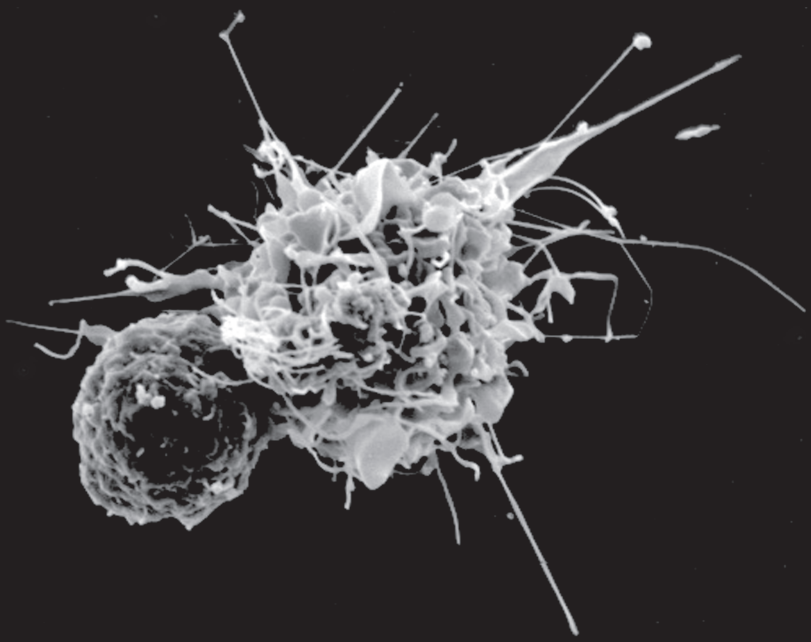
MECHANISMS OF DENDRITIC CELL MATURATION INDUCED BY INTRACELLULAR BACTERIA INFECTION



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Cover picture: Human dendritic cell (right) interacting with a CD4 T cell. Reproduced with permission of Dr. William Bowers. University of South Carolina (http://pathmicro.med.sc.edu/2004-fac/wbowers-pap.htm).

CONTENTS

ABSTRACT

Dendritic cells (DCs) are essential for the development of an immune response against pathogens such as *Listeria monocytogenes* and *Salmonella typhimurium*. This is mostly because of their unique capacity to stimulate naïve T cells. Before DCs become potent antigen presenting cells, they undergo a maturation process that enables them to efficiently stimulate naïve T cells. This process includes upregulation of costimulatory molecules such as CD80 and CD86 and production of cytokines. However, the pathway by which DCs mature can influence their capacity to induce effector functions in T cells. Thus, the aim of this thesis was to investigate the maturation and function of DCs during intracellular bacteria infection and its impact on T cell stimulation.

Conventional DCs expanded in number and upregulated costimulatory molecules in a subset- and tissue-specific manner after oral *Listeria* infection. Moreover, plasmacytoid DCs also expanded and upregulated CD86 and MHC-II although showing no tissue specificity. Conventional DCs produced significant amounts of IL-12. In addition, a complex CD11c-expressing population was identified, stratified in several subsets defined by production of TNF-α, iNOS and IL-12 alone or in combination. The production of these molecules was dependent on the subcellular compartment where *Listeria* was localized. Upregulation of CD80 and CD86 in DCs during orally acquired *Listeria* was differentially dependent on MyD88 and IFN-αβR. However, when the bacteria reached the blood stream directly, alternative pathways not mediated by MyD88 and IFN-αβR induced upregulation of costimulatory molecules. Remarkably, IFN-αβR$^{-/-}$ mice expressed higher levels of CD80 and CD86, which translated into stronger naïve T cell stimulation. However, despite the significance of IFN-αβR in the early anti-*Listeria* response, it had little impact in the development of memory T cells.

Similar to *Listeria*, expression of costimulatory molecules during *Salmonella* infection was only partially dependent on MyD88 and IFN-αβR. Expression of CD80 was controlled by MyD88, whereas the MyD88-independent upregulation of CD86 was supported by IFN-α/β. Furthermore, *Salmonella*-associated DCs upregulated CD86 and CD80 to some extent even in the simultaneous absence of both MyD88 and IFN-αβR. However, DCs that matured by direct contact with the bacteria, but in the absence of these two factors, were less competent at stimulating naïve T cells than their wild type counterpart due to a decreased capacity to process bacteria-derived antigens.

Taken together, these studies expand our understanding of DC function during bacterial infection. In addition, the identification of factors involved in DC maturation addressed here can help to design more efficient approaches in the future to eliminate bacterial infections.

Keywords: *Listeria*, *Salmonella*, dendritic cells, maturation, MyD88, IFN-α/β

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals

I. **Miguel A. Tam and Mary Jo Wick.** Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with *Listeria monocytogenes.* Cell. Microbiol. 2006 Jul; 8 (7): 1172-87.

II. **Miguel A. Tam and Mary Jo Wick.** Conditional roles of MyD88 and IFN-α/β in homeostatic regulation of dendritic cell maturation but not for development of protective CD8 T cell memory response against *Listeria*. Manuscript.

III. **Miguel A. Tam*, Malin Sundquist*, and Mary Jo Wick.** MyD88 and IFN-α/β are hierarchically required for functional maturation of dendritic cells and induction of CD4 T cells during infection. Submitted manuscript. *Authors contributed equally.

ABBREVIATIONS

APC	antigen presenting cell
CFU	colony forming unit
CTL	cytotoxic T lymphocyte
DC, cDC, pDC	dendritic cell, conventional DC, plasmacytoid DC
GFP, eGFP	green fluorescent protein, enhanced GFP
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
iv	intravenous
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
NLR	nucleotide-binding oligomerization domain-like receptor
OVA	ovalbumin
OT-I	$OVA_{257-264}$ peptide-specific TCR transgenic mice
OT-II	$OVA_{323-339}$ peptide-specific TCR transgenic mice
PAMP	pathogen-associated molecular pattern
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor

INTRODUCTION

Evolution imposes the challenge of the coexistence of many different forms of life. In some cases, peaceful coexistence turns into survival battles. This is the case of numerous microorganisms that constantly invade others. Humans, in an effort that could be defined as intelligent evolution, have used these microorganisms to understand how they invade us and, more importantly, how to prevent and eliminate these unwanted invasions. The use of some particular microorganisms, such as intracellular bacteria, has been of great significance in understanding this process. From these studies, we have learned that our most important self-preserving system is what we now know as the immune system. This system, although one of the most complex and refined in our body, can be divided into two main subsystems: the innate and the acquired immune system.

Each of these systems has its own cellular and soluble components that help to recognize and eliminate harmful processes such as microbial infections or tumors. The innate immune system reacts quickly to microbial invasion whereas the response of the acquired immune system takes longer. However, in the event of a second exposure to the same infection, the acquired immune system can remember the first encounter and reacts more promptly.

The cellular network of the innate immune system is mainly integrated by phagocytes. Some of these cells have the capacity to engulf material to be presented for recognition to cells of the acquired immune system. This interaction is a critical process in the development of an effective acquired immune response. In this thesis I use *Listeria monocytogenes* and *Salmonella typhimurium* to study the immune response against intracellular bacteria. Both *Listeria* and *Salmonella* are food-borne bacteria with a peculiar mechanism of

invasion that makes them very useful tools to understand the function of the immune system during intracellular bacterial infection. The role of one of the most important cells of the innate immune system, dendritic cells (DCs), in response to these bacteria, as well as their interaction with T cells, is the main focus of this thesis.

Listeria and *Salmonella* as infection models

Listeria and *Salmonella* share some pathogenic features, but also have many differences. As mentioned above, they are both intracellular bacteria. However, they have a very different life cycle inside a host cell. As a consequence of their particular mechanisms of evasion, the ensuing immune response has some properties unique to each pathogen. Table 1 summarizes some of the similarities and differences between these two microbes, which will be subsequently discussed.

The Gram Slam

Bacteria are one of the most common infectious agents known to date. The general classification of bacteria had its first breakthrough in 1884 when Hans Christian Gram published a staining method that could distinguish two large classes of bacteria (1). Bacteria that stain positive for Gram's stain contain a cell wall rich in peptidoglycans. These peptidoglycans are associated with the cytoplasmic membrane by lipoteichoic acids. On the other hand, Gram-negative bacteria have a thin inner wall also containing peptidoglycans adjacent to the cell membrane. However, an outer wall rich in lipopolysaccharides (LPS) surrounds the thin, peptidoglycan-containing inner wall. In contrast, Gram-positive bacteria lack LPS. Despite the obvious limitations of the Gram method

in the identification of specific bacteria, it remains useful today due to its ability to distinguish them based on the main components of their cell wall.

Table 1. Properties of *Listeria* and *Salmonella* and features of the immune response against them

	Listeria	*Salmonella*
Classification	Gram-positive	Gram-negative
Natural route of entry	Oral	Oral
Life cycle	Intracellular, escapes the phagocytic vacuole	Intracellular, colonizes the phagocytic vacuole
Immunodominant antigen	Listeriolysin O	Flagellin
Immunostimulatory molecules	Peptidoglycans, Lipoteichoic acid, DNA	LPS, Peptidoglycans, DNA
Important cytokines	TNF-α, IL-12, IFN-γ, IFN-α/β	TNF-α, IL-12, IFN-γ
Innate cells	Monocytes, Neutrophils, Macrophages, DCs	
Acquired immune response	Dominated by CD8 T cells	Dominated by CD4 T cells

Listeria monocytogenes

Listeria is a Gram-positive bacterium that poses a risk to certain groups in the human population, such as pregnant women, neonates and immunodeficient individuals. Since the bacteria is most often spread by the oral route, the first

symptoms may include nausea and diarrhea. In more serious complications, the bacteria can spread to the central nervous system and cause meningitis. In pregnant women, severe listeriosis can lead to miscarriage, stillbirth, premature delivery or infection of the newborn (2).

Despite being a serious threat to the groups mentioned above, the wide use of *Listeria* as an infection model arises mostly from the fact that mice infected with the bacteria develop a typical granulomatous disease. This infection and its resolution in animal models, which requires cellular immunity, could be compared to tuberculosis in humans (3). The safer manipulation of *Listeria* compared to *Mycobacterium tuberculosis* led to its increasing use as infection model in mice. Thus, since Mackaness first adopted *Listeria* as an infection model in the 1960s (4), it has helped to unravel many of the mechanisms of immunity against bacterial infection.

The way of the rocket: the life cycle of Listeria in a host cell

Listeria was first isolated from the blood of rabbits suffering of mononuclear leucocytosis and was originally named *Bacterium monocytogenes* (5). The bacteria, however, invade not only phagocytic cells such as monocytes, but virtually any nucleated mammal cell. A well-characterized intracellular cycle of *Listeria* begins with its attachment to, and internalization by, the host cell. In phagocytic cells a large battery of host receptors, that will be further discussed, aid these processes. In non-phagocytic cells, invasion can be mediated by bacterial invasins such as the internalins A and B. Invasion through internalin A is restricted to cells expressing E-cadherin, mostly epithelial cells (6), whereas internalin B mainly interacts with the hepatocyte growth factor receptor (7). Internalin A- and B-mediated entry into a cell is a specie-specific process. For example, internalin A binds E-cadherin of humans and guinea pigs, but not mice. Conversely, internalin B binds Met of humans and mice, but not guinea pigs (8, 9).

Once a bacterium is internalized by the host cell, it can escape into the cytosol before being killed in a lysosome. This is mainly mediated by a pore-forming cytolysin, listeriolysin O (LLO) (10, 11). In coordination with LLO, two other enzymes secreted by the bacterium, the phospholipases PI-PLC (12-14) and PC-PLC (15, 16), complete the destruction of the constraining vacuole. Bacterial liberation into the host cytosol is followed by polymerization of actin filaments. This is mediated by a bacterial protein named ActA (17). The bacteria use host actin to move within the cytosol (17-19). The ultimate goal of the pathogen is to launch itself out of the infected cell and spread to neighboring cells in a direct cell-to-cell fashion. The image of *Listeria* launching itself into adjacent cells is not inaccurate, as actin filaments of the host cell visually resemble rocket-powered motion as illustrated in figure 1.

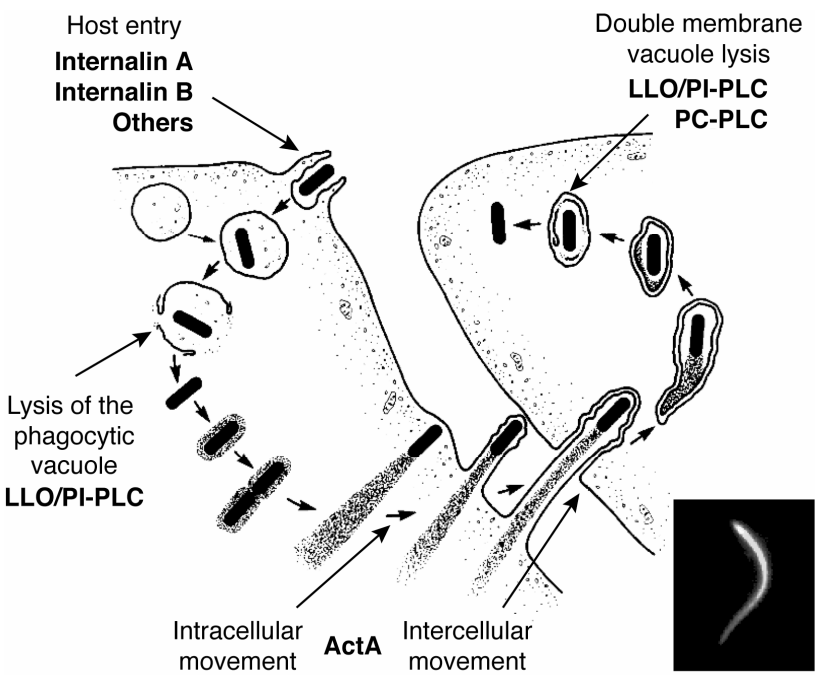


Figure 1. Typical life cycle of *Listeria*, adapted from Tilney and Portnoy (17). In the inset, *L. monocytogenes* moves in the cytoplasm of *Xenopus laevis* eggs by harnessing the force provided by the polymerization of actin filaments. (From Dr. Tim Mitchison's laboratory, Harvard University: http://mitchison.med.harvard.edu/research/researcharea.html?area=1).

Salmonella

Several hundred species of enteropathogenic bacteria are currently grouped under the genus *Salmonella*. The taxonomy of the group is complicated and diverse, comprising species with a wide range of hosts and pathogenicity (20). However, all *Salmonella* have the same route of spreading by ingestion of contaminated food or water. Curiously, although *Salmonella* can indeed be found in salmon (21), its name rather comes from one of the two scientists that first isolated it from pigs in 1885, Daniel E. Salmon, who at the time mistakenly believed it was the causative agent of the swine plague (22).

In humans, infection with *Salmonella* will cause a variable degree of illness, ranging from mild enteritis to severe systemic infections, depending on the particular serovar. *Salmonella enterica,* subspecie *enterica* serovar Typhi (*S. typhi*) has adapted to infect humans and is the cause of typhoid fever, an infection that is, tragically, often lethal. Since *S. typhi* has evolved into a host-specific pathogen, its transmission implicates ingestion of material contaminated with feces from infected people. Thus, typhoid fever is a health problem in places with poor sanitation. Partially because of this host-specific condition, another serovar of *Salmonella*, *Salmonella enterica* subspecie *enterica* serovar Typhimurium (*S. typhimurium*), emerged as a reliable bacterium to study Gram-negative bacterial infections in animal models. The fact that *S. typhimurium* causes a disease in mice that resembles typhoid fever in humans makes this bacterium an interesting model to understand the immune response against this type of infection (23).

Salmonella injects its way into a host cell and remains in vacuoles

Invasion of non-phagocytic mammal cells by *Salmonella* is a complex process that is not completely understood. However, some of the most important parts of this process have been revealed. Similar to other Gram-negative pathogens, *Salmonella* relies on specialized secretion systems to infect a host cell. The

most important secretion system of *Salmonella* is the type III secretion system (24). The function of this system is to inject effector molecules that promote internalization of *Salmonella* by the host. Some of these effector molecules, such as SipA and SipC, will facilitate bacterial engulfment by manipulating host cytoplasmic actin (25, 26). The changes induced by *Salmonella* in the eukaryotic cell are, however, reversible. Strikingly, the bacterium itself promotes the recovery of the normal cellular architecture after its internalization is complete (27). Once inside the host cytosol, engulfed in a vacuole that resembles an early phagosome, the bacteria will drive this vacuole away from maturation into a classical bactericidal compartment. Instead, *Salmonella* will interfere with this maturation process and turn the vacuole into a favorable niche for survival and replication (28). Thus, *Salmonella*'s strategy to survive within the host cell resides in preventing the internalized vacuole from becoming a degrading compartment. In contrast, *Listeria* escapes the vacuole before being killed.

Front line defense: the innate immune system

Once invading bacteria break through the intestinal barrier after oral infection, the fate of the bacteria inside the host will be the result of the coordinated action of different components of the immune system. The first line of defense against pathogens is provided by components of the innate immune system. Phagocytic cells, such as monocytes, macrophages and neutrophils are essential to control intracellular bacteria such as *Listeria* and *Salmonella*. Likewise, some of the molecules they secrete in response to the bacteria, such as TNF-α, IFN-γ, IL-12 and iNOS, are fundamental [(29) and reviewed in (30-32)]. Furthermore, the use of infection models such as *Listeria* and *Salmonella* has greatly furthered

our understanding of one of the most important component of the innate immune system in the defense against microbes: the DCs.

AC⁄DC

"**A**nother **C**ell/a **D**endritic **C**ell", in the semantic sense of "a different cell", could very well describe the function of DCs. The classical notion of AC/DC (Alternate Current/Direct Current) means that something can function with either type of electricity. Such plasticity is also applicable to DCs, considering the diversity of the stimuli that influence their function. DCs were revealed to modern science in 1973 (33). Since then we have learned that DCs are the most potent antigen presenting cell (APC) of the immune system, due to their superior capacity to stimulate naïve T cells compared to other APCs such as macrophages and B cells (34-36). Thus, the knowledge accumulated about the function of DCs during inflammation and other immune responses has increased enormously during the last 3 decades. However, as is often the case in science, every piece of new information generates new questions. Many of the unresolved questions about DC's plastic functionality arise from the fact that DCs are a population heterogeneous in phenotype and function.

DC types

In mice, the bulk population of DCs can be divided into two major categories depending on their phenotypic and functional properties: conventional DCs (cDCs) and non-conventional DCs. In addition, both kinds of DCs comprise more than one distinct subpopulation (figure 2).

Among cDCs, two groups can be distinguished according to their migratory capacity, one that migrates to a draining lymph node after collection of antigens, such as Langerhans cells for example, and other that is tissue-resident (37). Phenotypically, most murine cDCs can be identified by high

expression of the integrin CD11c and major histocompatibility complex (MHC) II molecules. Langerhans cells can be specifically recognized by expression of the C-type lectin langerin, a molecule that belongs to a family of important receptors (38, 39). In addition, several subsets of tissue-resident cDCs can also be identified by expression of surface molecules, such as CD8α and CD4 in the case of splenic cDCs for example (40). There is also evidence that the different cDC subsets can specialize in performing different functions. For example, under certain conditions $CD8\alpha^+$ DCs induce a T_H1-polarized response whereas $CD8\alpha^-$ DCs tend to induce a T_H2 response (41-43). In addition, $CD8\alpha^+$, but not $CD8\alpha^-$ DCs, preferentially stimulate cytotoxic T cells both by classical presentation of intracellular antigens (44) or by cross-presentation in MHC-I (45). In contrast, in a model system, it has been shown that the $CD8\alpha^-$ subset surpasses the $CD8\alpha^+$ DCs in presentation on MHC-II molecules. This correlates with higher expression of proteins involved in the MHC-II presentation pathway (46).

Non-conventional DCs include plasmacytoid DCs (pDCs) and other cells with DCs features. In addition to their plasmacytoid morphology, pDCs exhibit surface markers that are typically present in other cell types like B220 and Ly6C. They also express an intermediate level of CD11c and lack CD11b expression (47, 48). pDCs specialize in the production of type I interferons (IFN-α/β), particularly upon viral stimulation (49). Other cells with DCs attributes seem to be generated during inflammatory conditions. They have a mixed phenotype and appear to specialize in the production of molecules such as TNF-α, IL-12 and iNOS (50-53).

Thus, the phenotypic diversity and functional specialization of DCs adds to the complexity of their study (figure 2). As a consequence, the investigation of DC function during infection with bacteria such as *Listeria* and *Salmonella* requires the assessment of different subpopulations, which may have a different role during bacterial infection.

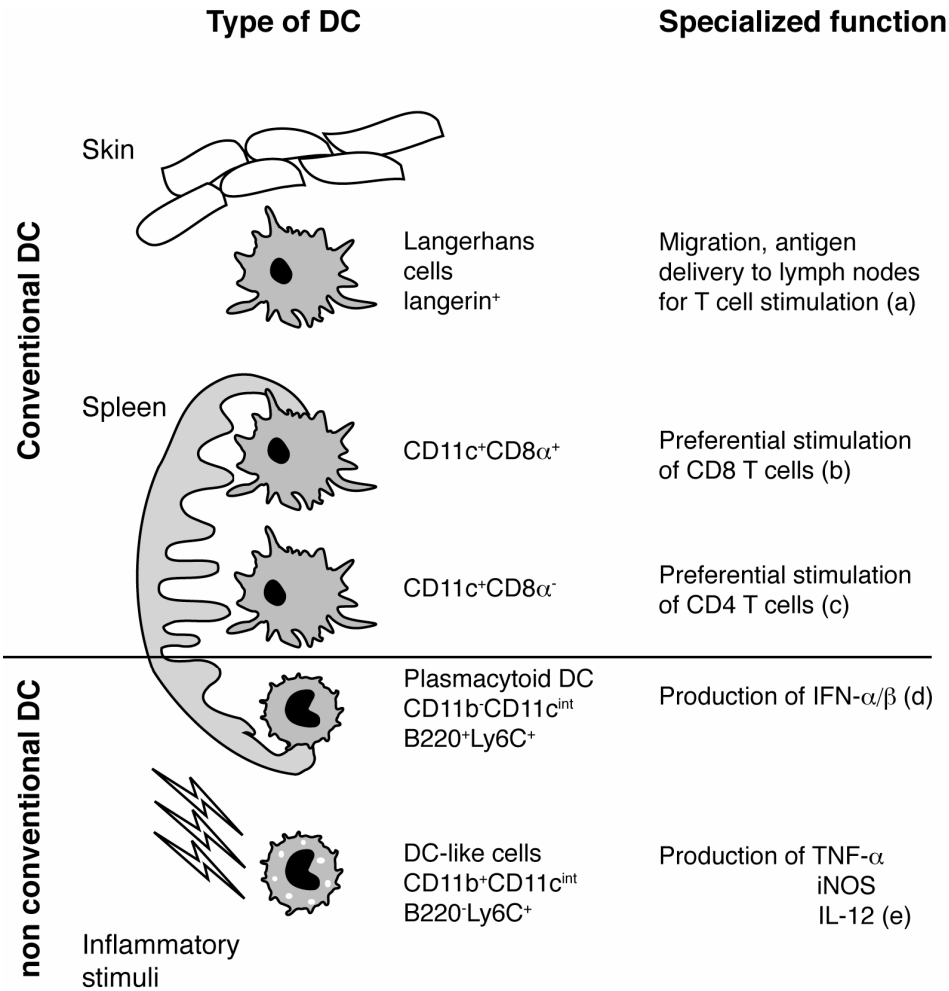


Figure 2. Examples of DC subpopulations and their surface phenotype and functions. References: a. (54), b. (44, 45), c. (46), d.(49), e. (50-53)

DC control of Listeria and Salmonella infection

Intestinal and splenic DCs are infected *in vivo* by *Listeria* (55-57). Although DCs are not the main reservoir for the bacteria (55, 56), accumulation of *Listeria* in the spleen could be dependent on $CD8\alpha^+$ DCs (56). Consistent with their prominent role as T cell stimulators, rather than pathogen eliminators, DCs seem to be less efficient than macrophages at killing *Listeria* (58). Furthermore,

using an *in vivo* DCs ablation model, Jung *et al* demonstrated that DCs are more potent stimulators of naïve T cells than macrophages (36). In the same study, the authors show the requirement of CD11c-expressing cells *in vivo* in eliciting an anti-*Listeria* CD8 T cell response.

DCs could access *Salmonella* acquired orally after bacteria traverse through specialized M cells (59) or directly by extending dendrites between the epithelial cell tight junctions (60-63). As in the case of *Listeria*, DCs are vital to initiate a T cell response during oral *Salmonella* infection (64) and DCs harboring *Salmonella* correlates with T cell stimulation *ex vivo* (64, 65). Furthermore, both *Listeria* and *Salmonella* induce profound changes in DC biology that influence their function as APCs. These changes include upregulation of costimulatory molecules (53, 55, 66, 67), production of inflammatory cytokines (53, 66, 68) and alteration of their tissue distribution and migratory pattern (64, 68, 69). Thus, DC contact with intracellular bacteria such as *Listeria* and *Salmonella*, or with bacteria-derived products, is strongly reflected in DC physiology. Some of these changes are part of the process of DC maturation, and will influence DC interaction with lymphocytes.

Antigen presentation and DC maturation

The ultimate function of an APC is to process a relatively complex antigen and present a fraction of it, a peptide, to the T cells. This interaction is the most important connection between the innate and the acquired immune systems. Antigens are presented by APCs in the molecular support called MHC. Several kinds of MHC molecules have been described. They are called classical molecules, such as MHC-I and MHC-II, and non-classical like, for example, CD1 molecules. MHC-I and II molecules present peptides in a process that is well characterized. Most cells are able to process and present antigens on MHC-I molecules to stimulate CD8 T cells whereas stimulation of CD4 T cells through presentation on MHC-II molecules is restricted to APCs such as DCs.

But the presentation of the antigen is just the tip of the iceberg. The fate of the T cells after their interaction with a DC will depend on the maturation and activation state of the latter. Thus, in order to potently activate naïve T cells, DCs have to undergo a process of maturation simultaneous with antigen processing and presentation.

DC maturation involves profound changes in DC physiology. These changes include phenotypic and functional alterations that influence the outcome of the DC-T cell interaction. Among the phenotypic changes that are commonly associated with mature DCs are the upregulation of costimulatory molecules such as CD80 and CD86, as well as upregulation of CD40 and MHC-II. Physiological changes include a transient increase in antigen sampling and processing, alteration of their migratory pattern, and secretion of soluble immunomodulators such as inflammatory cytokines (Reviewed in (70)). Thus, increased levels of costimulatory and MHC-II molecules have been used as a hallmark of DC maturation. However, these phenotypic changes do not necessarily translate into an increased capacity to stimulate T cells (70-73). Thus, it is important to highlight the distinction between phenotypic and functional maturation. As a consequence, future efforts aimed at identifying the mechanisms of DC maturation must address whether phenotypic changes influence the capacity of the DCs to induce T cell clonal expansion and effector functions.

Pathways of DC maturation

The profound changes that DCs undergo during the maturation process imply a complex regulation that is just beginning to be unveiled. As mentioned above, DCs can display phenotypic signs of maturation without the potential to induce T cell effector functions. A discrepancy between phenotypic and functional maturation has recently been associated with the pathway by which a DC enters the maturation cycle. For example, a DC that directly interacts with a microbial

product such as LPS becomes phenotypically and functionally mature. The latter includes production of cytokines and gaining capacity to induce effector functions in T cells (73). However, the direct maturation of DCs and its regulatory mechanism during infection with complex pathogens, such as intracellular bacteria, has not been addressed thus far and is one of the aims of this thesis.

Some of the cytokines produced by DCs have the potential to promote phenotypic maturation of DCs that do not directly interact with the pathogen (70). In addition, DCs are not the only cells in the body that can sense pathogens or their related products. Other cells, such as epithelial cells, can also produce inflammatory cytokines in response to microbial stimulation, indirectly influencing the maturation of DCs. However, DCs that mature indirectly through cytokine stimulation appear to be insufficient at inducing full activation of naïve T cells. For example, using mixed bone marrow chimeric mice, Spörri *et al* (73) constructed a system in which half of the DCs in the mixed chimera could sense the microbial product while the other half did not. In this setting, the DCs that could not directly sense the microbial product matured only indirectly. The authors showed that indirectly matured DCs displayed typical phenotypic maturation including increased CD40, CD86 and MHC after exposure to LPS or CpG. Furthermore, the indirectly matured DCs promoted CD4 T cell clonal expansion, but the CD4 T cells were, however, devoid of helper function (72, 73).

From these data several interesting and important questions arise. An obvious issue is whether there is biological relevance for the indirect maturation phenomenon. Due to the magnitude of the response of indirectly matured DCs, they may have a role in shaping the immune response. Although this must be tightly regulated they could, for example, induce effector functions in activated or memory CD4 T cells, possibilities not addressed in the work of Spörri *et al*. Indeed, inflammatory conditions enhance proliferation and differentiation of

activated CD4 T cells (74), and macrophages can efficiently stimulate activated, albeit not naïve, CD8 T cells (36). Thus, a possibility could be that the DCs that mature directly are the ones that stimulate naïve T cells to initiate the acquired immune response while those that mature indirectly have a supportive role at different stages of the infection. Alternatively, or even simultaneously, it could be a mechanism to induce tolerance to self-reactive clones of T cells more efficiently during infection and thus focus the immune response on the relevant foreign antigens (75, 76). Whichever the case, the existence of several possibilities warrants investigation of the mechanisms of both direct and indirect DC maturation.

Pathogen recognition and DC maturation

As discussed above, DCs can mature by direct recognition of microbial products or indirectly through the effect of inflammatory cytokines secreted upon exposure to the same microbial products. Thus, recognition of microbes and their products is an event intrinsically linked to DC maturation. DCs are equipped with a vast battery of receptors that efficiently recognize invading microorganisms. Some of the important receptor families are starting to be identified and characterized. Among these families, the Toll-like receptor (TLR) family is of crucial importance, due to the fact that TLRs can induce both direct and indirect DC maturation.

TLR signaling: the Toll bridge at work

Pathogenic microorganisms express a number of macromolecules inherent to their nature, commonly known as pathogen-associated molecular patterns (PAMPs). In turn, potential hosts can recognize these PAMPs through PAMP recognition receptors. Among these receptors, the TLRs contribute significantly to the orchestration of an efficient immune response against pathogens. TLRs

control the development of the immune response against pathogens by selectively triggering intracellular signals in response to specific PAMPs. These intracellular signals, when activated in DCs, critically contribute to their maturation into potent APCs.

The TLR family comprises 11 identified members in mice that are widely distributed among APCs including DCs. Although not comparable to T and B cell receptors, TLRs bring considerable specificity to the innate immune system. Some of them can associate as heterodimers, or even with non-TLR membrane clusters, to further diversify their recognition potential (77-79). Table 2 summarizes mouse TLRs and some of their identified ligands. TLRs are located both at the cell surface and intracellularly in endosomes. Regardless of their cellular location, engagement of TLRs leads to activation of transcription factors that promote transcription of inflammatory cytokines (80) and upregulation of costimulatory molecules (81-83).

Table 2. Murine TLRs and some of their ligands

TLR	Natural ligand (ref.)	TLR	Natural ligand (ref.)
1 and 2	Tri-acyl lipopeptides (84)	7	Single stranded RNA (85)
2 and 6	Di-acyl lipopeptides (86)	8	No natural ligand identified
3	Double stranded RNA (87)	9	CpG DNA (88)
4	LPS (89)	10	Not functional (90)
5	Flagellin (91)	11	Profilin-like protein (92)

Signal transduction from TLRs requires adaptor molecules. The protein MyD88 is the major adaptor molecule in the TLR signaling cascade (80). However, a MyD88-independent pathway, mediated by TRIF, makes an important contribution to TLR signaling. The TRIF pathway, which is activated by TLR3 and TLR4, results in the production of IFN-α/β (80). Thus, TLRs are key receptors in the identification of pathogens by the innate immune system. In

connection with this, TLRs also play an essential role in the maturation of DCs and activation of other APCs. Figure 3 summarizes some of the components in the intracellular signaling cascade of the most relevant TLRs in fighting infection with intracellular bacteria such as *Listeria* and *Salmonella*.

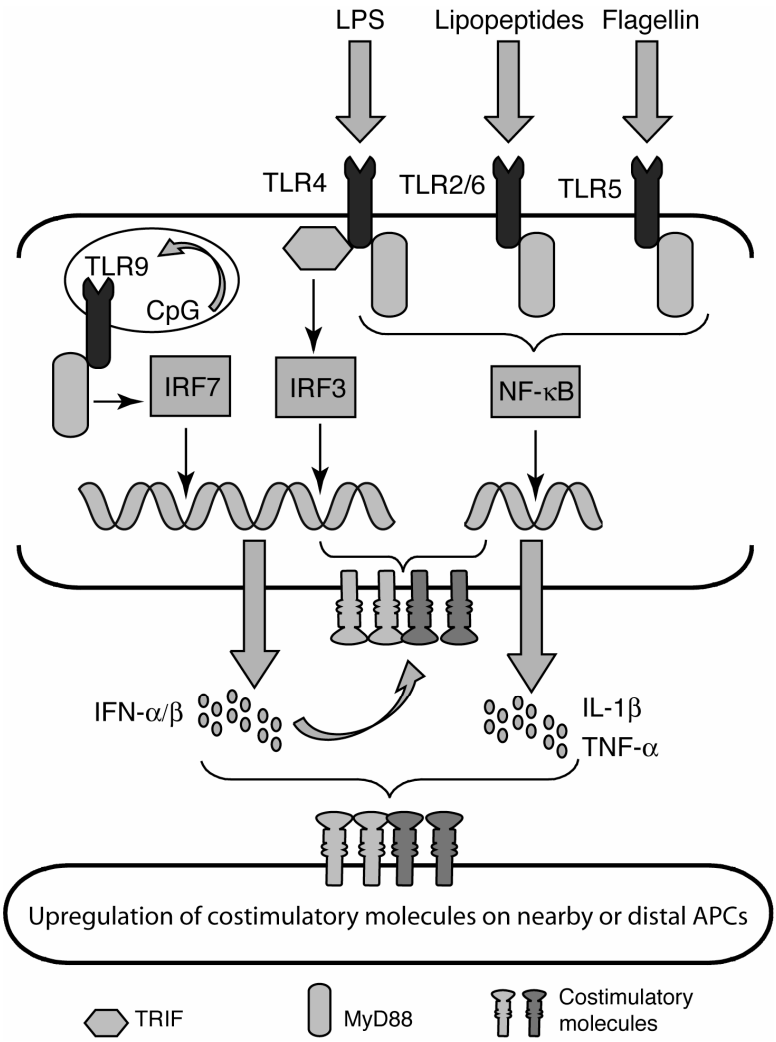


Figure 3. Possible TLRs involved in bacterial recognition and their functions. TLRs 2, 4, 5, 6 and 9 have the potential to recognize intracellular bacteria such as *Listeria* and *Salmonella*. Heterodimers formed by TLR2 and TLR6 could be involved in recognition of Gram-positive bacteria whereas TLR4 could be more relevant during Gram-negative infections. TLR5 could recognize both flagellated Gram-positive and Gram-negative bacteria.

TLRs during Listeria and Salmonella infection

Both *Listeria* and *Salmonella* express several PAMPs that can potentially be recognized by TLRs. In the case of *Listeria*, an important component of the cell wall, lipoteichoic acid, can be recognized with the involvement of TLR2 (93, 94). Likewise, the major component of *Salmonella*'s outer membrane, LPS, can be recognized by TLR4 (89). In addition, both *Listeria* and *Salmonella* express flagellin and contain CpG motifs in their DNA, molecules that can be recognized by TLR5 and TLR9, respectively (88, 91). Intracellular flagellin can also be recognized by another family of receptors that will be further discussed.

Supporting an important role of TLRs in the response against *Listeria*, two coincident studies reported increased susceptibility of MyD88 knockout mice infected with this bacterium (95, 96). Infected MyD88$^{-/-}$ mice had diminished serum levels of important anti-*Listeria* cytokines such as IL-12p40 and IFN-γ (95, 96). Moreover, production of TNF-α and iNOS was compromised in the spleen of *Listeria*-infected MyD88$^{-/-}$ mice (97), although serum levels of NO_2^- and NO_3^- were normal (95). In addition, MyD88-signaling mediates production of the antibacterial lectin RegIIIγ against oral *Listeria* (98). MyD88$^{-/-}$ mice are also more sensitive to *Salmonella* infection (99, 100). However, immunological parameters such as the cytokine profile are less studied in mice infected with *Salmonella*.

Infection with live virulent bacteria such as *Listeria* and *Salmonella* is a major challenge for the immune system. Thus, the existence of redundant mechanisms could be required to survive such threatening infections. Indeed, several studies agree that deficiency of a single TLR is not definitive in the outcome of an infection with either *Listeria* or *Salmonella* (95, 100, 101). As both bacteria express several PAMPs and have multiple mechanisms to subvert the immune system, it is not surprising that the response against them is not determined by a single receptor interacting with its ligand. Furthermore, despite

the importance of TLRs, increasing evidence highlights the significance of other families of receptors involved in the recognition of intracellular bacteria (102).

Other receptors: NLRs and beyond

Members of the nucleotide-binding oligomerization domain-like receptor (NLR) family recognize pathogens or their products that reach the host cell cytosol (103). The NLR family groups intracellular PAMP recognition receptors that activate different signaling pathways. One such pathway, triggered by Ipaf and NALP3, is the activation of inflammatory caspase (casp-1) through assembly of a multiprotein complex called the inflammasome (104). Active casp-1 catalyzes the conversion of procytokines to active IL-1β and IL-18, two potent inflammatory cytokines. An alternative pathway, initiated by Nod1 and Nod2, promotes direct transcription through activation of the transcription factor NF-κB (104). Similarly to the TLR-mediated pathways, this will result in an inflammatory response.

NALP3-dependent activation of IL-1β and IL-18 by macrophages infected *in vitro* with *Listeria* indicates a possible role of NLRs during *Listeria* infection (104, 105). Moreover, Nod2-deficient mice are more sensitive than wild type mice to oral *Listeria* infection (106). In addition to NLRs, other thus far unidentified intracellular receptors may also mediate the anti-*Listeria* innate immune response (107-109). These receptors activate the innate immune system, inducing the expression of several genes such as those encoding IFN-α/β, MHC-II and costimulatory molecules (108).

Although less studied than during *Listeria* infection, receptors other than TLRs may also be involved in the innate defense against *Salmonella*. Recently, two groups reported Ipaf-mediated intracellular recognition of *Salmonella* flagellin in a TLR5-independent fashion (103, 110, 111). Casp-1 activation through Ipaf-mediated flagellin recognition resulted in production of IL-1β and IL-18 (103, 110). It also remains possible that receptors other than

TLRs and NLRs can be involved in the host response against *Salmonella*, although this has not yet been reported.

Other families of receptors different from TLRs and NLRs are also important for the innate immune system. This includes the C-type lectin receptor family and the RIG-I-like receptor family. Members of both families can induce DC maturation and activation of the immune system. However, the identified members of these families capable of inducing DC maturation are restricted to recognizing fungi (dectin-1 as a C-type lectin receptor) (112) and double-stranded RNA (MDA5 and RIG-I as Rig-I-like receptors) (113). This specificity makes these particular receptors less relevant in antibacterial responses. Despite this, it is apparent that several families of receptors coexist and cooperate (114) and need to be considered when studying DC maturation. Such redundancy in the system underscores the need to study multiple pathways, especially when assessing *in vivo* responses to bacteria and DC maturation. In particular, the relative contribution of TLR-mediated and TLR-independent pathways to bacterial-induced DC maturation *in vivo* is an important issue that is not completely resolved at present and is a topic investigated in this thesis.

Acquired defense against *Listeria* and *Salmonella*

The role of the innate immune system is instrumental in eliminating both *Listeria* and *Salmonella*. A decisive step of this process is the initiation of an efficient acquired immune response. Both main cell types of the acquired immune system, T and B lymphocytes, are involved in eradicating these bacteria. However, although B cells have a role in eliminating both *Listeria* and *Salmonella*, (115-117), their impact is less significant when compared with the

input of T cells. Furthermore, due to their different life cycles, the extent and type of the T cell response against each of these bacteria is different.

The T cell solution

Complete elimination of *Listeria* mostly relies on the development of cytotoxic CD8 T lymphocytes (CTLs) (118). The proposed mechanisms for CD8 T cell-mediated immunity are the elimination of infected cells via perforin and granzymes, and the production of cytokines such as IFN-γ to activate phagocytes (119). In addition to classical MHC-I restricted CD8 T cells, CTLs restricted to non-classical MHC molecules also contribute significantly. The best characterized non-classical MHC-I anti-*Listeria* response is the one restricted to the presenting molecule called H2-M3 (120). These molecules present bacteria-derived peptides that contain a formylated amino terminal methionine residue. H2-M3-restricted CD8 T cells are cytolytic, produce IFN-γ and are sufficient to confer protection against a primary infection (121-123). Although both kinds of CTLs are important and not redundant (124) successive bacterial challenges are mainly cleared by expansion of classic MHC-I-restricted CTLs (125). The role of CD4 helper cells is less studied during listeriosis, but it is known that they contribute to providing a T_H1 environment (30). Moreover, CD4 T cells seem to control the development of CD8 memory T cells against *Listeria* (126-128).

In contrast to *Listeria*, the main population of T cells mediating protection against *Salmonella* is CD4 T cells. Both non-classical and classical MHC-I-restricted CD8 T cells are generated during *Salmonella* infection (129-131). However, their overall contribution to protective immunity is much less than that of CD4 T cells. As discussed above, CD4 T cells are less relevant in primary exposure to *Listeria*, but if not present then, subsequent exposures are detrimental to the host (127, 128). In contrast, CD8 T cells are dispensable

during primary infection with *Salmonella*, but are important for the memory response (132).

Thus, T cells are crucial for elimination of intracellular bacteria such as *Listeria* and *Salmonella*. Using these two bacteria as infection models, the knowledge about the generation of an immune response has extended considerably. Yet, the mechanisms regulating this process are still incompletely understood. In particular, the DC-T cell interface and the factors involved in DC maturation that in turn define the outcome of that interface, are largely unexplored. For example, despite the known importance of MyD88 in DC maturation, mice deficient in this adaptor molecule develop memory CD8 T cells against *Listeria* (133, 134). The features of this MyD88-independent mechanism remain unknown. Thus, understanding the relative contribution of multiple pathways on DC maturation, and the resulting consequences on DC interaction with other cells, is the focus of intense research and one of the aims of this thesis.

AIMS

The overall aim of this thesis is to study DC function, with special focus on the maturation process, during infection with Gram-positive and Gram-negative intracellular bacteria. To tackle this, three projects were designed and conducted, and are represented by the three papers included in this thesis.

Specific aims of the papers:

Paper I.

1. To characterize the expansion and expression of costimulatory and anti-bacterial molecules by different DC populations during *Listeria* infection.
2. To determine the influence of the intracellular compartment in which the bacteria is detected in the production of the anti-bacterial molecules.

Paper II.

3. To investigate the mechanism of *Listeria*-induced DC maturation by assessing the relative contribution of MyD88- versus IFN-αβR-derived signaling to DC-mediated T cell stimulation and development of T cell memory.

Paper III.

4. To determine the relative contribution of MyD88 and IFN-αβR in direct versus indirect maturation of DCs during *Salmonella* infection.

MATERIALS AND METHODS

The following section comprises general procedures and materials used in the experiments performed to obtain the results described in the section "results and discussion" of this thesis. Specific materials and methods used for experiments not shown in this thesis but shown in the individual papers can be found in the paper's respective material and methods section.

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). MyD88$^{-/-}$ mice, IFN-αβR$^{-/-}$, OVA$_{257-264}$ peptide-specific TCR transgenic mice (OT-I) and OVA$_{323-339}$ peptide-specific TCR transgenic mice (OT-II), all on C57BL/6 background, were kindly provided by S. Akira, J. Demengeot, N. Lycke and S. Schoenberger, respectively. MyD88$^{-/-}$ and IFN-αβR$^{-/-}$ mice were crossed to generate MyD88$^{-/-}$IFN-αβR$^{-/-}$ double knockout mice (called DKO mice). Mice were bred and maintained at the Laboratory for Experimental Biomedicine at Göteborg University. Mice were provided food and water *ad libitum*. Experiments were performed with 8-12 weeks old mice. All animal experiments were carried out following protocols approved by the government animal ethical committee and institutional animal use and care guidelines.

Bacteria

Listeria monocytogenes strains 10403s (papers I and II), 10403s LLO^{-} (EJL1) and 10403s ActA^{-} (EJL2) (both used in paper I), as well as the wild type 10403s and the ActA^{-} derivative expressing full length OVA (paper II) were all kindly provided by H. Shen. Bacteria were grown from glycerol stocks in Brain and

Heart Infusion medium overnight with shaking at 37°C. Bacteria from overnight cultures were centrifuged and resuspended in PBS (pH 7.4) at the desired final concentration. *S. typhimurium* χ8554, χ4550 expressing OVA-GFP, and the eGFP-expressing SM022 strains were grown in Lennox (χ8554) or Miller's Luria-Bertoni (OVA-GFP-χ4550, eGFP-SM022) broth overnight at 37°C (paper III). Strains χ8554 and χ4550 expressing OVA-GFP were kindly provided by R. Curtis III and eGFP-SM022 was from A. Zychlinsky.

Animal infections

Paper I. When mice were infected orally they were first given 100 µl of 1% $NaHCO_3$ followed 10 minutes later by administration of 2-6 x 10^9 CFU of wild type *Listeria* in 100-200 µl of PBS. In experiments where animals were infected iv, bacteria from overnight cultures were diluted in PBS and mice were given a single 150 µl injection in the lateral tail vein. Doses were 5 x 10^4 – 5 x 10^5 CFU for wild type *Listeria*, 3 x 10^6 – 1 x 10^7 CFU for ActA$^-$ *Listeria* and 5 x 10^8 – 5 x 10^9 CFU for LLO$^-$ *Listeria*. This was done to achieve equivalent bacterial burdens with the different bacterial strains.

Paper II. Oral administration of wild type *Listeria* to C57BL/6 mice was done as described for paper I. The different knockout mouse strains received different oral doses to achieve equivalent bacterial burdens at the time of sacrifice. C57BL/6 received 2 x 10^9 CFU, IFN-αβR$^{-/-}$ received 8 x 10^9 CFU and MyD88$^{-/-}$ received 2-8 x 10^7 CFU. In experiments where different mouse strains were injected iv with wild type *Listeria* or the OVA-expressing derivative, the doses administered were 2 x 10^3 - 3 x 10^4 CFU for C57BL/6 mice, 3 x 10^4 CFU for IFN-αβR$^{-/-}$ mice and 2-3 x 10^2 CFU for MyD88$^{-/-}$ mice. For experiments addressing the memory response, mice were infected iv with 5 x 10^6 CFU of

OVA-expressing ActA$^-$ *Listeria* followed by a challenge 4 weeks later with 2 x 10^5 – 1 x 10^6 CFU of OVA-expressing wild type *Listeria*.

Paper III. C57BL/6 received 2 x 10^8 CFU, IFN-αβR$^{-/-}$ received 2 x 10^8 - 1 x 10^9 CFU and MyD88$^{-/-}$ and DKO mice received 2 x 10^6 - 10^7 CFU when infected orally with *Salmonella* χ8554. When IFN-αβR$^{-/-}$, MyD88$^{-/-}$ and DKO mice were infected orally with eGFP SM022 *Salmonella*, doses were increased 10-fold to increase the number of GFP$^+$ events.

In all experiments, the bacterial dose administered was determined by reading the optical density at 600 nm and was confirmed by viable plating on corresponding agar plates. Likewise, the bacterial burden in tissues analyzed was determined by plating serial dilutions of organ suspensions on agar plates at the time of sacrifice.

Preparation of cell suspensions

In initial experiments, single cell suspensions from the mesenteric lymph nodes (MLN) and spleen were prepared by digestion with 1.6 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 2 mg/ml DNAse I (Sigma-Aldrich) in HBSS (Gibco, Life Technologies, Paisley, UK) for 45 minutes at 37°C. To study cytokine production in paper I, collagenase and DNAse were substituted by 0.45 mg/ml Liberase (135) (Roche, Basel Switzerland), and Liberase was used for the rest of the experiments. Tissue was disaggregated by repetitive pipetting and erythrocytes were lysed with a hypotonic solution of NH_4Cl. The cells were washed and resuspended in RPMI (Gibco, Life Technologies) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich). A fraction was stained with trypan blue (Gibco, Life Technologies) to calculate the number of viable cells by exclusion of the dye.

Cytokine detection by RT-PCR

Mice were infected with 2-6 x 10^5 CFU *Listeria* and 20 hours later the spleens were collected. Spleens from infected and naïve mice (5 per group) were pooled and a single cell suspension was prepared as described above. CD11c-expressing cells were separated using anti-CD11c magnetic beads and an AutoMACS (both from Miltenyi Biotec Bergisch Gladbach, Germany) following the manufacturer's instructions. RNA from both the negative and positive fractions was extracted using TriPure (Roche, Basel Switzerland). Genomic DNA was removed with the DNA-free kit (Ambion, Austin TX) and the remaining RNA was quantified and stored at -70°C until used in the reverse transcription reaction. 1 μg of RNA was transcribed using the Reverse Transcription System kit (Promega, Madison, WI) followed by amplification of the cDNA. PCR was standardized and performed using products from Promega. Specific primers and their target gene are listed in table 3. IFN-α genes were targeted at conserved sequences with primers designed to amplify all known members of the family.

Table 3. Primers for PCR amplification

Target	Sequence	Size in bp (ref.)
β-actin	GTG GGC CGC TCT AGG CAC CAA CTC TTT GAT GTC ACG CAC GAT TTC	540 (136)
GAPDH	TGC TGA GTA TGT CGT GGA GTC TA AGT GGG AGT TGC TGT TGA AGT CG	602 (137)
IL-12p40	CGT GCT CAT GGC TGG TGC AAA G CTT CAT CTG CAA GTT CTT GGG C	452 (136)
IFN-α	ATG GCT AGG CTC TGT GCT TTC TCT GAT CAC CTC CCA GGC ACA	500 (138)
IFN-β	CCA TCC AAG AGA TGC TCC AG GTG GAG AGC AGT TGA GGA CA	353 (139)

Flow cytometry

Single cell suspensions were stained in HBSS containing 3% FCS, 5 mM EDTA and 20 mM HEPES (Gibco Life Technologies). Samples were first blocked with anti-FcγRII/III monoclonal antibody (clone 2.4G2) for 15 minutes at 4°C. Cells were washed, antibody cocktails were added and the cells were incubated for 20 minutes at 4°C. 7-aminoactinomycin D (7AAD, Sigma-Aldrich) was always used to exclude non-viable cells, except when analyzing CFSE labeled T cells.

A 5- or 6-color staining strategy was used to study pDCs. First, non-viable cells were excluded using 7AAD. Subsequently, lymphocytes and CD11b-expressing cells were also excluded with a cocktail containing Allophycocyanin-conjugated anti- CD19, TCRαβ and CD11b. The remaining population was selected using anti-B220-PE-Cy7, anti-CD11c-FITC and biotinylated anti-Ly6C followed by streptavidin Allophycocyanin-Cy7. Thus, pDCs were identified as $7AAD^-$, $CD19^-$, $TCR\alpha\beta^-$, $CD11b^-$, $CD11c^{int}$, $B220^+$, $Ly6C^+$ cells. When expression of CD80, CD86 or MHC-II was assessed, cells were additionally stained with PE conjugated anti- CD80, CD86 or MHC-II.

Detection of intracellular cytokines by FACS was assessed directly *ex vivo*. Cell suspensions in RPMI supplemented with 10% heat-inactivated FCS, 2 mM sodium pyruvate, 20 M HEPES and 0.05 mM 2-mercaptoethanol (all from Gibco Life Technologies) were incubated for 4 hours at 37°C in the presence of 5 μg/ml of Brefeldin A (Sigma-Aldrich). Cells were stained for surface molecules, fixed with 2% formaldehyde (HistoLab Products AB, Göteborg, Sweden) and resuspended in permeabilization buffer (HBSS containing 0.5% BSA, 0.5% Saponin and 0.05% Azide). The antibodies required were added and incubated for 30 minutes at room temperature.

Cells were processed on either a LSR I or LSR II flow cytometer (BD Biosciences, San Diego, CA) using Cell Quest or DiVa software, respectively (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc,

Ashland, OR) for all experiments.

***Ex vivo* T cell stimulation**

Mice were infected iv with OVA-expressing wild type *Listeria* and after 48 hours, spleens were pooled and single cell suspensions were prepared (paper II). CD11c-expressing cells were magnetically enriched using anti-CD11c magnetic beads and an AutoMACS (Miltenyi Biotec). Cells were then stained and $CD11c^{high}$ cells were sorted at low pressure using a FACSAria cell sorter fitted with a 100 μm nozzle and DiVa software (BD Bioscience). Purity was > 98.5%.

CD8 T cells from OT-I mice were isolated using the CD8 T cell isolation kit from Miltenyi Biotec following the manufacturer's protocol. The procedure always rendered > 85% purity. OT-I cells were labeled with CFSE by incubating 10^7 cells in 1 ml of 1 μM CFSE diluted in PBS for 8 minutes. The reaction was stopped by addition of 1 ml of FCS. The cells were washed twice and resuspended in culture media. DCs and CFSE-labeled OT-I cells were incubated in RPMI containing gentamicin in 96 round-bottom well plates. After 3.5 days, co-culture supernatant was collected and stored at -20°C until assayed for IFN-γ content. The cells were harvested, stained and acquired in an LSR II flow cytometer.

***In vitro* DC-T cell assay.**

Flt3L-producing melanoma cells (140) were expanded in RPMI medium containing gentamicin. Medium was replaced after 24 hours and supernatant was harvested at 72 hours and stored at -20°C to be used as a Flt3L-rich supplement during *in vitro* culture of the *in vivo* expanded DCs. The melanoma cells were harvested by incubating them with 0.05% of Trypsin and 0.053 mM EDTA (Invitrogen), for 5 minutes at 37°C. Trypsinization was stopped by addition of medium containing 10% FCS and cells were centrifuged for 5 minutes. Cells were washed 3 times with PBS and resuspended in PBS at 2.5 x

10^6 cells/ml. Mice were injected with 0.25 x 10^6 live cells subcutaneously in each groin. Fourteen days later, spleens were removed, pooled and DCs were enriched using magnetic beads. The enriched $CD11c^+$ fraction was incubated with *Salmonella* χ4550 expressing OVA-GFP in antibiotic-free medium supplemented with 50% Flt3L supernatant for 2 hours in 6-well low adherence plates (Corning Inc. Acton, MA). GFP^+ cells were sorted as described above and serial dilutions were cultured with a fixed amount (256,000 cells) of CFSE-labeled $Ly5.1^+$ $CD4^+$ OT-II cells. Culture conditions were as described above for the *ex vivo* T cell stimulations.

Detection of cytokines in culture supernatants

Culture supernatant from DC-OT-I cell co-culture (paper II) and DC-OT-II co-culture (Paper III) was assessed for IFN-γ content using an IFN-γ ELISA set (BD Bioscience).

RESULTS AND DISCUSSION

The role of pDCs during viral infections has been well documented (49). However, their potential involvement in non-viral infections has not been studied. I thus set out to address the role of pDCs during infection with the intracellular bacterium *Listeria*. Using 4-color FACS analysis I found evidence that pDCs were reacting to *Listeria* infection. That is, an increase in a population that was $7AAD^{-}CD11c^{int}B220^{+}Ly6C^{+}$ was apparent in the MLN after 3 days of oral *Listeria* inoculation (figure 4A). In addition, an increase in a population that was also $CD11c^{int}$ and expressed CD8α was noticeable in the spleen after 5 days of infection (figure 4B). Interestingly, increased expression of CD8α by pDCs after microbial stimulation had been published (141).

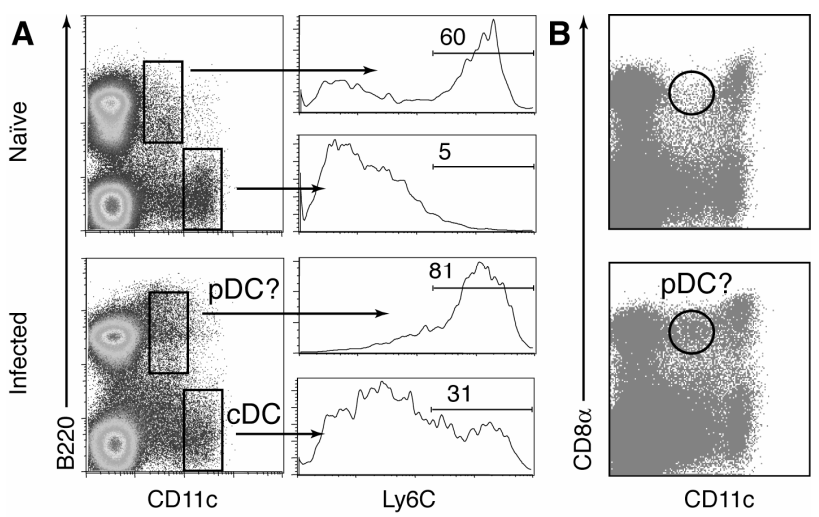


Figure 4. Cells with features of pDCs increase during oral *Listeria* infection. *A*, $B220^{+}CD11c^{int}$ cells increase in number in the MLN after 3 days of infection and stained positive for Ly6C, a molecule found in pDCs. *B*, $CD8\alpha^{+}CD11c^{int}$ splenocytes expand after 5 days of oral *Listeria* infection. The numbers in the histograms represent the percentage of $Ly6C^{+}$ cells gated on $CD11c^{int}$ or $CD11c^{hi}$ cells as indicated.

These data suggested that pDCs were rapidly expanding in response to orally acquired *Listeria*. However, careful analysis of these populations revealed that the CD11c^{int} population also contained B and T cells, as some of the CD11c^{int}B220^{+} cells were CD19^{+} and CD22^{+}, and some of the CD11c^{int}CD8α^{+} cells were TCR$\alpha\beta^{+}$. Additional experiments using an antibody reported to be specific for pDCs (142), mPDCA-1, confirmed the complexity of analysis of surface markers on pDCs, as several cell types expressed the antigen detected by the antibody mPDCA-1 in infected tissues (figure 5). This is in agreement with data showing upregulation of the antigen recognized by another antibody characterized as being specific for pDCs on B cells and DCs activated with IFN-α (143).

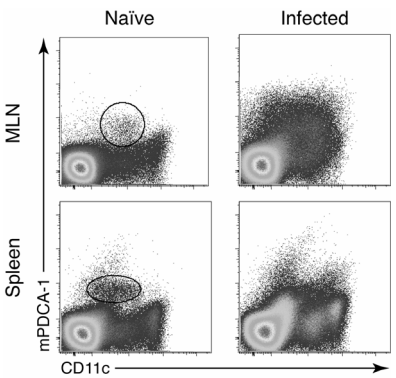


Figure 5. Dot plots show reactivity to the monoclonal antibody mPDCA-1 in naïve mice and mice infected orally with *Listeria* 3 days earlier. Circular gates in the left dot plots indicate the pDC population in steady state conditions.

Thus, "pDC-specific" antibodies recognized other cells and did not reliably identify only pDCs. Despite this, a fraction of CD11c^{int} cells that expanded in response to the infection were neither T cells nor B cells. In addition, anti-mPDCA-1 staining also indicated a potential increase of pDCs that could be masked by other cells with some shared phenotype. Thus, to be able to determine if typical pDCs were actually expanding in response to *Listeria* infection, I developed an exclusion strategy to eliminate T, B, and myeloid cells (figure 6 and figure 2A in paper I).

Once a reliable strategy to identify pDCs was developed, I investigated if this population expanded during the course of orally acquired *Listeria*.

Contrary to our initial thought, pDCs did not increase in the MLN or the spleen of infected mice 3 days after infection. However, the pDCs number doubled by day 5 post infection in both organs and remained elevated even after clearance of the infection (figure 2B in paper I).

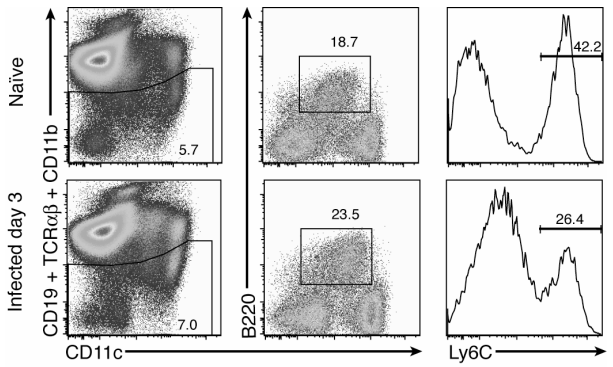


Figure 6. Identification of typical pDCs in the spleen of naïve and mice orally infected with *Listeria*. A cocktail of anti- CD19, TCRαβ and CD11b antibodies was employed to exclude T, B and myeloid cells (left dot plots). Cells negative for this cocktail were subsequently analyzed for CD11c, B220 and Ly6C to identify $CD19^{-}TCR\alpha\beta^{-}CD11b^{-}CD11c^{int}B220^{+}Ly6C^{+}$ pDCs. The numbers in the gates represent percentage of the populations. This figure is extracted from figure 2 in paper I.

Simultaneous analysis of cDCs revealed a subset-specific, tissue-specific expansion. In the MLN, the $CD8\alpha^{-}$ subset increased the most, while little expansion was observed by the $CD8\alpha^{+}$ subset. In the spleen, the $CD8\alpha^{+}$ and the $CD4^{-}CD8\alpha^{-}$ subsets increased the most, relative to their respective level in naïve mice. Figure 7 summarizes the changes in pDCs and total cDCs in the MLN and spleen of orally infected mice.

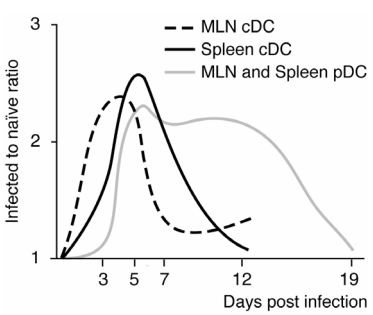


Figure 7. Diagram representing changes in pDCs and the total cDC population in the MLN and the spleen of mice over the course of a 19 day oral infection with *Listeria*. MLN cDCs peaked earlier than splenic cDCs. Both MLN and splenic pDCs peaked at day 5 post infection and remained elevated even after cDCs contracted.

Next, upregulation of costimulatory molecules on cDCs and pDCs was investigated. Similar to the findings with cDCs expansion, CD80 and CD86 were upregulated by cDCs in a subset-specific, tissue-specific manner. MLN cDCs upregulated both CD80 and CD86, whereas splenic cDCs preferentially upregulated CD86. In the MLN, $CD8\alpha^-$ cDCs showed the maximal upregulation of CD80 and CD86, while the $CD8\alpha^+$ subset showed little increase. Similarly, splenic $CD4^+$ ($CD8\alpha^-$) and $CD4^-CD8\alpha^-$ cDCs upregulated CD86 more than 5 fold, whereas in the $CD8\alpha^+$ subset the increase was approximately 2 fold. In addition, MLN and splenic pDCs greatly upregulated CD86 and MHC-II, but not CD80 (figure 8 and figures 3, 4 and 5 in paper I).

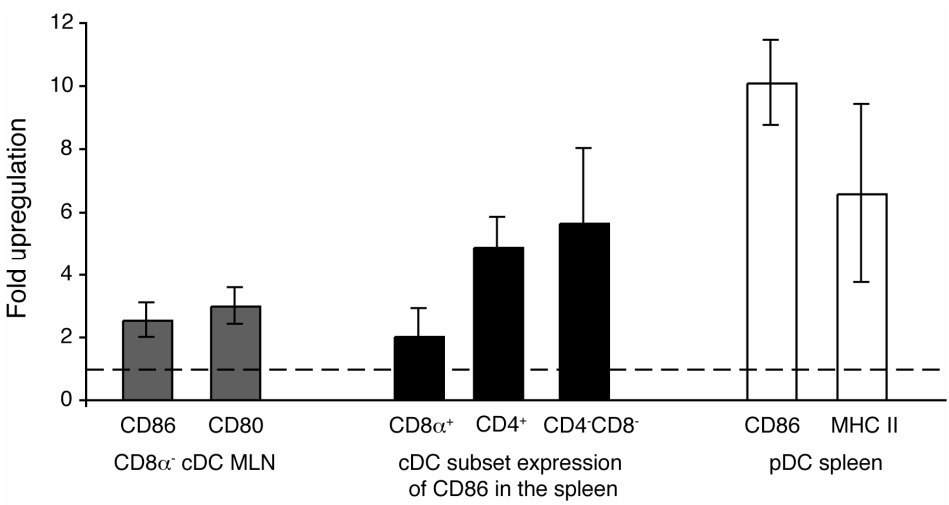


Figure 8. MLN and splenic cDCs upregulate costimulatory molecules in a tissue-specific, subset-specific manner while pDC upregulate CD86 and MHC-II similarly in both organs. The gray bars indicate upregulation of CD86 and CD80 in MLN $CD8\alpha^-$ cDCs. Upregulation of these molecules on the $CD8\alpha^+$ subset was minimal in the MLN.Black bars indicate upregulation of CD86 by three different splenic cDC subsets after 3 days of oral *Listeria* infection. Upregulation of CD80 in the spleen was marginal. The open bars indicate upregulation of CD86 and MHC-II on splenic pDCs. MLN pDCs showed a similar trend (not shown). Expression of CD80 was not increased on pDCs after infection. The dashed line represents naïve levels. This figure is a summary of the data shown in figures 3, 4 and 5 of paper I.

I next investigated additional features of DC maturation by assessing their cytokine production. Furthermore, I wished to explore the impact of the bacteria-host interface in the production of the cytokines. To this end it was necessary to use the LLO$^-$ and the ActA$^-$ *Listeria* mutants. These two deficient bacteria are less virulent than wild type *Listeria*. Since simultaneous analysis of mice infected with LLO$^-$, ActA$^-$ or wild type *Listeria* was desired, it was necessary to obtain mice with a similar bacterial burden in the tissue analyzed, regardless of the strain used for infection. It was practically not possible to increase the dose of the mutant bacteria, particularly LLO$^-$ *Listeria*, to recover similar numbers from orally infected mice. Thus, mice were infected iv.

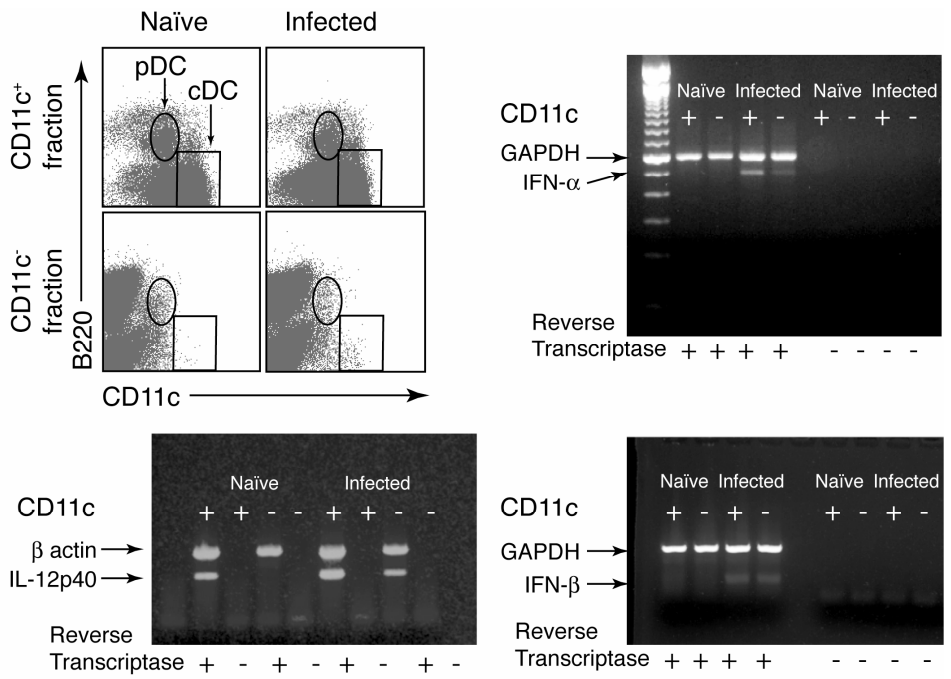


Figure 9. Increased expression of IL-12p40, IFN-α and IFN-β in CD11c-expressing splenocytes after 24 hours of iv *Listeria* infection. Dots plots show CD11c and B220 expression in the positive and negative fractions of cells magnetically separated using anti-CD11c beads from 5 pooled spleens of naïve or *Listeria*-injected mice. The lower left gel shows amplification of IL-12p40 cDNA in the CD11c$^+$ and CD11c$^-$ fractions, with and without reverse transcriptase for genomic DNA amplification control as indicated. To the right, amplification of IFN-α with universal primers (upper gel) and IFN-β (lower gel) is shown.

Initial experiments to detect cytokine production were performed by RT-PCR in CD11c-expressing cells magnetically enriched from the spleen of mice infected 24 hours earlier with wild type *Listeria*. The $CD11c^+$ fraction contained both cDCs and pDCs (figure 9, dot plots). $CD11c^+$ cells from infected mice showed increased expression of IL-12p40, IFN-α and IFN-β mRNA (figure 9). This approach provided initial evidence for DC cytokine production during *Listeria* infection. However, it was not optimal to identify which subpopulation (pDCs or cDCs) or cDC subset ($CD8\alpha^+$ or $CD8\alpha^-$) was producing the cytokines.

Although the previous experiments raised the possibility that pDCs were producing IFN-α/β during *Listeria* infection, numerous attempts to detect the cytokine by intracellular FACS staining were unsuccessful. Conversely, cDCs production of IL-12p40 by both $CD8\alpha^+$ and $CD8\alpha^-$ subsets was confirmed using FACS analysis (figure 6A and B in paper I). Furthermore, a $CD11c^{int}$ population different from pDCs that produced IL-12 and/or TNF-α was observed (paper I, figure 6C and D). The subset producing both TNF-α and IL-12 was not the same population described to produce TNF-α or iNOS (52), although they had a similar phenotype (figure 6E in paper I and (52)). Thus, these data suggest that an apparently homogeneous phenotypic population, which appears in response to acute listeriosis, consists of several different subsets characterized by functional specialization. The stratification of this population is represented in figure 10.

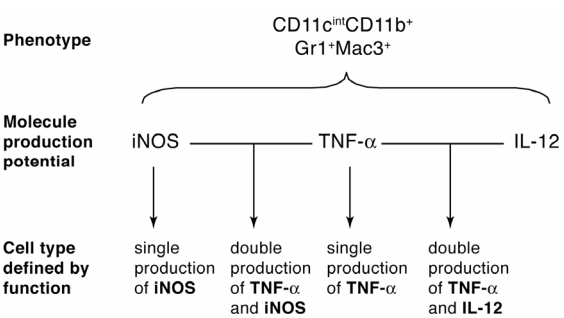


Figure 10. Cells with similar phenotype but different function appear in the spleen of mice during acute (iv) *Listeria* infection.

Production of these antimicrobial molecules was differentially dependent on the potential of the bacteria to escape the host cell. Wild type bacteria induced production of TNF-α, IL-12 and iNOS by splenocytes while ActA$^-$ bacteria induced TNF-α and iNOS, albeit at lower levels than wild type bacteria, but not IL-12. Finally, when the bacteria had compromised capacity to escape the phagosome (LLO$^-$ mutant), none of the three molecules was detected (figure 8 in paper I). Thus, production of antimicrobial molecules by the host cell depends on the intracellular compartment where the bacteria is detected, as illustrated in figure 11.

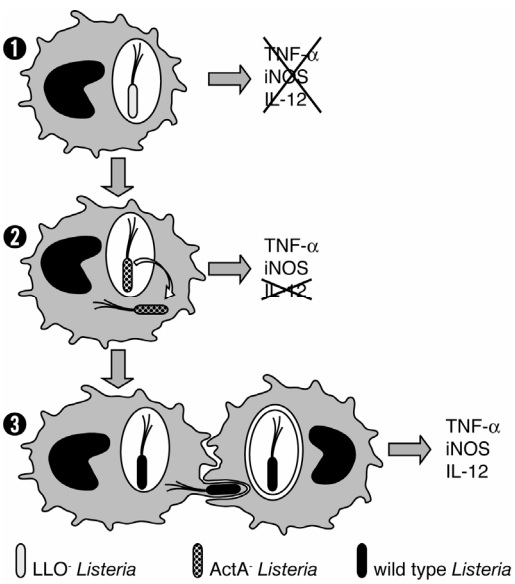


Figure 11. Distinct regulatory levels of cytokine production during *Listeria* infection.

1) Bacteria without the capacity to escape the phagosome (LLO$^-$) do not induce TNF-α, iNOS or IL-12

2) Bacteria that can escape the phagosome, but are unable to move by actin polymerization (ActA$^-$), induce TNF-α and iNOS

3) Wild type bacteria, able to escape the phagosome and to spread cell-to-cell, induce TNF-α, iNOS and IL-12

Next, the mechanism of costimulatory molecule upregulation during *Listeria* infection and its impact on T cell interaction was studied. As discussed previously, MyD88 is a major controller of bacteria-mediated DC maturation. In addition, other receptors that recognize *Listeria* induce production of IFN-α/β (107-109). IFN-α/β is a complex family of cytokines that greatly influences the immune system. Among their functions, a role of IFN-α/β on DC

maturation has been described under certain conditions (144). Furthermore, IFN-αβR$^{-/-}$ mice survive *Listeria* infection better than wild type mice (145-147), a somewhat surprising observation. Reduced bacteria-induced apoptosis was suggested as part of this mechanism (146). However, the impact of the lack of IFN-α/β signaling on DC maturation was not addressed in these studies. Thus, the obvious connection between *Listeria* recognition, MyD88 and IFN-αβR signaling, and DC maturation led us to study the contribution of these factors to DC immunocompetence during listeriosis (paper II).

In the MLN, cDCs upregulation of CD80 was dependent on MyD88 but occurred independently of IFN-αβR after 3 days of oral *Listeria* infection (paper II, figure 1A). In contrast, upregulation of CD86 was independent of both factors. Using the same strategy to detect pDCs as described in figure 6, I found that upregulation of CD86 in this population was partially dependent on both MyD88 and IFN-αβR (figure 1B in paper II). CD80 was not upregulated by pDCs in response to oral *Listeria* (paper I). In contrast with the MLN, splenic cDCs and pDCs upregulated CD86 in a MyD88-dependent fashion (figure 2 in paper II). In agreement with the observations in paper I, no major changes in CD80 expression were observed.

To rule out that the observed tissue specificity in the upregulation of CD80 and CD86 was related to the infection route, an iv route was subsequently used to mimic acute systemic infection without the complexity of bacterial traversal of the gastrointestinal barrier. When the bacteria reached the spleen in the absence of the mucosal barrier interface, CD80 and CD86 were upregulated independently of both MyD88 and IFN-αβR. Thus, the observation that cDCs from both infected MyD88$^{-/-}$ and IFN-αβR$^{-/-}$ mice could express high levels of costimulatory molecules led us to ask whether these signaling pathways would be needed simultaneously for this upregulation. To investigate this, MyD88$^{-/-}$ IFN-αβR$^{-/-}$ (DKO) mice were generated. These mice lack all the thus far described bacteria-induced TLR signaling pathways to produce inflammatory

cytokines. In addition, signaling from IFN-α/β induced by other intracellular receptors (107-109) is also blocked in these mice. However, 48 hours after iv infection, C57BL/6, MyD88$^{-/-}$ and DKO mice show similar levels of costimulatory molecules. Interestingly, IFN-αβR$^{-/-}$ mice have higher expression of CD80 and CD86 (figure 12).

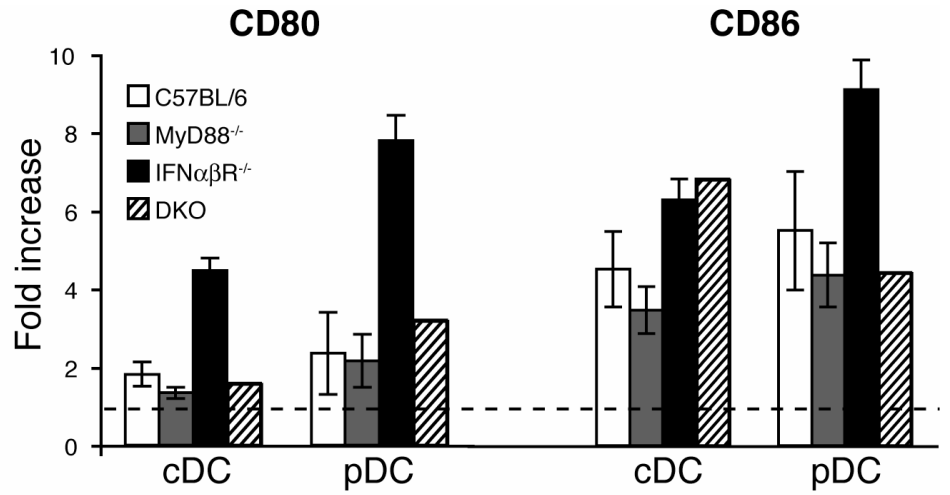


Figure 12. DCs from *Listeria*-infected mice have enhanced costimulatory molecule expression. Upregulation of CD80 and CD86 in cDCs and pDCs from C57BL/6, MyD88$^{-/-}$, IFN-αβR$^{-/-}$ and DKO mice 48 hours after iv *Listeria* infection is shown. The dashed line represents naïve levels. Bar graphs are from figure 3 in paper II.

Thus, the increased level of CD80 and CD86 in infected IFN-αβR$^{-/-}$ mice, that is restored to wild type levels in the simultaneous absence of MyD88, suggests a role for IFN-α/β in the regulation of the immunostimulatory capacity of DCs. Indeed, the elevation in costimulatory potential is reflected in a higher capacity of cDCs from infected IFN-αβR$^{-/-}$ mice to stimulate antigen-specific CD8 T cells compared to cDCs from wild type animals (figure 13).

The observation that IFN-α/β influences the costimulatory potential of DCs, and their capacity to activate naïve T cells, encouraged testing the hypothesis that a MyD88-independent anti-*Listeria* memory response could be mediated by IFN-α/β.

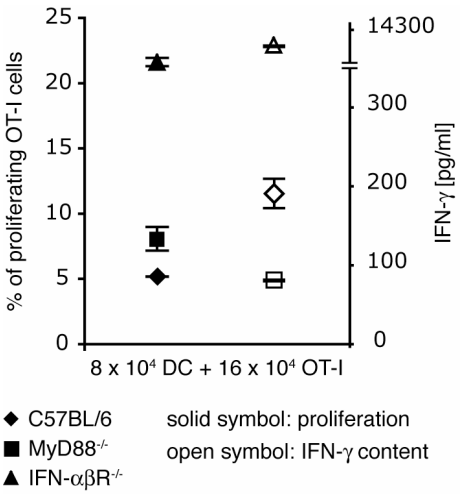


Figure 13. Enhanced capacity of IFN-αβR$^{-/-}$ DCs to stimulate CD8 T cells after *Listeria* infection. Symbols to the left show proliferation of OVA-specific TCR transgenic CD8 T cells induced by cDC sorted from C57BL/6, MyD88$^{-/-}$ and IFN-αβR$^{-/-}$ mice infected 48 hours earlier with OVA-expressing wild type *Listeria*. To the right the supernatant content of IFN-γ in the corresponding culture well is shown. Filled and open symbols of the same type correlate proliferation and IFN-γ content from the same wells. This figure summarizes data from figure 4 in paper II.

It has previously been published that MyD88$^{-/-}$ mice that survive *Listeria* infection are protected against secondary challenge (133, 134). Using OVA-specific MHC-I pentamers, I thus studied the development of anti-*Listeria* memory CD8 T cells in MyD88$^{-/-}$, IFN-αβR$^{-/-}$, DKO and wild type mice infected with OVA-expressing *Listeria*. To ensure survival of MyD88$^{-/-}$ and DKO mice, animals were first infected with an OVA-expressing ActA^{-} *Listeria* mutant. They were then challenged 4 weeks later with a fully virulent OVA-expressing *Listeria* strain. MyD88$^{-/-}$ mice developed lower numbers of specific memory CD8 T cells than IFN-αβR$^{-/-}$ and wild type mice (figure 14A). This defect was partially restored when mice simultaneously lacked MyD88 and IFN-αβR signaling (DKO mice, figure 14A). However, all four mouse strains efficiently cleared the challenge dose, which is lethal to naïve wild type mice.

The function of the OVA-specific CD8 memory T cells was next analyzed. As shown in figure 14B, the ratio of TNF-α-producing memory CD8 T cells generated in the DKO mice closely reflected the ratio of total OVA-specific memory cell (figure 14A and B). However, the differences in IFN-γ-producing, OVA-specific CD8 memory T cells were less dramatic than the difference in the ratio of total OVA-specific memory cell (figure 14A).

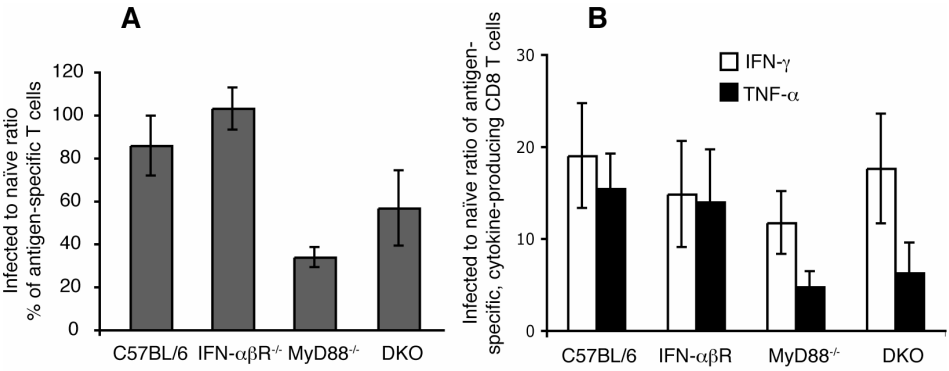
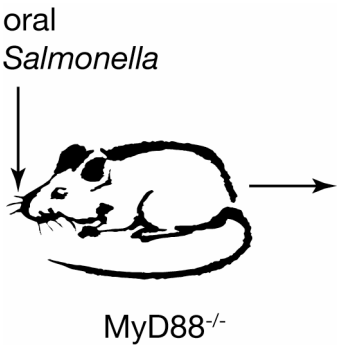


Figure 14. Diminished total memory CD8 T cells in MyD88$^{-/-}$ and DKO mice is compensated by IFN-γ-, but not TNF-α-, producing memory CD8 T cells. *A*. Infected to naïve ratio of total OVA-specific memory CD8 T cells after challenging with OVA expressing wild type *Listeria* C57BL/6, IFN-αβR$^{-/-}$, MyD88$^{-/-}$ and DKO mice infected 4 weeks earlier with OVA-ActA^{-} *Listeria*. *B*. Infected to naïve ratio of IFN-γ- and TNF-α-producing OVA-specific memory CD8 T cells in the same mice. Bar graphs are from figures 5 and 6 in paper II.

Thus, the ability to develop a normal pool of IFN-γ-producing, antigen-specific CD8 T cells could explain the capacity of MyD88$^{-/-}$ mice to clear a secondary challenge with the bacteria. This, however, is not mediated by IFN-α/β. Despite the role of IFN-α/β in the early response against *Listeria*, and its involvement in DCs immunostimulatory capacity, IFN-α/β seems to play a minor role in the development of an anti-*Listeria* memory response. This is apparent since DKO mice were as efficient as MyD88$^{-/-}$ mice at clearing secondary challenge with the bacteria.

We next addressed the role of these two important signaling pathways in *Salmonella*-induced DC maturation (paper III), based on previous studies in our group suggesting that multiple pathways were involved in DC maturation during *Salmonella* infection (66). Similar to *Listeria*, orally administered *Salmonella* induced upregulation of CD86, but not CD80, on DCs in the MLN and spleen of infected MyD88$^{-/-}$ mice. However, the onset of this MyD88-independent pathway required high bacterial load in the MLN (figure 15).

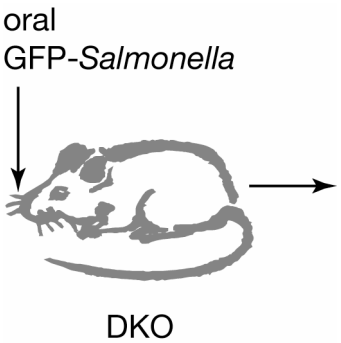


CD8α⁻ DC	Bacterial load	CD86 upregulation
SPLEEN	~10^5	High
MLN	~10^5	Absent
	>10^5	High

Figure 15. Upregulation of CD86 on CD8α⁻ DCs from MyD88$^{-/-}$ mice after oral *Salmonella* infection. MyD88-independent upregulation of CD86 in the MLN requires a higher threshold of bacterial load. This figure highlights results from figure 1 in paper III.

The MyD88-independent upregulation of CD86 on the bulk population of cDCs was indeed mediated by IFN-αβR signaling. Orally infected DKO mice failed to upregulate CD86 to levels shown by infected MyD88 deficient mice with similar bacterial burden (figure 3 in paper III). As only a very small percentage of DCs contain bacteria during infection, most of the DCs analyzed in the infected tissues are subject to indirect maturation through cytokine stimulation. We thus wanted to address the relative contribution of the MyD88 and IFN-αβR signaling pathways on direct maturation of DCs by bacterial contact compared to indirectly matured DCs.

To this end we infected MyD88$^{-/-}$, IFN-αβR$^{-/-}$ and DKO mice with a GFP-expressing *Salmonella*. Remarkably, *Salmonella*-associated DCs (GFP$^+$ DC) upregulated CD86 independently of both signaling pathways. In addition, CD80 was partially upregulated independently of these two factors. In sharp contrast, DCs non-associated with *Salmonella* (GFP$^-$ DC) were unable to upregulate costimulatory molecules (figure 16, see also figure 4A-C in paper III).



CD8α⁻ DC	CD80 upregulation	CD86 upregulation
GFP⁺	Moderate	High
GFP⁻	Absent	Absent

Figure 16. *Salmonella*-associated DCs upregulate CD86, and partially CD80, independently of MyD88 and IFN-αβR signaling. CD8α⁻ DCs contained the highest number of bacteria in Peyer's patches 2 days after oral administration of GFP-*Salmonella*. This figure highlights results from figure 4 in paper III.

This striking finding prompted us to ask whether DCs directly matured in the absence of MyD88 and/or IFN-αβR were competent stimulators of T cells. Focusing the experiments on *Salmonella*-driven direct DC maturation required access to a large number of *Salmonella*-associated DCs. To perform the experiments in figure 16, Peyer's patches from 20 mice were pooled in order to collect enough GFP^+ DC for reliable analysis. These numbers, however, were not sufficient for GFP^+ DC-T cell co-cultures experiments. To increase the number of GFP^+ DC it was therefore necessary to infect the mice iv. Thus, $MyD88^{-/-}$ and DKO mice were injected with a GFP-OVA-expressing *Salmonella* strain, and GFP^+ DC were isolated and cultured with OVA-specific CD4 T cells. These experiments revealed that $MyD88^{-/-}$ and DKO GFP^+ DC were less efficient than wild type GFP^+ DC at inducing T cell proliferation and production of IFN-γ (figure 5 in paper III).

To further investigate the mechanism, we compared the level of costimulatory and MHC-II molecules as well as some cytokines produced during infection between infected DKO and wild type mice. The defect in T cell stimulation could not be attributed to deficient expression of CD40, CD80, CD86 or MHC-II after iv bacterial administration (figure 6A in paper III). In

addition, both DKO and wild type DCs induced equal T cell proliferation and IFN-γ production when loaded *in vitro* with OVA peptide (figure 6B in paper III). Moreover, infected DKO and wild type mice produced a similar amount of cytokines with potential to induce DC maturation, such as TNF-α and IL-1β. However, infected wild type mice produced 7 times more IFN-γ than DKO mice (paper III, figure 6 C). These data indicate defective antigen presentation capacity in the absence of MyD88, a situation that has recently been described in another infection model (92). We thus designed an *in vitro* experiment where the influence of external factors, such as cytokines and other non-DC cell derived input, were absent. To this end, DCs were Flt3L-expanded *in vivo* in DKO and wild type mice, magnetically enriched from pooled spleens and infected *in vitro* with GFP-OVA-*Salmonella*. Sorted GFP^+ DKO DC induced lower T cell proliferation and IFN-γ production than their wild type counterparts (figure 17).

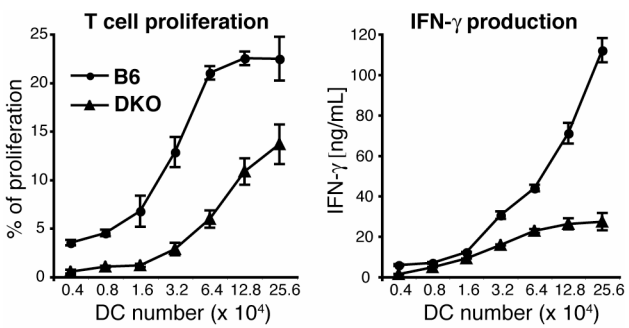


Figure 17. OT-II cells proliferation and IFN-γ production induced by DCs infected *in vitro* with GFP-OVA-expressing *Salmonella*. Plots are from figure 6 in paper III.

Thus, based on our data we propose a multilayered model for the regulation of the expression of costimulatory molecules during *Salmonella* infection (figure 18). The first level of regulation, which has a higher impact on costimulatory molecule expression, particularly CD80, is controlled by MyD88-derived signals. Second, in the absence of MyD88, IFN-α/β can ensure a high level of costimulatory molecules in the bulk population of DCs. A third level is

inherent to DCs that come in direct contact with the bacteria. The latter, however, is not sufficient to induce full stimulation of CD4 T cells.

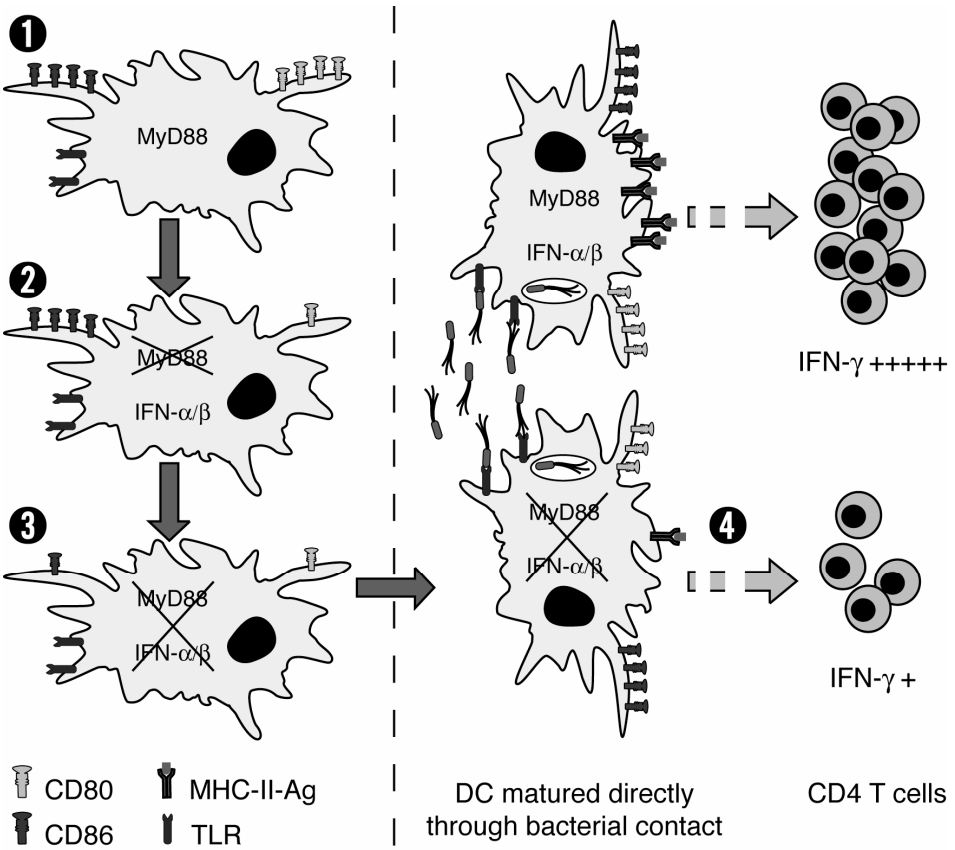


Figure 18. Hierarchical model of DC maturation controlled by MyD88 and IFN-αβR signaling pathways during *Salmonella* infection. 1) MyD88-derived signals control upregulation of costimulatory molecules. 2) In the absence of MyD88, IFN-α/β maintains a high level of CD86 in the bulk DC population. 3) The combined deficiency of MyD88 and IFN-αβR abrogates expression of costimulatory molecules. 4) DCs matured by direct bacterial contact still upregulate CD86 and CD80 even in the absence of MyD88 and IFN-α/βR. However, this is not sufficient to induce full activation of CD4 T cells, and appears to be due to poor presentation of bacterial-derived antigens.

CONCLUSIONS

The main objective of this work was to study the function of different DC subpopulations during infection with Gram-positive and Gram-negative intracellular bacteria. A particular focus was to investigate possible differential responses for described DC subpopulations and subsets in eliminating the infections. Furthermore, special efforts were aimed at understanding the mechanism of bacteria-induced DC maturation.

The work in this thesis thus started by characterizing the expansion, costimulatory molecule expression and production of anti-bacterial molecules by cDCs, pDCs and DC-related, CD11c-expressing cells during *Listeria* infection. I found that cDCs expanded and upregulated costimulatory molecules in a subset-specific and tissue-specific manner. In contrast to cDCs, pDCs expanded simultaneously in the MLN and spleen of infected mice. They also upregulated CD86 to a level found in naïve cDCs. As part of the maturation process, cytokine production by DCs was studied. Contrary to the general belief that macrophages are the main producers of IL-12 during listeriosis (148), two different cell types were identified as the main source of this cytokine. One of them was cDCs. The other was a population with a mixed phenotype that expressed an intermediate level of CD11c and an intermediate to high level of Gr1, Mac3 and CD11b. The complexity of this population was apparent, as cells could produce TNF-α, iNOS and IL-12 alone or in specific combinations. Finally, I determined that the intracellular compartment where the bacteria are found was crucial for the production of these molecules, a situation that could have strong implications in the development of the specific T cell response (149-153).

I also found that expression of costimulatory molecules was dependent on MyD88 and IFN-αβR signaling at different degrees in pDCs and cDCs of mice orally infected with *Listeria*. In particular, CD80 expression was highly dependent on MyD88, whereas CD86 showed partial dependence on MyD88 and IFN-αβR signaling. However, MyD88 and IFN-αβR dependence could be overcome by iv injection of the bacteria. Whether this is a mucosal-specific mechanism or is dependent of the number of bacteria seeding the organ at the time of analysis remains to be determined. Interestingly, iv infected IFN-αβR$^{-/-}$ mice showed increased upregulation of CD80 and CD86 compared to infected MyD88$^{-/-}$ and wild type mice. These values went down to MyD88$^{-/-}$ and wild type mice levels when IFN-αβR$^{-/-}$ mice simultaneously lacked MyD88-derived signaling (DKO mice). This suggests that IFN-α/β play a role in the regulation of costimulatory molecules, perhaps by downmodulating MyD88-derived factors. Whether IFN-α/β acts by downregulating the effect of MyD88-dependent cytokines or directly interferes in the intracellular MyD88 pathway, downstream of the IFN-αβR-signaling cascade, are possibilities open to future investigation. The increased level of costimulatory molecules in DCs from infected IFN-αβR$^{-/-}$ mice translated into a higher capacity to stimulate T cells. Thus, the lack of IFN-α/β signaling had an impact on the innate response to *Listeria*. In contrast, it had only a minor effect on the generation of anti-*Listeria* memory CD8 T cells.

Similar to *Listeria*, *Salmonella*-induced upregulation of CD80 in DCs was largely dependent on MyD88. However, CD86 could be expressed at high levels in the absence of MyD88. This upregulation was mediated by IFN-αβR signaling, since the bulk population of DCs was unable to express high levels of costimulatory molecules in infected DKO mice. However, *Salmonella*-associated DCs were able to upregulate CD86, and partially CD80, in the simultaneous absence of these two factors. Thus, DCs that mature by direct

contact with the bacteria undergo an intrinsic maturation program that is independent of factors important for cellular signaling, such as MyD88 and IFN-αβR. Nonetheless, the immunostimulatory capacity of DCs that mature in the absence of these factors is not optimal and they have a compromised capacity to stimulate naïve T cells compared to their wild type counterpart.

Thus, the process of DC maturation is fundamental for the development of an efficient immune response. Accordingly, the dissection of the different factors involved in the regulation of this process is of extreme importance. The identification of this factors and their relative contribution could lead to the improvement of tools and strategies, such as new therapies and vaccine design, that can improve public health in the future. Overall, the work presented in this thesis broadens the knowledge on DC function and maturation during bacterial infection. It is also my conviction that every piece of information made available to the scientific community is an important contribution in our search to understand nature. Therefore, an important goal of this thesis will be fulfilled if the information gathered here is useful to other researchers in future investigations.

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I really want to write acknowledgments, not farewells. But I am afraid that for most people I have to thank, an inevitable goodbye come along. And I have to start with the person who made this thesis a reality. That is my supervisor Mary Jo Wick. As I try to write this so many things come to my head that I just can't put in words all that I have to thank you for. Thank you for a very good supervision that always leaves space for independent thinking. Thanks for these years of education and scientific coaching, the initial and critical ones. Thank you for making me feel that I can count on you if I need it. By providing that feeling you help me to work without many otherwise unavoidable worries. I think I can summarize all if I thank you for taking me as a student. And I am sure you were aware of all the extras you were taking with that new student. I didn't want to get too "philosophical" (the PhD part is almost over), but there you have it again...

A huge thank to my lab mates Malin, Anna and Stina. Malin I can thank you now as I wanted when my paper was published "for all the help at the beginning of time". Sorry I couldn't pay off as much as I wanted showing you the sorter, it was bad timing. Thank you all for collaborations, technical help and more. I really think we all made an excellent group. I will miss that. I also want to thank the two former students from Mary Jo's lab that I met, Cecilia and Ulf for very good discussions and meetings.

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la Facultad de Biología en toda su historia (negro asere nosotros no te botamos, y tú no quisiste regresar, tal vez estabas siendo consecuente desde entonces).

Almost out of Cuba again I want to acknowledge the support of my colleges at the Ameijeiras Hospital and the Nutrition Institute in Havana. From there to Madrid, my Toll bridge to Europe, I want to thank Dr. Ascension Marcos and her group for a warming welcome.

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China, gracias por todo lo que has hecho y haces para salir adelante. Hemos pasado por tanto que el futuro apenas me asusta, solo un poco. De nuevo nos vamos juntos, con nuestras espinas y nuestras flores, pero juntos.

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