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Requirements for the Induction of Adaptive Immune Responses to Rotavirus

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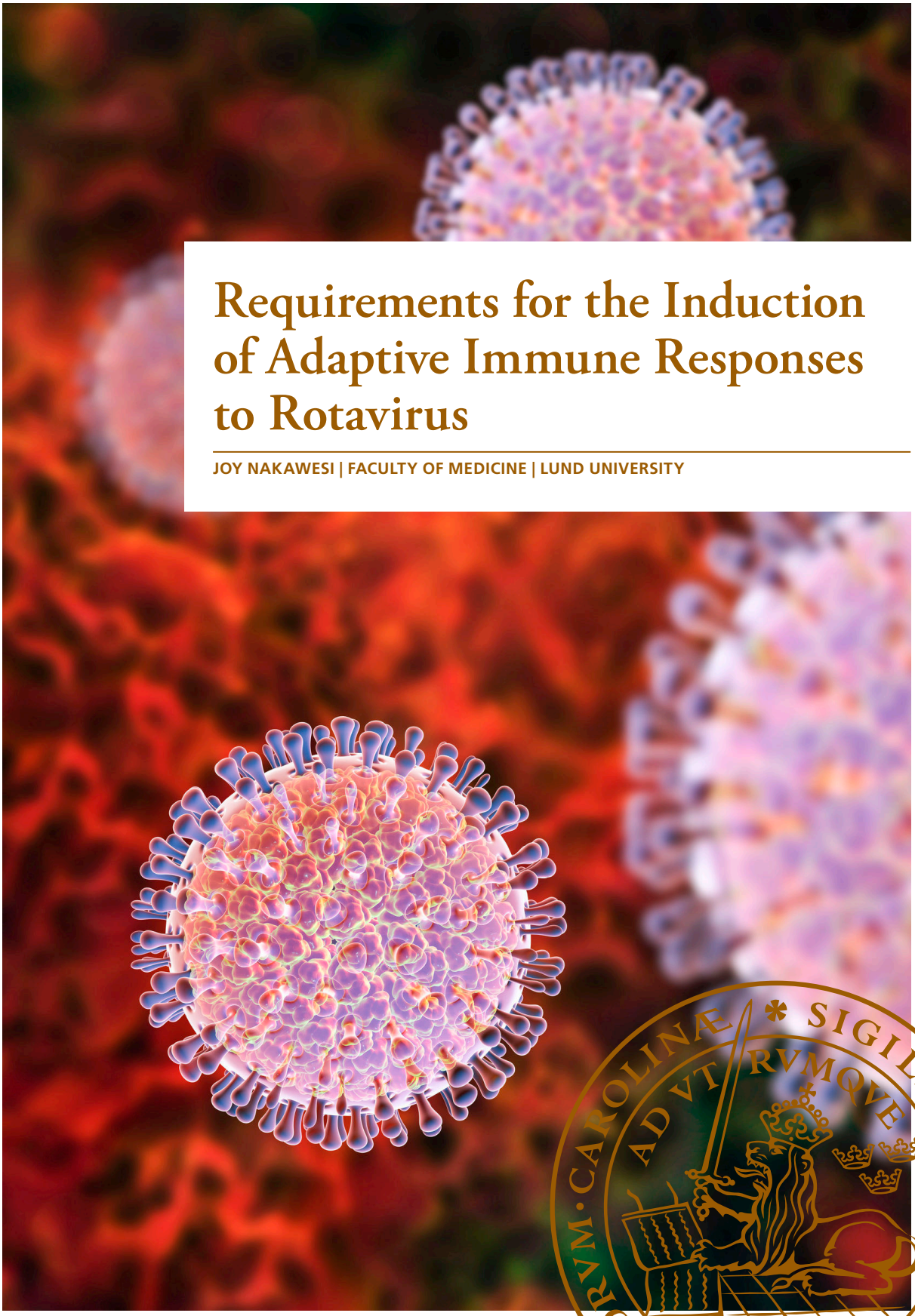

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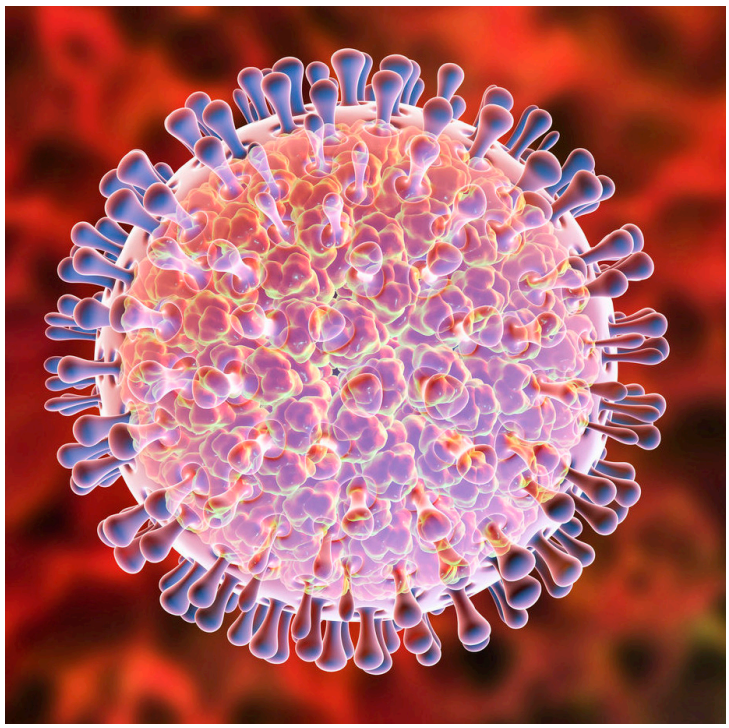

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Requirements for the Induction of Adaptive Immune Responses to Rotavirus

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Section for Immunology, Department of Experimental Medical Science

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DOCTORAL DISSERTATION

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Abstract

Rotavirus (RV) infections remain the leading cause of world-wide diarrhea-associated morbidity and mortality among children <5 years of age. Despite the global introduction of RV vaccines over a decade ago, RV infections result in >200,000 deaths annually mostly in the low-income countries of Africa and Asia. Efficient clearance of the primary RV infection and protection from future re-infections is mediated by adaptive immune responses.

The aim of the work presented in this thesis was to investigate the spatial, cellular and molecular requirements for the efficient induction of optimal adaptive immune responses towards primary RV infection.

Intestinal RV-specific IgA is the major correlate of protection from re-infection with RV. In Paper I, we demonstrated that Batf3-dependent cDC1 but not cDC2 (specific subsets of antigen-presenting dendritic cells) are required for the optimal induction of T cell-dependent RV-specific IgA responses in the mesenteric lymph nodes (mLNs). Additionally, cDC1-driven RV-specific IgA was dependent on the selective expression of the TGFβ-activating αvβ8 integrin by cDC1 while signaling via the type I interferon receptor on the dendritic cells was dispensable.

In Paper II, we investigated the major intestinal inductive site for the initiation of adaptive immune responses towards primary RV infection using lymphoid organ hypertrophy as a readout. We showed that the RV-induced hypertrophy was confined to the intestinal draining mLNs and resulted from increased recruitment of lymphocytes into the mLN and halted lymphocyte egress from the mLNs. Furthermore, the RV-induced hypertrophy of the mLNs was independent of antigen-specific recognition, type I interferon- and tumor necrosis factor α- receptor signaling.

Cytotoxic CD8 T cells mediate clearance of primary RV infection. In Paper III, we addressed the role of retinioc acid (RA) signaling in the development and function of CD8 T cells. Using the *CD4Cre.dnRAR*$^{lsl/lsl}$ mouse model, we showed that the absence of RA signaling in the developing thymocytes perturbed thymopoiesis and led to the accumullation of CD8SP thymocytes. Additionally, the abrogated RA signaling in peripheral CD8 T cells led to reduced expression of RA-controlled effector genes and impaired cytotoxic activity of the CD8 T cells.

In Paper IV, we investigated the molecular requirements for the activation and migration of intestinal cDC1 and cDC2 in response to poly(I:C), a TLR3-targeting adjuvant. We demostrated that poly(I:C) induced both cDC1 and cDC2 activation and migration from the small intestinal lamina propria to the mLNs in a TLR3-dependent manner despite the lack of TLR3 expression by cDC2. Furthermore, both cDC1 and cDC2 migration depended on tumor necrosis factor α while cDC1 showed a unique requirement for type I interferon signaling.

Collectively, the work included in this thesis helps to broaden our understanding of the requirements for the efficient induction of optimal RV-specific adaptive immune responses and provides important insights in the designing of better RV vaccines.

Key words: Rotavirus, classical dendritic cells, B cells, IgA, mesenteric lymph nodes, lymphoid organ hypertrophy, retinoic acid, CD8+ T cells, TLR3, Poly(I:C)

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To Daddy, Mama and Sandra

'And now these three remain: faith, hope and love. But the greatest of these is love.'
1Corinthians 13:13

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Mucosal Immunol. 2021 Jan;14(1):53-67.

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Joy Nakawesi, Konjit Getachew Muleta, Dragos-Christian Dasoveanu, Bengt Johansson-Lindbom, Katharina Lahl

European Journal of Immunology 2020 December 22; DOI: 10.1002/eji.202048990

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

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Eur J Immunol. 2020 Oct;50(10):1525-1536.

Abbreviations

Ag	Antigen
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
Batf3	Basic leucine zipper transcription factor ATF-like 3
BCR	B cell receptor
CSR	Class switch recombination
CTL	Cytotoxic T lymphocyte
cDC	Classical dendritic cell
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
dnRAR	Dominant-negative retinoic acid receptor
EC	Epithelial cell
FAE	Follicle-associated epithelium
FcR	Fc receptor
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid organs
GC	Germinal center
GI	Gastrointestinal
HEV	High endothelial venule
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
i.p	Intraperitoneally
ID2	Inhibitor of DNA binding 2
IEC	Intestinal epithelial cell
IFN	Interferon

ILFS	Isolated lymphoid follicles
ISG	Interferon-stimulated genes
IFNAR	Type I interferon receptor
KO	Knockout
LI	Large intestine
LN	Lymph node
LPS	Lipopolysaccharide
MAD5	Melanoma differentiation–associated protein-5 receptor
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
mLN	Mesenteric lymph nodes
PAMP	Pathogen-associated molecular pattern
poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
pLN	Peripheral lymph nodes
PPs	Peyer's Patches
RA	Retinoic acid
RIG I	Retinoic acid-inducible gene 1–like receptor
RAG	Recombination-activating gene
RV	Rotavirus
sIgA	Secretory IgA
SED	Subepithelial dome
SI	Small intestine
SiLP	Small intestine Lamina Propria
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine 1 phosphate receptor
T1D	Type 1 diabetes
TD	T cell-dependent
TCR	T cell receptor

TGFβ	Transforming growth factor beta
Tfh	Follicular helper T cell
Th	Helper T cell
TI	T cell-independent
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TLR	Toll-like receptor
TLP	Triple-layered particle
Treg	Regulatory T cell
IRF	Interferon regulatory factor
VAD	Vitamin A deficient
VP	Viral protein
VLP	Virus-like particle
WT	wild type
WHO	World Health Organisation
Zbtb46	Zinc finger and BTB domain containing 46

1.The Gastrointestinal tract

The human gastrointestinal tract (GI tract) extends from the mouth to the anus and is composed of multiple organs of the digestive system, including the mouth, esophagus, stomach, small intestine and large intestine. The GI tract is about 9m long, making it the body's largest surface to the environment and a major site of entry for many microorganisms. The GI tract is designed to perform the dual roles of food digestion and nutrient uptake while maintaining immune homeostasis, i.e., discriminate between the invasive harmful pathogens and the harmless antigens (Ags) derived from commensal microbiota and food (immunity against the bad and tolerance towards the good). The GI tract is anatomically composed of different layers; the inner mucosal layer, which consists of a single layer of absorptive and secretory epithelial cells, the underlying lamina propria (LP), and a thin layer below the LP called the muscularis mucosa. The remaining layers in the GI tract include the submucosal layer which contains the lymphatic vessels, nerves, and connective tissue. Finally, the GI tract contains a smooth muscle layer and an outer thick fibrous serosa which separates the intestine from the surrounding peritoneal cavity[1–3].

The intestine

The intestine is a long continuous tube-like organ extending from the pyloric sphincter of the stomach to the anus. It is divided into the small and large intestine. The small intestine (SI) has the primary role in digestion and absorption of nutrients from food. In humans, it is about 6 - 7m long, beginning from the pylorus and ends at the ileocecal valve. It is compartmentalised into the duodenum (about 20 - 25cm long), the jejunum (2.5m long) and the ileum (3m long). The large intestine (LI) is ~1.5m long and begins at the caecum, followed by the proximal colon, transverse colon, distal colon, the rectum and terminates at the anus. The SI surface is characterised by multiple long finger-like projections named villi, which extend into the lumen and serve to increase the surface area for food digestion and nutrient absorption. The villi become progressively shorter towards the end of the SI and are absent from the caecum and colon whose main function is water and salt reabsorption from the undigested food passed on from the SI.

Along the entire intestine, the epithelial cells (ECs) are derived from multi-potent stem cells that reside in the intestinal crypt invaginations known as crypts of Lieberkühn. These give rise to various mature and specialised ECs including the absorptive

enterocytes, Paneth cells, goblet cells and neuroendocrine cells. The absorptive enterocytes are covered with a brush-border consisting of projections called microvilli. The Paneth cells, which are mainly concentrated in the ileum, produce a range of antimicrobial peptides, which serve to maintain a sterile crypt environment. The goblet cells are the mucus-secreting cells, and they comprise at least 25% of the ECs in the LI and 10% or less in the SI. The mucus layer (glycocalyx) serves as an antimicrobial physical barrier between the lumen and the lamina propria[1,4–6].

The immune inductive sites of the intestine

Immune inductive sites are the main locations for the priming of adaptive immune responses in the body. The immune inductive sites of the intestine consist of the Peyer's patches (PPs) and the Isolated lymphoid follicles (ILFs) – which together make up the gut-associated lymphoid tissue (GALT) - and the mesenteric lymph nodes (mLNs) (**Figure 1**).

Mesenteric lymph nodes

The mLNs are the intestinal draining lymph nodes[7]. They are the largest LNs in the body and their main function is to filter and scan lymph coming from the SI and LI for Ags and either generate adaptive immune responses against the pathogenic Ags or tolerance in the case of harmless Ags. Ag arriving in the mLNs is either free (passively drained via the lymphatics)[8,9] or loaded onto the dendritic cells (DCs) migrating from the intestinal LP via the afferent lymphatics in a CCR7-dependent manner[10]. mLNs consist of three main compartments, the cortex (contains the B cell follicles), the paracortex (contains the T cell zone) and the medullar sinus (contains macrophages and APCs). Naïve lymphocytes migrate to the mLNs from the blood via the high endothelial venules (HEVs). Upon activation and differentiation into effector cells, lymphocytes primed in the mLNs home to the intestinal effector sites (i.e., the LP and the overlying epithelium) via the efferent lymphatics[11,12].

Peyer's patches

Mature PPs are macroscopically visible secondary lymphoid organs that are distributed along the SI. They are rare in the duodenum but their size and numbers increase from the jejunum to the ileum[13]. The epithelium overlying the PPs has a less distinct brush-border and is known as the follicle-associated epithelium (FAE). The FAE contains specialised intestinal ECs called microfold (M) cells that mediate uptake and transport of particulate Ags from the intestinal lumen into the underlying subepithelial dome (SED). The SED of the PPs contains large and numerous B cell follicles (which always contain germinal centers (GCs)) surrounded by smaller T cell areas. The SED is also rich in cDCs, which are the main APCs in the GALT. PPs are important sites for T cell-dependent IgA induction and class switch recombination (CSR) in the gut. Unlike the mLNs, PPs lack afferent lymphatics[1,14–17].

Isolated lymphoid follicles

ILFs are small aggregations of lymphoid structures distributed in tandem on the antimesenteric wall of the SI from the duodenum to the ileum. About 100 - 200 ILFs exist in 8 - 20 weeks old C57BL/6 mice. ILFs are smaller than the PPs and lack the T cell zones. Similar to PPs, ILFs are predominantly filled with B cells, contain GCs and are covered by the FAE containing M cells[18]. Different studies have documented the nonredundant roles of ILFs as great reservoirs for intestinal IgA induction against a broad range of Ags and enteric microbiota (reviewed in[19]). A study by Tsuji et al demonstrated that cross-talks between the RORγt$^+$ lymphoid inducer cells, stromal cells, bacteria, DCs, and B cells are essential for the formation of ILFs. The authors further showed that ILFs but not PPs, are the sites for the induction of Activation-induced cytidine deaminase (AID) and T cell-independent IgA CSR[20].

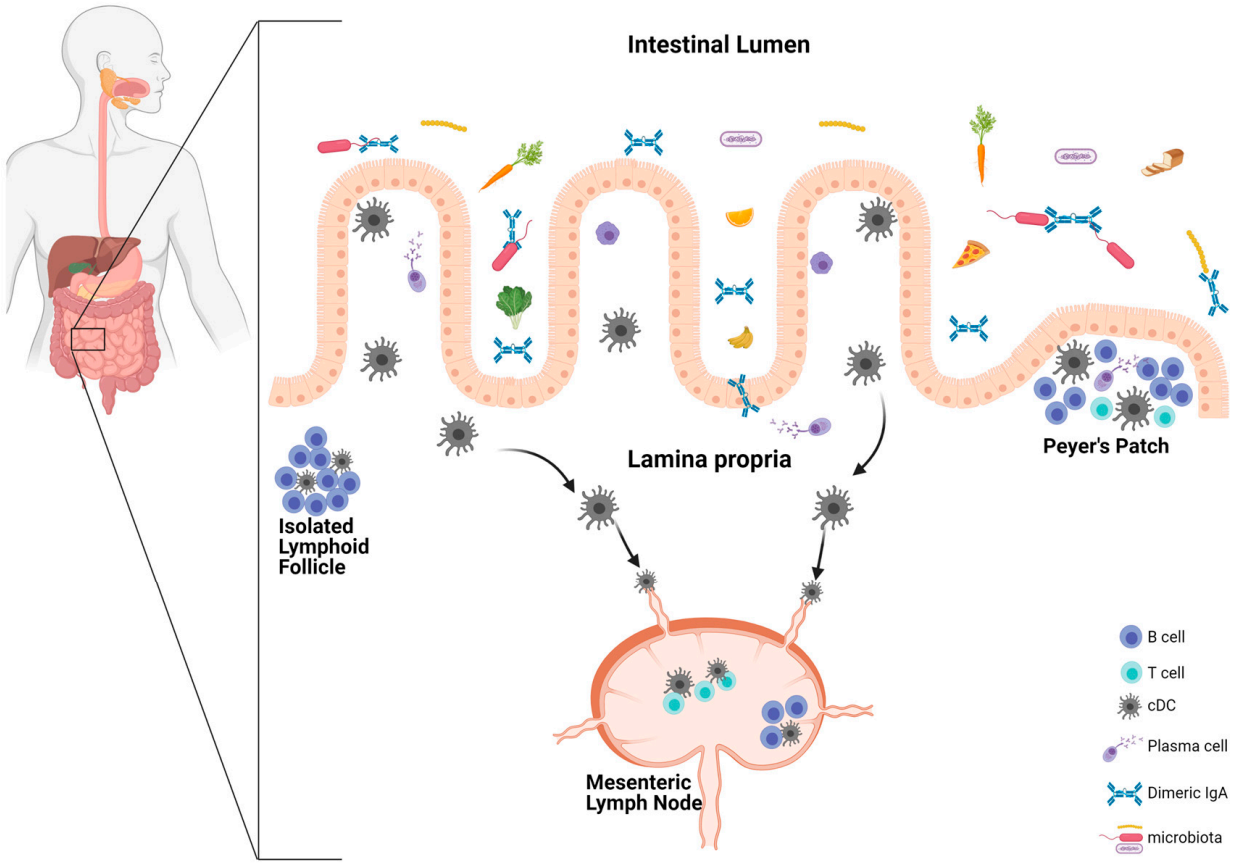

Figure 1. Structure of the intestinal immune system. The intestinal lamina propria is covered by a single layer of epithelial cells which separate the luminal contents (which include the microbiota and food Ags) from the underlying lamina propria. The lamina propria contains several types of immune cells including cDC, macrophages, IgA producing plasma cells, B and T cells. The Peyer's Patches, Isolated lymphoid follicles and draining mLNs represent the immune inductive sites of the intestine. Depicted are cDC present in the lamina propria migrating to the mLNs via afferent lymphatics. *Figure created using BioRender.com*

2. Intestinal classical dendritic cells and their role in the initiation of adaptive immune responses

Dendritic cells (DCs) were first discovered by Ralph M. Steinman and Zanvil A. Cohn in 1973 as 'large stellate cells with distinct properties from other mononuclear phagocytes, granulocytes and lymphocytes' in preparations of adherent mouse splenocytes on glass and plastic surfaces[21]. Decades later, over 90,000 studies on DC biology and function have been made and for this discovery, Ralph M. Steinman was awarded the Alfred Nobel's prize in Medicine in 2011[22].

DCs are present in all mammalian peripheral tissues and they are described as motile sentinel professional antigen-presenting cells (APC) that play key roles in innate immunity and in the initiation and regulation of adaptive immune responses[23]. The classical DCs (cDCs) express the DC-specific transcription factor Zinc finger and BTB domain-containing protein 46 (ZBTB46) and depend on FMS-like tyrosine kinase 3 (FLT3) ligand for their development[24–27].

In the intestinal mucosa, cDCs are distributed throughout the intestinal LP, GALT (PPs and ILFs) and the intestinal draining mLNs. cDCs in the LP continuously sample the intestinal environment for Ags derived from food and microorganisms or self Ags (dead/damaged cells). After acquisition of Ags, cDCs migrate from the intestinal LP to the mLNs via afferent lymphatics in a chemokine receptor 7 (CCR7)-dependent manner to present the processed Ags to naïve lymphocytes[10,28–32].

Subsets and functions of intestinal cDC

cDCs are generally identified as $CD11c^{+}MHCII^{+}$ and lack expression of the macrophage-associated markers CD64 and F4/80. In the mouse intestinal LP, three main cDC subsets have been identified, which are classified based on their expression of the αE (CD103) integrin and CD11b. These include $CD103^{+}CD11b^{-}$ (cDC1), $CD103^{+}CD11b^{+}$ and $CD103^{-}CD11b^{+}$ (collectively cDC2), and a minor $CD103^{-}CD11b^{-}$ cDC subset. These cDC subsets differ in their transcription factor requirements for development and maintenance (**Figure 2**). They have also been shown to have distinct roles in maintaining intestinal immune homeostasis[1,33–35].

cDC1

While cDC1 share CD103 expression with a subset of cDC2 in the intestinal LP, all cDC1 express the chemokine receptor XCR1, the C-type lectin receptor DNGR-1 (CLEC9A), and the CD8αα homodimer. For their development and differentiation, cDC1 depend on the Interferon regulatory factor 8 (IRF8), Basic leucine-rich zipper transcription factor ATF-like 3 (BATF3), and Inhibitor of DNA binding 2 (ID2) transcription factors[36–40]. *Irf8* is required for the specification of the pre-cDC1 precursors in the bone marrow and *Batf3* maintains pre-cDC1 commitment to the cDC1 lineage via autoactivation of the *Irf8* gene[41]. *Batf3*^{KO} mice lack cDC1 at steady-state[36].

The *Xcr1*-DTA, *Clec9a*-DTR, *Cd11c-cre.Irf8*$^{fl/fl}$, *Zbtb46-cre.Irf8*$^{fl/fl}$, and *Batf3*^{KO} mouse models have been widely utilised to examine the in vivo functions of cDC1[36,38,39,42–44]. cDC1 were shown to be critical in the maintenance of T cell homeostasis in the SiLP and its overlying epithelium[38,42]. cDC1 support T cell homeostasis through various mechanisms: Migratory cDC1 in the mLNs appear to be the major source of cDC-derived retinoic acid (RA), which induces gut homing CCR9 and $\alpha_4\beta_7$ receptors on the responding T cells[38]. Upon arrival in the mLNs, they also preferentially express high levels of the TGFβ-activating αvβ8 integrin, which is important in the induction of regulatory T cells (Tregs)[45]. Additionally, cDC1 play a dominant role in cross-presenting epithelial-derived Ags to CD8 T cells at steady-state[29] and they are required for the optimal induction of CD8 T cell responses towards viruses and tumours[36,46]. Finally, we (discussed in detail in paper 1 in this thesis) have also shown that cDC1 are essential for mounting optimal RV-specific IgA antibody responses in the mLN[47].

cDC2

The intestine harbours two subgroups of cDC2, CD103$^+$CD11b$^+$ and CD103$^-$ CD11b$^+$ populations. Collectively, both subgroups express high levels of signal regulatory protein α (SIRPα/CD172a) and the DC inhibitory receptor 2 (DCIR2)[48]. The Interferon regulatory factor 4 (IRF4), Interferon regulatory factor 2 (IRF2), neurogenic locus notch homolog protein 2 (Notch2), RelB and Krüppel-like factor 4 (KLF4) transcription factors partially regulate development, function, and maintenance of cDC2[40,48–52].

Mouse models like *Cd11c-cre.Irf4*$^{fl/fl}$ and *hulangerin*-DTA (among others) have been utilised to study the role of cDC2 in intestinal immunity[48,53]. Mice lacking CD103$^+$CD11b$^+$ cDC2 show selectively reduced intestinal Th17 cell numbers. This finding suggested a key role for CD103$^+$CD11b$^+$ cDC2 in intestinal Th17 homeostasis[48,49,53,54]. Intestinal Th2 responses against *Trichuris muris* worms and *Schistosoma mansoni* eggs do not develop in mice lacking the IRF4-dependent cDC2[55]. CD103$^+$CD11b$^+$ and CD103$^-$CD11b$^+$ cDC2 are essential for the induction of Th2 cell responses in the SI and colon, respectively, during infection with parasites[55]. CD103$^+$CD11b$^+$ cDC2 produce high amounts of IL-23 in response to

Citrobacter rodentium infection[52] and upon challenge with bacterial flagellin[56]. This cDC2-driven IL-23 is critical for driving IL-22 production by innate lymphoid cells type 3 (ILC3) which promotes intestinal barrier integrity by inducing the production of antimicrobial peptides from epithelial cells[52,56]. cDC2 present in the PPs SED were reported to drive IgA CSR against oral Ags and commensal microbiota[17,57–59].

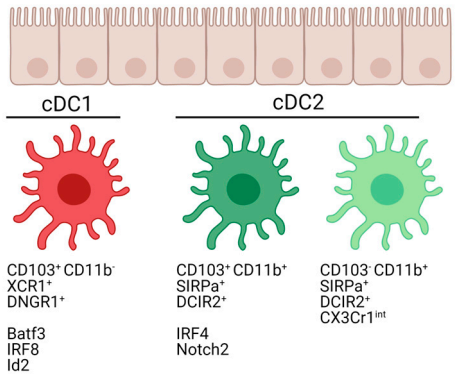

Figure 2. Intestinal classical dendritic (cDC) subsets. The murine intestinal cDCs are mainly divided into three major subsets based on their expression of CD103 and CD11b. $CD103^{+}CD11b^{-}$ belong to the cDC1 lineage and $CD103^{+}CD11b^{+}$ and $CD103^{-}CD11b^{+}$ represent the cDC2 lineage. cDC1 and cDC2 differ in their transcription factor requirements for development and maintenance. *Figure created using BioRender.com*

Antigen sensing by intestinal cDC

cDCs sense and sample their local environments via pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) found on microorganisms or damage-associated molecular patterns (DAMPs), which are components released by damaged/dead cells. The PRRs are either membrane-bound as for example Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which sense extracellular bacteria and parasites, or cytoplasmic as for example NOD-like receptors and RIG-1-like receptors, which sense viruses and intracellular bacteria.

cDC1 and cDC2 vary in their expression patterns of PRRs. cDC1 express high levels of TLR3, an endosomal PRR that detects viral dsRNA[60–62] and DNGR-1, a CLR that binds filamentous actin, a DAMP exposed by dead cells[63,64]. On the other hand, cDC2 uniquely express high levels of TLR5, a membrane-bound PRR that detects bacterial flagellin[59,65]. This unique expression of PRRs by cDC subsets allows for activation of different arms of the immune system. Targeting of Ags to $CLEC9A^{+}$ cDC1 enhances antibody responses in the absence of adjuvants and TLR signaling pathways[63]. A study by Schulz et al demonstrated that TLR3 expression by APC permits cross-priming of cytotoxic T lymphocytes (CTLs) against viruses that do not directly infect DCs[60]. The expression of TLR5 by cDC2 was shown to be essential in the induction of long-lived T cell-dependent antibody responses against

bacterial flagellin[59]. Uematsu and colleagues showed that $CD11c^+$ cells in the intestinal LP detect and induce innate immune responses against pathogenic bacteria in a TLR5-dependent manner[65]. Collectively, all cDCs express a wide range of PRRs that enable them to recognise and respond to a broad range of microorganisms and apoptotic/damaged cells in the body tissues.

Antigen uptake by intestinal cDC

As professional APCs, cDCs are distributed throughout the intestinal LP, which they constantly survey for Ags. Several mechanisms by which cDCs acquire Ags in the intestinal LP have been proposed. First, cDCs directly acquire Ags from apoptotic or damaged IEC within the intestinal LP[29]. In the case of luminal Ags, an initial study by Rescigno et al reported that DCs present in the intestinal LP express tight junction proteins that enable them to send their dendrites between IEC directly into the lumen to sample bacteria while preserving the epithelial barrier integrity[66]. Several follow-up studies further confirmed this mechanism[67,68]. Further, using the *cx3cr1*$^{GFP/GFP}$ mice, Niess et al showed that LP cDCs acquire luminal Ags by forming CX_3CR1-dependent transepithelial dendrites[69]. cDCs have also been shown to acquire luminal Ags and bacteria indirectly via the M-cells located in the FAE of the PPs[70,71]. Finally, studies also reported that luminal Ags can be transported via the goblet cells present in the intestinal epithelium to cDCs in the intestinal LP[72,73].

Upon Ag encounter, cDCs take up the foreign or self Ags via several mechanisms. These include receptor-mediated endocytosis via Fc receptors, TLR and CLR (for example mannose receptor), and the larger materials are taken up via phagocytosis. These Ags are processed within the cell vesicles and the peptides are loaded on MHC molecules for presentation to naïve lymphocytes in the mLNs[74].

Intestinal cDC activation and migration to mLNs

At steady-state, intestinal cDCs are generally immature (resting). Immature cDCs are characterised by high endocytosis and/or phagocytic activity, and low expression of surface MHCII, but instead high levels of intracellular MHCII molecules within the lysosomal cell compartment. Immature cDCs also have a low T cell activation potential. Acquisition of Ags by cDCs leads to immediate cDC maturation. This is accompanied by increased surface expression of MHCII molecules, co-stimulatory molecules (CD80/86), and CD40. Finally, the activated-Ag loaded cDCs upregulate the chemokine receptor CCR7 which drives their migration from the intestinal LP to the draining mLNs via the afferent lymphatics[10,27–29,31,32,74,75].

In the mLNs, the cDCs process the Ags into peptides which are then loaded onto surface MHCII or MHCI molecules for presentation to naïve CD4 T cells and CD8

T cells, respectively[74]. The Ag is presented to naïve B cells in its native form[76–79]. The outcome of this cDC-naïve lymphocyte interaction may lead to immunity (in the case of Ags derived from pathogens)[28,80] or tolerance (in the case of Ags derived from food, commensal microbiota, and self)[30,31,44].

cDC induction of adaptive immunity

cDC in priming different types of effector T cells

Pioneering studies by Steinman et al revealed that lymphoid DCs have the potential to stimulate T cells in vitro[81]. Indeed, the constitutive or conditional ablation of DCs in mice confirmed their role in the priming of naïve T cell responses[82,83]. Optimal activation and induction of protective T cell responses by cDCs is believed to rely on three distinct signals. Signal 1 involves the presentation of cognate Ag in the context of MHC molecules by cDC to the T cell receptor (TCR). The second signal is provided by the upregulation of costimulatory molecules (CD80/CD86) by cDC that bind to CD28 on T cells. Finally, signal 3 is provided by the instructing cytokines which determine the type of effector T cell response induced[74,84]. Direct stimulation of cDCs via PRRs is critical for the induction of protective T cell responses. cDCs indirectly activated by exposure to inflammatory signals are able to only induce T cell proliferation, but fail to direct full T cell differentiation as these T cells lack effector functions[85]. T cell encounter of cognate Ag presented on MHC molecules on activated cDC but without costimulatory signals contributes to peripheral tolerance[86,87]. Ags presented on MHCII molecules elicit CD4 T cell responses, which can be polarised towards either Th1, Th2, Th17 or Tregs depending on the polarising cytokines released. Ags presented on MHC1 molecules elicits CD8 T cell responses.

Th1

Th1 cells require IL-12 cytokine for their differentiation[88,89] and are critical for immunity against intracellular bacteria and viruses. Fujimoto et al showed that both cDC1 and cDC2 can induce Th1 responses in vitro[90]. An in vivo study by Luda et al demonstrated that cDC1 are essential for the generation and survival of steady-state Th1 cells in the intestinal mucosa[38]. Th1 cells play key roles in phagocyte-dependent host responses. Th1 cells produce IFNγ, TNF and IL-2 cytokines which promote the activation of macrophages, the production of opsonising and complement-fixing antibodies by B cells and thus induction of cell mediated immunity[91,92].

Th2

Naïve CD4 T cells differentiate into Th2 cells in the presence of IL-4 cytokine[93]. These cells produce IL-4, IL-5, IL-13, IL-9, and IL-25, which are critical for immunity against extracellular parasites (such as helminths) and allergic inflammatory responses[94]. IL-4 production by Th2 cells mediates IgE class switching in B cells[95]. IRF4-dependent cDC2 are essential for driving Th2 responses against *Trichuris muris* worms and *Schistosoma mansoni* eggs at the intestinal mucosa[55]. Furthermore, this study also revealed a specific functional heterogeneity among the intestinal cDC2 subpopulations in driving Th2 responses. $CD103^{+}CD11b^{+}$ cDC2 were shown to drive Th2 responses in the small intestine whereas $CD103^{-}CD11b^{+}$ cDC2 perform this role in the colon[55].

Th17

Th17 cells are the most abundant Th cells in the intestine at steady-state. Th17 cell differentiation requires TGFß, IL-6 and IL-21 cytokines[96–98]. Th17 cells are proinflammatory cells that secrete IL-17A, IL-17F, IL-21, and IL-22 cytokines and provide immunity against several extracellular pathogens[99]. A study by Persson et al demonstrated that IRF4-dependent $CD103^{+}CD11b^{+}$ intestinal cDC2 are essential for the generation and differentiation of Th17 cells in the mLNs in vivo[48]. Similarly, Denning et al showed that only the $CD103^{+}CD11b^{+}$ cDC2 efficiently induced Th17 cells in vitro[100]. In both studies, Th17 polarisation was linked to the capacity of cDC2 to produce IL-6 cytokine[48,100].

Tregs

Tregs control the proinflammatory responses of effector Th cells[101]. Tregs suppress T cell activation against self and harmless Ags such as commensal microbiota[102,103]. Natural Tregs are derived from the thymus during T cell development whereas naïve CD4 T cells exposed to TGFß give rise to inducible Tregs in the periphery. Transport of Ags from the intestinal LP to the mLNs by migratory cDCs is critical in the induction of Tregs that mediate oral tolerance against food Ags[31]. cDCs isolated from the SiLP and mLNs were shown to induce the generation of Tregs via a TGFß- and RA-dependent mechanism[104,105]. Furthermore, mice that lack the TGFß-activating αvß8 integrin on all cDCs have reduced Tregs in the colon in vivo. Cells isolated from these mice fail to induce Tregs in vitro[45]. These results demonstrate the critical role for cDCs in the provision of bioactive TGFß as an essential cytokine for the induction of Tregs.

Cytotoxic CD8 T cells

Ags presented on MHC class I molecules prime naïve CD8 T cells, which then clonally expand and differentiate into cytotoxic T lymphocytes (CTL). CTL provide immunity against viral infections, intracellular bacterial infections and cancer via their production of perforin, granzymes and IFNγ[106]. Terminal CTL differentiation

requires the cytokine IL-2[107]. Migratory cDC1 have the unique ability to cross-present epithelial-derived Ag (from apoptotic ECs) to naïve CD8 T cells in the mLNs at steady-state[29]. Cross-presentation is a unique ability of cDC1 to acquire, process and present exogenous Ags on MHCI to naïve CD8 T cells. This process is critical for immunity against tumours and viruses that do not readily infect the APCs but rather infect other peripheral tissue cells. Mice lacking cDC1 displayed deficiencies in cross-presenting Ags to CD8 T cells in vivo[38]. Similarly, it has been shown that cDC1 are essential for the optimal induction of CD8 T cell responses during Rotavirus infection[47,108]. $CD103^-$ lymph-borne DCs were also shown to efficiently cross-present Ags and prime naïve CD8 T cells in vitro[28].

cDC induction of humoral immunity

cDCs have been greatly recognised for their capacity to process and present Ag on MHC molecules to prime naïve T cells. On the other hand, B cells can only recognise Ag via the B cell receptor (BCR) in its native and unprocessed form.

cDCs sense and take up Ags using a variety of receptors. The type of receptor used in Ag uptake determines the fate of the Ag. Ag uptake via the activating Fc receptors (FcγRI, FcγRIII, FcγRIV) recruits the degradative pathway into the lysosome which allows Ag processing into peptides. Ag uptake via the inhibitory FcγRIIB receptor recruits a non-degradative pathway that retains the Ag in its native form. An initial study by Wykes et al showed that DCs directly interacted and transferred Ag in its native form to B cells[109]. The study also showed that DCs could retain the native Ag for at least 48 hours both in vivo and in vitro. In another study, Bergtold and colleagues showed that immune complexes (ICs) internalised via the FcγRIIB receptor on DCs were stored and recycled to the cell surface for direct presentation to B cells in their native form[79]. Using mAb to deliver Ag in vivo, Chappell et al showed that Ag uptake via the DC inhibitory receptor 2 (DCIR2), which is uniquely expressed by cDC2, induced robust IgG1 humoral responses[110]. Similarly, a study by Caminschi et al demonstrated that targeting of Ag directly to the DNGR-1 (CLEC9A) receptor, which is uniquely expressed by cDC1 using CLEC9A mAb significantly enhanced antibody responses in vivo[63]. Complement receptors (CR1, CR2, CR3 and CR4) have also been implicated in the presentation of native Ag to B cells[78,77]. Collectively, cDCs are well equipped to efficiently capture and retain Ags or ICs in their native unprocessed form to induce the activation of naïve B cells.

Mucosal IgA induction

Approximately 80% of the body's total plasma cells (PCs) are in the intestinal mucosa where they constantly secrete dimeric Immunoglobulin A (IgA). In humans, 3 – 5g/day of dimeric IgA is secreted under steady-state conditions. Secretory IgA (sIgA) serves as an immunological barrier at the intestinal mucosa by neutralising

and suppressing microbial toxins and growth, respectively. sIgA coats the intestinal microbiota thus preventing microbial attachment to the IEC[111]. Homeostatic (natural) IgA is induced by constant stimulation with the commensal microbiota present in the intestinal mucosa. Indeed, germ-free mice and neonates before microbial colonisation have significantly reduced IgA-secreting PCs[112,113]. cDCs present in the intestinal LP continuously sample luminal microbial Ags and cDCs carrying commensal bacterial Ags induce IgA class switching. The cDC-mediated IgA induction occurs either via T cell-dependent (TD) or T cell-independent (TI) pathways in the GALT and mLNs[57,111,114].

T cell-dependent IgA induction

TD IgA induction primarily occurs in the GCs within the B cell follicles of the PPs and mLNs[111,115]. TD IgA induction involves interactions between Ag-specific B cells expressing CD40 and Ag-specific CD4 T cells (follicular helper T cells, Tfh) expressing CD40L[115] (**Figure 3A**). To receive CD4 T cell help, first the naïve CD4 T cells are activated by cDCs presenting cognate Ags on MHCII, while providing costimulatory signals and IL-6 and IL-21 cytokines to promote pre-Tfh differentiation. These pre-Tfh cells then migrate to the T-B cell border in a CXCR5 dependent manner[58,114]. Second, naïve B cells in the B cell follicles are activated by cDCs carrying cognate Ag in its native form[109,114]. The activated Ag-specific B cells migrate to the T-B cell border in a CCR7 dependent manner in pursuit of T cell help[58,78,114]. Interactions between the Ag-specific B cells and pre-Tfh cells at the T-B cell border leads to full Tfh differentiation. The Tfh cells deliver signals including CD40L, IL-21 and IL-4 which promote B cell survival, proliferation, and differentiation. Cross-linking of the CD40 on B cells and CD40L on Tfh cells induces the activation of Activation-induced cytidine deaminase (AID), an RNA editing enzyme that initiates CSR in the activated B cells[111,114,115]. The cDCs also provide bioactive TGFß which, together with CD40-CD40L is essential for the IgA CSR[116]. This GC reaction also promotes somatic hypermutations within the BCR to increase the antibody's affinity for the Ag, thus producing highly specific monoclonal antibodies[57,78,111,114].

T cell-independent IgA induction

The TI IgA induction mainly occurs in the intestinal LP and the ILFs independent of CD40L and CD4 T cell help[117–119]. The Ag-loaded cDCs directly induce TI IgA synthesis through the upregulation of B cell-activating factor (BAFF, also known as B lymphocyte stimulator protein) and a proliferation-inducing ligand (APRIL)[120] (**Figure 3B**). BAFF and APRIL expressed by cDCs as soluble or membrane-bound, directly bind to different receptors on B cells. BAFF binds to BAFF receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and the B cell maturation antigen (BCMA). APRIL binds to TACI with low affinity and BCMA with high affinity[121]. The cross-linking of BAFF and APRIL on the Ag-loaded cDCs to their receptors on B cells activates a CD40-like

pathway that promotes B cell survival and induce AID expression which initiates CSR in the activated B cells. The cytokine TGFß is also essential in the TI IgA class switching[111,117,120,122–124].

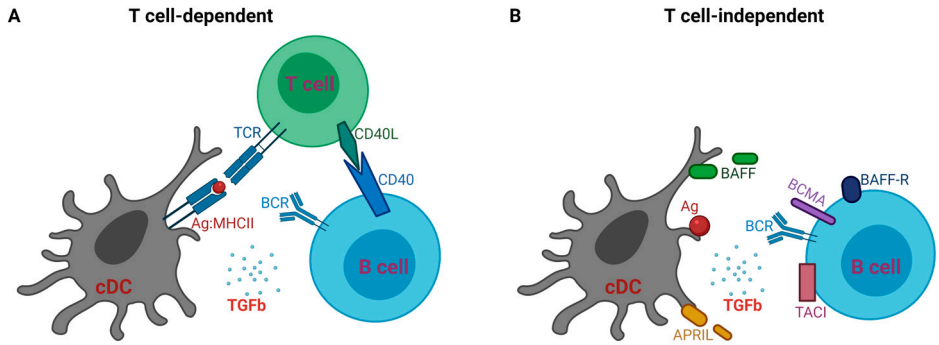

Figure 3. Induction of mucosal IgA by TD and TI pathways. cDCs have the capacity to retain and recycle Ag in its native form on their surface to prime naïve B cells. In the TD manner, the CD40-CD40L interaction between Ag-specific B and T cells plus the provision of bioactive TGFβ induces IgA CSR within the germinal centers. In the TI manner, cDCs upregulate soluble or membrane-bound BAFF and APRIL which bind to BCMA, TACI and BAFF-R on activated B cells. Interactions between these ligand-receptor signals plus bioactive TGFβ induce IgA CSR. *Figure created using BioRender.com*

Role of TGFß in IgA induction

The transforming growth factor beta (TGFß) is the principal cytokine required for IgA CSR in activated B cells[125]. Bioactive TGFß engages the heterotetrameric TGFß receptor (TGFßR) complex. Cross-linking of the TGFß-TGFßR on the activated B cells initiates Cα gene transcription, which leads to IgA isotype class switching[111,125]. TGFß was shown to specifically induce IgA class switching in lipopolysaccharide (LPS)-stimulated murine splenic B cell cultures in vitro[126,127]. TGFß only induces a small proportion of activated B cells to give rise to IgA-secreting PCs. However, the addition of IL-2 and IL-5 cytokines significantly enhances IgA secretion in LPS-stimulated B cell cultures in vitro[126,128].

TGFß Activation

TGFß is a pleiotropic cytokine that is ubiquitously secreted by many cell types in the body in an inactive (latent) form. The latent TGFß must be activated to exert its biological effects. After it has been synthesised, the TGFß homodimer interacts with a latency-associated peptide (LAP) forming a complex called small latent complex

(SLC). The SLC remains in the cell until it is bound by another protein called latent TGFß binding protein (LTBP) forming a larger complex called large latent complex (LLC). This LLC then gets secreted into the extracellular matrix[129]. To release the active TGFß, the LTBP and LAP must be cleaved from the LLC.

Physical processes including heat, acidic conditions, reactive oxygen species as well as biological processes such as proteolysis (mediated by proteases such as plasmin and metalloproteases) and integrins can be used to activate latent TGFß[130,131].

Integrins such as αvß6 (which is restricted to a subset of epithelial cells) and αvß8 (which is mainly expressed by immune cells such as T cells and cDC) activate TGFß[45,132,133]. The αvß6 and αvß8 integrins bind to an RGD amino acid sequence present in the LAP thus releasing the active TGFß[131]. An initial study by Munger et al identified a role for the αvß6 integrin in locally regulating TGFß function in vivo[132]. Further, Mu et al reported that the αvß8 integrin is essential for activating latent TGFß and maintaining epithelial homeostasis[133]. A study by Travis et al showed that the αvß8 integrin expressed on the cDCs is critical in providing bioactive TGFß as cells from mice that lack the integrin on all cDCs failed to induce Tregs in vitro, an effect that depends on TGFß activity[45].

Role of retinoic acid signaling in adaptive immune responses

Retinoic acid (RA) is a vitamin A metabolite that plays key roles in regulating mucosal immune responses and a variety of biological processes in the body. Vitamin A is obtained from the diet through the consumption of foods containing vitamin A precursors (such as ß-carotene present in plant foods) or vitamin A in the form of retinyl esters (present in foods of animal origin). In the intestinal lumen, the dietary vitamin A is absorbed by enterocytes and is converted to retinol, which can be transported to the liver for long-term storage[134,135]. RA is generated from retinol in two enzymatic reactions. The first is a reversible reaction in which retinol is oxidised to retinal by the ubiquitously expressed alcohol dehydrogenase enzyme. In the second reaction, retinal is irreversibly converted to RA by retinal dehydrogenase enzymes[135,136].

RA plays important roles in humoral immune responses and is essential for IgA production by B cells. Studies by Tokuyama et al showed that RA enhances IgA production in LPS-stimulated splenocytes[137,138]. Vitamin A deficient (VAD) mice have significantly reduced IgA-secreting cells following Influenza vaccination, but the administration of oral RA corrected and re-established the mucosal IgA responses in these mice[139]. In combination with lactoferrin (a monomeric glycoprotein that is abundant at mucosal surfaces), RA was shown to significantly enhance IgA production by peritoneal B-1 cells in vitro[140]. Further, cDC-derived RA in the PPs and mLNs is essential in the generation of gut-homing receptors α4ß7 and CCR9 on IgA-secreting PCs[141,142].

RA plays important roles in the shaping of peripheral T cell responses. A study from the Belkaid group showed that cDCs present in the SiLP are essential in promoting the de novo generation of Tregs in a RA-dependent manner[105]. Just like in B cells, cDC-derived RA imprints gut-homing receptors on effector T cells in the mLNs[143–145]. In addition, RA modulates Th1/Th2 responses in vivo. A study by Iwata et al demonstrated that RA directly suppresses Th1 development and directly enhances Th2 development[146]. In agreement with this study, Stephensen et al showed that the disruption of the retinoid X receptor in T cells produces a bias towards the Th1 phenotype in vivo[147]. Further, different studies have shown that RA regulates the Th17/Treg balance mainly in the intestinal mucosa. At steady-state, RA was shown to increase Treg development by enhancing TGFß-driven Smad3 signaling and inhibit Th17 development by inhibiting the expression of IL-6 and IL-23 receptors[148]. In an experimental model of DSS-induced colitis, RA was shown to attenuate intestinal inflammation by enhancing the production of IL-22 by innate lymphoid cells (ILC3) and γδ T cells[149] and via inhibition of NF- κB activation[150]. In line with these studies, Okayasu and colleagues showed that vitamin A inhibits the development of DSS-induced colitis and colon cancer in mice[151].

Lymphoid organ hypertrophy

Efficient immune surveillance involves the continuous recirculation of lymphocytes between blood, lymphoid organs, and lymph in search for Ags. Naïve B and T cells enter the lymph nodes from the blood via HEV, from where they travel into the B cell follicles in a CXCR5- and into the T cell zone in a CCR7-dependent manner, respectively. Under steady-state conditions, it is estimated that the lymph node median dwell times are approximately 6 - 8 hours for CD4 T cells, 8 - 12 hours for CD8 T cells, and up to 24 hours for B cells. Lymphocyte exit from the lymph nodes occurs via the efferent lymphatics[152–155]. Lymphocyte recirculation and lymph node cellularity are tightly controlled under steady-state conditions[156] but this rapidly changes upon infection and/or inflammation leading to transient hypertrophy of the local responding lymph nodes[157–161].

Earlier studies in sheep revealed that injection of antigenic material into the cannulated lymph nodes was always followed by an acute but transient fall in lymphocyte output in the efferent lymph – a phenomenon known as cell shutdown which is usually accompanied by lymph node hypertrophy[157,158,160]. Several important players have been implicated in the coordination of lymph node hypertrophy. Type I IFNs (α/ß) were shown to directly sequester both B and T cells in the lymphoid organs leading to blood lymphopenia during Vesicular stomatitis virus infection in mice[162]. Lymphocyte egress from the lymphoid organs requires the sphingosine 1 phosphate receptor 1 (S1PR1) and a sphingosine 1 phosphate (S1P) gradient[152,163–165]. A study by Shiow and colleagues showed that IFNα/ß

inhibits lymphocyte responsiveness to the S1P gradient by the rapid induction of CD69, which forms a complex with and negatively regulates the S1PR1. Furthermore, the authors demonstrated that $CD69^{-/-}$ cells are poorly retained within the lymphoid organs after infection or TLR stimulation. This study thus established that CD69 acts downstream of IFNα/ß to inhibit the S1PR1-mediated lymphocyte egress from the lymphoid organs[166]. Tumour necrosis factor (TNF) induces hypertrophy of the draining lymph nodes during bacteria infection[161]. TNFα is required for the efficient maturation and migration of local DCs to activate adaptive immune responses during viral infections[167]. In line with this, the tumour necrosis factor superfamily member 14 (TNFSF14 or LIGHT), a secreted protein of the TNF family, was shown to enhance lymph node hypertrophy by promoting the migration of DCs and the influx of lymphocytes into the draining lymph nodes but not cell egress after immunisation with a strong adjuvant[168]. In contrast to the studies above, Schulz et al reported that the hypertrophy of the *Salmonella*-infected PPs is independent of type I IFN, TNFα, and CD69[159]. This matched our findings (discussed in detail in paper 2) that the hypertrophy of mLNs upon oral Rotavirus infection is independent of type I IFN and TNFα[169].

Other mechanisms implicated in driving hypertrophy of the lymph nodes include signaling via the ß2-adrenergic receptors (ß2-AR). The expression of ß2-AR by lymphocytes was reported to control lymphocyte egress from the lymph nodes. Nakai et al demonstrated that agonist stimulation of ß2-AR on lymphocytes inhibits their egress from the lymph nodes causing rapid lymphopenia in blood. The authors showed that ß2-AR physically interacts with CCR7 and CXCR4 and enhances or strengthens these retention-promoting signals thus inhibiting lymphocyte egress leading to hypertrophy of the lymph nodes[170].

Different studies have established that halted lymphocyte egress from the responding lymphoid organs is the major driver for lymphoid organ hypertrophy[159,169,171]. For many decades now, lymphocyte egress from lymphoid organs has been attributed to the IFNAR/CD69/S1PR1 axis. This does not seem to be the case for the global cell shutdown seen in the context of localised infections at the intestinal mucosa[159,169]. To reconcile their findings with the proposed role for the IFNAR/CD69/S1PR1 axis in lymphocyte egress from the lymphoid organs, Schulz and colleagues hypothesized that two distinct mechanisms of lymphocyte retention must exist, which differ in their requirement of CD69. Indeed, Ag-specific $CD69^{+/+}$ cells were efficiently retained in the PPs compared to $CD69^{-/-}$ cells. These results thus suggested that the CD69-dependent retention mechanisms require Ag-specific lymphocyte activation, whereas other cells within the same compartment remain unaffected[159]. However, to better understand the physiological consequences of infection-induced lymphoid organ hypertrophy, further mechanistic investigations are needed.

3. Rotavirus

Rotaviruses were first discovered in humans in 1973 by electron microscopy of duodenal biopsy samples from children suffering from acute severe diarrhoea. The name was adapted from the Latin word '*rota*' (wheel) and was assigned to the newly discovered virus because of its distinct morphological appearance[172]. Rotavirus (RV) is classified as a genus within the Reoviridae family. The virion has a non-enveloped triple-layered protein capsid surrounding a genome composed of 11 segments of dsRNA. The RNA segments encode six structural viral proteins (VP 1,2,3,4,6, and 7) (**Figure 4**) and six non-structural proteins (NSP 1,2,3,4,5, and 6). The RV genus is divided into five serological groups (A - E). All groups infect animals, only groups A - C infect humans. RV group A causes more than 90% of the infections in humans[172–176].

Rotaviruses are the leading cause of acute, severe, dehydrating gastroenteritis in children under 5 years of age globally with an estimated >25 million outpatient visits and >2 million hospitalisations annually[177]. Within the first year of life, three quarters of the children in the developing countries of Africa and Asia acquire their first RV episode but this is delayed in the developed countries until 2 - 5 years of age[177]. Virtually every child will have been infected with RV between 2 and 3 years of age regardless of the social-economic status. Although child-RV infections occur in every country, over 90% of the deaths occur in low-income countries[178–180].

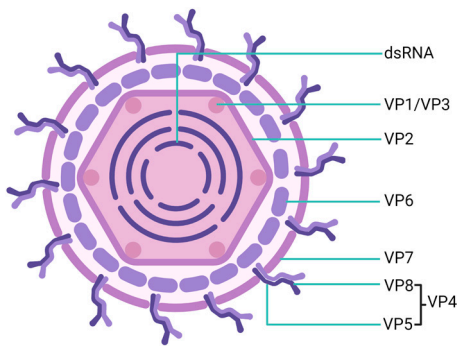

Figure 4. The Rotavirion triple layered particle. The Rotavirion consists of a non-enveloped triple layered capsid, which surrounds 11 segments of dsRNA. The genome encodes six structural viral proteins (VP 1,2,3,4,6, and 7) and six non-structural proteins (NSP 1,2,3,4,5, and 6). *Figure created using BioRender.com*

Global burden

The primary RV infection accounts for most of the clinical significance as the severity of the disease decreases with each re-infection. In 2004, 527,000 (475000 - 580000) RV-associated deaths were registered[177]. According to a study conducted by Tate and colleagues in 2013, the proportion of diarrheal deaths due to RV slightly decreased from 43% to 37% over the 14-year study period from 2000 to 2013[180]. RV alone resulted in 215,000 diarrheal deaths in children <5 years in the year 2013. The largest number of RV deaths occurred in sub-Saharan Africa, where the number ranged from 250,000 (range, 217000 – 282000) deaths in 2000 to 121,000 (range, 111000 – 131000) deaths in 2013[180]. In 2015, RV was still reported as the leading cause of diarrhoea deaths in children <5 years globally (199000, 95% uncertainty interval (UI) 165000 – 241000). Nearly 23% of RV deaths occurred in people older than 5 years (52,697 deaths, 47400 – 57700)[178]. As of 2016, a study conducted by Troeger et al showed that RV still resulted in 128,500 deaths (95% UI, 104500 - 155600) among children under 5 years worldwide and was responsible for >258 million episodes of diarrhoea among children <5 years old (95% UI, 193 million to 341 million)[179].

Transmission

RV has a short incubation period of 1 to 3 days and it is rapidly shed in the stools and vomitus of the infected individuals. The virus is mainly transmitted via the fecal-oral route directly from person-person, or indirectly via contaminated food, water, and other environmental surfaces. RV is very stable and can survive for days to weeks on contaminated fomites. In tropical regions, the RV infections may occur throughout the year whereas the infections usually peak during winter in the temperate climates. While a very low number of infectious particles is required to cause infection, RV-infected children have been reported to excrete about 100 billion virus particles per gram of stool[177,181–184].

Rotavirus entry and detection by host cells

RV primarily infects and replicates in the mature, non-dividing epithelial cells at the tip of the small intestinal villi[173,174,185]. The infectious rota virion is a triple-layered particle (TLP) that attaches to the host cells via the capsid protein VP4. First, the VP4 protein is proteolytically cleaved by trypsin-like proteases into VP5 and VP8 subunits. Attachment is then mediated by VP8 which interacts with binding partners on the host cell surface (mainly the enterocytes), including sialoglycans (such as gangliosides GM1 and GD1a), histo-blood group antigens (HBGAs), integrins (α2β1, α4β1, αvβ, and αxβ2) and the heat shock cognate protein (hsc70)[175,176,184,186,187,188] Following attachment of the RV to the host cellular

receptors, the TLPs are internalized into the cytoplasm by receptor-mediated endocytosis. Upon cellular uptake, RV replication and assembly occurs in the cytoplasmic viroplasm, and the newly produced RVs are released from the infected host cells through cell lysis or Golgi-independent non-classical vesicular transport[175,176,188].

Upon entry into the host, RV is recognized by PRRs in the enterocytes or immune cells (macrophages, DCs, or B cells). The PRRs include RA-inducible gene 1 (RIG-I)–like receptors, TLR3, and the melanoma differentiation–associated protein-5 receptors (MDA5). TLR3 and MDA5 both recognise dsRNA. TLR3 has been reported to have a role in the age-dependent resistance to RV infection in mice[189] and signaling via MDA5 in the RV-infected enterocytes is required for induction of IFN-β production thus restricting RV replication[190].

Immunity against Rotavirus

RV infection induces both innate and adaptive immune responses. RV primarily targets the IECs. Following RV infection of the IEC, the innate immune system is rapidly triggered to suppress RV replication and provide an antiviral state[191,192]. A study in both suckling and adult mice deficient of the IFN λ receptor in IEC showed that those mice were highly susceptible to oral RV infection, demonstrating a critical role for type III IFNs in the IEC anti-RV host defence[193]. In line with this, Jian Da Lin et al showed that both type I IFN and type III IFN are not only required for the optimal anti-RV protection of the GI tract in suckling mice, but independently, both IFN types contribute to the innate anti-RV defences in the intestinal mucosa and cooperate to restrict the extra-intestinal RV replication in other tissues[194]. Furthermore, Hernández et al demonstrated a synergistic cooperation between ILC3-derived IL-22 and IEC-derived IFN λ for activation of STAT1 which is required for optimal transcription of interferon-stimulated genes (ISG) and for restricting RV replication[195]. However, the RV NSP1 has been shown to antagonise the anti-RV innate immune responses by targeting the interferon regulatory factors (IRF) 3, 5 and 7 for degradation thus inhibiting the interferon-mediated STAT1 activation[109,196–199]. Although the innate anti-RV immunity is important, efficient RV clearance and protection from re-infection are mediated by the adaptive immune responses.

In the late 1980's, passive transfer experiments showed that RV-specific antibodies can protect neonatal mice against RV-induced diarrhoea[200,201]. Blutt et al demonstrated a critical role for IgA in the establishment of anti-RV immunity[202]. $IgA^{-/-}$ mice on both the BALB/c and C57BL/6 background failed to develop protective immunity against multiple RV re-exposures[202]. Intestinal RV-specific IgA is the major correlate of protection from re-infection against the natural RV infection[203,204]. The anti-RV IgA response is heavily dependent on $CD4^{+}$ T cell help[47,205,206]. As opposed to mucosal protection, the clearance of primary RV

infection is mainly mediated by cytotoxic $CD8^+$ T cells. β2-microglobulin$^{-/-}$ mice, that lack the MHC class I-restricted $CD8^+$ T cells, were significantly delayed in clearing the initial RV infection, just as the nude mice (deficient of all T cells) and the αβ and αβ/γδ $TCR^{-/-}$ mice[205–207].

Rotavirus vaccines

The World Health Organisation (WHO) considers vaccination to be the best strategy to decrease the disease burden of RV. Vaccine efforts were thus focused on the development of a live attenuated RV strain of human and/or animal origin that can replicate in the human gut[177]. In 1998, the first RV vaccine (RotaShield®, Wyeth Lederle) a rhesus-human reassortant tetravalent was licenced in the United States of America. However, in less than a year, the manufacturer withdrew the vaccine from the market following reports of excess intussusception (intestinal blockage) in infants within a period of two weeks after vaccination[208]. Subsequently, two oral RV vaccines were developed; RotaTeq™, a pentavalent bovine-human reassortant that had 74% (95% CI: 67 - 79) efficacy against RV gastroenteritis of any severity and 98% (95% CI: 90 - 100) efficacy against severe gastroenteritis in all the clinical phase trials[177,209] and Rotarix™, a monovalent human RV vaccine had 87% (95% CI: 80 - 92) protection against any and 96% (95% CI: 90 - 99) protection against severe gastroenteritis in all the clinical phase trials[177,210]. Rotarix™ is administered to children at 2 and 4 months of age and RotaTeq™ is administered at 2, 4 and 6 months of age. In 2009, the WHO strongly recommended the inclusion of RV vaccines into the nationwide immunisation programmes in all countries[174,177].

A study by Burnett et al following the global impact of RV vaccination on childhood hospitalisations and mortality from diarrhoea during the first 10 years after the introduction of the RV vaccines into the national immunisation schedule in 57 countries, observed a reduction in the disease burden of RV in these countries[211]. The RV disease-associated hospitalisations decreased by a median of 67% (with a range of 18 - 84%)[211]. The RV vaccines have low efficacy in the developing countries of Africa and Asia[212–214], but a recent study on the impact of RV vaccine in sub-Saharan Africa observed that the introduction of the RV vaccines was partly responsible for the significant reduction in the burden of RV-associated diarrhoea. The proportion of RV-positive cases significantly reduced from 42% (95% CI: 38 - 46) pre-vaccination period to 21% (95% CI: 17 - 25) post-vaccination[215]. The introduction of the RV vaccine in South Korea was also reported to decrease the nation's economic burden from $17.3 million in 2009 to $9.6 million 2012[216]. Finally, using a decision-analytic model to strongly support the WHO recommendation for the introduction of the RV vaccines in countries with high <5 mortality rates and limited health resources, Atherly and colleagues estimated that RV vaccination would prevent 2.46 million childhood deaths and 83 million

disability-adjusted life years from 2011 to 2030, with annual reductions of 180,000 childhood deaths at peak vaccine uptake[217].

Rotavirus and autoimmunity

Autoimmunity develops because of a break in tolerance to self Ags by the host's immune system. Sex, age, genetics, immune regulation, and environmental factors contribute to the development of autoimmune responses. Viruses and bacteria are considered to be the main environmental triggers and mechanisms that include molecular mimicry, bystander activation, epitope spreading and cryptic Ags have been proposed to explain the breakdown of self-tolerance by pathogens[218,219]. Over the last few years, the role of RV infections as potential triggers for autoimmune diseases has been a focus of interest with special attention paid to celiac disease and type 1 diabetes (T1D)[220–222].

A high frequency of RV infections was reported to positively correlate with the increased risk of celiac disease in genetically predisposed individuals[223,224]. In addition, vaccination against the RV infection prevented/reduced the prevalence of celiac disease in children[221,222]. Studies in animals and humans have shown that RV infections trigger T1D through molecular mimicry to the Glutamic Acid Decarboxylase (GAD) and tyrosine phosphate IA-2 Ags[219,225,226]. Studies in Finland, which has the highest incidence of T1D worldwide, showed that vaccination against RV does not significantly affect the onset, increase or decrease of T1D in children and adoloscents[221,222]. However, these findings were not corroborated in a recent study by Rogers et al that showed that RV vaccination reduced the incidence of T1D in children aged 0 - 4 years in the United States of America between 2001 - 2017[227].

Alterations in the intestinal microbiome composition are greatly implicated in the pathogenesis of inflammatory bowel disease (IBD). In a study using the metagenomic DNA sequencing of fecal samples obtained from IBD patients (both Crohn's disease and ulcerative colitis), Norman et al. observed an abnormal enteric virome in these patients compared to the healthy controls – a contributing factor to the incidence of IBD[228]. Signaling via the mitochondrial antiviral protein (MAVs) was shown to protect mice from experimental colitis[229]. In agreement with these observations, a study by Yang and colleagues showed that mice administered with inactivated RV were protected from colitis while the pre-treatment of mice with antivirals resulted in severe colitis[230].

In summary, different studies have documented a potential role of RV vaccination and/or infection in affecting the prevalence and/or incidence of autoimmune diseases and IBD, but further mechanistic investigations are still required in this field.

Present investigation

Aims of the thesis

The overall aim of this thesis work was to investigate the requirements as well as the location for the induction of optimal adaptive immune responses towards the enteric oral primary Rotavirus infection.

Specifically, the aims of the included studies were:

I. To investigate the cellular and molecular requirements for the induction of optimal Rotavirus-specific IgA antibody responses during primary Rotavirus infection.

II. To investigate the location and the role of various mediators in driving lymphoid organ hypertrophy in the context of oral Rotavirus infection in adult mice

III. To assess the impact of retinoic acid signaling on $CD8^+$ T cell development, phenotype, and function

IV. To investigate the molecular requirements and differences between cDC1 and cDC2 activation and migration from the SiLP to the mLNs in response to the TLR3 agonist, poly(I:C)

Summary and discussion of the papers

Paper 1

αvβ8 integrin-expression by BATF3-dependent dendritic cells facilitates early IgA responses to Rotavirus

Intestinal RV-specific secretory IgA is the major correlate for long-term protection against natural RV infection. IgA can be generated by TD and TI pathways, both facilitated by intestinal cDC and the cytokine TGFβ. The role of cDC in facilitating the induction of steady-state IgA against the commensal microbes has been studied, but very little is known about the mechanisms that induce IgA during intestinal viral infections and the division of labour between the different cDC subsets for the induction of B cell responses. The aim of this study was therefore to investigate the cellular and molecular requirements for the induction of optimal RV-specific IgA antibody responses during primary RV infection.

Key findings

- Batf3-dependent cDC1 but not cDC2 are required for the optimal induction of anti-RV-specific IgA responses in the mLNs.
- Generation of IgA^+ B cell responses in the mLNs requires $CD4^+$ T cells but not $CD8^+$ T cells.
- Signaling via the type I interferon receptor either on all dendritic cells or specifically on cDC1 is dispensable for the induction of B cell responses during RV infection.
- β8 expression is preferentially confined to the migratory cDC1 in the mLNs and this expression pattern is conserved during RV infection.
- αvβ8 integrin expression by the cDC1 is dispensable for the generation of steady-state mucosal immune responses but is essential for the optimal induction of RV-specific IgA responses.

Discussion

In this study, we show for the first time that cDC1 facilitate the generation of IgA^+ B cell responses during primary RV infection while cDC2 are dispensable. These data further suggest that IgA class switching is not restricted to the cDC2 compartment as has been previously discussed[17,59] but rather depends on the context and nature of the Ag. We also observed that the mLNs are the major inductive site for the initiation of RV-specific IgA immune responses. This is in agreement with other studies by Li et al[231] and is discussed in more detail in paper 2 in this thesis. RV primarily infects the villus ECs of the small intestine and cDC1 have a unique ability of presenting epithelial-derived Ags to $CD8^+$ T cells in the mLNs[29]. Analysis of cDC1-deficient *Batf3*^{KO} mice confirmed that optimal anti-RV specific IgA responses depend on the presence of cDC1 in the mLNs, which correlated with a marked delay of secretory RV-specific IgA increases in the fecal samples of these mice.

Furthermore, we explored the various mechanisms involved in the cDC1 induction of optimal anti-RV specific IgA responses in the mLNs. Even though cDC can facilitate IgA production via both TD and TI pathways[122,232], we found that deletion of the $CD4^+$ T cells severely ablated both the total and RV-specific IgA cells in the mLNs. In contrast, the deletion of $CD8^+$ T cells did not affect the B cell response. These results further confirmed that the IgA anti-RV response heavily depends on T cell help[47,205,206] and that the decreased $CD8^+$ T cell responses seen in the absence of cDC1 have no secondary effect on the IgA class switching in this model. Using CD11c.cre - and XCR1.cre -$IFNAR^{flox}$ mice, we found that signaling via the type I IFN receptor on either all cDCs or only cDC1 was dispensable for the induction of anti-RV IgA immune responses in the mLNs and RV clearance in mice. While previous evidence demonstrated that stimulation of DCs with type I IFNs enhances humoral immunity[233], type I IFN does not seem to exert its effects through cDCs in our model. Similar results were previously observed in the case of Norovirus, another enteric viral infection[234].

IgA CSR requires the TGFβ cytokine[235,236]. In this study, we generated a novel tdTomato fluorescent β8 reporter mouse model to assess the expression of the TGFβ-activating αvβ8 integrin by cDCs. Analysis of the cDC subsets in these mice confirmed that the β8-expression is confined to the migratory cDC1 in the mLNs. This expression pattern was conserved during RV infection. Interestingly, deletion of the integrin only in cDC1 significantly reduced the total and RV-specific IgA plasmablast numbers in the mLNs during RV infection but had no effects on the steady-state intestinal immune homeostasis.

Taken together, these results show that BATF3-dependent cDC1 are essential for the induction of optimal TD-anti-RV IgA immune responses in the mLNs during the primary RV infection in adult mice.

Paper 2

Rotavirus infection causes mesenteric lymph node hypertrophy independently of type I interferon or TNFα in mice

Lymphoid organ hypertrophy is a central component of immune responses to inflammation and local infection. It is associated with alterations in lymphocyte circulation between the blood and the secondary lymphoid organs and is essential for the efficient induction of adaptive immune responses[155,159,160,237,238]. However, the mechanisms involved in driving the accumulation of lymphocytes in various lymphoid organs during inflammation and/or infection are still poorly defined. In this study, we sought to investigate the location and the role of various mediators in driving lymphoid organ hypertrophy in the context of oral Rotavirus infection of the intestine in adult mice.

Key findings

- The RV infection-induced hypertrophy is primarily confined to the mLNs and results from the accumulation of all major lymphocyte populations.
- Lymphocyte accumulation during RV infection in the mLNs does not require Ag-specific activation.
- RV-induced mLN hypertrophy is a consequence of both increased lymphocyte recruitment and their enhanced retention within the mLNs without substantial local lymphocyte proliferation.
- The enhanced lymphocyte sequestration in the mLNs in response to RV is independent of type I IFN and TNFα.

Discussion

Prior to the start of this work, we[47] (paper 1) and others[239] had shown that oral RV infection induces a transient ~3-fold increase of B lymphocytes in the mLNs of adult mice. As a follow-up study, we used C57BL/6 mice to analyse the location and kinetics of the hypertrophy of lymphoid organs following oral RV infection of the intestine. We confirmed that RV-induced hypertrophy was confined to the gut draining mLNs with a very small effect in the PPs and no response in the more distal lymphoid tissues including the spleen. Further analysis of the lymphocyte populations revealed that all major populations efficiently accumulated (both naïve and Ag-experienced/effector cells) in the mLNs following oral RV infection.

Lymphoid organ hypertrophy can result from increased recruitment of naïve lymphocytes into the organ, from robust activated-lymphocyte proliferation within the organ, or from the halted egress of the lymphocytes from the lymphoid organ[166,171,237,240–242]. Here, using congenically labelled wildtype mice, we found that RV infection leads to increased retention and recruitment of lymphocytes into the mLNs without proliferation of the activated Ag-specific lymphocytes substantially contributing to the overall mLN cellularity. These results are in part in agreement with a study by Schulz et al. that reported that PPs hypertrophy upon *Salmonella* infection was primarily due to lymphocyte retention, while proliferation and recruitment were not contributing to the PPs cellularity to a measurable extent[159].

In this study, we also demonstrated that lymphocyte sequestration within the mLNs during RV infection is independent of the IFNAR-CD69-S1PR1 pathway. Engagement of the Ag-receptor on lymphocytes leads to the downregulation of S1PR1/3[243]. Analysis of RV-infected SW_{HEL} mice (a B-cell receptor-specific mouse model that contains B cells specific for hen egg lysozyme) revealed that Ag-specific recognition is dispensable for B cell accumulation in the mLNs. Further, RV-induced mLN hypertrophy did not require signaling via the IFNAR despite an observed reduction of CD69 expression by the lymphocytes in IFNAR-deficient mice. TNFα and signaling via the TNF receptors1/2 were also dispensable for the hypertrophy of mLNs during RV infection.

Collectively, these results show that lymphoid organ hypertrophy in the context of oral RV infection is primarily confined to the mLNs and that Ag-specific recognition, type I IFN and TNFα are not required to coordinate the events involved in the mLN response.

Paper 3

Retinoic acid signaling affects thymic and peripheral CD8 T cell phenotype and function

Retinoic acid (RA), a vitamin A metabolite, has been shown to play a role in controlling T cell responses in the periphery, for example by increasing the generation of peripheral FoxP3$^+$ Tregs[145,244] as well as the differentiation and lineage stability of CD4$^+$ T helper cells[245] and the induction of gut-homing receptors on T and B cells[141,143,144]. Its role in CD8$^+$ T cell development is however poorly defined. We used *CD4Cre.dnRAR$^{lsl/lsl}$* mice, in which RA signaling in developing thymocytes and peripheral T cells is abrogated to assess the impact of RA signaling on CD8$^+$ T cell development, phenotype, and function. As a relevant in vivo readout, we assessed the CD8$^+$ T cell response to intestinal RV infection.

Key findings

- The absence of RA signaling in developing thymocytes leads to perturbed thymopoiesis.
- RA signaling-impaired naive CD8$^+$ T cells display enhanced survival and expansion upon TCR stimulation.
- RA signaling regulates gene expression of key effector genes in a similar manner in splenic and mLN primed CD8$^+$ T cells.
- RA signaling is required for the cytotoxic activity of CD8$^+$ T cells.

Discussion

Phenotypic analysis of the developing thymocytes revealed that T cell development in *CD4Cre.dnRAR$^{lsl/lsl}$* mice was skewed towards more CD8SP thymocytes and that the majority of these cells were of a CD24^{lo}CD62L^{hi} mature phenotype[246]. Furthermore, these CD24^{lo}CD62L^{hi} CD8SP cells displayed a CD44^{hi}CD122^{hi} phenotype that was earlier described for cells described as "virtual memory CD8$^+$ T cells"[247]. A study by Miller et al showed that the transcription factor EOMES was upregulated in CD8-memory phenotype T cell precursors during their maturation in the thymus[247]. In line with this, EOMES-expressing cells were substantially enriched within the CD24^{lo}CD62L^{hi} CD8SP compartment of the *CD4Cre.dnRAR$^{lsl/lsl}$* mice. Further, we found that abrogated RA signaling in peripheral CD8$^+$ T cells led to reduced gene expression of key effector genes such as several granzyme family members (GZMA, GZMB and GZMK). Indeed, the

cytotoxic activity was significantly reduced in the *CD4Cre.dnRAR*$^{lsl/lsl}$ mice immunized with OVA peptide. Finally, to assess the role of RA signaling in the generation of effector CD8$^+$ T cells in the context of a natural enteric virus, *CD4Cre.dnRAR*$^{lsl/lsl}$ mice were orally infected with RV. CD8$^+$ T cells mediate clearance of the primary RV infection[206,207]. Despite the similar numbers of RV-specific tetramer$^+$ cells in the mLNs, *CD4Cre.dnRAR*$^{lsl/lsl}$ mice had significantly lower numbers of RV-specific tetramer$^+$ cells in small intestinal epithelium and lamina propria. This was expected based on the known role of RA to induce gut-homing receptors on effector lymphocytes in the mLNs. Importantly, CD8$^+$ effector cells in the mLNs of *CD4Cre.dnRAR*$^{lsl/lsl}$ mice showed reduced expression of granzyme A (GzmA) suggesting that the impaired cytotoxic activity of the effector CD8$^+$ T cells in the absence of RA is likely due to impaired production of granzymes. As expected, the *CD4Cre.dnRAR*$^{lsl/lsl}$ mice showed delayed clearance of the virus compared to the wildtypes.

Collectively, these results show RA plays a role in CD8SP thymocyte homeostasis in the thymus and in the acquisition of cytotoxic activity by the peripheral CD8$^+$ T cells through effector gene regulation.

Paper 4

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

One hallmark of intestinal cDC function is their ability to migrate from the SiLP to the draining mLNs in response to stimulation via PRR, where they induce adaptive immune responses. Intestinal cDC subsets differ in their expression of different PRR, for example, TLR3 is specifically expressed by cDC1 while cDC2 uniquely express TLR5[90,248]. This suggests that cDC subsets can recognise different Ags, thus fulfilling different immune functions, possibly allowing for cDC subset-specific targeting in vaccination. Hence, we sought to investigate the molecular requirements and differences between cDC1 and cDC2 activation and migration from the SiLP to the mLNs in response to the TLR3 agonist, poly(I:C).

Key findings

- Poly(I:C)-induced intestinal cDC migration depends on TLR3 signaling.
- Cell-intrinsic TLR3-sensing is dispensable for cDC migration.
- cDC migration in response to poly(I:C) is independent of MyD88 but requires TNF-receptor signaling.
- cDC subsets differ in type I IFN signaling requirements in response to poly(I:C).

Discussion

In this study, we analysed the molecular requirements for the activation and migration of intestinal cDC subsets in response to poly(I:C), a synthetic analog of dsRNA. Poly(I:C) is a good model for studying enteric viral infections since it mimics dsRNA viruses such as RV. We found that poly(I:C) induced activation and migration of both cDC1 and cDC2 from the SiLP to the mLNs in a strictly TLR3-dependent manner despite cDC2 expressing virtually no TLR3 themselves. Poly(I:C) induced upregulated expression of type I interferons and TNFα in the SiLP. Examination of the role of these cytokines in poly(I:C)-induced cDC migration revealed that migration of both cDC1 and cDC2 was dependent on TNFα while type I interferon signaling induced activation and migration was more essential for cDC1 as compared to cDC2. Taken together, our findings reveal common and differing pathways in regulating cDC subset migration in response to poly(I:C), a TLR3-targeting adjuvant.

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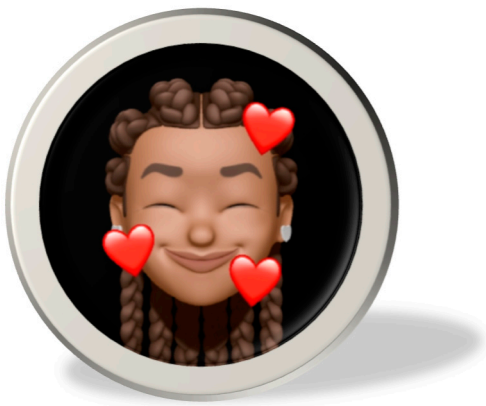

Then Samuel took a stone and set it up between Mizpah and Shen, and called its name ***Ebenezer,*** *saying,* ***"Thus far the LORD has helped us."*** 1 Samuel 7:12.

Great is THY faithfullness.

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