The role of intestinal dendritic cells and the microbiota during oral *Salmonella* infection

María Fernández

Department of Microbiology and Immunology
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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maria.fernandez@gu.se

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ABSTRACT

The intestinal pathogen *Salmonella* causes millions of infections per year worldwide. The immune response to these bacteria involves interactions between several cell types via specific molecules and is under the influence of the intestinal microbiota.

Dendritic cells (DC) initiate immune responses including those to *Salmonella*. Toll-like receptors and CD40 can act synergistically on DC activation but their cooperativity during bacterial infection had not been addressed. *Salmonella*-infected mice lacking MyD88, CD40 or both (DKO) showed that synergistic effects of CD40 and MyD88 do not influence host survival, bacterial burden in intestinal tissues or serum levels of IFN-γ and IL-10 during infection. However, cooperativity between CD40 and MyD88 influenced IL-10 production in DC-T cell co-cultures using killed *Salmonella* as the antigen. Moreover, cooperative effects of CD40 and MyD88 on T cell effector functions such as proliferation and IFN-γ production were influenced by the complexity of the antigen.

Although some studies had addressed the role of DC subsets in infection, the influence of the $CD103^+CD11b^+$ DC in *Salmonella* infection was unknown. Studies using mice with a reduced $CD103^+CD11b^+$ DC population in mesenteric lymph nodes (MLN) and small intestine lamina propria showed no alterations in *Salmonella* colonization of intestinal tissues or spleen. Moreover, mechanisms important in host survival to *Salmonella* infection such as IFN-γ production analyzed by flow cytometry and antibody production analyzed by ELISA were not altered. This suggests that the absence of $CD103^+CD11b^+$ DC has a limited effect on the host response to *Salmonella* infection.

Interactions between *Salmonella* and the microbiota at an early phase of colonization have been reported, but the role of the microbiota later during infection was poorly understood. *Salmonella*-infected germ-free (GF) and antibiotic treated mice (ABX) revealed a higher bacterial burden in the MLN, which seems to be due to increased intestinal bacterial translocation to MLN caused by the lack of the microbiota. Furthermore, higher IFN-γ in MLN of GF and ABX relative to controls was detected by flow cytometry despite similar IL-12 levels six days post infection. While the higher IFN-γ in MLN of ABX mice correlated to the severity of infection, a lack of immune signals provided by the microbiota from birth may influence IFN-γ production in GF mice.

These studies provide further information about the role of DC and the microbiota during *Salmonella* infection, which could be used for the generation of vaccines or treatments for this infection.

Keywords: *Salmonella*, dendritic cells, NF-κB, MyD88, CD40, CD103, IRF4, microbiota

SAMMANFATTNING PÅ SVENSKA

Salmonella orsakar varje år miljontals infektioner. Immunsvaret mot denna bakterie involverar interaktioner mellan flera olika typer av immunceller däribland dendritiska celler och T-celler. Även de bakterier som normalt finns i våra tarmar – tarmfloran – har visats påverka hur vi kan bli infekterade av patogener så som *Salmonella*.

Dendritiska celler (DC) är viktiga för att starta ett effektivt immunsvar, inklusive det mot *Salmonella*. Det har visats att Toll-like receptors (TLR) kan verka synergistiskt med CD40 för att aktivera DC, men dessa molekylers roll vid en bakteriell infektion hade ännu inte belysts. Genom att använda möss som saknar MyD88, CD40 eller båda (DKO) visar vi att dessa synergistiska effekter inte påverkar mössens överlevnad, antalet *Salmonella* som återfinns i tarmen eller serumnivåer av IFN-γ och IL-10 efter infektion. Däremot påverkade CD40 och MyD88 DC förmåga att aktivera T celler eftersom DC från DKO inducerade mindre delning av T-celler än MyD88$^{-/-}$ DC. Genom att använda olika antigen (proteiner, peptider eller bakterier) i samodlingarna kunde vi påvisa att delningen av T-celler och produktion av IL-10 påverkas av antigenets komplexitet.

Den roll olika subgrupper av DC spelar vid infektioner har tidigare studerats men betydelsen av CD103^{+}CD11b^{+} DC vid en *Salmonella*-infektion har dock hittills inte studerats utförligt. Vi visar att i möss med en minskad mängd av denna DC subgrupp i tarmdränerande lymfnoder (MLN) inte får fler *Salmonella* i tarmvävnad eller mjälte efter infektion. Inte heller mekanismer viktiga för att överleva en *Salmonella*-infektion såsom IFN-γ och antikroppsproduktion är försvagade. Detta tyder på att CD103^{+}CD11b^{+} DC spelar en mindre roll vid en *Salmonella* infektion.

Tidigare studier belyser betydelsen av tarmfloran i ett tidigt skede av en *Salmonella* infektion, men dess roll under den senare delen av infektionen är till stor del okänd. Våra försök med möss, som från födseln saknar tarmflora (GF) och antibiotika behandlade möss (ABX) visar ett högre antal *Salmonella* bakterier i deras MLN jämfört med kontroll möss med normal tarmflora från födseln. Detta tycks bero på ökad bakteriell translokation från tarm till MLN i djur som saknar tarmflora. Vidare observerades en högre IL-12-oberoende IFN-γ produktion i MLN av GF och ABX. Även om det i ABX möss tycks vara en påföljd av infektionens svårighetsgrad, verkar ytterligare effekter av bristen på immunsignaler som annars ges av tarmfloran från födseln, påverka IFN-γ produktion i GF-möss.

Dessa studier ger ytterligare information om den roll som DC och tarmfloran har vid en *Salmonella* infektion, som skulle kunna användas för att skapa nya vaccin mot eller behandlingar av denna infektion.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Wenzel UA, Fernández-Santoscoy M, Tam MA, Tegtmeyer P and Wick MJ.
Synergy between CD40 and MyD88 does not influence host survival to *Salmonella* infection.
Front Immunol (2015) **6**:460.

II. Fernández-Santoscoy M, Wenzel UA, Yrlid U, Cardell S, Bäckhed F and Wick MJ.
The normal gut microbiota reduces colonization of the mesenteric lymph nodes and IL-12-independent IFN-γ production during *Salmonella* infection.
Submitted

III. Fernández-Santoscoy M, Wenzel UA, Yrlid U, Persson EK, Agace WW and Wick MJ.
The influence of intestinal CD103$^+$CD11b$^+$ dendritic cells on oral *Salmonella* infection.
Manuscript

Reprints were made according to the journal requirements

ABBREVIATIONS

NK	Natural killer
DC	Dendritic cells
APC	Antigen-presenting cells
MDP	Monocyte/DC precursor
CDP	Common DC precursor
Flt3L	Fms-like tyrosine kinase 3 ligand
pDC	Plasmacytoid DC
pre-DC	DC progenitors
cDC	Conventional DC
MHC	Major histocompatibility complex
PRR	Pathogen recognition receptors
PAMP	Pathogen associated molecular patterns
TLR	Toll-like receptors
MyD88	Myeloid differentiation factor 88
CCR7	Chemokine receptor 7
IFN	Interferon
LN	Lymph nodes
NF-κB	Nuclear factor κB
NOS	Nitric oxide synthase
LP	Lamina propria
PP	Peyer's patches
MLN	Mesenteric lymph nodes
siLP	Small intestine lamina propria
TGF-β	Transforming growth factor β
CX_3CR1	CX3 chemokine receptor 1
Ig	Immunoglobulin
Tregs	Regulatory T cells
GF	Germ-free
IEL	Intraepithelial lymphocytes
S. typhimurium	*Salmonella enterica* Serovar typhimurium
TTSS	Type 3 secretion system
SPI	*Salmonella* pathogenicity island
NADPH	Nicotinamide adenine dinucleotide phosphate
SCV	*Salmonella*-containing vacuole
OVA	Ovalbumin
WT	Wildtype
CONV-R	Conventionally raised mice
CFU	Colony forming units
ELISA	Enzyme-linked immunosorbant assay
RT-PCR	Real time polymerase chain reaction
DKO	Double knockout

1 INTRODUCTION

The ability to survive infection with a pathogen relies on both physical barriers to keep microbial invaders from penetrating into the body and on the function of a number of cells that fight the pathogen once it enters the body. The term *immunity* refers to the resistance to infection while the hosts' defense mechanisms against infection are called immune responses. There are two types of immune responses: innate and adaptive. While innate immunity provides a rapid non-specific response to invading microorganisms, adaptive immunity is antigen-specific and develops memory, which provides long-lasting protection against a second encounter with the same antigen.

The cells of the innate and adaptive arms of the immune system have specific yet overlapping roles to ensure host survival to infection. Macrophages and neutrophils are phagocytic cells whose main function is to take up and kill microorganisms. Natural killer (NK) cells contribute to innate immune responses with the production of cytokines, which are soluble proteins that regulate the function of other cells. Dendritic cells (DC) share a common precursor with macrophages and are also phagocytic, although their main function is to initiate adaptive immune responses through the activation of naïve T cells. In contrast to these cell types, which belong to the innate immune system, T and B cells are part of the adaptive immune system. T cells have either effector functions that facilitate clearance of pathogens or tolerogenic responses against the hosts' own proteins. And finally, B cells play an important role in the defense against pathogens through the production of antibodies.

Much progress has been made in the understanding of the general functions of DC and the microbiota in immunological processes. However, their specific role in the context of an infection with a bacterial pathogen is not completely understood. Therefore, the main purpose of this thesis was to investigate the impact of DC and the intestinal microbiota on *Salmonella* infection.

1.1 Dendritic cells

DC are a member of the family of hematopoietic cells with the ability to present antigens to T cells, so-called antigen-presenting cells (APC). Despite that DC are not necessarily the most abundant APC in tissues, their special niche comes from their unique ability to prime naïve T cells and thus initiate adaptive immune responses. Importantly, DC are the APC with the capacity to migrate from peripheral tissues to draining lymph nodes, which is a critical feature of their ability to prime naïve T cells. They also have an important role as mediators of tolerance, thus helping avoid undesired reactions to i.e. self antigens and food proteins (1-3). Due to their unique ability to prime naïve T cells, DC constitute the link between innate and adaptive immunity (4,5). DC were first described by Steinman and Cohn in the early seventies (6) for which Steinman was awarded the Nobel Prize in Physiology or Medicine in 2011.

Origin and classification

Monocytes/macrophages and DC originate from a common bone marrow progenitor called monocyte/DC precursor (MDP). MDP give rise to monocytes and common DC precursors (CDP). CDP, through the growth factor Fms-like tyrosine kinase 3 ligand (Flt3L), give rise to plasmacytoid DC (pDC) and circulating DC progenitors (pre-DC) that finally differentiate into conventional DC (cDC) (Figure 1)(7,8).

DC are usually identified in the different organs by their coexpression of CD11c and major histocompatibility complex II (MHC-II). However, macrophages present in peripheral tissues also express these proteins (9,10), making it more challenging to separate macrophages and DC by the sole use of these surface proteins. This problem is overcome by the identification of macrophages through other molecules that DC do not express. For instance, it has been shown that the expression of CD64 distinguishes macrophages from DC (11). In addition, bona fide

DC lack expression of the macrophage marker F4/80 (12). Moreover, macrophages express CX_3CR1 at higher levels than DC (13,14).

It has been suggested that classification of DC according to their ontogeny is an accurate criteria conserved across tissues and species (15), therefore they are usually classified into pDC and cDC. pDC are best characterized for their role in viral infections (16) and were not studied in this thesis.

Conventional DC

These cells are also known as classical DC because they are experts in performing the classical DC function, which is the initiation of adaptive immune responses against foreign antigens or the induction of tolerance to self-antigens. cDC achieve this goal through their advanced antigen presentation machinery and their remarkable capacity to migrate to the lymph nodes and activate T cell responses (17). cDC classification into subsets is generally based in the expression of CD8α or CD103 and CD11b (Figure 1)(18,19).

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

CD8 expression by DC was first detected in the spleen and thymus (20). However, the presence of $CD8\alpha^+$ cDC is not restricted to lymphoid organs. In fact, there is a population of CD103 (integrin $\alpha_E\beta_7$)-expressing cDC in peripheral tissues that do not express CD11b and share similar functions and developmental requirements with the $CD8\alpha^+$ cDC (21). The development of both $CD103^+CD11b^-$ and $CD8\alpha^+$ DC depends on the transcription factors BATF3, Id2, IRF8 and NFIL3 (22-25). A key feature of these populations of cDC is their ability to efficiently cross-present antigens to $CD8^+$ T cells (26). In addition, it has been shown that these DC preferably induce a Th1 response that depends on the production of IL-12 (27,28). It has recently been suggested that XCR1 can be used as a universal marker for cross-presenting murine DC regardless of their expression of CD8 and CD103 (29).

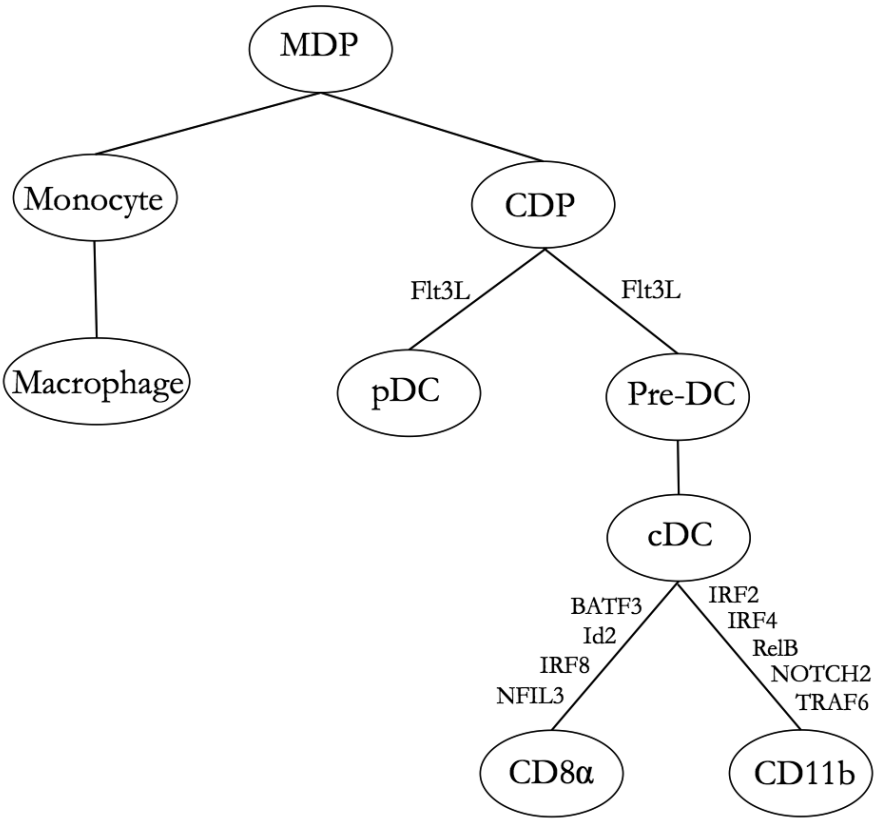

Figure 1. DC ontogeny. The names given downstream of cDC are transcription factors described to be involved in the development of the different lineages.

CD11b⁺ cDC

This population is less characterized compared to CD8α$^+$ cDC and it is also more heterogeneous. CD11b$^+$ DC express high levels of SIRPα (30). Transcription factors that have been demonstrated to be necessary for the development of CD11b$^+$ DC include IRF2, IRF4, RelB, and NOTCH2 (19) as well as TRAF6 (31). In contrast to CD8α$^+$ DC, CD11b$^+$ DC preferentially prime CD4$^+$ T cells (32-34).

DC as initiators of adaptive immunity

Pathogen recognition

Cells involved in innate immune responses, including DC, are equipped with pattern recognition receptors (PRR). PRR recognize molecular patterns expressed by pathogenic microorganisms, which are commonly known as pathogen-associated molecular patterns (PAMP). PRR in mammals include Toll-like receptors (TLR), RIG-I-like receptors, NOD-like receptors, AIM2-like receptors, C-type lectin receptors and intracellular DNA sensors (35). The best characterized family of PRR is TLR which are thought to have originated 700 million years ago (36). TLR have an extracellular N-terminal ligand recognition domain, a single transmembrane helix and a C-terminal cytoplasmic signaling domain (37). These signaling domains are called TIR and interact with different adaptor proteins such as the myeloid differentiation factor 88 (MyD88), TIRAP, TRIF, TRAM and SARM (38,39). All TLRs associate with MyD88 with the exception of TLR3, which associates with TRIF (40). In addition to the MyD88-dependent signaling pathway downstream of TLR4, this receptor can associate with TRAM and TRIF instead of MyD88 (41), which provides a MyD88-independent signaling pathway downstream of TLR4 (Figure 2). Thus far 12 TLRs have been identified in mice (TLR1-9, TLR11-13). Ligation of TLRs with PAMP activates signaling cascades that lead to transcription of genes involved in antimicrobial host defense; the use of different adaptor proteins triggers different signaling cascades (42).

DC maturation and migration

Antigen-PRR ligation on DC leads to a process in which the cell undergoes phenotypic and functional changes. This process is known as DC maturation and is necessary for the transformation of naïve DC into powerful APC capable of activating adaptive immune responses. There are three signals that DC deliver to naïve T cells in order to activate them: antigen loaded in MHC molecules, costimulation and cytokines (43). During DC maturation, DC endocytic capacity is enhanced and then eventually down-regulated

(44). In addition, DC lysosomal proteolysis is also enhanced allowing antigen processing (45). Furthermore, DC upregulate the costimulatory molecules CD80 and CD86 that trigger CD28 in T cells (46). Finally, DC produce cytokines that instruct T cell differentiation.

Mature DC are generally identified by their high expression of MHC-II, the costimulatory molecules CD80, CD86 and CD40 as well as the chemokine receptor 7 (CCR7). However, a DC with a mature phenotype is not necessarily immunogenic and can instead induce tolerance (47).

DC can also undergo a maturation process that is independent of PRR ligation and that is known as indirect maturation. In this process, pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and type I interferons (IFN) produced by hematopoietic cells induce DC maturation. (48-50). However, indirectly activated DC are poorly immunogenic due to their inability to produce inflammatory cytokines (51). In addition, it has been shown that indirectly activated DC induce T cell clonal expansion but not direct Th1 cell differentiation (52).

In order to prime T cells, DC must reach the T cell zones of lymphoid organs. Upon receiving maturation signals, DC migrate via lymph to draining lymph nodes (LN). As mentioned above, mature DC are characterized by upregulation of CCR7, which mediates DC migration to secondary lymphoid organs. Studies performed in CCR7-deficient mice showed an impaired ability of DC to migrate to LN (53). However, the sole expression of CCR7 is not sufficient for DC migration. Studies have shown that additional signals such as cysteinyl leukotrienes, prostaglandin E_2 and CD38 are necessary for CCR7 binding to its ligands CCL19 and CCL21 (54). On the other hand, factors that negatively regulate DC migration include platelet-activating factor and adenosine (55).

The canonical and non-canonical NF-κB signaling pathways

The nuclear factor -κB (NF-κB) family of transcription factors is involved in the regulation of many immunological processes such as the production of proinflammatory cytokines, chemokines and other proteins including nitric oxide synthase (NOS) and MHC molecules (56). There are 5 NF-κB members in mammals: RelA (also called p65), RelB, c-Rel, NF-κB1 p50 and NF-κB2 p52 (57). NF-κB transcription factors are activated through signaling pathways that are dependent or independent of the adaptor protein MyD88 (Figure 2).

In the MyD88-dependent signaling pathway, MyD88 recruits IRAK1, IRAK2 and IRAK4. The IRAK proteins become phosphorylated and then associate with TRAF6. Later on TRAF6 becomes polyubiquitinated and activates TAK and TAK-1 proteins (TABs) that consequently phosphorylate the IKK complex and finally activate MAP kinases and NF-κB. This process, also known as the classical or canonical TIR pathway, results in the nuclear accumulation of NF-κB dimers consisting of RelA and NF-κB1 p50. This ultimately leads to the production of inflammatory cytokines and costimulatory molecules among other outcomes (39,42,58).

On the other hand, the MyD88-independent signaling or non-canonical pathway relies on the activation of the NF-κB-inducing kinase (NIK), which induces phosphorylation of IKKα. IKKα phosphorylates p100, which becomes polyubiquitinated. This signaling pathway results in the accumulation of RelB/p52 dimers in the nucleus (59) leading to, for instance, the production of IFN-inducible genes (60) and lymphoid organogenesis (56,57).

The canonical NF-κB signaling pathway responds to numerous stimuli derived from different receptors. Contrarily, the non-canonical pathway responds to specific receptors such as members of the TNF receptor superfamily (57). One example is CD40, a costimulatory molecule expressed on antigen presenting cells (APC)

like dendritic cells and B cells. Its ligand CD154 (CD40L) is expressed mainly by activated T and B cells (61). CD40/CD154 engagement triggers both the canonical and non-canonical NF-κB pathways (62) that have been shown to act synergistically in DC. For instance, there is evidence suggesting that the non-canonical NF-κB pathway activated by CD40 signaling is involved in DC survival mechanisms as well as cross-presentation of antigen to $CD8^+$ T cells (63). Some studies have investigated synergistic signals in DC that drive T cells responses using TLR-agonists or bacterial or parasite extracts (64,65). Using α-galactoceramide as an antigen, another study showed that DC upregulate CD80 and CD86 independently of CD40 ligation. However, regardless of their mature phenotype, DC require CD40 ligation to induce T cell responses (66).

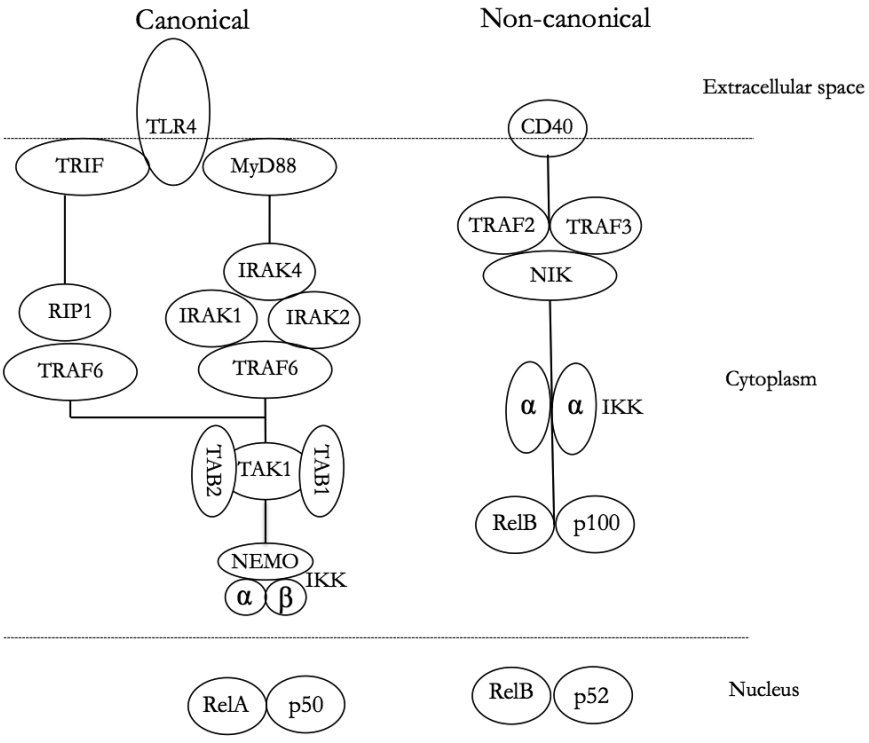

Figure 2. Canonical and non-canonical NF-κB activation pathways

Intestinal DC

The intestine is constantly exposed to numerous antigens and microbial populations and the intestinal immune system has the crucial task of distinguishing, for example, pathogenic microorganisms from beneficial ones such as the intestinal microbiota. Intestinal DC play a major role in this task by activating defense mechanisms against invading pathogens or inducing tolerance to self-proteins (30,67,68). In intestinal tissue, DC are located in intestinal lamina propria (LP), Peyer's patches (PP), isolated lymphoid follicles and mesenteric lymph nodes (MLN).

Subsets of Intestinal DC

Intestinal DC can be classified into subsets according to their expression of the integrin CD103. Intestinal $CD103^+$ DC have been described in small intestine LP (siLP), colonic LP, intestinal lymph, PP and MLN (13,69). A remarkable function of this subset is the preferential ability to drive $Foxp3^+$ regulatory T cell (Tregs) differentiation via the transforming growth factor β (TGF-β) and retinoic acid, where DC can generate the latter from dietary vitamin A (70,71). Nonetheless, $CD103^+$ DC can also drive effector T cell differentiation (13,69,72). Another key feature is that intestinal $CD103^+$ DC promote intestinal T cell homing. For instance, they induce the upregulation of CCR9 in $CD8^+$ T cells, which gives them the capacity to migrate to the small intestine (73,74).

$CD103^+$ intestinal DC can be further classified according to expression of CD11b and their localization, developmental requirements and function (30,75). $CD103^+CD11b^-$ DC express high levels of CD8α and are the most abundant subset in colonic LP (76). Their development depends on the transcription factors BATF3, IRF8 and Id2 (30). Phenotypically, these cells display prominent "spiny" dendrites and function-wise, have been shown to induce IFN-γ production by T cells (77,78).

$CD103^+CD11b^+$ DC are the major subset in the siLP (23,75) and their development is mediated by the transcription factors IRF4 and

NOTCH2 (34,79). Compared to the $CD103^+CD11b^-$ DC, $CD103^+CD11b^+$ DC display shorter and more evenly-spaced protrusions (77). These cells have been shown to drive Th17 cell differentiation through the production of IL-6 (79). In response to flagellin, $CD103^+CD11b^+$ DC are an important source of IL-23 (80). In addition, production of this cytokine by $CD103^+CD11b^+$ DC was shown to be important in the resolution of and infection with *Citrobacter rodentium* (81). Moreover, a study showed that $CD103^+CD11b^+$ stimulated by flagellin induce the development of immunoglobulin A (IgA)-producing cells (82).

On the other hand, the $CD103^-$ intestinal DC subset has not been studied in detail and the transcription factors involved in their development are yet to be defined (30). Recent studies have shown that these cells are indeed *bona fide* DC as their development is regulated by Flt3L and they express the DC-specific transcription factor Zbtb46 (12,77). $CD103^-$ DC can present antigen and activate $CD4^+$ T cells and are also capable of cross-presenting antigen to $CD8^+$ T cells (77). These DC are also classified according to CD11b expression. Both $CD103^-$ DC subsets induce differentiation of IL-17-producing T cells; however only the $CD103^-CD11b^+$ DC induce differentiation of IFN-γ-producing T cells (77). Moreover, a significant proportion of the $CD103^-CD11b^+$ DC express CCR2 and are involved in inducing Th17 cell differentiation (12). In addition, $CD103^+CD11b^-$ DC in the MLN regulate T cell responses to flagellin through TLR5 (83).

$CD103^-$ DC can be further classified according to their expression of the CX3 chemokine receptor 1 (CX_3CR1) giving rise to two populations, one that expresses CD11b and an intermediate level of CX_3CR1 and the other that does not express CX_3CR1 or CD11b (77).

Intestinal DC migration to MLN

In the MLN there are two DC populations that express different levels of MHC-II. Resident DC that enter the MLN directly from the blood express an intermediate level of MHC-II. On the other

hand, migratory DC coming from the intestine via lymph express high levels of MHC-II (79). Intestinal DC migrate via lymph from the LP to the MLN through a CCR7-dependent process (13,73,77,84-86). DC migration occurs during both inflammation and steady-state conditions (87). DC were first identified in lymph by thoracic duct cannulation in rats whose MLN were previously surgically removed (88-90). Studies have shown that $CD103^+$ DC are reduced in MLN of CCR7 deficient mice (73). In addition, $CD103^+$ DC have been identified as the most abundant DC subset in intestinal lymph (13,77). Furthermore, a recent study showed that $CD103^-CD11b^+CX_3CR1^{int}$ DC also migrate in lymph, which distinguishes them from CX_3CR1^{high} macrophages that are unable to migrate to MLN (13,77). However, the latter is controversial as studies have suggested that after antibiotic treatment and therefore disruption of the microbiota, DC expressing CX_3CR1 migrate to the MLN, and these cells were described as either being positive or high for expression of CX_3CR1 (91,92).

1.2 Intestinal Microbiota

Composition

The microbiota is a population composed of several types of bacteria, fungi, viruses and other eukaryotic species that live in symbiosis with a multicellular organism (93). In humans the microbiota resides in skin and mucosal areas such as the upper respiratory tract, vagina and the gastrointestinal tract (94). For every cell in the human body there are ten microbes in the gastrointestinal tract, which makes it the most colonized surface, harboring 100-fold more bacteria than the skin (95). Over 99% of the composition of the human intestinal microbiota is bacterial and consists of more than a thousand species (96), most of them being obligate anaerobes (97). According to the analysis of fecal samples, the majority of the bacterial species that constitute the intestinal microbiota belong to two phyla, Bacteroidetes and Firmicutes (98).

Mammals acquire their microbiota during birth and later on through their interaction with other organisms and the environment (99). Several factors influence the composition of the microbiota throughout life including the geographic location, diet, genetics, disease and medication (100). Among these the diet has received special attention. For instance, studies have shown that a diet rich in fat and refined carbohydrates leads to a microbiota composition different from diets rich in fibers (101).

Features

Host-microbiota mutualism

The microbiota indeed contributes to host homeostasis. However, overstimulation of intestinal immune responses by the microbiota can result in inflammation in which the mutualistic relationship with the host is broken. Therefore it is important to keep it compartmentalized. There is evidence showing that signaling through TLR is needed for efficient commensal compartmentalization as commensals are found in the spleen of MyD88 and TRIF knockouts (102). Indeed, some studies have linked DC migration and intestinal microbiota. For example, studies have shown that the absence of MyD88 signaling induces goblet cell-mediated commensal translocation from the colonic lumen that is followed by commensal transport to the MLN by CX_3CR1^+ cells (91,92).

Specalized cells and molecules of the host, such as the mucus and antimicrobial peptides, restricts translocation of intestinal commensals across the intestinal epithelium with the MLN being a "mucosal firewall" that prevents further systemic bacterial penetration (103). IgA, which is mostly induced by the intestinal microbiota, is a molecule important in keeping intestinal commensals compartmentalized (104). It has been shown that, depending on the microbiota species, both low and high-affinity IgA are needed to prevent commensal invasion (105). The need to

keep the microbiota compartmentalized is extended to infection conditions. For instance, a recent study showed that the liver acts as a firewall that prevents commensals from entering the blood during intestinal infection with a pathogen (106).

Additionally, the intestinal immune system prevents unnecessary immune responses to the microbiota through processes mediated by Tregs (93,107). It has been shown that the specificity of intestinal Tregs is highly influenced by the composition of the microbiota (108). Furthermore, several microbiota species such as *Clostridia* are involved in the expansion and differentiation of Tregs (109,110). In addition, the capsular polysaccharide-A present in *Bacteroides fragilis* promotes proliferation of Tregs in a MyD88-dependent manner(111). It has been proposed that commensals induce Tregs through metabolic products such as short chain fatty acids (112). The role of the microbiota in inducing the development of Tregs is illustrated in the defective function of Tregs in the MLN of germ-free (GF) mice (113). In addition, colonization of GF mice results in the development of Tregs in the colonic LP (114).

Metabolic processes

One of the most important features of our mutualistic relationship with the microbiota is its contribution to host metabolism. For instance, it has been shown that the intestinal microbiota stimulates the production of triglycerides by the host and promotes their storage (115). In addition, the intestinal microbiota is also involved in the metabolism of secondary bile acids and inhibits the synthesis of hepatic bile acids in the liver (116). Furthermore, the microbiota plays a role in the metabolism of toxic compounds such as pyrolysates and helps in the biotransformation of drugs and their metabolites (117).

Role in development of lymphoid structures

The development of secondary lymphoid organs such as PP and MLN occurs before birth and is therefore independent of the intestinal microbiota. However, the maturation of these tissues is

related to postnatal microbial colonization (118). In addition, the development of tertiary lymphoid tissues such as isolated lymphoid follicles and cryptopatches is also induced by the microbiota (119).

Effects on host immune cells

Effector T cell responses are also affected by the microbiota. For instance, in the absence of the intestinal flora there is a reduction of the number of inducible Foxp3$^+$ T cells in the colonic LP (109). Furthermore, ATP-dependent and independent mechanisms that influence the development of Th17 cells are also dependent on the microbiota (120,121). Regarding B cells, a study showed that development of B cells in LP is influenced by microbial colonization, as less Igλ^+ B cells are found in LP of GF mice (122). Moreover, the absence of the intestinal microbiota leads to reduced numbers of IgA-producing B cells as well as immature development of germinal centers in PP (123).

In addition to the effects of the intestinal microbiota on T and B cells, it is also implicated in the regulation of LP phagocytes. For example, the microbiota promotes expression of pro-IL1β by macrophages and neutrophils and enhances IL-10 production by macrophages (107). Furthermore, microbial compounds are necessary to drive steady-state development of myeloid cell populations and the numbers of bone marrow granulocytes and monocytes correlate positively with the complexity of the microbiota (124). Finally, there is some evidence that the intestinal microbiota plays a role in the recruitment, development and activation of intraepithelial lymphocytes (IEL), specially TCRγδ IEL (125,126).

Associated pathologies

Studies have shown that the impact of the microbiota in host metabolic processes, mainly those related to fat storage, can lead to metabolic disorders such as obesity, insulin resistance and diabetes (127-131). In addition, other studies have linked some metabolites

produced by the intestinal microbiota with cardiovascular diseases such as atherosclerosis (132,133).

The use of antimicrobial products has been recently encouraged due to the importance of, or perhaps obsession with, cleanliness that exists especially in western countries. The indiscriminate and prolonged used of these products, as well as other factors, produces alterations in the composition of the gut microbiota that has been shown to have an impact in the development of allergies (134,135). Moreover, reductions in the complexity of the intestinal microbiota are associated with inflammatory bowel diseases (134,136,137). Finally, there is some evidence indicating that the dysbiosis of the microbiota leads to altered host immune responses that are linked to cancer (93).

Microbiota and infection with intestinal pathogens

Protection vs. promotion

The ability of the microbiota to inhibit colonization by invading pathogens is a phenomenon first described five decades ago and known as colonization resistance (138,139). The microbiota can inhibit pathogen outgrown through microbe-microbe interactions such as competition for nutrients and space as well as the release of bactericidal compounds called bacteriocins (140). Moreover, microbiota-derived metabolic products such as acetate and short-chain fatty acids provide protection by, for example, inhibiting pathogen growth or avoiding the absorption of toxins (141,142).

The microbiota is also involved in epithelial cell renewal, which is important for keeping the intestinal barrier intact. In addition, the microbiota plays a role in the regulation of the mucus layer, intestinal permeability as well as production, quantity and quality of antimicrobial peptides (92,143). Additional studies have also suggested that the microbiota offers protection against infection through the regulation of innate lymphoid cells (107). For instance,

a study showed that colonization of mice with commensal segmented filamentous bacterium (SFB) induces IL-17 and IL-22 production and increases resistance to the pathogen *Citrobacter rodentium* (121).

Some studies have revealed a role of the microbiota in the promotion of parasitic infections. For example, hatching of *Trichuris muris* eggs in the intestine is dependent on the microbiota (144). In addition, the microbiota can also promote viral infections as shown in studies where the microbiota enhanced the pathogenesis of poliovirus, reovirus and mouse mammary tumor virus (145,146). Regarding bacterial pathogens, a study showed that the increased levels of sialic acid induced by the microbiota promote *Clostridium difficile* expansion (147).

As mentioned earlier, alterations in the composition of the microbiota can lead to homeostasis disturbance. This also involves the promotion of intestinal infections. The inflammatory responses that the host mounts alter the composition of the microbiota and this is enhanced by pathogenic invasion (148). For instance, a study showed that the chances of invasion by a pathogen are increased with the presence of commensal species that are related to the pathogen i.e. hosts that harbor high numbers of commensal *Escherichia coli* species are more susceptible to infection with *Salmonella* (149). It has also been reported that the accumulation of commensal Enterobacteriaceae during inflammation aggravated the effects of *Toxoplasma gondii* infection (150,151). Another study showed that the parasite *Heligmosomoides polygyrus* increases the proportion of *Lactobacillus* among the microbiota and this in turn promotes the infection by the parasite (152). Finally, using the streptomycin mouse model where caecum and colon colonization as well as colonic inflammation are enhanced (153,154), it was demonstrated that *Salmonella enterica* serovar typhimurium *(S. typhimurium*) induce inflammation to alter microbiota composition and outcompete commensal growth (155).

Besides altering the microbiota composition, some pathogens have other strategies to subvert the competition. For example, *S. typhimurium* has the ability to use tetrathionate as an electron acceptor, which constitutes an advantage for the pathogen over fermenting gut microbes (156). In addition, *S. typhimurium* subverts calprotectin-induced zinc sequestration through a high affinity zinc transporter, which promotes its growth over that of the microbiota (157).

1.3 *Salmonella* infection

Theobald Smith discovered *Salmonella* in 1885; nevertheless the genus is named after his mentor Daniel Elmer Salmon, who received credit for the discovery (158).

Salmonella are facultative intracellular, Gram-negative bacteria that infect a broad range of hosts and cause a variety of diseases from gastroenteritis to typhoid fever (159). There are two species of *Salmonella*: *S. bongori* and *S. enterica*, and the latter is further divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (160). *S. enterica* subspecies serovars Typhi and Paratyphi are the etiological agents of human typhoid fever and cause more than 20 million cases and 200 000 fatalities worldwide per year (161,162). Certain areas in Asia and Africa have a higher typhoid fever burden due to limited access to clean water and poor sanitation (163) whereas in developed countries it has become predominantly a travel-associated disease (164). A number of multi-drug resistant strains have appeared in the last years limiting the treatment options for typhoid fever (165,166) and increasing the need for safe and effective vaccines. *S. typhimurium* causes a mild gastroenteritis in humans whereas in susceptible mice it causes a systemic infection similar to typhoid fever, which makes it a suitable model for the study of this disease (167).

Salmonella's invasion mechanisms

The infection is initiated by ingestion of contaminated food or water. *Salmonella* has an acid tolerance response that allows survival in the stomach despite the low pH (168) and results in bacteria reaching the intestine. *Salmonella* then crosses the epithelial barrier by transcytosis after penetrating mainly the M cells of the PP (169,170). M cells are specialized epithelial cells that constitute an antigen sampling system due to their ability to transport microorganisms and macromolecules to the intestinal lumen (171). *S. typhimurium* increase M cell numbers by inducing transdifferentiation of enterocytes into M cells (172) and can also induce death of the M cell allowing microorganisms to cross to the lumen (169).

Due to the type 3 secretion system (T3SS) encoded in *Salmonella* pathogenicity island 1 (SPI-1), *Salmonella* can penetrate via non-phagocytic cells such as epithelial cells. The T3SS apparatus is assembled as a consequence of *Salmonella* sensing the environmental conditions of the small intestine (173). It consists of two main protein complexes. More than 20 proteins form a needle-like complex through which effector molecules are injected into the host cell cytosol. Another set of proteins form the translocon, which forms a translocation pore in the cell membrane of the host facilitating the injection of the effector molecules (174). These effector proteins induce reorganization of the actin cytoskeleton that leads to macropynocytosis by host cells allowing bacterial internalization (175).

S. typhimurium lacking a functional T3SS can also reach the basolateral side of the epithelium through an alternative pathway. For example, it has been shown *in vitro* that ileal phagocytes expressing CX_3CR1 sample bacteria from the intestinal lumen through the formation of transepithelial dendrites (176). These are able to cross the epithelial layer by opening the tight junctions between epithelial cells and take up microorganisms without disrupting the epithelial barrier (177). The transepithelial dendrites increase in the terminal ileum during *Salmonella* infection.

However, *Salmonella*-dendrite association is an infrequent process *in vivo* (178). Another study using the streptomycin mouse model showed that *S. typhimurium* lacking TTSS required $CD11c^+CX_3CR1^+$ phagocytes to cross the epithelium in an early phase of the infection and the process was MyD88-independent (179). Additional studies *in vitro* have provided evidence of another M-cell independent translocation pathway, in which virulent *S. typhimurium* alters the distribution of intercellular tight junction proteins disrupting epithelial barrier integrity and promoting bacterial translocation (180,181).

Tissue colonization by *Salmonella*

Once *Salmonella* reach the intestine, they seed PP and the LP. *Salmonella* can be detected in PP at early time points after oral infection followed by MLN (182), and 48 hours post infection the PP are more colonized than the MLN (49,183). How *Salmonella* reach the MLN from the LP and/or PP has been an area of active research for quiet some time. It has been suggested that *Salmonella* travel from the intestine to the MLN via the lymph inside cells or as free bacteria (184). However, the proportion of *Salmonella* reaching the MLN extracellularly versus the proportion reaching the MLN transported inside cells is poorly understood. There is evidence suggesting that *Salmonella* reach the MLN inside DC. For instance, DC have been shown to transport intestinal commensals to the MLN (89). In addition, DC expansion in mice through injection of Flt3L-secreting cells resulted in higher *S. Typhimurium* counts in MLN (185) and depletion of $CD11c^+$ cells as well as infection of mice with impaired DC migration resulted in less *S. Typhimurium* in MLN (185,186). Furthermore, using the streptomycin mouse model (153) a study showed that the $CD103^+CD11b^+$ subset of DC was the only DC subset found to contain *Salmonella* in the MLN early after oral infection despite the other subsets carrying *Salmonella* in the LP (75). Moreover, there is some evidence of CX_3CR1-expressing cells being involved in *Salmonella* transport to the MLN in the absence of commensals (91). However, as mentioned earlier,

this is controversial as cells expressing high levels of CX_3CR1 have been reported to be non-migratory macrophages (77), while cells with an intermediate expression of CX_3CR1 are migratory and can be found in lymph (77). Further studies are necessary to elucidate more specifically the role of DCs and subsets thereof in transporting *Salmonella* from the LP to the MLN.

Systemic dissemination

Salmonella infection is not confined to intestinal tissues and they are also found in systemic tissues such as spleen (187-189), liver (190-193), bone marrow (194) and gall bladder (195) mainly inside macrophages, DC and neutrophils (196). However, the mechanisms through which *Salmonella* reaches systemic organs are unclear. Initial studies showed that *Salmonella* could only be found in systemic organs such as spleen and liver when PP and MLN were heavily infected (182). Since the efferent lymph of the MLN empties into the blood, *Salmonella* could spread from MLN to systemic tissues via lymph-blood (196,197). This is supported by studies demonstrating that the surgical removal of MLN resulted in higher numbers of *S. typhimurium* reaching systemic sites and supports that the MLN limits bacterial systemic dissemination during early stages of the infection (103,185,198). However, alternative mechanisms may exist. For example, one study showed that *S. typhimurium* was found in the bloodstream inside $CD18^+$ phagocytic cells independently of M cell invasion within 5 minutes after oral gavage (199). This suggests almost immediate access of intestinal bacteria to the blood. Moreover, recent experiments using a pool of tagged *S. typhimurium* strains showed that the infection of liver and spleen was caused by a different pool of bacteria than the one colonizing PP and MLN (186). This further supports that *Salmonella* in systemic tissues access the blood without necessarily colonizing intestinal lymphoid organs. Moreover, a study on systemic dissemination of *Yersinia pseudotuberculosis* showed similar results (200). Thus, *Salmonella* colonization of systemic organs may originate from both bacteria that colonized PP and MLN and bacteria that reached the blood directly from the

intestine. Further studies are needed to determine the relative contribution of the different routes that *Salmonella* exploits to disseminate systemically.

Innate immunity to *Salmonella*

The innate immune system constitutes the first line of defense against *Salmonella* and other bacterial infections; it becomes active rapidly after the infection initiates and its main goal is to stop bacterial penetration and/or replication.

The mucus layer and antimicrobial peptides

The mucus blanket that covers the gut epithelium functions as a physical protective barrier against invasion by commensals and intestinal pathogens. Its main components are glycoproteins called mucins, which are secreted by goblet cells (201). During *S. typhimurium* infection, goblet cells release mucus into the gut lumen from mucus-filled vacuoles whose formation is induced by IFN-γ-receptor-signaling (202). Furthermore, TNF-α is another cytokine thought to be involved in the regulation of mucus production in *S. typhimurium* infection (203). Many bacterial species have the ability to degrade mucus and reach the epithelial layer; *S. typhimurium* does so by binding preferentially to a mucin called Mucus-Rs, which could constitute the first site of bacterial interaction in the intestine (204). It must be taken into consideration, however, that the thickness of the mucus blanket in the ileum, where *Salmonella* mainly penetrates in models with intact microbiota, is half as thick as in the colon (205).

Antimicrobial peptides, which are short polypeptides that provide protection against microorganisms (206,207), are other molecules that are also part of the innate immune system. Most antimicrobial peptides target the cell membrane and thereafter cause cell damage by, for example, inhibiting protein synthesis (208). In the gastrointestinal tract epithelial cells, mainly Paneth cells, are the

predominant cells expressing antimicrobial peptides (209,210). An example of an antimicrobial peptide that offers protection against *S. typhimurium* is WRWYCR, which inhibits DNA repair enzymes (211). In addition, expression of peptides such as RegIIIβ are increased during *S. typhimurium* infection (212). However, RegIIIβ kills commensals but not *S. typhimurium* which could promote infection instead of preventing it (212).

In addition to the mucus layer and the production of antimicrobial peptides, the intestinal microbiota also promotes protection against invading pathogens like *Salmonella* as discussed in section 1.2.

Recognition of *Salmonella* by innate immune cells

As mentioned in section 1.1, DC and other phagocytes are equipped with PRR such as TLR that allow them to recognize invading pathogens. *Salmonella* express TLR ligands such as bacterial lipoproteins, lipopolysaccharide, flagellin and CpG DNA, which are recognized by TLR-2, TLR-4, TLR-5 and TLR-9, respectively. Mice lacking TLR2 and TLR4 show a greater susceptibility to infection. However, when TLR9 is also absent, mice are less susceptible to infection due to poor *Salmonella* replication inside macrophages. This indicates that TLR signaling is necessary to activate certain *Salmonella* virulence factors (213).

Phagocyte accumulation in intestinal tissues

Myeloid cells such as neutrophils, monocytes and macrophages play a crucial role in controlling the infection at early stages through phagocytosis-mediated killing and the induction of inflammation. Neutrophils are the first cells to respond to *Salmonella* infection (214). They accumulate rapidly in PP and MLN of infected mice (183,215) and their recruitment seems to be mediated by the chemokine CXCL2 (216). The main function of neutrophils is to kill extracellular bacteria to confine and reduce bacterial replication (197). The importance of neutrophils is illustrated with the fact that neutropenia increases susceptibility to *Salmonella* infection (217,218).

Inflammatory monocytes are phagocytic cells that together with neutrophils are rapidly recruited to *Salmonella*-infected tissues (183,216). Although these cells are not as efficient in phagocytizing *Salmonella* as neutrophils, their main function is also to control initial bacterial replication (183). Macrophages resident in tissues are also phagocytic and defend the host against pathogens through antimicrobial functions. Infected macrophages kill *Salmonella* mainly through lysosomal enzymes and reactive oxygen and nitrogen intermediates (197), which are critical to host survival to *Salmonella* infection (219). Moreover, inactivation or depletion of macrophages leads to increased growth of *Salmonella* (220,221).

NK cells are also part of innate immunity and play a role during *Salmonella* infection by contributing to early production of IFN-γ (222-224). In fact, it has been shown that there is a crosstalk between NK cells and macrophages during *Salmonella* infection in which macrophages activate NK cells and the latter produce IFN-γ that activates macrophages (225). However, although it has been shown that NK cells alone can produce enough IFN-γ during an early stage of the infection to control bacterial growth (223), their ablation does not prevent the mice from clearing the infection (226).

DC are also phagocytic cells but, as discussed above, their most important function is as APC rather than killing bacteria to control replication and spread. *Salmonella* that cross the epithelium overlying PP encounter a population of DC expressing CCR6 that play an important role in activating *Salmonella*-specific T cells (227). Approximately 10% of cells in PP are DC (171) and *Salmonella*-associated DC are found in this tissue shortly after infection (50). As discussed before, DC maturation is crucial for the activation of naïve T cells. During *Salmonella* infection DC maturation occurs as a consequence of direct association with bacteria or via an indirect pathway mediated by TNFRI signaling. DC in PP, MLN and spleen up-regulate costimulatory molecules in *Salmonella*-infected mice and gain a mature phenotype (49,50,228).

Phagocyte killing of Salmonella and the Salmonella-containing vacuole

Phagocytes kill bacteria through antimicrobial substances such as proteases, lysozyme and reactive oxygen and nitrogen species produced by Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and iNOS, respectively (219,229,230). Inflammatory monocytes are the predominant producers of iNOS and its production is dependent on IL-12 and IFN-γ (183,231). When *Salmonella* are engulfed by phagocytes, they reside in intracellular compartments known as *Salmonella*-containing vacuoles (SCV). *Salmonella* are able to survive inside SCV thanks to the TTSS encoded in SPI-2 (232,233), which inhibits trafficking of vesicles containing antimicrobial substances as well as phagosome-lysosome fusion (234). Macrophages kill bacteria through the acidification of the SCV. However, a recent study showed that *Salmonella* acidifies its own cytoplasm, which in turn activates the secretion of SP1-2 effectors that enable bacterial survival inside macrophages (235). *Salmonella* replication inside macrophages is followed by escape from the infected macrophage and infection of additional macrophages (236).

Although macrophages and neutrophils use similar mechanisms to kill intracellular bacteria, *Salmonella* can survive and replicate inside macrophages while neutrophils and inflammatory monocytes have a greater capacity to kill *Salmonella* (231). This might be explained by the basic environment in neutrophil phagosome which differs from the acidified phagosome in macrophages (237). In addition, while neutrophils and inflammatory monocytes kill *Salmonella* through NADPH oxidase, macrophages produce a non-fatal oxidative stress and use NADPH oxidase-independent mechanisms to kill bacteria (231).

The role of cytokines in immunity to *Salmonella*

IFN-γ activates cells such as macrophages and NK cells, facilitates antigen processing and presentation on MHC-I and -II, directs Th1 differentiation of T cells and is involved in leukocyte recruitment by stimulating chemokine production (238). IFN-γ plays an important

role in host survival to *Salmonella* as neutralization of this cytokine in mice prevents bacterial clearance even with a sub-lethal dose of the pathogen (239). Furthermore, IFN-γ knockout mice show higher bacterial counts in all the organs and fail to upregulate MHC-II to the same extent as wildtype mice (240). As stated above, NK cells are the most important source of IFN-γ at early stages of the infection (223), whereas when the adaptive immune responses are activated $CD4^+$ T cells become the main producers of this cytokine (228). In addition, IFN-γ-producing $CD4^+$ T cells also have a role during innate immune responses as they become activated via an antigen-independent mechanism mediated by IL-18 (241). Moreover, a study using the streptomycin mouse model showed that IFN-γ also controls mucus accumulation/secretion by goblet cells during *Salmonella* infection (202).

Since IFN-γ is such an important cytokine in the control of *Salmonella* infection, cytokines such as IL-12 and IL-18 that have IFN-γ-inducing properties are also crucial. These two cytokines are produced mainly by macrophages and their neutralization results in decreased survival (242). Other cytokines that play an indirect role are IL-15 and IL-24. While IL-15 neutralization results in poor development of NK cells and therefore diminished IFN-γ production (243), IL-24 induces IFN-γ and nitric oxide production by neutrophils (244).

TNF-α is another crucial cytokine in the control of *Salmonella* infection. Its main functions are the attraction of inflammatory cells, induction of apoptosis and reinforcement of some IFN-γ functions (184). Furthermore, *Salmonella*-induced TNF-α mediates indirect DC maturation during infection (49). Studies performed in TNF-α knockout mice showed higher susceptibility to *Salmonella* infection (245).

Some cytokines associated with Th17 responses are also important. For example, IL-6 has been shown to be upregulated during *Salmonella* infection (246,247) and is required to control inflammatory responses such as the level of proinflammatory

cytokines (248). IL-17 is also upregulated during *Salmonella* infection and plays a role in neutrophil recruitment. Moreover, IL-17 knockout mice have higher *Salmonella* counts in systemic organs (249). IL-17-producing $CD4^+$ T cells are detected in mucosal tissues after *Salmonella* infection (250) and $CD4^+$ Th17 cells and γδ T cells produce IL-17 that contributes in the defense responses (251). Regarding IL-22, IL-23-dependent IL-22 is associated with protection against systemic *Salmonella* infection in the absence of IL-12 (252). Moreover there is some evidence that IL-22 is also involved in the production of the microbial peptide lipocalin-2 during *Salmonella* infection(253).

Adaptive immunity to *Salmonella*

Adaptive immune responses to *Salmonella* infection start when activated DC prime naïve T cells by the presentation of *Salmonella* antigens in lymphoid tissues. However, *Salmonella* can evade the initiation of adaptive immune responses through processes that are SPI-2 TTSS-mediated. These include inhibiting the loading of bacterial peptides on MHC molecules, which leads to poor T cell activation (254-256). Nonetheless, adaptive immunity is ultimately needed, as innate immune responses control bacterial replication during early stages of the infection but, as underscored by the importance of T cells described below, are not sufficient for bacterial clearance.

T cells and immunity to *Salmonella*

The importance of T cells in immune responses to *Salmonella* has been known for a long time. Experiments in mice lacking or having inactivated T cells showed that these cells are indispensable to clear a primary *Salmonella* infection (257-260).

Resistance to *Salmonella* is mediated mainly by $CD4^+$ T cells (257,259) that are mostly skewed towards Th1 cells capable of secreting IFN-γ (197,228,261). Indeed, one of the main roles of T

cells during *Salmonella* infection is the production of IFN-γ, which activates macrophages and increases their bactericidal capacity (262). Activated Th1 cells can be detected already one week after the infection starts (263,264). Furthermore, *Salmonella*-specific memory T cells are important contributors in secondary infections (265-267); IFN-γ-secreting $CD4^+$ T cells persist in mice for at least 6 months after the first challenge (264,267). Regarding Th17 responses, there is some evidence that they play a role in immunity to *Salmonella*. For instance, the production of cytokines associated with Th17 cells, such as IL-17, IL-22 and IL-23, is augmented in *Salmonella* infection (268). Furthermore, it has been shown that the absence of IL-17 promotes *Salmonella* dissemination to MLN and systemic tissues and impairs recruitment of neutrophils (251,269). In addition, another study documented that flagellin-specific $CD4^+$ T cells differentiated into Th1 and Th17 lineages (250).

Although their role is smaller, $CD8^+$ T cells also contribute in combating *Salmonella* infection. *Salmonella*-specific $CD8^+$ T cells are induced after infection (267). Studies performed using *Salmonella* expressing ovalbumin (OVA) protein have shown that expansion of OVA-specific $CD8^+$ T cells peaks around three weeks post infection (270,271). Furthermore, $CD8^+$ T cells are also contributors to the production of IFN-γ (228,241,266). Some studies have suggested that MHC-I-restricted $CD8^+$ T cells are important only in secondary infections (257,272). However, another study showed that these cells play a protective role via cytolytic granule release during primary infection but do not contribute significantly during a second response (273). Finally, it has been shown that months after immunization, memory $CD8^+$ T cells are detected in response to restimulation with *Salmonella* lysate or attenuated strains (266,274).

B cells and antibody responses

Less emphasis has been put on the role of B cells in *Salmonella* infection in comparison to T cells. One reason could be the thinking that antibodies play an important role in the immune

responses against extracellular rather than intracellular pathogens like *Salmonella* (275). However, it is important to consider that there are specific times when *Salmonella* is extracellular, for example when they are released from an infected cell before they infect another (276). However, a study showed that antibody/complement-mediated killing of *Salmonella* takes from 5 to 10 minutes, which is enough time for some bacteria to escape from antibodies by re-entering into intracellular spaces (277). Nonetheless, the presence of anti-*Salmonella* antibodies overcomes *Salmonella*'s ability to evade the initiation of adaptive immune responses. For example, binding of opsonized *Salmonella* to Fcγ receptors expressed on DC promotes phagosome-lysosome fusion, which leads to bacterial degradation and peptide presentation even in the presence of TTSS-derived effector proteins (278).

Experiments performed in Igμ knockout mice, which lack B cells and therefore antibody production, showed that B cells play an important role in infection with virulent strains of *Salmonella*. In contrast, these cells were dispensable in infections with attenuated strains (279). However, B cell-derived antibodies produced during an infection with an attenuated strain were necessary for protection against a secondary challenge with a virulent strain (280).

In addition, the role of B cells in *Salmonella* infection is also reflected in their crosstalk with T cells. One study showed that human B cell lines are able to present *Salmonella* antigens to human T cells (281). Furthermore, in another study, T cells from B cell deficient mice showed reduced ability to release IL-2 and IFN-γ in the context of a *Salmonella* infection (282).

2 AIMS

The overall aims of this thesis were to expand the knowledge on the role of DC in oral *Salmonella* infection as well as analyze the impact of the microbiota on early stages of oral *Salmonella* infection.

Specific aims of the thesis:

I. To investigate the synergistic effect that MyD88-dependent NFκB signaling and CD40-dependent NFκB2 signaling in DC have in the T cell response against *Salmonella*.

II. To address the influence of the intestinal microbiota in *Salmonella* colonization in different tissues as well as in cytokine production in response to the infection.

III. To determine the role of intestinal $CD103^{+}CD11b^{+}$ DC in oral *Salmonella* infection.

3 KEY METHODOLOGY

This section provides a broad description of the methods used in the studies contained in this thesis. For more detailed information consult the Materials and Methods section of the individual papers.

3.1 Mice

The different susceptibility to *Salmonella* infection between mouse strains is associated with the allele *Slc11a1* (previously known as *Nramp1*) (283). The mouse strain C57BL6/J, which is the genetic background of the mice used throughout these studies, has a mutation *Slc11a1* and is susceptible to *Salmonella* infection.

In paper I the mouse strains used were C57BL6 wildtype controls (WT) as well as mice deficient in CD40, MyD88, or both by generating a double knockout strain for the study. In paper II, germ-free (GF), conventionally raised mice (CONV-R) and WT mice with or without antibiotic treatment were used. Finally, in paper III, the studies were performed in Cre^+ (IRF4 knockouts) and Cre^- (control) mice. Cre^+ is the designation for CD11c-cre.$Irf4^{fl/fl}$ and CD11c-cre.$Irf4^{fl/-}$ mice, which lack expression of IRF4 in CD11c cells. These mice have alterations in the number of $CD103^+CD11b^+$ intestinal DCs (79). Cre^- refers to $Irf4^{fl/fl}$ and $Irf4^{fl/-}$ mice whose CD11c cells express IRF4 and are therefore used as controls (79).

All experiments were performed following protocols approved by the government animal ethics committee (permits 311-2010 and 212-2013) and institutional animal care and use guidelines. Animals were bred and housed in the Laboratory for Experimental Biomedicine, University of Gothenburg.

3.2 *Salmonella* strains and infections

The *S. typhimurium* strains used in these studies were the SR11 derivatives χ4550 (228,266) (papers I and II) and χ8554 (183) as well as the WT strain SL1344 (paper III) (49).

The SR11 derivative χ4550 has a reduced virulence compared to the WT strain due to deletions in the genes encoding adenylate cyclase and the cyclic AMP receptor protein (266). On the other hand, the derivative χ8554 has a comparable virulence to the WT strain. Both χ4550 and χ8554 contain the vector pYA3259 into which the model antigen ovalbumin (OVA) was cloned (228) and were chosen in anticipation of the need to eventually analyze T cell responses in future experiments.

Strains were grown overnight at 37°C. The bacterial concentration was determined spectrophotometrically at OD_{600} and diluted to the desired concentration in sterile PBS. The bacterial doses were administered intragastrically or IP. The actual bacterial dose administered, as well as the bacterial burden in *Salmonella*-infected organs at the time of sacrifice, were determined by plating serial dilutions of bacterial or cell suspensions on LB agar plates and counting CFU.

3.3 Cell suspensions

For determining bacterial burden in *Salmonella*-infected organs, the analysis of cells by flow cytometry as well as cytokine and nucleic acid quantification, the preparation of single cell suspensions of the different organs is first needed. To this end, PP, small intestine, MLN and spleen were incubated in a digestion solution containing collagenase, DNase and Dispase. After filtration, the concentration of the cell suspensions was determined.

3.4 Flow cytometry

The study of immunological processes *in vivo* generally requires the analysis of distinct cell types in different tissues that can be specifically identified by expression of surface molecules. Flow cytometry is a technique that allows identification and quantification of cell populations in complex cell mixtures (284). In paper I, multicolor flow cytometry was used to analyze T cell proliferation. In paper II, this technique facilitated the study of IFN-γ production by NK, NKT, $CD4^+$ and $CD8^+$ cells. Finally, in paper III the study of diverse cell populations such as DC, myeloid cells and T cells was possible thanks to flow cytometry.

3.5 Cytokine analysis

In paper I, IFN-γ and IL-10 were measured in serum samples and in supernatants from DC and T cell co-cultures using the enzyme-linked immunosorbant assay (ELISA). This technique is extensively used worldwide and provides a sensitive and reliable analysis of a single analyte (285,286).

In papers II and III different cytokines were measured in lysates of whole organs. For this purpose the organs were incubated in a solution containing saponin and the supernatants were then collected and analyzed. IL-12 (paper II) and IL-6 and IL-17 (paper III) were analyzed using a bead-based multiplex immunoassay, which allows the simultaneous detection and quantification of multiple analytes in a shorter period of time compared to other techniques (287).

3.6 Gene expression

In paper I, the expression of the genes A20, ABIN-2, c-Rel, NIK, TICAM2 and β-catenin was measured through real-time polymerase chain reaction (RT-PCR), also known as quantitative polymerase chain reaction (qPCR). RT-PCR is a technique that has been used for more than 30 years and it is still considered the most powerful tool for nucleic acid quantification(288).

Before qPCR analysis, the cells of interest were collected and total RNA was extracted and reverse transcribed into cDNA. The sequences of the primers used are reported in Table 1. Differential gene expression was assesed with the $2^{\Delta\Delta_{Ct}}$-method using HPRT as a housekeeping gene.

Table 1. Oligonucleotide primers used for qPCR

	Primer sequence	
Gene	Forward	Reverse
HPRT	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
TICAM-2	GAAGATCGAAGAGCCTCGTG	GTGATTGAGACGCCTTAGCC
NIK	CTGCAACCTGACGGCCTA	CTCCGTGCCAGGAATGTAGT
β-catenin	GCAGCAGCAGTTTGTGGA	TGTGGAGAGCTCCAGTACACC
A20	TCATCGAATACAGAGAAAATAAGCAG	AGGCACGGGACATTGTTCT
ABIN-2	GAC GCA CTT CTG GAT CAG GT	CGCTCCGTAAGTCTTTCAACTT
cRel	TTGCAGAGATGGATACTATGAAGC	CACCGAATACCCAAATTTTGAA

4 RESULTS AND DISCUSSION

Infectious diseases are the principal cause of death worldwide. The discovery of new infectious pathogens, the reappearance of old ones and their continual acquisition of resistance mechanisms have revealed the need for developing new tools to help win the battle against infectious diseases. This has awakened the interest in researchers to decipher the details involved in host-pathogen interactions with the goal of preventing and treating infections through the development of vaccines and new antibiotics.

The appearance of *Salmonella* strains resistant to a variety of antibiotics including fluoroquinolones has been documented (289). Although the infection has been broadly studied for a long time, and great progress has been achieved in the field, there are still some loose ends. Understanding the complete picture of the mechanisms occurring during the infection will indeed provide tools for the generation of more effective vaccines.

4.1 Role of DC in *Salmonella* infection

Nobody could argue against the importance of DC in infection with any microbe including *Salmonella*. In fact, these cells have been demonstrated to be so important that numerous researchers in the *Salmonella* field have focused their investigations on DC. Studies have attempted to elucidate the details from localization and migration of DC during infection to their interactions with other cells and their death.

In papers I and III we attempted to contribute to the knowledge of the impact of DC during *Salmonella* infection. In paper I we focused in signaling pathways that occur in DC and their influence on activation of adaptive immunity. On the other hand, in paper II we studied the consequences of a diminished population of $CD103^+CD11b^+$ DC on the host response to *Salmonella* infection.

Paper I

Studies investigating the cooperative influence of CD40 costimulation and MyD88-dependent signaling using TLR ligands, protein antigen and other molecules showed that both the canonical and non-canonical pathways act synergistically in DC and optimize T cell activation (64-66,290). Nevertheless, the importance of synergistic signals in DC–T cell activation during an infection with a live bacterial pathogen where several bacterial ligands activate TLR and other receptors had not yet been investigated. Therefore, the aim of Paper I was to investigate whether cooperation between CD40 and MyD88 in DC influenced the host response against *S. typhimurium* with focus on the T cell response. Mice lacking both CD40 and MyD88 were first generated, $CD40^{-/-}MyD88^{-/-}$ mice (hereafter referred to as double knockouts, DKO), and $CD40^{-/-}$, $MyD88^{-/-}$, DKO and WT mice were orally infected with *S. typhimurium* and the host response was analyzed.

MyD88$^{-/-}$ and DKO mice are more susceptible to infection with *S. typhimurium*

The first observation was that DKO and $MyD88^{-/-}$ succumbed to the infection, whereas $CD40^{-/-}$ and WT survived. The consequences of MyD88 deficiency in *Salmonella* infection have been the focus of previous studies. For instance, it has been reported that $MyD88^{-/-}$ mice are highly susceptible to infection with *S. typhimurium* due to defects that involve cytokine production, intestinal barrier integrity and induction of T cell responses, among others (50,216,291,292). Furthermore, DKO and $MyD88^{-/-}$ mice displayed higher bacterial burdens in intestinal tissues as well as in the spleen compared to $CD40^{-/-}$ and WT mice. This is supported by studies in which higher *Salmonella* CFU were reported in $MyD88^{-/-}$ mice compared to WT (291,293). Insufficient phagocyte recruitment could explain the impaired ability of DKO and $MyD88^{-/-}$ to control bacterial replication. However, it had been previously reported that the recruitment of neutrophils and monocytes is not impaired in $MyD88^{-/-}$ mice (216) and no differences in the number of these cells between infected $CD40^{-/-}$, $MyD88^{-/-}$ and DKO mice were

observed. This indicates that the increased *S. typhimurium* CFU in DKO and MyD88$^{-/-}$ mice were not due to altered neutrophil and inflammatory monocyte recruitment to sites of infection. However, although *Salmonella* uptake by macrophages is a MyD88-independent process, MyD88 deficiency in macrophages results in a reduced ability to kill *Salmonella* (294). This suggests that the defective macrophage function in these mice could contribute to the higher CFU observed. This, however, needs to be addressed. Thus, the data so far support that MyD88 deficiency results in increased susceptibility to *S. typhimurium* infection and DKO mice succumbed earlier that MyD88$^{-/-}$ mice. However, whether synergistic effects of CD40 and MyD88 in DC were responsible for the augmented susceptibility of DKO mice to bacterial infection was not clear at this point.

DKO and MyD88$^{-/-}$ mice induce a differential cytokine profile and reduced T cell proliferation

The Th1 dominance in the immune response to *Salmonella* has been known for quiet some time (295). Considering the importance of IFN-γ in bacterial clearance (240,265), it was next investigated whether DKO and MyD88$^{-/-}$ mice showed abnormal production of IFN-γ. While IFN-γ was detectable in serum of CD40$^{-/-}$ and WT mice, IL-10 but not IFN-γ was present in serum of DKO and MyD88$^{-/-}$ mice. The presence of IL-10 in serum of DKO and MyD88$^{-/-}$ mice could be sign of induction of Tregs (296). Interestingly, it has been shown that blocking the CD40 signaling pathway induces Treg development (297). The data suggest that in the absence of MyD88, IL-10 production by T cells is favored over IFN-γ production. This is supported by a study showing that *Salmonella*-infected mice produce high levels of IL-10 in the absence of Th1 cells (298). In addition, a recent study showed that MyD88 signaling is necessary to instruct CD4^{+} T cells to differentiate into Th1 cells instead of Tregs (299). The fact that similar results were obtained in the serum of DKO and MyD88$^{-/-}$ mice suggests that the abnormal cytokine profile observed in these

mice is likely due to the lack of MyD88 expression rather than cooperative effects of MyD88 and CD40.

The time frame when DKO and MyD88$^{-/-}$ mice started to succumb to *S. typhimurium* infection (7-10 days p.i.) raised the possibility that defective activation of adaptive immunity could be involved in the increased susceptibility to infection. However, no differences in the number of DC or T cells were found between CD40$^{-/-}$, MyD88$^{-/-}$ and DKO mice at days 6 and 13 p.i. In addition, no differences in the upregulation of CD80 and CD86 in DC from CD40$^{-/-}$, MyD88$^{-/-}$ and DKO mice were found. These results support that alterations in DC or T cell numbers, or expression of costimulatory molecules on DCs, in both MyD88$^{-/-}$ and DKO mice did not underlie the altered susceptibility to infection.

As discussed in section 1.3, T cells are the main contributors to IFN-γ production during later stages of *S. typhimurium* infection (228). Therefore, the next step was the analysis of DC-induced IFN-γ and IL-10 production by antigen-specific CD4$^+$ T cells. This was done using heat-killed *S. typhimurium* expressing the model antigen OVA (HKS_{OVA}). As the goal of the study was to analyze possible cooperative effects of CD40 and MyD88 during the complex but real situation of bacterial infection, the use of HKS_{OVA} provided the tool to track a defined "model" antigen in the background of all of the other antigens and components of the bacteria. To analyze whether the complexity of the antigen influences CD40 and MyD88 cooperation, similar experiments were also performed using the purified antigens OVA protein and $OVA_{323-339}$ peptide for comparison with HKS_{OVA}. In general, no differences in DC ability to induce IFN-γ between DKO and MyD88$^{-/-}$ DC were found except for a slight difference when $OVA_{323-339}$ peptide was used. However, DC capacity to induce IFN-γ production as well as OT-II cell proliferation in CD40$^{-/-}$ and WT mice was greater than that in DKO and MyD88$^{-/-}$ mice. Nonetheless, the data showed that DC from DKO mice induced lower OT-II proliferation to OVA protein and $OVA_{323-339}$ peptide, but not HKS_{OVA}, compared to DC from MyD88$^{-/-}$ mice. This

suggests a synergy between CD40 and MyD88 on T cell proliferation in DC-T cell co-cultures containing a less complex antigen. Finally, DC from DKO mice pulsed with HKS_{OVA}, but not with OVA protein or $OVA_{323-339}$ peptide, induced higher production of IL-10 than DC from MyD88$^{-/-}$ mice. This differs from the *in vivo* data that showed similar levels of IL-10 in the serum of MyD88$^{-/-}$ and DKO mice. This could be due to the fact that *in vivo* data are influenced by numerous cell types that interact and IL-10 production by a specific cell type could be masked by the production by other cells. Indeed, IL-10 has been reported to be produced by cells other than T cells (300).

Collectively, data from the co-culture experiments suggest that CD40 and MyD88 cooperate in some DC-induced T cell effector functions, such as IL-10 production and proliferation, while a cooperative effect on IFN-γ production is less apparent. Moreover, the data suggest that cooperativity of CD40 and MyD88 in DCs on ensuing T cell functions is affected by antigen complexity. The data also indicate that responses to a single purified antigen may not be detectable when a complex mix of antigens such as bacteria (HKS_{OVA}) is used, as they might be masked in the presence of other antigens and microbial signals (i.e. PAMP). Furthermore, it may be difficult to extrapolate effects observed *in vitro* to *in vivo* responses as an infection involves interaction of many cell types and molecules whereas a co-culture system is very defined.

Gene regulation requires MyD88-dependent signaling

DC-T cell interactions activate signaling pathways in both cells that result in the production of cytokines. Thus, the expression of genes associated with the canonical (A20, cRel and β-Catenin) and non-canonical (ABIN-2, NIK and TICAM-2) NF-κB signaling pathways in co-cultures of DC pulsed with HKS_{OVA} and OT-II T cells were next investigated. DC from CD40$^{-/-}$ and WT mice activated expression of these genes in co-cultures and did so to a greater extent than DC from MyD88$^{-/-}$ or DKO mice. In addition, no differences in gene expression were found in DC from MyD88$^{-/-}$

compared to DKO mice. This is consistent with the data from the co-culture experiments where T cell responses were not altered when HKS_{OVA} was used as the antigen.

Altogether, the findings in Paper I suggest that the defects observed in MyD88$^{-/-}$ and DKO mice, namely poor capacity to control *Salmonella* infection, lack of IFN-γ production and reduced induction of NF-κB-dependent and –independent genes, are due to the MyD88 deficiency rather than a cooperative effect between MyD88 and CD40 *per se*. However, the *in vitro* data from co-culture experiments revealed synergistic effects of MyD88 and CD40 in processes that DC induce in T cells, such as proliferation and production of some cytokines. A more precise investigation of the synergistic effects of MyD88 and CD40 *in vivo* would require mice that lack expression of MyD88 and CD40 only in a specific type of cell. In addition, experiments where other bacteria or virus are used and/or the use of, for example, BALB/c mice, which are biased towards Th2-dominated immune responses (301), could help decipher more synergistic effects of MyD88 and CD40 during microbial infection.

Paper III

Since their discovery, many studies have shown that DC constitute a heterogeneous population of cells that differ in location, function, phenotype and transcription factors that drive their ontogeny. In the context of *Salmonella* infection, some progress has been made in deciphering the role of different DC subsets during infection with the use of mouse models that lack a specific subset of DC.

In paper III we studied the impact of the CD103^{+}CD11b^{+} DC subset in *Salmonella* infection with the use of mice that display a reduced population of these cells (herein designated as Cre^{+} mice) and compared them to controls with intact DC populations (Cre^{-} mice). It has been reported that under steady-state conditions, Cre^{+}

mice have a 50% reduction in splenic $CD4^+$ DC and siLP $CD103^+CD11b^+$ DC. In addition they have a 50% reduction in resident MHC-II^{int} $CD103^+CD11b^+$ DC and 90% in migratory MHC-II^{high} $CD103^+CD11b^+$ DC in the MLN (79).

$CD103^+CD11b^+$ DC and *Salmonella* tissue colonization

First, Cre^+ and Cre^- mice were orally infected with *S. typhimurium* and the CFU in intestinal organs and spleen were analyzed 3 days p.i. Even with their defective DC compartment, Cre^+ mice had similar CFU in PP, siLP, MLN and spleen to Cre^- mice. Furthermore, neutrophil and inflammatory monocyte recruitment to these tissues was similar in Cre^+ and Cre^- mice. As no differences in bacterial burden in PP and siLP were found between Cre^+ and Cre^- animals, the data suggest that initial bacterial penetration is not altered with a reduced population of $CD103^+CD11b^+$ DC. Furthermore the similar CFU in spleen suggest an unaltered systemic dissemination of *S. typhimurium* in Cre^+ mice.

Regarding MLN, a previous study using the streptomycin mouse model (153) showed that infected $Flt3L^{-/-}$ mice displayed less bacterial dissemination to MLN (75). Since $Flt3L^{-/-}$ mice lack $CD103^+CD11b^+$ DC in the LP, the authors concluded that $CD103^+CD11b^+$ DC are the first DC subset to transport *S. typhimurium* to the MLN after oral infection (75). Therefore, it could be expected that Cre^+ mice, which have reduced $CD103^+CD11b^+$ DC in the LP and MLN, display a lower bacterial burden in the MLN. This was not observed. However, the Cre^+ mice used in paper III were not streptocycin-pretreated as in Bogunovic et al. Furthermore, Bogunovic et al. looked at the different DC subsets containing *S. typhimurium* 18 and 24 hours p.i. The time point examined in Paper III was 72 hours p.i. and perhaps this was no longer the case. Moreover, they identified bacteria in $CD103^+CD11b^+$ DC in the MLN, which could represent bacteria transported from the LP to MLN inside $CD103^+CD11b^+$ DC or bacteria that travelled cell-free to the MLN and were taken up by $CD103^+CD11b^+$ DC in this tissue. Indeed, ~50% of the *Salmonella* that reach the MLN of rats travel cell-free from the LP (Yrlid et al.,

unpublished data). Furthermore, our results showing similar CFU in the MLN of Cre^+ and Cre^- mice are consistent with data obtained using huLangerin-DTA mice, where $CD103^+CD11b^+$ DC are ablated using a diphtheria toxin transgene (302). In this paper, a similar bacterial burden in MLN was found in huLangerin-DTA mice and controls 48 hours p.i. (302). However, a caveat is that these data were generated using the streptomycin mouse model (153), which, as discussed previously, may influence bacterial penetration to the MLN (92).

Alterations in the abundance of DC have been reported in *Salmonella*-infected mice using molecules other than CD103 and CD11b to identify subsets (49,303). We therefore proceeded to analyze the DC compartment according to the expression of CD103 and CD11b in Cre^+ and Cre^- mice infected with *S. typhimurium*. We observed that, similar to that in steady state conditions (79), the MLN of infected Cre^+ displayed a reduced population of $CD103^+CD11b^+$ DC at 3 and 6 days p.i. Moreover, DC subsets in the MLN of Cre^+ and Cre^- mice upregulated CD80 and CD86 to a similar extent.

Production of IFN-γ, IL-17 and IL-6 is not impaired in *Salmonella*-infected Cre^+ mice

The role of $CD103^+CD11b^+$ DC in priming T cell responses during *Salmonella* infection has not been investigated in depth. It is only known that the ablation of this DC subset in huLangerin-DTA mice does not alter CD44 expression on *Salmonella*-specific T cells (302). To begin to investigate the role of $CD103^+CD11b^+$ DC in adaptive immune responses to *S. typhimurium,* changes in the T cell compartment of Cre^+ and Cre^- mice infected with *S. typhimurium* were investigated at 6 and 13 days p.i. Similar numbers of TCR-β^+, $CD4^+$ and $CD8^+$ T cells in PP, siLP and MLN of Cre^+ and Cre^- mice were found.

It has been reported that $CD103^+CD11b^+$ DC are necessary for Th17 differentiation, as Cre^+ mice display less IL-17^+OT-II cells than Cre^- mice after administration of the protein antigen OVA

(79). Similar results were obtained by examining PMA+ionomycin-treated T cells from the LP of huLangerin-DTA mice (302). That is, a lower frequency of IL-17-producing T cells was found when $CD4^+$ cells T cells from the LP of huLangerin-DTA were stimulated *ex vivo* with PMA+ionomycin compared to T cells from controls (302). In contrast to these data, we found no difference in the frequency of IL-17-producing $CD4^+$ and $CD8^+$ T cells between Cre^+ and Cre^- mice infected with *S. typhimurium*. Furthermore, a similar frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells was found in Cre^+ and Cre^- mice infected with *S. typhimurium*. This is consistent with data showing similar IFN-γ^+CD4^+ T cell numbers in siLP of Cre^+ and Cre^- or huLangerin-DTA mice and controls stimulated with PMA and ionomycin (79,302). Furthermore, Persson et al. found that $CD103^+CD11b^+$ DC promote Th17 differentiation in the MLN and, in *ex vivo* experiments using sorted cells, that $CD103^+CD11b^+$ DC are a predominant source of IL-6, which is necessary for Th17 cell differentiation (79). Based on these data, IL-17 and IL-6 were measured in lysates of PP, siLP and MLN of naïve and *Salmonella*-infected Cre^+ and Cre^- mice. However, no differences were found in the production of these cytokines in whole organ lysates of *Salmonella*-infected Cre^+ and Cre^- mice at days 3 and 6 p.i. These data suggest that IL-6 production by other cells seems to be enough to drive Th17 differentiation during *S. typhimurium* infection. Altogether, the data suggest that the reduced $CD103^+CD11b^+$ DC subset in Cre^+ mice does not have a major impact on the induction of effector T cells during *Salmonella* infection.

$CD103^+CD11b^+$ DC influence the production of serum IgM during *Salmonella* infection

Intestinal DC play a role in production of intestinal IgA (89,304,305). Furthermore, $CD103^+CD11b^+$ have the capacity to induce the development of IgA^+ cells due to the synthesis of retinoic acid (82). In addition, $CD103^+CD11b^+$ DC have been shown to induce OVA-specific IgG in serum (72). As antibodies are induced during *Salmonella* infection (280,306), we measured

Salmonella-specific IgA in feces and IgM and IgG in serum of Cre^+ and Cre^- mice at days 3, 6 and 13 p.i. Similar low *Salmonella*-specific IgA was found in the feces of Cre^+ and Cre^- mice up to day 13 p.i. On the other hand, higher IgM, but not IgG, was detected in the serum of Cre^+ mice compared to Cre^- mice at day 6 p.i., which was not apparent at day 13 p.i. *Salmonella*-specific serum IgG was detectable above background only at day 13 p.i. and did not differ between Cre^+ and Cre^- mice.

Overall, the data in paper III show that the absence of intestinal $CD103^+CD11b^+$ DC has little if any effect on *Salmonella* burden in intestinal tissues and spleen, or mechanisms important in host survival to *Salmonella* infection such as IFN-γ and IL-17 production as well as antibodies. However, it is important to consider the constraints of the mouse model used. For example, reduction of the $CD103^+CD11b^+$ DC in Cre^+ mice is due to a survival disadvantage of this DC subset (79) rather than targeted ablation as in huLangerin-DTA mice (302). Indeed, the death of $CD103^+CD11b^+$ DC in Cre^+ mice could be potentiated by the infectious environment, since it has been shown that *Salmonella* induces death of DC in the MLN of infected mice (307). The Irf4-Cre mouse model may thus not be the optimal to study the role of $CD103^+CD11b^+$ DC in *Salmonella* infection. Instead, other models with a definite knockout of the subset, such as huLangerin-DTA mice or NOTCH2 knockouts, which also display a selective depletion of the $CD103^+CD11b^+$ DC subset (34), may be better suited for infection studies. Moreover, to further investigate the role of the intestinal $CD103^+CD11b^+$ DC in *Salmonella* infection, more refined experiments investigating T cell responses could be performed. For instance, the dynamics of T cell subset differentiation in primary and secondary *Salmonella* infection of Cre^+ mice could be analyzed through the expression of the T cell transcription factors Tbet, Foxp3, GATA-3 and RorγT and different times post infection. In addition, similar experiments as the ones presented here could be performed with mouse models that lack other DC subsets, such as $BATF3^{-/-}$ mice that lack $CD103^+CD11b^-$ DC (21), and even mice with two DC subsets

knocked out, to be able to define distinct as well as overlapping functions of each intestinal DC subset in *Salmonella* infection.

4.2 Role of the intestinal microbiota in *Salmonella* infection

A very important feature of the intestinal microbiota is that it offers the host protection against pathogens. However, as discussed above, the microbiota can also promote infection. Although some studies have reported interactions between *Salmonella* and intestinal commensals at an initial colonization stage, little was known about the effects of the microbiota at later stages of the infection. Thus, the aim of Paper II was to address the influence of the intestinal microbiota one week post *Salmonella* infection.

Paper II

Higher bacterial burden in MLN of GF and ABX mice

Germ-free (GF) and conventionally raised (CONV-R) mice were orally infected with *S. typhimurium* and CFU in different organs 3 and 6 days p.i were analyzed. The results suggested that the initial bacterial penetration and seeding of intestinal as well as systemic tissues (represented by the spleen) were not altered in the absence of the intestinal microbiota. This is supported by data showing that PP, siLP and spleen of GF and CONV-R mice had similar bacterial loads. However, a higher bacterial burden in the MLN of GF mice compared to CONV-R mice was observed at both time points. As discussed before, myeloid cells such as inflammatory monocytes and neutrophils infiltrate PP and MLN early after infection and are crucial for bacterial clearance (308). The recruitment of these cell populations in GF mice was thus assessed. Similar numbers of inflammatory monocytes and neutrophils in GF and CONV-R mice during *Salmonella* infection were detected, which indicates that the recruitment of these cells is not altered in the absence of the microbiota. However, it has been reported that neutrophils from GF mice are less efficient at killing *Streptococcus pneumoniae* and

Staphylococcus aureus (309) and other studies showed that macrophages of GF mice are less functional than those from CONV-R (310,311). Therefore, whether inflammatory monocytes, macrophages and neutrophils recruited in *Salmonella*-infected GF mice are fully functional remains to be determined. Moreover, as discussed in sections 1.2 and 1.3, antimicrobial peptides play a role in defense against intestinal pathogen colonization (206) and could contribute to the higher CFU in the MLN of GF mice. However, cells that produce these peptides are located mainly in the intestine, which would be predicted to influence CFU in the siLP as well as the MLN. However, no differences in CFU in the siLP of GF and CONV-R mice were found. This suggests that differences in antimicrobial peptides in GF and CONV-R mice are a not major contributor to differences in CFU in the MLN of GF and CONV-R mice.

The intestinal microbiota protects the host against infection by pathogenic microorganisms through the so called "colonization resistance", which involves competition of intestinal pathogens and the microbiota for space and nutrients (140). This phenomenon could explain the higher *S. typhimurium* burden in MLN of GF mice. To test this hypothesis, an alternate stragety was used to diminish the intestinal microbiota, namely, treating mice with antibiotics (herein called ABX mice) (312). After antibiotic treatment for three days, ABX mice were orally infected wtih *S. typhimurium* infection and CFU in intestinal tissues were compared with infected controls. Similar to the results with GF and CONV-R mice, ABX mice displayed a higher bacterial burden in the MLN compared to control mice. The fact that both models with a diminished or absent microbiota displayed higher bacterial burdens in the MLN could suggest that the lack of competition between *Salmonella* and the microbiota results in a higher degree of colonization in MLN. However, if this was the case, it would be expected to also find higher CFU in siLP as has been reported previously (313,314). This, however, was not the case. Moreover, the colonization resistance phenomenon has been mostly associated with the ability of *Salmonella* to colonize the colon using the

streptomycin mouse model (155,315). Additional studies have provided data in the context of increased CFU in the MLN of mice with reduced microbiota. For example, based on higher CFU in the MLN of ABX mice or MyD88$^{-/-}$ mice infected with non-invasive *Salmonella* compared to infected controls, Diehl et al concluded that the microbiota regulates trafficking of bacteria to the MLN in a MyD88-dependent fashion (91). Consistent with this, antibiotic treatment that diminishes the intestinal microbiota results in increased penetration of commensals through goblet cell-associated antigen passages, allowing increased bacterial access to the MLN (92). This suggests that the higher bacterial burden in the MLN of GF and ABX mice is a consequence of the lack of signals generated from the microbiota that normally prevent bacterial penetration to the MLN.

Higher IFN-γ production in the MLN of GF and ABX mice

A higher bacterial burden in the MLN of GF and ABX mice compared to controls was detected at 6 days p.i., which is in the time frame of initiation of adaptive immunity. The focus of the study was therefore turned to the T cell response. Characterization of the T cell compartment in the MLN of *S. typhimurium*-infected GF and CONV-R mice showed that GF mice have fewer TCRβ^{+}, CD4^{+} and CD8^{+} T cells compared to CONV-R. This is consistent with data on T cell numbers in GF mice under steady state conditions (134,136). No alterations in T cell numbers were found in the MLN of ABX mice compared to controls. However, both GF and ABX displayed an elevated frequency of IFN-γ-producing NK, NKT, CD4^{+} and CD8^{+} T cells in the MLN. To determine whether the increase in IFN-γ-producing cells was a consequence of the elevated bacterial burden in the MLN, the number of CFU were plotted against the frequency of IFN-γ-producing cells for each individual mouse to determine if a correlation existed. Whereas for ABX mice a positive correlation was observed, no correlation was apparent for GF mice. This indicates that the increase in IFN-γ-producing cells in the MLN of ABX could simply be a consequence of the higher CFU in this tissue. Moreover, it

suggests that, in addition to the severity of the infection, other mechanisms influence the production of IFN-γ in GF mice. One possibility is IL-12, which stimulates the production of IFN-γ (316). We thus measured the concentration of IL-12 in the MLN of infected animals to determine if differences in this cytokine could contribute to the higher frequency of IFN-γ-producing cells in the MLN of GF and ABX mice. However, no differences in the level of IL-12p70 were found in MLN lysates of GF and ABX mice compared to their controls at this time point. Overall the data suggest that the absence of the intestinal microbiota in GF mice, or the diminished microbiota in ABX mice, promotes *Salmonella* translocation to MLN inducing inflammation that is reflected in increased IFN-γ production.

IP infection of GF and ABX mice does not result in elevated bacterial burden or IFN-γ-producing cells in the MLN

To further confirm the role of the intestinal microbiota in IFN-γ production, GF, ABX and their controls were infected IP with *S. typhimurium*. *Salmonella* infections via the IP route mimic the systemic phase of the infection, bypassing the penetration of bacteria through intestinal cells and consequently also the effects of the microbiota in the initial phase of the infection. In contrast to the results obtained with an oral infection, no differences in the bacterial burden in the MLN of GF or ABX relative to their respective controls were found. Furthermore, IP-infected GF, ABX and their controls displayed a similar frequency of IFN-γ-producing NK, $CD4^+$ and $CD8^+$ T cells in the MLN. Thus, in contrast to an oral infection route, infecting GF or ABX mice with a non-oral route does not result in increased bacterial burden or IFN-γ-producing cells in the MLN. This supports an influence of the intestinal microbiota on penetration of oral pathogens to the MLN and the ensuing inflammatory response.

Altogether, the data in paper II shows that a diminished or absent intestinal microbiota results in a higher bacterial burden in the MLN as well as an increased frequency of IFN-γ-producing cells at

day 6 p.i., suggesting a role of the microbiota in the host response to oral *Salmonella* infection. To continue to investigate the role of the intestinal microbiota in immune responses to *Salmonella* infection, the production of other cytokines important in immunity to *Salmonella*, such as TNF-α, could be addressed. Furthermore, memory responses to *Salmonella* infection in the absence of the microbiota have not been examined and constitute an interesting piece of the puzzle of the host-microbiota-*Salmonella* interactions.

5 CONCLUSIONS

Paper I

- Synergistic effects of CD40 and MyD88 do not influence host survival to *Salmonella* infection, bacterial burden in intestinal tissues or serum levels of IFN-γ and IL-10 during infection.
- Cooperativity between CD40 and MyD88 in DC on T cell functions such as proliferation and production of IL-10 is observed in co-cultures and is influenced by the complexity of the antigen.

Paper II

- The MLN of GF and ABX mice display a higher bacterial burden, likely due to the increased intestinal bacterial translocation to the MLN due to the lack of the microbiota.
- While the higher IFN-γ production in ABX mice seems to be a consequence of the severity of the infection, additional effects of the lack of immune signals provided by the microbiota from birth may influence IFN-γ production in GF mice.

Paper III

- Reduced $CD103^+CD11b^+$ DC in the MLN does not influence *Salmonella* colonization of intestinal tissues or spleen, or mechanisms important in host survival to *Salmonella* infection such as IFN-γ production and antibody production.

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