of dendritic cells has been in the development of DC based vaccination strategies (Palucka, Banchereau, & Mellman, 2010). However, these efforts have faced several challenges. Cell-based therapies have inherent challenges related to the cost of generating DCs from PBMC samples *ex vivo* and also in stimulating effective immune responses at the proper locations when infused into the patient. Additionally, such strategies do not take into account the normal division of labor between DC subsets *in vivo*. These challenges, along with the previous success of antibody-based DC targeting strategies to augment vaccine responses in mice (Bonifaz et al., 2004), have suggested that this approach might better leverage DC subsets in tailored vaccination approaches (Tacken & Figdor, 2011; Tacken, de Vries, Torensma, & Figdor, 2007). Several transcriptomic and functional studies have now provided convincing evidence that counterparts of murine dendritic cell subsets are present in human lymphoid and nonlymphoid tissue (Haniffa et al., 2012; Poulin et al., 2010; Watchmaker et al., 2014). These subsets often express characteristic phenotypic markers, such as DNGR1 (Poulin et al., 2010) and langerin (Watchmaker et al., 2014; Welty et al., 2013), providing potentially targetable receptors for antibody-based therapeutics.

Beyond vaccination strategies aimed at promoting anamnestic responses in the adaptive immune compartment, numerous recent studies have indicated the importance of non-myeloid innate immune cells, in particular the recently canonized innate lymphoid cell subsets (Spits et al., 2013), in sustaining or

preventing numerous complex diseases, including metabolic syndrome, inflammatory bowel disease, colon cancer, graft-versus-host disease, and asthma (Buonocore et al., 2010; Halim et al., 2014; Hanash et al., 2012; Huber et al., 2012; Munneke et al., 2014; X. Wang et al., 2014). Although the exact mechanisms and mediators remain to be defined, it is clear that DCs exert direct control over the effector actions of ILCs in many circumstances (Briseño et al., 2014). Thus, dendritic cell based therapeutics represent a potential mechanism to directly target ILC-dependent pathways, broadening the scope of such therapies from the initiation phase to the effector phase of the disease. Because antibody-based targeting strategies rely on delivery to surface receptors of differentiated dendritic cells, further understanding of DC subset functions at the cellular level, as opposed to merely their ontogeny, will be a key step to realizing success with this approach.

References

Acharya, M., Mukhopadhyay, S., Païdassi, H., Jamil, T., Chow, C., Kissler, S., et al. (2010). αv Integrin expression by DCs is required for Th17 cell differentiation and development of experimental autoimmune encephalomyelitis in mice. *The Journal of Clinical Investigation*, *120*(12), 4445–4452. doi:10.1172/JCI43796

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., et al. (2008). ATP drives lamina propria T(H)17 cell differentiation. *Nature*, *455*(7214), 808–812. doi:10.1038/nature07240

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*, *500*(7461), 232–236. doi:10.1038/nature12331

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. *Science (New York, NY)*, *331*(6015), 337–341. doi:10.1126/science.1198469

Axelrod, J. D. (2010). Delivering the Lateral Inhibition Punchline: It's All About the Timing. *Science Signaling*, *3*(145), pe38–pe38. doi:10.1126/scisignal.3145pe38

Bajana, S., Roach, K., Turner, S., Paul, J., & Kovats, S. (2012). IRF4 Promotes Cutaneous Dendritic Cell Migration to Lymph Nodes during Homeostasis and Inflammation. *The Journal of Immunology*, *189*(7), 3368–3377. doi:10.4049/jimmunol.1102613

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., & Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infection and Immunity*, *76*(3), 907–915. doi:10.1128/IAI.01432-07

Barnden, M. J., Allison, J., Heath, W. R., & Carbone, F. R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunology and Cell Biology*, *76*(1), 34–40. doi:10.1046/j.1440-1711.1998.00709.x

Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., et al. (2003). Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infection and Immunity*, *71*(5), 2839–2858.

Basu, R., O'Quinn, D. B., Silberger, D. J., Schoeb, T. R., Fouser, L., Ouyang, W., et al. (2012). Th22 Cells Are an Important Source of IL-22 for Host Protection against Enteropathogenic Bacteria. *Immunity*, *37*(6), 1061–1075. doi:10.1016/j.immuni.2012.08.024

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., et al. (2009). Cross-presentation of viral and self antigens by skin-derived

CD103+ dendritic cells. *Nature Immunology*, *10*(5), 488–495. doi:10.1038/ni.1724
Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., et al. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, *441*(7090), 235–238. doi:10.1038/nature04753
Bobr, A., Igyarto, B. Z., Haley, K. M., Li, M. O., Flavell, R. A., & Kaplan, D. H. (2012). Autocrine/paracrine TGF-β1 inhibits Langerhans cell migration. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.1119178109
Bobr, A., Olvera-Gomez, I., Igyarto, B. Z., Haley, K. M., Hogquist, K. A., & Kaplan, D. H. (2010). Acute ablation of Langerhans cells enhances skin immune responses. *Journal of Immunology (Baltimore, Md : 1950)*, *185*(8), 4724–4728. doi:10.4049/jimmunol.1001802
Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., et al. (2009). Origin of the lamina propria dendritic cell network. *Immunity*, *31*(3), 513–525. doi:10.1016/j.immuni.2009.08.010
Bonifaz, L. C., Bonnyay, D. P., Charalambous, A., Darguste, D. I., Fujii, S.-I., Soares, H., et al. (2004). In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *The Journal of Experimental Medicine*, *199*(6), 815–824. doi:10.1084/jem.20032220
Brandl, K., Plitas, G., Mihu, C. N., Ubeda, C., Jia, T., Fleisher, M., et al. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature*, *455*(7214), 804–807. doi:10.1038/nature07250
Bray, J. R., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325. doi:10.2307/1942268
Briseño, C. G., Murphy, T. L., & Murphy, K. M. (2014). Complementary diversification of dendritic cells and innate lymphoid cells. *Current Opinion in Immunology*, *29C*, 69–78. doi:10.1016/j.coi.2014.04.006
Buonocore, S., Ahern, P. P., Uhlig, H. H., Ivanov, I. I., Littman, D. R., Maloy, K. J., & Powrie, F. (2010). Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature*, *464*(7293), 1371–1375. doi:10.1038/nature08949
Bursch, L. S., Wang, L., Igyarto, B., Kissenpfennig, A., Malissen, B., Kaplan, D. H., & Hogquist, K. A. (2007). Identification of a novel population of Langerin+ dendritic cells. *The Journal of Experimental Medicine*, *204*(13), 3147–3156. doi:10.1084/jem.20071966
Cassani, B., Villablanca, E. J., Quintana, F. J., Love, P. E., Lacy-Hulbert, A., Blaner, W. S., et al. (2011). Gut-tropic T cells that express integrin α4β7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology*, *141*(6), 2109–2118. doi:10.1053/j.gastro.2011.09.015
Caton, M. L., Smith-Raska, M. R., & Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *The Journal of*

Experimental Medicine, *204*(7), 1653–1664. doi:10.1084/jem.20062648
Cerovic, V., Houston, S. A., Scott, C. L., Aumeunier, A., Yrlid, U., Mowat, A. M., & Milling, S. W. F. (2013). Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunology*, *6*(1), 104–113. doi:10.1038/mi.2012.53
Chang, S.-Y., & Kweon, M.-N. (2010). Langerin-expressing dendritic cells in gut-associated lymphoid tissues. *Immunological Reviews*, *234*(1), 233–246. doi:10.1111/j.0105-2896.2009.00878.x
Chappert, P., Bouladoux, N., Naik, S., & Schwartz, R. H. (2013). Specific gut commensal flora locally alters T cell tuning to endogenous ligands. *Immunity*, *38*(6), 1198–1210. doi:10.1016/j.immuni.2013.06.005
Chikwava, K., & Jaffe, R. (2004). Langerin (CD207) staining in normal pediatric tissues, reactive lymph nodes, and childhood histiocytic disorders. *Pediatric and Developmental Pathology : the Official Journal of the Society for Pediatric Pathology and the Paediatric Pathology Society*, *7*(6), 607–614. doi:10.1007/s10024-004-3027-z
Chitnis, A. B. (1995). The role of Notch in lateral inhibition and cell fate specification. *Molecular and Cellular Neurosciences*, *6*(6), 311–321.
Chow, A., Brown, B. D., & Merad, M. (2011). Studying the mononuclear phagocyte system in the molecular age. *Nature Publishing Group*, *11*(11), 788–798. doi:10.1038/nri3087
Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., et al. (2012). Gut Immune Maturation Depends on Colonization with a Host-Specific Microbiota. *Cell*, *149*(7), 1578–1593. doi:10.1016/j.cell.2012.04.037
Chung, Y., Chang, S. H., Martinez, G. J., Yang, X. O., Nurieva, R., Kang, H. S., et al. (2009). Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling. *Immunity*, *30*(4), 576–587. doi:10.1016/j.immuni.2009.02.007
Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*, *37*(Database issue), D141–5. doi:10.1093/nar/gkn879
Coombes, J. L., & Powrie, F. (2008). Dendritic cells in intestinal immune regulation. *Nature Publishing Group*, *8*(6), 435–446. doi:10.1038/nri2335
Coombes, J. L., Siddiqui, K. R. R., Arancibia-Cárcamo, C. V., Hall, J., Sun, C.-M., Belkaid, Y., & Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of Experimental Medicine*, *204*(8), 1757–1764. doi:10.1084/jem.20070590
Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C.-A., Ventre, E., et al. (2010). The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *The Journal of Experimental Medicine*, *207*(6), 1283–1292. doi:10.1084/jem.20100223

Crozat, K., Tamoutounour, S., Vu Manh, T.-P., Fossum, E., Luche, H., Ardouin, L., et al. (2011). Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8α+ type. *The Journal of Immunology*, *187*(9), 4411–4415. doi:10.4049/jimmunol.1101717

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., et al. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*, *421*(6924), 744–748. doi:10.1038/nature01355

Cullender, T. C., Chassaing, B., Janzon, A., Kumar, K., Muller, C. E., Werner, J. J., et al. (2013). Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. *Cell Host and Microbe*, *14*(5), 571–581. doi:10.1016/j.chom.2013.10.009

Denning, T. L., Norris, B. A., Medina-Contreras, O., Manicassamy, S., Geem, D., Madan, R., et al. (2011). Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses Are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization. *The Journal of Immunology*, *187*(2), 733–747. doi:10.4049/jimmunol.1002701

Denning, T. L., Wang, Y.-C., Patel, S. R., Williams, I. R., & Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature Immunology*, *8*(10), 1086–1094. doi:10.1038/ni1511

DeNucci, C. C., Mitchell, J. S., & Shimizu, Y. (2009). Integrin function in T-cell homing to lymphoid and nonlymphoid sites: getting there and staying there. *Critical Reviews in Immunology*, *29*(2), 87–109.

Diehl, G. E., Longman, R. S., Zhang, J.-X., Breart, B., Galan, C., Cuesta, A., et al. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature*, *494*(7435), 116–120. doi:10.1038/nature11809

Dieu, M. C., Vanbervliet, B., Vicari, A., Bridon, J. M., Oldham, E., Aït-Yahia, S., et al. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *The Journal of Experimental Medicine*, *188*(2), 373–386.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., et al. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science (New York, NY)*, *315*(5808), 107–111. doi:10.1126/science.1136080

Edelson, B. T., Bradstreet, T. R., Wumesh, K. C., Hildner, K., Herzog, J. W., Sim, J., et al. (2011). Batf3-Dependent CD11blow/− Peripheral Dendritic Cells Are GM-CSF-Independent and Are Not Required for Th Cell Priming after Subcutaneous Immunization. *PloS One*, *6*(10), e25660. doi:10.1371/journal.pone.0025660.g004

Edelson, B. T., KC, W., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., et al. (2010). Peripheral CD103+ dendritic cells form a unified subset

developmentally related to CD8alpha+ conventional dendritic cells. *The Journal of Experimental Medicine*, *207*(4), 823–836. doi:10.1084/jem.20091627

Elinav, E., Strowig, T., Kau, A. L., Henao-Mejia, J., Thaiss, C. A., Booth, C. J., et al. (2011). NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. *Cell*, *145*(5), 745–757. doi:10.1016/j.cell.2011.04.022

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., & Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science (New York, NY)*, *298*(5597), 1424–1427. doi:10.1126/science.1077336

Feng, D. X., Liu, D. P., Huang, Y., Wu, L., Li, T. C., Wu, M., et al. (2001). The expression of human alpha -like globin genes in transgenic mice mediated by bacterial artificial chromosome. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(26), 15073–15077. doi:10.1073/pnas.251550598

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., et al. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science (New York, NY)*, *311*(5757), 83–87. doi:10.1126/science.1117729

Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., & Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, *99*(1), 23–33.

Fujimoto, K., Karuppuchamy, T., Takemura, N., Shimohigoshi, M., Machida, T., Haseda, Y., et al. (2011). A New Subset of CD103+CD8 + Dendritic Cells in the Small Intestine Expresses TLR3, TLR7, and TLR9 and Induces Th1 Response and CTL Activity. *The Journal of Immunology*, *186*(11), 6287–6295. doi:10.4049/jimmunol.1004036

Gao, Y., Nish, S. A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J. S., et al. (2013). Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. *Immunity*, *39*(4), 722–732. doi:10.1016/j.immuni.2013.08.028

Garrett, W. S., Lord, G. M., Punit, S., Lugo-Villarino, G., Mazmanian, S. K., Ito, S., et al. (2007). Communicable Ulcerative Colitis Induced by T-bet Deficiency in the Innate Immune System. *Cell*, *131*(1), 33–45. doi:10.1016/j.cell.2007.08.017

Gautier, E. L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., et al. (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature Immunology*, *13*(11), 1118–1128. doi:10.1038/ni.2419

Geem, D., Medina-Contreras, O., McBride, M., Newberry, R. D., Koni, P. A., &

Denning, T. L. (2014). Specific Microbiota-Induced Intestinal Th17 Differentiation Requires MHC Class II but Not GALT and Mesenteric Lymph Nodes. *The Journal of Immunology*. doi:10.4049/jimmunol.1303167

Ghilardi, N., Kljavin, N., Chen, Q., Lucas, S., Gurney, A. L., & De Sauvage, F. J. (2004). Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. *Journal of Immunology (Baltimore, Md : 1950)*, *172*(5), 2827–2833.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.-M., et al. (2006). Langerhans cells arise from monocytes in vivo. *Nature Publishing Group*, *7*(3), 265–273. doi:10.1038/ni1307

Godinez, I., Keestra, A. M., Spees, A., & Baumler, A. J. (2011). The IL-23 axis in Salmonella gastroenteritis. *Cellular Microbiology*, *13*(11), 1639–1647. doi:10.1111/j.1462-5822.2011.01637.x

Gong, S., Zheng, C., Doughty, M. L., Losos, K., Didkovsky, N., Schambra, U. B., et al. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature*, *425*(6961), 917–925. doi:10.1038/nature02033

Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M. G., et al. (2014). Segmented Filamentous Bacteria Antigens Presented by Intestinal Dendritic Cells Drive Mucosal Th17 Cell Differentiation. *Immunity*, 1–14. doi:10.1016/j.immuni.2014.03.005

Gottschalk, R. A., Corse, E., & Allison, J. P. (2012). Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *The Journal of Immunology*, *188*(3), 976–980. doi:10.4049/jimmunol.1102964

Griffin, A. J., & McSorley, S. J. (2011). Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunology*, *4*(4), 371–382. doi:10.1038/mi.2011.2

Grubin, C. E., Kovats, S., deRoos, P., & Rudensky, A. Y. (1997). Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides. *Immunity*, *7*(2), 197–208.

Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., et al. (2010). Skin-draining lymph nodes contain dermis-derived CD103-dendritic cells that constitutively produce retinoic acid and induce Foxp3+ regulatory T cells. *Blood*, *115*(10), 1958–1968. doi:10.1182/blood-2009-09-245274

Gutcher, I., Donkor, M. K., Ma, Q., Rudensky, A. Y., Flavell, R. A., & Li, M. O. (2011). Autocrine transforming growth factor-β1 promotes in vivo Th17 cell differentiation. *Immunity*, *34*(3), 396–408. doi:10.1016/j.immuni.2011.03.005

Hacker, C., Kirsch, R. D., Ju, X.-S., Hieronymus, T., Gust, T. C., Kuhl, C., et al. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. *Nature Immunology*, *4*(4), 380–386. doi:10.1038/ni903

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., et al. (2011). Intestinal Tolerance Requires Gut Homing and Expansion of FoxP3+ Regulatory T Cells in the Lamina Propria. *Immunity*, *34*(2), 237–246.

doi:10.1016/j.immuni.2011.01.016
Haley, K., Igyarto, B. Z., Ortner, D., Bobr, A., Kashem, S., Schenten, D., & Kaplan, D. H. (2012). Langerhans cells require MyD88-dependent signals for Candida albicans response but not for contact hypersensitivity or migration. *The Journal of Immunology*, *188*(9), 4334–4339. doi:10.4049/jimmunol.1102759
Halim, T. Y. F., Steer, C. A., Mathä, L., Gold, M. J., Martinez-Gonzalez, I., McNagny, K. M., et al. (2014). Group 2 Innate Lymphoid Cells Are Critical for the Initiation of Adaptive T Helper 2 Cell-Mediated Allergic Lung Inflammation. *Immunity*, 1–11. doi:10.1016/j.immuni.2014.01.011
Hall, J. A., Grainger, J. R., Spencer, S. P., & Belkaid, Y. (2011). The Role of Retinoic Acid in Tolerance and Immunity. *Immunity*, *35*(1), 13–22. doi:10.1016/j.immuni.2011.07.002
Han, D., Walsh, M. C., Cejas, P. J., Dang, N. N., Kim, Y. F., Kim, J., et al. (2013). Dendritic Cell Expression of the Signaling Molecule TRAF6 Is Critical for Gut Microbiota-Dependent Immune Tolerance. *Immunity*, *38*(6), 1211–1222. doi:10.1016/j.immuni.2013.05.012
Hanash, A. M., Dudakov, J. A., Hua, G., O'Connor, M. H., Young, L. F., Singer, N. V., et al. (2012). Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity*, *37*(2), 339–350. doi:10.1016/j.immuni.2012.05.028
Haniffa, M., Shin, A., Bigley, V., McGovern, N., Teo, P., See, P., et al. (2012). Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells. *Immunity*, *37*(1), 60–73. doi:10.1016/j.immuni.2012.04.012
Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., & Weaver, C. T. (2005). Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*, *6*(11), 1123–1132. doi:10.1038/ni1254
Hataye, J. (2006). Naive and Memory CD4+ T Cell Survival Controlled by Clonal Abundance. *Science (New York, NY)*, *312*(5770), 114–116. doi:10.1126/science.1124228
Hepworth, M. R., Monticelli, L. A., Fung, T. C., Ziegler, C. G. K., Grunberg, S., Sinha, R., et al. (2013). Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature*. doi:10.1038/nature12240
Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., et al. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science (New York, NY)*, *322*(5904), 1097–1100. doi:10.1126/science.1164206
Hoeffel, G., Wang, Y., Greter, M., See, P., Teo, P., Malleret, B., et al. (2012). Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *The Journal of Experimental Medicine*, *209*(6), 1167–1181. doi:10.1084/jem.20120340

Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J., & Carbone, F. R. (1994). T cell receptor antagonist peptides induce positive selection. *Cell*, *76*(1), 17–27.

Honda, K., & Littman, D. R. (2012). The microbiome in infectious disease and inflammation. *Annual Review of Immunology*, *30*, 759–795. doi:10.1146/annurev-immunol-020711-074937

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., & Murphy, K. M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science (New York, NY)*, *260*(5107), 547–549.

Hu, W., Troutman, T. D., Edukulla, R., & Pasare, C. (2011). Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. *Immunity*, *35*(6), 1010–1022. doi:10.1016/j.immuni.2011.10.013

Huber, S., Gagliani, N., Zenewicz, L. A., Huber, F. J., Bosurgi, L., Hu, B., et al. (2012). IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature*, 1–6. doi:10.1038/nature11535

Ichikawa, E., Hida, S., Omatsu, Y., Shimoyama, S., Takahara, K., Miyagawa, S., et al. (2004). Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(11), 3909–3914. doi:10.1073/pnas.0400610101

Igyarto, B. Z., Haley, K., Ortner, D., Bobr, A., Gerami-Nejad, M., Edelson, B. T., et al. (2011). Skin-Resident Murine Dendritic Cell Subsets Promote Distinct and Opposing Antigen-Specific T Helper Cell Responses. *Immunity*, *35*(2), 260–272. doi:10.1016/j.immuni.2011.06.005

Igyarto, B. Z., Jenison, M. C., Dudda, J. C., Roers, A., Müller, W., Koni, P. A., et al. (2009). Langerhans cells suppress contact hypersensitivity responses via cognate CD4 interaction and langerhans cell-derived IL-10. *Journal of Immunology (Baltimore, Md : 1950)*, *183*(8), 5085–5093. doi:10.4049/jimmunol.0901884

Inaba, K., Swiggard, W. J., Steinman, R. M., Romani, N., Schuler, G., & Brinster, C. (2009). Isolation of dendritic cells. *Current Protocols in Immunology / Edited by John E Coligan [Et Al]*, *Chapter 3*, Unit 3.7. doi:10.1002/0471142735.im0307s86

Ishifune, C., Maruyama, S., Sasaki, Y., Yagita, H., Hozumi, K., Tomita, T., et al. (2014). Differentiation of CD11c+CX3CR1+ cells in the small intestine requires Notch signaling. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.1401671111

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*, *139*(3), 485–498. doi:10.1016/j.cell.2009.09.033

Ivanov, I. I., Frutos, R. de L., Manel, N., Yoshinaga, K., Rifkin, D. B., Sartor, R. B., et al. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host and*

Microbe, *4*(4), 337–349. doi:10.1016/j.chom.2008.09.009
Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., et al. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*, *126*(6), 1121–1133. doi:10.1016/j.cell.2006.07.035
Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J. L., et al. (2008). Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *The Journal of Experimental Medicine*, *205*(9), 2139–2149. doi:10.1084/jem.20080414
Jaensson-Gyllenbäck, E., Kotarsky, K., Zapata, F., Persson, E. K., Gundersen, T. E., Blomhoff, R., & Agace, W. W. (2011). Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunology*, *4*(4), 438–447. doi:10.1038/mi.2010.91
Janeway, C. A. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symposia on Quantitative Biology*, *54 Pt 1*, 1–13.
Jantsch, J., Chikkaballi, D., & Hensel, M. (2011). Cellular aspects of immunity to intracellular Salmonella enterica. *Immunological Reviews*, *240*(1), 185–195. doi:10.1111/j.1600-065X.2010.00981.x
Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., et al. (2010). Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *The Journal of Experimental Medicine*, *207*(6), 1247–1260. doi:10.1084/jem.20092140
Jung, S., Unutmaz, D., Wong, P., Sano, G.-I., De los Santos, K., Sparwasser, T., et al. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*, *17*(2), 211–220.
Kaplan, D. H., Jenison, M. C., Saeland, S., Shlomchik, W. D., & Shlomchik, M. J. (2005). Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity*, *23*(6), 611–620. doi:10.1016/j.immuni.2005.10.008
Kaplan, D. H., Kissenpfennig, A., & Clausen, B. E. (2008). Insights into Langerhans cell function from Langerhans cell ablation models. *European Journal of Immunology*, *38*(9), 2369–2376. doi:10.1002/eji.200838397
Kaplan, D. H., Li, M. O., Jenison, M. C., Shlomchik, W. D., Flavell, R. A., & Shlomchik, M. J. (2007). Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. *The Journal of Experimental Medicine*, *204*(11), 2545–2552. doi:10.1084/jem.20071401
Kaser, A., Ludwiczek, O., Holzmann, S., Moschen, A. R., Weiss, G., Enrich, B., et al. (2004). Increased expression of CCL20 in human inflammatory bowel disease. *Journal of Clinical Immunology*, *24*(1), 74–85. doi:10.1023/B:JOCI.0000018066.46279.6b

Khan, M. A., Bouzari, S., Ma, C., Rosenberger, C. M., Bergstrom, K. S. B., Gibson, D. L., et al. (2008). Flagellin-Dependent and -Independent Inflammatory Responses following Infection by Enteropathogenic Escherichia coli and Citrobacter rodentium. *Infection and Immunity*, *76*(4), 1410–1422. doi:10.1128/IAI.01141-07

Kim, J. M., Rasmussen, J. P., & Rudensky, A. Y. (2006). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature Immunology*, *8*(2), 191–197. doi:10.1038/ni1428

Kinnebrew, M. A., Buffie, C. G., Diehl, G. E., Zenewicz, L. A., Leiner, I., Hohl, T. M., et al. (2012). Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. *Immunity*, *36*(2), 276–287. doi:10.1016/j.immuni.2011.12.011

Kinnebrew, M. A., Ubeda, C., Zenewicz, L. A., Smith, N., Flavell, R. A., & Pamer, E. G. (2010). Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. *The Journal of Infectious Diseases*, *201*(4), 534–543. doi:10.1086/650203

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., et al. (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity*, *22*(5), 643–654. doi:10.1016/j.immuni.2005.04.004

Kobayashi, T., Walsh, P. T., Walsh, M. C., Speirs, K. M., Chiffoleau, E., King, C. G., et al. (2003). TRAF6 is a critical factor for dendritic cell maturation and development. *Immunity*, *19*(3), 353–363.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jäger, A., Strom, T. B., et al. (2007). IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*, *448*(7152), 484–487. doi:10.1038/nature05970

Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annual Review of Immunology*, *27*, 485–517. doi:10.1146/annurev.immunol.021908.132710

Kumamoto, Y., Linehan, M., Weinstein, J. S., Laidlaw, B. J., Craft, J. E., & Iwasaki, A. (2013). Dermal Dendritic Cells Drive T Helper 2 Cell-Mediated Immunity. *Immunity*, *39*(4), 733–743. doi:10.1016/j.immuni.2013.08.029

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., et al. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of Experimental Medicine*, *201*(2), 233–240. doi:10.1084/jem.20041257

Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S., & Lowry, R. P. (1992). Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *The Journal of Experimental Medicine*, *176*(4), 1215–1220.

Larsen, C. P., Steinman, R. M., Witmer-Pack, M., Hankins, D. F., Morris, P. J., & Austyn, J. M. (1990). Migration and maturation of Langerhans cells in skin

transplants and explants. *The Journal of Experimental Medicine*, *172*(5), 1483–1493.
Lathrop, S. K., Bloom, S. M., Rao, S. M., Nutsch, K., Lio, C.-W., Santacruz, N., et al. (2011). Peripheral education of the immune system by colonic commensal microbiota. *Nature*, *478*(7368), 250–254. doi:10.1038/nature10434
Lee, Y. K., Menezes, J. S., Umesaki, Y., & Mazmanian, S. K. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl 1*, 4615–4622. doi:10.1073/pnas.1000082107
Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Seminars in Cell & Developmental Biology*, *9*(6), 583–589. doi:10.1006/scdb.1998.0266
Lewis, K. L., Caton, M. L., Bogunovic, M., Greter, M., Grajkowska, L. T., Ng, D., et al. (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity*, *35*(5), 780–791. doi:10.1016/j.immuni.2011.08.013
Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., et al. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science (New York, NY)*, *324*(5925), 392–397. doi:10.1126/science.1170540
Lochner, M., Berard, M., Sawa, S., Hauer, S., Gaboriau-Routhiau, V., Fernandez, T. D., et al. (2011). Restricted Microbiota and Absence of Cognate TCR Antigen Leads to an Unbalanced Generation of Th17 Cells. *The Journal of Immunology*, *186*(3), 1531–1537. doi:10.4049/jimmunol.1001723
Longman, R. S., Diehl, G. E., Victorio, D. A., Huh, J. R., Galan, C., Miraldi, E. R., et al. (2014). CX3CR1+ mononuclear phagocytes support colitis-associated innate lymphoid cell production of IL-22. *The Journal of Experimental Medicine*, *211*(8), 1571–1583. doi:10.1084/jem.20140678
Lozupone, C., & Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005
Macpherson, A. J., Slack, E., Geuking, M. B., & McCoy, K. D. (2009). The mucosal firewalls against commensal intestinal microbes. *Seminars in Immunopathology*, *31*(2), 145–149. doi:10.1007/s00281-009-0174-3
Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., et al. (2006). Transforming growth factor-β induces development of the TH17 lineage. *Nature*, *441*(7090), 231–234. doi:10.1038/nature04754
Manicassamy, S., Reizis, B., Ravindran, R., Nakaya, H., Salazar-Gonzalez, R. M., Wang, Y.-C., & Pulendran, B. (2010). Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science (New York, NY)*, *329*(5993), 849–853. doi:10.1126/science.1188510
Mashayekhi, M., Sandau, M. M., Dunay, I. R., Frickel, E. M., Khan, A.,

Goldszmid, R. S., et al. (2011). CD8α(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity*, *35*(2), 249–259. doi:10.1016/j.immuni.2011.08.008

Maynard, C. L., Elson, C. O., Hatton, R. D., & Weaver, C. T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. *Nature*, *489*(7415), 231–241. doi:10.1038/nature11551

Mazzini, E., Massimiliano, L., Penna, G., & Rescigno, M. (2014). Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1^{+} macrophages to CD103^{+} dendritic cells. *Immunity*, *40*(2), 248–261. doi:10.1016/j.immuni.2013.12.012

McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., et al. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*, *483*(7389), 345–349. doi:10.1038/nature10863

Melton, A. C., Bailey-Bucktrout, S. L., Travis, M. A., Fife, B. T., Bluestone, J. A., & Sheppard, D. (2010). Expression of αvβ8 integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice. *The Journal of Clinical Investigation*, *120*(12), 4436–4444. doi:10.1172/JCI43786

Merad, M., Ginhoux, F., & Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nature Publishing Group*, *8*(12), 935–947. doi:10.1038/nri2455

Meredith, M. M., Liu, K., Darrasse-Jeze, G., Kamphorst, A. O., Schreiber, H. A., Guermonprez, P., et al. (2012). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *The Journal of Experimental Medicine*, *209*(6), 1153–1165. doi:10.1084/jem.20112675

Miller, J. C., Brown, B. D., Shay, T., Gautier, E. L., Jojic, V., Cohain, A., et al. (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nature Immunology*, *13*(9), 888–899. doi:10.1038/ni.2370

Moon, J. J., Chu, H. H., Hataye, J., Pagán, A. J., Pepper, M., McLachlan, J. B., et al. (2009). Tracking epitope-specific T cells. *Nature Protocols*, *4*(4), 565–581. doi:10.1038/nprot.2009.9

Moon, J. J., Chu, H. H., Pepper, M., McSorley, S. J., Jameson, S. C., Kedl, R. M., & Jenkins, M. K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*, *27*(2), 203–213. doi:10.1016/j.immuni.2007.07.007

Moussion, C., & Girard, J.-P. (2011). Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature*, *479*(7374), 542–546. doi:10.1038/nature10540

Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J. L., Munger, J. S., Kawakatsu, H., et al. (2002). The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *The Journal of Cell Biology*, *157*(3), 493–507. doi:10.1083/jcb.200109100

Muller, P. A., Koscsó, B., Rajani, G. M., Stevanovic, K., Berres, M.-L., Hashimoto, D., et al. (2014). Crosstalk between Muscularis Macrophages and Enteric Neurons Regulates Gastrointestinal Motility. *Cell*, *158*(2), 300–313. doi:10.1016/j.cell.2014.04.050

Munneke, J. M., Bjorklund, A. T., Mjosberg, J. M., Garming-Legert, K., Bernink, J. H., Blom, B., et al. (2014). Activated innate lymphoid cells are associated with a reduced susceptibility to graft versus host disease. *Blood*. doi:10.1182/blood-2013-11-536888

Murphy, K. M. (2011). Comment on “Activation of -Catenin in Dendritic Cells Regulates Immunity Versus Tolerance in the Intestine.” *Science (New York, NY)*, *333*(6041), 405–405. doi:10.1126/science.1198277

Naik, S. H., Perié, L., Swart, E., Gerlach, C., van Rooij, N., De Boer, R. J., & Schumacher, T. N. (2013). Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature*, 1–5. doi:10.1038/nature12013

Naik, S. H., Sathe, P., Park, H.-Y., Metcalf, D., Proietto, A. I., Dakic, A., et al. (2007). Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nature Immunology*, *8*(11), 1217–1226. doi:10.1038/ni1522

Nelson, R. W., McLachlan, J. B., Kurtz, J. R., & Jenkins, M. K. (2013). CD4+ T Cell Persistence and Function after Infection Are Maintained by Low-Level Peptide:MHC Class II Presentation. *The Journal of Immunology*, *190*(6), 2828–2834. doi:10.4049/jimmunol.1202183

Niess, J. H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B. A., et al. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science (New York, NY)*, *307*(5707), 254–258. doi:10.1126/science.1102901

Nikolich-Zugich, J., Slifka, M. K., & Messaoudi, I. (2004). The many important facets of T-cell repertoire diversity. *Nature Reviews Immunology*, *4*(2), 123–132. doi:10.1038/nri1292

Nurieva, R., Yang, X. O., Martinez, G., Zhang, Y., Panopoulos, A. D., Ma, L., et al. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*, *448*(7152), 480–483. doi:10.1038/nature05969

Onai, N., Obata-Onai, A., Schmid, M. A., & Manz, M. G. (2007a). Flt3 in regulation of type I interferon-producing cell and dendritic cell development. *Annals of the New York Academy of Sciences*, *1106*, 253–261. doi:10.1196/annals.1392.015

Onai, N., Obata-Onai, A., Schmid, M. A., Ohteki, T., Jarrossay, D., & Manz, M. G. (2007b). Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nature Immunology*, *8*(11), 1207–1216. doi:10.1038/ni1518

Ouyang, W., Oh, S. A., Ma, Q., Bivona, M. R., Zhu, J., & Li, M. O. (2013). TGF-β cytokine signaling promotes CD8+ T cell development and low-affinity CD4+ T cell homeostasis by regulation of interleukin-7 receptor α expression. *Immunity*, *39*(2), 335–346. doi:10.1016/j.immuni.2013.07.016

Pabst, O., & Mowat, A. M. (2012). Oral tolerance to food protein. *Mucosal Immunology*. doi:10.1038/mi.2012.4

Païdassi, H., Acharya, M., Zhang, A., Mukhopadhyay, S., Kwon, M., Chow, C., et al. (2011). Preferential expression of integrin αvβ8 promotes generation of regulatory T cells by mouse CD103+ dendritic cells. *Gastroenterology*, *141*(5), 1813–1820. doi:10.1053/j.gastro.2011.06.076

Palucka, K., Banchereau, J., & Mellman, I. (2010). Designing vaccines based on biology of human dendritic cell subsets. *Immunity*, *33*(4), 464–478. doi:10.1016/j.immuni.2010.10.007

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y.-H., et al. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature Immunology*, *6*(11), 1133–1141. doi:10.1038/ni1261

Perona-Wright, G., Mohrs, K., & Mohrs, M. (2010). Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. *Nature Immunology*, *11*(6), 520–526. doi:10.1038/ni.1866

Persson, E. K., Uronen-Hansson, H., Semmrich, M., Rivollier, A., Hägerbrand, K., Marsal, J., et al. (2013). IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity*, *38*(5), 958–969. doi:10.1016/j.immuni.2013.03.009

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., et al. (2013). Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity*, 1–14. doi:10.1016/j.immuni.2012.10.016

Poulin, L. F., Reyal, Y., Uronen-Hansson, H., Schraml, B. U., Sancho, D., Murphy, K. M., et al. (2012). DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. *Blood*, *119*(25), 6052–6062. doi:10.1182/blood-2012-01-406967

Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., et al. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8 + dendritic cells. *The Journal of Experimental Medicine*, *207*(6), 1261–1271. doi:10.1084/jem.20092618

Proctor, J. M., Zang, K., Wang, D., Wang, R., & Reichardt, L. F. (2005). Vascular development of the brain requires beta8 integrin expression in the neuroepithelium. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *25*(43), 9940–9948. doi:10.1523/JNEUROSCI.3467-05.2005

Reis e Sousa, C. (2006). Dendritic cells in a mature age. *Nature Reviews Immunology*, *6*(6), 476–483. doi:10.1038/nri1845

Reis e Sousa, C., Stahl, P. D., & Austyn, J. M. (1993). Phagocytosis of antigens by Langerhans cells in vitro. *The Journal of Experimental Medicine*, *178*(2), 509–519.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., et al. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Publishing Group*, *2*(4), 361–367. doi:10.1038/86373

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., & Austyn, J. M. (1995). Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *The Journal of Experimental Medicine*, *181*(6), 2237–2247.

Romani, N., Koide, S., Crowley, M., Witmer-Pack, M., Livingstone, A. M., Fathman, C. G., et al. (1989). Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *The Journal of Experimental Medicine*, *169*(3), 1169–1178.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., et al. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nature Biotechnology*, *19*(8), 746–750. doi:10.1038/90795

Sallusto, F., Cella, M., Danieli, C., & Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *The Journal of Experimental Medicine*, *182*(2), 389–400.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martínez, D., Hernanz-Falcón, P., et al. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature*, *458*(7240), 899–903. doi:10.1038/nature07750

Satpathy, A. T., Briseño, C. G., Lee, J. S., Ng, D., Manieri, N. A., KC, W., et al. (2013). Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nature Immunology*, *14*(9), 937–948. doi:10.1038/ni.2679

Satpathy, A. T., KC, W., Albring, J. C., Edelson, B. T., Kretzer, N. M., Bhattacharya, D., et al. (2012a). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *The Journal of Experimental Medicine*. doi:10.1084/jem.20120030

Satpathy, A. T., Wu, X., Albring, J. C., & Murphy, K. M. (2012b). Re(de)fining the dendritic cell lineage. *Nature Immunology*, *13*(12), 1145–1154. doi:10.1038/ni.2467

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H. C., et al. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. *The Journal of Experimental Medicine*, *196*(11), 1415–1425.

Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., et al. (2013). IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity*, *38*(5), 970–983. doi:10.1016/j.immuni.2013.04.011

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. doi:10.1128/AEM.01541-09

Scholz, F., Badgley, B. D., Sadowsky, M. J., & Kaplan, D. H. (2014). Immune mediated shaping of microflora community composition depends on barrier site. *PloS One*, *9*(1), e84019. doi:10.1371/journal.pone.0084019

Schraml, B. U., van Blijswijk, J., Zelenay, S., Whitney, P. G., Filby, A., Acton, S. E., et al. (2013). Genetic Tracing via DNGR-1 Expression History Defines Dendritic Cells as a Hematopoietic Lineage. *Cell*, *154*(4), 843–858. doi:10.1016/j.cell.2013.07.014

Schuler, G., & Steinman, R. M. (1985). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *The Journal of Experimental Medicine*, *161*(3), 526–546.

Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W., & Pabst, O. (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *The Journal of Experimental Medicine*, *206*(13), 3101–3114. doi:10.1084/jem.20091925

Semmrich, M., Plantinga, M., Svensson-Frej, M., Uronen-Hansson, H., Gustafsson, T., Mowat, A. M., et al. (2011). Directed antigen targeting in vivo identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic T-cell responses. *Mucosal Immunology*, 1–11. doi:10.1038/mi.2011.61

Shaw, M. H., Kamada, N., Kim, Y. G., & Nunez, G. (2012). Microbiota-induced IL-1 , but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *The Journal of Experimental Medicine*. doi:10.1084/jem.20111703

Sogin, M. L., Morrison, H. G., Huber, J. A., Mark Welch, D., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences of the United States of America*, *103*(32), 12115–12120. doi:10.1073/pnas.0605127103

Sonnenberg, G. F., Monticelli, L. A., Alenghat, T., Fung, T. C., Hutnick, N. A., Kunisawa, J., et al. (2012). Innate Lymphoid Cells Promote Anatomical Containment of Lymphoid-Resident Commensal Bacteria. *Science (New York, NY)*, *336*(6086), 1321–1325. doi:10.1126/science.1222551

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., et al. (2013). Innate lymphoid cells — a proposal for uniform nomenclature. *Nature Publishing Group*, *13*(2), 145–149. doi:10.1038/nri3365

Steinman, R. M., & Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *The Journal of Experimental Medicine*, *137*(5), 1142–1162.

Steinman, R. M., & Cohn, Z. A. (1974). Identification of a novel cell type in

peripheral lymphoid organs of mice. II. Functional properties in vitro. *The Journal of Experimental Medicine*, *139*(2), 380–397.
Steinman, R. M., & Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *75*(10), 5132–5136.
Steinman, R. M., Hawiger, D., & Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annual Review of Immunology*, *21*, 685–711. doi:10.1146/annurev.immunol.21.120601.141040
Steinman, R. M., Lustig, D. S., & Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *The Journal of Experimental Medicine*, *139*(6), 1431–1445.
Sun, C.-M., Hall, J. A., Blank, R. B., Bouladoux, N., Oukka, M., Mora, J. R., & Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *The Journal of Experimental Medicine*, *204*(8), 1775–1785. doi:10.1084/jem.20070602
Tacken, P. J., & Figdor, C. G. (2011). Seminars in Immunology. *Seminars in Immunology*, *23*(1), 12–20. doi:10.1016/j.smim.2011.01.001
Tacken, P. J., de Vries, I. J. M., Torensma, R., & Figdor, C. G. (2007). Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nature Publishing Group*, *7*(10), 790–802. doi:10.1038/nri2173
Takada, K., & Jameson, S. C. (2009). Naive T cell homeostasis: from awareness of space to a sense of place. *Nature Publishing Group*, *9*(12), 823–832. doi:10.1038/nri2657
Tamoutounour, S., Guilliams, M., Montanana Sanchis, F., Liu, H., Terhorst, D., Malosse, C., et al. (2013). Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity*, *39*(5), 925–938. doi:10.1016/j.immuni.2013.10.004
Tamoutounour, S., Henri, S., Lelouard, H., de Bovis, B., de Haar, C., van der Woude, C. J., et al. (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *European Journal of Immunology*, *42*(12), 3150–3166. doi:10.1002/eji.201242847
Thornton, A. M., Korty, P. E., Tran, D. Q., Wohlfert, E. A., Murray, P. E., Belkaid, Y., & Shevach, E. M. (2010). Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced Foxp3+ T Regulatory Cells. *The Journal of Immunology*, *184*(7), 3433–3441. doi:10.4049/jimmunol.0904028
Tubo, N. J., Pagán, A. J., Taylor, J. J., Nelson, R. W., Linehan, J. L., Ertelt, J. M., et al. (2013). Single Naive CD4+ T Cells from a Diverse Repertoire Produce Different Effector Cell Types during Infection. *Cell*, *153*(4), 785–796. doi:10.1016/j.cell.2013.04.007
Tussiwand, R., Lee, W.-L., Murphy, T. L., Mashayekhi, M., KC, W., Albring, J. C., et al. (2012). Compensatory dendritic cell development mediated by BATF-

IRF interactions. *Nature*, 1–9. doi:10.1038/nature11531
Ubeda, C., Lipuma, L., Gobourne, A., Viale, A., Leiner, I., Equinda, M., et al. (2012). Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *The Journal of Experimental Medicine*, *209*(8), 1445–1456. doi:10.1084/jem.20120504
Uematsu, S., Fujimoto, K., Jang, M. H., Yang, B.-G., Jung, Y.-J., Nishiyama, M., et al. (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nature Immunology*, *9*(7), 769–776. doi:10.1038/ni.1622
Uematsu, S., Jang, M. H., Chevrier, N., Guo, Z., Kumagai, Y., Yamamoto, M., et al. (2006). Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nature Publishing Group*, *7*(8), 868–874. doi:10.1038/ni1362
Van Maele, L., Carnoy, C., Cayet, D., Songhet, P., Dumoutier, L., Ferrero, I., et al. (2010). TLR5 signaling stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and mucosa. *Journal of Immunology (Baltimore, Md : 1950)*, *185*(2), 1177–1185. doi:10.4049/jimmunol.1000115
Vander Lugt, B., Khan, A. A., Hackney, J. A., Agrawal, S., Lesch, J., Zhou, M., et al. (2013). Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nature Immunology*, *15*(2), 161–167. doi:10.1038/ni.2795
Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., et al. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*, *31*(3), 502–512. doi:10.1016/j.immuni.2009.06.025
Veldhoen, M., Hocking, R. J., Flavell, R. A., & Stockinger, B. (2006). Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nature Immunology*, *7*(11), 1151–1156. doi:10.1038/ni1391
Voedisch, S., Koenecke, C., David, S., Herbrand, H., Förster, R., Rhen, M., & Pabst, O. (2009). Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar Typhimurium and limit systemic disease in mice. *Infection and Immunity*, *77*(8), 3170–3180. doi:10.1128/IAI.00272-09
Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., & Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *The Journal of Experimental Medicine*, *176*(1), 47–58.
Wang, X., Ota, N., Manzanillo, P., Kates, L., Zavala-Solorio, J., Eidenschenk, C., et al. (2014). Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. *Nature*, 1–16. doi:10.1038/nature13564
Waskow, C., Liu, K., Darrasse-Jeze, G., Guermonprez, P., Ginhoux, F., Merad, M., et al. (2008). The receptor tyrosine kinase Flt3 is required for dendritic

cell development in peripheral lymphoid tissues. *Nature Immunology*, *9*(6), 676–683. doi:10.1038/ni.1615
Watchmaker, P. B., Lahl, K., Lee, M., Baumjohann, D., Morton, J., Kim, S. J., et al. (2014). Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nature Immunology*, *15*(1), 98–108. doi:10.1038/ni.2768
Weiner, H. L., da Cunha, A. P., Quintana, F., & Wu, H. (2011). Oral tolerance. *Immunological Reviews*, *241*(1), 241–259. doi:10.1111/j.1600-065X.2011.01017.x
Wells, H. G., & Osborne, T. B. (1911). The Biological Reactions of the Vegetable Proteins. *The Journal of Infectious Diseases*, *8*(1), 66–124.
Welty, N. E., Staley, C., Ghilardi, N., Sadowsky, M. J., Igyarto, B. Z., & Kaplan, D. H. (2013). Intestinal lamina propria dendritic cells maintain T cell homeostasis but do not affect commensalism. *The Journal of Experimental Medicine*, *210*(10), 2011–2024. doi:10.1084/jem.20130728
Wendland, M., Willenzon, S., Kocks, J., Davalos-Misslitz, A. C., Hammerschmidt, S. I., Schumann, K., et al. (2011). Lymph node T cell homeostasis relies on steady state homing of dendritic cells. *Immunity*, *35*(6), 945–957. doi:10.1016/j.immuni.2011.10.017
Williams, J. W., Tjota, M. Y., Clay, B. S., Vander Lugt, B., Bandukwala, H. S., Hrusch, C. L., et al. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nature Communications*, *4*, 1–12. doi:10.1038/ncomms3990
Wirtz, S., Neufert, C., Weigmann, B., & Neurath, M. F. (2007). Chemically induced mouse models of intestinal inflammation. *Nature Protocols*, *2*(3), 541–546. doi:10.1038/nprot.2007.41
Worbs, T., Bode, U., Yan, S., Hoffmann, M. W., Hintzen, G., Bernhardt, G., et al. (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *The Journal of Experimental Medicine*, *203*(3), 519–527. doi:10.1084/jem.20052016
Worthington, J. J., Czajkowska, B. I., Melton, A. C., & Travis, M. A. (2011). Intestinal dendritic cells specialize to activate transforming growth factor-β and induce Foxp3+ regulatory T cells via integrin αvβ8. *Gastroenterology*, *141*(5), 1802–1812. doi:10.1053/j.gastro.2011.06.057
Wu, H.-J., Ivanov, I. I., Darce, J., Hattori, K., Shima, T., Umesaki, Y., et al. (2010). Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*, *32*(6), 815–827. doi:10.1016/j.immuni.2010.06.001
Wu, L., D'Amico, A., Winkel, K. D., Suter, M., Lo, D., & Shortman, K. (1998). RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. *Immunity*, *9*(6), 839–847.
Wu, Y., Cain-Hom, C., Choy, L., Hagenbeek, T. J., de Leon, G. P., Chen, Y., et al. (2011). Therapeutic antibody targeting of individual Notch receptors.

Nature, *464*(7291), 1052–1057. doi:10.1038/nature08878
Yang, Y., Torchinsky, M. B., Gobert, M., Xiong, H., Xu, M., Linehan, J. L., et al. (2014). Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature*, *510*(7503), 152–156. doi:10.1038/nature13279
Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., et al. (2007). Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. *The Journal of Cell Biology*, *176*(6), 787–793. doi:10.1083/jcb.200611044
Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., et al. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, *14*(3), 282–289. doi:10.1038/nm1720
Zhou, L., Ivanov, I. I., Spolski, R., Min, R., Shenderov, K., Egawa, T., et al. (2007). IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature Publishing Group*, *8*(9), 967–974. doi:10.1038/ni1488
Zigmond, E., Bernshtein, B., Friedlander, G., Walker, C. R., Yona, S., Kim, K.-W., et al. (2014). Macrophage-Restricted Interleukin-10 Receptor Deficiency, but Not IL-10 Deficiency, Causes Severe Spontaneous Colitis. *Immunity*, *40*(5), 720–733. doi:10.1016/j.immuni.2014.03.012