Dendritic Cell Maturation and Death during *Salmonella* Infection

The role of pro-inflammatory cytokines and MyD88

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To my family

Dendritic Cell Maturation and Death during *Salmonella* infection
The role of pro-inflammatory cytokines and MyD88

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Abstract

The costimulatory molecules CD80 and CD86 are required for dendritic cells (DC) to induce tolerance and immunity. They have overlapping but distinct effects on costimulation and are upregulated during DC maturation. This thesis investigates the regulation of CD80/CD86 expression in vivo on DC during *Salmonella* infection.

After oral *Salmonella* infection, DC in Peyer´s patches (PP), mesenteric lymph nodes (MLN) and spleen upregulated costimulatory molecules almost simultaneously despite differential seeding of these organs with bacteria. Costimulatory molecules were also induced on TNF/iNOS-producing $CD11c^{int}CD11b^{+}$ DC that accumulated in infected organs. The $CD11c^{int}CD11b^{+}$ DC were efficient at bacterial uptake but, in contrast to conventional DC, failed to process and present *Salmonella* Ag on MHC-II.

Using different gene-deficient mice, the pathways controlling CD80/86 upregulation on DC during *Salmonella* infection were dissected. Upregulation of CD80 was strictly dependent on the Toll-like receptor adaptor MyD88, whereas upregulation of CD86 was mediated by both MyD88-dependent and -independent factors. The pro-inflammatory cytokine TNF was identified as one MyD88-dependent factor required for optimal upregulation of CD80/86 in the MLN. In the absence of MyD88, upregulation of CD86 was mediated by type I interferons. However, the contribution of type I interferons to CD86 upregulation in wild type mice is only marginal, since mice lacking the type I interferon receptor (IFN-αβR) showed no major defects in CD80/86 upregulation. Despite the abrogated upregulation of CD80/86 on DC of $TNFR1^{-/-}$, $MyD88^{-/-}$ or $MyD88^{-/-}$IFN-$\alpha\beta R^{-/-}$ mice, DC directly associated with bacteria upregulated costimulatory molecules independently of these factors.

Pro-inflammatory signaling not only upregulated costimulatory molecules on DC during *Salmonella* infection, but also mediated DC death. Thus, MyD88-dependent production of TNF induced DC death in *Salmonella*-infected mice. $CD8\alpha^{+}$ DC were most susceptible to infection-induced cell death as assessed directly ex vivo by Annexin-V and 7AAD staining, whereas recruited $CD11c^{int}CD11b^{+}$ DC were completely resistant.

Thus, the inflammatory environment imprints a distinct pattern of costimulatory molecules on DC, with MyD88-dependent factors controlling the upregulation of CD80. However, MyD88-dependent factors also induce DC death during *Salmonella* infection, which is likely to have a negative impact on anti-bacterial immunity.

Keywords: Dendritic cells, costimulatory molecules, bacterial infection, pro-inflammatory cytokines, Toll-like receptors, Ag presentation, cell death

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Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III).

I. Malin Sundquist and Mary Jo Wick. TNF-α-dependent and -independent maturation of dendritic cells and recruited $CD11c^{int}CD11b^{+}$ cells during oral *Salmonella* infection.
J. Immunol. 175:3287-98 (2005).

II. Miguel A. Tam*, Malin Sundquist* and Mary Jo Wick. MyD88 and IFN-αβ differentially control maturation of bystander but not *Salmonella*-associated dendritic cells or $CD11c^{int}CD11b^{+}$ cells during infection.
Submitted manuscript. *Authors contributed equally.

III. Malin Sundquist and Mary Jo Wick. *Salmonella* induces apoptosis of $CD8\alpha^{+}$ dendritic cells in the draining lymph node via MyD88-dependent production of TNF.
Manuscript.

Paper I is printed with permission from the publisher.

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Abbreviations

7AAD	7-aminoactinomycin D
Ag	Antigen
c-FLIP	Cellular FADD-like interleukin-1-converting enzyme inhibitory protein
CLIP	MHC class II-associated invariant chain peptide
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocyte
CTLA-4	CTL-associated antigen-4
DC	Dendritic cells
eGFP	Enhanced green fluorescent protein
FADD	Fas-associated death domain protein
FAE	Follicle-associated epithelium
Flt3L	Flt3 ligand
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HLA-DM	Human leukocyte antigen-DM
IFN	Interferons
IL	Interleukin
ILF	Isolated lymphoid follicle
iNOS	Inducible NO synthase
int	Intermediate
i.p.	Intraperitoneal
IRF	IFN-regulatory factor
IRAK	IL-1 receptor-associated kinases
i.v.	Intravenous
LCMV	lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LT-α	Lymphotoxin-α
MAL	MyD88-adaptor-like
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation-associated gene-5
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MyD88	Myeloid differentiation factor 88
NLR	NOD-like receptor
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
Nramp1	Natural resistance associated macrophage protein 1
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PP	Peyer´s patches
RIG-I	Retinoic acid-inducible gene I
RIP-1	Receptor-interacting protein-1
RLR	RIG-1-like receptor
SARM	Sterile α- and armadillo-motif-containing protein
SED	Subepithelial dome
SPI	*Salmonella* pathogenicity island
STAT	Signal transducers and activators of transcription
TACE	TNF-converting enzyme
TAP	Transporter associated with antigen processing
Th	T helper
TIR	Toll/IL-1 receptor homology domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter inducing IFN-β
TSLP	thymic stromal lymphopoietin
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

Introduction

The immune system can be compared to an army where different units are strategically positioned to optimize host defense against invading pathogens. Dendritic cells (DC) belong to the innate immune system and are widely distributed throughout the body, particularly dense at sites of pathogen entry. This makes them ideally situated to detect and phagocytose invading microorganisms. Other cells of the innate immune system, such as phagocytes and NK cells, are rapidly summoned at sites of infection by the host's alarm system, chemokines and pro-inflammatory cytokines. In contrast, T and B cells of the adaptive immune system are contained within a relatively small area of the host, circulating between the lymphoid organs, until activated by a DC that has encountered a pathogen for which the T cell is specific.

The innate immune system is evolutionary old compared to the adaptive immune system, which developed in jawed vertebrates. Pathogen recognition by the innate immune system relies on germline-encoded receptors, which recognize conserved molecular patterns on different classes of microorganisms. Upon ligand binding an inflammatory response is initiated that limits the invasion of the pathogen. Thus, the innate immune system constitutes a first-line of defense against pathogens. The adaptive immune system can via gene rearrangements and somatic diversifications generate receptors of almost unlimited specificity. The space restriction of naïve T cells to lymphoid organs increases the likelihood of a T cell meeting a DC presenting the specific antigen (Ag). Furthermore, the adaptive immune system is capable of clonal expansion and generation of long lasting memory cells that quickly eradicate pathogens upon second encounter. However, the innate immune system plays a crucial instructive role, influencing the magnitude, duration, and type of adaptive response generated.

Pattern-recognition receptors

The immune system has the intricate task of specifically recognizing and eradicating a diverse range of pathogens without harming the host. To distinguish self from invading pathogens, the immune system relies on pattern-recognition receptors that recognize evolutionary stable molecular patterns expressed by microbes. It is intriguing that our adaptive immune system, which can generate T and B cells of almost unlimited specificity, uses a limited number of pattern-recognition receptors to detect invading pathogens and induce DC maturation, a process that is a pre-requisite for most adaptive immune responses. A distinction can be made between receptors that bind microbial structures and those that, in addition to binding, initiate a signaling cascade that alerts the host to the infection and modulates the ensuing immune response. Among the latter are pattern-recognition receptors such as the Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1-like receptors (RLRs), and C-type lectin receptors (CLRs).

Toll-like receptors

The evolutionary conserved TLRs are a family of trans-membrane proteins that so far have 11 members in humans and 13 members in mice. This is a relatively low number compared to the 222 TLRs present in sea urchin (1). Each TLR recognizes distinct pathogen-associated molecular patterns (PAMPs) derived from bacteria, viruses, protozoa and fungi (Table 1). TLR1, 2, 4 and 6 are expressed at the plasma membrane

and recognize a broad range of microbe-derived lipid structures (2). TLR5 and 11 specialize in proteins, recognizing flagellin and a profilin-like protein, respectively. A third class of TLRs, including TLR3, 7, 8 and 9 are localized intracellularly in the endoplasmic reticulum or endosomes where they detect nucleic acids from viruses or bacteria (2).

Table 1. Location, ligands and adaptor usage by TLRs.

Toll-like receptors	Location	Ligands	Ligand-carrying microorganisms	Adaptor molecules
TLR2/1	Plasma membrane	Triacyl lipopeptides	Bacteria, parasites	MAL/MyD88
TLR2/6	Plasma membrane	Diacyl lipopeptides, LTA, zymosan	*Mycoplasma*, Gram-positive bacteria, yeast	MAL/MyD88
TLR4	Plasma membrane	LPS	Gram-negative bacteria	MAL/MyD88, TRAM/TRIF
TLR5	Plasma membrane	Flagellin	Flagellated bacteria	MyD88
TLR11	Plasma membrane	Profilin-like protein	*Toxoplasma*	MyD88
TLR3	ER/endosomes	ds RNA	Viruses	TRIF
TLR7	ER/endosomes	ss RNA	RNA viruses	MyD88
TLR8	ER/endosomes	ss RNA	RNA viruses	MyD88
TLR9	ER/endosomes	CpG DNA	Bacteria, viruses	MyD88

ER, endoplasmic reticulum; LTA, lipoteichoic acids; ds, double-stranded; ss, single-stranded.

TLRs are expressed as homo- or heterodimers on a vast variety of immune- and non-immune cells. The extracellular domain is composed of leucine rich repeats and participates in recognition of PAMPs. Intracellular signaling is initiated by the cytoplasmic TIR domain, which is homologous to the cytoplasmic domain of the IL-1 receptor (3). Ligand binding, either directly or via accessory molecules, is believed to induce a conformational change that brings the intracellular TIR domains in the dimer together (4). The conformational change allows recruitment of TIR domain-containing adaptor proteins. So far, five adaptors have been described that are differentially used by the TLRs (Table 1). MyD88 is utilized by all TLRs except TLR3, which signals via TRIF (5). In addition, TLR4 can signal via both MyD88 and TRIF. Some TLRs require a bridging adaptor, Mal or TRAM, to recruit MyD88 or TRIF, respectively (Table 1) (5). The fifth adaptor, SARM, is a negative regulator of the TRIF-dependent signaling pathway of TLR3 and TLR4 (6).

The MyD88-dependent pathway signals via members of the IRAK family and TRAF6, leading to activation of MAP-kinases and NF-κB (Fig. 1)(5, 7). This results in production of pro-inflammatory cytokines such as TNF, IL-1 and IL-6. Downstream of TLR7, 8 and 9 the MyD88-dependent pathway activates IRF7 in addition to NF-κB, which leads to production of type I interferons (type I IFN) (8, 9). The TRIF-dependent pathway of TLR3 and TLR4 leads to activation of IRF3 and IRF7 via TRAF3, as well as activation of NF-κB (10-13). This leads to production of type I IFN and certain chemokines such as IP-10 and CCL5 (6, 10, 11, 14, 15).

Thus, TLR-mediated detection of microbes activates NF-κB and IRFs, which control the expression of several genes involved in host defense, including pro-inflammatory cytokines and chemokines that attract phagocytes and other immune cells to the site of infection. Depending on the cell type being infected, the TLR-mediated response may differ. Furthermore, the distinct signaling pathways used by different TLRs and the synergy between TLR ligands during microbial infection may contribute to the development of adequate responses to different pathogens.

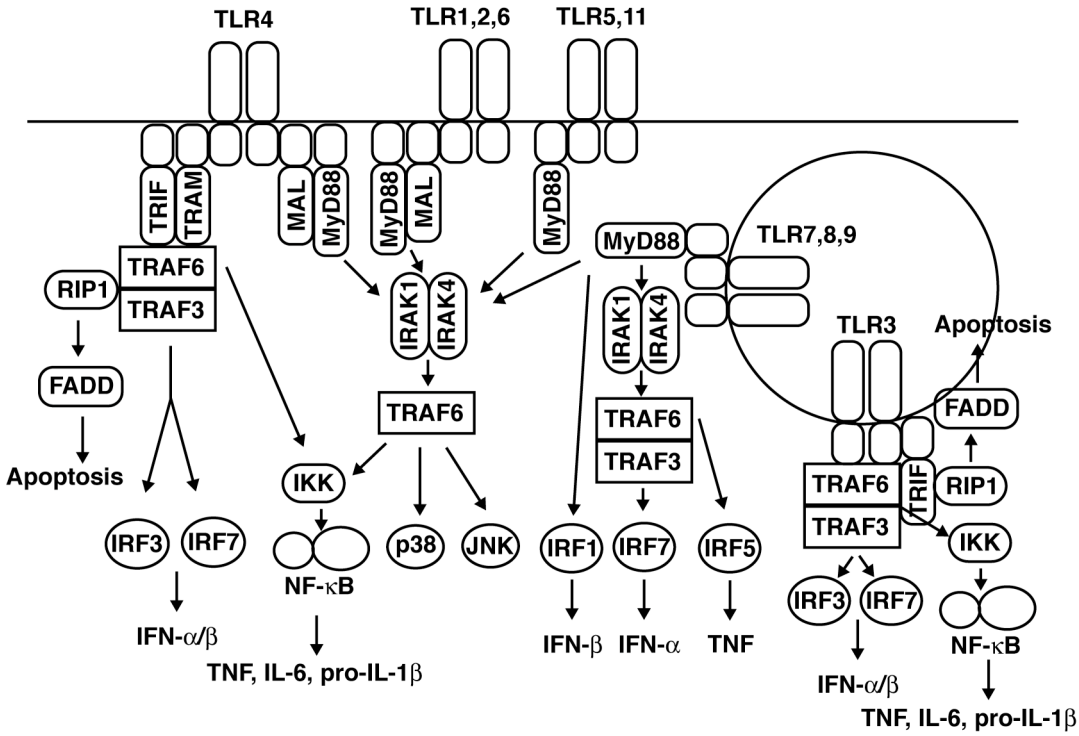

Figure 1. *TLR signaling pathways*. Signaling via MyD88 leads to activation of NF-κB and the MAP-kinases p38 and JNK via IRAK1/4, TRAF6 and the inhibitor of NF-κB kinase (IKK) complex. TLR3 and TLR4 signals via TRIF, which leads to activation of IRF3/7 and NF-κB. TRIF also recruits FADD via RIP-1, activating the apoptosis pathway. MyD88-dependent activation of NF-κB controls the production of pro-inflammatory cytokines such as TNF, IL-6 and pro-IL-1β, whereas activation of IRFs leads to production of type I IFN. TRIF-dependent activation of IRFs and NF-κB leads to production of type I IFN and chemokines such as CCL5 and IP-10.

TLR-independent pathogen recognition

Since the discovery of TLRs, other receptors mediating microbe detection have been identified. Among these are the intracellular NLRs and RLRs as well as the surface expressed CLRs. The NLR family has over 30 members in humans and is characterized by the presence of a NACHT domain and leucine-rich repeats (16). However, ligands have only been identified for a minority of the NLRs. The NLRs NOD1 and NOD2 recognize different substructures of bacterial peptidoglycan, which leads to activation of NF-κB (16). On the other hand, ligand recognition by the NLRs IPAF and NALP3 promotes assembly of the inflammasome and the subsequent Caspase-1-mediated cleavage of pro-IL1β, pro-IL18 and pro-IL33 into their mature forms. IPAF recognizes bacterial flagellin (17, 18) whereas the NALP3 inflammasome is activated by several stimuli such as bacterial RNA, bacterial toxins, uric acid crystals and ATP (19-21). Several of the ligands for the NALP3 inflammasome have in common that they mediate K^+ efflux (22). Interestingly, the TLR and NLR pathways cooperate in the induction of IL-1β production. Thus, TLR signaling is required for production of pro-IL1β whereas NLR signaling activates Caspase-1, which cleaves pro-IL1β.

RLRs such as RIG-1 and MDA5 are located in the cytoplasm where they mediate detection of viral RNA, resulting in production of type I IFN (23). RIG-1 is essential for the response to a set of RNA viruses including *Flaviviridae*, whereas MDA5 detects picornaviruses (24, 25). RIG-1 was shown to distinguish viral ssRNA from host RNA in the cytosol based on the presence of a 5´-triphosphate moiety (26).

The CLRs encompass a large family of surface expressed pattern-recognition receptors that recognize glycans. Although the majority are primarily involved in Ag uptake, some CLRs such as Dectin-1 have been shown to trigger intracellular signaling cascades upon ligand binding. Dectin-1 binds fungal β-glucans, which recruits the tyrosine kinase Syk and initiates a signaling cascade that results in NF-κB activation and production of cytokines (27, 28). Thus, the host relies on several types of pattern-recognition receptors that are localized in different cellular compartments to detect microbial infections.

Pro-inflammatory cytokines

The recognition of microbes by pattern-recognition receptors results in production of pro-inflammatory cytokines, which orchestrate innate immune defenses. Pro-inflammatory cytokines can also synergize with TLR signaling pathways to reinforce or suppress signaling. Two pro-inflammatory cytokines, TNF and type I IFN, are particularly relevant for this thesis and will be reviewed in more detail below.

TNF

TNF is a powerful pro-inflammatory cytokine that is essential for surviving of infections with bacterial pathogens, intracellular parasites, as well as some viruses including lymphocytic choriomeningitis virus (LCMV) (29). During infection, TNF induces production of chemokines and other pro-inflammatory cytokines such as IL-1β that mediates leukocyte recruitment (29-32). In addition, TNF contributes to granuloma formation, which is believed to prevent the spread of virulent bacteria (29). TNF also inhibits the replication of intracellular pathogens and can directly kill infected cells. Indeed, TNF was originally identified based on its ability to induce tumor cell death (33). However, TNF could not be used in treatment of cancer patients because of the severe side effects, which range from influenza-like syndromes to septic shock. On the contrary, pharmaceuticals that block the activity of TNF are used today to treat chronic autoimmune disorders such as rheumatoid arthritis and Crohn´s disease (34).

TNF is produced as a membrane-bound protein (35). Proteolytic cleavage by the metalloproteinase TACE releases a soluble form of TNF (36). Both the membrane-bound and soluble forms of TNF are arranged in trimers and are capable of signaling through the receptors, TNFR1 and TNFR2. TNFR1 is constitutively expressed in most tissues and is responsible for the majority of the biological activities of TNF (37, 38). In addition to TNF, the closely related cytokine lymphotoxin α (LT-α) utilizes TNFR1 for signaling. Binding of TNF to TNFR1 leads to recruitment of the adaptor protein TRADD to the intracellular death domain. TRADD can then assemble two signaling complexes depending on the location of the receptor. At the plasma membrane, TRADD associates with TRAF2 and RIP-1, which leads to rapid activation of NF-κB and MAP kinases (37). If internalized, a cytoplasmic complex composed of TRADD, RIP-1, FADD and Caspase-8 is formed, which mediates TNF-induced apoptosis (39, 40). Activation of NF-κB induces the expression of several anti-apoptotic proteins, including c-FLIP that inhibits the activation of Caspase-8 (41). Thus, the two pathways balance each other, and TNF can only induce apoptosis in cells that contain low levels of c-FLIP (41). In summary, TNF is crucial for host defense. However, if unregulated, it can cause chronic inflammation, generalized wasting and, at high levels, septic shock.

Type I interferons

While TNF is crucial for combating bacteria, immunity to viral infections is typically coordinated by type I IFN. These pleiotropic cytokines are composed of 13 IFN-α proteins and 1 IFN-β that share a common receptor (42). Upon ligand binding, STAT1 and STAT2 are recruited to the receptor and become phosphorylated, after which they dissociate from the receptor and translocate to the nucleus. In the nucleus STAT1/STAT2 associate with IRF9 and initiate transcription of IFN-α/β-inducible genes (42). Indeed, micro array data have revealed that type I IFN regulate the expression of hundreds of genes (43). Some of these genes directly confer an "antiviral state" to host cells. For example, type I IFN arrest the cell cycle, mediate degradation of viral RNA, block mRNA translation and sensitize cells for apoptosis (44). In addition, type I IFN modulate immune responses by NK cells and cytotoxic T lymphocytes (CTLs), which are responsible for killing infected host cells. Briefly, type I IFN induce the expression of chemokines that attract and activate these cytotoxic cells (45, 46). Furthermore, type I IFN induce the expression of cytokines such as IL-15, which promotes the expansion of NK cells and CTLs and the maintenance of memory CD8 T cells (47).

Host detection of viral infection relies on the recognition of viral nucleotides in endosomes (by TLR3, 7, 8 or 9) or in the cytosol (by RIG-1 or MDA5). However, IFN-β can also be induced via the TRIF-dependent pathway of TLR4 after recognition of LPS from Gram-negative bacteria. A role for type I IFN in bacterial infection came from studies showing that mice lacking the type I IFN receptor were more susceptible to infection with the Gram positive bacterium *Listeria monocytogenes* (48-50). The ability of LPS to induce production of type I IFN via the TLR4-TRIF pathway implies a role for these cytokines during infection with Gram-negative bacteria.

Dendritic cells

DC constitute an important link between the innate and adaptive immune system, since they are the only cells capable of efficiently activating naïve T cells. These remarkable cells were discovered in mouse spleen in 1973 by Steinman and Cohn (51, 52). A few years later Steinman went on to show that DC were at least a 100-fold better at activating T cells in a mixed leukocyte reaction compared to other antigen-presenting cells such as macrophages and B cells (53). Later, the superiority of DC in activating T cells was extended to Ag-specific responses in vitro (54) and in vivo (55). More recently, the crucial role of DC in generating anti-microbial T cell immunity in vivo was demonstrated using DC-ablated mice (56). Equally important, DC have a role in inducing peripheral tolerance to self Ag. This ability of DC is mediated both by clonal deletion of T cells during Ag presentation in the steady state (57, 58) and by directing regulatory T cell differentiation (59). Thus, DC are crucial for the control of adaptive immune responses and, as will be discussed below, innate signals are instructive in this process.

Antigen processing and presentation

DC present endogenous Ag on MHC-I for CD8 T cells, whereas exogenous Ag can be presented on MHC-II for CD4 T cells or cross-presented on MHC-I for CD8 T cells. Peptides derived from endogenous Ag for presentation on MHC-I are generated by the proteasome or other enzymes in the cytosol, and transported into the endoplasmic reticulum via TAP. They are then loaded onto MHC-I by a protein complex including

tapasin, which physically links MHC-I to TAP as well as the chaperone calreticulin (60). After peptide loading, MHC-I molecules are transported to the cell surface.

In contrast, peptide loading onto MHC-II occurs in late endosomal compartments after cleavage of the stabilizing invariant chain and HL-DM-mediated exchange of the invariant chain peptide, CLIP, present in the peptide-binding groove (60). Peptides are generated from internalized Ag in endosomes/lysosomes by proteolysis. For efficient generation of immunogenic peptides, the activity of lysosomal proteases needs to be tightly controlled (61). Indeed, the superiority of DC over other phagocytic cells in Ag presentation depends partially on their less aggressive lysosomal degradation, which favors Ag presentation over microbe killing (62).

In addition to direct presentation of peptides derived from endogenous Ag on MHC-I, DC have the ability to cross-present exogenous Ag to CD8 T cells. The mechanism of cross-presentation is not entirely clear and multiple pathways have been described. For example, cross-presentation can be TAP-dependent or TAP-independent, although TAP-dependent mechanisms appear to dominate. The TAP-dependent pathway of cross-presentation may involve peptide exchange on recycling MHC-I molecules in endosomes or on the cell surface and involves Cathepsin S (63). In contrast, TAP-dependent pathways involve the transport of Ag from endosomes to the cytoplasm, and the translocation machinery, Sec61, has been implicated in this process (64). Thereafter the direct MHC-I presentation pathway may process the Ag Indeed, a recent study suggested that the mode of Ag internalization might control access to the cross-presentation pathway. Thus, Ag uptake via the mannose receptor results in proteasome-dependent cross-presentation, whereas internalization via the scavenger receptor or pinocytosis targets Ag to lysosomes and efficient presentation to CD4 T cells (65). Further, type I IFN can promote cross-presentation of viral Ag by DC during infection (66).

DC induce tolerance to self Ag in the steady state

DC are present in virtually all tissues at a low frequency, particularly at sites of pathogen entry. Despite the low frequency of DC, their size and dendritic extensions allows them to form vast networks, facilitating the surveillance of the host for invading pathogens. In the steady state DC continuously process and present captured tissue Ag to specific T cells. For example, gut DC constitutively transport fragments of apoptotic cells in the lymph from the gut to the mesenteric lymph nodes (MLN) (67). In addition, tissue Ag are presented by DC in the draining lymph nodes (68-70). Steady state presentation of Ag by DC induces, in most cases, T cell tolerance (57, 58, 71). However, T cell immunity to self Ag has also been reported after presentation by steady-state DC (70, 72).

DC maturation

When the host detects a microbial infection, DC undergo a maturation process, which enhances their ability to process and present Ag. During maturation, the phagocytosing capacity of DC is transiently increased before being shut down (73). Furthermore, DC upregulate costimulatory molecules that are required for T cell priming, and produce cytokines that direct T cell differentiation. MHC class II molecules are translocated from endosomal compartments to the cell surface and lysosmal Ag processing is increased (74-76). Mature DC upregulate the chemokine receptor CCR7, which is required for homing of tissue-resident DC to secondary lymphoid organs (77, 78). The phenotypic changes associated with DC maturation can

be induced after the direct recognition of microbial products via TLRs or indirectly via pro-inflammatory cytokines such as TNF, IL-1β or type I IFN. Importantly, DC with a mature phenotype are not necessarily immunogenic (58, 79, 80), and may induce tolerance in some settings (80, 81). Therefore, DC maturation and immunogenicity are two different things, and conclusions about DC immunogenicity cannot be drawn solely based on phenotype.

What is required to generate immunogenic DC?

It is known that certain microbial infections or simply the administration of proteins mixed or directly conjugated to adjuvants, can induce strong immunity. However, the mechanism by which pathogens and adjuvants act on DC to generate immunogenicity is not known. Studies using mixed bone-marrow chimeras and purified TLR ligands showed that only DC capable of directly sensing the TLR ligand could induce proliferation and IFN-γ production by Ag-specific T cells (79). DC lacking expression of the relevant TLR upregulated costimulatory molecules to a similar extent as their TLR-expressing counterparts, but were not able to induce T cell effector functions (79).

In addition to TLR ligands, CD40 triggering can promote T cell immunity. Thus, administration of an Ag targeted to DC results in tolerance unless administered together with an agonistic anti-CD40 antibody (82, 83). In the maturation process, DC upregulate CD40, which is engaged by CD40L on T cells during antigen presentation. The triggering of CD40 induces many responses in DC such as cytokine production, increased survival and licenses DC for CTL priming (84-92). Interestingly, the life span of DC influences their immunogenicity. Thus, enhancing the life span of DC by retroviral expression of a caspase-inhibitor under the CD11c-promoter results in DC accumulation, chronic lymphocyte activation and autoimmunity (93). Although several pathways of generating immunity are known, the series of events during which a DC acquires immunogenicity needs to be elucidated.

DC influence T cell differentiation

DC can affect T cell differentiation in several ways and different maturation stimuli may trigger distinct maturation programs in DC that direct a variety of T cell differentiation pathways. It has been known for some time that production of IL-12p70 by DC supports differentiation of CD4 T cells towards Th1 cells (94). Th1 cells express the transcription factor T-bet and are crucial in combating intracellular infections due to their production of cytokines such as IFN-γ that augments phagocyte killing (95). Interestingly, the potency of different TLR ligands to induce IL-12p70 varies widely (96) and different combinations of TLR ligands synergize to induce large amounts of IL-12p70 by DC (97). Furthermore, CD40 triggering boosts the production of IL-12 by DC, possibly as a positive feed-back mechanism provided by the T cells at the time of Ag-presentation (86). Despite the well-known role of IL-12 in Th1 differentiation, other molecules may also influence Th1 differentiation fate. Indeed, Th1 differentiation can occur in the absence of IL-12 (94, 98). A recent study showed that in the absence of IL-12, Th1 differentiation was dependent on the Notch ligand Delta 4, which was upregulated on splenic $CD8\alpha^-$ DC after LPS injection (99). Another study identified CD70 signaling as a mechanism of IL-12-independent Th1 differentiation exclusively in $CD8\alpha^+$ DC (100).

T cell differentiation towards Th2 cells requires the expression of the transcription factor GATA-3. It was recently shown that Notch-induced signaling directly regulates the expression of GATA-3, and thus Th2 differentiation fate (101,

102). Thereafter, autocrine production of IL-4 has an important role in sustaining Th2 responses. In addition to IL-4, Th2 cells produce IL-5 and IL-13, and are important in the defense against multicellular parasites (95).

A third subset of T helper cells that produce IL-17, Th17 cells, was recently described (95). Th17 cells express the transcription factor RORγt and recruit phagocytes to mucosal surfaces to combat infections with extracellular bacteria and fungi (95). TGF-β and IL-6 provide instructive signals for Th17 cell differentiation (103, 104). Similar to Th1 cells, production of IL-12 sustains and amplifies the differentiation towards Th17.

DC are not only important for inducing T cell immunity, but also for maintaining peripheral tolerance. For example, DC in the presence of TGF-β1 instruct T cells to differentiate into $Foxp3^+$ regulatory T cells that protect against autoimmune disease (59). Taken together, innate stimulation of DC triggers a maturation process that influences their ability to direct T cell differentiation. Thus, innate signals provided at the site of Ag uptake may imprint DC in different ways enabling them to provide adequate instructions for T cell differentiation.

Ag presentation in vivo

The visualization of Ag presentation to T cells in vivo has been accomplished by two-photon microscopy of lymph nodes ex vivo and intravital microscopy of lymph nodes. Such studies have revealed that T cells migrate rapidly along collagen networks ensheathed by lymph node stromal cells (105). The collagen fibers provide guidance and a foothold for the T cells allowing them to scan the DC networks of the lymph node. After the appearance of Ag-bearing immunogenic DC in the lymph node, many brief contacts are formed between these DC and T cells, similar to the scanning that takes place in the steady state (106, 107). Indeed, a DC can form up to 5000 contacts with T cells per hour (108, 109). This induces the expression of activation markers such as CD69 in the T cells (106, 107). After a few hours, more stable contacts are formed between DC and specific T cells and the vigorous motility of the T cells is reduced (106, 107, 110). The T cell arrest on DC lasts about 1.5 days and is probably required for formation of the immunological synapse, the special contact site between the DC and T cell. The immunological synapse is important for signal integration of activated T cells, and many signaling molecules involved in T cell activation are concentrated in the synapse (111). Directed secretion of cytokines into the synapse has also been described (112). After the formation of stable DC-T cell contacts, T cells are trapped in the lymph node for 2-3 days until they are egress (113). The scanning behavior of T cells probably enhances the possibility of Ag-specific T cells to encounter a DC presenting the respective Ag. However, chemokines may also be involved in this. Indeed, it has been shown that CD8 T cells, which require "T cell help" for optimal activation, are recruited to DC-CD4 T cell pairs via the production of CCR5 ligands (110).

DC subsets

DC are a heterogeneous group of cells where different subsets have both overlapping and distinct functions. A gross division can be made between DC that reside in secondary lymphoid organs and DC that constitutively travel to lymph nodes from peripheral tissues via lymph. The spleen, which lacks afferent lymphatics is devoid of tissue-derived DC. The lymphoid organ-resident DC are blood-derived and are present in all secondary lymphoid organs, with a particularly high frequency in the spleen. Conventional DC that reside in secondary lymphoid organs can be identified

based on a high expression of the integrin CD11c and MHC-II. DC in peripheral tissues include Langerhans cells and interstitial DC, which have a lower expression of CD11c (114-116). Non-conventional DC such as TNF/iNOS-producing DC and plasmacytoid DC also express an intermediate level of CD11c. These cells resemble DC phenotypically but specialize in production of TNF and inducible NO synthase (iNOS) as well as large production of type I IFN, respectively.

Lymphoid organ-resident DC

DC resident in the lymphoid organs of mice are often divided into three phenotypic subsets: $CD8\alpha^+$, $CD8\alpha^-CD4^+$ and $CD8\alpha^-CD4^-$. The latter two subsets are often lumped together as $CD8\alpha^-$ DC, which constitute about 75% of splenic DC (117). In addition, a fourth subset lacking expression of CD8α, CD4 and CD11b has been described in the Peyer´s patches (PP) of the gut (118) and will be discussed below. $CD8\alpha^+$ and $CD8\alpha^-$ DC localize to distinct regions of the spleen and lymph nodes in the steady state. $CD8\alpha^+$ DC are mainly localized in the T cell zones of the spleen and lymph nodes, whereas $CD8\alpha^-$ DC are predominantly present in the surrounding areas (118-121). However, in response to infection or injection of TLR ligands, $CD8\alpha^-$ DC also migrate to the T cell zones (119, 122). The differential distribution of DC subsets in lymphoid organs is probably due to a differential expression of chemokine receptors. Indeed, $CD8\alpha^-$ DC uniquely express CCR6 (123-125), which localizes this subset to a distinct area in PP (126).

As a further reinforcement of the heterogeneity of DC, different DC subsets require the expression of distinct transcription factors for their development. Thus, $CD8\alpha^+$ DC and plasmacytoid DC, but not $CD8\alpha^-$ conventional DC, are dependent on IRF-8 for their development (127). In contrast, IRF-4 is required for development of $CD8\alpha^-$, but not $CD8\alpha^+$ DC (128). Even in the steady state, $CD8\alpha^+$ and $CD8\alpha^-$ DC preferentially express IRF-8 or IRF-4, respectively (125).

Moreover, the DC subsets are differentially equipped with pattern-recognition receptors involved in Ag uptake and DC maturation (Table 2). For example, $CD8\alpha^+$ and $CD8\alpha^-$ DC differ in the expression of CLRs such as DEC-205, DCIR2, DC-SIGN, Dectin-1 and Langerin (table 2). DC subsets also express different sets of TLRs, which, as discussed above, mediate microbe recognition and DC maturation (table 2). The subset-specific expression of pattern-recognition receptors might restrict the DC subsets involved in the response to certain pathogens. Indeed, HIV-1 exploits DC-SIGN expressed by DC in the subepithelium for transmission to T cells (129). However, Langerhans cells present in the epidermis lack DC-SIGN and utilize Langerin to capture HIV-1 (130). In contrast to DC-SIGN, Langerin targets HIV-1 to Birbeck granules where the virus is degraded, preventing further transmission to T cells (130).

DC subsets not only differ in Ag uptake, but also in Ag processing. Thus, $CD8\alpha^+$ are specialized for cross-presentation of Ag to CD8 T cells (82, 131-133). On the other hand, $CD8\alpha^-$ DC may be superior at presenting Ag on MHC-II for CD4 T cells (82, 134). This subset-specific specialization in Ag-presentation has been demonstrated both with steady state and immunogenic DC. One exception may be Fc-receptor-mediated Ag uptake, in which case Ag is cross-presented equally well by both subsets (135). The differential capacity of DC subsets in Ag presentation to CD4 and CD8 T cells is associated with increased expression of proteins involved in Ag processing for MHC-II and MHC-I, respectively (82). Thus, $CD8\alpha^+$ DC express a higher level of proteins involved in Ag processing for MHC-I and $CD8\alpha^-$ DC

preferentially express proteins involved in Ag processing for MHC-II. Further, CD8α$^+$ DC are specialized in phagocytosis of apoptotic cells (136), and may thus cross-present cell-associated Ag. This could be important in viral infections that do not target DC.

Table 2. Differential expression of pattern-recognition receptors among DC subsets

	Lymphoid organ-resident		Tissue-derived		Non-conventional	
Receptors	**CD8α$^+$ DC**	**CD8α$^-$ DC**	**LC[1]**	**dermal DC[1]**	**pDC**	**Tip-DC[1]**
TLRs						
TLR3	+	-	+	+	-	n.d.
TLR4	+	+	-	+	-	n.d.
TLR7	-	+	+	+	+	n.d.
TLR9	+	+	-	-	+	n.d.
TLR11	+	-	n.d.	n.d.	n.d.	n.d.
CLRs						
DEC-205	++	-	++	+	-	-
DCIR2	-	+	-	-	n.d.	n.d.
Dectin-1	-	+	-	+	+	n.d.
Langerin	+	-	++	-	-	-
DC-SIGN	-	+	-	+	++	n.d.

[1]Expression of TLR3-9 on Langherhans cells and dermal DC as well as expression of Langerin in Tip-DC was determined on human cells. The table is based on the references (82, 125, 130, 137-145).
LC, Langerhans cells; pDC, plasmacytoid DC; Tip-DC, TNF/iNOS-producing DC; n.d., not determined.

Tissue-resident DC

DC resident in peripheral tissues include Langerhans cells located in the skin epidermis and in mucosal epithelia, dermal DC that reside in the skin dermis and interstitial DC that are the dermal DC counterpart in tissues other than the skin. Langerhans cells can be identified based on high expression of the CLR Langerin whereas dermal and interstitial DC can be distinguished from CD8α$^-$ lymphoid organ-resident DC based on the expression of an intermediate level of CD11c, DEC-205 and CD8α (114, 116). Tissue-resident DC constitutively migrate to the draining lymph node and upregulate costimulatory molecules and MHC-II upon arrival (146). It has been assumed that tissue-resident DC are responsible for presenting Ag encountered in the skin and at mucosal sites after migration to the draining lymph node. However, this view is now changing (147, 148). As will be discussed below, the involvement of tissue-derived DC in priming Ag-specific T cells after microbial infection of the skin or mucosa is very limited (132, 149-151). The Ag cargo appears to be delivered to resident DC in the lymph node, which in turn activate specific T cells. Langerhans cells and dermal DC reach the draining lymph nodes in two waves, with the dermal DC arriving first after application of an inflammatory stimulus (114, 149). The arrival of dermal DC coincides with Ag presentation by lymph node-resident DC (149), suggesting that dermal DC transport the Ag to the lymph node and then transfer it to lymph node-resident DC. As an alternative to Ag transfer between DC, small soluble Ag may also flow freely in the lymph and reach DC present in the T cell areas (152).

The development of Langerhans cells-ablated mice has made it possible to selectively dissect the role of these cells in vivo. Such studies revealed that Langerhans cells are dispensable for contact hypersensitivity (114, 153). One study even noted an enhanced reaction in the absence of Langerhans cells (153), suggesting that Langerhans cells may have an inhibitory effect after exposure to irritating substances.

Plasmacytoid DC

Plasmacytoid DC specialize in producing large quantities of type I IFN, particularly during viral infections (154). They are non-conventional DC expressing a low to intermediate level of CD11c and MHC-II, and are $CD11b^{-}B220^{+}$. In the steady state they morphologically resemble plasma cells with their abundant endoplasmic reticulum and round shape. However, exposure to inflammatory stimuli converts plasmacytoid DC to a more dendritic shape, during which they also acquire some Ag-presenting skills (154).

TNF/iNOS-producing DC

During *Listeria* infection, Serbina et al. reported a DC subset producing TNF and iNOS that was recruited to the spleen via a CCR2-dependent mechanism (155). The DC were $CD11c^{int}CD11b^{int}Ly6C^{+}$, distinguishing them from conventional splenic DC and plasmacytoid DC. Moreover, the TNF/iNOS-producing DC expressed a high level of MHC-II and costimulatory molecules and stimulated allogenic T cells in a mixed leukocyte reaction as well as conventional splenic DC (155). At the same time, Geissman et al. reported a subset of $CCR2^{+}$ murine monocytes recruited to sites of inflammation that differentiated into CD11c-expressing cells in vivo (156). From these data it was speculated, although not yet shown, that the TNF/iNOS-producing DC develop from $CCR2^{+}$ monocytes under inflammatory conditions. Support for this came from Naik et al., who transferred $CCR2^{+}$ monocytes into recipients undergoing GM-CSF-dependent inflammation (157). Under these conditions the transferred monocytes developed into $CD11c^{int}CD11b^{hi}Ly6C^{+}$ DC expressing a high level of MHC-II and had a similar capacity to activate allogenic T cells as conventional splenic DC. Although the capacity of the monocyte-derived DC to produce TNF and iNOS was not assessed in this study, these cells could represent the TNF/iNOS-producing DC observed during *Listeria* infection (155, 158). In addition, TNF/iNOS-producing DC or phenotypically similar cells ($CD11c^{int}CD11b^{+}$ cells) have been shown to accumulate during infection, inflammation and in autoimmune disorders (157, 159-163). Recently, a role for TNF/iNOS-producing DC in the steady state induction of IgA-secreting B cells in the gut has been described (164). The function of TNF/iNOS-producing DC during infection and other inflammatory conditions need to be elucidated, particularly their contribution to priming of pathogen-specific T cells.

DC ontogeny

One of the main characteristics of DC ontogeny is the large flexibility within the system. For example, commitment to the DC lineage occurs quite late, since DC are generated from both common myeloid and common lymphoid progenitors of the bone marrow (Fig. 2) (165, 166). Downstream of these progenitors, clonogenic precursors committed to the generation of conventional and plasmacytoid DC has recently been described (167, 168). However, a clonogenic precursor that forms monocytes/ macrophages and conventional DC, but not plasmacytoid DC, has also been identified (169). The DC/plasmacytoid DC precursor and the macrophage/DC precursor are phenotypically different and, while the former is dependent on Flt3L to generate DC, the latter requires M-CSF or GM-CSF (167-169). Mice lacking GM-CSF or M-CSF have normal or slightly reduced numbers of lymphoid organ-resident DC (170). In contrast, Flt3L-deficient mice show a 90% reduction in the number of lymphoid organ-resident DC and plasmacytoid DC (171, 172). Furthermore, the DC/plasmacytoid DC precursor is more effective than the macrophage/DC precursor in generating lymphoid organ-resident DC (167-169). Thus, the DC/plasmacytoid

precursor probably represents the main intermediate DC progenitor for the steady state generation of DC resident in lymphoid organs.

Committed DC precursors then leave the bone marrow and reach the blood, where they have a very short half-life (173). After entering secondary lymphoid organs, DC precursors in the spleen are committed to the formation lymphoid organ-resident DC, but not plasmacytoid DC (Fig. 2) (157). A fraction of the intra-splenic precursors are even committed to generate either CD8α^+ or CD8α^- subsets (157). Final differentiation to lymphoid organ-resident DC thus occurs from these local progenitors that are distinct from monocytes (157).

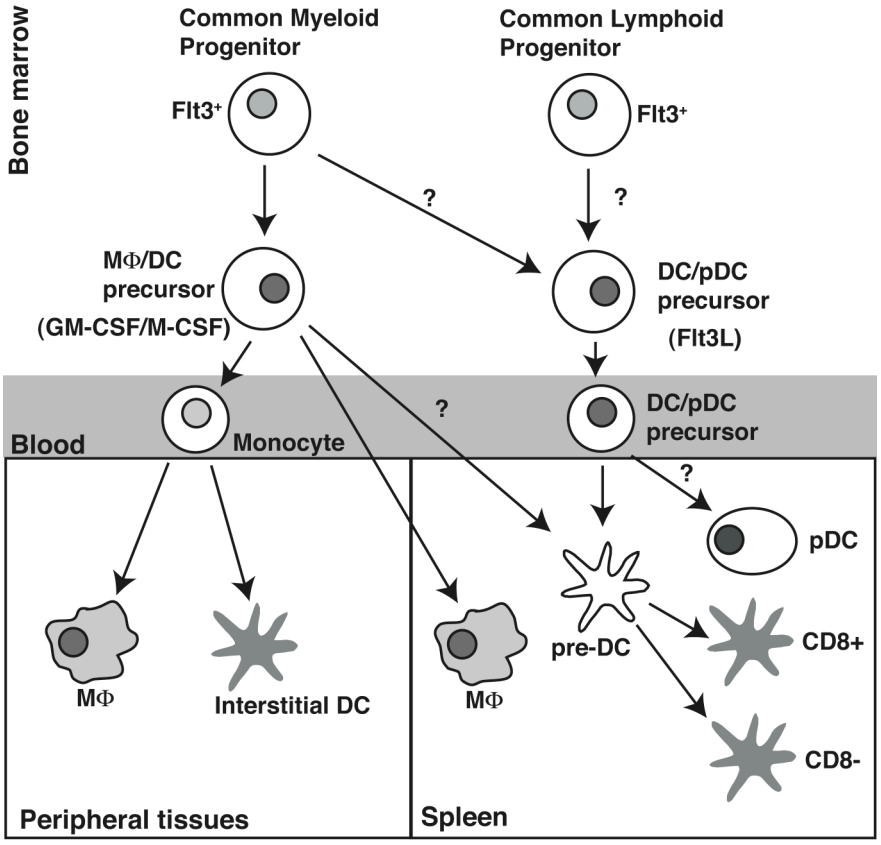

Figure 2. *DC ontogeny*. DC can be generated from common myeloid and lymphoid progenitors as long as they express Flt3. Downstream of these, two precursors that generate DC and pDC or DC and MΦ, have been identified. The DC/pDC precursor requires Flt3L whereas the MΦ/DC precursor requires GM-CSF/M-CSF. DC precursors committed to the generation of CD8α^+ and CD8α^- DC are present in the spleen. Monocytes that develop from the MΦ/DC precursor give rise to macrophages and interstitial DC in peripheral tissues. pDC, plasmacytoid DC; MΦ, macrophage.

The half-life of conventional DC in lymphoid organs was originally determined to be extremely short, only 1.5-3 days, based on BrdU labeling (174, 175). However, these studies assumed that DC were terminally differentiated, non-dividing cells. This has been challenged by studies showing that DC resident in secondary lymphoid organs are capable of proliferation (173, 176). Thus, in vivo 5% of the DC in the spleen are in cell cycle at any time (176). Indeed, studies using parabiotic mice with joined bloodstreams showed that DC underwent a limited number of cell divisions in the spleen or lymph nodes in 10-14 days (half life 5-7 days), after which they were replenished by blood-borne precursors (173).

In contrast to lymphoid organ-resident DC, Langerhans cells and interstitial DC of peripheral tissues seem to derive from monocytes in the steady state (177, 178). Furthermore, during inflammation, monocytes develop into a special DC subset described above, the inflammatory $CD11c^{int}CD11b^{+}$ DC that produce TNF and iNOS, the function of which during anti-microbial immunity remains unclear (157).

DC in gut-associated lymphoid tissues

The intestinal immune system must tolerate colonization by commensal bacteria and at the same time initiate protective immune responses against pathogenic bacteria that invade via the gut. Further, the intestinal immune system is constantly bombarded with food Ag, to which tolerance need to be maintained. Thus, in the steady state the gut-associated lymphoid tissue (GALT) is quite immunosuppressive and GALT-DC, relative to DC in other lymphoid compartments, have some unique characteristics. For example, DC from MLN and PP induce expression of the gut-homing receptors $\alpha4\beta7$ and CCR9 on T cells, which allows T effector cells to home to the gut (179, 180). The imprinting of gut-tropism is dependent on the vitamin A metabolite retinoic acid, which is produced by a subset of gut DC that express the αE integrin CD103 (181, 182). DC-derived retinoic acid also induces $\alpha4\beta7$ and CCR9 on B cells, and together with IL-6 or IL-5, promotes class-switching to IgA in activated B cells (183).

Several of the functions specific for GALT-DC have been attributed to their production of retinoic acid. Thus, retinoic acid produced by GALT-DC together with TGF-β induce expression of Foxp3 in naïve T cells and promote their differentiation into regulatory T cells with gut-homing potential (184-186). Furthermore, retinoic acid can inhibit the TGF-β- and IL-6-driven differentiation into pro-inflammatory Th17 cells (187). The promotion of gut-homing regulatory T cells and the prevention of Th17 differentiation by retinoic acid may be important mechanisms that sustain the immunosuppressive environment of the gut.

Other mechanisms for steady state immunosuppression in the gut exist. For example, intestinal epithelial cells may release factors such as thymic stromal lymphopoietin (TSLP) that conditions nearby DC to become less inflammatory (188). Thus, human monocyte-derived DC conditioned in vitro by intestinal epithelial cells secreted IL-10 and IL-6, but not IL-12, upon exposure to *Salmonella* and induced allogenic T cells to differentiate into Th2 instead of Th1 cells (188). Similarly, $CD11b^{+}$ DC resident in the subepithelial dome (SED) of PP, just beneath the follicle-associated epithelium (FAE), produce low levels of IL-12p70 and high levels of IL-10 upon activation, skewing T cell differentiation to Th2 (189). Further, plasmacytoid DC from PP produce much less type I IFN than splenic plasmacytoid DC when stimulated with influenza virus or CpG DNA (190). However, treatment of splenic plasmacytoid DC with IL-10, TGF-β and prostaglandin E2, all of which can be found in mucosal tissues, inhibited their production of type I IFN. Thus, immunomodulatory factors present in the GALT conditions local DC to become less inflammatory and perform "gut-specific functions" such as inducing gut-tropism in T and B cells and promoting IgA production. How these immunosuppressive factors are controlled and balanced in the steady state as well as during pathogen invasion are important to study.

DC subsets and anti-microbial adaptive immunity

The DC subsets seem to be differentially involved in priming of CD4 and CD8 T cells during infection with virus, parasites and bacteria. For example, during virus infection of the skin, Ag presentation to specific CD8 T cells is almost exclusively carried out

by lymph node-resident CD8α$^+$ DC, and not by skin-derived Langerhans cells or dermal DC (132, 149-151). This has been shown for herpes simplex virus-1, vaccinia virus and influenza virus, which all are cytolytic and have developed several strategies to evade the Ag-presentation machinery. Importantly, a recent study by He et al. showed that intradermal injection of lentivectors, which are not cytolytic and stably transfect DC without apparent alteration of DC function, leads to activation of specific CD8 T cells predominantly by skin-derived DC in the draining lymph node (191). In contrast, the same study showed that vaccinia virus was presented by lymph node-resident CD8α$^+$ DC, confirming previous results by Belz et al. (150, 191). These data fit with a model where skin-derived DC transfer viral Ag from the site of infection to the draining lymph node. However, the skin-derived DC are not able to activate CD8 T cells if infected with a cytolytic virus. Rather, these viruses are presented by lymph node-resident CD8α$^+$ DC specialized in the uptake of apoptotic cells and cross-presentation (82, 131-133, 136).

To complicate the picture, the route of infection influences the DC subset(s) involved in anti-viral CD8 T cell priming. In contrast to the skin, infection of the lung with influenza virus or herpes simplex virus-1 allows both lymph node-resident CD8α$^+$ DC and a DC subset derived from the lung (CD8α$^-$CD11b$^-$F4/80$^+$DEC205$^+$) to induce proliferation of specific CD8 T cells in the draining lymph node (192). The reason for the differential involvement of DC subsets in skin infection versus lung infection is not known. It could involve an increased capacity of migratory DC from the lung to cross-present viral Ag relative to skin-derived DC. Interestingly, a CD103$^+$ DC population in the lung that lack expression of CD8α and CD11b has recently been shown to be able to cross-present innocuous Ag to CD8 T cells (193).

Less is known about the DC subsets involved in priming of anti-viral CD4 T cell responses. After intravaginal infection with herpes simplex virus-2, CD11b$^+$CD8α$^-$ DC, but not CD8α$^+$ DC or Langerhans cells induce cytokine production in pre-activated CD4 T cells (194). In contrast, during oral infection with the reovirus type 1 Lang, CD8α$^+$ as well as CD8α$^-$CD11b$^-$ DC in PP induce proliferation in virus-primed CD4 T cells (195).

The role of DC subsets in initiating T cell responses to parasites has also been studied. For instance, during malarial infection with *Plasmodium chabaudi*, both CD8α$^+$ and CD8α$^-$ DC of the spleen present malaria Ag on MHC-II, as detected by hybridomas. However, only CD8α$^-$ DC can induce proliferation and IL-4/IL-10 production by transgenic Ag-specific CD4 T cells at the peak of infection (196). This study highlights the well-known notion that Ag presentation does not necessarily translate into T cell priming. While *P. chabaudi* causes a systemic infection the parasite *Leishmania major* infects the skin. This allows the relative contribution of skin-derived versus lymph node-resident DC in Ag presentation to be determined in the *Leishmania* model. Three studies have identified CD8α$^-$ DC in the draining lymph node as the main presenters of *Leishmania* Ag on MHC-II to a T cell hybridoma early after infection (197-199). The Ag presenting CD8α$^-$ DC did not derive from the skin as determined by FITC painting (197). Furthermore, Ag presentation occurred in two phases with one peaking early (day 1) and one being detectable later (day 21 and 28) after *Leishamania* infection (198). León et al. studied late phase Ag presentation to CD4 T cells during *Leishmania* infection (115). They performed extensive flow cytometric and monocyte transfer experiments to identify two subsets of Ag presenting DC that they propose are monocyte-derived dermal DC (that migrate to the draining lymph node) and monocyte-derived DC (that reach the lymph node directly

from the blood). The latter phenotypically resembles the TNF/iNOS-producing DC. During *Leishmania* infection, late Ag presentation (4 wk) was mediated by the "monocyte-derived dermal DC" and not by the "lymph node-resident monocyte-derived DC", as detected by cytokine-production in pre-activated CD4 T cells or a T cell hybridoma. The capacity of conventional DC subsets to present *Leishmania* Ag was not evaluated and, importantly, the authors do not formally show that the "dermal DC" derive from the skin.

During bacterial infection, $CD8\alpha^+$ DC induce proliferation of specific CD8 T cells in the spleen after i.v. infection with *Listeria* (200), whereas both $CD8\alpha^+$ and $CD8\alpha^-$ DC present mycobacterial Ag on MCH-II to a T cell hybridoma after i.v. infection (201). While both $CD8\alpha^+$ and $CD8\alpha^-$ DC can process and present *Salmonella* Ag in vitro (202, 203), their contribution to priming of CD4 and CD8 T cells in vivo during infection remains to be investigated.

In general, the available data suggest that $CD8\alpha^+$ DC may be primarily involved in the activation of CD8 T cells, particularly during infection with cytolytic viruses. In contrast, CD4 T cells appear predominantly, but not exclusively, to be activated by $CD8\alpha^-$ DC during viral, parasite or bacterial infection. Furthermore, local differentiation of monocytes into DC at the site of infection may contribute to Ag presentation at later stages.

Salmonella typhimurium

Salmonella enterica encompasses a large group of enteropathogenic bacteria that cause a spectrum of diseases, ranging from fairly mild enteritis to possibly fatal Typhoid fever, in a variety of hosts. Typhoid fever in humans is caused by *Salmonella enterica* serovars Typhi and Paratyphi and results in severe systemic illness leading to an estimated 20 million cases and 200,000 deaths worldwide per year, particularly in developing countries (204). A major health problem is the rapid emergence of antibiotic resistant strains (205). *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) causes gastroenteritis in humans but systemic illness resembling human Typhoid fever in susceptible mice (206). Therefore, murine *S. typhimurium* infections are commonly used as a model to understand the pathogenesis of mucosal pathogens able to cause systemic disease.

Host entry of *Salmonella*

Salmonella are Gram negative, facultative intracellular bacteria that infect via the oral route through contaminated food or water. To establish an infection, the bacteria must survive the acidic milieu of the stomach, traverse the gut mucus layer and compete with the intestinal flora to reach the gut epithelium (207, 208). Bacterial invasion occurs mainly in the distal ileum, and to a lesser extent, in the caecum (209). *Salmonella* are believed to preferentially enter through M cells located in the FAE overlying PP (210). M cells, which are specialized for transcytosis of a wide range of luminal particles, lack overlying mucus and have a very thin glycocalix (211-213). This makes the M cells rather accessible to intestinal microbes. Upon contact, *Salmonella* induce actin rearrangements in the M cells, which facilitates bacterial uptake (210, 211).

The uptake of *Salmonella* is mediated by a type III secretion system encoded in *Salmonella* pathogenicity island 1 (SPI-1) (214). The SPI-1 type III secretion system is a syringe-like machinery that injects soluble effector molecules into host cells that

induces bacterial uptake, even in non-phagocytic cells (215, 216). The expression of the SPI-1 type III secretion system is induced by environmental conditions in the distal ileum such as low oxygen and pH (217). Upon arrival at the intestinal epithelium the bacteria already express 10-100 assembled secretion systems and a pool of effector molecules ready to be injected (218, 219). The injection of SPI-1 effectors is extremely fast, occurring within minutes after bacterial contact with the host cell, and induces rapid uptake of bacteria (216, 219). Although required for invasion of non-phagocytic cells, invasion-deficient *Salmonella* lacking a functional SPI-1 type III secretion system are able to colonize the PP after oral infection, albeit less efficiently (214, 220, 221).

The M cell route into PP is believed to be the main site of entry for *Salmonella*. However, M cells are also present in the FAE overlying isolated lymphoid follicles (ILFs) that are small lymphoid aggregations in the small intestine consisting of a B cell follicle and an overlying SED that contains DC. In addition, small clusters of M cells have been observed interspersed on tips of small intestinal villi (222, 223). Indeed, a substantial number of bacteria can be cultured from the lamina propria after removal of PP (224). A recent study showed that *Salmonella* is able to infect ILFs in the small intestine, which induced the ILFs to increase in size and recruit neutrophils (224). Further, *Salmonella* has been detected in villous M cells, even in $TNF^{-/-}LT\alpha^{-/-}$ mice that lack both PP and ILFs (223).

The lamina propria, which lies beneath the villous gut epithelium, contains a dense network of DC and macrophages that express the chemokine receptor CX_3CR_1 (225-228). Some of these DC extend trans-epithelial dendrites into the gut lumen in a CX_3CR_1-dependent manner (225-227, 229). Oral infection with *Salmonella* increases the formation of trans-epithelial dendrites, and it has been proposed that lamina propria DC can sample pathogens and/or commensals in the gut lumen. However, invasive *Salmonella* can breach the intestinal epithelium independently of CX_3CR_1 (225). Further, the importance of trans-epithelial dendrites for pathogen uptake was recently challenged by a study showing that Balb/c mice are unable to form trans-epithelial dendrites, even after challenge with *Salmonella* (227). A fourth pathway for *Salmonella* entry where bacteria penetrate directly through the absorptive enterocytes in the gut epithelium, has been described in calves (230, 231). The relative contribution of these pathways to *Salmonella* invasion requires further study.

Fate of *Salmonella* in the GALT

PP in the small intestine are inductive sites for gut-oriented immune responses. A dense network of DC is present in the SED, just beneath the FAE, strategically localized for Ag uptake (Fig. 3). These DC are $CD11b^+$ or $CD11b^-CD8\alpha^-$, and express the chemokine receptor CCR6 (118, 232, 233). DC belonging to the $CD11b^-CD8\alpha^-$ subset are also present in the FAE in the steady state (118). *Salmonella* can be detected in PP 6 hr after oral infection and have been shown to co-localize with $CD11c^+$ DC in the SED shortly after oral infection (209, 234). Although the DC subset responsible for *Salmonella* uptake in PP after oral infection has not been identified, *Salmonella* infection induces subset-specific redistribution of DC in PP. Thus, $CCR6^+$ DC that are localized in the SED in the steady state migrate toward the FAE in response to *Salmonella* infection (233). It is possible that DC recruited to the FAE in the early stages of infection, participate in bacterial uptake. It has also been postulated that recruited DC may have an advantage over DC constitutively present close to the FAE in inducing pathogen-specific immunity, since they may not have been conditioned by intestinal epithelial cells to induce Th2 or tolerogenic responses

(235). DC present in the SED have also been shown to migrate to the T cell area of PP in response to *Salmonella* infection (236). Inflammatory responses are rapidly induced in infected PP. Thus, 2-3 days after oral infection, monocytes and neutrophils are recruited to infected PP and participate in host defense (237, 238).

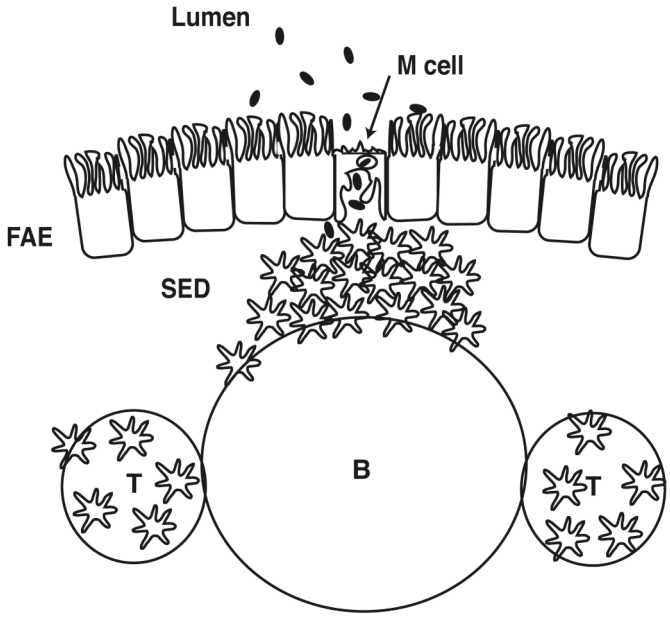

Figure 3. *DC localization in PP*. Bacteria enter through M cells in the FAE, and are rapidly captured by DC present in the underlying SED. DC are also present in the T cell areas. B, B cell follicle; T, T cell area.

Less is known about the fate of *Salmonella* in the lamina propria, the effector site of the GALT. However, a few hours after infection *Salmonella* can be found inside $CX_3CR_1^+$ DC beneath the gut epithelium (225, 226). DC constitutively traffic from lamina propria to the MLN, which is an inductive site of the GALT, and TLR ligands or pro-inflammatory cytokines enhance the migration of lymph-borne DC (67, 239, 240). Thus, although it remains to be directly shown, *Salmonella* in the lamina propria probably traffic to the MLN in the lymph either as free bacteria or in phagocytes. *Salmonella* may also leave PP in efferent lymph and traffic to the MLN. Bacteria can be detected in the MLN 24-48 hr after oral *Salmonella* infection (209). At this time point, bacteria appear mainly to be associated with $CD11c^+$ DC (238, 241, 242). However, about 2 days after infection, neutrophils and monocytes recruited to the MLN participate in the innate response to *Salmonella* by phagocytosing bacteria and producing TNF and iNOS (237, 238).

Systemic *Salmonella* infection

In the steady state, MLN act as a firewall to prevent commensal bacteria from reaching systemic sites (241). However, MLN are not able to contain infection with virulent *Salmonella*. Whether the systemic spread of *Salmonella* is a passive process that requires a certain bacterial threshold in MLN or whether *Salmonella* manipulates the host to mediate egress in efferent lymph is not known. Interestingly, it was recently shown that *Salmonella* can promote the mobility of phagocytes via expression of the SPI-2 gene srfH, which correlated with an increased ability of *Salmonella* to disseminate systemically (243). SPI-2 encodes a type III secretion system and several effector proteins required for bacterial survival in phagosomes (244). The seeding of systemic sites such as the spleen, liver and bone marrow with

bacteria is often lagging behind that of PP and MLN, suggesting that *Salmonella* reach systemic sites *after* leaving the MLN. The efferent lymph of the MLN empties into the blood via the thoracic duct. In cattle, where the efferent lymph leaving MLN and the venules of the distal ileum can be cannulated, it was recently shown that *Salmonella* predominantly translocates from GALT in the efferent lymph and not in venules. Although, *Salmonella* was associated with MHC-II$^+$ cells in the intestinal mucosa, the majority of the bacteria in the efferent lymph appeared to be extracellular (245). It has also been reported that *Salmonella* can be found in CD18$^+$ phagocytes in the blood as early as 15 min after oral infection (243, 246). This extremely rapid translocation of *Salmonella* has been speculated to occur after direct dissemination from the intestinal mucosa to the blood. However, other studies have failed to detect viable *Salmonella* in the blood at 1, 3, 6, 24, or 48 hr after oral infection (209), and the cannulation of venules of the distal ileum in cattle did not reliably detect *Salmonella* (245). Thus, the direct translocation of *Salmonella* into the blood is controversial. Although the gavage method used in this thesis should minimize the possibility, it cannot be eliminated that small amounts of bacteria may be inhaled and enter the host through the respiratory epithelium directly into the blood during oral inoculation of mice.

After reaching the blood stream, *Salmonella* are found mainly in the spleen, liver and bone marrow where they reside in monocytes/macrophages, neutrophils and DC (202, 203, 237, 247-251). The DC subset(s) harboring *Salmonella* in the spleen after i.v. infection might depend on the dose and time after injection. For example, 1 hr after infection the majority of the bacteria are found in CD8α^+ DC (251), whereas 4 hr after infection with a 10^3-10^4 higher dose, the DC subsets are roughly equally efficient at taking up *Salmonella* expressing GFP as determined by flow cytometry (202). After about 3 days neutrophils and monocytes are recruited to the spleen and liver, where they participate in the innate immune defense against *Salmonella* (237, 248, 252).

Innate defense against *Salmonella*

The concerted action of many cells such as neutrophils, monocytes/macrophages, DC, NK cells and NKT cells contribute to the innate defense against *Salmonella*. A few days after infection, infected neutrophils and monocytes/macrophages form well-defined pathological lesions that are separated by normal tissue (248, 250). The majority of the bacteria appear to be confined to these lesions that are believed to prevent bacterial dissemination. The proper formation of lesions is dependent on cytokines such as TNF and IFN-γ, and mice lacking any of these cytokines show abnormal dissemination of bacteria (253-255).

Phagocyte killing of *Salmonella* is primarily mediated by the phagocyte NADPH oxidase early during infection and iNOS at later stages, which produce reactive oxygen or nitrogen species, respectively (256, 257). The localization of NADPH oxidase to bacteria-containing phagosomes is dependent on TNFR1 (258), whereas expression of iNOS in phagocytes is mediated by IFN-γ, IL-12 and IL-18 (237, 254). However, the expression of SPI-2 virulence genes allows *Salmonella* to resist phagocyte killing. The SPI-2 secretion system prevents NADPH oxidase and iNOS to contact the phagosome (258-260). Furthermore, the SPI-2 secretion system alters the trafficking of lysosomes to avoid phagosome-lysosome fusion (261, 262). On the other hand, the activation of macrophages by IFN-γ increases their microbicidal activity and enhances the fusion rate of phagosomes with lysosomes (263). TNF and IFN-γ, as well as the cytokines that induce their expression, are crucial in many ways for the innate immune system to control the replication of *Salmonella*.

Despite these multiple defense mechanisms, the innate control of *Salmonella* infection depends mainly on the virulence of the bacteria and the susceptibility of the host (264). A major locus controlling host susceptibility in mice is the gene encoding Nramp1 (265). Mice with the susceptible allele such as C57BL/6 mice, succumb to infection with virulent *Salmonella* whereas mice with the resistant allele usually clear an infection. The resistant allele of Nramp1 also confers protection against infection with *Leishmania donovani* and *Mycobacterium bovis* (265). Nramp-1 is mainly expressed in monocytes/macrophages and neutrophils where it is recruited to the membrane of phagosomes (266, 267). There it is believed to function as a transporter of divalent metal ions, such as $Fe^{2+}$and Mn^{2+}(268, 269). Thus, after phagocytosis of bacteria or parasites that reside in phagosomes, Nramp1 may limit bacterial access to divalent ions. This may influence the microbes in many ways, but overall, it results in a reduced growth rate of *Salmonella* (265, 270).

Salmonella induces death of phagocytes

An important feature of the interaction between phagocytes and *Salmonella* is that under certain conditions host cell death is induced. Thus, co-incubation of macrophages or DC with *Salmonella* can induce rapid and massive cell death in vitro (271-276). Culture conditions that induce bacterial expression of SPI-1 as well as high bacteria to cell ratios increase the *Salmonella*-induced macrophage death (17, 277). The cell death has been described as apoptosis, necrosis or "programmed necrosis" (278, 279). However, the frequently observed plasma membrane leakage would argue that the rapid *Salmonella*-induced cell death is a form of necrosis. Lower bacteria to macrophage ratios induce more apoptosis-like death (271, 273).

The rapid *Salmonella*-induced macrophage death is dependent on Caspase-1 and a functional bacterial SPI-1 type III secretion system (17, 271-276, 280). The SPI-1-encoded gene SipB was believed to mediate cell death by directly binding to caspase-1 (272). However, it was recently shown that it is cytosolic flagellin that activates Caspase-1 via assembling the IPAF inflammasome (17, 18). The flagellin-mediated Caspase-1 activation is indirectly dependent on SipB since it is an integral part of the SPI-1 type III secretion system.

What is the in vivo relevance of these data? It has been hypothesized that *Salmonella*-induced phagocyte death would facilitate the systemic spread of infection. For example, a frequently cited paper by Monack et al. showed that Caspase-$1^{-/-}$ mice had a 1000-fold higher 50% lethal dose than wild type mice and a significantly reduced systemic dissemination of bacteria after oral infection supports the hypothesis (281). However, these data were recently reevaluated using Caspase-$1^{-/-}$ mice on another genetic background, which showed that Caspase-1-deficient mice were highly susceptible to oral *Salmonella* infection (280, 282). In vivo, *Salmonella* infection has been shown to induce cell death of phagocytes in the liver (248). However, the mechanism of *Salmonella*-mediated cytotoxicity in vivo is not known. Indeed, the interaction of phagocytes with *Salmonella* is very different in vivo compared to in vitro. For example, in vitro incubation of macrophages with *Salmonella* results in a massive uptake of bacteria, whereas in vivo, the majority of phagocytes within lesions harbor a relatively small number of bacteria (mean=2.8 bacteria/phagocyte in the liver, bacterial count: 6.3 $\log_{10}$), despite a progressive increase in bacterial load (250). Rather, the increase in bacterial load in vivo manifests as an increase in the number and size of bacteria-containing neutrophil/monocyte/macrophage lesions (250), which indicates that to form new lesions *Salmonella* has to spread from already established ones. Thus, induction of necrosis could be a way of *Salmonella* to facilitate its spread.

Ag presentation and activation of adaptive immunity

The innate immune system cannot eradicate virulent *Salmonella*, but keeps the infection in check until adaptive immunity is initiated. CD4 T cells are absolutely required for the control of *Salmonella* infection (283-286). However, CD8 T cells and B cells strongly contribute to protective immunity. Thus, B cell-deficient or MHC-I-deficient mice are able to clear infections with attenuated strains, but are not protected against rechallenge with virulent *Salmonella* (287-289). Likewise, protective immunity cannot be transferred to naïve recipients with T cells alone, but requires transfer of both T cells and serum antibodies (290).

The activation of *Salmonella*-specific CD4 T cells requires Ag presentation by DC (233). Splenic as well as liver DC can process and present *Salmonella*-encoded Ag to specific CD4 and CD8 T cells (202, 203, 237). On the other hand, there are also several reports showing that *Salmonella* can interfere with Ag presentation in infected cells (242, 291-296). One of these studies showed that the yej operon can interfere with the MHC-I presentation pathway both in vivo and in vitro (296). Furthermore, the PhoP-PhoQ regulatory system, which controls the expression of several genes required for *Salmonella* virulence and intracellular survival, reduces Ag presentation to T cells (295). Interestingly, targeting of IgG-coated bacteria to Fcγ receptor III on DC leads to lysosomal degradation and efficient presentation of bacterial Ag on MHC-I and MCH-II (294). This suggests that Fcγ receptor III-mediated uptake may target bacteria for rapid lysosomal degradation. *Salmonella* could also evade Ag presentation by inducing death of infected DC (276). *Salmonella* therefore can directly or indirectly interfere with the Ag presentation machinery. However, the extent to which Ag presentation is inhibited depends on the virulence of bacteria, the multiplicity of infection and the bacterial growth conditions.

Polyclonal T cell responses to *Salmonella* have been studied during sub-lethal infections, which can be achieved in susceptible mice infected with attenuated strains or in resistant mice infected with virulent strains. Considering that virulent *Salmonella* can interfere with Ag presentation, it is possible that the T cell response differs in the two models. We have analyzed the polyclonal T cell response to *Salmonella* in susceptible mice infected with an attenuated strain (297). Up to 6 months after immunization we could detect CD4 and CD8 T cells that produce IFN-γ or TNF in response to restimulation with *Salmonella* lysate. Importantly, a substantial portion of the activated *Salmonella*-specific T cells acquired the capacity to migrate to the liver, which is a site of extensive bacterial replication (297).

Adoptive transfer of OVA-specific OT-I T cells to resistant mice during sub-lethal infection with virulent *Salmonella* expressing OVA has revealed that CD8 T cells undergo a delayed expansion, peaking around day 21, followed by a protracted contraction phase (298). This pattern is in marked contrast to viral or *Listeria* infection, where CD8 T cells rapidly expand within the first few days of infection, after which a steep contraction phase follows (298, 299). Even memory CD8 T cells generated during infection with *Listeria*-OVA showed a delayed expansion upon challenge with *Salmonella*-OVA (298). Similarly, infection of resistant mice with attenuated *Salmonella*-OVA showed maximal expansion of endogenous OVA-specific CD8 T cells 21 days after infection (300). On the other hand, adoptively transferred OVA-specific DO11.10 cells expand modestly (~4-fold) in the draining lymph node after s.c. injection of 10^8 attenuated *Salmonella*, peaking 5 days after infection. However, the transferred OT-II cells failed to persist or produce IFN-γ 10 days after infection of susceptible mice (301). A similar study showed that after oral infection with attenuated *Salmonella*-OVA, DO11.10 cells expand in PP, but not

MLN, with a somewhat delayed kinetics compared to the previous study (peak day 7-10) (302).

Adoptively transferred flagellin-specific CD4 T cells (SM1) are rapidly activated (6-9 hr after infection) and expanded (detectable 3 days after infection) in PP and MLN during lethal infection of susceptible mice. However, they fail to expand in the spleen or liver after oral infection (303). Strikingly, i.v. infection with a high dose of virulent *Salmonella* (10^4-10^6) is required for SM1 T cell proliferation in the spleen 3 days after infection, at which time point the bacterial burden in the spleen reaches 10^6-10^{10} CFU. Lower doses of *Salmonella* fail to activate SM1 T cells in the spleen despite a considerable bacterial burden days 3-5 after infection. A similar trend is observed after oral infection, during which a dose of 10^8-10^{10} virulent *Salmonella* is required to induce proliferation of flagellin-specific CD4 T cell in the MLN (304). Furthermore, transferred SM1 T cells expand poorly and fail to persist in sub-lethal infections of susceptible mice infected with avirulent *Salmonella* (305). An explanation for these results came from studies showing that *Salmonella* downregulate the expression of flagellin when switching to the intracellular life style (306, 307), restricting the expression of flagellin to the PP (308). Others have suggested that virulent *Salmonella* evade Ag presentation during lethal infections. For example, transferred OT-I, OT-II or SM1 T cells failed to proliferate in the spleen 3 days after i.v. infection with 10^5 virulent *Salmonella* grown to logarithmic phase, which increases bacterial virulence (292). In contrast, SPI-2 mutant strains induced T cell proliferation (292).

In summary, although a detailed analysis of the activation and expansion of *Salmonella*-specific CD4 T cells during sub-lethal infections is lacking, the available data suggest that virulent *Salmonella* may evade Ag presentation during infection. In sublethal infections this is detected as a delayed expansion of specific T cells whereas in lethal infections proliferation of specific CD4 T cells require unusually high bacterial loads or is not detected (292, 304). Interestingly, formation of germinal centers, which is required for affinity maturation of B cells, is also delayed during infection of susceptible mice with attenuated *Salmonella* (309). During acute infection, switched antibodies are derived from T-dependent extrafollicular B cells whereas germinal center formation does not commence until ~1 month after infection when the bacteria are cleared. Treatment with antibiotics allowed earlier formation of germinal centers, suggesting that the presence of bacteria inhibits germinal center formation (309).

Aims of the thesis

The overall aim of the thesis was to investigate how upregulation of costimulatory molecules on DC is regulated during *Salmonella* infection. The specific aims were:

I. To study the kinetics and mechanism of costimulatory molecule upregulation on DC subsets in PP, MLN and spleen after oral *Salmonella* infection

II. To investigate the role of MyD88 and type I IFN in direct and indirect costimulatory molecule upregulation by DC subsets during *Salmonella* infection

III. To investigate whether *Salmonella* induces DC apoptosis in vivo during oral infection and, if so, to analyze the mechanism of infection-induced DC death

Results and comments

Subset- and organ-specific expression of costimulatory molecules in the steady state and during *Salmonella* infection (I)

The costimulatory molecules CD80 and CD86 are required both for immunity and tolerance (310-314). Several studies suggest that they have overlapping but distinct roles in costimulation. This is partially explained by their differential affinities for the receptor CD28, which enhances T cell receptor signaling, and the inhibitory receptors CTLA-4 and PD-1 (315-318). Thus, blocking CD80 or CD86 can have different outcomes in models of autoimmune disease (319-325). In addition to CD80 and CD86, signaling through CD40 on DC has strong immunomodulatory effects. Engagement of CD40 with CD40L expressed by T cells boosts IL-12 production by DC, increases DC longevity and licenses DC for CD8 T cell activation (84-92).

In Paper I the upregulation of CD80, CD86 and CD40 on DC subsets in PP and MLN of the GALT, as well as in systemic sites such as the spleen, was carefully dissected after oral infection with *Salmonella*. In the steady state, all DC expressed a low level of CD80 and CD86 with one notable exception: CD8α^+ DC of the spleen and MLN, but not PP, constitutively expressed an intermediate level of CD86 (Table 3). It has been reported that constitutive expression of costimulatory molecules is required for maintaining self tolerance, possibly by sustaining a population of regulatory T cells (312). The localization of CD8α^+ DC in the T cell areas of spleen and lymph nodes in the steady state, their ability to phagocytose apoptotic cells as well as their superiority in cross-presentation, makes them ideally situated for tolerance induction (82, 118-121, 136).

Table 3. Expression of costimulatory molecules on DC subsets of naïve or *Salmonella*-infected mice

		naïve			day 5		
Organ	**DC**	**CD80**	**CD86**	**CD40**	**CD80**	**CD86**	**CD40**
spleen	CD8α^+	2800±200[a]	3200±200	600±50	2900±700	5300±700	2000±500
	CD8α^-	2600±200	1000±200	400±30	5200±800	5300±1100	900±80
MLN	CD8α^+	2800±200	3400±300	1300±300	3400±400	4400±1000	2400±180
	CD8α^-	2100±300	2600±200	800±200	5700±1200	6100±2500	1600±400
PP	CD8α^+	700±600	800±600	200±200	1400±600	1300±400	800±400
	CD8α^-	3100±600	800±200	500±70	5500±800	1900±100	1000±300

[a]Values represent the mean of the median fluorescence intensities for CD80, CD86 or CD40 of 4-6 mice±SD.

Five days after *Salmonella* infection, costimulatory molecules were upregulated on DC in PP, MLN and spleen (Table 3). CD8α^+ DC preferentially upregulated CD86 and CD40, while the level of CD80 showed little or no increase. In contrast, CD8α^- DC greatly upregulated CD80 and CD86, but the level of CD40 was consistently lower than that on CD8α^+ DC in the spleen and MLN. Indeed, both steady state and *Salmonella*-induced expression of costimulatory molecules was similar on DC subsets in the spleen and MLN. In sharp contrast, DC in PP expressed lower levels of CD86 and CD40 in the steady state and only showed a small upregulation of these molecules after *Salmonella* infection. However, CD80 was induced in PP DC to a similar level as MLN and splenic DC.

In summary, costimulation provided by CD8α^+ DC may preferentially involve CD86 over CD80 during *Salmonella* infection. In contrast, CD8α^- DC express similar levels of both CD80 and CD86. Furthermore, DC in PP show a restricted pattern of costimulatory molecules upregulation.

Recruitment of CD11c^{int}CD11b^{+} DC to infected organs (I, III)

High expression of costimulatory molecules was not restricted to conventional DC after *Salmonella* infection. A population of CD80^{hi} CD11c^{int}CD11b^{+} cells accumulated in PP, MLN and spleen after *Salmonella* infection (Fig. 4A-C). While performing these studies, a paper describing a similar population of cells being recruited to the spleen of *Listeria*-infected mice was published (155). These cells were named Tip-DC due to their production of TNF and iNOS. After comparing phenotypic markers as well as their capacity to produce TNF and iNOS (Fig. 4D,E), the data strongly suggested that our CD11c^{int}CD11b^{+} cells were similar to the TNF/iNOS-producing DC observed by Serbina et al. during *Listeria* infection (155). The CD11c^{int}CD11b^{+} cells induced during *Salmonella* infection expressed a similar, high level of CD80 as conventional CD8α^{-} DC of spleen, MLN and PP. Furthermore, in the spleen, but not MLN during infection, CD11c^{int}CD11b^{+} cells upregulated CD86 to a level similar to that on conventional DC.

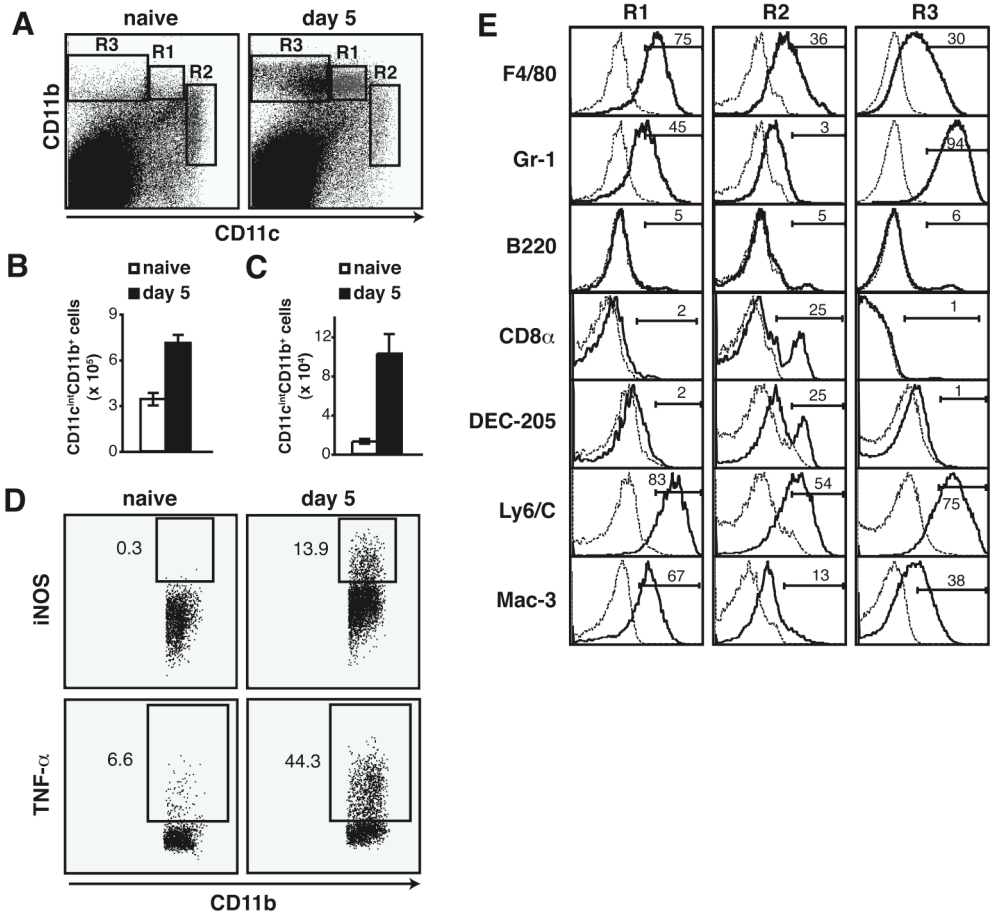

Figure 4. *TNF/iNOS-producing CD11c^{int}CD11b^{+} DC accumulate in spleen and MLN after* Salmonella *infection.* Mice were orally infected with *Salmonella* and after 5 days splenic and MLN cells were analyzed by flow cytometry. *A*, CD11c and CD11b expression on gated live cells from MLN of naïve and infected mice. *B* and *C*, Absolute number of CD11c^{int}CD11b^{+} cells (R1) in spleen (B) and MLN (C) of naïve or infected mice. *D*, Production of TNF and iNOS by gated CD11c^{int}CD11b^{+} cells in spleen. *E*, Histograms show the expression of the indicated markers on gated R1 (CD11c^{int}CD11b^{+}), R2 (DC) or R3 (CD11c^{-}CD11b^{+}) splenic cells 5 days after infection.

Strikingly, the accumulation of $CD11c^{int}CD11b^{+}$ DC was considerably greater in the GALT compared to the spleen. The number of $CD11c^{int}CD11b^{+}$ DC increased 8-fold 5 days after infection in the MLN, but only 2-fold in spleen (Fig. 4B,C). In PP, the percent $CD11c^{int}CD11b^{+}$ DC increased 6-fold (data not shown). Thus, in the spleen, the $CD11c^{int}CD11b^{+}$ DC constituted a minor population with the number of conventional DC being ~4-fold higher. In contrast, the number of $CD11c^{int}CD11b^{+}$ and conventional DC was roughly equal in the MLN at the peak of infection.

The recruited $CD11c^{int}CD11b^{+}$ DC in PP and MLN were more frequently associated with *Salmonella* than conventional DC, as determined by flow cytometry. However, despite their apparent efficiency in bacterial uptake, the $CD11c^{int}CD11b^{+}$ DC were not able to process and present a *Salmonella*-encoded Ag on MHC-II in vitro. In contrast, conventional DC from MLN efficiently induced proliferation of Ag-specific CD4 T cells after in vitro co-incubation with *Salmonella*. Thus, during *Salmonella* infection, TNF/iNOS-producing DC with a high expression of costimulatory molecules are recruited to infected organs, particularly within the GALT. The $CD11c^{int}CD11b^{+}$ DC effectively phagocytose bacteria, but do not appear to participate in priming of naïve T cells during *Salmonella* infection.

Mechanism of costimulatory molecule upregulation during *Salmonella* infection (I, II)

In Paper I the role of TNFR1 in upregulation of costimulatory molecules during *Salmonella* infection was investigated. These studies showed that DC in the MLN required TNFR1 for optimal upregulation of CD80, CD86 and CD40. In contrast, splenic DC were not dependent on TNFR1 for upregulation of costimulatory molecules, and even showed a significantly increased expression of CD86 on $CD8\alpha^{+}$ DC in the absence of TNFR1. In contrast, the $CD11c^{int}CD11b^{+}$ DC were not significantly dependent on TNFR1 for upregulation of costimulatory molecules. Since $CD11c^{int}CD11b^{+}$ DC are recruited from the blood (155, 156), they may have been subjected to a systemically produced mediator such as IL-1β, which can influence the expression of costimulatory molecules.

In addition to its effects on costimulatory molecules, TNFR1 signaling was responsible for recruiting $CD11c^{int}CD11b^{+}$ DC as well as inducing production of IL-1β in the MLN. However, the TNFR1-mediated upregulation of costimulatory molecules and IL-1β production was dependent on the bacterial burden in the MLN. Thus, at a higher bacterial load, upregulation of costimulatory molecules was partially restored in TNFR1-deficient mice. This was coincident with production of IL-1β in the MLN of $TNFR1^{-/-}$ mice.

In Paper II, we investigated the role of MyD88-dependent and MyD88-independent signaling pathways for costimulatory molecule upregulation during *Salmonella* infection. MyD88 controls the production of many pro-inflammatory cytokines, including TNF after oral *Salmonella* infection (Paper 3, Fig 3D). In addition, the IL-1 receptor requires MyD88 for signaling. Thus, *Salmonella*-infected $MyD88^{-/-}$ mice are deficient both in TNF production and IL-1β signaling, lacking both pathways identified in Paper I as possible mediators of costimulatory molecule upregulation on DC.

MyD88 was absolutely required for upregulation of CD80 on conventional DC in spleen and MLN after *Salmonella* infection. In addition, MyD88 was required for optimal upregulation of CD86 and CD40 on DC in MLN. In contrast, splenic DC readily upregulated CD86 in the absence of MyD88. However, the upregulation of CD40 on splenic $CD8\alpha^{-}$ and $CD11c^{int}CD11b^{+}$ DC was inhibited in infected MyD88-

deficient mice. Similar to our results on TNFR1, a higher bacterial load could overcome the defective upregulation of CD86 and CD40, but not CD80, on DC in the MLN. Thus, upregulation of CD80 on DC is strictly controlled by the MyD88-dependent pathway whereas upregulation of CD86 and CD40 can occur independently of MyD88. Furthermore, the MyD88-dependent upregulation of CD80 was not due to defective IL-1β signaling in these mice, since Caspase-1$^{-/-}$ mice showed unimpeded upregulation of CD80 on DC after *Salmonella* infection.

The MyD88-independent pathway of TLR3 and TLR4 signals via TRIF and leads to production of type I IFN (5). Since type I IFN can induce upregulation of costimulatory molecules (326), we tested whether type I IFN were responsible for the MyD88-independent upregulation of CD86 and CD40. Indeed, the upregulation of CD80, CD86 and CD40 was completely abrogated in mice doubly deficient for MyD88 and IFN-αβR. Thus, MyD88 is absolutely required for upregulation of CD80 on DC after oral *Salmonella* infection, but in the absence of MyD88, type I IFN mediate upregulation of CD86 and CD40. However, the contribution of type I IFN to upregulation of CD86 and CD40 in wild type mice is likely small, since DC in IFN-αβR$^{-/-}$ mice showed no major defects in costimulatory molecule upregulation after *Salmonella* infection. Furthermore, low, but significantly elevated, levels of IFN-α, but not IFN-β, could be detected in the spleen of infected C57BL/6 and MyD88$^{-/-}$ mice. If normalized to protein content, infected MyD88$^{-/-}$ mice had a significantly higher level of IFN-α in the spleen than infected C57BL/6 mice (data not shown). This indicates that MyD88-dependent factors may inhibit the production of type I IFN in wild type mice. Indeed, it has been shown that TNF can inhibit the production of IFN-α in response to influenza virus (327).

Maturation of *Salmonella*-containing DC (I, II)

Having found that MyD88-dependent and -independent pro-inflammatory cytokines contribute to the upregulation of costimulatory molecules on DC during infection, we next investigated how direct bacterial association influences the expression of costimulatory molecules on DC in vivo. To accomplish this, mice were orally infected with *Salmonella* expressing eGFP, and after 3 days the expression of costimulatory molecules by eGFP^{+} and eGFP^{-} DC in PP and MLN was compared. In wild type mice, *Salmonella*-containing DC had similar expression of CD80, CD86 and CD40 as the bulk population of DC, which were not associated with bacteria. However, in the absence of pro-inflammatory cytokines, i.e. in the PP or MLN of TNFR1$^{-/-}$, MyD88$^{-/-}$ or MyD88$^{-/-}$IFN-αβR$^{-/-}$ mice, only DC harboring bacteria were able to upregulate costimulatory molecules. Interestingly, eGFP^{+} DC from TNFR1$^{-/-}$ mice expressed a higher level of CD80 and CD86, but not CD40, compared to eGFP^{+} DC of wild type mice (Table 4). This suggests that TNFR1 signaling might retard the upregulation of costimulatory molecules on DC directly associated with bacteria. Consistent with the dominant role of MyD88 in upregulation of CD80, eGFP^{+} DC in MyD88$^{-/-}$ or MyD88$^{-/-}$IFN-αβR$^{-/-}$ mice had a compromised upregulation of CD80. In contrast, CD86 was upregulated to a level 2-3 times higher than that of eGFP^{+} DC from IFN-αβR$^{-/-}$ mice. Thus, in the absence of TNFR1, DC directly associated with *Salmonella* upregulate both CD80 and CD86. In the absence of MyD88, which controls expression of CD80, *Salmonella*-containing DC are restricted to upregulate CD86, but do so to a higher level than wild type mice (Table 4).

Table 4. The role of TNFR1, MyD88 and IFNαβR for upregulation of CD80 and CD86 on $eGFP^+$ and $eGFP^-$ DC in PP and MLN after oral infection with *Salmonella*-eGFP.

Mouse strain	DC	CD80	CD86
C57BL/6	eGFP-	++	++
	eGFP+	++	++
$TNFR1^{-/-}$	eGFP-	+/-	+/-
	eGFP+	++++	+++
$MyD88^{-/-}$	eGFP-	+/-	+/-
	eGFP+	+	++++
$MyD88^{-/-}IFN\alpha\beta R^{-/-}$	eGFP-	+/-	+/-
	eGFP+	+	++++

We were surprised and intrigued to find that *Salmonella*-containing DC were able to upregulate costimulatory molecules independently of both MyD88 and type I IFN. One explanation could be that not all signaling in the MyD88-independent pathway (via TRIF) is abrogated in the absence of the type I IFN receptor. Indeed, TRIF signaling activates NF-κB (6, 15, 328-330), in addition to IRF-3 and IRF-7 that regulate production of type I IFN. It is unlikely that IL-1β induced by the IPAF inflammasome after recognition of cytosolic flagellin is responsible for the observed upregulation of costimulatory molecules in *Salmonella*-associated DC, since the absence of MyD88 abrogates signaling through the IL-1 receptor. Interestingly, $MyD88^{-/-}IFN\text{-}\alpha\beta R^{-/-}$ mice that received an i.v. injection of *Salmonella* were able to overcome the defective upregulation of costimulatory molecules observed on the bulk population of DC after oral infection. The i.v. route of infection preferentially facilitated upregulation of CD86 in both wild type and $MyD88^{-/-}IFN\text{-}\alpha\beta R^{-/-}$ mice analyzed 12 hr after injection. The low expression of CD80 after i.v. infection could be due to that CD80 usually requires a longer time than 12 hr to be upregulated (331, 332).

When analyzing the ability of *Salmonella*-associated DC from MyD88-deficient mice to activate OVA-specific OT-II cells after i.v. infection with *Salmonella* expressing OVA and GFP, we found that their ability to prime T cells was compromised compared to wild type mice. Indeed, TLR signaling is crucial for phagosome maturation, and absence of MyD88 results in a delayed kinetics and lower levels of MHC-II presentation of bacterial antigens (333).

Salmonella induces DC death in vivo via MyD88-dependent production of TNF (III)

In addition to the effects on costimulatory molecules, we found that signaling through TNFR1 and MyD88 induced DC death in the MLN during *Salmonella* infection. $CD8\alpha^+$ DC were particularly sensitive, and 3 days after infection 40% of these cells stained positive for Annexin-V and 7AAD. In sharp contrast, the TNF/iNOS-producing $CD11c^{int}CD11b^+$ cells were completely resistant to *Salmonella*-induced cell death. Indeed, these cells probably contribute to the infection-induced death of $CD8\alpha^+$ DC, since they are a source of TNF during *Salmonella* infection. Furthermore, the death of $CD8\alpha^+$ DC coincides with the massive recruitment of $CD11c^{int}CD11b^+$ cells to the MLN.

General discussion

Costimulatory molecules: an imprint of the inflammatory milieu?

This thesis work has revealed some of the pathways that control the upregulation of CD80 and CD86 on DC during *Salmonella* infection. Thus, infection-induced upregulation of CD80 is strictly controlled by MyD88, whereas upregulation of CD86 is redundantly mediated by both MyD88-dependent and -independent factors. We have evidence that TNF, via TNFR1, is one of the MyD88-dependent factors responsible for the upregulation of costimulatory molecules on DC during *Salmonella* infection, particularly in the MLN. We also have indirect evidence suggesting that IL-1β is another MyD88-dependent factor that may contribute to costimulatory molecule upregulation, since TNFR1$^{-/-}$ DC in MLN containing a high bacterial load upregulate CD80 and CD86 coincident with IL-1β production. However, Caspase-1$^{-/-}$ mice, which cannot synthesize active IL-1β, showed no obvious defects in infection-induced costimulatory molecule upregulation during *Salmonella* infection. Thus, several MyD88-dependent factors contribute to upregulation of CD80 and CD86 during infection. In contrast, the MyD88-independent upregulation of CD86 was solely mediated by type I IFN. However, both MyD88-dependent and independent factors contribute to the upregulation of CD86 in wild type mice, since infection-induced upregulation of CD86 was only marginally reduced in mice lacking the type I IFN receptor. Upregulation of CD80 on DC, however, was strictly controlled by MyD88 and could not be induced by type I IFN.

Adding to the complexity, the mediators that control expression of CD80 or CD86 appear to cross-regulate each other, since upregulation of CD86 was enhanced in splenic DC in the absence of MyD88 or TNFR1. Interestingly, TNF has been shown to inhibit the release of IFN-α by plasmacytoid DC in response to influenza virus, and patients treated with TNF antagonists overexpress IFN-α-regulated genes in their blood leukocytes (327). Furthermore, type I IFN can inhibit the MyD88-dependent upregulation of B7RP-1, another member of the B7 costimulatory molecule family (334). Thus, the MyD88-dependent and -independent pathways cross-regulate each other.

Previous studies have shown that TRIF-induced type I IFN, but not MyD88, mediates upregulation of CD80, CD86 and CD40 on macrophages after exposure to LPS in vitro (335). In addition, TRIF, but not MyD88, mediated upregulation of CD86 on DC after i.v. injection of LPS (335). At a first glance these results appear contradictory to our results on *Salmonella*. However, the study by Hoebe et al. used rough LPS lacking O-polysaccharides. Recent studies have revealed that TLR4 can distinguish between rough and smooth LPS (336). In addition, lipid A, which lacks both the O- and core polysaccharides of LPS, preferentially signals via TRIF. This is in contrast to smooth LPS that utilizes both TRIF and MyD88 as adaptors (14). Therefore, the study by Hoebe et al. may be biased towards TRIF-dependent signaling by the use of rough LPS (14, 335, 336). On the contrary, we are using an infection model with a strain of *Salmonella* that expresses smooth LPS. Accordingly, we see a greater dependency on MyD88 for upregulation of costimulatory molecules, particularly CD80, whereas TRIF-mediated production of type I IFN is only mediating upregulation of CD86 in the absence of MyD88.

We found that the regulation of costimulatory molecule expression differs in conventional and CD11c^{int}CD11b^{+} DC, the latter being capable of upregulating CD80 in the absence of MyD88, albeit to a reduced level. Furthermore, the regulation of costimulatory molecule expression differed in the GALT compared to systemic sites.

Thus, GALT DC preferentially relied on the MyD88-dependent pathway to induce expression of CD80 and CD86. Indeed, we found that MyD88-independent upregulation of costimulatory molecules on DC in the MLN and PP required a very high bacterial burden and was mediated by type I IFN, which selectively upregulated CD86. In contrast, splenic DC readily upregulated CD86 in the absence of MyD88 during *Salmonella* infection. Consistent with this, an elevated level of IFN-α was detected in the spleen, but not the MLN, after *Salmonella* infection. In contrast to *Salmonella* infection, clearance of reovirus type 1 Lang from PP after oral infection requires type I IFN, but not MyD88 (337).

Our results show that during *Salmonella* infection, MyD88-dependent factors induce upregulation of CD80 on DC. Considering that CD80 binds more strongly than CD86 to both CD28 and CTLA-4 (315, 316), and preferentially concentrates CTLA-4 in the immunological synapse (317), it is likely that such an imprint will influence the ensuing immune response. In addition, it is possible that different pathogens, which generate different inflammatory cues, will imprint distinct maturation phenotypes on DC that can be interpreted by T cells at the time of Ag-presentation. Thus, a T cell that encounters a DC with highly expressed costimulatory molecules will know that the DC has been exposed to an inflammatory environment. More specifically, a T cell that encounters a DC with high expression of CD80 will know that the DC has been subjected to an infection that induces MyD88-dependent signaling. Thus, the upregulation of costimulatory molecules on DC may reflect the pro-inflammatory environment elicited by distinct pathogens.

TNF/iNOS-producing CD11c^{int}CD11b^{+} cells: DC or monocytes?

Monocytes can give rise to Langerhans cells or interstitial DC in peripheral tissues that have the capacity to cross-prime CD8 T cells (115, 155-157, 177, 178, 338-341). This concept has been clearly established in the human system, where the majority of DC studied to date have been generated from monocytes cultured with GM-CSF and IL-4. However, during inflammatory conditions in mice, monocytes appear to generate a certain DC subset, the CD11c^{int}CD11b^{+} TNF/iNOS-producing DC (115, 155-157, 339).

We found that during *Salmonella* infection, CD11c^{int}CD11b^{+} DC accumulated in infected lymphoid organs, phagocytosed bacteria more efficiently than conventional DC, and produced TNF and iNOS. Furthermore, the CD11c^{int}CD11b^{+} DC expressed a high level of costimulatory molecules and MHC-II, yet were unable to process and present a *Salmonella*-encoded Ag on MHC-II for specific T cells. Previous studies have only evaluated the T cell stimulatory capacity of TNF/iNOS-producing DC in mixed leukocyte reactions, in which case they are as efficient as conventional DC in inducing allogenic T cell proliferation (155, 157). It will be important to evaluate the capacity of TNF/iNOS-producing DC to initiate Ag-specific immunity in other infection models. Thus, although CD11c^{int}CD11b^{+} DC resemble conventional DC phenotypically (155, 342), their function appear more similar to monocytes, which also produce TNF and iNOS and fail to process and present *Salmonella* Ag on MHC-II (237).

The subset of monocytes (CCR2^{+}Ly6C/Gr1^{+}) thought to give rise to TNF/iNOS-producing DC, require CCR2 to leave the bone marrow (340), and therefore fail to accumulate in the spleen of *Listeria*-infected CCR2$^{-/-}$ mice. However, transfer of CCR2$^{-/-}$ monocytes into the blood results in accumulation of TNF/iNOS-producing DC in the spleen and peripheral tissues (340). In contrast, adjuvant-induced recruitment and subsequent differentiation of circulatory Gr1^{+} monocytes into Ag-

presenting DC in the dermis and epithelium is dependent on CCR6/CCL20 (338). We found that recruitment of $CD11c^{int}CD11b^{+}$ cells to the MLN, but not the spleen, was dependent on TNFR1 and MyD88. Interestingly, induction of CCL20 and recruitment of inflammatory cells to the liver after infection with *Popionibacterium acnes* followed by LPS injection is completely dependent on TNFR1-mediated NF-κB activation (343). Thus, *Salmonella*-induced TNF is probably required for optimal expression of chemokines that guide the $CD11c^{int}CD11b^{+}$ cells to the MLN.

The capacity to produce TNF and iNOS might be viewed as a marker of monocyte origin. Thus, bone marrow-derived DC obtained with GM-CSF/IL-4, which promotes DC differentiation from monocytes or a common monocyte/DC precursor, retain the capacity to produce TNF and iNOS in response to TLR ligands (168, 169, 344). In contrast, bone marrow-derived DC obtained with Flt3L, which is required for development of DC resident in secondary lymphoid organs, are unable to produce TNF/iNOS upon stimulation (125, 167, 168, 344).

In summary, monocytes have the capacity to differentiate into DC capable of activating naïve T cells (338). However, whether TNF/iNOS-producing DC, which may represent one end point of many for monocyte differentiation, are able to prime naïve T cells is not clear. Indeed, the results in this thesis suggest that they have a poor capacity to process and present a *Salmonella*-encoded Ag.

Why are the DC subsets variably susceptible to infection-induced cell death?

During oral *Salmonella* infection, $CD8\alpha^{+}$ DC, and to a lesser extent $CD8\alpha^{-}$ DC, in the MLN undergo MyD88- and TNFR1-dependent cell death. Production of TNF in MLN was absolutely dependent on MyD88. This suggests that MyD88-induced production of TNF mediates DC death during *Salmonella* infection. In sharp contrast, $CD11c^{int}CD11b^{+}$ DC were resistant to infection-induced cell death, at least during the early stages of infection. Indeed, $CD8\alpha^{+}$ DC are more susceptible to apoptosis than $CD8\alpha^{-}$ DC in response to a range of infections (196, 345, 346). Furthermore, $CD8\alpha^{+}$ DC had a higher frequency of Annexin-V^{+} $7AAD^{+}$ cells than $CD8\alpha^{-}$ DC when analyzed directly ex vivo. This indicates that the $CD8\alpha^{+}$ DC might be, in general, more sensitive to different apoptosis-inducing stimuli. $CD8\alpha^{+}$ DC also have a faster turn-over rate than $CD8\alpha^{-}$ DC in vivo (173-175). The resistance of $CD11c^{int}CD11b^{+}$ cells to infection-induced cell death could be due to their relatedness with monocytes/macrophages. Indeed, autocrine production of TNF induces long-term survival of macrophages after treatment with LPS (347).

One reason for the higher susceptibility of $CD8\alpha^{+}$ DC to infection-induced death could be lower expression of anti-apoptotic molecules in this subset compared to the $CD8\alpha^{-}$ or $CD11c^{int}CD11b^{+}$ DC. Bcl-2 is an anti-apoptotic molecule that has been implicated in regulating DC longevity, and conventional DC in the steady state spleen express differential levels of Bcl-2 (348). Furthermore, the expression of anti-apoptotic proteins varies between conventional DC and longer-lived plasmacytoid DC (349). Indeed, DC apoptosis is crucial to prevent autoimmune reactions (93, 350). Resistance to apoptosis in DC could lead to sustained presentation of self Ag, which can promote immunogenic T cell responses without apparent DC maturation (351). In addition, CD40 ligation increases DC longevity, and can switch a tolerogenic response into efficient T cell priming (82, 83).

Despite being more susceptible to infection-induced cell death, $CD8\alpha^{+}$ DC clearly have an important role in Ag presentation during infection with several pathogens, particularly during viral infection (132, 149-151, 195, 200). It is possible

that CD8α$^+$ DC could present Ag before dying during infections that induce their death. As an example of this, CD8α$^+$ DC are able to induce proliferation of LCMV-specific CD8 T cells 1-2 days after infection (200), despite that LCMV induces death of both CD8α$^+$ and CD8α$^-$ DC 3 days after infection (352).

DC death: evasion strategy or host defense?

We found that the MyD88-dependent production of TNF was responsible for the induction of DC death during *Salmonella* infection. Signaling through TNFR1 leads to activation of NF-κB via TRADD and RIP-1 (37). However, upon receptor internalization, TRADD is able to recruit FADD and pro-caspase 8 to initiate apoptosis (39, 40). Activation of NF-κB leads to expression of anti-apoptotic proteins and TNF can only induce apoptosis in cells with low levels of anti-apoptotic proteins (41). Interestingly, *Salmonella* can inhibit the anti-apoptotic NF-κB pathway via a virulence protein, AvrA, encoded by SPI-1 (353). Therefore, *Salmonella* can induce apoptosis in infected cells by inhibiting the NF-κB-mediated upregulation of anti-apoptotic molecules in response to TNF. The *Yersinia* effector YopJ is an ortholog of AvrA. YopJ has been shown to inhibit proteasomal degradation of IκB resulting in inhibition of NF-κB signaling and increased susceptibility to apoptosis (354). In addition, *Salmonella* AvrA is related to an "avirulence" protein in plants that mediates apoptosis of infected cells as a host defense strategy to limit the spread of infection (355). Considering that AvrA-mediated cell death can be expected to be limited to infected cells, and given the low percentage of DC directly associated with *Salmonella*, AvrA-mediated cell death is unlikely to mediate the death of 40% of CD8α$^+$ DC in the MLN during infection. We cannot, however, exclude that the inhibition of NF-κB by AvrA could influence cell death in DC directly associated with bacteria. Thus, although *Salmonella*-induced DC death is mediated by a host factor involved in host defense, bacteria such as *Salmonella* and *Yersinia* can subvert this response to induce apoptosis in infected cells.

In addition to the relatively unexplored effects of AvrA, *Salmonella* has been shown to rapidly induce macrophage and DC death upon in vitro co-culture. This very rapid form of cell death is dependent on the host factor Caspase-1 and the bacterial SPI-1 secretion system (17, 271-276, 280). Caspase-1 is generally not involved in apoptosis, but rather in inflammatory responses. This suggests that *Salmonella* and other bacteria that utilize Caspase-1 for induction of cell death might subvert the normal function of Caspase-1. Despite the well-studied effects of Caspase-1 on *Salmonella*-induced phagocyte death in vitro, we could not detect an increased survival of MLN DC in Caspase-1-deficient mice 3 days after infection. It is possible that Caspase-1-mediated cell death acts at another time point or another organ after *Salmonella* infection. Indeed, ileal loop experiments revealed that Caspase-1-deficient mice contained fewer TUNEL$^+$ cells in PP 1 hr after *Salmonella* injection compared to wild type mice (281).

DC death could be a way for the host to limit an ongoing immune response (356). The general susceptibility of CD8α$^+$ DC to different apoptosis-inducing stimuli, and the autoimmune reactions that occur in mice containing DC with a prolonged life span, would argue for such an explanation (93, 196, 345, 346). In order to be protective, host-mediated DC death would, of course, require that Ag-specific adaptive responses were initiated before death of the DC, as is the case during LCMV infection (200, 352). In addition, *Salmonella*-induced apoptosis of macrophages allows bystander DC to take up Ag and, in turn, activate anti-bacterial T cell

responses (271). This suggests that macrophage apoptosis could facilitate the initiation of adaptive immunity during *Salmonella* infection.

In contrast, apoptosis of DC can be expected to have detrimental effects on the ensuing immune response, particularly if bacterial evasion strategies result in delayed presentation of bacterial Ag. Indeed, virulent *Salmonella* appear to actively interfere with Ag presentation in infected cells (242, 291-296), a conclusion that is also supported by results in this thesis. Moreover, the activation of CD8 T cells is markedly delayed in *Salmonella* infection compared to infection with *Listeria* (298). Therefore, evasion of Ag presentation combined with infection-induced DC death could be an efficient way for *Salmonella* to prevent adaptive immune responses.

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