Activation and migration characteristics of intestinal dendritic cell subsets

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Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

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Ph.D. thesis

Agnès Garcias López

Preface

The presented work has been submitted to the Department of Health Technology at the Technical University of Denmark (DTU) in order to fulfill the requirements of the PhD degree. The presented research was conducted first at the Veterinary Institute, later at the Micro-and Nanotechnology Department and finally at the Department of Health Technology. The work was carried out from November 2016 to October 2019 under the supervision of Associate Professor Katharina Lahl and Associate Professor Vasileios Bekiaris. In addition, three months of research within the stated period were conducted at the Monash Biomedicine Discovery Institute, Melbourne, Australia, at Associate Professor Meredith O'Keeffe's lab.

This thesis consists of an introduction to the research topic, followed by the main manuscript together with complementary results, discussion and conclusion of the major findings of this work. Finally, a brief description of future perspectives is included.

Agnès Garcias López, October 31st, 2019

Abstract

The intestine is continuously challenged to generate protective immunity against harmful antigens, such as pathogens, and tolerance against harmless materials, such as food. Dendritic cells (DCs) are key regulators of innate and adaptive immune responses and play important roles in the generation of immunity to intestinal antigens. DCs acquire antigen in the periphery and migrate to draining lymph nodes where they prime immune responses. Different DC subsets differ in their capacity to induce distinct immune responses and this is thought to be in part due to differential expression of pattern recognition receptors (PRRs). Targeting specific DC subsets has been exploited in vaccination strategies in order to develop more efficient, targeted therapies. However, little is known about the specific requirements for activation and migration of different DC subsets.

Herein, we hypothesized that distinct intestinal DC subsets differ in their migratory patterns following stimulation with poly(I:C), a synthetic analog of dsRNA signaling through TLR3. Although TLR3 is highly expressed in cDC1, poly(I:C) induced migration of cDC1 and cDC2 equally in a cell-extrinsic, TLR3 dependent manner. TLR3 activation by poly(I:C) induced early expression of pro-inflammatory cytokines, including type I IFNs, TNF-α and IL-1β. By using different genetic mouse models, we found that TNF-α was required for migration of both cDC1 and cDC2 in response to poly(I:C). However, we also detected a previously unrecognized role for intrinsic type I IFN signaling in cDC1 but not cDC2 activation and migration in response to poly(I:C). Stimulation with R848, a TLR7 ligand, showed similar results, suggesting type I IFN as a signal required for activation and migration of cDC1 in response to different TLR ligands. In contrast, IL-1β signaling was dispensable for migration. In addition, we found that pDCs were not required as a cellular source for type I IFN and TNF-α in the context of poly(I:C) injection. Preliminary studies suggest a role for macrophages instead, but future experiments are needed to confirm these facts.

Collectively, these findings suggest distinct requirements for migration of DC subsets in response to poly(I:C). Future experiments assessing the functionality of *cis* vs *trans*-activated DCs will elucidate whether the observed differences on DC subset migration translate into different immune responses.

Dansk resumé

Tarmene bliver konstant udfordret af fremmede antigener og er derfor nød til at generere beskyttende immunitet mod skadelige antigener såsom patogener, og tolerance mod ufarlige antigener såsom fødevare. Dendritceller (DC'er) spiller en stor rolle i reguleringen af både det uspecifikke og specifikke immunrespons, og har stor betydning for dannelsen af immunitet overfor antigener i tarmen. DC'er optager antigener i periferien og migrerer derefter til de drænende lymfeknuder, hvor de igangsætter det primære immunrespons. Forskellige undergrupper af DCer adskiller sig i deres evne til at inducere forskellige immunresponser. Dette menes at være delvis på grund af forskelle i udtrykkelsen af "pattern recognition receptors" (PRR'er). Man har udnyttet, at man kan ramme specifikke DC-undergrupper i vaccinationsstrategier til at udvikle mere effektive og målrettede behandlingsformer, dog er der meget begrænset viden om de specifikke krav til aktivering og migrering af forskellige DC-undergrupper.

Heri antog vi, at forskellige DC-undergrupper i tarmen varierer i deres migrationsmønstre efter stimulering med poly(I: C), en syntetisk analog af dsRNA, som signalerer gennem TLR3. Selvom TLR3 er højt udtrykt i cDC1, inducerede poly(I: C) tilsvarende migrering af både cDC1 og cDC2, begge afhængigt af ekstern TLR3 signalering. Aktivering af TLR3 ved hjælp af poly(I: C) inducerede i første omgang ekspression af pro-inflammatoriske cytokiner, så som type I interferoner (IFN'er), TNF-α og IL-1β. Ved brug af forskellige genetiske musemodeller fandt vi at TNF-α var nødvendig for migrering af både cDC1 og cDC2 som et respons på poly(I: C) stimulering. Derudover opdagede vi en endnu ikke beskrevet rolle for type I IFN-signalering i aktivering og migrering af cDC1, men ikke cDC2, i respons på poly(I: C). Stimulering med R848, en TLR7-ligand, gav lignende resultater, hvilket antyder at type I IFN-signalering er nødvendig for aktivering og migrering af cDC1 som respons på forskellige TLR-ligander. I modsætning til dette var IL-1β-signalering ikke nødvendig for migration. Derudover fandt vi at pDC'er ikke var påkrævet som en cellulær kilde til type I IFN og TNF- α i forbindelse med poly(I: C) injektion. Indledende forsøg antyder en rolle for makrofager i stedet, men fremtidige eksperimenter er nødvendige for at bekræfte dette.

Samlet set tyder disse fund på forskellige krav til migrering af DC-undergrupper som respons på poly(I: C). Fremtidige eksperimenter, der vurderer funktionaliteten af *cis* vs *trans*-aktiverede DC'er, vil undersøge, om de observerede forskelle på migrering af DC-undergrupper bliver omsat til forskellige immunresponser.

Acknowledgements

What a journey! This thesis contains a lot of love from all the amazing people that have helped me through, so be prepared for a long list of names.

I want to start acknowledging my supervisor, Katharina Lahl. This journey started in a Danish class, and I think that was the best outcome one could get from taking a language course. I am very thankful for the opportunity you have given to me, where I have been able to explore science at its finest, from hating it to loving it on the same day. Thanks for dealing with all my emotions, certainly not easy, and keep pushing me to get the best out of me. I need to thank you for always being at the other side of the phone, helping me fix Fortessas in the middle of the night. Starting in a new group has come with challenges, but I can only be grateful for the freedom you have given to me during my project as well as the opportunity to go to many international conferences. Lastly, I thank you for taking me as your first PhD student, and I hope I will be an example of how (maybe not) to do a PhD for future generations.

I would also like to thank the members of my group Katrine, Anna, Xiao and Isabel. Katrine, thanks for all the help with the mice –and everything else. Skål to the most fun island of the lab! Anna, thanks for being my reference at the beginning of my PhD; I learned a lot from being next to you, certainly missing our joined experiments. Isabel, the latest acquisition of our lab: woho! I can just say how sad I am that you had to start just when I finish, please let's plan it better for future collaborations! Thanks for being here the last 5 months; you've been helping both professionally and personally. To all of the Lahl group at both sides of the bridge: keep it up, I am sure we are taking off soon!

I want to thank the groups of William Agace, Bengt Johansson and Vasileios Bekiaris for fruitful discussions in our joined lab meetings. To Nicole and Telma, who were very kind at giving me the first round of feedback on the very early draft of my thesis. Nicole, thank you so much for inspiring me and always having the most accurate answers to all my questions. I need to thank my favorite PhD crew: Simone, John and Rasmus. I love working in the lab together, sharing frustrations, listening to amazing music and having the best time. Who said being a PhD is not fun? Simone, thanks for that lunch break that brought us together, I really enjoy our conversations and how we understand each other. Please keep being as cheery and fun. Rasmus, my type I IFN buddy! Thanks for all your expertise on mouse stuff and being so helpful and kind any time I freaked out - often actually…To John, thank you for all your wisdom and the incredible help with setting up my qPCRs. Thank you for being always available and answering any of my questions. Special thanks to Darshana for being my mentor in teaching me most of my lab skills. Thank you thank you thank you!

To Amalie, Jeppe, Nadia and Sara for sharing the drama and the beauty of the PhD journey. Thanks for all the conversations as well as the fun parties.

To my most beloved ones. I would like to start thanking my family for supporting me at every single step I've taken so far. I wish I could be closer to you, but I guess a 3h flight is not as bad. To my girls from Mallorca: Joana, Laura, Maria and Marta, for being family and understanding, supporting and never judging. I love you. Special mention to Marta, who I admire and look up as a scientist. Thanks for all your love through this process.

To my tipas molonas: Anna, Barbara, Cris, Effy, Elia, Julia, Lea, Lucia and Maria, thanks for understanding and taking me out whenever I needed it. It has been very difficult for me to cut my social life to focus on my writing, and you ladies have made this very smoothly by helping me all the way. A special mention goes to Cris and Lucia. Cristinita: thanks again for your support and help through the writing process. You are always there, no matter the distance. Lucia, the author of all the beautiful figures presented in this thesis as well as the front page. Thank you for being involved in the process and wanting to be part of such a special thing for me. You cannot imagine how much this means to me. For all the GAES moments, laughs and cries together, I am immensely thankful. Os quiero mucho.

Special thanks to Johan Musaeus Vøgg Bruun for his guidance and support through the PhD. You are probably the person who has understood me the most regarding the emotions involved in the PhD. Thank you so much for sharing and understanding such feelings. Not least, thank you for your help on the statistics.

To a very special person in my life: Carme Riera. My partner in crime, who understands me like no one and supports me through every single step I take. Because we can do everything we want. T'estim infinit.

Finally, to my boyfriend Johan Pedersen. Mi amor, you have been a tremendous support through the whole process, in all aspects. Thanks for understanding me, supporting me and taking care of everything else so I could fully focus in my writing. Thanks for believing in me and making me believe in myself, and for always bringing the best Agnès out. I think we will make great things together and I cannot wait to start this new chapter with you. I love you.

To all the people that have been part of it, I thank you all. I am immensely grateful.

Love,
Agnès Garcias López, (soon to be) PhD

List of abbreviations

Ag	Antigen
AID	Activation-induced cytidine deaminase
AIDS	Acquired Immune Deficiency Syndrome
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
AMP	Antimicrobial peptides
APRIL	A proliferation-inducing ligand
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine tri-posphate
BAFF	B-cell activating factor
Bcl-6	B-cell lymphoma 6
BCR	B-cell receptor
BCZ	B cell zone
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
CARD	Caspase recruitment domains
CCL	Chemokine (C-C motif) ligand
cDC	Classical dendritic cell
CDP	Common DC precursors
cGAMP	Cyclic-GMP-AMP
cGAS	cGAMP synthase
CLR	C-type lectin receptors
cMoP	Common monocyte precursors
CSF-2	Colony stimulating factor 2
CSF2R	CSF-2 receptor
CSR	Class-switch recombination
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
CXCR	C-X-C Motif Chemokine Receptor
DAMP	Damaged-associated molecular pattern
DC	Dendritic cell
d-KO	double Knock Out
DN	Double negative
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DT	Diptheria Toxin
ER	Endoplasmic reticulum
FAE	Follicle-associated epithelium
FcR	Fc receptors
Flt3L	FMS-like tyrosine kinase 3 ligand

FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GAP	Goblet cell passages
GC	Germinal center
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine tri-phosphate
HEV	High endothelial venues
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
i.p.	Intraperitoneally
IBD	Inflammatory bowel disease
ID2	Inhibitor of DNA binding 2
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN	Interferon
IFR	Interfollicular region
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicles
iNOS	Inducible nitric-oxide synthase
IRF	IFN regulatory factor
ISG	IFN stimulated genes
Klf4	Kruppel-like factor 4
KO	Knockout
LEC	Lymphatic endothelial cells
LGP2	Laboratory of genetics and physiology 2
LI	Large intestine
LN	Lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTi	Lymphoid tissue inducer
M cells	Microfold cells
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation-associated protein 5
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
MLR	Mixed-leukocyte reaction

MNP	Mononuclear phagocytes
mRNA	Messenger ribonucleic acid
mTNF-α	Transmembrane TNF-α
MyD88	Myeloid differentiation primary response 88
NET	Neutrophil extracellular trap
NFIL3	Nuclear Factor interleukin 3 regulated
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
Notch2	neurogenic locus notch homolog protein 2
nTreg	Natural T regulatory cell
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PGE2	Prostaglandin E_2
pIgR	Polymeric Ig receptor
poly(I:C)	Polyinosinic:polycytidylic acid
PPs	Peyer's patches
pre-μDCs	Pre mucosal DCs
PRR	Patern recognition receptor
pTreg	Peripherally-differentiated T regulatory cell
RA	Retinoic acid
RALDH1	Retinaldehyde dehydrogenase 1
Rbp-J	Recombination signal binding protein for immunoglobuling kappa J
REGIIIγ	Regenerating islet-derived protein 3 gamma
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RORγt	Retinoic-acid-receptor-related orphan nuclear receptor gamma
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SED	Subepithelial dome
SEM	Standard error mean
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutations
SI	Small intestine
sIgA	Secretory IgA
SILT	Solitary intestinal lymphoid tissue
SPF	Specific-pathogen-free
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
sTNF-α	Soluble TNF-α
SAA	Serum amyloid A
TACE	TNF-α-converting enzyme

TCR	T-cell receptor
TCZ	T cell zone
TD	T cell-dependent
TED	Transepithelial dendrites
Tfh	T follicular helper cell
tg	Transgenic
TGF	Transforming growth factor
TGFβRII	TGF-β receptor
Th	T helper
TI	T cell-independent
Tip-DC	TNF and iNOS-producing DC
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
VAD	Vitamin A deficient
WT	Wild type
Zbtb46	Zinc finger transcription factor zDC
Zeb2	Zinc Finger E-Box Binding Homeobox 2

List of figures

All the figures in this thesis were produced by the author, except whenever stated.

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1. Background

1.1 The intestine

Architecture of the gut

The intestine is a continuous tube that expands from the end of the stomach – pylorus – to the anus and is broadly subdivided into the small (SI) and large (LI) intestine. These two different regions differ in their anatomy because they have distinct physiological functions. The SI is responsible for food digestion and absorption of nutrients, while the LI is where water reabsorption occurs.

Small intestine

The SI is the first section of the intestinal tract, placed between the pylorus and the ileocecal valve. It is divided into three main segments: the duodenum, the jejunum and the ileum (Figure 1). The duodenum and the jejunum are characterized by finger-like projections termed villi. On top, a layer of absorptive epithelial cells covers the villi with membrane projections called microvilli. This "brush border" contains enzymes needed for digestion and increases the surface area of the SI to 30 m^2 [2]. Hence, the SI is the major digestion and nutrient absorption site. The ileum, with shorter villi, has a lesser role in nutrition.

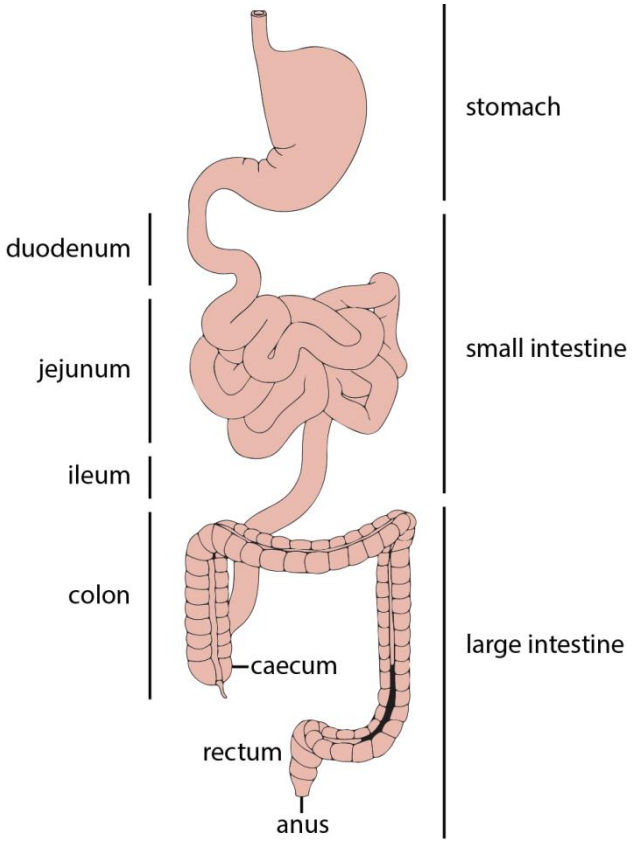

Figure 1. The intestinal tract. The intestinal tract consists of a long tube divided into SI and LI. The SI starts at the end of the stomach and consists of the duodenum, the jejunum and the ileum. The LI consists of the caecum, the colon (ascending and transversal), rectum and finishing at the anus[1].

Large intestine

The undigested contents from the SI then enter the LI. The main segments of the LI are caecum, colon, rectum and anus (Figure 1). The LI lacks a brush border and its main role is water reabsorption and elimination of indigestible foodstuffs. Moreover, the LI constitutes a complex reservoir of beneficial microorganisms, known as commensal microbiota. These commensals contribute to nutrition of the host by fermenting indigestible carbohydrates such as dietary fibers to absorbable metabolites such as short-chain fatty acids (SCFA). Additionally, commensals provide protection by competing for niches with pathogens.

Intestinal wall

The intestinal tract consists of distinct layers (Figure 2). The mucus protects the mucosa, which is the layer in direct contact with the lumen. The mucosa consists of a single cell layer of specialized intestinal epithelial cells termed epithelium, an underlying connective tissue called lamina propria (LP) and a thin muscle layer named muscularis mucosa. The LP functions as scaffold for the villi and contains blood supply, lymph drainage and nervous system for the mucosa. The LP also contains large amounts of immune cells. The muscularis mucosa separates the LP to the submucosa, which consists of a highly vascularized connective tissue. Finally, the serosa is the thick fibrous layer that separates the intestine from the peritoneal cavity.

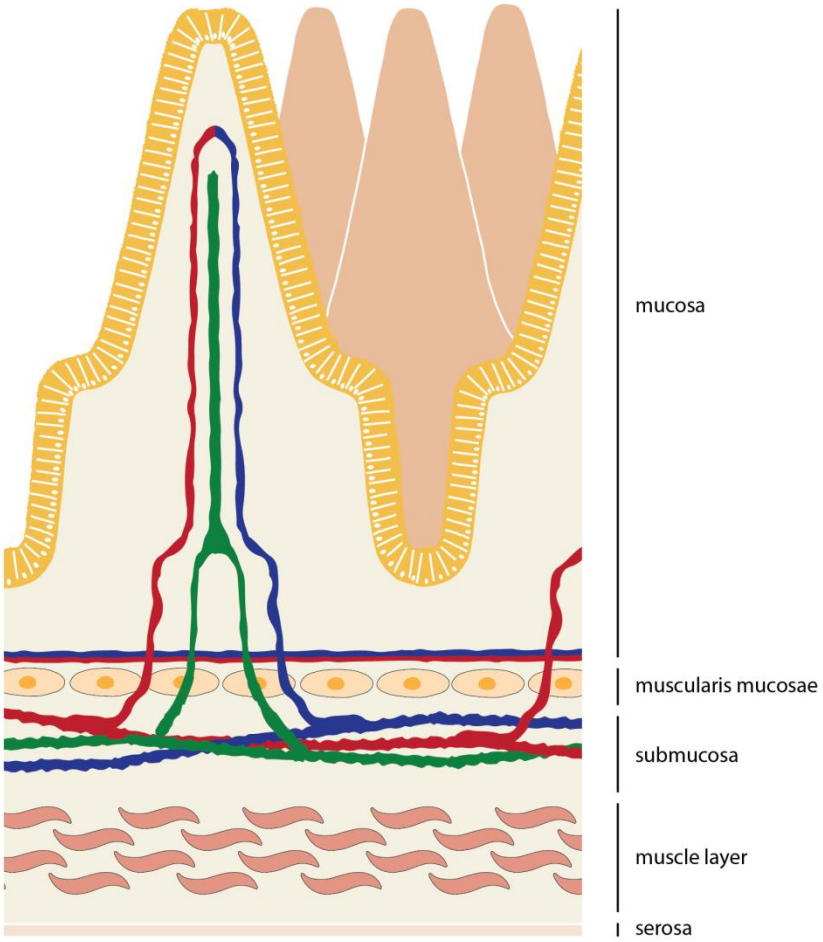

Figure 2. The intestinal wall. The intestinal mucosa is in direct contact with the lumen and consists of the epithelium, the LP and the muscularis mucosa. Below the muscularis mucosa, we find the submucosa, which is highly vascularized, providing with blood and lymphatic supply. The underlying muscle layer is responsible for the peristaltic movements to transport the food along the intestine. Finally, the serosa is the layer of tissue separating the intestine from the peritoneal cavity.

Tolerance vs Immunity

The intestine is continuously exposed to foreign antigens from the daily-ingested food as well as from the community of commensal bacteria. As a consequence, the intestine is constantly challenged to generate tolerance against harmless materials and protective immunity against harmful antigens. Tolerance needs to be generated towards nutrients as well as the commensal bacteria, which consists of approximately 10^{14} microorganisms from around 500 different species[1]. At the same time, the thin epithelial layer is constantly self-renewed. However, the exposure of the intestine to the outside increases the risk of pathogen entry and the immune system needs to establish protective immunity against it. Therefore, the intestine contains a large number of both innate and adaptive immune cells that allow for the generation of the proper immune response tailored to the specific Ag.

1.2 The intestinal immune system

In higher animals, the immune system consists of two arms: the innate and the adaptive. The innate immune system, evolutionary ancient, provides immediate protection by detecting invariant features of invading microbes. Contrarily, the adaptive immune system is temporally delayed as it uses antigen-specific receptors that are newly generated. It is the combination of both the innate and the adaptive immune systems that recognize and eliminate invading pathogens with the maximal efficacy and minimal self-damage, as well as leading to protection from re-infection by the same pathogen (Figure 3)[3].

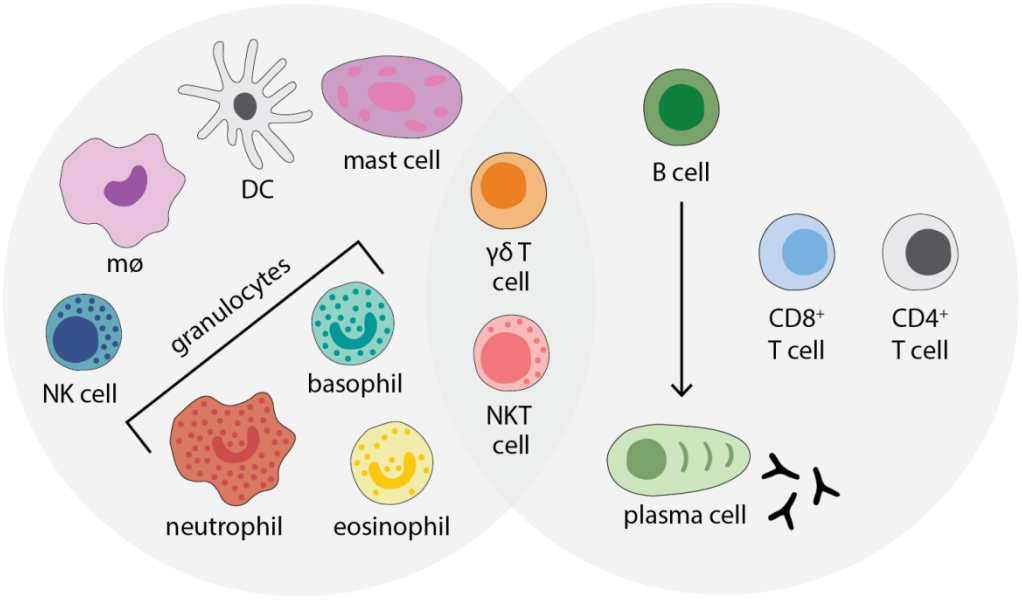

Figure 3. The innate and adaptive immune system. The innate immune system is the first line of defense. The innate immune cells include dendritic cells (DC), macrophages (mø), Natural Killer (NK) cells, mast cells and granulocytes (neutrophils, basophils and eosinophils). The adaptive immune system is delayed and consists of B and T cells which carry highly antigen-specific receptors. Although Natural Killer T (NKT) cells and γδT cells are developed as cells of the adaptive immune system, they function as innate-like cells, hence their placement at the interface.

Innate immune system

The innate immune system is the first line of defense upon pathogen invasion. It is triggered by pattern recognition receptors (PRRs) that serve as sensors of common microbial structures known as pathogen-associated molecular patterns (PAMPs)[4]. Additionally, these receptors can sense damaged-associated molecular patterns (DAMPs) produced by a cell in response to injury. PRRs are expressed mainly by hematopoietic immune cells. However, non-immune cells such as non-hematopoietic mesenchymal stromal cells or epithelial cells can also express PRRs[5,6]. The activation of the innate immune system happens quickly, within minutes to hours after infection. It detects the nature of infection, provides the first line of host defense and determines the class of adaptive immune response to be initiated. The cells contributing to innate immunity in the gut are described below.

Intestinal epithelial cells (IECs): the intestinal epithelium is a single cell layer that separates the lumen from the LP. Epithelial cells are increasingly recognized as contributors to immune regulation as they express a vast range of innate immune receptors that are pivotal for intestinal homeostasis[7–10]. Several subsets of IECs comprise the intestinal epithelium, and they appear to play distinct immune functions (summarized in Figure 4). For example, goblet cells produce mucus, a gel-like substance that covers the intestinal epithelium and acts as a chemical barrier to trap and prevent bacteria from direct contact to the epithelium. Germ-free mice have very poor mucus production but toll-like receptor (TLR) ligands restores the mucus production, suggesting a major role of microbial sensing in mucus production[11,12]. In addition, Paneth cells are the major producers of antimicrobial peptides (AMP) such as lysozyme, defensins and regenerating islet-derived protein 3 gamma (REGIIIγ), and their production is largely dependent on the intrinsic expression of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and myeloid differentiation primary response 88 (MyD88)[13,14]. Production of REGIIIγ is also dependent on interleukin (IL)-22, which is produced by innate lymphoid cells (ILCs) and $CD4^+$ T cells in response to pathogen invasion[15]. These studies confirm that microbial recognition by IECs is essential for intestinal immune homeostasis.

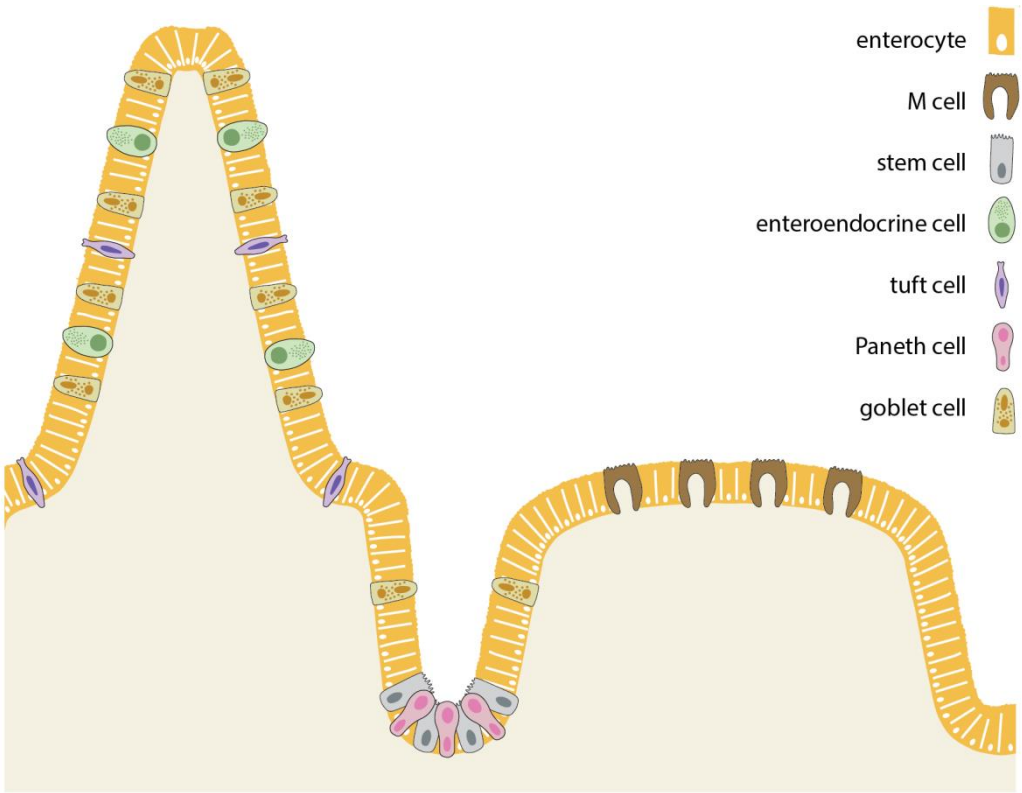

Figure 4. The intestinal epithelium. Several types of epithelial cells constitute the intestinal epithelium and all differentiate from the stem cells present at the crypt. Paneth cells are responsible for protecting the stem cell niche by production of AMP; goblet cells produce mucus; tuft cells produce IL-25 in response to parasitic infections; enterocytes are responsible for nutrient absorption; microfold (M) cells are specialized epithelial cells present at the follicle-associated epithelium (FAE). Enteroendocrine cells secrete hormones that play crucial roles in regulating processes such as control of glucose levels, food intake and stomach emptying.

Innate lymphoid cells (ILCs): recently described as lymphocyte populations of the innate immune system, ILCs also populate the intestinal mucosa. ILCs lack recombined antigen-specific receptors but rapidly respond to inflammatory mediators produced by IEC or cells of the myeloid lineage, such as macrophages and dendritic cells (DCs). Although previous studies have shown the limited expression of PRRs by ILCs, recent studies have proven that ILCs can express functional PRRs such as TLR3[16,17]. ILCs are classified into three distinct groups that mirror those of effector T helper (Th) cells from the adaptive immune system: group 1, 2 and 3 ILCs. Accordingly, group 1 ILCs (ILC1) are dependent on transcription factor T-bet and produce interferon (IFN)-γ, and are generally involved in the clearance of intracellular pathogens[18,19], like Th1 cells. ILC1 additionally include classical natural killer (NK) cells. Mirroring Th2 cells, group 2 ILCs (ILC2) express the transcription factor GATA3 and secrete IL-5 and IL-13 in response to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which are secreted by IEC and thought to be involved in the clearance of parasitic infections[18,20]. Lastly, group 3 ILCs (ILC3) include lymphoid tissue inducer cells (LTi), responsible for secondary lymphoid tissue organogenesis during embryogenesis, and those ILCs analogous to the Th17 cell lineage. ILC3 depend on the expression of transcription factor retinoid-related orphan receptor gamma t (ROR-γt) and produce cytokines IL-17A and/or IL-22 in response to IL-23, IL-1α and IL-1β, among others[18]. Production of IL-22 by a subset of ILC3s plays a crucial role during intestinal *Citrobater rodentium* infection[15,21]. Taken together, ILCs are key players in maintaining intestinal homeostasis[22–24].

Mononuclear phagocytes (MNP): MNP consist of intestinal macrophages and classical DCs (cDCs), both known to be involved in antigen sampling and presentation. Distinction of these populations has been controversial, since they share the expression of surface markers such as major histocompatibility complex (MHC)-II and CD11c[25]. However, recent studies have shown that the surface molecule CD64 is expressed in intestinal macrophages and absent in cDCs[26], leading CD64 to be a useful marker to identify intestinal macrophages[27]. Accordingly, intestinal macrophages can now be more accurately identified by the combined expression of F4/80, CD64 and CX3CR1[28]. In contrast, DCs express high levels of CD11c and MHC-II and, although some subsets can express intermediate levels of CX3CR1, they lack F4/80 and CD64[29]. As DCs are the main subject of this thesis, they will be discussed in detailed in Chapter 1.3.

Intestinal macrophages are the most abundant MNP in healthy intestinal LP and represent the largest pool of macrophages in the body[30]. As major phagocytic cells, the main function of macrophages is to engulf and clear pathogens, cellular debris and bacterial products as well as production of mediators for epithelial cell renewal[31]. In steady state, they are constantly replenished by the Ly6C^{hi} blood monocytes which undergo a local differentiation into tissue-resident macrophages, where they lose Ly6C^{hi} and upregulate F4/80, CD64, CX3CR1 as well as CD11c and MHC-II, becoming highly phagocytic (Figure 5)[25,26]. Additionally, tissue-resident macrophages secrete IL-10, an anti-inflammatory cytokine that plays a pivotal role in maintaining intestinal homeostasis. IL-10 induces survival and expansion of T regulatory (Treg) cells and it is responsible for the hyporesponsiveness of macrophages to TLR ligands[28,32–34]. Accordingly, IL-10 receptor deficiency leads to severe spontaneous colitis in mice and is responsible for a type of early onset of

inflammatory bowel disease (IBD) in children[35,36]. In addition, macrophages sense intestinal microbes by MyD88 and NLRC4 inflammasome, which leads to production of IL-1β. Macrophage-derived IL-1β in steady state mantains intestinal homeostasis by maintenance of Th17 cells and crosstalk with ILC3[37–39]. Under inflammatory conditions, however, blood-derived monocytes accumulate at the site of inflammation and respond to TLR ligands and pro-inflammatory cytokines (Figure 5). In physiological conditions, macrophages do not migrate to mesenteric lymph nodes (mLNs) and are unable to prime naïve $CD4^+$ T cells[33,40]. However, some monocyte-derived MNPs can upregulate C-C chemokine receptor type 7 (CCR7) and migrate to lymph nodes during certain inflammatory conditions, a topic under intensive investigation[41]. Collectively, macrophages play crucial roles in maintaining intestinal homeostasis.

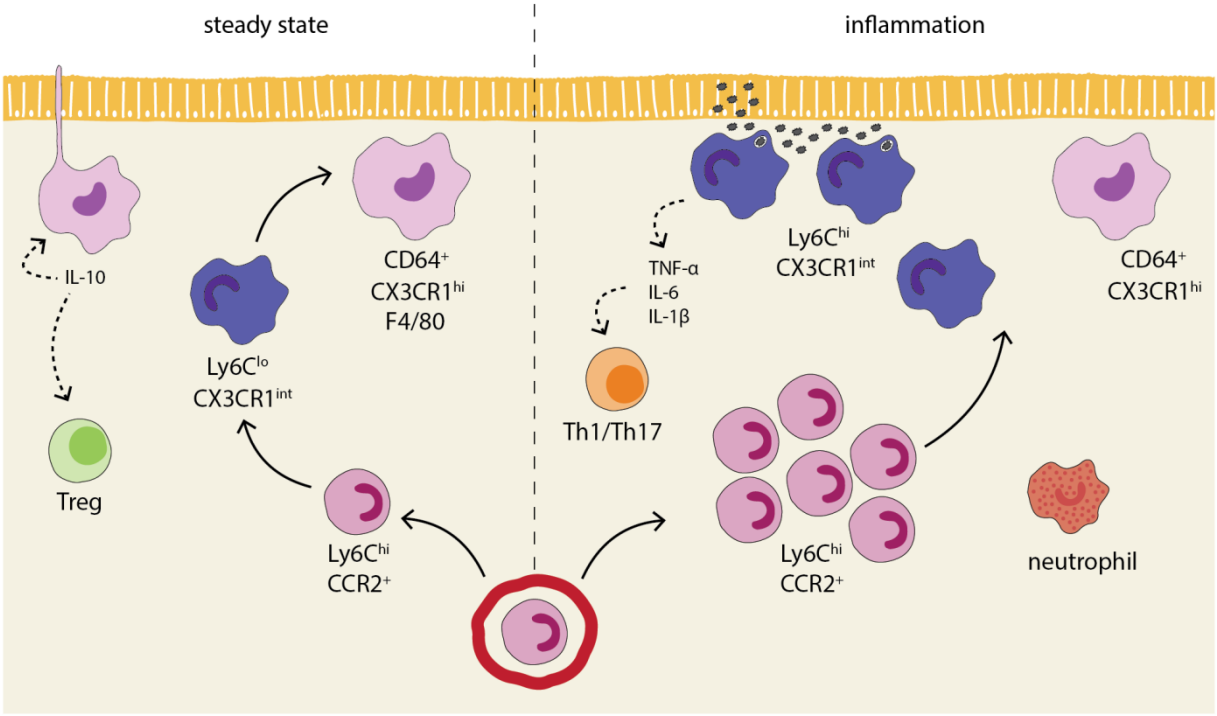

Figure 5. Intestinal macrophages in health and inflammation. In steady state, intestinal macrophages are constantly replenished by blood-born monocytes ($Ly6C^{hi}CCR2^+$). Once in the intestine, they undergo a local differentiation into tissue-resident macrophages, characterized by loss of Ly6C and upregulation of CD64, F4/80 and CX3CR1. Production of IL-10 by macrophages maintains them hyporesponsive to TLR ligands in an autocrine manner and maintains a population of Tregs. Under inflammatory conditions, there is an increased recruitment of monocytes that differentiate into $CX3CR1^{int}$ cells, which are responsible for pro-inflammatory cytokine production and recruitment of other immune cells such as neutrophils.

Plasmacytoid DCs (pDCs): pDCs are fully differentiated in the bone marrow (BM) and seed the intestine in a CCR9-dependent manner. In contrast to the general role of type I IFN production that characterizes pDCs, intestinal pDCs produce only low levels of type I IFN at steady-state due to the unique intestinal microenvironment, characterized by the presence of tolerogenic mediators such as IL-10, transforming growth factor (TGF)-β and prostaglandin E_2 (PGE_2)[42]. In contrast, intestinal pDCs have been shown to induce oral tolerance by triggering differentiation of Treg cells[43]. In addition, pDCs can also trigger immunoglobulin (Ig) A induction in a T cell independent pathway[44].

Unlike DC, pDCs do not migrate to the mLNs, but can drive DC migration in response to TLR ligands via production of by Type I IFN and tumor necrosis factor (TNF)-α[45,46]. In addition, they are thought to play protective roles to commensal bacteria such as *B. fragilis* as well as in food allergy[47–49].

Granulocytes: although usually associated with allergy and parasitic infections, granulocytes are also present in healthy intestine. Eosinophils account for up to 30% of the myeloid population and they are thought to play important roles in tissue repair[28,50]. Mast cells represent 2-3% of total cells in human intestinal LP and they produce mediators involved in epithelial barrier integrity and peristalsis[51–53]. They seem to play a role in interaction with the enteric nervous system as well as tissue remodeling[51–53]. In contrast, neutrophils are present in low numbers in healthy gut but increase upon pathogen invasion. The main role of neutrophils is to kill microbes invading the mucosa and to prevent their systemic spread by the release of degradative enzymes, production of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs), composed of chromatin laced with antimicrobial peptides. NETs released by neutrophils can trap and kill extracellular bacteria such as *Shigella spp*[54–56]. Of note, neutrophils can also contribute to tissue damage during infection[56].

Adaptive immune system

Initiation of the adaptive immune response is a slower process than that of innate immunity. This is mainly because it relies on antigen (Ag)-specific recognition from a highly diverse repertoire of receptors, requiring Ag-specific clonal expansion before contributing to immunity. Adaptive immunity is mediated by B and T cell lymphocytes, and the receptors are called B-cell receptor (BCR) and T-cell receptor (TCR), respectively.

B and T cells develop in the bone marrow and thymus, respectively. While B cells can directly recognize and bind Ags through the BCR, T cells recognize Ags through the TCR only if presented on a MHC molecule. The BCR consists of a membrane-bound Ig, formed by a pair of heavy and light chains, as a result of a random rearrangement of Ig subgenes. Activated B cells in germinal centers (GC) express activation-induced cytidine deaminase (AID) enzyme, responsible for creating somatic hypermutations (SHM) that will lead to highly specific antibodies against pathogens[57]. In addition, Ag recognition promotes class-switch recombination (CSR) of the constant region to tailor the effector response to the Ag encountered (leading to IgM, IgD, IgG, IgA or IgE). By contrast, TCR specificity remains static after the DNA rearrangement in the thymus[58].

Inductive sites

The inductive sites of the intestine are the main location where priming of adaptive immune responses occurs. They consist of organized lymphoid structures including the mLNs and the gut-associated lymphoid tissues (GALT), which comprise solitary intestinal lymphoid tissue (SILT, including mature isolated lymphoid follicles (ILFs) and colonic patches) and Peyer's patches (PPs).

mLNs: The mLNs consist of a chain of lymph nodes draining the SI and colon. Distinct nodes of the mLNs are known to drain different segments. While the central nodes of the mLNs have shown to drain the SI, the most distal node from the mLN chain together with another node at the opposite end of the mLNs drain the colon[59]. In contrast, the transverse colon and the descending colon together with the rectum are drained by the duodenopancreatic and the caudal lymph node, respectively[59].
The segregation of mLNs draining different sections of the intestine likely provides a mechanism by which immune responses in the distinct sites of the intestine can be independently controlled. Accordingly, a recent study has shown that different nodes within mLNs are immunologically specific to the site they drain by containing distinct subpopulations of stromal cells and DCs[60].

GALT: The gut-associated lymphoid tissues are lymphoid tissue aggregates that lie at the mucosa and submucosa. They contain B cells, T cells and DCs similarly to lymph nodes, but they lack encapsulation. DCs in GALT lie in a region called subepithelial dome (SED), which is separated from the lumen by a follicle-associated epithelium (FAE). The FAE contains specialized epithelial cells, called microfold (M) cells, which lack microvilli and enzymes but instead take up and transport Ags from the lumen to the underlying DCs (Figure 6)[1]. However, M cells are also the site of entry of many pathogens such as *Salmonella*, *Yersinia* and some viruses[61,62]. The best characterized GALT are PPs that can be seen macroscopically. SILTs are smaller and can only be seen microscopically.

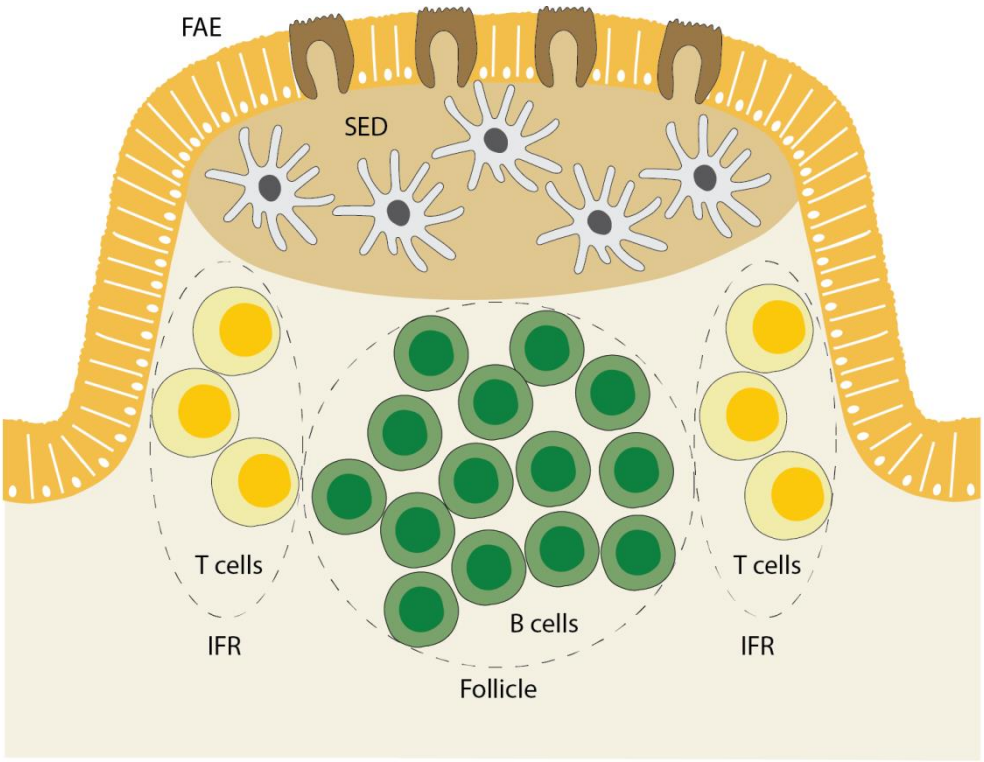

Figure 6. Structure of PPs. PPs are the best characterized GALT. They consist of a FAE containing M cells. Ag taken up by M cells is transferred to underlying DCs present at the SED. Ag-bearing DCs will then move to the interfollicular regions (IFR) and interact with T cells. DC can also interact with B cells at the follicle.

T cell activation

Generation of T cell responses generally occurs in the mLNs. The structure of mLNs resembles that of other lymph nodes (LNs), including a subcapsular sinus, a cortex where B cell follicles lie, and a paracortical area, populated by T cells. B cells express C-X-C Motif Chemokine Receptor (CXCR)5 and travel to B-cell follicles guided by chemokine CXCL13 expressed by follicular dendritic cells, whereas T cells are guided to the T cell zone (TCZ) by chemokine (C-C motif) ligand (CCL)19 and CCL21 produced by fibroblastic reticular cells[63]. Lymph, containing Ag as well as DCs, travels from the intestine to the LNs through afferent lymphatics. DCs migrate to the mLNs in a CCR7-dependent manner, following a gradient of CCL19 and CCL21 produced by lymphatic endothelial cells (LEC). Once in the LNs, DCs will localize mainly to the TCZ[63,64]. In parallel, naïve B and T cells circulating in blood enter the mLNs through high endothelial venules (HEV) in a process dependent of $\alpha_4\beta_7$-Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) interaction[65]. Naïve $CD8^+$ T cells are activated by cognate Ag presented on MHC-I molecules by DCs, leading to generation of cytotoxic T lymphocyte (CTL) responses. By contrast, naïve $CD4^+$ T cells recognize their Ag presented on MHC-II molecules, and will subsequently develop into different Th effector cells (e.g. Th1, Th2, Th17, Tregs and T follicular helper (Tfh), summarized in Figure 7), depending on the stimuli received by the presenting DC.

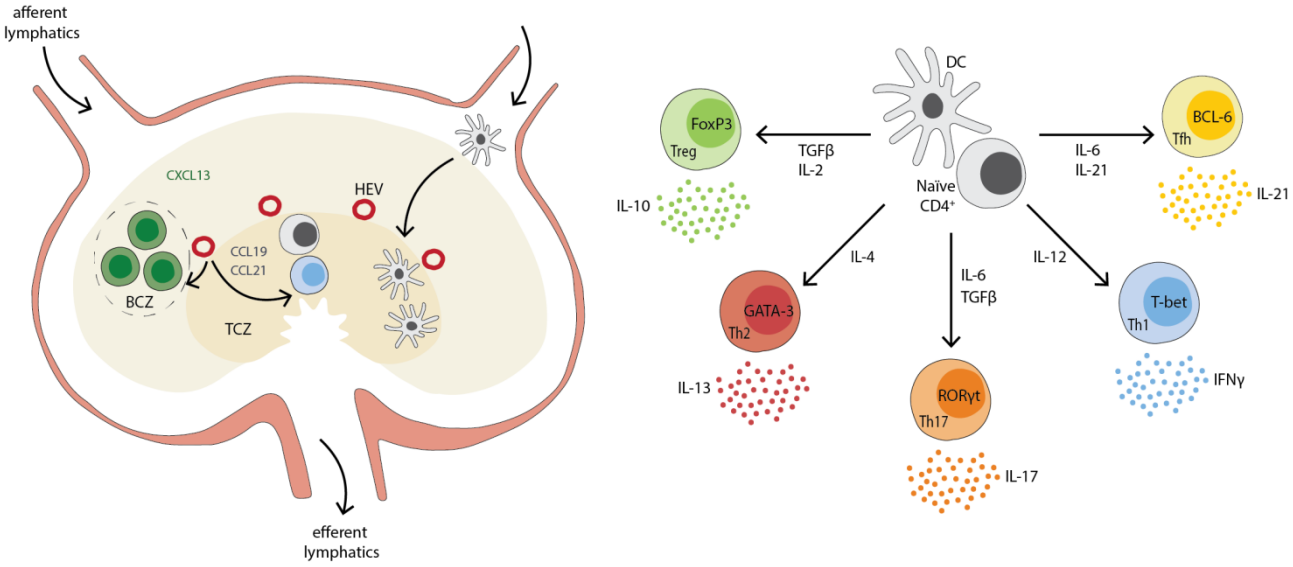

Figure 7. Location of immune cells in the mLNs and distinct Th effector cell lineages in mice. Left: Migratory DCs enter the mLNs through afferent lymphatics and are recruited to the TCZ in a CCR7-dependent manner, following the CCL19 and CCL21 gradient. Naïve B and T cells enter the mLNs from the blood via HEV. B cells are recruited to the B cell zone (BCZ) by a CXCL13 gradient whereas T cells are recruited to the TCZ via the CCR7-CCL19/21 axis. Right: Recognition of cognate antigen by naïve $CD4^+$ T cells on MHC-II molecules on DCs leads to differentiation of distinct T cell lineages. Intracellular pathogens induce Th1 cells; Tfh cells provide help to B cells for antibody production; Th17 maintain tissue homeostasis by clearing extracellular bacteria; Th2 are responsible for parasite clearance and Tregs suppress the rest of the Th cells and induce tolerance.

B cell activation

PPs are the main sites where B cells differentiate into IgA-secreting plasma cells. IgA class switching is a complex process and can be achieved either through T cell-dependent (TD) or T cell-

independent (TI) pathways. TD-IgA responses require the help from $CD4^+$ activated T cells through binding of CD40L, as well as the cytokines TGF-β, IL-5 and IL-6[66–68]. Bioavailable TGF-β is thought to be primarily provided by DCs, which express the integrin $\alpha_V\beta8$ responsible for TGF-β activation[66]. In addition, IL-5 and IL-6 play complementary roles by inducing differentiation and secretion of IgA, respectively[69,70]. PPs have constant germinal center reactions, showing the continual immune stimulation of the intestine. Accordingly, PPs are the main site for TD-IgA induction (Figure 8).

In contrast, TI-IgA responses are GC-independent and depend on A proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF)[71,72]. Both APRIL and BAFF can be expressed by DCs and IECs in response to commensal microbiota and TLR stimulation[73–75]. Additionally, IECs also produce TSLP, which induces DCs to produce more APRIL and IL-10, contributing further to the IgA induction[68]. Inducible nitric-oxide synthase (iNOS), an enzyme induced also in DCs by commensal bacteria and TLR ligands seems to play a role in both TD and TI-IgA induction[76]. Moreover, pDCs can also induce TI-IgA by production of membrane-bound BAFF and APRIL, which are induced by type I IFNs produced in low levels by GALT stromal cells (Figure 8)[44].

It is still unclear whether IgA^+ plasma cells in the gut are primarily primed through TD or TI pathways. Mice deficient in CD40, which lack TD-IgA induction, showed normal levels of IgA^+ plasma cells in the LP[77]. However, SHM was not achieved, leading to induction of low affinity IgA[77,78]. Accordingly, ILFs that consist of mainly B cells, together with DCs and LTi cells, a type of ILC3, are thought to be important sites for TI-IgA class-switch in mice[79].

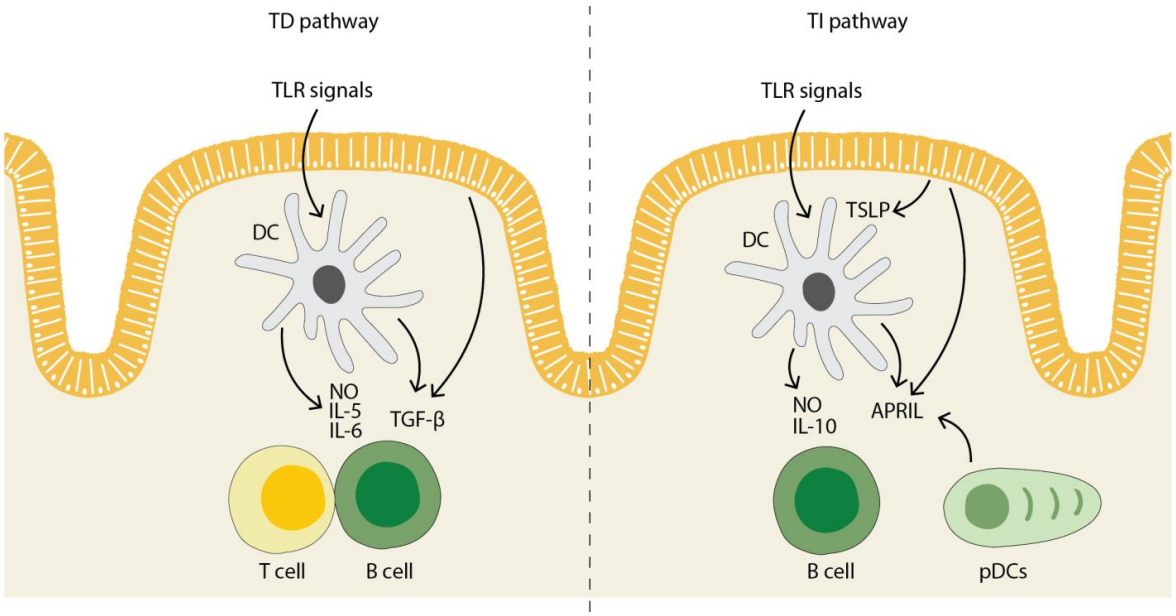

Figure 8. Induction of IgA by TD and TI pathways. Upon TLR stimulation by commensal bacteria, TD-IgA induction requires the help from T cells by CD40L interaction, together with TGF-β, IL-5 and IL-6. Alternatively, TI-IgA induction can be achieved by APRIL and BAFF produced by DCs and IECs. Additionally, TSLP from IECs induces DC to produce IL-10, which has been shown to further contribute to TI-IgA induction. pDCs can also contribute to TI-IgA-induction by providing membrane-bound APRIL. Production of NO by DCs plays a role during both TD- and TI-IgA induction.

Gut-homing receptors imprinted in lymphocytes

The compartmentalization of the intestinal immune system is specific, as B and T cells primed in intestinal sites return to the mucosa. This is achieved by the induction of gut-homing receptors such as CCR9 and $\alpha_4\beta_7$. The ligands of such receptors, CCL25 and MAdCAM-1, are highly expressed in the SI and the HEV of mLNs, allowing homing of lymphocytes back to the intestine. For T cells, expression of CCR9 and $\alpha_4\beta_7$ is induced by DCs in the mLNs and PPs in a process dependent on retinoic acid (RA)[80–82]. Although early studies suggested that only $CD103^+$ DCs were able to metabolize RA due to their unique expression of the enzyme retinaldehyde dehydrogenase 1 (RALDH1), a recent study has shown that all migratory DC subsets have the capability of inducing gut-homing receptors[83]. In addition, stromal cells as well as IECs express RALDH1 and can produce RA[84,85]. Of note, G protein-coupled receptor 15 (GPR15) is involved in homing of T cells to the colon, but its expression on effector vs regulatory T cells seems to differ between mice and humans[86,87].

Intestinal activated B cells home to the intestine by expression of $\alpha_4\beta_7$. Imprinting gut-homing receptors in B cells also depends on RA. Accordingly, DCs seem to be responsible for imprinting expression of CCR9 through the metabolism of RA[67]. Gut homing of B cells is important for protection against intestinal infections such as rotavirus, as mice lacking β_7 integrin show decreased protection[88]. Additionally, CCR10 is required for IgA-producing B cells to home to the colon in a mechanisms dependent of its ligand CCL28[89].

Effector sites

The effector sites of the intestine comprise the intraepithelial compartment and the LP. However, unlike the inductive sites, they are characterized by the diffuse distribution of lymphocytes among non-immune cells and connective tissue, e.g. the cell matrix.

Intraepithelial lymphocytes (IELs): IELs are specialized T cells present at the base of and in between IECs. The majority of these T cells express CD8, either the conventional heterodimer CD8αβ or the unconventional homodimer CD8αα. In addition, the vast majority of IELs in mice express the γδTCR[90]. The minority of IELs that express CD4 express the αβTCR and their numbers increase towards the colon[91]. IELs interact with IECs by the expression of integrin CD103, which is thought to maintain the population at the epithelium[90]. Although IELs are though to play important protective roles, their exact functions are still unknown. Of note, γδTCR IELs may be involved in certain cases of pathology, as epithelium from celiac disease patients show increased numbers of γδTCR IELs[92,93].

Effector T cells in LP: Within the LP, most of the T cells are $CD4^+$, with smaller proportions of $CD8\alpha\beta^+$ T cells, at least in mice. Differences of the type of effector T cells can be seen along the length of the intestine, likely due to the difference in luminal contents. The most common T cells present in a healthy gut are $CD4^+$ T cells, particularly Th17 cells, INF-γ-producing Th1 cells and forkhead box (Fox)$p3^+$ Treg cells[94].

Th17: Th17 cells are the most abundant $CD4^+$ effector memory T cells in the LP, accounting for 30%-40%. In general, Th17 cells play crucial roles in response to extracellular bacteria and fungi and their development depends on the combined actions of TGF-β and IL-6, which induce recruitment of signal transducer and activator of transcription (STAT)3 and RORγ[95]. The development of Th17 cells is largely dependent on microbiota, as germ-free mice show low numbers of Th17 in LP[96]. However, steady state development of Th17 is likely to be a complex and multifactorial process[94]. Dietary compounds such as long-chain fatty acids can also drive Th17 differentiation[97]. The major cytokines secreted by Th17 are IL-17A, IL-17F and IL-22[98]. The cytokine IL-17A drives IECs to secrete granulocyte-colony stimulating factor (G-CSF), which in term drives recruitment of neutrophils to keep barrier integrity[98,99]. Both IL-17A and IL17F drive the expression of β-defensins by IECs, but IL-17F seems to be more important for the protection of colonic epithelial cells during *C.rodentium* infection[99]. IL-22 stimulates IECs to produce antimicrobial peptides such as REGIIIγ and reinforce tight junctions[15,100].

Th1: Classical Th1 cells are developed in response to intracellular bacteria or viruses through the activation of STAT1 and STAT4 by type I (IFN-α/β) and II (IFN-γ) IFNs and IL-12, respectively. This, in turn, promotes expression of transcription factor T-bet and IFN-γ, the two major signatures of Th1 cells[94]. Although present in healthy gut, their function in steady state remains unclear. In the intestines, IL-27 is thought to play a role in Th1 cell differentiation, as mice deficient for IL-27 receptor display reduced $CD4^+$ IFN-γ^+ T cells in the LP[94].

$FoxP3^+$ Tregs: Tregs constitute a large proportion of the intestinal LP. Their main function is to maintain immune tolerance to dietary antigens and commensal microbiota as well as suppressing tissue damage by effector T cells during infections[101,102]. The intestine harbors both natural Tregs derived from the thymus (nTregs) and peripherally differentiated Tregs (pTregs). The pTregs can be broadly divided into specialized $FoxP3^+CD4^+$ Tregs to food antigens in the small intestine ($FoxP3^+ROR\gamma t^-$) and to commensal microbiota in the colon ($FoxP3^+ROR\gamma t^+$)[102]. Naïve $CD4^+$ T cells can also develop into the $Foxp3^-CD4^+$ Treg1 subset, which seems to exhibit the strongest immunosuppressive capacity by secretion of high amounts of IL-10. SCFA, RA and TGF-β are crucial for the development of pTregs[102].

LP B cells: most of the B cells in the LP are IgA-secreting plasma cells and their numbers increase at the distal end of the intestinal tract. IgA is produced as a dimeric or multimeric form and is secreted mostly as a dimer into the lumen through translocation mediated by the polymeric Ig receptor (pIgR), which is expressed on the basolateral membrane of IECs[103]. A fraction of secretory IgA (sIgA) is transported to the liver through the blood stream. In the liver, sIgA is transported to the bile and secreted to the intestinal lumen[104]. In humans, 3-5g of sIgA is secreted daily in the intestinal lumen, being the most produced Ig of the body[104]. Mice lacking the J-chain or the pIgR showed decrease protection against intestinal toxins and higher susceptibility to *Salmonella* infections, respectively[105,106]. Interestingly, humans with IgA deficiency have enriched bacteria from taxa with potentially inflammatory features[107]. Therefore, IgA may have a role not only in neutralizing toxins and protection against pathogens but also as non-inflammatory immune

exclusion in the gut. Unlike mice, with only one isotype, humans have two IgA isotypes: IgA1 and IgA2. IgA1 is predominant in the small intestine whereas IgA2 increases significantly in the colon. IgA2 is more resistant to bacterial proteases than IgA1 and, thus, the presence of this isotype in the colon may be due to its special adaptation to the bacterial-rich environment of the colon[108].

1.3 Intestinal dendritic cells

DCs are defined as large stellate cells that can efficiently present Ags on MHC molecules and activate naïve T cells[109,110]. DCs were first described by Nobel laureate Ralph Steinman in the early 1970s, who named the cells after the Greek word "*Dendron*", which means tree, due to their tree-like shape[1]. DCs are part of the MNP system, together with macrophages and monocytes. DCs are characterized by the unique ability to migrate from tissue to draining lymph nodes where they present antigens to naïve T cells, generating adaptive immune responses[111]. This capability makes DCs key players in linking innate and adaptive immune responses.

Despite the functional and developmental differences between macrophages and DCs, the separation of these two populations has been controversial. This is because surface markers once proposed to be unique for DCs are shared by macrophages, including CD11c and MHC-II[25,28,112]. Yet, markers such as F4/80 and CD64 are expressed in intestinal macrophages[26]. Hence, in addition to their different ontogeny, the expression of CD64, among other macrophage markers, allows for the identification of intestinal macrophages[26,27]. DCs are further subdivided into two main groups: the cDCs and the pDCs. In general, murine cDCs are identified as $CD45^{+}Lineage^{-}CD11c^{hi}MHC\text{-}II^{+}CD64^{-}F4/80^{lo}$ [113].

Defining cDC subsets in the gut

In mice, there are four main intestinal cDC subsets based on the expression of CD11b and CD103 integrins: cDC1 are $CD103^{+}CD11b^{-}$, cDC2 comprise both $CD103^{+}CD11b^{+}$(here referred as cDC2) and $CD103^{-}CD11b^{+}$ (here referred as $CD103^{-}$ cDC2), and $CD103^{-}CD11b^{-}$ (double negative, DN) comprises the last subset (Figure 9). Type I (cDC1) and Type II (cDC2) nomenclature was assigned based on Guilliams et al[29]. Additional cell markers are used to further characterize cDC subsets in different species and/or tissues. For example, cDC1 also express XCR1, CD8α and Clec9A, which suggests their similarity to LN-resident $CD8\alpha^{+}$ DCs[29,114,115]. In contrast, cDC2 express SIRPα, similar to $CD11b^{+}$ LN-resident DCs[114]. Interestingly, human intestinal cDC subsets share developmental, phenotypic and functional characteristics with mouse intestinal cDC subsets, highlighting the relevance for translational investigation of the function of gut cDC subsets in intestinal immunity[116].

The distribution of cDC subsets varies depending on the anatomical location. The SI contains mainly cDC2, with minor contribution by the rest of the subsets. Contrary, cDC1 are highly represented in the colon (Figure 9). Interestingly, the presence of specific subsets in different anatomical location seems to contribute to the regional immune specialization of the intestine[59,60]. Indeed, a recent study has shown that cDC subsets migrating from different sections of the intestine are immunologically different, with SI-derived DCs preferentially giving rise to tolerogenic responses, whereas DCs from more distal parts leading to pro-inflammatory T cell responses[60]. All four subsets can be found at different sites of the intestine, including the LP and GALT. However, as ILFs and colonic patches cannot be removed from the LP preparations, the exact origin of all subsets is still unclear. Studies on RORγt-deficient mice, which lack ILFs, PPs and colonic patches,

displayed normal numbers of all cDC subsets but DN, suggesting that DN cDC exclusively locate to GALT whereas cDC1 and the two subsets of cDC2 derive from the LP[83].

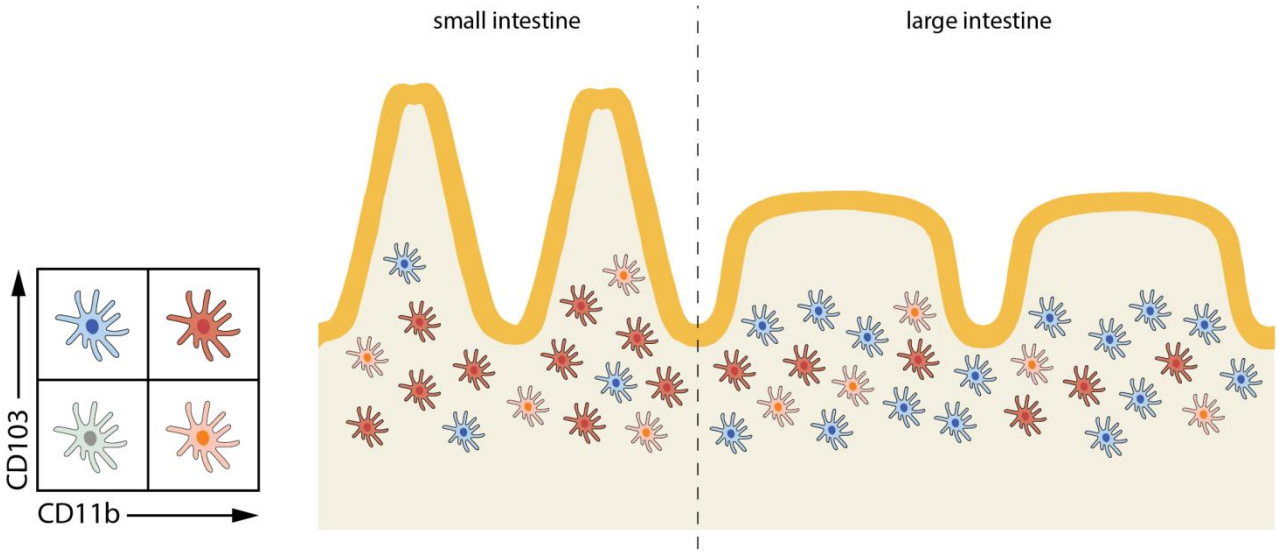

Figure 9. Intestinal dendritic cell subsets. There are four different intestinal DC subsets characterized by the differential expression of CD103 and CD11b: cDC1 are $CD103^{+}CD11b^{-}$ (blue), cDC2 are $CD103^{+}CD11b^{+}$ (red), $CD103^{-}CD11b^{+}$ are considered $CD103^{-}$cDC2 (orange), and lastly the $CD103^{-}CD11b^{-}$, named herein double negative (DN, grey). The SI contains higher numbers of cDC2, whereas the colon contains more cDC1. The DN are thought to be mainly present in GALT tissue and are not represented in the figure.

Ontogeny of intestinal cDCs

cDCs develop in the BM in a process called hematopoiesis. In the BM, a multipotent hematopoietic stem cell (HSC) undergoes several differentiation steps, leading to common monocyte precursors (cMoP) and common DC precursors (CDP). While intestinal macrophages and monocytes differentiate from cMoP, DCs originate from CDP. Subsequently, CDPs are thought to generate pre-cDCs and pre-pDCs, and pre-pDCs fully differentiate into pDCs in the BM (Figure 10). Fully differentiated pDCs together with monocytes and pre-DCs leave the BM and seed lymphoid and non-lymphoid tissues, where they will further differentiate under the influence of soluble mediators such as granulocyte macrophage colony-stimulating factor (GM-CSF) and/or macrophage colony-stimulating factor (M-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L). The pre-cDCs are thought to further develop into pre-cDC1 and pre-cDC2 that are committed to cDC1 and cDC2 development, respectively[117].

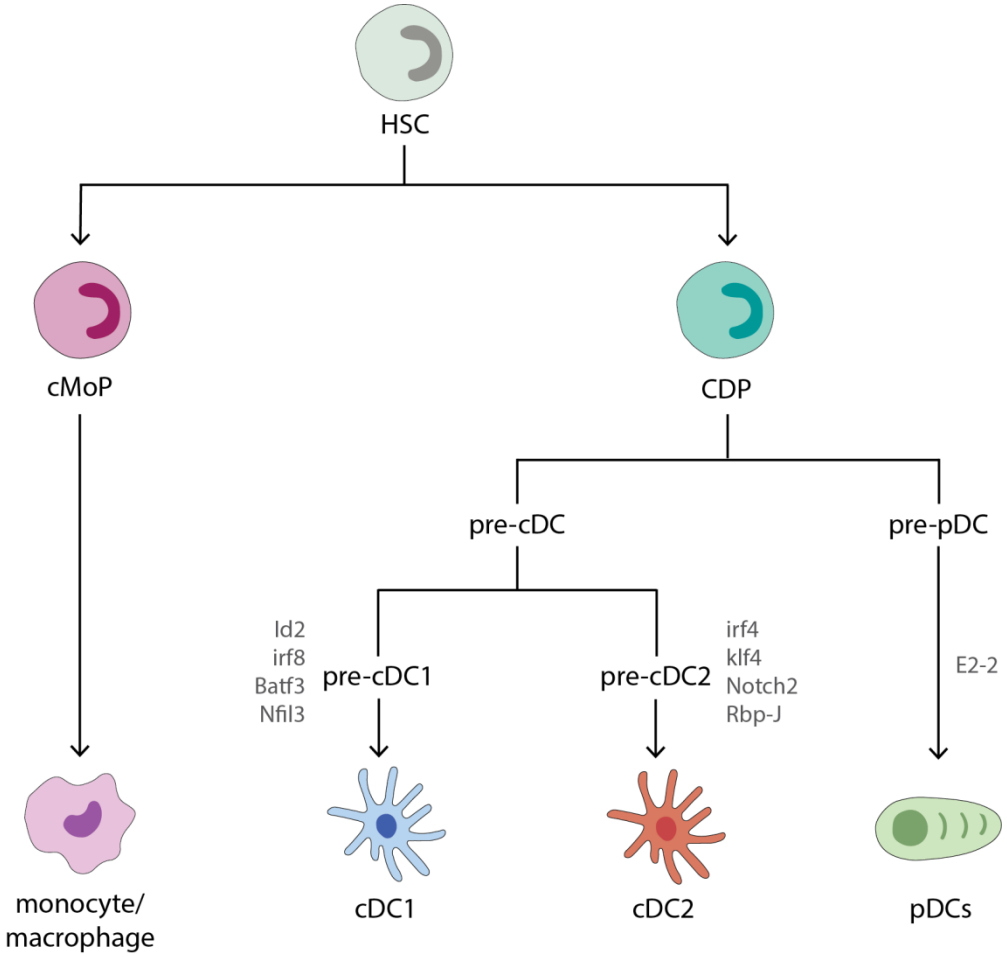

Figure 10. Development of DCs. Schematic view of the development of DCs, pDCs and monocytes/macrophages from the bone marrow to their fully differentiated stages. Transcription factors required for each subset are written along the arrows.

Whether specific precursors in the BM preferentially lead to intestinal DCs is still unclear. Zeng et al identified a population of DC progenitors that gave rise to both CD103$^+$ DC subsets in the intestine as well as splenic CD8α$^+$ DCs when adoptively transferred[118]. Importantly, their development depended on RA[118]. These progenitors, called pre-mucosal DCs (pre-μDCs) also maintained the ability to differentiate into pDCs and some DCs in the spleen[118]. Another study showed that the development of cDC2 in the intestine as well as CD11b$^+$ DCs in the spleen from pre-DCs was dependent on RA, as shown in vitamin A deficient (VAD) mice[119]. Although the relation of the pre-μDCs to pre-DCs is still unclear, RA seems to play a specific but essential role for development of intestinal cDCs.

Transcriptional control of cDC subsets

The study of mice lacking different transcription factors has shown the importance of such transcription factors in the development of distinct cDC subsets. For example, cDC1 require IFN regulatory factor 8 (IRF8), BATF3, nuclear factor Interleukin 3 regulated (NFIL3) and inhibitor of DNA binding 2 (ID2) transcription factors for their development and are thus developmentally linked to lymph node-resident CD8α$^+$ DCs[120]. In contrast, cDC2 require the transcription factor

IRF4 (Figure 10)[121]. Interestingly, other transcription factors such as neurogenic locus notch homolog protein 2 (Notch2), recombination signal binding protein for immunoglobulin kappa J (Rbp-J) and kruppel-like factor 4 (Klf4) are required for the development of a fraction of $IRF4^+$ cells, indicating that cDC2 comprises a heterogeneous population. The requirements for the $CD103^-$ cDC2 subset development are, however, less clear. The $CD103^-$ cDC2 subset are *bona-fide* DCs because they express DC-specific markers such as CD26 and messenger ribonucleic acid (mRNA) of the transcription factor zinc finger transcription factor zDC (Zbtb46), and lack macrophage markers such as CD64 and F4/80[112]. In addition, they migrate to mLNs and are able to drive Th17 responses[83]. A recent study has shown that Zinc Finger E-Box Binding Homeobox 2 (Zeb2) is highly expressed by $CD103^-$ cDC2 and that their development is affected by Zeb2 absence[122]. These findings suggest that further studies of specific transcription factors are required for this subset of intestinal cDCs.

Although specific for cDC subset development, most of these transcription factors can affect other cell types. For example, IRF8 is known to affect functionality of pDCs and its absence leads to additional defects in monocyte and B-cell lineages[123,124]. Recent studies have proposed new transcription factors required for the development of different cDC subsets[116]. For example, the transcription factors B-cell lymphoma 6 (Bcl-6) and B lymphocyte-induced maturation protein-1 (Blimp-1), associated with B and T cell differentiation, have been suggested to play a role in development of cDC1 and cDC2, respectively[116]. As these transcription factors are essential for other cell types, such as Bcl-6 for Tfh cells, Cre-lox mouse systems represent valuable tools and will contribute to further characterize the exact transcription factor requirements in specific cDC subsets.

Growth factors required for cDC development

DC development is also dependent on soluble mediators. As other DCs, intestinal DCs depend on the growth factor Flt3L for their development, demonstrated by drastically lowered levels of intestinal cDC1 and cDC2 in Flt3-deficient mice[125]. Accordingly, exogenous administration of Flt3L increases numbers of cDC1 and cDC2, with greater impact on cDC1 numbers[83,121]. In addition, colony stimulating factor 2 (CSF-2) is also involved in cDC homeostasis[126,127]. Deficient mice in either CSF-2 or CSF-2 receptor (CSF2R) showed a reduction of cDC2, while $CD103^-$ cDC2 were unaffected[125,126]. Finally, a recent study has shown that cDC2-intrinsic TGF-β signaling is required for the development of this subset in the intestine[128]. Indeed, mice deficient in TGF-β receptor (TGFβRII) displayed reduced levels of intestinal cDC2, reflecting a defective differentiation from $CD103^-$ cDC2 intermediates, rather than a loss of CD103 expression[128].

Functionality of DCs

Antigen uptake

Several mechanisms have been proposed as to how cDCs acquire different luminal antigens. However, whether the different mechanisms activate different adaptive immune responses is still unknown. In the PPs, M cells can translocate Ag to the underlying DCs at the SED. Subsequently,

these DCs migrate to the IFR where they prime Ag-specific T cells (Figure 11A)[129]. In addition, populations of monocyte-derived CX3CR1$^+$ cells that express lysozyme are able to extend dendrites through M cell pores and capture and kill *Salmonella* [130,131].

In the LP, CX3CR1$^+$ macrophages extend transepithelial dendrites (TEDs) through the expression of tight junction molecules to capture luminal Ags (Figure 11B)[132,133]. Additionally, a proportion of activated CD103$^+$ DCs is recruited to the epithelium and directly captures luminal Ag by TEDs (Figure 11C)[40,134]. However, CD103$^+$ DCs have a poorer phagocytic capacity compared to macrophages. Hence, cDC2 have shown to get soluble Ag from macrophages by trogocytosis, which is the passage of soluble Ag together with membrane from macrophages by establishing gap junctions between cDC2 and macrophages (Figure 11D)[135]. Of note, CX3CR1-deficient mice, which lack TEDs, showed normal T-cell priming in the mLNs in response to orally administrated soluble Ag, indicating that CX3CR1-dependent Ag uptake is dispensable[40]. In addition, goblet cells have been shown to transfer soluble Ag to DCs by goblet cell passages (GAP) (Figure 11E)[136].

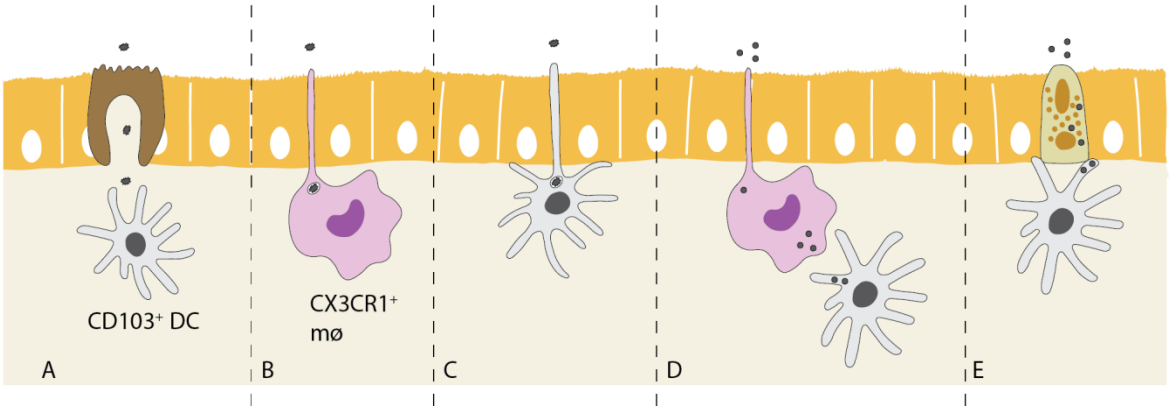

Figure 11. Mechanisms of Ag uptake in the intestine. In GALT, M cells can translocate Ag or live bacteria from the lumen to underlying DCs (A). In the LP, both macrophages (B) and DCs (C) are able to extend TED and capture Ag directly from the lumen. Ag transfer has also been seen from macrophages (D) and goblet cells (E) to DCs via gap junctions and GAP, respectively.

DCs can internalize luminal Ag by phagocytosis, macropinocytosis and receptor-mediated endocytosis[137]. Phagocytosis allows for engulfment of pathogenic bacteria, apoptotic or necrotic cells into a phagosome[138]. Receptors such as CD36 and $\alpha_v\beta3$ are involved in mediating the phagocytosis of apoptotic bodies[139–141]. In contrast, macropinocytosis is used by cDCs to capture Ags from the extracellular fluid[142,143]. Finally, receptor mediated-endocytosis is mediated by PRRs, such as C-type lectin receptors (CLRs), or Fc receptors (FcR), which internalize immune complexes[142,144].

Antigen processing and presentation in the intestine

Upon internalization, DCs can process Ag in several ways depending on the nature of the Ag. Ultimately, the Ag can be presented in an MHC-I or MHC-II context. Most soluble and particulate Ags are targeted to MHC class II compartments for presentation to CD4$^+$ naïve T cells by cDCs. In contrast, MHC-I is generally used in any cell type to present endogenous proteins, which marks

infected cells to be killed by cytotoxicity. In addition to these classical pathways of processing and presenting Ag, DCs have an alternative pathway that allows them to present exogenous peptides in an MHC-I context. This process, named cross-presentation, enables DCs to instruct naïve $CD8^+$ T cells to become CTL to kill virally infected or cancer cells even if the DC is not infected[145]. Cross-presentation is a feature of cDC1 and represents a valuable tool to prime naïve T cells with desired Ag[146], a feature of high interest for vaccine development.

Conditioning of intestinal DCs

Intestinal cDCs differ from non-intestinal cDCs in their functionality, and this might be due to the unique microenvironment they are exposed to. Accordingly, metabolites derived from commensal microbiota, IECs and stromal cells are known to directly condition DCs. For example, SCFA including butyrate and acetate are metabolized by commensals from fiber-rich diets and have shown to educate DCs. While both butyrate and acetate induce RA-producing DCs which in turn induce IL-10-secreting Tregs, only acetate induces production of IgA[147,148]. However, the mechanisms of how these different SCFA act on DCs are still unknown. Butyrate also induces RA production on IECs, which will in turn induce tolerogenic DCs[149]. Furthermore, segmented filamentous bacteria (SFB) are commensal microbes that attach to the IECs, inducing their production of serum amyloid A (SAA)[150]. Subsequently, SAA induces DCs to produce IL-6 and IL-23[150]. IECs also produce TGF-β and TSLP, inducing a tolerogenic phenotype on DCs. In addition, stromal cells can produce TGF-β, RA, and PGE_2, and stromal cell-derived TGF-β is important for imprinting gut-homing receptors on T cells in the mLNs[85,151]. Collectively, these findings demonstrate an intricate interplay between microbiota, IECs and DCs.

1.4 Pathogen sensing and signaling

PRRs are germ-line encoded receptors of the innate immune system that recognize constitutive and conserved products of microbial origin, called PAMPs[4]. These PAMPS are present in viruses, bacteria, fungi and protozoa and range from lipoproteins to carbohydrates, lipopolysaccharides and nucleic acids. In addition, PRRs recognize DAMPs from the host, which are usually released by dying or dead cells. Of note, both pathogenic and non-pathogenic microbes produce PAMPs, hence PRRs are not able to distinguish between pathogens and commensal bacteria. Other mechanisms such as compartmentalization as well as anti-inflammatory cytokines play a role in defining these differences[152].

PRRs are predominantly expressed in innate immune cells including macrophages and DCs, but non-immune cells such as endothelial cells and fibroblasts can also express PRRs and contribute to innate immunity[153,154]. Activation of PRRs induces signals that can in turn activate the adaptive immune system. This occurs through triggering maturation of DCs that will generate the appropriate immune response to the Ag acquired in the periphery[155].

Classes of PRRs

Most of the PRRs are classified into five different classes based on protein domain homology. The classes consist of TLRs, CLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma 2(AIM2)-like receptors (ALRs). In addition, there is the recently described cytosolic DNA sensor cGAS-STING, although it does not share protein homology with any of the other PRRs families[156]. Herein, I describe briefly the different PRR families, focusing on nucleic acid sensing and particularly on TLRs as this is the main focus of this thesis.

RLRs

RLRs are cytosolic sensors that recognize intracellular RNA. There are three main members identified: RIG-I, Melanoma Differentiation-Associated protein 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). The structure of RIG-I and MDA5 is characterized by a N-terminal two tandem caspase recruitment domain (2CARD) responsible for signal transduction, a DExH-box helicase domain and a C-terminal repressor domain[157]. The third member, LGP2, lacks the 2CARD domain and thus the signaling activity, but is able to regulate RIG-I and MDA5 by binding to RNA. Although both RIG-I and MDA5 can recognize some viruses simultaneously[158], they generally recognize different double-stranded RNA (dsRNA). While RIG-I recognizes mainly 5'ppp-dsRNA, which is a short dsRNA generated during viral replication, MDA5 recognizes long dsRNA[158]. In steady state, the CARD region is subjected to auto-inhibition. Upon ligand binding, a conformational change releases CARD, which will lead to the formation of a CARD tetramer and filament formation along the dsRNA. The tetramer recruits MAVS present at the membrane of mitochondria, leading to formation of prion-like structure signaling complex[159]. Downstream adaptor molecules and kinases will ultimately induce the expression of IFNs and pro-inflammatory

cytokines through activation of IRF3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), respectively.

NLRs

NLRs are a family of cytosolic receptors that recognize mainly bacterial PAMPs, but also viral-derived PAMPs and endogenous DAMPs. Although there are 22 members in humans and 34 in mice, NOD1 an NOD2 are the prototypical members of the NLR family[156]. NLRs consist of C-terminal ligand-binding leucine-rich repeats (LRR), a central NACHT domain, and a single (NOD1) or a tandem (NOD2) N-terminal CARD domain. All NLRs induce inflammasome formation upon activation, except for NOD1 and NOD2, which induce pro-inflammatory cytokine expression[160]. The inflammasome is a protein complex formation that, upon activation by pathogens, leads to caspase-1 activation, which in turn induces the proteolytic processing of pro-IL-1β and pro-IL-18 into their active forms[161]. Several NLRs that activate inflammasomes recognize a broad and distinct range of PAMPs including lipopolysaccharide (LPS), bacterial and viral RNA, ATP and uric acid[161,162]. NOD1 and NOD2 signaling, in contrast, occurs in close association with endosomal membranes. Upon activation, NOD1 and NOD2 signal through RIP2 to activate NF-κB, inducing the expression of pro-inflammatory cytokines[160].

CLRs

CLRs constitute a heterogeneous group of soluble and transmembrane receptors that generally recognize carbohydrates from bacteria, viruses and fungi[163]. Based on their structure, CLRs are subdivided into 17 different groups, but dectin-1 and dectin-2 are the best characterized CLRs[156]. Dectin-1 and dectin-2 recognize β-glucan and α-mannose, which are components of fungal cell walls and hyphae, respectively. A study showed that activation of DCs through dectin-1 and dectin-2 confers protective immunity against *Candida albicans*[164]. Dectin-1 also recognizes mycobacterial species, sIgA and mucins[165,166]. Upon ligand binding, Dectin-1 promotes phagocytosis and the initiation of signal transduction that leads ultimately to the induction of pro-inflammatory cytokines via NF-κB[138]. Other CLRs include MINCLE, which is expressed by macrophages and is responsible for detection of necrotic cells; and Clec9A, expressed by $CD8\alpha^{+}$DCs and responsible for cross-presentation of necrotic cells[167,168].

ALRs and other DNA sensors

ALRs form a family of cytosolic receptors that sense intracellular DNA. Upon DNA binding, the first characterized ALR AIM2 interacts with adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and in turn induces inflammasome activation[156]. The inflammasome helps caspase-1 to cleave pro-IL-1β and pro-IL-18 into their mature forms, which are then secreted by unknown mechanisms. Inflammasome can also lead to pyroptotic cell death[169].

Another member of ALRs is IFI16. IFI16 recognizes several relevant DNA viruses such as herpes simplex virus (HSV) and human immunodeficiency virus (HIV)[170,171]. Although few studies have shown that IFI16 also localizes to nucleus depending on the cell type, signaling ultimately occurs in the cytosol via inflammasome or STING activation[170,172,173]. However, little is known on how this receptor can differentiate between self and non-self DNA.

The cytosolic DNA sensors Cyclic-GMP-AMP (cGAMP) synthase (cGAS) and stimulator of interferon genes (STING) are the most recently described PRRs. Upon DNA binding, cGAS catalyzes the production of cGAMP from adenosine tri-phosphate (ATP) and guanosine tri-phosphate (GTP). Subsequently, cGAMP binds and activates STING, inducing type I IFN production. STING is a membrane protein of the endoplasmic reticulum (ER) and partially localizes to mitochondria[174]. Activation by cytosolic DNA leads to STING dimerization and subsequent translocation to perinuclear region. There, STING recruits TBK1, which phosphorylates STING and IRF3, resulting ultimately in type I IFN production[174]. Of note, STING can also function as direct DNA sensor in the cytosol. Additionally, both cGAS and STING have been shown to be important for protection against RNA viruses in a different mechanism from their DNA virus sensing role[174].

Toll-like receptors

TLRs were the first PRRs identified and are the best characterized family of PRRs. The TLR family comprises 10 members in humans (TLR1-TLR10) and 12 members in mice (TLR1-TLR9, TLR11-13)(Table 1). The localization of TLRs differs within the cell: TLRs present at the cell surface generally recognize microbial components, whereas intracellular TLRs present in endosomes recognize nucleic acids (Table 1). TLRs are type I transmembrane receptors, characterized by an ectodomain with LRR responsible for ligand recognition, a transmembrane domain, and a cytosolic Toll/IL-1 receptor (TIR) domain[175]. Ligand binding induces TLRs to homo- or heterodimerize, which in turn recruits adaptor molecules via TIR domains, inducing signal transduction. Some TLRs, however, such as TLR4, require co-receptors for downstream signaling[175].

Table 1. List of different TLRs in mice[163,176].

TLR	Ligand	Origin of the ligand	Location	Function	Dimerization
TLR1	Triacyl lipoproteins	Bacteria	Cell surface	Activates NFκB through TIRAP, MyD88, IRAKs, TRAF6	Heterodimerizes with TLR2
TLR2	Lipoproteins, LTA, PGN, mannans	Bacteria, viruses, parasites, fungi, self	Cell surface	Activates NFκB through TIRAP, MyD88, IRAKs, TRAF6	Heterodimerizes with TLR1 or 6 or CD36, homodimer in endosomes
TLR3	dsRNA	Virus	Cell surface	Activates NFκB through TRIF-TAK1 and type I IFN through TRIF-TRAF3	Homodimer in endosomes
TLR4	LPS	Bacteria, viruses, self	Cell surface, endosome	Activates NFκB through TIRAP, MyD88, IRAKs, and TRAF6, and induces type I IFN through TRAM-TRIF-IRF3	Homodimer
TLR5	Flagellin	Bacteria	Cell surface	Activates NFκB through MyD88, IRAKs, TRAF6	Homodimer
TLR6	Diacyl lipoproteins, zymosan, β-glucan	Bacteria, viruses, fungi	Cell surface	Activates NFκB through TIRAP, MyD88, IRAKs, TRAF6	Heterodimerizes with TLR2
TLR7 (human TLR8)	ssRNA	Virus, bacteria, self	Endosome	activates NFκB through MyD88, TRAF6, induces type I IFN via MyD88	Homodimer in endosomes
TLR9	CpG DNA	Bacteria, viruses, fungi, self	Endosome	Activates NFκB through MyD88, TRAF6, induces type I IFN via MyD88	Homodimer in endosomes
TLR10	Unknown	Unknown	Endosome	-	-
TLR11	Profilin-like molecule	Protozoa	Cell surface	-	-
TLR12			Cell surface	-	Heterodimerizes with TLR11[177]
TLR13	rRNA[178]	Bacteria	Cell surface	-	Heterodimerizes with TLR2[179]

Signaling pathways of TLRs

Upon ligand binding, TLRs recruit a set of adaptor molecules to drive the expression of IFNs and pro-inflammatory cytokines. Based on the adaptor molecule recruited, there are two distinct signaling pathways: MyD88-dependent and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways.

MyD88-dependent pathway: except for TLR3, all TLRs require the adaptor molecule MyD88 for downstream signaling. However, some differences exist in the requirement of MyD88 depending on the TLRs. For TLRs present at the cell surface, such as TLR2, TLR4 or TLR5, ligand binding leads to MyD88 recruitment via TIR domains[180]. Of note, TLR4 requires an additional adaptor, TIRAP, for signal transduction[175]. MyD88 recruits IRAK kinases through its death domain, forming the so-called myddosome complex. Subsequently, activation of IRAK kinases leads to recruitment of the E3 ubiquitin ligase TRAF6, which interacts with the TAK1/TAB complex. TAK1 then activates two different pathways: the IKK complex-NF-κB pathway and the MAPK pathway. In the IKK pathway, TAK1 binds and activates the three-IKK complex via phosphorylation of IKKβ. The activated IKK complex phosphorylates the inhibitor IkBα, releasing NF-κB. NF-κB is then translocated to the nucleus and induces expression of pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β (Figure 12). MAPK kinases are also activated by TAK1, which in turn mediates activation of AP-1 transcription factors to regulate inflammatory responses[175,180].

Alternatively, MyD88 activation through intracellular TLRs such as TLR7 and TLR9 leads to production of type I IFNs. While the myddosome induces NF-κB-dependent pro-inflammatory cytokine production, a complex formed by the myddosome together with TRAF3, IKKα, OPNi, and Dock2 leads to downstream activation of transcription factor IRF7, which then translocates to the nucleus and induces type I IFN production (Figure 12). Of note, TLR9 signaling occurs in two different endosomal compartments: first in early endosomes where it triggers NF-κB activation, and then in lysosome-related organelles where it induces IRF7 activation[180,181].

TRIF-dependent pathway: this pathway is only used by TLR4 and TLR3. While TLR4 requires the co-adaptor TRAM for TRIF recruitment, TLR3 directly recruits TRIF[182]. Generally, TLR-ligand binding recruits TRIF, which in turn interacts with both TRAF6 and TRAF3 (Figure 12). TRAF6 recruits the kinase RIP-1, which activates the TBK1 complex, leading to activation of both NF-κB and MAPK kinases and production of pro-inflammatory cytokines. In parallel, TRAF3 recruits the IKK kinases TBK1, IKKi and IKKε, which phosphorylate IRF3. Activated IRF3 forms homodimers that translocate into the nucleus, inducing type I IFN expression[180].

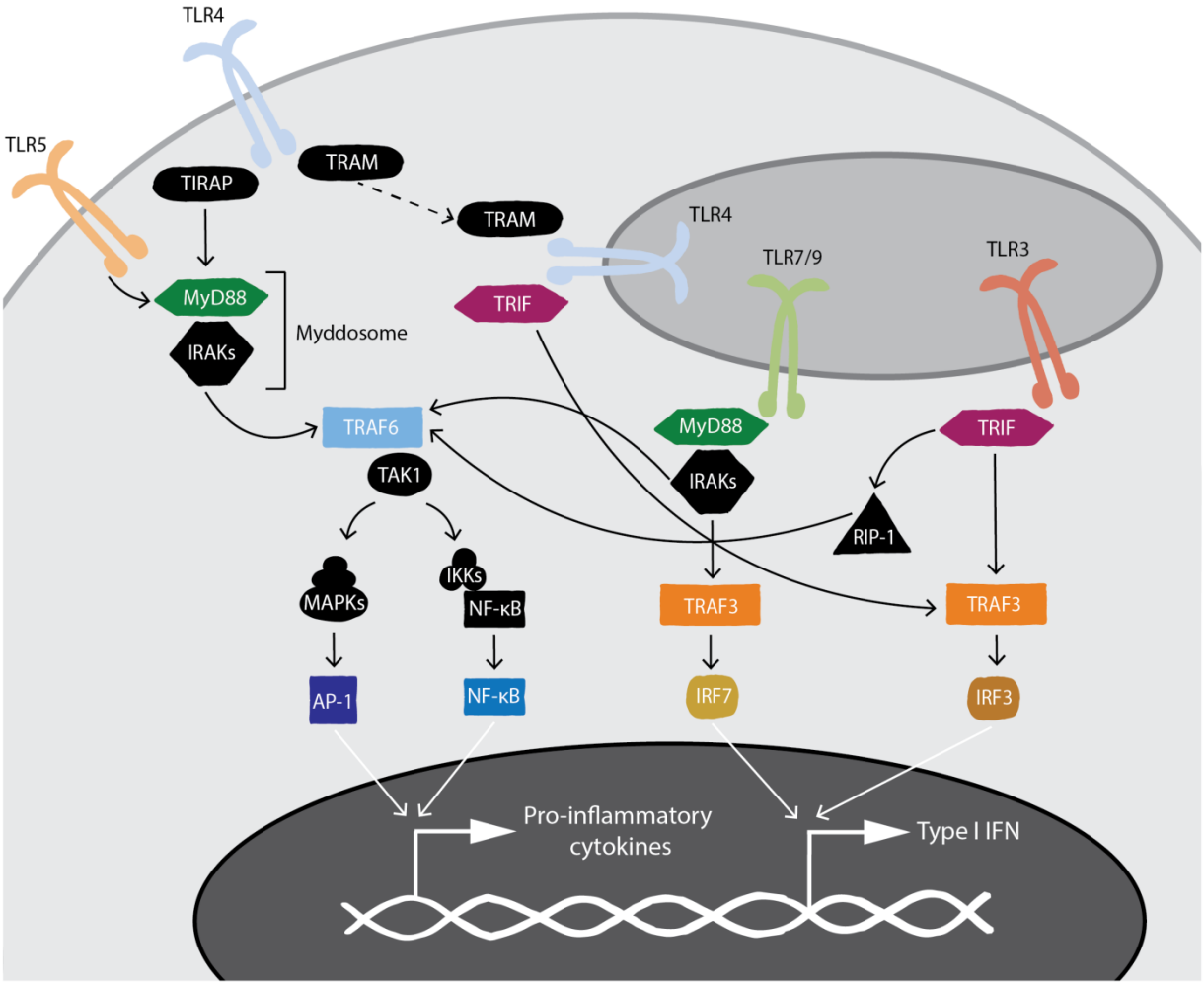

Figure 12. MyD88 and TRIF-dependent TLR signaling. Cell surface TLRs such as TLR5 and TLR4 signal through the adaptor molecule MyD88, leading to activation of IRAKs and TRAF6, inducing ultimately expression of pro-inflammatory cytokines. Activation of endosomal TLRs such as TLR7, TLR9 and TLR3 also lead to production of type I IFN in a process dependent of TRAF3. Of note, TLR4 can also induce the expression of type I IFN by recruiting TRAF3 in the endosome. TLR4 requires TIRAP and TRAM adaptors to bind to MyD88 and TRIF, respectively. Figure is simplified and shows the main distinct features of the signaling pathways.

PRRs in the intestinal immune system

TLRs are generally the first PRRs to recognize pathogens in the intestine. They play an important role in both the course and the outcome of an infection. Despite the crucial role of TLRs in pathogen recognition, they can also have detrimental effects by causing inflammation, leading to disruption of intestinal homeostasis.

IECs express several PRRs that enable the recognition of microbes[183,184]. However, IECs need to distinguish between commensals and pathogens. Accordingly, the polarized nature of the IECs allows for differential expression of TLRs, leading to a different response depending on the anatomical location. For example, TLR9 ligands sensed at the basolateral membrane induce activation of NF-κB, whereas apical exposure stabilized its inhibition through IκB[185]. In addition, microbial sensing of Paneth cells via intrinsic expression of MyD88 and NOD2 induces the

expression of AMP such as REGIIIγ and defensins, respectively[13,14,186]. MyD88 signaling via NF-κB is also crucial for pIgR expression on IECs, enabling the translocation of sIgA to the lumen[103,187].

PRRs also activate cellular pathways that lead to the elimination of pathogens. For example, NLRP3 and NLRC4–mediated IL-18 production in macrophages is important for *C. rodentium* infection clearance, as mice deficient for both PRRs display higher intestinal inflammation upon infection[188]. In addition, NLRP6 is involved in keeping the balance of commensal microbes by the inflammasome-dependent production of IL-18, AMP production and mucus secretion by goblet cells[189]. Finally, variants of NOD2 are known to be associated with Crohn's disease, highlighting the importance of PPRs in maintaining intestinal homeostasis[190].

TLRs and adaptive immune responses: role for DCs

TLRs are key components of the innate immune system that, upon activation, trigger multiple pro-inflammatory steps that lead ultimately to the elimination of the pathogen. In addition, TLRs are responsible for activating DCs, which activate critical signals involved in the initiation of adaptive immune responses[191]. Accordingly, the instruction of adaptive immunity occurs via TLR triggering in DCs, which are central at initiating adaptive immune responses. Upon TLR stimulation, peripheral DCs mature and migrate to local draining lymph nodes, where they present the processed Ag to naïve T cells in the context of MHC molecules. TLR activation is responsible for all the maturational changes of the DCs, including the upregulation of CCR7, which imprints their migratory capacity to the lymph nodes[192].

TLRs in DCs

Several studies have shown that DC subsets have different TLR expression patterns. In mice, Edwards et al showed that all splenic DC subsets expressed most of the TLRs to similar levels, but TLR3 is preferentially expressed in $CD8\alpha^+$ while TLR5 and TLR7 are absent in the same subset[193]. Luber et al confirmed the specific expression of TLR7 by $CD4^+$ DCs, and TLR3 for $CD8\alpha^+$ DCs[194]. Although less is known in the intestinal context, several studies have shown that the picture might not differ from other organs[195]. For example, TLR5 is expressed by cDC2, while cDC1 highly express TLR3[116,195,196]. By contrast, pDCs express TLR7 and TLR9[193]. Accordingly, the differential expression of TLRs by distinct DC subsets is thought to influence the adaptive immune responses generated by DC subsets. In line with this, cDC2 are required for the induction of adaptive immune responses against soluble flagellin, a ligand for TLR5[197]. In addition, the specific expression of TLR3 by cDC1 has been shown to promote cross-presentation, a unique ability of this subset[198].

TLR3 and intestinal immunity

TLR3 recognizes dsRNA, which is generally found as intermediate of viral replication during viral infections. TLR3 is specifically and highly expressed in cDC1 compared to other DC subsets[116,195]. Engagement of TLR3 often correlates with cross-presentation[198,199]. Indeed, Schulz et al showed that splenic $CD8\alpha^+$ DCs were activated by dsRNA present in virally infected cells and not from uninfected cells, and that TLR3 activation was required to mount a CTL response[198]. In addition,

Jelinek et al found that TLR3 was essential for DC activation after dsRNA stimulation and that dsRNA served as adjuvant for the generation of Ag-specific CTL and protection against challenge with influenza virus[200]. Therefore, targeting TLR3 on cDC1 may serve as a potential tool to develop protection against viral infections, among others.

Cytokines

Engagement of TLRs by the respective ligands generally leads to downstream signaling, culminating with the expression of soluble mediators. These mediators, including pro-inflammatory cytokines, chemokines and IFNs can lead to further auto-activation (autocrine) and the activation of neighboring cells (paracrine). This activation is mediated by the expression of the respective receptors for these mediators in the target cells. Here, I describe three of these mediators that are relevant for my project.

Type I IFN

Type I IFNs consist of several members, including 13 subtypes of IFN-α and one single IFN-β and are mainly produced in response to viral and bacterial infections. All type I IFNs signal through the same heterodimeric IFNα/β receptor composed of the IFNAR1 and IFNAR2 subunits[201]. Upon type I IFN binding, IFNAR triggers the JAK-STAT signaling axis, leading to expression of type I IFN stimulated genes (ISGs). Although virtually all cells can produce type I IFNs upon infection, the cellular source varies depending on the type of virus[202]. Once expressed, type I IFNs can act in an autocrine as well as paracrine manner, which induces a second wave of ISG expression of antiviral factors, such as PKR and 2´-5´OAS, involved in blocking viral replication[163]. Type I IFNs have been shown to play important roles in $CD8\alpha^+$ DC activation upon TLR3 engagement[199]. In fact, one study suggested that autocrine type I IFN signaling rather than direct TLR3 signaling accounted for activation of DCs, highlighting the importance of type I IFN in DC functionality[203].

In the intestine, both macrophages and DCs are responsible for constitutive production of type I IFN, which is thought to be dependent on microbial signals, as shown by germ-free mice whose type I IFN levels increase upon exposure to normal specific-pathogen-free (SPF) flora[204]. Interestingly, microbial dsRNA sensing through TLR3 is thought to be involved in the constitutive production of IFN-β[205]. In steady state, type I IFNs produced by stromal cells in GALT have shown to promote production of APRIL and BAFF by pDCs, leading thus to TI-IgA production[44]. Furthermore, type I IFNs play crucial roles in response to viral infections, such as rotavirus, as well as against bacterial infections, such as *Listeria* and *Salmonella*[206]. Type I IFNs have been suggested to maintain intestinal homeostasis during acute-DSS colitis[207,208]. Particularly, poly(I:C) treatment, among other adjuvants, ameliorated DSS colitis in a TLR3-dependent, type I IFN response[209,210]. In addition to acute colitis, mouse models of chronic colitis induced by adoptive naive T cell transfer into RAG-deficient hosts have shown similar protective roles of type I IFNs[211–213]. Collectively, these findings suggest a role for type I IFN not only during infections but also during immune homeostasis in the intestine.

Type III IFNs

Type III IFNs, or IFN-λ or IL-28 and IL-29, consists of up to four members in humans and two functional orthologues in mice (IFN-λ2 and IFN-λ3). Similar to type I IFNs, IFN-λ is also induced upon viral infections, which are generally recognized by cytosolic receptors signaling through MAVS. However, its induction is favored when signaling through MAVS occurs in peroxisomes, as opposed to mitochondria for type I IFNs[214]. Interestingly, splenic $CD8\alpha^+$ DCs and pDCs have been shown to produce IFN-λ in response to poly(I:C) in a TLR3 and IFNAR-dependent manner[215]. In the intestine, poly(I:C) is thought to induce additional IFN-λ production by other immune cells, although the exact cellular source remains unknown[216].

This family of IFNs signals through IFN-λR, a unique heterodimeric receptor consisting of IFN-λR1 and IL-10R2, the latter shared with other IL-10 family cytokines[217]. Despite engaging different receptors, IFN-λ signaling events overlap with those of type I IFNs. Accordingly, IFN-λ also signals through JAK-STAT, leading to activation of STAT1 and STAT2 and expression of ISG. The difference between type I and III interferons is thought to rely on the compartmentalization of their response[218]. Unlike IFNAR, which is expressed broadly in most of the cells, IFN-λR expression is mainly restricted to IECs, although other cells such as NK cells and pDCs have been reported to express it as well[219–221]. Hence, IFN-λ is thought to play a role in limiting replication of virus in IECs whereas type I IFNs are thought to be involved in avoiding systemic spread of the virus[222]. Therefore, IFN-λ might provide a localized antiviral protection at barrier sites such as the intestinal epithelium without activating a systemic pro-inflammatory immune response.

TNF-**α**

TNF-α belongs to the TNF superfamily, which consists of 19 ligands and 29 receptors[223]. The TNF-α protein is generally produced as transmembrane-bound protein (mTNF-α), whose cleavage by a TNF-α-converting enzyme (TACE) results in the release of the soluble form (sTNF-α). Both mTNF-α and sTNF-α are bioactive and can signal through the two receptors TNF receptor 1 and 2 (TNFR1 and 2)[224]. However, while TNFR1 is ubiquitously expressed on most cell types of the body and preferentially binds sTNF-α; TNFR2 expression is restricted to hematopoietic cells, endothelial cells and neurons and binds mainly to mTNF-α[224,225].

Signaling via TNFR1 is involved in the regulation of cell survival. Upon sTNF-α binding, TNFR1 recruits several proteins, leading to a subsequent signaling cascade that culminates in the activation of NFκB and expression of antiapoptotic functions, besides autoregulation of TNF-α in a positive feedback loop[226]. Additionally, depending on the cellular context, TNFR1 signaling can also induce cell death via apoptosis or necroptosis.

In the intestine, a tight regulation of TNF-α expression is crucial for maintaining intestinal homeostasis. In addition to monocyte-derived cells, which seem to be the main producers, TNF-α is also produced constitutively by Paneth cells and it is thought to be involved in the tight regulation of IECs shedding into the lumen during intestinal epithelium renewal[190,227]. Increased levels of TNF-α have shown to induce increased epithelial shedding, altering thus the barrier integrity due to

increased apoptosis[228–230]. Moreover, TNFR2 signaling in IECs has been shown to drive wound healing during intestinal inflammation by activating the epithelial Wnt/β-catenin pathway, which is very important for maintaining the intestinal crypt-villus architecture[231,232].

During intestinal infection with bacteria such as *Clostridium difficile*, TNF-α levels are generally increased compared to healthy controls[233,234]. Interestingly, TNF-α driven inflammation is fully dependent on microbiota, since inflammation was completely absent in germ-free mice that overproduce TNF-α[235]. Accordingly, LPS from microbiota induces immune cells to produce TNF-α, which will act via TNFR1 on IECs and induce their necroptosis and shedding into the lumen. Contrary, during viral infections such as rotavirus, TNF-α on IECs seems to reduce the total viral RNA levels[236]. Collectively, TNF-α signaling plays a crucial role in induction of necroptotic cell death during bacterial infection whereas viral dsRNA induces cell death in a TNF-independent manner, as shown in *Tnfr1* knockout (KO) mice[236].

Mouse models overexpressing TNF-α develop intestinal inflammation resembling human IBD. Indeed, anti-TNF-α is the gold standard treatment in IBD patients[224]. However, this treatment only works for a specific subgroup of patients, indicating that several other mechanisms are involved in the development of the disease.

1.5 Dendritic cells and their potential for mucosal vaccination

Vaccination is one of the most successful medical interventions in history, leading to significantly reduced mortality and morbidity across the globe and even to the eradication of the small pox virus. However, infectious diarrhea is still the leading cause of death among children under the age of 5[237]. In developing countries, implementation of rotavirus vaccination as well as the improvement in sanitation strategies, safe water and reduction in childhood malnutrition has helped decrease diarrhea-associated mortality[238]. However, there are still financial, logistic and biological challenges. Biologically, the low efficacy of the two approved vaccines in the developing world might be due to the greater diversity of circulating rotavirus serotypes[239]. In addition, children might suffer from micronutrient malnutrition, HIV/AIDS or are co-infected with other enteropathogens[240,241], of which there is still no vaccination against. Accordingly, there is a need for understanding the mechanisms of efficient vaccination to develop new and better vaccines that can overcome most of these challenges.

Vaccine-induced T cell immunity is required for effective protection against intracellular pathogens such as HIV, tuberculosis and also against cancer. However, most of the currently available vaccines confer protection by the induction of antigen-specific, long-lived antibodies. DCs are key players in linking innate and adaptive immune responses. Activation of DCs through PRRs leads to their interaction with both B and T cells, thus shaping both the humoral and cellular immune responses[242]. Therefore, activation of DCs represents a potential strategy in vaccines designed to induce cellular immunity[243].

Activation of DCs

DCs are generally immature during homeostatic conditions, characterized by high expression of intracellular MHC-II in late endosome-lysosomal compartments, low expression of costimulatory molecules and low expression of chemokine receptors[137]. Immature DCs are highly phagocytic and are constantly sampling foreign and self-Ag. Upon Ag encounter, Ag-bearing DCs decrease their phagocytic capacity and increase their motility. Subsequently, DC activation leads to upregulation of costimulatory molecules, MHC-molecules and chemokine receptors such as CCR7, which drives their migration to the draining lymph nodes, allowing DCs to interact with T cells and initiate the generation of immune T cell responses[242,244].

Upon activation, DCs must provide three signals to induce proper T cell adaptive immune responses. Signal 1 is provided by the increased expression of MHC molecules presenting peptides to the naïve T cells. Signal 2 is provided by a variety of costimulatory molecules (e.g. CD86 and CD80), which engage CD28 and others on T cells and transmit signals for T-cell proliferation and survival. Lastly, signal 3 consists of mediators such as pro-inflammatory cytokines that act on T cells, promoting their differentiation into effector T cells. Importantly, the combination of all three signals defines the tailored T cell effector response[245].

Cis vs *trans*-activation

Although DC activation is generally thought to be induced by direct TLR engagement, soluble mediators such as TNF-α and type I IFNs have been shown to induce DC activation, characterized by up-regulation of costimulatory molecules and increased expression of MHC molecules[246–249]. Accordingly, activation of DCs by direct TLR engagement is known as *cis*-activation, whereas maturation of DCs by pro-inflammatory cytokines is known as *trans*-activation[245]. The relevance of this difference is thought to rely on the capacity of the *cis* vs *trans*-activated DCs at inducing adaptive immune responses. Indeed, previous studies have shown that only *cis*-activated DCs provide all signals required for priming effector T cell responses[250–252]. In contrast, *trans*-activated DCs have shown to only provide with signal 1 and 2, which generally leads to clonal expansion of non-polarized T cells[253].

In line with this, *trans*-activated DCs seem to induce expansion of Tregs and are, in fact, superior to *cis*-activated DCs[254,255]. Accordingly, induction of Tregs could control the intensity of the immune response during infection, avoiding excessive inflammation. However, this could also be detrimental during vaccination strategies, due to suppression of immune responses[255]. Another hypothesis is that *trans*-activated DCs presenting self-Ags could prevent generation of self-reactive effector T cells during infection. Given that *trans*-activated DCs are likely to outnumber *cis*-activated DCs during infection, self-reactive non-tolerant T cells that had escaped central or peripheral tolerance would thus more likely encounter its cognate Ag on *trans*-activated DCs rather than on *cis*-activated DC[256]. Collectively, these findings suggest that *cis* and *trans*-activated DCs play distinct roles during infection and that this needs to be considered when designing DC-targeted vaccines.

Adjuvants are defined as substances used in combination with a specific Ag that produced a more robust immune response than the Ag alone[257]. In line with this, some vaccines contain adjuvants that enhance their induction of protective immunity. Several distinct compounds have been tested as adjuvants, including mineral salts, microbial products and microparticles. In the intestine, cholera toxin (CT) has shown to be a potent mucosal adjuvant, inducing potent mucosal memory[258]. In addition, R848 and poly(I:C) are synthetic ssRNA and dsRNA, respectively, and have been used to study the mechanisms involved in viral infections. Surprisingly, the mechanisms of action of such adjuvants are still not fully understood. Accordingly, more research is required to understand the mechanisms of action of adjuvants, leading to development of optimal mucosal vaccines[257,259].

DC migration

Migration from peripheral tissues to lymphoid organs is a key aspect of DCs. Migration of DCs into lymphatics is dependent on CCR7, which interacts with its ligands CCL19 and CCL21 found on LEC. Thus, DCs migrate by the CCR7-CCL21 axis in a process called haptotaxis[64,260].

The continuous, steady-state migration of DCs from the intestine to the mLNs can significantly increase upon TLR stimulation. Yrlid et al showed that upon R848, a ligand for TLR7, migration of all DC subsets was significantly increased in mLNs[45]. This indicates that although DCs seem to be

hyporesponsive to TLR4 ligands, intestinal DCs are still able to respond to other TLR ligands, and these responses may lead to different migratory patterns for different intestinal cDC subsets[195]. Indeed, Flores-Langarica and co-workers showed that upon flagellin stimulation, which is a ligand for TLR5, numbers of cDC2 but not cDC1 increased in the mLNs, and this increased migration was responsible for the antibody response[261]. These findings suggest that the dynamics of intestinal DC migration change after immune stimulation, and that this can occur in a DC subset specific manner.

Resident vs migratory

DCs either seed the mLNs as precursors from the blood or as peripherally matured DCs from the afferent lymph. Thus, it is important to distinguish these different DCs. The main approach to distinguish between resident and migratory DCs is the level of expression of MHC-II and CD11c. Accordingly, LN-resident DCs are $CD11c^{hi}MHC^{int}$ whereas migratory DCs are $CD11c^{int}MHC^{hi}$ [262]. However, DC activation in response to inflammatory stimuli such as TLR ligands leads to a merge of resident and migratory DCs on the basis of these markers, hampering their faithful discrimination. Of note, LN-resident cDC1 uniquely express CD8α and thus can be distinguished from migratory cDC1 on the basis of CD8α expression[263,264]. However, there is no equivalent marker to distinguish LN-resident cDC2 from migratory cDC2.

In addition, previous studies with CCR7-deficient mice led to identification of CD103 as a surface marker for migratory DCs[81,265]. In agreement with these findings, Cerovic et al showed that 75 to 85% of $CD11c^{+}MHC\text{-}II^{+}$ cells present in intestinal-derived lymph were $CD103^{+}$ [83]. Although LN-resident DCs might express low levels of CD103, high expression of CD103 is a good marker for identifying migratory DCs.

Several studies showed that $CX3CR1^{+}$ macrophages were absent in intestinal-derived lymph[40,83]. Indeed, macrophages display a very poor migratory capacity and lack CCR7 expression. Of note, the population of $CX3CR1^{+}$ cells migrating in the lymph found by Diehl et al are probably $CD103^{-}$ cDC2 that express intermediate levels of CX3CR1[83,266]. However, under certain inflammatory conditions, monocytes can give rise to $CX3CR1^{int}$ cells with the capacity to migrate to the mLNs[28,41,267].

Role of DCs in T cell responses *in vivo*

Migratory cDCs play crucial roles in the initiation of adaptive immune responses due to their unique ability to prime naïve T cells in mLNs. In the intestine, different subsets of cDCs have been linked to prime different adaptive T cell responses. This is probably due to a combination of intrinsic properties of the different DC subsets together with the expression of a large repertoire of PRRs that partially differs between different DC subsets, allowing for tailoring a specific immune response to the Ag acquired[196,261,268].

$CD8^{+}$ cytotoxic T cells

The ability to cross-prime $CD8^{+}$ T cells is conferred to cDC1. Consistent with their developmental relationship to LN-resident $CD8\alpha^{+}$ cross-presenting DCs, cDC1 isolated from afferent lymph

appear to be superior at cross-presenting Ag to $CD8^+$ T cells *in vitro*[83]. Furthermore, *in vivo* studies have shown that cDC1 play a unique, dominant role at presenting intestinal epithelium cell-derived antigens to $CD8^+$ T cells both in steady state[269] and during rotavirus infection[270]. This might explain the reduction in $CD8^+$ T cell numbers seen by Luda et al and Ohta et al, suggesting that reduction of cDC1 numbers is the cause of reduction of $CD8^+$ T cells[271,272]. This is further confirmed by the fact that cDC1 are the main source of RA, and absence of RA results in reduced induction of gut-homing receptors such as CCR9 and $\alpha_4\beta_7$ and in turn, reduction of $CD8^+$ T cells in the SI[271].

$CD4^+$ effector and regulatory T cells

Th1 induction

Th1 cells are constitutively present in the steady state intestine and are required to help in clearance of intracellular pathogens and viruses. Fujimoto et al showed that both cDC1 and cDC2 can drive Th1 differentiation *in vitro*[273]. By contrast, Luda et al showed that cDC1 were required for generation and survival of Th1 cells in steady state[271]. In addition, mice lacking cDC1 failed to mount Th1 cell responses to *Trichuris muris* infection[271]. These findings were further confirmed by Ohta et al, who showed that $CD4^+$ T cells from XCR1-DTA mice displayed reduced IFN-γ mRNA levels[272]. Although not yet known, the role for cDC1 in driving Th1 responses might be due to their higher capability of producing IL-12[274].

Th2 induction

Th2 cells are virtually absent in the steady state intestine. The generation of Th2 responses is thus actively induced by parasitic infections or allergic stimuli. A recent study by Mayer et al showed that upon infection with *Trichuris muris* and *Schistosoma mansoni* eggs, mice lacking IRF4-dependent DCs, which have reduced numbers of both cDC2 and $CD103^-$ cDC2 subsets, did not develop Th2 responses[275]. Interestingly, they found that while cDC2 were responsible for induction of Th2 towards *Schistosoma mansoni* eggs in the SI, this role was fulfilled by the $CD103^-$ cDC2 subset when the eggs were delivered directly to the colon[275]. Impaired survival and lack of migration of DCs seemed to be the reason for deficient Th2 responses in these mice[275]. $CD11b^+$ migratory DCs are likely involved in priming Th2 responses to different parasites and distinct subsets fulfil the same role at different anatomical compartments. Additionally, Tussiwand et al showed that Klf4 expression in IRF4-dependent DCs was required for Th2 responses to several stimuli, but did not affect Th1 nor Th17 responses *in vivo*[276]. Collectively, these findings demonstrate a functional specialization of cDC2 and $CD103^-$cDC2 subsets at driving intestinal Th2 responses.

Th17 induction

Th17 cells are the most abundant effector Th cells present in the intestine at steady state. Mice specifically lacking IRF4 in all CD11c-expressing cells, which have reduced numbers of cDC2, showed impairment in driving intestinal Th17 cells, suggesting that intestinal cDC2 have a major role in intestinal Th17 differentiation *in vivo*[121]. However, MHC-II-deficient cDC2, which lack the ability to engage T cells via their TCR, showed normal numbers of Th17 cells, indicating that cognate Ag presentation is dispensable for Th17 polarization[277]. Instead, the polarization appears to

be linked to the capacity of cDC2 to produce IL-6[121,278]. Of note, these mice still have Th17 cells, indicating that other subsets additionally contribute to Th17 generation[112,121]. Indeed, Panea et al showed that macrophages are in part responsible for driving Th17 responses to SFB[279].

Tregs and oral tolerance

Induced regulatory T (iTreg) cells are abundant in the intestinal mucosa and play an important role in maintaining intestinal homeostasis. Induction of iTreg cells requires CCR7, mLNs and FoxP3, indicating that intestinal migratory DCs play a crucial role in inducing tolerance to Ag[33,280–282]. *In vitro* studies have shown a synergistic role for RA and TGF-β in inducing iTregs by $CD103^+$ DCs[281,282]. Similarly, $CD103^+$ DCs are responsible for differentiation of naïve T cells into Tregs by mechanisms involving RA and TGF-β *in vivo*. IRF8-dependent migratory cDC1 express high levels of RALDH2, the enzyme required for metabolizing retinal into RA. Of note, RA is also involved in imprinting gut-homing receptors, and migration of newly induced iTregs is also required for oral tolerance. In addition, cDC1 are unique in their expression of $\alpha_v\beta_8$, the integrin that activates latent TGF-β[283]. Therefore, cDC1 appear to be the main subset responsible for iTreg induction. Interestingly however, depletion of these cells had no effect on the induction of oral tolerance, indicating that there must be redundancy in the contribution of distinct DC subsets[271,272,284,285].

Role of DCs in B cell responses *in vivo*

The vast majority of intestinal B cells are IgA-secreting plasma cells. Early *in vitro* studies of co-culture systems with B cells, T cells and DCs demonstrated the role of DCs in IgA induction[286,287]. Ag-carrying DCs have shown to induce IgA CSR by both TD- and TI-pathways [68,288]. TD-IgA induction is largely performed in the GC of the PPs. In line with this, PPs $CD11b^+$ cDC2 at the SED acquire microbial-derived Ag and migrate into IFR, where they prime T cells. Subsequently, primed T cells provide the help to B cells to induce IgA. This TD-IgA by $CD11b^+$ cDC2 is driven by the higher expression of IL-6 by this DC subset, compare to the rest[70]. Interestingly, a recent study showed that cDC2 but not cDC1 were required for induction of IgA in the mLNs in response to soluble flagellin[261]. Lack of migratory cDC2 in mLNs was the reason for the absence of IgA-producing plasma cells in the SI, mLNs and BM. Of note, RA is required for the imprinting the gut-homing phenotype of IgA^+ B cells, which derives mainly from DCs and stromal cells in the mLNs[82,84,85].

In contrast, TI-IgA generation by DCs occurs through DC-derived active TGF-β as well as APRIL and BAFF, driving CSR to IgA^+ B cells[75]. This IgA contributes to the homeostatic, low affinity IgA, since it does not undergo somatic recombination. However, TI-IgA is still important in maintaining the commensal bacterial homeostasis[289]. In addition, GALT-generated TNF and iNOS-producing DCs (Tip-DCs), which express iNOS (and in turn produce NO) induce TD-IgA by induction of TGF-β receptor on B cells via NO[290]. In addition, Tip-DCs can also induce TI-IgA via stimulating DC production of BAFF and APRIL. Interestingly, NO induces DC expression of CCR7, leading to their migration to the mLNs[291]. However, Tip-DCs seem to derive from inflammatory monocytes, because they are absent in CCR2-deficient mice[292].

DCs as potential candidates for mucosal vaccination

The oral polio vaccine was the first mucosal immunization introduced in 1950s by Sabin. Despite the benefits of mucosal vaccination being needle-free and conferring more efficient protective immunity than systemic immunizations, only few mucosal vaccines are commercially available.

This is due to unique features of mucosal tissues that make vaccine delivery particularly challenging. First, vaccines get diluted in the mucosal secretions, trapped in the mucus gel or are attacked by proteases and nucleases. In line with this, relatively high doses are required and it is very difficult to determine exactly the dose that crosses the mucosa. Additionally, the intestine generally induces tolerance to most Ags. Therefore, the induction of an effective mucosal immune response requires a potent Ag that is able to break tolerance. Accordingly, the use of several PAMPs together might be more successful at mimicking the danger signals from viruses, inducing thus a potent mucosal immunity[293].

Optimal protection of mucosal surfaces is achieved by induction of sIgA, which is poorly induced by conventional injected vaccines[294]. IgA induction mainly takes place in GALT, where DCs in the SED capture Ag and present it to T cells, leading in turn to TD-IgA production. Additionally, mLNs have shown to play important roles for the generation of memory IgA^+ B cells, and reduction of migratory DCs in the mLNs have shown to reduce the amount of IgA produced in response to soluble flagellin[261]. Thus, migratory DCs in the mLNs might also be important sites for IgA-induction. Finally, compartmentalization in the gut is achieved by imprinting gut-homing receptors that allow B and T cells to return to the mucosa. The imprinting of gut homing receptors is mediated by DCs and is dependent on RA.

Collectively, given that DCs play crucial roles in both TD and TI-IgA generation in the gut and that they are uniquely capable of inducing potent T cell responses from a naïve responder pool, they are formidable candidates as mucosal vaccine targets.

2. Hypothesis, aim and presented work

We hypothesize that in addition to their distinct functionality, different intestinal DC subsets require distinct mediators to become activated and migrate in response to different TLR ligands.

The overall aim of my thesis is to assess the molecular requirements for activation and migration of intestinal mouse cDC1 and cDC2 subsets to the mLNs in response to poly(I:C).

Experiments included preparations of both mLNs and SI to assess DC migration. Indeed, an increase in DC numbers in the mLNs correlated with a decrease of DCs in the SI. The focus of this thesis is on migratory DCs and thus all data presented herein show DC numbers from mLNs.

Due to the difficulty of separating resident vs migratory DCs in the mLNs after poly(I:C) injection, both resident and migratory DCs were considered for analysis.

Manuscript included in the thesis:

Migration of intestinal dendritic cell subsets upon intrinsic and extrinsic TLR3 stimulation

Agnès Garcias López, Vasileios Bekiaris, Katarzyna Müller Luda, Julia Hütter, Konjit Getachew Muleta, Joy Nakawesi, Isabel Ulmert, Knut Kotarsky, Bernard Malissen, Meredith O'Keeffe, Bernhard Holzmann, William Agace and Katharina Lahl

BioRxiv, doi: https://doi.org/10.1101/785675

The thesis also contains complementary results developed in parallel to the results presented in the manuscript.

Migration of intestinal dendritic cell subsets upon intrinsic and extrinsic TLR3 stimulation

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Running Title: Poly(I:C)-induced migration of both major dendritic cell subsets depends on TLR3

Keywords: Dendritic cells, TLR3, migration, activation, type I interferon

Abstract

Initiation of adaptive immunity to particulate antigens in lymph nodes largely depends on their presentation by migratory dendritic cells (DCs). DC subsets differ in their capacity to induce specific types of immunity, allowing subset-specific DC-targeting to influence vaccination and therapy outcomes. Faithful drug design however requires exact understanding of subset-specific versus global activation mechanisms. cDC1, the subset of DCs that excel in supporting immunity towards viruses, intracellular bacteria and tumors, express uniquely high levels of the pattern recognition receptor TLR3. Using various genetic models, we show here that both the cDC1 and cDC2 subsets of cDCs are activated and migrate equally well in response to TLR3 stimulation in a cell extrinsic and TNFα dependent manner, but that cDC1 show a unique requirement for type I interferon signaling. Our findings reveal common and differing pathways regulating DC subset migration, offering important insights for the design of DC-based vaccination and therapy approaches.

Introduction

Dendritic cells (DCs) are the major antigen-presenting cells in the body, which, upon migration to secondary lymphoid organs, initiate and shape naïve T cell responses to peripherally acquired antigen. DCs are divided into two major subsets referred to as cDC1 and cDC2 (Guilliams et al., 2014). In the intestine, migratory cDC1 are defined as $XCR1^{+}CD103^{+}CD11b^{-}$, while cDC2 can be divided into a major $XCR1^{-}CD103^{+}CD11b^{+}$ and a minor $XCR1^{-}CD103^{-}CD11b^{+}$ subset. Although both subsets present mucosa-derived antigen in the draining lymph nodes (LNs), cDC1 and cDC2 differ in their capacity to induce specific immune responses (Eisenbarth, 2018). While cDC1 are generally implicated in viral defense and cross presentation of exogenous antigens to MHCI-restricted $CD8^{+}$ T cells and MHCII-restricted $CD4^{+}$ T_H1 cells (Hildner et al., 2008), cDC2 are highly effective at inducing T_H17 and T_H2 responses (Persson et al., 2013; Schlitzer et al., 2013; Williams et al., 2013). Specific targeting of DC subsets is thus of high relevance for DC-based strategies for vaccination and therapeutic approaches against different types of antigen.

Antigen-targeting to specific DC subsets using antibody-mediated delivery to differentially expressed surface receptors can indeed shape the resulting type of immunity (Dudziak et al., 2007). One family of molecules expressed differentially by DC subsets is toll-like receptors (TLRs)(Denning et al., 2011; Edwards et al., 2003), suggesting that differential engagement of DC subsets could also be achieved by using adjuvants specifically activating one subset but not the other. In support of this idea, the induction of fully functional cytotoxic $CD8^{+}$ T lymphocytes depends on simultaneous uptake of antigen together with cell-intrinsic stimulation of pattern recognition receptors expressed by the presenting DC (cis-activation)(Desch et al., 2014). TLR3 is an endosomal receptor that recognizes double-stranded RNA (dsRNA), a molecular pattern associated with viral infections (Alexopoulou et al., 2001; Matsumoto et al., 2002). As several studies have demonstrated that TLR3 is preferentially expressed in cDC1 (Davey et al., 2010; Edwards et al., 2003; Jelinek et al., 2011; Luber et al., 2010) and promotes cross-presentation of antigen with high efficiency (Mandraju et al., 2014; Rizzo et al., 2016; Schulz et al., 2005), targeting TLR3 is a promising strategy in cancer-immunotherapy and vaccination against viruses. A hallmark of DCs is to migrate to the draining LNs to present peripherally acquired antigen. In response to the TLR7-stimulating agent R848, plasmacytoid (pDC)-derived TNFα drives cDC migration from the small intestinal lamina propria (SI LP) to the mesenteric LNs (mLNs), while type I interferon (IFN) regulates DC activation (Yrlid et al., 2006). Subset-specific requirements were not assessed. Most TLR3 driven transcriptional changes in splenic DCs after stimulation with the double-stranded (ds)RNA mimic polyinosinic:polycytidylic acid (poly(I:C)) result from secondary effects through the type I IFN receptor on cDCs (Pantel et al., 2014). This suggests that migration in response to poly(I:C) may also depend on type I IFN signaling. Here we have analyzed in detail the major cellular and molecular players involved in the activation and migration of intestinal cDC subsets in response to poly(I:C) *in vivo* and provide novel insights regarding cis- and trans-regulation of these processes.

Results and Discussion

Poly(I:C)-induced intestinal DC migration depends on TLR3 signaling

We first set out to analyze in detail the expression of TLR3 by immune cells of the spleen, mLNs and small intestine lamina propria (SI-LP) and confirmed that only cDC1 DCs expressed high amounts of TLR3 in all

organs (Fig. 1A). While macrophages also expressed low levels of TLR3, cDC2 were almost entirely negative and B and T cells showed no expression (Fig. 1A and Supplemental Fig. 1A). Importantly, stimulation with poly(I:C) did not change TLR3 expression across subsets (Fig. 1A). These results are consistent with *in vitro* data on the differential abilities of the cDC subsets to poly(I:C) stimulation (Jelinek et al., 2011) and therefore we hypothesized that poly(I:C) would drive migration of cDC1 preferentially *in vivo*. To test this, we quantified $CD103^+$ cDC1 and cDC2 in the mLN after intraperitoneal injection of poly(I:C), based on the knowledge that CD103 expression by cDC in the mLN defines those which are derived from CCR7 dependent migration from the intestinal mucosa (Hagerbrand et al., 2015; Johansson-Lindbom et al., 2005). Consistent with this idea, the numbers of both migratory cDC1 and cDC2 increased after administration of poly(I:C), peaking at 12 hours post-injection and returning to steady state levels after 24h (Fig. 1B). Interestingly, cDC2 migrated almost as efficiently as cDC1, with only a small disadvantage being seen at early time points.

Although retinoic acid-inducible gene 1 (RIG-I)-like helicases that signal through mitochondrial antiviral-signaling protein (MAVS) can also sense poly(I:C) (Jensen and Thomsen, 2012), DC migration of both subsets was completely abrogated in TLR3-deficient mice (Fig. 1C) and in mice deficient for the TLR3 adapter TRIF (TIR-domain-containing adapter-inducing interferon-β) (Supplemental Fig. 1B). As DC migration and activation, both crucial events for the induction of immunity, can be regulated independently (Jones et al., 2016; Yrlid et al., 2006), we also measured the expression of the costimulatory molecule CD86 as a surrogate marker for DC activation. Again, activation of both DC subsets was also entirely depended on TLR3 and TRIF expression (Fig. 1D and Supplemental Fig. 1C), showing that poly(I:C) induces migration and activation of both cDC1 and cDC2 in a strictly TLR3-dependent manner. Our findings are in accordance with previously published data showing that *in vitro* activation with poly(I:C) is abrogated in bone-marrow (BM)-derived DCs from TLR3-deficient mice (Jelinek et al., 2011). However as cDC2 themselves express virtually no TLR3, our data indicate that TLR3 stimulation can act in both cell-intrinsic and extrinsic manners on cDCs *in vivo*.

Cell-intrinsic TLR3-sensing is dispensable for DC migration

Non-hematopoietic cells express TLR3 and support immune cell survival, maturation and function. TLR3 expression in intestinal epithelial cells is required for optimal clearance of rotavirus (Pott et al., 2012) and epithelial cells have previously been implicated in driving DC migration to the draining LNs during viral infection (Ye et al., 2019). To determine whether TLR3-dependent sensing in non-hematopoietic cells could induce intestinal DC migration in response to poly(I:C), we reconstituted irradiated wild-type mice with TLR3-deficient BM and treated the mice with poly(I:C). The results showed that while DCs migrated well in response to poly(I:C) in WT recipients of WT BM, there were no significant increases in mLN DC numbers in recipients of TLR3-deficient BM after administration (Fig. 2A). Thus TLR3-expression within the hematopoietic compartment is required to drive efficient DC migration in response to poly(I:C).

As cDC1 uniformly expressed TLR3, we explored the role of this subset in sensing poly(I:C) for driving DC migration directly, by generating a mouse model that allows for cell-specific re-expression of TLR3 in a TLR3 KO background. To this end, a floxed transcriptional termination cassette was inserted into the coding sequence of the TLR3 gene ($TLR3^{OFF}$), abolishing TLR3 expression. Expression of TLR3 by cDC1s could then be restored in cDC1.$TLR3^{ON}$ mice in which the TLR3 stop codon was deleted using XCR1-driven cre recombinase (XCR1.cre (Janela et al., 2019)) (Fig. 2B and Supplemental Fig. 2A). Poly(I:C)-induced DC

migration and activation of both cDC1 and cDC2 occurred in cDC1.TLR3^{ON} mice, but to a lesser extent compared to WT mice (Fig. 2C,D). As expected, DC migration was absent in TLR3^{OFF} mice (Fig. 2C,D). These findings suggest that while cDC1-restricted TLR3 expression can drive poly(I:C) induced DC migration, other TLR3-expressing cells contribute to optimal DC migration in response to poly(I:C). Of note, careful analysis of XCR1-driven re-expression of TLR3 revealed that re-expression of TLR3 also occurred in ~20% of CD64^{+}CD11b^{+}XCR1^{-} macrophages in the intestine, but not in spleen macrophages (Supplemental Fig. 2A). This phenomenon is not specific for the TLR3 locus, as XCR1.cre could also drive YFP expression by some intestinal macrophages when crossed to ROSA-STOP-YFP (data not shown). We therefore examined whether off-target re-expression of TLR3 by intestinal macrophages might account for the restored DC migration in cDC1.TLR3^{ON} mice. However, migration of both cDC1 and cDC2 was entirely normal after administration of poly(I:C) to CCR2-deficient mice that lack most intestinal macrophages (Bain et al 2014) (Supplemental Fig. 2B). Finally, we could not detect any migration or activation of either DC subset if TLR3 expression was restricted to intestinal epithelial cells of TLR3^{OFF} mice using villin-cre (villin.TLR3^{ON}, Supplemental Fig. 2C). Together, these data suggest that cDC1-specific TLR3 expression can drive DC migration in response to poly(I:C), although a contributory role for a residual population of CCR2-independent, TLR3 expressing intestinal macrophages in cDC1.TLR3^{ON} mice is likely.

The fact that expression of TLR3 restricted to XCR1-expressing cDC1 can drive the migration and activation of cDC2 indicates a cell extrinsic effect of poly(I:C) on this subset. To confirm this, we generated mixed-BM chimeras in which WT recipients on a CD45.1/.2 congenic background were reconstituted with a 50:50 mix of CD45.1^{+} WT and CD45.2^{+} TLR3-deficient BM. Under these conditions, administration of poly(I:C) induced the activation and migration of TLR3-deficient cDC1 and cDC2 to the same extent as their WT counterparts in the same hosts (Fig. 2E,F), indicating that both cDC1 and cDC2 can respond to TLR3 stimulation in a cell extrinsic manner. This is presumably driven by the TLR3-competent bone marrow derived cells of WT origin present in the mixed chimeras. Interestingly, cDC1 themselves do not appear to play an essential role in this process, as complete deficiency of cDC1 DCs in BATF3^{KO} mice (Edelson et al., 2010) did not abrogate the activation and migration of cDC2 in response to poly(I:C), showing that hematopoietic cells other than cDC1 can also contribute (Figure 2G,H). Macrophages are a potential candidate for this role, as they express and respond to TLR3-stimulation (Zhou et al., 2010) and we attempted to explore their involvement by generating macrophage-specific TLR3^{ON} mice using LysM.cre (McCubbrey et al., 2017) to delete the TLR3 stop codon in TLR3^{OFF} mice. However this approach was unsuccessful, as TLR3 was re-expressed by ~50% of cDC1 of LysM.TLR3^{ON} mice and thus the role of macrophages in responding to TLR3 *in vivo* requires further investigation (Supplemental Fig. 2D).

Taken together, these results show that the hematopoietic compartment is responsible for TLR3-dependent migration and activation of DCs, but that these processes can occur in a cell-extrinsic manner, with cDC1-derived signals not being essential, despite the high levels of TLR3 expression by these cells.

DC migration in response to poly(I:C) is independent of MyD88, but requires TNF receptor signaling

The cell-extrinsic effect of TLR3 on DC migration in response to poly(I:C) suggests that inflammatory mediators produced following TLR3 signaling on TLR3^{+} target cells might play a key role in this process. We therefore measured the expression of cytokines that have been implicated in DC activation and migration by qPCR analysis of whole SI tissue samples at different times after administration of poly(I:C). This showed

increased levels of mRNA for TNF-α, IL-1β, IFN-α, and IFN-β after 2 and 4 hours after poly(I:C) injection (Figure 3A).

Steady state migration of intestinal DCs depends on MyD88 signaling through NFκB (Baratin et al., 2015; Hagerbrand et al., 2015) and although TLR3 signaling itself does not require MyD88, the IL1 receptor signals through MyD88 (Dinarello, 2009). However, the activation and migration of both cDC1 and cDC2 occurred normally in poly(I:C) treated MyD88^{KO} mice (Figure 3B, C). Although TNF receptor 1 (TNFR1) signaling is not important for intestinal DC migration in the steady state, it is required for DCs to migrate in response to R848 (Hagerbrand et al., 2015). TNFR1 signaling is important for the induction of the CD8 T cell response towards mouse hepatitis virus, and expression on DCs alone is sufficient to confer protection (Ding et al., 2011). As we found TNFα to be upregulated in the intestine after injection with poly(I:C), we examined its role in poly(I:C) induced DC migration. Indeed, there were no significant increases in migration of either cDC1 or cDC2 in response to poly(I:C) in either TNFR1^{KO} mice or mice treated *in vivo* with a blocking anti-TNFR1 antibody, while there was minimal DC activation in poly(I:C) treated TNFR1^{KO} mice (Figures 3D-F). These results are consistent with previous studies on skin DCs (Suto et al., 2014) and show that TNFR1 signaling is a crucial secondary signal that mediates the extrinsic response of DCs to TLR3-sensing *in vivo*.

DC subsets differ in type I IFN signaling requirements for migration and activation in response to poly(I:C)

In addition to elevated expression of TNF-α and IL-1β, type I IFNs were significantly upregulated in the intestine after poly(I:C) injection (Figure 3A). Previous studies have shown a prominent role for type I IFN in the activation and maturation of splenic DCs in response to poly (I:C), acting via the type I IFN receptor on DCs (Pantel et al., 2014). Conversely, the TNFα dependent migration of intestinal DCs in response to R848 does not require type 1 IFNR signaling (Yrlid et al., 2006) and we therefore tested directly the role of type I IFN in the activation and migration of intestinal DCs in response to poly(I:C).

Type I IFN receptor-deficient mice (IFNAR^{KO}) showed defective migration of both cDC1 and cDC2 in response to poly(I:C) (Figure 4A) and similar results were found in mice lacking IFNAR in all CD11c-expressing cells, although cDC2 were partially resistant to the effects of deletion of IFNAR in these mice (Figure 4B). The activation of both DC subsets as assessed by CD86 expression was also greatly diminished in CD11c.IFNAR^{KO} mice (Figure 4C). Conversely, while cDC1-specific deletion of the IFNAR in XCR1.IFNAR1^{KO} mice abrogated the poly(I:C) induced migration and activation of cDC1, this had no effect on either parameter in cDC2 (Figures 4B, C). Deletion of IFNAR in cDC2 in huCD207.IFNAR1^{KO} mice had no effect on the migration or activation of either DC subset, apart from a small decrease in CD86 upregulation by cDC2 (Figures 4B, C), consistent with previous findings that type I IFN may be more important for activation than migration (Yrlid et al., 2006). These data suggest that type I IFN has few if any direct effects on cDC2 migration in response to poly(I:C).

Mixed BM chimeras using a 50:50 combination of WT and XCR1.IFNAR1^{KO} BM showed that the requirement for type I IFN signaling in cDC1 migration was cell intrinsic, contrasting with what we had observed for TLR3-signaling. Poly(I:C) induced migration of cDC2 remained intact in these chimeras (Figure 4D). A similar defect in cDC1 migration was seen in XCR1.IFNAR1^{KO} mice treated orally with R848, the ligand for TLR7 expressed mostly by cDC2, indicating a need for type I IFN signaling in cDC1 regardless of whether stimulation occurred in a direct or indirect manner (Figure 4E). cDC2 migration was also induced by R848 in huCD207.IFNAR1^{KO} mice lacking type I IFN signaling specifically on cDC2, although this was somewhat

reduced in comparison with that in WT mice (Fig. 4F). Thus, type I IFN signaling plays a global role in the migration of cDC1 in response to TLR stimulation, but has little or any effect on cDC2 migration in response to either TLR3 or TLR7 stimulation.

As well as type I IFN, poly(I:C) also induces cDC1 to express IFN-λ in a manner that requires IFNAR on DCs (Lauterbach et al., 2010). IFN-λ is a type III IFN that drives thymic stromal lymphopoietin expression by M cells in response to nasal vaccination with an influenza vaccine, which in turn drives cDC1 migration from the respiratory tract to the mediastinal lymph nodes (Ye et al., 2019). We therefore tested whether IFN-λ was required for the poly(I:C) induced migration of DCs, using mice deficient for IL28R, the receptor for IFN-λ. However the activation and migration of both cDC1 and cDC2 in response to poly(I:C) were normal in these animals (Figure 4G,H).

Our data reveal a previously unappreciated differential role for type I IFN in cDC migration from the intestine to the mLNs. While IFNAR signaling drives maturation of both major subsets of migratory DCs, only cDC1 critically depend on direct type I IFN signals for migration in response to poly(I:C). Thus, the mechanisms inducing DC migration may be subset specific. Although the migration and upregulation of CD86 in both cDC1 and cDC2 were entirely TLR3-dependent, this occurred in a cell-extrinsic manner and the cells responding directly to TLR3 remain to be identified. Consistent with previous reports in other models ((Longhi et al., 2009; Pantel et al., 2014; Yrlid et al., 2006); we found that TNFα and type I IFN signaling played important roles as secondary mediators in TLR3-mediated intestinal DC migration. Interestingly, the ability of DCs to induce proliferation by naïve $CD4^+$ T cells also does not require cell intrinsic expression of pattern recognition receptors by DCs, whereas the functional polarization of T cells depends on direct sensing of the pathogen-associated molecular pattern by the presenting DC (Desch et al., 2014; Spörri and Reis e Sousa, 2005). Our data showing that the migration and upregulation of costimulatory molecules by DCs in response to TLR stimuli *in vivo* can result from trans-activation of DCs therefore suggest that differences in migration capacities may not be linked to the polarization of T cell responses. However it is important to note that not all TLR ligands can induce migration in trans, as signaling through TLR5, that is only expressed on cDC2, does not induce cDC1 migration *in vivo* (Flores-Langarica et al., 2017). Taken together, these findings indicate that TLR based adjuvants and targeting need to be examined individually for their impact on specific DC subsets if their effects on the immune system are to be understood in depth. Future research aiming at better understanding the role of potently migrating and activating trans-activated DC subsets, as well as the signals responsible, will be critical for the design of selective immune interventions in vaccination and therapy.

Acknowledgements

We thank Prof. Dan Kaplan for sharing huCD207-cre mice and Prof. Ulrich Kalinke for sharing $IFNAR^{flox}$ mice. We thank the Lahl, Agace, Bekiaris and Johansson-Lindbom laboratories for many fruitful discussions, Matthias Schiemann and Immanuel Andrä for flow cytometry support, and Allan Mowat for editing of the manuscript. KL was supported by a Vetenskaprådet Young Investigator Award, the Ragnar Söderberg Foundation Fellowship in Medicine, a Lundbeck Foundation Research Fellowship, the Åke Wiberg Foundation, the Carl Trygger Foundation, and the Crafoord Foundation. JH was supported by a MARIE CURIE Fellowship as part of the EU-funded project HC Ørsted Postdoc. AGL received travel support from the Niels Bohr Foundation.

Author contributions

AGL, VB, WA, and KL conceived and designed the project; AGL, VB, JH, MO'K, WA and KL designed experiments; AGL, VB, KM-L, JH, KGM, JN, IU, KK, and KL performed experiments and analysed the data; BM and BH provided essential tools; AGL and KL wrote the manuscript; WA and KL supervised the study and acquired funding; all authors reviewed the manuscript.

Experimental procedures

Mice

All animal animals were house under specific pathogen-free conditions at the Danish Technical University (Denmark), Lund University (Sweden) or Monash University (Australia). The experiments were performed under the appropriate national licenses and guidelines for animal care. Both male and female mice were used between 8 and 16 weeks of age as no obvious age differences were detected. CD11c.cre mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J (Caton et al., 2007)) allow floxed gene deletion in CD11c-expressing cells, huCD207.cre mice drive floxed gene deletion in Langerhans cells and intestinal cDC2 (Welty et al., 2013), XCR1.cre mice permit to specifically delete floxed genes in cDC1 (Janela et al., 2019), villin.cre (B6.Cg-Tg(Vil-cre)997Gum/J) mice excise floxed genes in intestinal epithelial cells (Madison et al., 2002) and Rosa26-STOP-YFP mice allow tracking of cre specificity (B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J). We used "switch-on" mutants carrying a floxed stop cassette in the endogenous locus prior to the gene of interest, allowing for re-expression of the targeted gene in the presence of cre for MyD88 (Gais et al., 2012), TLR3 and TRIF (unpublished, manuscript in preparation) (both generated at TU Munich, Germany). IFNAR floxed mice were obtained from U. Kalinke (Kamphuis et al., 2006). BATF3^{KO} (B6.129S(C)-Batf3^{tm1Kmm}/J) were maintained at DTU, TNFR1^{KO} (crossed out from TNFR1/2^{KO} (Peschon et al., 1998)), IFNAR^{KO} (Cucak et al., 2009) and CCR2^{KO} (B6.129S4-Ccr2^{tm1Ifc}/J) at Lund University and IL28RA^{KO} (Ank et al., 2008) (kindly provided by Sean Doyle, Zymogenetics/BMS) at Monash University. All mice were on the C57Bl/6J background (B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ for CD45.1 bone marrow donors) and littermates were used as controls.

In vivo treatments

Mice were injected with PBS or 100μg pIC (Sigma-Aldrich) in PBS intraperitoneally (i.p.) and mLNs and small intestinal lamina propria (SI LP) were collected 12-14 h later if not indicated otherwise. For TLR7 stimulation, 20μg of R848 (Invivogen) in PBS was given orally. αTNFα (XT3.11, BioXcell) was blocked with 0.5mg on day -1 and 0.5mg at the time of stimulation.

Cell isolation

Isolation of mLN and splenic DCs was performed by digesting the tissue with collagenase IV (0.5 mg/mL, Sigma-Aldrich) and DNase I (12.5 μg/mL) diluted in R10 media (RPMI 1640 + 10% FCS) for 40 min at room temperature. Remaining tissue was mashed and filtered through 70 μm cell strainer with R10. For spleens, red blood cells (RBC) were lysed using RBC lysing buffer, containing ammonium chloride, potassium bicarbonate, EDTA and MiliQ water. The SI-LP cell isolation was performed as described previously(Luda et al., 2016).

Flow cytometer

Ca/Mg-containing PBS with 2% FCS was used as buffer during the entire staining procedure. Non-specific binding was blocked with rat anti-mouse CD16/CD32 Fc block (2.4G2, BD Biosciences) for 20 minutes at 4°C. Dead cells identified as propidium iodide^{+} (Sigma Aldrich) or by Aqua LIVE/DEAD Fixable Dead Cell Staining Kit (Life Technologies) and cell aggregates (identified on FSC-A versus FSC-H scatterplots) were excluded from analyses. DCs were identified by using the following antibodies: α-CD3 (145-2C11), α-CD19 (eBio1D3), α-NK1.1 (PK136), α-B220 (RA3-6B2), α-CD64 (X54-5/7.1), α-CD103 (M290), α-CD11b (M1/70), α-CD11c (HL3), α-CD8a (53-6.7), α-CD86 (GL1), α-MHC-II I (IA/I-E) (M5/114.15.2), α-CD45.1 (A20), α-CD45.2 (104), α-IFNAR (MAR1-5A3), α-XCR1 (ZET), α-SiglecH (551), and α-TLR3 (11F8). Intracellular staining was performed using the FoxP3 Fixation/Permeabilization Kit (eBioscience) according to the manufacturer's instructions. Data was acquired on a FACS Aria II or LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Adoptive Transfers

Bone marrow (BM) chimeras were generated by intravenous injection of BM (5 x 10^6) cells into irradiated (9 Gy) recipients. Analysis of BM chimeras was performed 6-8 weeks after cell transfer. In all mixed BM chimeras, WT cells were identified by CD45.1 expression.

Real-Time PCR

Total RNA was isolated from small intestine (SI) using the RNeasy kit (QIAGEN). cDNA was generated using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad), using SsoFast™EvaGreen® Supermix (Bio-Rad). The expression of all genes was normalized to the mean of beta-actin, Reep5 and GAPDH. Primer sequences are specified in Suppl. Table 1. Undetectable values were calculated based on the highest possible Cq +1 (=41cyles).

Statistical Analysis

Statistics were performed using two-way ANOVA considering treatment and experiment as factors for the analysis. Wherever indicated in the figure legends, Mann-Whitney U test was applied to compare two groups (e.g.: different treatments (n=2) within the same genotype), and Kruskal-Wallis test was applied to compare more than 2 groups (e.g.: different genotypes (n=3) within the same treatment). Statistical significance was estimated by using R Studio.

R Scripts:

- Two-way ANOVA: aov(value ~ genotype + day, data = Data)
    - Post-Hoc test Tukey: TukeyHSD(Data_anova2, which="genotype")
- Mann-Whitney U test: wilcox.test(value ~ genotype, data = Data, exact = FALSE)

Genotype accounts for analysis between different genotypes within the same treatment. Using treatment instead of genotype allows for analysis between different treatments within a genotype. Data refers to the data to be analyzed.

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Figure Legends

Fig.1: TLR3 expression by mononuclear phagocytes and migration of cDCs in response to poly(I:C).

A. Left: representative flow cytometry plots of spleen, mLN and SI-LP DC subsets and macrophages in C57BL/6 mice. All populations were gated on live, lineage (CD3, CD19, NK1.1) negative, single cells. DC in spleen and mLN were further pre-gated as $CD11c^{+}MHCII^{+}$ cells and in SI-LP as $CD11c^{+}MHCII^{+}CD64^{-}$ cells. Macrophages in spleen were further pre-gated as $CD11c^{int}$ and $CD11b^{+}$ or $CD11b^{-}$, while macrophages in SI-LP were further gated as $CD11c^{+}CD64^{+}$ cells. Histograms: Intracellular TLR3 staining of the indicated DC and macrophage populations 12 hours after i.p injection of PBS or 100µg poly(I:C) into wild type mice and by bulk DC in resting $TLR3^{OFF}$ mice (KO). Right: Quantification of TLR3 expression by DC subsets and macrophages in C57BL/6 mice 12h after i.p. injection of PBS or poly(I:C). Data shown are means ± 1 sem pooled from two independent experiments with 3 mice per group.
B. Kinetics of intestinal cDC1 and cDC2 migration after i.p. injection of 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from two to four independent experiments with 2-3 mice per group. Differences between cDC1 and cDC2 are not significant.
C. Total numbers of cDC1 and cDC2 in the mLNs of WT and $TLR3^{OFF}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with 3 mice per group. Two-way ANOVA, *$p<0.05$.
D. Activation of cDC subsets in mLNs of WT and $TLR3^{OFF}$ mice by poly(I:C). Results shown are fold change in CD86 expression 12h after injection of 100µg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled

from three independent experiments with 3 mice per group. Two-way ANOVA, *p<0.05, ***p<0.0005.

Fig. 2: Cellular requirements for TLR3 mediated DC migration in response to poly(I:C)

A. Total numbers of cDC1 and cDC2 in the mLNs of WT recipients reconstituted for 8 weeks with either WT or $TLR3^{OFF}$ BM 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem from one experiment with 5-8 mice per group. Mann Whitney U test,*p<0.05, **p<0.005.

B. Schematic diagram of generation of cell specific $TLR3^{ON}$ mice in which $TLR3^{OFF}$ was created using a floxed STOP codon and TLR3 then re-expressed using cell specific cre promoters to delete the STOP codon.

C. Total numbers of cDC1 and cDC2 in the mLNs of WT, $TLR3^{OFF}$ and $XCR1.TLR3^{ON}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with 3-4 mice per group. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005. Open circles were used to mark those poly(I:C) injected mice that also did not show upregulation of CD86 (panel D); these were not excluded from statistics.

D. Activation of cDC subsets in mLNs of WT, $TLR3^{OFF}$ and $XCR1.TLR3^{ON}$ mice by poly(I:C). Results shown are fold change in CD86 expression 12h after i.p. injection of 100µg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three independent experiments with 3-4 mice per group. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005.

E. Fold change of total number of cDC1 and cDC2 in the mLNs 12h after i.p. injection of 100µg poly(I:C) versus PBS derived from the indicated BM in 50:50 WT:$TLR3^{OFF}$ mixed BM chimeras. Data shown are means ± 1 sem pooled from two independent experiments with 7 mice per group. Two-way ANOVA, not significant.

F. Activation of cDC subsets in mLNs of 50:50 WT:$TLR3^{OFF}$ mixed BM chimeras by poly(I:C). Results shown are fold change in CD86 expression 12h after i.p. injection of 100µg poly(I:C) versus PBS and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from two independent experiments with 3-7 mice per group. Two-way ANOVA, not significant

G. Total number of cDC1 and cDC2 cells in $BATF3^{KO}$ mice 12 after i.p. injection of PBS or 100 µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from two representative experiment out of 3 with 2-4 mice per group. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005.

H. Activation of cDC2 subset in mLNs of $BATF3^{KO}$ mice by poly(I:C). Results are shown as mean fluorescent intensity of CD86 12h after i.p. injection of PBS or 100µg poly(I:C) in WT and $BATF3^{KO}$ mice. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005.

Fig.3: Role of cytokines and MyD88 in response of DC to poly(I:C)

A. Expression of cytokine mRNA in total SI LP of WT C57BL/6 mice at indicated times after i.p. injection of 100µg poly (I:C). Each point represents mean of qPCR triplicates for every gene as assessed by

RT-qPCR and measured relative to mean of three housekeeping genes (Reep5, β-actin, GAPDH). Data shown are means ± 1 sem pooled from three independent experiments with 2-4 mice per group. Two-way ANOVA, *p<0.05.

B. Total number of cDC1 and cDC2 in the mLNs of WT and MyD88^{OFF} 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from four independent experiments with 3-4 mice per group (only two including WT). Two-way ANOVA, *p<0.05, ***p<0.0005. Open circles were used to mark those poly(I:C) injected mice that also did not show upregulation of CD86 (panel C); these were not excluded from statistics.
C. Activation of cDC subsets in mLNs of WT and MyD88^{OFF} mice by poly(I:C). Results shown are delta MFI of CD86 expression 12h after i.p. injection of PBS or 100µg poly(I:C) over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 sem pooled from two independent experiments with 3-4 mice per group. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005.
D. Total number of cDC1 and cDC2 in the mLNs of TNFR1^{KO} mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with 1-3 mice per group. Two-way ANOVA, not significant.
E. Activation of cDC subsets in mLNs of WT and TNFR1^{KO} mice by poly(I:C). Results shown are fold change in CD86 expression 12h after injection of 100µg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three independent experiments with 1-3 mice per group. Two-way ANOVA, *p<0.05, **p<0.005.
F. Total number of cDC1 and cDC2 in the mLNs of C57BL/6 mice pre-treated with TNF-α antibody-blocking and 12h after i.p. injection of 100µg poly(I:C). Control mice were treated with the isotype antibody (IgG). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with 2-4 mice per group. Two-way ANOVA, **p<0.005, ***p<0.0005

Fig.4: Type I IFN signaling in migration and activation of DC subsets

A. Total number of cDC1 and cDC2 cells in the mLNs of WT and IFNAR^{KO} mice 12h after i.p. injections of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from four independent experiments with 3-5 mice per group. Two-way ANOVA, **p<0.005, ***p<0.0005.
B. Total number of cDC1 (top) and cDC2 (bottom) cells in the mLNs of WT, CD11c.IFNARKO^{KO}, XCR1.IFNAR^{KO} and huCD207.IFNAR^{KO} mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from five independent experiments with 2-3 mice per group for WT vs CD11c.IFNAR^{KO}; five independent experiments with 3-5 mice per group for XCR1.IFNAR^{KO}, and three independent experiments 3-5 mice per group for huCD207.IFNAR^{KO}. Two-way ANOVA within littermates, *p<0.05, **p<0.005, ***p<0.0005.
C. Activation of cDC1 (top) and cDC2 (bottom) in the mLNs of WT, CD11c.IFNAR^{KO}, XCR1.IFNAR^{KO} and huCD207.IFNAR^{KO} mice by poly(I:C). Results shown are fold change in CD86 expression 12h after i.p. injection of 100µg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem pooled from three out of five independent experiments with 2-3 mice per group for WT vs CD11c.IFNAR^{KO}; four out of five independent experiments with 3-5 mice per group for XCR1.IFNAR^{KO}, and three

independent experiments 3-5 mice per group for huCD207.IFNAR^{KO}. Two-way ANOVA within littermates, *p<0.05, ***p<0.0005.

D. Fold change of total number of cDC1 and cDC2 in the mLNs 12h after i.p. injection of 100µg poly(I:C) versus PBS derived from the indicated BM in 50:50 WT:XCR1.IFNAR^{KO} mixed BM chimeras. Data shown are means ± 1 sem pooled from two independent experiments with 3-9 mice per group. Two-way ANOVA, *p<0.05.
E. Total number of cDC1 and cDC2 in the mLNs of XCR1.IFNAR^{KO} mice 12h after oral gavage of PBS or 20µg R848. Data shown are mean numbers of cells ± 1 sem pooled from two independent experiments with 3-5 mice per group. Two-way ANOVA, *p<0.05.
F. Total number of cDC1 and cDC2 in the mLNs of huCD207.IFNAR^{KO} mice 12h after oral gavage of PBS or 20µg R848. Data shown are mean numbers of cells ± 1 sem pooled from two independent experiments with 3-5 mice per group. Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005.
G. Total number of cDC1 and cDC2 in the mLNs of IL28R^{KO} mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from three independent experiments with 3 mice per group. Two-way ANOVA, **p<0.005.
H. Activation of cDC1 and cDC2 in the mLNs of IL28R^{KO} mice by poly(I:C). Results shown are delta MFI of CD86 expression 12h after i.p. injection of PBS or 100µg poly(I:C) over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 sem pooled from three independent experiments with 3 mice per group. Two-way ANOVA, ***p<0.0005.

Figure 1

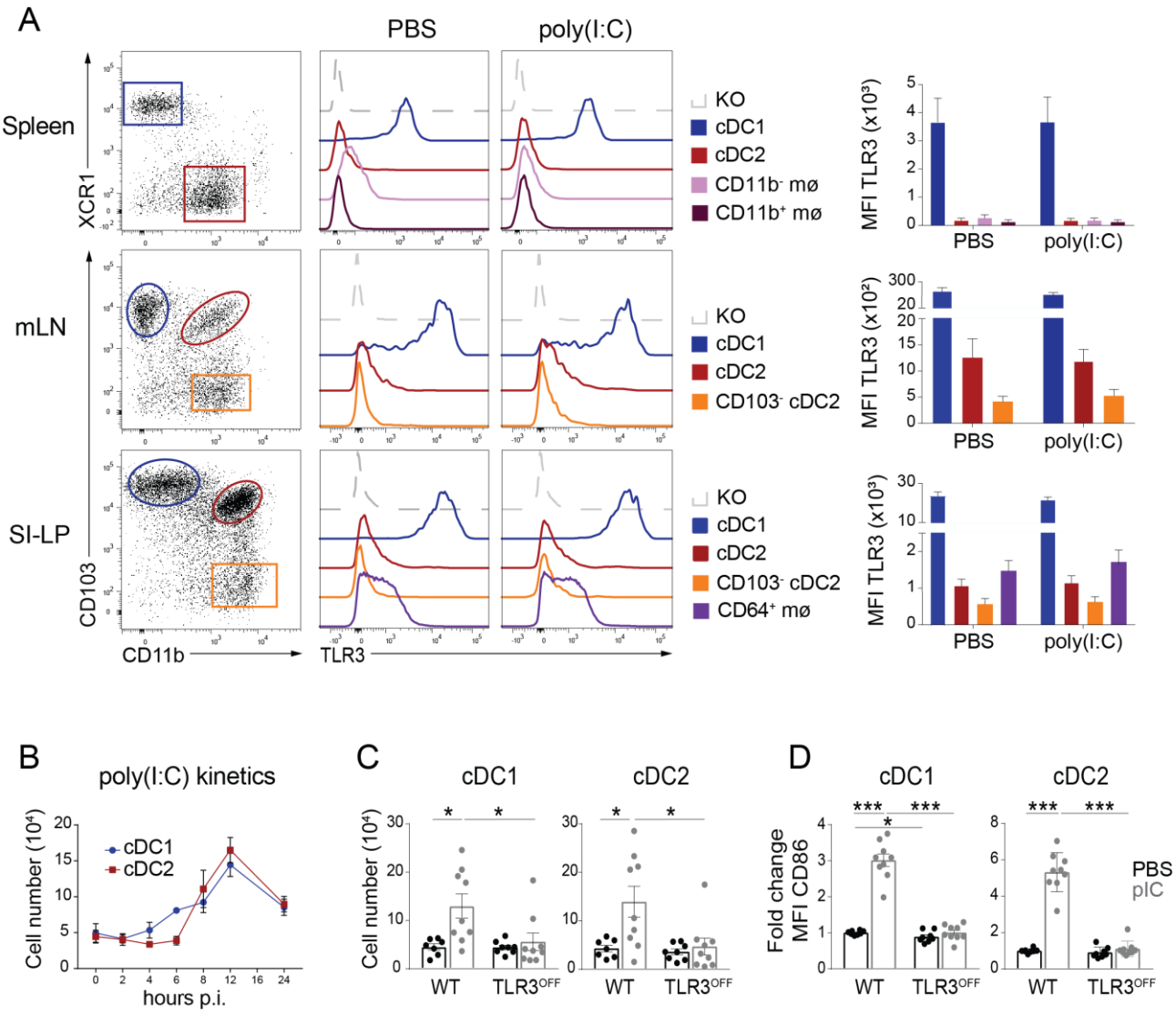

A
PBS
poly(I:C)
Spleen
mLN
SI-LP
XCR1
CD103
CD11b
TLR3
KO
cDC1
cDC2
CD11b⁻ mø
CD11b⁺ mø
CD103⁻ cDC2
CD64⁺ mø
MFI TLR3 (x10³)
MFI TLR3 (x10²)
B
poly(I:C) kinetics
Cell number (10⁴)
hours p.i.
C
cDC1
cDC2
WT
TLR3OFF
D
Fold change MFI CD86
PBS
pIC

Figure 2

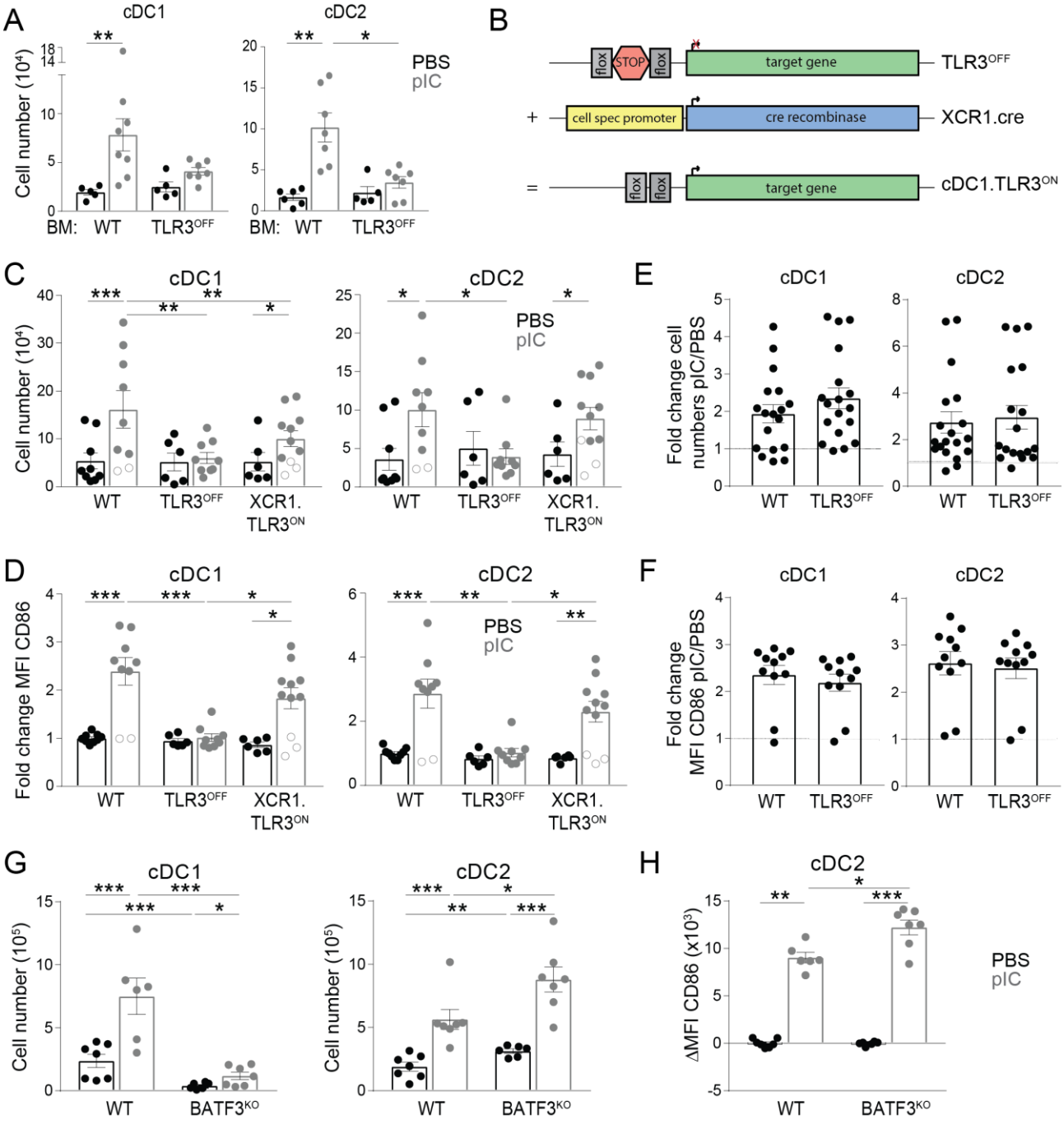

A
cDC1
cDC2
Cell number (10⁴)
PBS
pIC
BM: WT
TLR3OFF
B
flox
STOP
target gene
TLR3OFF
cell spec promoter
cre recombinase
XCR1.cre
cDC1.TLR3ON
C
cDC1
cDC2
Cell number (10⁴)
WT
TLR3OFF
XCR1. TLR3ON
D
Fold change MFI CD86
E
Fold change cell numbers pIC/PBS
F
Fold change MFI CD86 pIC/PBS
G
Cell number (10⁵)
BATF3KO
H
ΔMFI CD86 (x10³)

Figure 3

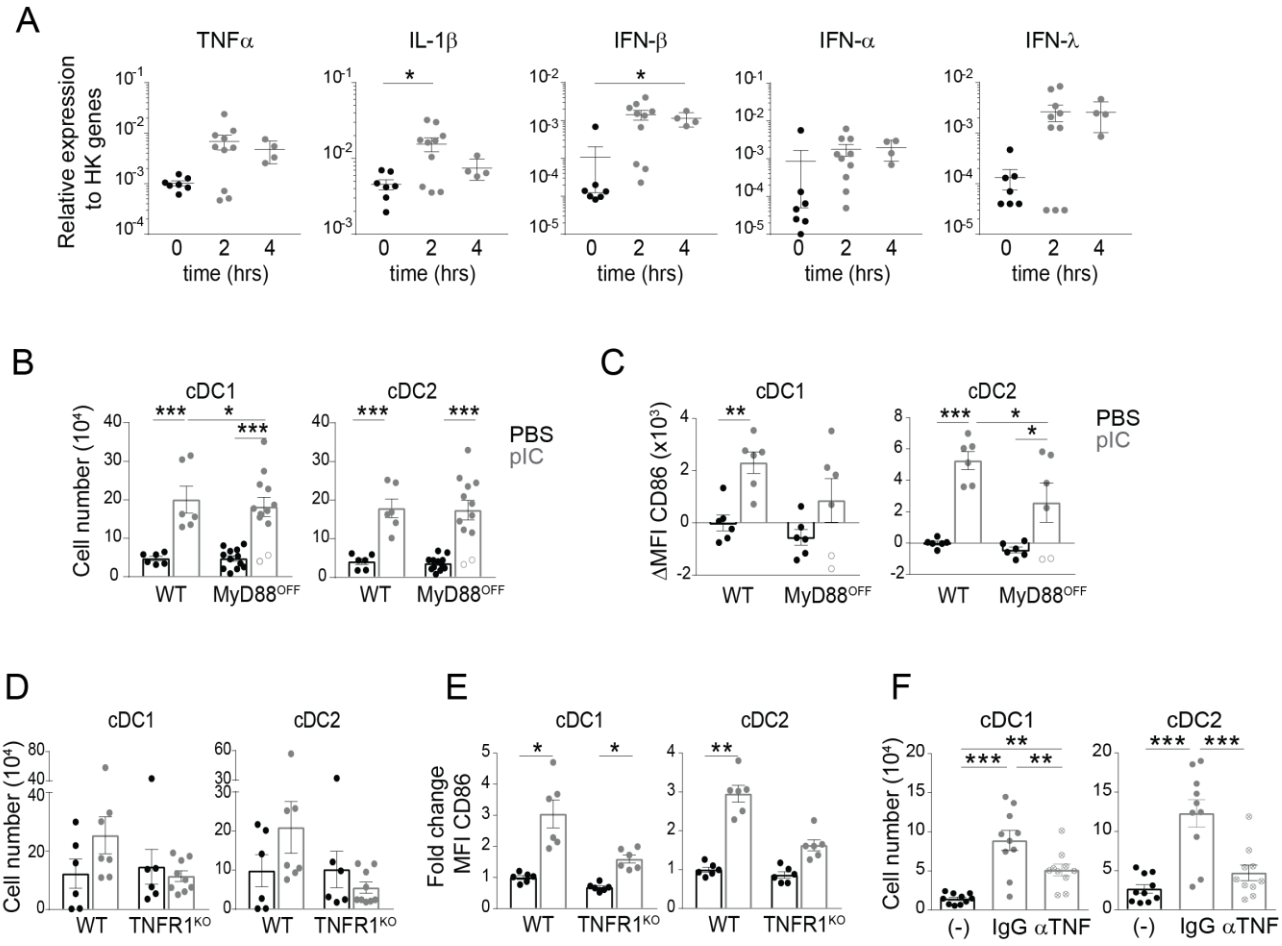
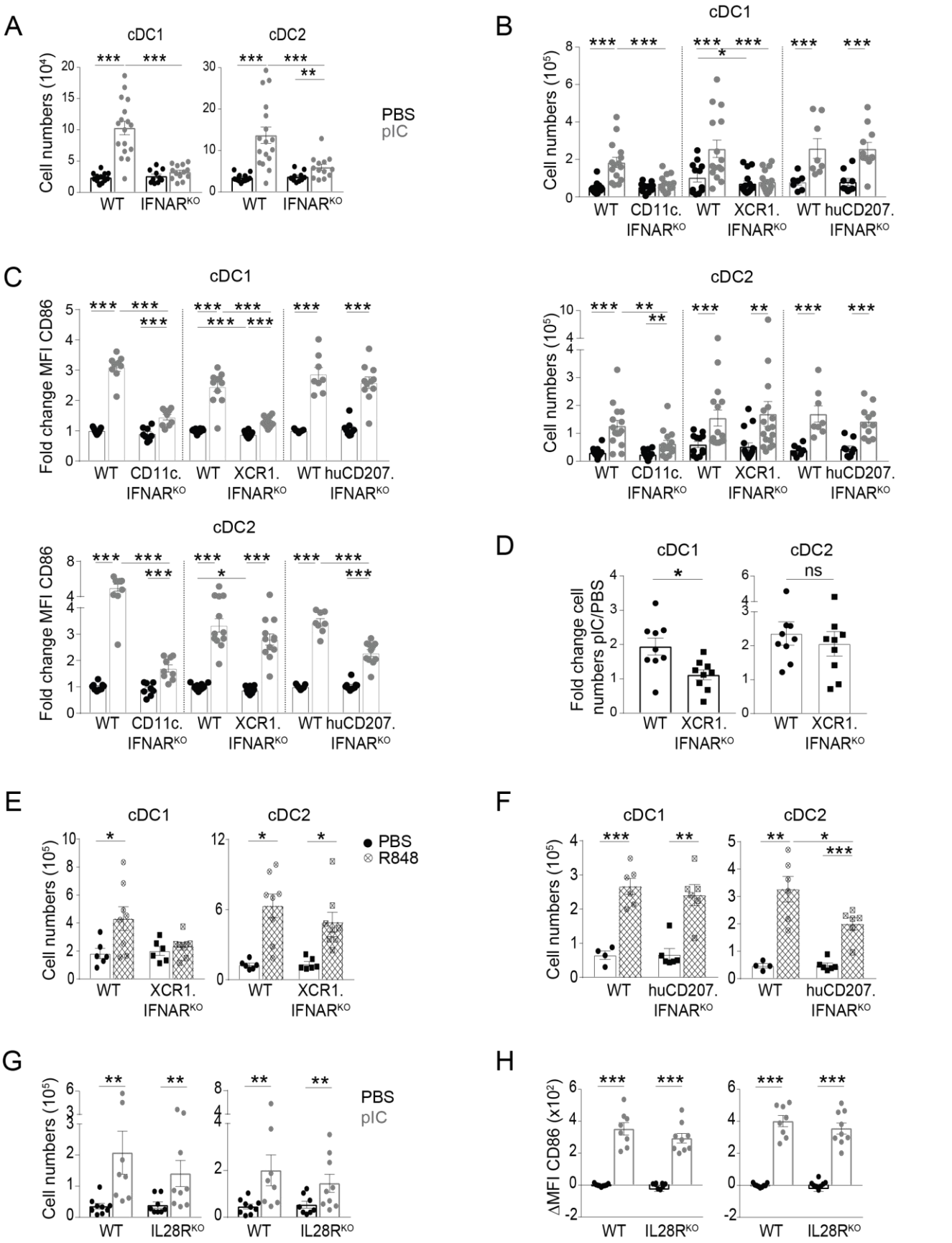

A
TNFα
IL-1β
IFN-β
IFN-α
IFN-λ
Relative expression to HK genes
time (hrs)
B
cDC1
cDC2
Cell number (10⁴)
PBS
pIC
WT
MyD88OFF
C
ΔMFI CD86 (x10³)
D
TNFR1KO
E
Fold change MFI CD86
F
(-)
IgG
αTNF

Figure 4


A
cDC1
cDC2
Cell numbers (10⁴)
PBS
pIC
WT
IFNAR^KO
B
cDC1
Cell numbers (10⁵)
WT
CD11c. IFNAR^KO
XCR1. IFNAR^KO
huCD207. IFNAR^KO
C
cDC1
Fold change MFI CD86
cDC2
Cell numbers (10⁵)
D
cDC1
cDC2
Fold change cell numbers pIC/PBS
ns
E
cDC1
cDC2
Cell numbers (10⁵)
PBS
R848
F
cDC1
cDC2
Cell numbers (10⁵)
G
Cell numbers (10⁵)
PBS
pIC
IL28R^KO
H
ΔMFI CD86 (x10²)

Supplementary Data

Suppl.1:

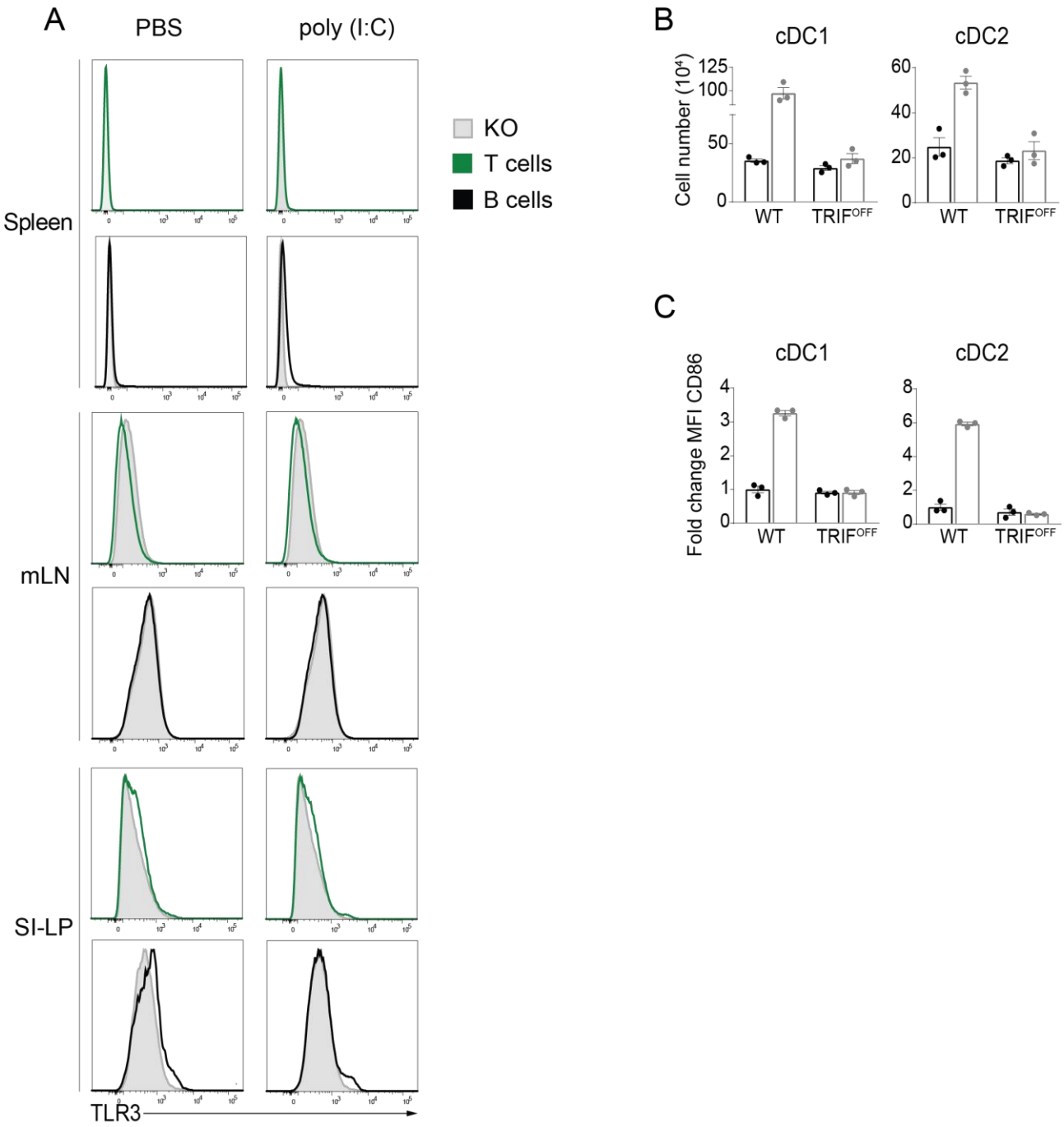
A. Intracellular TLR3 staining of B and T cells 12 hours after i.p injection of PBS or 100µg poly(I:C) into wild type mice and by B and T cells from resting $TLR3^{OFF}$.
B. Total numbers of cDC1 and cDC2 in the mLNs of WT and $TRIF^{OFF}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem of one experiment representative of three with 3 mice per group. Mann Whitney U test, not significant.
C. Activation of cDC subsets in mLNs of WT and $TRIF^{OFF}$ mice by poly(I:C). Results shown are fold changes in CD86 expression 12h after injection of 100µg poly(I:C) as assessed by MFI normalized to FMO and relative to expression by DCs in untreated WT. Data shown are means ± 1 sem of one experiment representative of three with 3 mice per group. Mann Whitney U test, not significant.

Suppl.2:

A. Intracellular TLR3 staining of spleen and SI LP in the indicated DC and macrophage populations from WT, $TLR3^{OFF}$ and XCR1.$TLR3^{ON}$ mice.
B. Total number of cDC1 and cDC2 in the mLNs of WT and $CCR2^{KO}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem from one experiment with 4 mice per group. Mann Whitney test, *$p<0.05$.
C. Total number of cDC1 and cDC2 in the mLNS of WT, $TLR3^{OFF}$ and Villin.$TLR3^{ON}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 sem pooled from four independent experiments with 3 mice per group. Two-way ANOVA, **$p<0.005$, ***$p<0.0005$.
D. Intracellular TLR3 staining of spleen cDC1 from WT, $TLR3^{OFF}$ and LysM.$TLR3^{ON}$ mice. Data shows one representative mouse per genotype from two independent experiments with 3-4 mice per group.

Table 1: Primer Sequences for RT-PCR

Supplemental Figure 1



Supplemental Figure 2

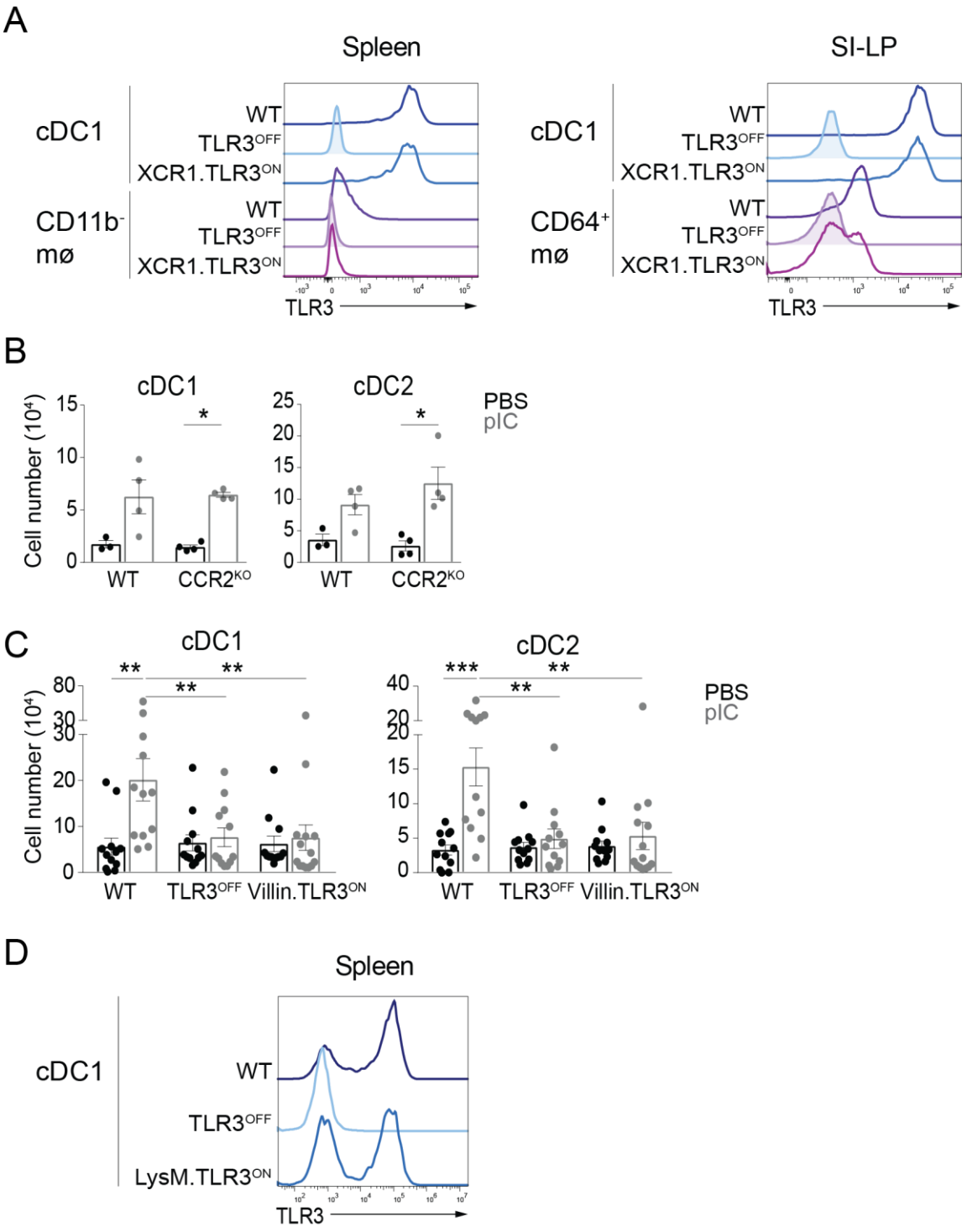


Table 1: Primer Sequences for RT-PCR

	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1β	GACAGTGATGAGAATGACCTGTT	TGGAAGGTCCACGGGAAAGACA
TNF-α	TGTCTACTGAACTTCGGGGTGA	TCTTTGAGATCCATGCCGTTG
IFN-α	TGCAATGACCTCCATCAGCA	TTCCTGGGTCAGAGGAGGTTC
IFN-β	CTGGAGCAGCTGAATGGAAAG	CTCCGTCATCTCCATAGGGAT
IFN-λ	GTTCAAGTCTCTGTCCCCAAAA	GTGGGAACTGCACCTCATGT
Reep5	GCCATCGAGAGTCCCAACAA	AGCATCTCAGCCCCATTAGC
β-actin	ccgggacctgacagacta	GTTTCATGGATGCCACAGGAT
GAPDH	cctgcaccaccaactgctta	TCATACTTGGCAGGTTTCTCCA

4. Complementary methodology

4.1 Mouse models overview

This thesis is based on studies using a series of different mouse models. In Table 2, I summarize the different mouse models, with their phenotype and the reason of their use.

Table 2. Overview of mouse models used in this thesis.

MODELS	CRE	PHENOTYPE	AIM	REFERENCES
SWITCH-ON				
TLR3-LSL	XCR1	Only cDC1 express TLR3	Role of TLR3 in cDC1 only	[295],Manuscript
	Villin	Only intestinal epithelial cells express TLR3	Role of TLR3 in IECs only	[296], Manuscript
TRIF-LSL	CD11c	Only CD11c-expressing cells, e.g. cDC, macrophages and pDCs, express TRIF	Role of TRIF signaling in CD11c-expressing cells	[297], Manuscript
MyD88-LSL	CD11c	Only CD11c-expressing cells, e.g. cDC, macrophages and pDCs, express MyD88	Role of MyD88 signaling in CD11c-expressing cells	[297,298]
	huCD207	Only Langerhan cells and intestinal cDC2 express MyD88	Role of intestinal cDC2 in intesitnal cDC migration	[277,298]
CONSTITUTIVE KO				
IFNAR^{KO}	-	All cells lack type I IFN signaling	Role of type I IFN signaling in intestinal cDC migration	[299]
CCR2^{KO}	-	No recruitment of monocytes from blood to tissue	Role of newly recruited intestinal monocytes in intestinal cDC migration	[300]
BATF3^{KO}	-	Lack of cDC1 development	Role of cDC1 in intestinal cDC migration	[301]
TNFRI^{KO}	-	All cells lack TNF signaling	Role of TNF signaling in intestinal cDC migration	crossed out from TNFR1/2^{KO}([302])
IL28R^{KO}	-	All cells lack type III IFN signaling	Role of type III IFN signaling in intestinal cDC migration	[303]
CONDITIONAL KO				
IFNAR$^{fl/fl}$	CD11c	CD11c-expressing cells, e.g. cDC, macrophages and pDCs, lack type I IFN signaling	Role of type I IFN signaling in CD11c-expressing cells in intestinal cDC migration	[297,304]
	XCR1	cDC1 lack type I IFN signaling	Role of cDC1-intrinsic type I IFN in intestinal cDC migration	[295,304]
	huCD207	Langerhan cells and intestinal cDC2 lack type I IFN signaling	Role of cDC2-intrinsic type I IFN in intestinal cDC migration	[277,304]
INDUCIBLE KO				
BDCA2-DTR	DT	Depletion of pDCs	Role of pDCs in intestinal cDC migration	[305]

4.2 Depletion of pDCs

BDCA2-DTR (transgenic, tg) mice were injected with diphtheria toxin (DT, EMD Milipore) intraperitoneally (i.p.) at 200 ng/mouse per injection. pDCs were depleted on days -2 and -1 prior to poly(I:C) injection. Mice not injected with DT were administered PBS as control. Mice were used between 8-16 weeks of age.

4.3 Treatment with different poly(I:C) formulations

The synthetic dsRNA poly(I:C) was purchased from Sigma-Aldrich whereas High molecular weight (HMW) and Low molecular weight (LMW) poly(I:C)s were purchased from InvivoGen (San Diego, CA, USA). All poly(I:C)s were injected i.p. into C57BL/6 (wild type,WT) and $TLR3^{OFF}$ mice at 100µg/mouse, diluted in PBS.

4.4 Statistical analysis

Total cell numbers varied between experiments performed on different days. Regular statistical tests masked the effect observed between different treatments across experiments. Accordingly, wherever possible, we employed a two-way ANOVA, with both treatment and day of experiment considered as factors for the analysis. Some of the preliminary results consist of only one experiment. In this case, Mann-Whitney U test was applied to compare two groups (e.g.: different treatments (n=2) within the same genotype), and Kruskal-Wallis test was applied to compare more than 2 groups (e.g.: different genotypes (n=3) within the same treatment). Statistical significance was estimated by using R Studio.

R Scripts:

- Two-way ANOVA: aov(value ~ genotype + day, data = Data)
    - Post-Hoc test Tukey: TukeyHSD(Data_anova2, which="genotype")
- Kruskal Wallis test: kruskal.test(value ~ genotype, data = Data)
    - Post-Hoc test Dunn: Library(FSA)[306], dunnTest(value ~ genotype, data = Data, method="bh")
- Mann-Whitney U test: wilcox.test(value ~ genotype, data = Data, exact = FALSE)

Genotype accounts for analysis between different genotypes within the same treatment. Using treatment instead of genotype allows for analysis between different treatments within a genotype. Data refers to the data to be analyzed.

5. Complementary results

This section contains a set of complementary results to the manuscript. Of note, the figures are numbered following the thesis' figures.

5.1 TLR3-mediated recognition of different poly(I:C) formulations

Poly(I:C) is a synthetic dsRNA recognized mainly by endosomal TLR3[307]. However, other cytosolic PRRs such as MDA5 and RIG-I can also recognize dsRNA[308]. There are several poly(I:C) formulations that differ in their molecular size and some studies have shown that they induce different responses, depending on the cell type[309]. For example, different poly(I:C)s generate phenotypically mature DCs, but with different functional properties[310]. Particularly, one study showed that RIG-I and MDA5 selectively recognize short and long dsRNA, respectively[311]. However, little is known about the involvement of TLR3 in recognizing different dsRNA lengths.

Given that DC migration in our model was dependent on TLR3, we performed preliminary studies to assess whether different formulations of poly(I:C) induce intestinal cDC migration similarly and whether all depend on TLR3 signaling. We tested three different commercially-available poly(I:C) formulations: poly(I:C) from Sigma-Aldrich (SA, undefined molecular weight); HMW poly(I:C) and LMW poly(I:C), the latter two from InvivoGen. Consistent with our previous findings (Manuscript, Figure 1C&D), SA poly(I:C)-induced cDC migration was fully dependent on TLR3 for both DC subsets (Figure 13A). Similarly, cDC migration in response to LMW-poly(I:C) seemed to depend fully on TLR3. Interestingly however, HMW-poly(I:C) induced partial migration of both cDC1 and cDC2 DCs in the absence of TLR3, suggesting a TLR3-independent manner of HMW-poly(I:C)-induced DC migration (Figure 13A). Additionally, all poly(I:C)s generated maturation of cDCs, assessed by expression of CD86 (Figure 13B). However, while SA and HMW poly(I:C)-induced maturation was fully TLR3-dependent, HMW poly(I:C) induced CD86 expression on cDCs in a TLR3-independent manner (Figure 13B). Large data-spread within the HMW-poly(I:C) group in this one experiment prevents a definitive conclusion. These results are however in line with previous findings that different poly(I:C)s may use different pathways and thus elicit different responses.

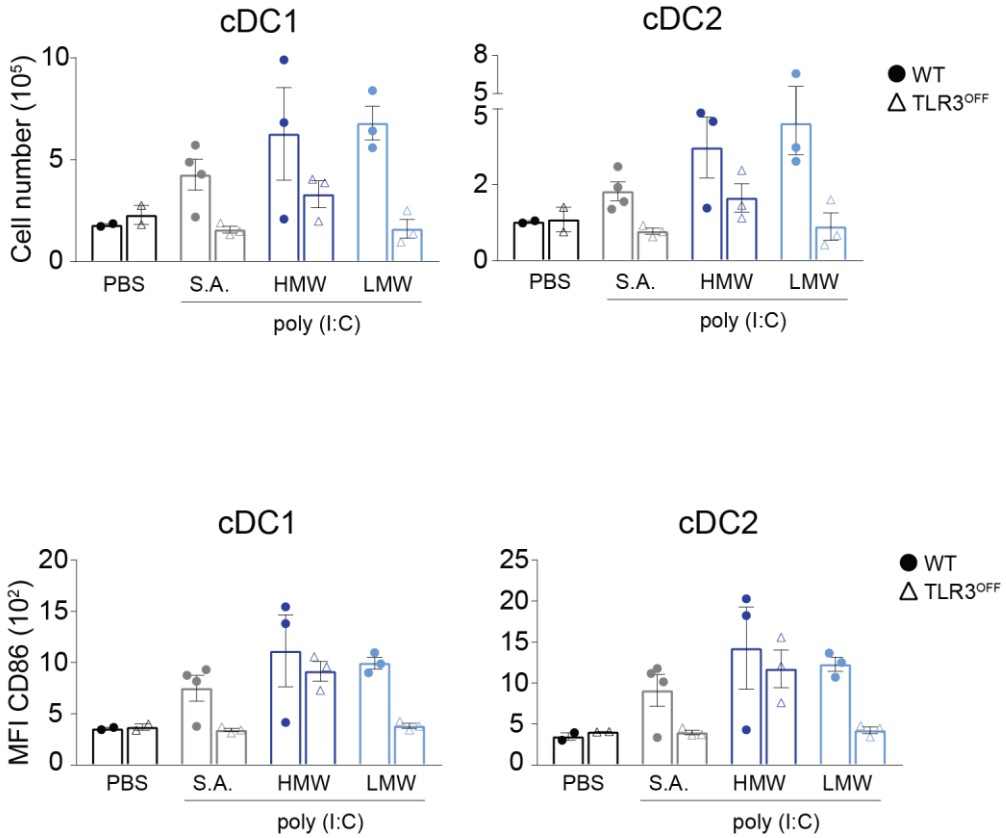


Figure 13. Migration and activation of intestinal cDC1 and cDC2 in response to different poly(I:C) formulations. A) Total number of cDC1 and cDC2 in the mLNs of C57BL/6 and TLR3^{OFF} mice 12h after i.p. injection of PBS or 100µg of given poly(I:C). Data shown are mean numbers of cells ± 1 SEM of one experiment with 3 mice per group; **B)** Activation of cDC1 and cDC2 subsets from A, expressed by mean fluorescent value (MFI) of CD86. Data shown are means ± 1 SEM from experiments in A. Mann-Whitney U test within the same genotype, different treatments, not significant; Kruskal-Wallis test within the same treatment, different genotypes, not significant. SEM: standard error mean.

5.2 Macrophages in intestinal cDC migration

As shown in our manuscript, cDCs in mice lacking TLR3 do not migrate in response to poly(I:C) (Manuscript Figure 1C). We further showed that expression within the cDC1 population was able to drive migration of both cDC1 and cDC2 subsets in response to poly(I:C) (Manuscript Figure 2C). However, we detected TLR3 expression in about 20% of intestinal but not spleen macrophages (Manuscript Sup. Figure 2A). Hence, we wanted to test whether TLR3 in macrophages was sufficient to drive intestinal cDC migration in response to poly(I:C). We used the newly developed switch on model that allows for re-expression of TLR3 only in LysM-expressing cells, e.g: macrophages (TLR3-LSL x LysM CRE, Figure 14A). Surprisingly, numbers of both cDC1 and cDC2 increased in mLNs of LysM.TLR3^{ON} mice after poly (I:C) injection (Figure 14B), suggesting that poly(I:C) sensed by macrophages alone is enough to drive intestinal cDC migration. Activation of cDCs assessed by CD86 expression showed that both cDC1 and cDC1 became activated in response to poly(I:C) (Figure 14C). However, further characterization of the model showed that approximately 50% of cDC1 in the spleen also re-expressed TLR3 (Manuscript Sup. Figure 2D). In line with our results showing that TLR3 on cDC1 is able to drive DC migration (Manuscript Figure

2C), the re-expression of TLR3 in cDC1 in this model may account for the entire phenotype, with macrophages playing little or no role. A definitive assessment of the role of macrophages in poly(I:C)-induced cDC migration thus requires new models allowing for complete macrophage depletion, a model unfortunately not currently available.

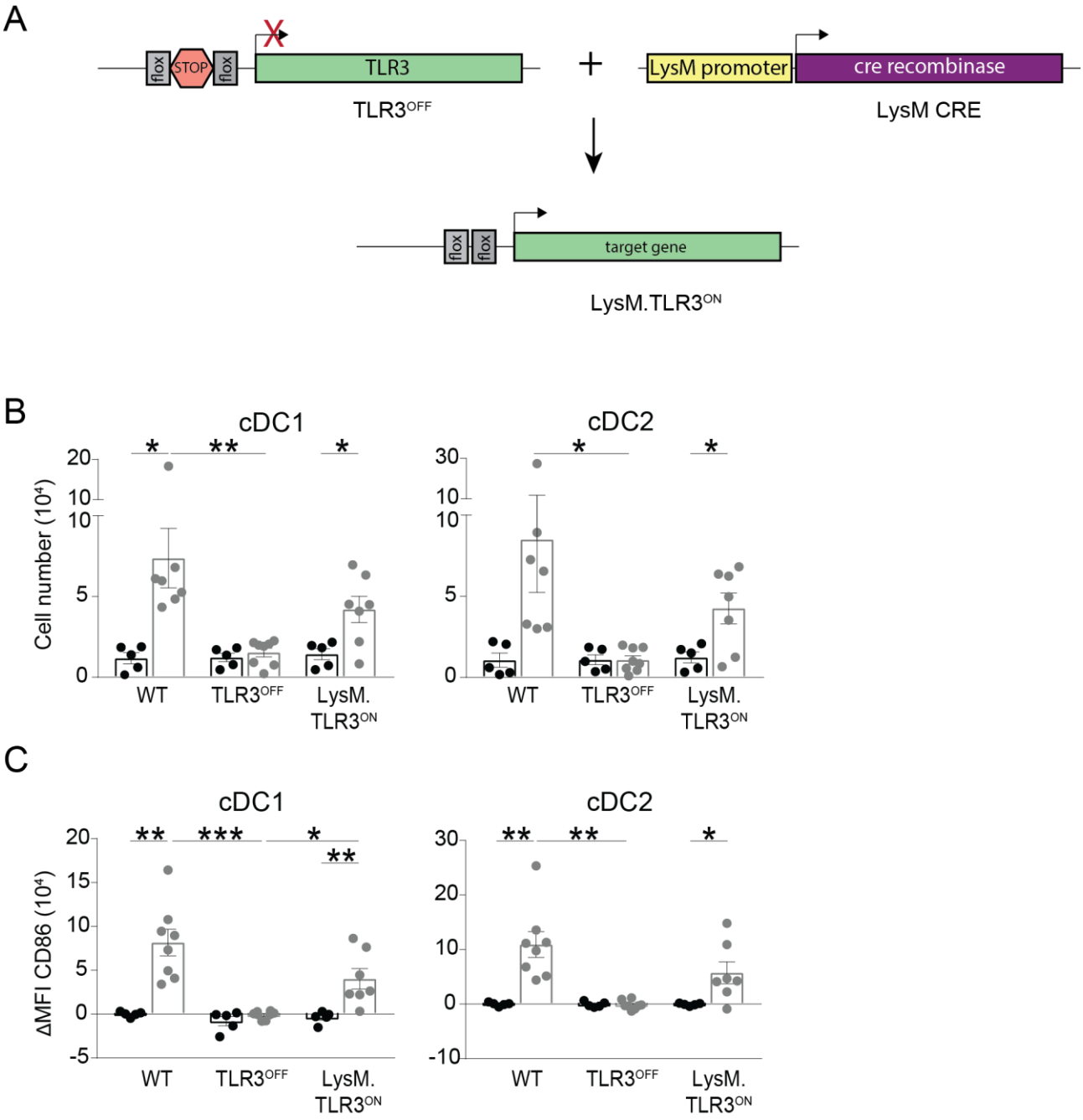


Figure 14. Role of TLR3-expressing macrophages in migration and activation of intestinal cDC1 and cDC2 in response to poly(I:C). A) Graphical representation of the new TLR3-LSLxLysM CRE mouse model generated by crossing $TLR3^{OFF}$ mice, which contain a floxed STOP codon in front of the transcriptional start site of the TLR3 gene, with mice expressing recombinase CRE under the LysM promoter, known to target macrophages; **B)** Total number of cDC1 and cDC2 in the mLNs of WT, $TLR3^{OFF}$ and LysM.$TLR3^{ON}$ mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 SEM pooled from two independent experiments with 3-4 mice per group; **C)** Activation of cDC1 and cDC2 subsets from A, expressed by ΔMFI of CD86 expression over the mean of all

untreated WT CD86 MFI values. Data shown are means ± 1 SEM from experiments in B. Two-way ANOVA test, *p<0.05, **p<0.005, ***p<0.0005.

5.3 Type I IFN signaling for migration in response to poly(I:C)

Type I IFN signaling is essential for many cell functions and they were upregulated 2h after poly(I:C) injection (Manuscript Figure 3A). We found that type I IFN was required for migration of both cDC1 and cDC2 in response to poly(I:C) (Manuscript Figure 4A). However, DCs lacking type I IFN signaling completely may have unknown developmental defects, as previously shown to be de case for pDCs[312]. Accordingly, we acutely blocked IFNAR signaling in WT mice by injection of the blocking antibody MAR1[313] prior to poly(I:C) injection. In agreement with our findings with specific IFNAR^{KO} (Manuscript Figure 4B&C), blocking type I IFN affected cDC1 migration in response to poly(I:C), while having little effect on cDC2 migration (Figure 15). These findings confirm our results using subset-specific IFNAR^{KO} mice, suggesting a specific role for type I IFN for cDC1 and not cDC2 migration in response to poly(I:C).

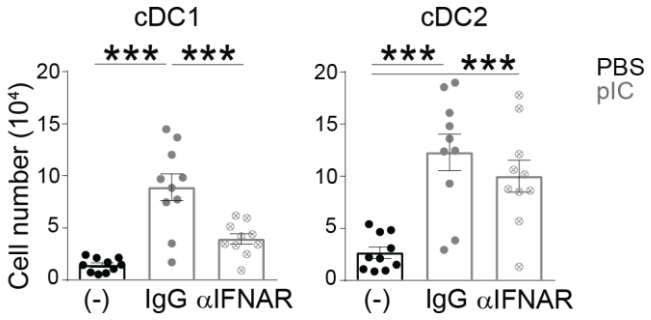


Figure 15. Role of type I IFN signaling in intestinal cDC1 and cDC2 migration in response to poly(I:C). Total number of cDC1 and cDC2 in the mLNs of C57BL/6 mice pre-treated with IFNAR antibody-blocking and 12h after i.p. injection of 100µg poly(I:C). Control mice were treated with the isotype antibody (IgG). Data shown are mean numbers of cells ± 1 SEM pooled from three independent experiments with 2-4 mice per group. Two-way ANOVA test, ***p<0.0005.

5.4 Role of pDCs in intestinal cDC migration

pDCs are a class of DCs that produce large amounts of type I and III IFNs against viral infections[314]. Accordingly, they are considered professional IFN-producing cells. We found that cDC1 but not cDC2 depended on type I IFN signaling for migration in response to poly(I:C) (Manuscript Figure 4B&C). In addition, pDCs are essential for inducing intestinal cDC migration to the mLNs in response to R848 by production of TNF-α and type I IFN[45]. Consequently, we wanted to assess whether pDCs are a main source of type I IFN and play a crucial role in intestinal cDC migration in response to poly(I:C). While pDCs do not express TLR3, they could still act as important secondary signal amplifiers. We used the BDCA2-DTR model, in which injection of DT leads to pDC depletion[305]. As previous DTR mouse models showed lower LN size and cellularity, we used tg mice as controls[315]. pDCs were gated as CD11c^{int}B220^{+}SiglecH^{+} and cDC were gated on CD11c^{+}MHC-II^{+} and further characterized by expression of CD103 and CD11b (Figure 16A). Injection of DT twice prior to poly(I:C) injection lead to efficient depletion of pDCs (Figure 16B).

In the absence of pDCs, poly(I:C) increased migration of both cDC1 and cDC2 as efficient as in untreated mice (Figure 16C). Additionally, maturation of DCs was also not affected by the absence of pDCs (Figure 16D). These findings suggest that pDCs do not play an essential role in intestinal cDC migration.

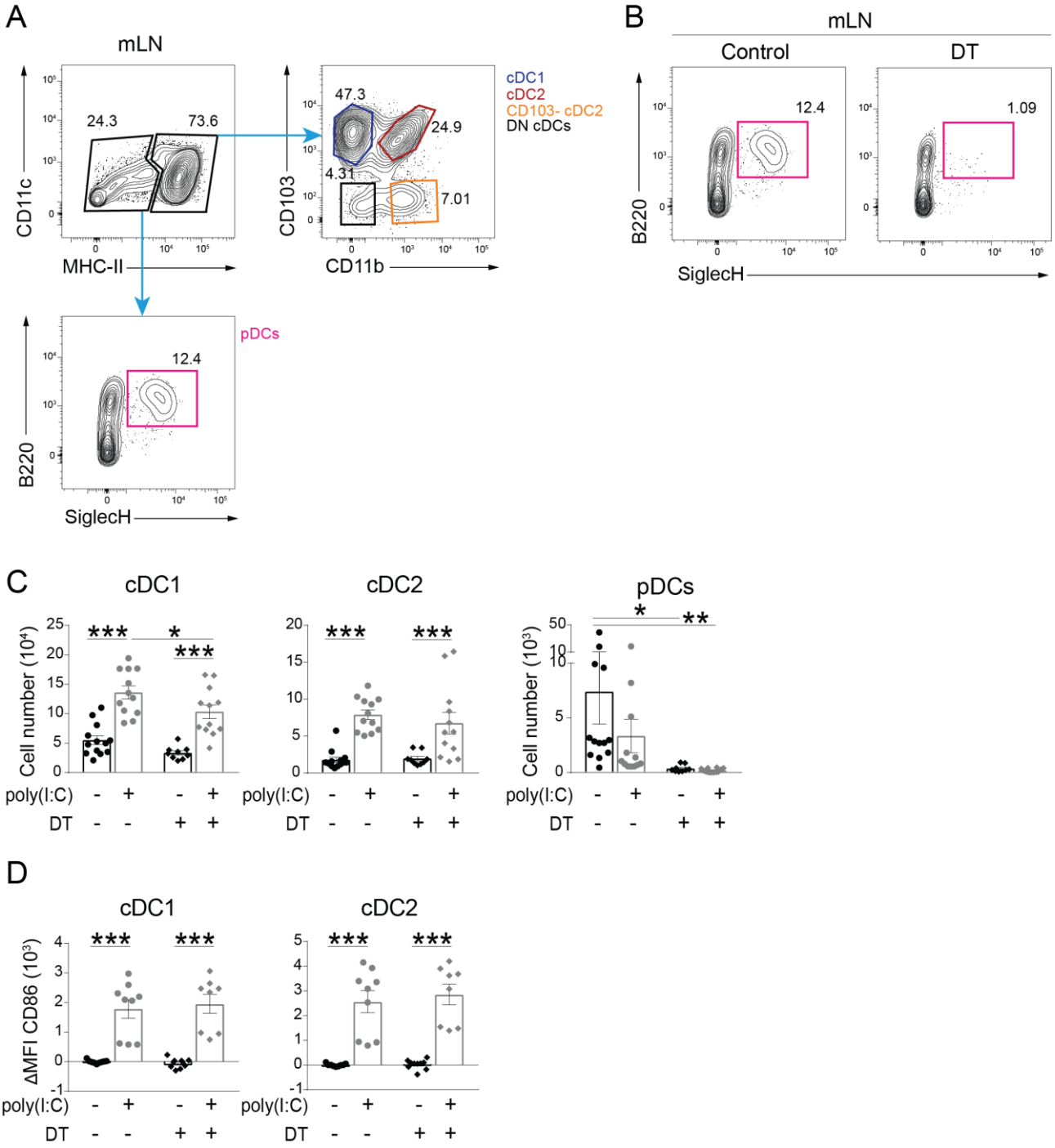


Figure 16. Role of pDCs in intestinal cDC1 and cDC2 migration in response to poly(I:C). A) Representative flow cytometry plots of mLN cDCs and pDCs in BDCA2-DTR mice. Cells are pre-gated on live, Lin (CD3, CD19, NK1.1, CD64)$^-$, cDCs are gated as CD11c^{hi}MHC^{hi} and further subdivided into four different subsets based on CD103 and CD11b expression; pDCs are further gated as CD11c^{int}B220$^+$SiglecH$^+$; **B)** Representative flow cytometry plot of mLN pDCs; **C)** Total number of cDC1, cDC2 and pDCs in the mLNs of BDCA2-DTR mice 12h after i.p. injection of

poly(I:C) and/or DT. Mice that did not receive poly(I:C) nor DT were injected i.p. with PBS. Data shown are mean numbers of cells ± 1 SEM pooled from four independent experiments with 2-5 mice per group; **D)** Activation of cDC1 and cDC2 subsets expressed by ΔMFI of CD86 expression over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 SEM from three out of four experiments in C. Two-way ANOVA test, *p<0.05, **p<0.005, ***p<0.0005.

5.5 MyD88 signaling in intestinal cDC migration

Steady state migration of intestinal DCs depends on MyD88 signaling[316,317]. In our manuscript, we showed that poly(I:C) injection induced the expression of several cytokines 2h post injection, including IL-1β (Manuscript Figure 3A). IL-1β signals through IL-1R and MyD88[318]. Consequently, we next wanted to assess whether migration and activation of either DC subset in response to poly(I:C) also depended on MyD88. Complete deficiency of MyD88 ($MyD88^{OFF}$) did not affect DC migration or activation of either DC subset in response to poly(I:C) (Figure 17A). Interestingly however, re-expression of MyD88 in $CD11c^+$ cells ($CD11c.MyD88^{ON}$) showed a tendency towards increased migration of both cDC subsets in response to poly(I:C) compared to WT (Figure 17A). This increased DC migration might be a consequence of altered homeostasis due to absence of MyD88 in cells such as IECs[319] or T cells[320]. Maturation of cDC seemed not to be affected by lack of MyD88 (Figure 17B), although results are inconclusive due to large data spread. In contrast, R848 induced migration of both cDC1 and cDC2 to similar levels in WT mice, and was abrogated in absence of MyD88 (Figure 17C). Interestingly, MyD88 signaling only in $CD11c^+$ cells was sufficient to restore cDC migration in response to R848 to levels compared to treated WT (Figure 17C). Collectively, these data indicate that poly(I:C), as opposed to R848, induces migration of cDC1 and cDC2 in a MyD88-independent manner, suggesting that any soluble mediators driving DC migration in a TLR3-extrinsic manner are also MyD88-independent.

Dendritic cells can be directly (*cis*) or indirectly (*trans*) activated by cell-intrinsic PAMP recognition or pro-inflammatory cytokines, respectively[245]. Both activation pathways lead to maturation of DCs characterized by higher expression of MHC-II and costimulatory molecules such as CD80 and CD86, among others. We found that poly(I:C) can drive TLR3-dependent DC migration and activation cell-extrinsically, meaning that direct recognition was dispensable for the migrating cell. We next wanted to investigate whether migration of *trans*-activated DCs was a unique phenomenon triggered in response to TLR3-TRIF activation. Accordingly, we switched the system and expressed MyD88 specifically in cDC2, and investigated cDC1 and cDC2 migration and activation triggered by R848. We used R848, which is a synthetic ssRNA that signals through TLR7, because cDC2 were previously shown to be capable of sensing R848 through TLR7[321]. Interestingly, MyD88 signaling restricted to cDC2 led to only partially increased migration and activation of cDC1 and cDC2 compared to untreated mice (Figure 17E&F), confirming a previous study showing a major role for pDCs at driving DC migration in response to R848. These data indicate that intestinal cDC2 might not be the main responders to R848, but are able to respond partially in a cell-intrinsic manner. Of note, this is preliminary data and further experiments need to be performed in order to confirm such results.

Together with the results on XCR1.TLR3^{ON} mice in response to poly(I:C)(Manuscript Figure 2C), these findings suggest that while poly(I:C) and R848 can be sensed directly by cDC1 and cDC2, respectively, both adjuvants drive migration and activation of all subsets. However, cDC1 and cDC2 differ in their requirements for type I IFN for migration in response to poly(I:C) and R848. These differences might be due to cell-intrinsic properties of the specific subsets rather than nature of TLR ligands, since cDC1 seem to require type I IFN independent of the nature of the TLR ligand.

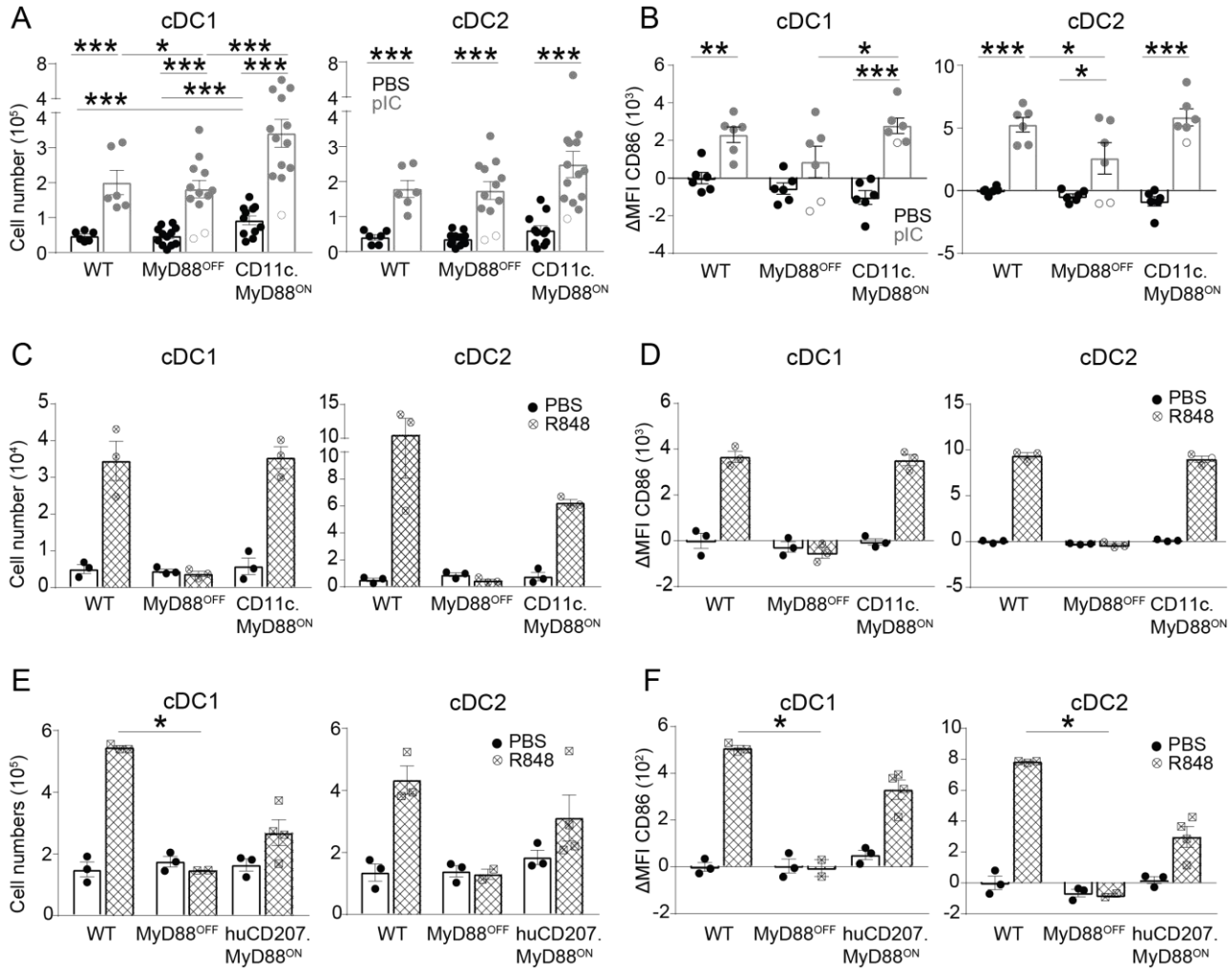


Figure 17. Role of MyD88 signaling in intestinal cDC1 and cDC2 migration in response to poly(I:C) and R848. A) Total number of cDC1 and cDC2 in the mLNs of WT, MyD88^{OFF} and CD11c.MyD88^{ON} mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 SEM pooled from four independent experiments with 3-5 mice per group. Open circles refer to poly(I:C) injected mice that did not show upregulation of CD86 (in B) these were not excluded from statistics; **B)** Activation of cDC1 and cDC2 subsets expressed by ΔMFI of CD86 expression over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 SEM from two out of four experiments in A; **C)** Total number of cDC1 and cDC2 in the mLNs of WT, MyD88^{OFF} and CD11c.MyD88^{ON} mice 12h after oral gavage of PBS or 20µg R848. Data shown are mean numbers of cells ± 1 SEM from one experiment with 3 mice per group; **D)** Activation of cDC1 and cDC2 subsets expressed by ΔMFI of CD86 expression over the mean of all untreated WT CD86 MFI values. Data shown are means ± 1 SEM from experiment in A. **E)** Total number of cDC1 and cDC2 in the mLNs of WT, MyD88^{OFF} and huCD207.MyD88^{ON} mice 12h after oral gavage of PBS or 20µg R848. Data shown are mean numbers of cells ± 1 SEM from one experiment with 2-4 mice per group; **F)** Activation of cDC1 and cDC2 subsets expressed by ΔMFI of CD86 expression over the mean of all untreated WT CD86 MFI values. Data

shown are means ± 1 SEM from experiment in E. Two-way ANOVA test in A) and B), *p<0.05, **p<0.005, ***p<0.0005. Mann-Whitney U test within the same treatment, not significant; Kruskal-Wallis test for the same genotypes but different treatments,*p<0.05.

5.6 Cell-extrinsic requirement of TLR3

In our manuscript, we show that poly(I:C)-induced DC migration depends on TLR3 in hematopoietic cells. This can occur in a cell-extrinsic manner, as shown by migration of $TLR3^{OFF}$ DCs in mixed BM chimeras (Manuscript Figure 2E&F). Mixed BM chimeras contain cDC1 derived from WT mice that could account for poly(I:C) sensing, driving the migration of the $TLR3^{OFF}$ DC counterparts. This hypothesis is strengthened by our results in $TLR3.XCR1^{ON}$ mice, where DC migration of both cDC1 and cDC2 is comparable to WT in response to poly(I:C)(Manuscript Figure 2C). Accordingly, we wanted to assess whether the TLR3 requirement for cDC migration in response to poly(I:C) was specific to its expression on cDC1. Due to the lack of mouse models and the difficulty of generating one that met our needs, we set up mixed BM chimeras with 50:50 $BATF3^{KO}$ and $TLR3^{OFF}$ BM into WT hosts (Figure 18A). Since the $BATF3^{KO}$ BM cannot give rise to cDC1[301], these mice have only half the amount of cDC1 compared to the rest of cells, and those are exclusively derived from $TLR3^{OFF}$ BM. In this way, the cDC1 present in the system will not express TLR3, allowing thus to study cDC1 migration in response to poly(I:C) when all cDC1 lack TLR3. We used $BATF3^{HET}$ BM as control groups (Figure 18A).

Flow cytometry analysis of the injected BM confirmed that proportions of injected BM from the different donors were very similar (Figure 18B). Reconstitution analysis after 8 weeks showed that cDC subsets were equally reconstituted from both BM in the mLNs from control mice (Figure 18C). In contrast, 70 to 80% of cDC2 and the minor population of $CD103^{-}$ cDC2 in the double KO (d-KO) mice derived from $BATF3^{KO}$ BM (Figure 18C). These results could suggest that $TLR3^{OFF}$-derived cells are competitively at a disadvantage over $BATF3^{KO}$. Another, more likely explanation is that $BATF3^{KO}$ BM gives rise to more of the other DC subsets because precursors cannot develop into classical cDC1. This last explanation is in line with our findings in $BATF3^{KO}$ mice (Manuscript Figure 2G), where there is an overall increase in cDC2 numbers.

Figure 10D shows that in response to poly(I:C), cell numbers in mLNs of control mice were increased for both $BATF3^{HET}$ and $TLR3^{OFF}$, confirming our previous findings (Manuscript Figure 2G&H). Further, cDC1 and cDC2 numbers of d-KO mLNs were increased in response to poly(I:C) compared to steady state (Figure 18D). Although these preliminary results need to be confirmed with further experiments, these findings suggest that cDC1 can also migrate in response to poly(I:C) in the absence of TLR3 on all DCs available in the system.

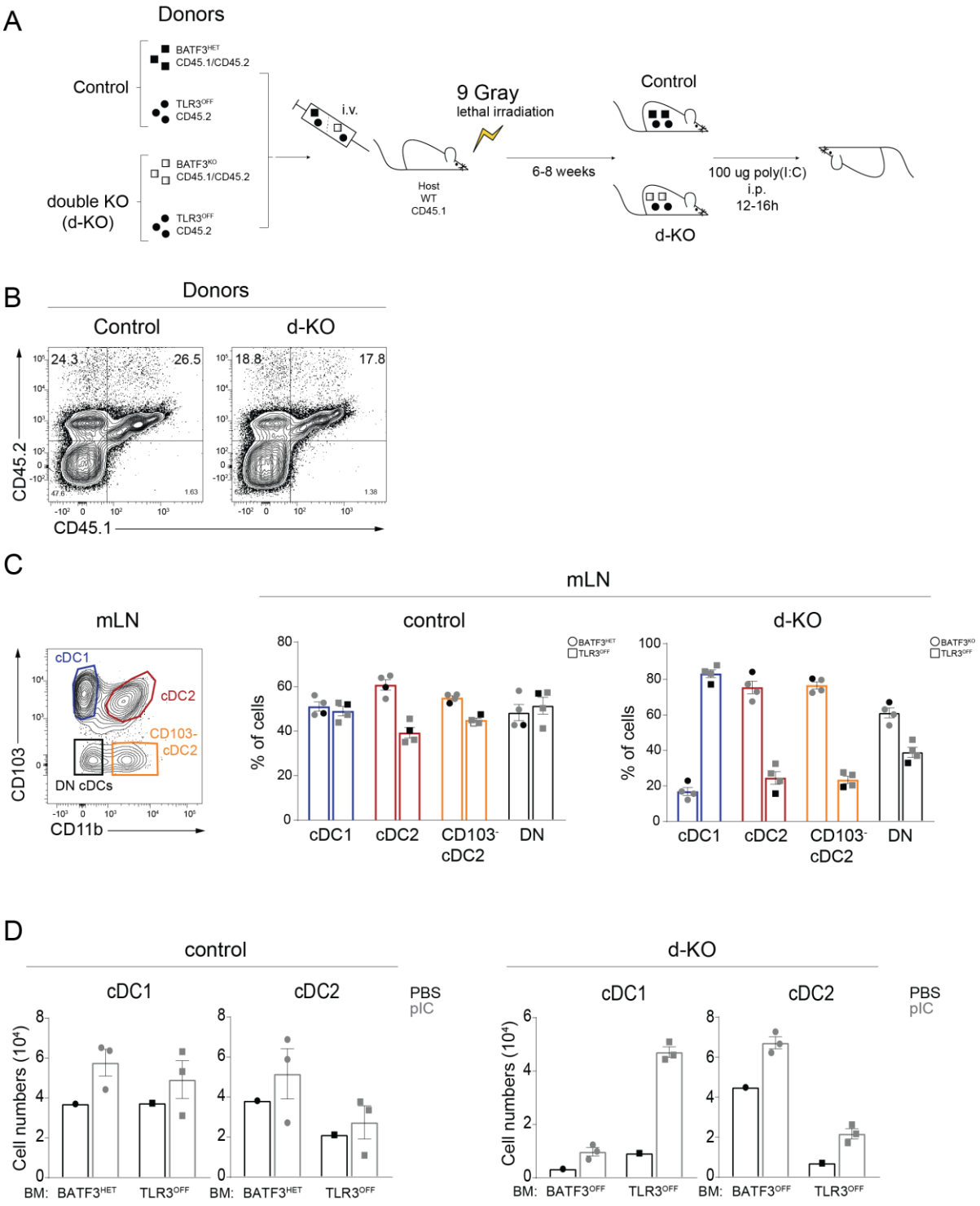


Figure 18. Role of cell-extrinsic TLR3 in intestinal cDC1 and cDC2 migration in response to poly(I:C). A) Experimental design; d-KO: double knock out; i.v.: intravenously, i.p. intraperitoneally; **B)** Representative flow cytometry plots of BM cells from donors. Cells are pre-gated on Live cells; **C)** Left: representative flow cytometry plot of mLN cDC subsets. Right: percentage of mLN cDC subsets in control and d-KO derived from $BATF3^{KO}$ (circle) and $TLR3^{OFF}$ (square) mice. Black represents PBS-treated and grey represents poly(I:C)-treated mice; **D)** Total number of cDC1 and cDC2 in the mLNs of control and d-KO mice 12h after i.p. injection of PBS or 100µg poly(I:C). Data shown are mean numbers of cells ± 1 SEM from one experiment with 1-3 mice per group. SEM: standard error mean.

6. Discussion

Intestinal DCs play a crucial role in the induction of adaptive immune responses to either maintain tolerance to commensals and food proteins or to develop protective immunity against pathogens. A key aspect of DCs in priming T cell responses is their effective migration from the intestine to the mLNs. However, while migration of intestinal DCs is likely to be influenced by microbial stimuli via TLRs and/or inflammatory cytokines, the exact mechanisms of how different DC subsets migrate to different stimuli remains poorly understood. In the intestine, different DC subsets differ in their expression of PRR. For example, intestinal cDC2 express high levels of TLR5, whereas cDC1 express TLR3[196,273]. This difference in expression is likely to be physiologically relevant. For example, systemic immunization with soluble flagellin induces increased migration of cDC2 but not cDC1 from the intestine to the mLNs. In addition, cDC2 but not cDC1 are responsible to drive antibody responses to flagellin[197]. In line with this, we speculated that different TLR ligands might induce different DC subset migration patterns. In this thesis, I study the molecular requirements for intestinal DC migration to the mLNs in response to poly(I:C) and dissect the migratory differences between the two main DC subsets, cDC1 and cDC2.

6.1 Poly(I:C) as a model for intestinal viral infection

Diarrhea caused by enteric viral infection remains the leading cause of death among children under 5 years of age[237]. Although vaccination against rotavirus as well as implementation of sanitation actions have helped reducing the cases of hospitalized children, better understanding of the immune mechanisms against viruses are needed to improve and broaden vaccination strategies.

Poly(I:C) is a synthetic analogue of dsRNA that mimics dsRNA viruses such as reoviruses as well as intermediates of viral replication of ssRNA and some DNA viruses[6,322,323]. Intraperitoneal injection of poly(I:C) induces small intestinal damage in a mechanism dependent on TLR3 in IECs[324]. Enteric viral infections such as rotavirus are characterized by shortening of the villi, loss of small intestinal villus cells and concomitant diarrhea, and systemic dsRNA is found in mice and humans infected with rotavirus[325,326]. Collectively, peritoneal injection of poly(I:C) comprises a good model for studying enteric viral infection.

The enteropathy caused by poly(I:C) has also been used to study other diseases such as celiac disease[16,327]. This is because recent studies have suggested that early life infections with reoviruses lead to break of oral tolerance by DCs, leading to development of celiac disease[328]. This is thought to be dependent on the direct effect of type I IFN on DCs, leading to activation of food protein-specific Th1 responses by DC production of IL-12 and expression of IRF-1[328,329]. In addition, a longitudinal study found a correlation between celiac disease and rotavirus infection, and a recent population cohort study suggests an association of rotavirus vaccination with decrease of celiac disease prevalence[330,331]. Given that poly(I:C) mimics reoviruses infections and induces a strong type I IFN response that affects DCs directly, poly(I:C) injection represents a relevant model to study the role of DCs in the development of celiac disease after viral infections.

Additionally, rotavirus infection has been associated with type I diabetes incidence, as rotavirus displays molecular mimicry with T cell epitopes in pancreatic β-cell autoantigens[332]. Although still not clear in the human context, several studies performed with animal models have shown that heterologous rotavirus induces pancreas pathology[333]. Of note, a study using weanling mice showed that pancreatic apoptosis was TLR3-dependent after rotavirus infection[334]. Moreover, previous hypothesis suggesting a role for rotavirus vaccination in decreasing type 1 diabetes incidence have been confirmed by two recent studies[335,336]. Although a recent Finnish population-based cohort study also showed a negative correlation between rotavirus vaccination and type I diabetes or celiac disease incidence, the study was performed in a too small cohort with a too short period of follow-up in order to draw firm conclusions[331].

Collectively, these recent findings suggest an important role of dsRNA viruses in the development of autoimmune diseases. Accordingly, poly(I:C) injection represents a useful model to better understand the mechanism of pathology exert by dsRNA viruses in the intestine.

6.2 TLR3 sensing of poly(I:C)s with different molecular weight

Poly(I:C) is a dsRNA-like complex of synthetic polymers, and different formulations vary in the distribution of strand lengths, affecting their biological functions[309,310,337]. However, very little is known on the receptor requirement for sensing these different poly(I:C)s. Kato et al have shown that poly(I:C), generally recognized by MDA5, was converted into a RIG-I ligand after shortening of the dsRNA by enzymes[311]. Another study has shown that different dsRNA lengths induce distinct immune functions in a cell-dependent manner[309]. For example, short poly(I:C) induced greater amounts of TNF-α and IFN-β in myeloid cells, while long poly(I:C) did so in fibroblasts[309]. Additionally, only one study has shown how different poly(I:C)s exert distinct maturation profiles in DCs and has suggested that these differences might be due to alternative, TLR3-independent ways of sensing dsRNA[310]. However, very little is known about the involvement of TLR3 in sensing different dsRNA lengths.

Our preliminary studies assessing the effect of different poly(I:C) formulations showed that all three poly(I:C)s induced increased DC migration to mLNs. However, unlike the rest, HMW poly(I:C) showed a minor dependency on TLR3 as shown in TLR3-deficient mice. Zhou et al showed that HMW poly(I:C) exhibited the highest efficiency in activating TLR3 signaling, measured by the resulting type I IFN response. Moreover, HMW induced the expression of TLR3, MDA5 and RIG-I[337]. Accordingly, our results indicate that HMW poly(I:C) might induce intestinal DC migration by inducing the expression of RIG-I and MDA5, which could then be responsible for the TLR3-independent migration observed in response to HMW poly(I:C). Nevertheless, most studies performed are based on *in vitro* experiments, and the definition of short and long dsRNA varies. In addition, previous studies suggest that poly(I:C) from the same supplier but different batches generated firmly contrasting innate immune responses[309]. In our studies, we observed a great variability between experiments, and differences in poly(I:C) batches might have influenced DC migration. SA poly(I:C) showed the highest efficiency in inducing maturation of DC in a previous

study[310], and this is the poly(I:C) we have used for all experiments. These observations indicate the necessity of understanding the exact mechanisms driving activation of immune responses in response to different dsRNA lengths for the design of better, more specific adjuvants.

6.3 DC migration in response to poly(I:C)

By using a range of different mouse models, we found that the accumulation of different DC subsets in the mLNs was differentially regulated by TNF-α and type I IFNs in response to poly(I:C). Similar to the scenario using R848[45], poly(I:C)-induced migration of both DC subsets depended on TNF-α. Interestingly, one study showed that TNF-α is abrogated in TLR3-deficient mice after poly(I:C) injection, indicating that TNF-α may be produced by the cell directly sensing poly(I:C) through TLR3[338]. This might explain why DC migration is disturbed in TLR3-deficient mice. In steady state, migration of intestinal DCs is independent of TNF-α[339]. It seems thus that TNF-α might be a common regulator for intestinal DC migration in response to different TLR ligands.

In contrast to TNF-α, systemic type I IFN induction by poly(I:C) is fully dependent on MDA5 and is mainly produced by non-hematopoietic cells, although splenic DCs also contribute to type I IFN production in a TLR3-dependent manner[340,341]. However, despite normal levels of systemic type I IFN in TLR3-deficient mice after poly(I:C) stimulation, both the systemic type I IFN response together with a local production by DCs are required for the adjuvant effect of poly(I:C)[340]. Collectively, these results suggest that the cellular source of type I IFN and TNF-α might be a cell directly sensing poly(I:C) in a TLR3-dependent manner. In addition to cDC1, macrophages could be potential candidates as they can express TLR3. Preliminary data from sorted intestinal cells indicate that macrophages may indeed be the main type I IFN producers (data not shown). Further studies are required to elucidate whether macrophages are also the main TNF-α producers.

Interestingly, while TNF-α was essential for the migration of both cDC1 and cDC2, type I IFN signaling was only required for cDC1 to migrate, being dispensable for cDC2. Given that cDC1 start to migrate earlier (6h) than cDC2 (8h) in response to poly(I:C), direct poly(I:C)-sensing via cDC1 could induce a type I IFN response that would act in an autocrine manner, speeding up their migration via direct activation. Along these lines, cDC2 migration might be delayed overall due to the dependency on secondary signals.

6.3 pDCs in intestinal DC migration

pDCs are the main type I IFN producing cells upon viral infection. Yrlid et al showed that pDCs produce TNF-α and type I IFNs upon oral R848 administration, which are essential for intestinal DC migration and activation, respectively[45]. However, we found that pDCs are dispensable for cDC1 and cDC2 migration in response to poly(I:C). pDCs express the endosomal receptors TLR7 and TLR9, but not TLR3, suggesting a minimal involvement of pDCs in the TLR3-dependent poly(I:C)-induced DC migration.

Interestingly, poly(I:C) injection ameliorated intestinal inflammation due to IFN-β production by pDCs in a DSS-induced colitis model[210]. Moreover, such production by pDCs was TLR3 and TLR7 dependent. However, DDS-induced colitis is a complex scenario, and disruption of the barrier integrity might lead to translocation of viruses present in the healthy intestine that might directly activate pDCs. Of note, pDCs express MDA5 and RIG-I, sensors also able to sense poly(I:C)[194]. Collectively, while pDCs do not play a role in poly(I:C)-induced DC migration, they might be involved in type I IFN responses in more complex scenarios such as during intestinal inflammation or enteric viral infections.

6.4 MyD88 signaling in poly(I:C)-induced DC migration

Poly(I:C) sensed via TLR3 induces the expression of type I IFN and pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β[307]. Indeed, IL-1β was increased in the small intestine after 2h of poly(I:C) injection. IL-1β signals through IL-1R, which signals via the molecular adaptor MyD88. We show however that neither cDC1 nor cDC2 require MyD88 signaling to migrate in steady state or in response to poly(I:C). The finding that MyD88 is dispensable for steady state migration of intestinal DCs is in contrast with a previously published observation of 50–60% reduction in $CD103^+$ DC migration in the absence of MyD88[339]. Of note, steady state conditions vary extensively between different animal facilities, influenced by microbiota, enteric viruses as well as dietary conditions. All these factors might account for the differences we see in steady state DC migration in the absence of MyD88.

Pang et al showed that signaling via IL-1R was particularly important for lung cDC1 to migrate to mediastinal lymph nodes after Influenza A infection[342]. Moreover, IL1R and MyD88 signaling in DCs was sufficient to drive their activation and migration to the lymph node[342]. However, the *in vivo* scenario during infection with a live pathogen varies substantially compared to immunization with a TLR ligand. In line with this, while classical immunization by injection of a model Ag together with an adjuvant has shown a requirement for direct PAMP recognition by DCs via TLRs; live pathogens can evade the direct mechanisms of recognition in DCs, rendering them unable to prime T cells[343].
The increased expression of IL-1β seen at transcriptional level does not necessarily correlate with its activation. TLR ligands have been shown to activate macrophages, leading to a metabolic reprogramming characterized by accumulation of succinate[344]. The increased concentration of succinate leads to HIF1α activation, which can directly binds to the gene promoter of IL-1β, inducing its expression[345]. Accordingly, the slight increase in IL-1β levels might be due to a direct activation of macrophages by poly(I:C).

Interestingly, MyD88 re-expression on DCs allowed for even increased DC migration compared to WT in response to poly(I:C). This increased cellularity might be a consequence of altered homeostasis in the absence of MyD88 signaling in other cells, such as IECs. MyD88 signaling is known to induce the expression of AMPs such as REGIIIγ[186]. Consequently, lack of MyD88 signaling in IECs might lead to reduced AMP production and a subsequent increase of bacterial

colonization of the epithelial surface, leading to an increased DC migration and higher adaptive immune responses to the microbiota.

6.5 Type I IFN in the regulation of maturation and migration of cDC1

As a mimic of viral infection, poly(I:C) is known to induce great amounts of type I IFNs. Effective migration of DCs to lymph nodes is a key aspect controlled by type I IFN signaling[260]. Webb et al recently demonstrated a role of type I IFN signaling in inducing migration of lung DCs in response to a pathogen-associated Ag[346]. However, previous studies have shown a specific role of type I IFN in maturation and not migration of DCs in response to TLR ligands and during viral infections[45,347]. Of note, type I IFN is involved in many immunological processes besides cell migration, and the use of mouse models completely lacking IFNAR does not address which specific DC subset requires type I IFN signaling[348]. Here, we used specific deletion of IFNAR in either cDC1 or cDC2 to study the cell intrinsic requirement of type I IFN, as well as a blocking anti-IFNAR antibody to exclude any type I IFN-driven homeostatic effects. We observed a previously unrecognized role for type I IFN signaling on cDC1 but not cDC2 migration in response to poly(I:C). Surprisingly, this requirement was the same for mice stimulated with R848, in contrast to what has been described previously when assessing bulk DCs[45].

Type I IFN is known to play a key role in DC activation and induction of adaptive immune responses *in vivo*[246,347,349]. In line with these studies, Pantel et al showed that cell-intrinsic type I IFN signaling rather than TLR3 signaling was required for splenic DC maturation in response to poly(I:C)[203]. However, no distinction of the DC subsets was performed. Here, we found that maturation of cDC1, but not cDC2, was significantly affected by the lack of intrinsic type I IFN signaling after poly(I:C) stimulation. These data suggest a unique role of intrinsic type I IFN signaling to induce both maturation and migration of cDC1 in response to poly(I:C) and R848. Further studies with other TLR ligands will help unravel whether this is a common feature for cDC1 in response to TLR signaling.

TLR ligands have been shown to regulate DC activation by inducing changes in their glycolytic metabolism. Accordingly, metabolic conversion to aerobic glycolysis is essential for DC maturation and function, as inhibition of glycolysis leads to immature DCs[350,351]. In line with this, Pantel et al showed that type I IFN was responsible for upregulation of all pathways associated with DC immunogenicity, particularly the metabolic switch from oxidative phosphorylation to glycolysis. Additionally, type I IFN signaling upregulated the expression of Hif1α, which might play a role in DC survival by suppressing production of ROS species as well as maintaining intracellular ATP levels.

DC maturation and migration are very intimately related processes. In contrast to previous studies[45,347], we found that type I IFN is required for both migration and activation of cDC1, strongly suggesting a tight correlation between maturation and migration. Interestingly, Guak et al showed that an early switch to glycolysis in DCs in response to TLR agonists was responsible for

CCR7 oligomerization and thus migration to the lymph nodes[352]. Blocking of glycolysis resulted in a defect of DC migration and motility[352]. Accordingly, cDC1 might uniquely depend on type I IFN signaling to switch to glycolysis in response to poly(I:C), and the absence of type I IFN signaling may lead to a lack of CCR7 oligomerization and thus no migration. If this scenario is true, other signals must drive the metabolic change and consequently the CCR7 oligomerization in cDC2 in response to poly(I:C). Metabolic analysis of sorted intestinal DC subsets from subset-specific $IFNAR^{KO}$ mice after poly(I:C) stimulation will reveal whether other signals than type I IFN produced in response to poly(I:C) are able to induce a metabolic switch in cDC2.

6.6 TLR3 expression across cell types

TLR3 is broadly expressed not only by hematopoietic immune cells but also by non-hematopoietic cells, such as epithelial cells at mucosal surfaces, mast cells in the peritoneal cavity, or lymphatic endothelial cells[5,10,353]. Recent studies have shown that ILCs also express TLR3 and may in fact be responsible for the small intestinal damage caused by poly(I:C)[16]. Hence, our finding that TLR3-deficient mice have a defect in DC migration might be due to the overall deletion of TLR3 in a set of cells rather than a single cell type.

Indeed, mixed BM chimeras in which cDC1 did not express TLR3 showed that DC migration occurred normally in response to poly(I:C) compared to control mice. We speculate that macrophages might be potential candidates to directly sense poly(I:C) through TLR3. Unfortunately, we have been unable to test this hypothesis due to the lack of a proper mouse model. Yet, the intestinal damage caused by poly(I:C) will lead to recruitment of $Ly6C^{hi}$ monocytes in large numbers. The $CX3CR1^{int}$ macrophages can produce large amounts of TNF-α and IL-6, among other pro-inflammatory cytokines, potentially inducing DC migration. However, our results in CCR2-deficient mice show no difference in DC cellularity in response to poly(I:C) compared to WT, indicating that newly recruited monocytes are not required for DC migration in the context of an otherwise WT background.

In addition, ILCs in the intestinal LP have shown to respond rapidly to poly(I:C) by producing TNF-α[16]. Hence, TNF-α produced by ILCs could be responsible for driving migration of DCs in response to poly(I:C). Of note, this study did not distinguish between different groups of ILCs[16]. Sorting of different groups of ILCs present in the intestine after poly(I:C) injection may help to identify whether ILCs are the main producers of TNF-α and identify the specific group responsible.

Of note, several studies have shown that murine mast cells, whether bone marrow-derived or isolated *in vivo*, express TLR3 both at the membrane and intracellularly[353]. However, as mast cells were shown to primarily produces chemokines like RANTES, MIP-1α and MIP-1β in response to poly(I:C) injection (all involved in T cell recruitment rather than DC migration[353]), they are unlikely to drive our observed phenotype.

6.7 *Cis* vs *trans*-activation of DCs in response to poly(I:C)

DC activation or maturation is here defined as a phenotypical feature characterized by the increased expression of the costimulatory molecule CD86. However, maturation does not equal immunogenicity, the latter referring to the capacity of DCs to prime full T cell differentiation, requiring proper positioning and suitable cytokine expression profiles in addition to co-stimulation. Accordingly, while activation of DCs can be achieved by both direct and indirect signals, *in vivo* immunogenic DCs can be obtained only by direct cell-intrinsic activation of TLR ligands[354]. In our research, we have not addressed the question of whether migrating DC subsets activated directly or indirectly differ in their functional capacity. Future functional studies addressing the capability of *cis* vs *trans*-activated DC subsets to induce optimal adaptive immune responses could lead to new insights into whether different molecular requirements for different DC subsets translate into subset-specific effector immune responses.

7. Conclusion

In this thesis, I have used poly(I:C) as a mimic of viral dsRNA to study the molecular requirements for migration and activation of intestinal cDC1 and cDC2 to the mLNs.

Herein, I have shown that poly(I:C) is a potent adjuvant that triggers increased intestinal DC migration compared to steady state. Migration in response to poly(I:C) depended entirely on TLR3 signaling. Surprisingly, cDC1 and cDC2 migrated equally in response to poly(I:C) despite the prominent expression of TLR3 only in cDC1.

Poly(I:C) induced the early expression of several cytokines, including TNF-α and type I IFNs. Indeed, we saw that migration of both cDC1 and cDC2 was dependent on TNF-α. In addition, we have identified a previously unknown role for intrinsic type I IFN signaling in inducing migration and activation of cDC1 but not cDC2 in response to poly(I:C). Similar results were obtained when using R848, suggesting type I IFN as a specific signal for cDC1 migration and activation in response to R848 and poly(I:C). Whether or not specific signals exist that drive cDC2 migration remains unknown.

Our experiments show that TLR3 in cDC1 is sufficient to drive DC migration. However, preliminary studies suggest that cells other than DCs expressing TLR3 can also drive DC migration in a cell-extrinsic manner. In contrast to published data on R848-induced migration, pDCs are not required for poly(I:C)-induced DC migration, as depletion of this cell type did not affect normal migration and activation of intestinal DCs. Preliminary studies point to macrophages as the potential cell source of type I IFN. However, whether they are also the main source of TNF-α is still unknown. Unfortunately, the lack of a suitable mouse model to dissect whether macrophages at all contribute to DC migration in response to poly(I:C) leaves this question still unanswered.

Finally, we show that *cis*- and trans-activated DCs seem to migrate with similar efficiency. Previous studies have shown that immune responses primed by *cis*-activated DCs differ from those of trans-activated DCs. Unfortunately, a very complex experimental set up would be needed to address this: a readout for a uniquely cDC2-induced immune reaction in response to poly(I:C). We are not aware of a system to model such a scenario. Alternatively, we could set up mixed BM chimeras from TLR3OFF and WT donors, sort the *in vivo* activated DC subsets according to donor origin and test their ability to cross-prime *in vitro*. Future research of this kind will clarify whether our observed differences on DC subset migration translate into different immune responses.

8. Future perspectives

Data shown in this thesis elucidate that distinct intestinal DC subsets differ in the specific mediators required for migration to the mLNs in response to poly(I:C). While type I IFN was shown to play an important, specific role in poly(I:C)-induced cDC1 migration and activation, specific signals for cDC2 are still unknown. Although we found that TNF-α was required for both cDC1 and cDC2 migration, the use of full $TNFRI^{KO}$ mice did not allow for studying the TNF-α requirement for a specific subset. ThereTherThe use of mouse models specifically targeting a given DC subset will lead to a better understanding of the DC-specific signals required for their migration. Additionally, the use of other adjuvants such as flagellin, known to target cDC2 specifically, could be tested using such models to dissect the signals required for cDC2 migration and activation. Of note, TLR7 expression in intestinal cDC2 requires further study, as the situation in lung cDC2 highly responding to R848 might not be the same for intestinal cDC2.

An interesting question that remains still unanswered is the cellular source of type I IFN and TNF-α. Preliminary studies performed on sorted cell populations 3-4h after poly(I:C) injection suggest macrophages as main source of type I IFN. Several repeats of such experiments, sorting specific cell populations, will give new insights on the cellular source(s) of type I IFN and TNF-α.

DC migration is a key aspect for mounting adaptive immune responses. Despite our observation on similar migration capabilities of both *cis* and *trans*-activated DCs, previous studies have shown that differentially activated DC induce distinct immune responses. In line with this, functional studies assessing the immunogenicity of differently activated DCs subsets will lead to a better understanding of migration vs immunogenicity. Experiments such as *in vitro* mixed-leukocyte reaction (MLR) with sorted intestinal DC subsets activated in *cis* and *trans*, as well as *in vivo* T cell transfers will help answer these questions.

The relevance of DC activation relies on their medical application. *Cis*-activated DCs are uniquely capable of priming optimal immune responses. Particularly, cDC1 can, due to their unique ability to cross-present Ag, induce potent cellular immunity towards tumors. Importantly, DC subset specific TLR3 expression is conserved across mouse and man[116]. The approach of *ex vivo* antigen-loaded DC-based vaccines has already been shown safely and effectively induce tumor-specific $CD4^+$ T cells and CTLs. However, such a method is expensive, labor-intensive and operation process-complex. Accordingly, the next generation of DC-based vaccines will involve direct *in vivo* targeting of DCs to generate effective immunity. Better understanding of the requirements and consequences in response to defined vaccines is crucial to faithfully predict immune-regulatory versus immunogenic outcomes upon intervention.

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