RECRUITMENT, DIFFERENTIATION, AND FUNCTION OF MONOCYTES DURING *SALMONELLA* INFECTION

Anna Rydström

UNIVERSITY OF GOTHENBURG

Department of Microbiology and Immunology
The Sahlgrenska Academy
Sweden 2008

Supervisor: Professor Mary Jo Wick

Opponent: Dr. Philip R Taylor, School of Medicine, Cardiff, UK

**Printed by Chalmers Tekniska högskola AB Reproservice,
Göteborg, Sweden 2008**

ISBN 978-91-628-7495-7

Till mormor och morfar

ABSTRACT

Monocytes are a heterogeneous population in the blood with an enormous plasticity whose fate and functions are dictated by the microenvironment. They are phenotypically and functionally related to neutrophils and dendritic cells (DCs) and share an overlapping expression pattern of surface molecules with these cells. The presence of phagocytic cells including neutrophils, monocytes/macrophages and DCs in infected tissues is critical to host survival. However, how these cells respond to bacterial infections regarding differentiation and effector functions is not fully understood. The overall aim of this thesis was to examine the recruitment, function and differentiation of monocytes and neutrophils in the blood, Peyer's patches (PP) and mesenteric lymph nodes (MLN) during oral *Salmonella* infection.

Ly6C^{hi} monocytes and neutrophils rapidly accumulated in the blood, PP and MLN of mice orally infected with *Salmonella*. The recruitment of neutrophils and monocytes was not diminished in infected TLR4$^{-/-}$ mice, but was reduced in MyD88$^{-/-}$ mice and almost absent in MyD88$^{-/-}$TLR4$^{-/-}$ mice. The chemokine receptors CCR2 and CXCR2 were expressed by monocytes and neutrophils, respectively, in the blood and their cognate ligands CCL2 and CXCL2 were produced early during infection in infected organs. Furthermore, the production of these chemokines was dependent on MyD88/TLR4, indicating a critical role of these signaling pathways in myeloid cell recruitment. Upon migration into the organs, neutrophils and monocytes formed inflammatory foci and one to two percent of the cells phagocytosed *Salmonella*. In addition, monocytes were the major producers of TNFα and iNOS, which are important for controlling *Salmonella* infection.

The upregulation of MHC-II and costimulatory molecules on monocytes initiated the investigation of whether they differentiated into DCs and became antigen-presenting cells. However, activated monocytes were unable to present antigens to T cells ex vivo although they differentiated into DCs after in vitro culture. Furthermore, *Salmonella* added to in vitro cultures inhibited monocyte differentiation to DCs by inducing cytokines via a MyD88-dependent pathway. This suggests a mechanism for the incapacity of monocytes to present antigens in vivo.

Collectively, these studies reveal MyD88/TLR4-dependent recruitment of phagocytes to infected intestinal tissues. They also suggest a major role for monocytes in eliminating bacteria and producing pro-inflammatory cytokines but not for inducing adaptive immunity during *Salmonella* infection. Increased knowledge of monocytes improves the chances to find therapies against a broad spectrum of diseases ranging from atherosclerosis to infectious diseases, where monocytes have opposing roles of either being beneficial or detrimental to the host.

Keywords: *Salmonella*, monocyte, neutrophil, chemokine, Toll-like receptor, differentiation

Original papers

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

I. Anna Rydström and Mary Jo Wick. Monocyte recruitment, activation, and function in the gut-associated lymphoid tissue during oral *Salmonella* infection. J. Immunol. 2007 May 1;178(9):5789-801.

II. Anna Rydström and Mary Jo Wick. MyD88 is required to recruit neutrophils and monocytes to intestinal lymphoid tissues during oral *Salmonella* infection. *Manuscript*

III. Anna Rydström and Mary Jo Wick. Toll-like receptor signalling blocks the differentiation of immature monocytes to dendritic cells. *Manuscript*

Paper I is printed with permission from the publisher.

Table of contents

ABBREVIATIONS

7AAD	7-aminoactinomycin D
Ag	antigen
CCL	chemokine ligand
CCR	chemokine receptor
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
cDC	conventional dendritic cell
DC	dendritic cell
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
FAE	follicle associated epithelium
Flt3L	Flt3 ligand
GALT	gut associated lymphoid tissue
GM-CSF	granulocyte/ macrophage colony-stimulating factor
HEV	high endothelial venules
hi	high
IFN	interferons
ICAM	intracellular adhesion molecule
IL	interleukin
int	intermediate
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRF	
i.v.	intravenous
LPS	lipopolysaccaride
LT-α	lymphotoxin-α
mAb	monoclonal antibody
MAdCAM-1	mucosal peripheral node addressins cell adhesion molecule
M cell	microfold cell
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MLN	MLN
MLR	mixed lymphocyte reaction
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor kappa B
NK	natural killer
Nramp	natural resistance associated macrophage protein 1
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
pDC	plasmacytoid DC
PP	PP
PSGL-1	P-selectin glycoprotein ligand 1
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
SARM	sterile α- and armadillo-motif-containing protein
SED	subepithelial dome
SPI	*salmonella* pathogenicity island
Th	T helper
TLR	Toll-like receptors
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRAM	TRIF-related adaptor molecule

Introduction

A functioning immune system is extremely important for our survival. The innate immune system is the front line of defense that first recognizes invading pathogens, initiates an immune response and subsequently activates the adaptive immune system. A close interaction and collaboration between the two systems is needed to eradicate the pathogen from the host. In addition, however, the immune system needs to be tightly regulated since an exaggerated immune response can lead to autoimmunity and tissue destruction.

With recent data showing that monocytes are a more adaptive, heterogeneous population of innate cells than was previously appreciated, a new interest in monocytes has emerged. During the last few years, intense research on monocytes has been carried out in many experimental systems. It was discovered that the enormous plasticity of monocytes can lead to development into distinct cell populations with various functions. The final fate of monocytes is primarily dictated by the tissue microenvironment and the status of the host (steady state or inflammatory conditions). In addition, while monocytes are a crucial part of the host defense during many infections and in wound healing, they can be harmful and cause or exacerbate diseases such as atherosclerosis and multiple sclerosis. Hence, deeper knowledge of monocytes is beneficial for the development of vaccines and drugs against bacterial infections as well as find therapies against diseases when their effects are detrimental to the host.

Innate and adaptive immunity are necessary to clear an infection with the intracellular bacteria *Salmonella* but the contribution of monocytes to the host defense is not known. In this thesis, I have examined the recruitment, function and differentiation of monocytes during *Salmonella* infection.

Salmonella

Infection

The first strain of *Salmonella,* which are Gram-negative, facultative intracellular bacteria, was discovered in 1885 and today over 2500 serotypes (strains) have been identified. *Salmonella* can infect several species and transmission occurs through

contaminated food or water. In humans, *S. enterica* Serovar Typhi (*S.* Typhi) and *S. enterica* Serovar Paratyphi (*S.* Paratyphi*)* cause typhoid fever and typhoid fever-like illnesses, respectively, while other serovars such as Typhimurium and Enteriditis cause a localized gastroenteritis. Typhoid fever is a severe systemic disease where one of ten dies if left untreated, and *S.* Paratyphi gives similar although milder disease. The estimated number of typhoid fever illnesses was around 20 million cases during the year 2000, leading to 200,000 deaths (1). The incubation period for *S.* Typhi varies between 1-3 weeks and symptoms include sustained fever, malaise, anorexia, headache, constipation or diarrhea, rose-coloured spots on the chest and enlarged spleen and liver. Most people show symptoms 1-3 weeks after exposure. After recovery from typhoid fever, a small number of persons continue to carry the bacteria and can be a source of infection for others. An emerging threat is the development of multi-antibiotic resistant strains that have become prevalent in several areas of the world (2). Typhoid fever almost exclusively exists in the undeveloped part of the world due to poor sanitation. In contrast, serovars causing gastroenteritis are common in industrialized countries but are only a severe threat to immunocompromised people, the elderly and young children. The symptoms include fever, abdominal pain, diarrhea and nausea and usually appear 12–72 hours after infection and last 4–7 days.

S. Typhimurium infection in mice gives a systemic disease and is widely used as a model for human typhoid fever. In this model, intestinal inflammation is not observed (3) and mouse strains differ in their susceptibility to *Salmonella* infection, which is attributed to differences at the Slc11a1 (Nramp1) locus (4). For example, the C57BL/6 mouse strain used throughout this thesis are of the Nramp1 susceptible genotype (5) and, depending on the orally administred dose, will succumb to the infection after 6-8 days. In contrast, it is more difficult to establish infection in an Nramp1 resistant strain (6).

Structure of the GALT

Gut-associated lymphoid tissue (GALT) is a general term for all lymphoid tissue in the gut including Peyer´s patches (PP), mesenteric lymph nodes (MLN), and isolated lymphoid follicles.

PP are covered by a specialized follicle-associated epithelium (FAE) that lacks goblet cells but contains interspersed microfold (M) cells. M cells are broad cells without an overlaying glycocalyx and are spezialized in transcytosing antigens and

particles from the lumen and delivering them to leukocytes enfolded in pockets in the basolateral surface of M cells (7). Under the FAE lies the sub-epithelial dome (SED) (Figure 1). This region is enriched in dendritic cells (DCs) (8-10) that are ready to engulf particles delivered from the M cells and to migrate to the T cell area to initiate an adaptive immune response. Beneath the SED is the B cell follicle which is sandwiched between T cell areas that are called inter-follicular regions (IFRs). The IFRs contain high endothelial venules (HEV), the exit and entry point for cell migration to and from the blood. Lymphocytes that are primed in the PP exit through the draining lymphatics to the MLN, where they reside for a period of further differentiation before they migrate into the bloodstream and back to the mucosa (11).

Efferent lymphatics from Peyer´s patches and from the rest of the intestine drain into the MLN (MLN). The MLN form a chain-like structure of lymph nodes and is the largest lymph node in the body. A capsule envelopes each lymph node and underneath the capsule lies the subcapsular sinus followed by the inner medulla. The B cell-follicles are located along the outer edges of a lymph node in an area called the cortex, and the paracortex, which contains the T cell zones including DCs and macrophages, lies beneath and between the B cell follicles. Afferent lymph vessels drain into the subcapsular sinuses and the lymph seeps through the cortex into the medullary sinuses where it leaves the node in efferent lymphatics. Conduits large enough to carry proteins, such as antigens and chemokines, run from the subcapsular sinuses through the T cell zones to the HEV. Blood enters the lymph nodes through HEVs and leaves the node in a singular vein close to the efferent lymphatics (12).

Invasion and dissemination of *Salmonella*: studies in mouse models

Invasive enteric bacteria appear to have at least two basic strategies for translocating across an intact mucosal barrier, either via M cells to PP or through the villus epithelium. *Salmonella* mainly enters host tissues via PP in the distal ileum and caecum, although bacteria can be found in the entire intestine (13). M cells are the target cell for *Salmonella* invasion of PP, and after transcytosis bacteria are found within DCs in the SED (13-15) (Fig. 1). Invasion via PP is crucial for the onset of a rapid adaptive immune response. For example, $CCR6^{+}$ DCs were found to be recruited to the FAE within 6 h after *Salmonella* infection and induce activation of specific CD4 T cells (16). DCs have also been shown to migrate from the SED to IFRs in response to *Salmonella* to initiate adaptive immune responses (16, 17). In

addition, protective IgA responses were induced specifically by *Salmonella* invading PP but not by *Salmonella* taken up by DCs in lamina propria (18). However, *Salmonella* can also penetrate the intestine outside PP and invade lamina propria (13, 19, 20).

Salmonella could enter the lamina propria in three different ways: 1) via M cells interspersed in the villus epithelium; 2) through or between enterocytes or 3) via DCs that breach the villus epithelium through dendrite extensions (19, 21-23). The latter route of entry was revealed in studies showing that CX_3CR1^+ DCs in the lamina propria can actively take up *Salmonella* by extending dendrites through the epithelial wall, thereby sampling the lumen, and the bacteria are subsequently found within these cells in lamina propria (19, 22, 23)(Fig. 1). These studies suggest that this is the main pathway by which non-invasive *Salmonella* enter the lamina propria (19, 22, 24). However, dendrite formation does not seem to be required for penetration of non-invasive pathogens to cross the intestinal epithelium, as lamina propria DCs in BALBc mice, which are unable to form dendrites, harbored fungi after oral challenge with non-invasive fungi (25). Moreover, invasive *Salmonella* could cross the epithelium independent of dendrite formation and was found within phagocytes in the lamina propria, thus questioning the importance of direct DC uptake (19). Thus, several routes appear to be involved in the passage of *Salmonella* across the intestinal epithelium although most occurs via PP (7, 13, 15, 19, 20, 26).

Despite early immune responses in PP, however, *Salmonella* disseminate further and are found in MLN 24-48h after oral inoculation and later in the spleen and liver (13, 15, 27, 28). Exactly how *Salmonella* disseminate systemically is poorly understood. The bacteria may spread either through PP (15) or lamina propria via lymph to MLN (19, 20) (Fig. 1). The latter pathway was shown to mediate systemic infection in mice when *Salmonella* was prevented from colonizing PP (20).

Salmonella are found within DCs in PP, lamina propria and MLN within the first 24-48 hours after oral infection, suggesting that *Salmonella* is transported inside DCs to the MLN (15, 19, 29-31)However *Salmonella* may also disseminate extracellularly in the lymph. For example, in calves it was shown that *Salmonella* are found free in the lymph (32), which empties into the blood via the thoracic duct. This could thus be a way for the bacteria to access the organs like the spleen and liver.

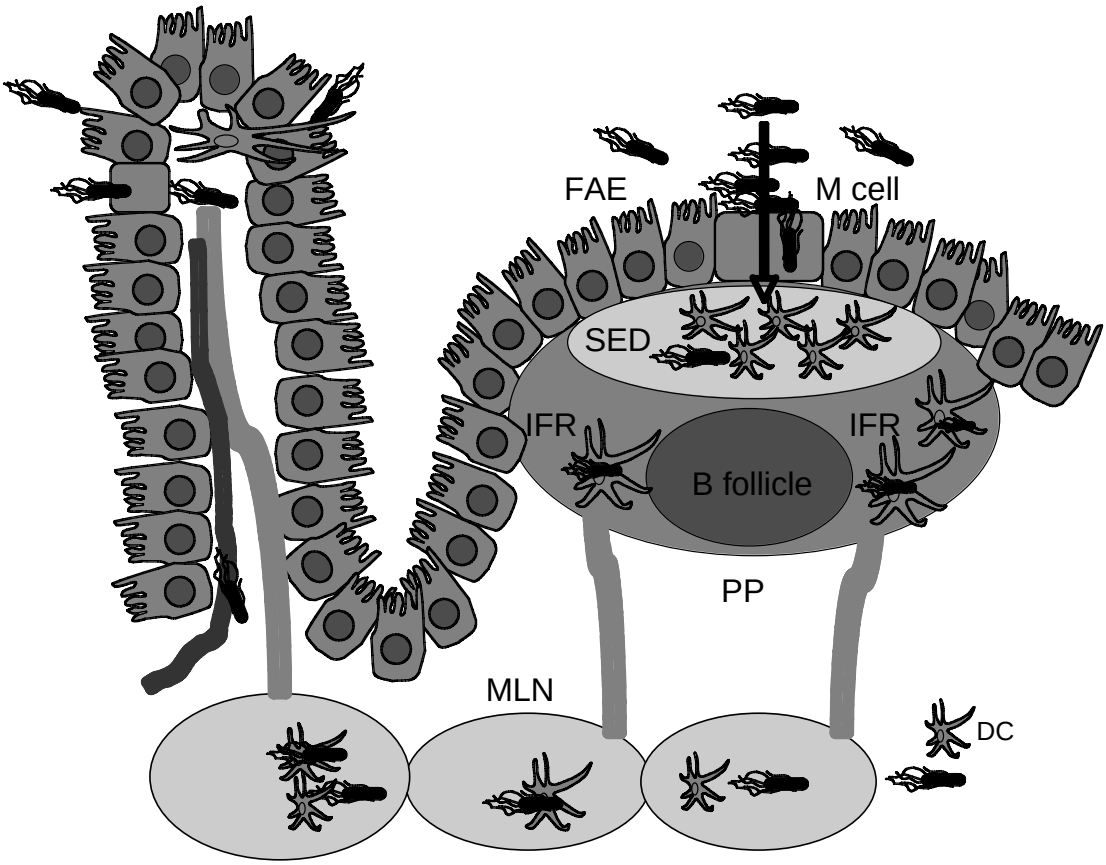

Figure 1. Infection route in the gut. *Salmonella* invades primarly via M cells in PP but passage via intestinal epthelial cells could also occur. It is also suggested that DCs directly sample the lumen with their dendrites and take up *Salmonella*. Subsequently, bacteria are found in the MLN. FAE, follicle-associated epithelium; SED, sub epithelial dome; IFR, intrafollicular region

Direct bacterial dissemination via blood from lamina proria is also suggested by studies showing that $CD18^+$ phagocytes containing *Salmonella* were detected in the blood 15 min after oral inoculation with an invasion-deficient strain (33, 34). The bacteremia was dependent on CD18-expressing phagocytes and the bacteria could promote migration of phagocytes and increase dissemination by producing the SrfH protein (34). However, since bacteria are sequentially found in the PP/laminia propria, followed by the MLN and spleen, the CD18-dependent pathway seems to be a minor contributior to the systemic spread of bacteria (13, 28). In conclusion, how *Salmonella* disseminate systemically is still not clarified, but the bacteria may enter the blood by exiting the MLN in the lymph or egress directly from lamina propria to the blood inside $CD18^+$ cells.

Infection beyond the GALT

Once bacteria cross the intestinal barrier and enter host tissues, the host response is aimed at preventing bacterial spread. This is accomplished by building structures, called granulomas, to physically contain the bacteria. During the first days after i.v. or i.p. infection, *Salmonella* grow within discrete foci dominated by neutrophils containing bacteria. Later on the neutrophils are replaced by infiltrating mononuclear phagocytes and the foci become granulomas in the spleen and liver.

Salmonella predominantly reside within red pulp and marginal zone macrophages in the spleen, and within $CD18^{+}$ phagocytes in the liver. In the spleen and liver, most of the bacteria are intracellular (35, 36). Intracellular *S. typhimurium* evade killing and at the same time they also exert a cytotoxic effect, either direct or indirect, on the infiltrating phagocytes (35, 37). Cell death can mediate the spread of *Salmonella* by releasing bacteria into the extracellular environment where they can invade new cells (35, 37, 38).

Granulomas are formed to prevent the uncontrolled spread of bacteria. However, an increase in *Salmonella* numbers leads to the formation of new granulomas rather than an increase in the number of bacteria per cell or the expansion of the already formed granuloma (38). Thus, *Salmonella* evade the host cell and migrate away to initiate new foci. Indeed, concomitantly infecting mice with two different strains of *Salmonella* revealed that only bacteria from the same strain were found in each lesion and that one strain always outnumbered the other strain in the blood in mice that died from bacteremia (39, 40). Thus, each bacterium acts independently, causing a localized immune response in each lesion. The data also indicate that as few as one bacterium can escape beyond the intestinal tissue to give a systemic infection (40). Finally, if the host cannot control the infection, *Salmonella* will, following extensive replication within splenic and hepatic phagocytes, re-enter the bloodstream and cause infected animals to succumb to septic shock and multiple organ failure.

Salmonella and virulence factors

To survive intracellularly and colonize the host, *Salmonella* has evolved strategies for its uptake and to counteract killing. In particular, *Salmonella* uses two distinct type III secretion systems to translocate virulence proteins from the bacteria to host cells and

thus promote bacterial uptake and intracellular survival (41). The type III secretion system encoded by genes located on *Salmonella* pathogenicity island-1 (SPI-1) is used by *Salmonella* to invade epithelial cells and M cells (42). After uptake, *Salmonella* reside in specialized membrane-bound compartments called *Salmonella*-containing vacuoles that protect the bacteria from degradation and promote their growth (43). *Salmonella* pathogenicity island-2 (SPI-2) encodes a second type III secretion system necessary for intracellular survival and bacterial replication (43). Proteins encoded by SPI-2 block the transport of iNOS and Phox to the bacteria-containing vacuole and in this way circumvent killing by reactive oxygen and reactive nitrogen species (44, 45). Proteins encoded by SPI-2 also prevent fusion of the vacuoles with lysosomes. In addition, *Salmonella* subverts antigen presentation to T cells by using SPI-2 encoded genes to avoid degradation in DC (46). Intracellular growth is important for *Salmonella* since mutants that can not survive intracellularly are highly attenuated *in vivo* (35, 47). *Salmonella* can also hinder activation of the adaptive immune response to some of its antigens. For example, the flagellar subunit protein FliC is down regulated in vivo. The down regulation is induced by conditions encountered inside host cells and as a result, priming of FliC-specific T cells occurred in PP where *Salmonella* still transcribe fliC, and less in the MLN or spleen (48).

The Innate immune response to *Salmonella*

Neutrophils, monocytes/macrophages and NK cells belong to the innate immune system and are crucial for the protection against *Salmonella*. During the earliest stages of infection, before cells are recruited to the infection site, resident phagocytes such as macrophages are involved in controlling the infection. In support of this, administering silica, which impairs macrophage function, resulted in increased early growth of *Salmonella* in vivo (49). Resident phagocytes are also the first cells harbouring bacteria in the spleen and liver (36, 38). After the initial stage, however, recruited phagocytes contribute to the defense. Early evidence indicating that an influx of bone marrow-derived cells, most likely neutrophils and monocytes, mediate protection early during infection comes from studies demonstrating increased *Salmonella* growth in the spleen and liver of mice that received whole body irradiation (50). Attempts to define the role of neutrophils in protection were performed in depletion studies using the Gr-1 antibody directed against Ly6G/C. Gr-

1-treated mice were more susceptible to *Salmonella* (51, 52). However, the bacterial load was increased already day two post infection in PP and spleen, before most neutrophils have entered the infection site. Resident cells depleted by the Gr-1 treatment may thus be involved in the protection. Furthermore, expression of Ly6G/C is not specific to neutrophils, and other cells such as $Ly6C^{hi}$ monocytes could also have been depleted in these experiments. Thus, the specific contribution of neutrophils to restrict bacterial growth was not fully clarified and was more specifically addressed in this thesis (Paper I).

Neutrophils and macrophages are the main cell types that harbor *Salmonella* during infection in mouse models (35, 53). After recruitment to infected organs these cells amplify the inflammatory response initiated by resident cells by producing inflammatory mediators such as cytokines and chemokines. They also exert the important function of phagocytosing and killing *Salmonella.* Even though *Salmonella* has evolved a number of mechanisms to evade killing, the ability of phagocytes to reduce the growth of bacteria through expression of Nramp1, NADPH oxidase (phox) and inducible nitric oxide synthase (iNOS) during infection is crucial for host survival.

Mouse strains differ in their susceptibility to *Salmonella* infection. This is attributed to the mouse genotype at the Nramp1 locus (presently called Slc11a1), and strains such as C57BL/6 that have a mutation in the Nramp1-encoding gene are highly susceptible (5). Nramp1 is a late endocytic/lysosomal protein that is situated in the membrane of *Salmonella*-containing vacuoles of monocytes/ macrophages and neutrophils (54). Nramp1 is a divalent ion transporter and, althought its exact function in bacterial killing is still not known, it likely causes iron efflux from the phagosome and starves the bacteria from this essential growth factor. Nramp1 expression can be induced in phagosomes by LPS, TNFα and IL-1 (55). The importance of functional Nramp1 in recruited bone marrow cells to suppress the early stages of infection was shown nearly 30 years ago. In these studies, the growth of *Salmonella* in the spleen and liver was slowed in x-irradiated Nramp1 susceptible mice reconstituted with Nramp1 resistant bone marrow in the first days after infection of the reconstituted mice (6).

In addition to Nramp1, production of reactive oxygen species by NADPH oxidase as well as reactive nitrogen species and nitric oxide (NO) by iNOS are

important for host defense to eliminate intracellular bacteria. By infecting NADPH oxidase$^{-/-}$ and iNOS$^{-/-}$ mice with *Salmonella* i.v., it was shown that early killing of bacteria was dependent on NADPH oxidase while iNOS was dispensable. However, later during infection, iNOS-deficient mice were unable to control bacterial growth and eventual bacterial clearence was dependent on NO production (56, 57). iNOS is induced by bacterial products and pro-inflammatory cytokines, particularly dual stimulation with LPS and IFNγ, and the production is further enhanced by TNFα (58). Despite the role of iNOS for control of *Salmonella* infection, NO produced by iNOS induces immunosuppression in adjacent lymphocytes (59). Seven days after infection with *Salmonella*, the response of spleen cells to B- and T-cell mitogens was profoundly suppressed although it was restored after 21 days (60, 61). This could be due to NO production by cells of the monocyte-macrophage lineage since mature splenic macrophages and immature monocytes were responsible for suppression in vitro (62). In addition, immunosuppression was released by blocking NO by aminoguanidine, which also led to increased bacterial load and bacteremia (63).

Adaptive responses

DCs are the antigen presenting cells that initiate the adaptive immune response by priming naïve T cells (64, 65), and the absence of these cells in vivo results in a severely compromised capacity to activate T cells during bacterial infection, including *Salmonella* (16, 66). DCs from mouse spleen, liver, MLN or grown from precursors in bone marrow can indeed phagocytose *Salmonella* and process and present the antigens to activate CD4 and CD8 T cells (16, 53, 67-73). In addition, after i.v. infection of mice, splenic DCs contained *Salmonella* expressing GFP-OVA and could activate OVA-specific CD4 and CD8 T cells upon ex vivo co-culture with OVA-specific T cells (69, 71). In addition, injection of *Salmonella*-loaded DCs into naive mice activated CD4 and CD8 T cells in vivo (53). DCs can also phagocytose macrophages that have undergone *Salmonella*-induced apoptosis and present a bacteria-encoded antigen present in the macrophages on MHC class I and class II (74). Thus, using direct and indirect mechanisms, DCs can process *Salmonella* for antigen presentation to CD4 and CD8 T cells.

Despite that T cells are clearly activated and involved in combating *Salmonella* infection, the suppression of early bacterial growth requires bone marrow

derived cells and cytokines, but not T cells. T cells, however, are definitely needed in the control and clearance beyond the initial stages of *Salmonella* infection (75-80). For example, atymic mice, which lack T cells, were unable to control the growth of several attenuated *Salmonella* strains in BALBc mice despite that one strain caused a T-cell independent antibody response against LPS (75). Furthermore, the importance of CD4 T cells rather than B cells was demonstrated in infection studies in CD28-deficient mice, which can not deliver costimulation by CD80 and CD86. These mice could not resolve a primary infection with attenuated *Salmonella* (76).

Although B cells and CD8 T cells may be dispensible for a primary infection, they play an important role for survival against a secondary infection, particularly for infections by the oral route (76, 77, 79, 81, 82). While antibodies alone are not sufficient for protection, they are needed for rapid clearance of a secondary infection with a virulent strain, and a role for antibodies is particularly evident in infections by the oral route (76, 77, 82). Transfer of both serum and immune cells including T cells from immunized mice was needed for protection against infection with a virulent *Salmonella* strain (79). Furthermore, mice without B cells can survive a primary infection with a less virulent strain but do not survive secondary challenge with a virulent strain even after transfer of immune serum (77, 82). Thus, B cells do have a role in combating *Salmonella* infection, particularly virulent strains and during infection by the oral route (76, 77, 82). The antibodies produced may be important in controlling bacterial replication by acting as opsonins, as suggested by the uncontrolled bacterial growth in mice strains that can not mount isotype-switched antibody responses (77, 82). Mucosal IgA also hasa role in protecting against oral *Salmonella* infection and fecal shedding of bacteria (18). Taken together, the available data support that DCs are critical to initiating adaptive immunity during *Salmonella* infection and that CD4 T cells but not B cells are crucial to clear a primary infection. Furthermore, antibodies produced by B cells are an important mechanism to defend against orally acquired bacteria and, similar to CD8 T cells, are needed to resolve a secondary challenge with a virulent strain.

Cytokines: small key players during *Salmonella* infection

Two major players required to control an infection with *Salmonella* are TNFα and IFNγ. The cytokines involved in regulating production of IFNγ, particularly IL-12 and IL-18, are also important during *Salmonella* infection. While IFNγ mainly activates phagocytic cells and increase their killing capacity, TNFα attract phagocytes to the infection site, induces apoptosis and enhances some functions induced by IFNγ. Thus, the inflammatory cytokines IL-12, IL-18, IFNγ, TNFα and IL-1β are all important for host resistance to *Salmonella*. Indeed, numerous studies in gene deficient mice or mice treated with Abs against TNFα, IFNγ, IL-12, and IL-18 showed increased susceptibility and decreased survival after *Salmonella* infection (78, 83-89). In addition, GM-CSF, IL-6 and IL-1β have been found to be produced in response to *Salmonella* both in vivo and in vitro (28, 90).

IFNγ and IFNγ inducing cytokines

IFNγ is produced by NK cells and T cells after stimulation with IL-12 and IL-18 (85-87). During acute *Salmonella* infection, NK cells, NKT cells and T cells are early sources of IFNγ that is rapidly produced in the PP, MLN and spleen (91-95). The key functions of IFNγ are to activate macrophages, including up-regulation of MHC-II and iNOS induction, and to mediate the formation of inflammatory foci (59, 81, 96, 97). The lack of phagocyte activation in anti-IFNγ treated or IFNγ-deficient mice results in uncontrolled bacterial growth and death (81, 83, 93).

IL-12 and IL-18 are produced by DCs and macrophages/monocytes after activation by, for example, TLR ligation and their main function is to induce and enhance IFNγ production, respectively (70, 98). Three distinct heterodimers of bioactive IL-12 have been found. The classical IL-12p70 consists of the induced p40 subunit and the constitutively expressed p35 subunit. The rather newly discovered IL-23 is formed by association of IL-23p19 with IL-12p40 (99). IL-27 is the endproduct of the p28 subunit association with EB13, another molecule in the IL-2/IL-6 superfamily. IL-12p70 in particular, but also IL-23 alone, can induce IFNγ, but in synergy with IL-27 and IL-18 they promote a much stronger IFNγ response. Similar

to IL-18, IL-27 needs to synergize with IL-12 for production of IFNγ (99, 100). During *Salmonella* infection, IL12p19 gene expression was not upregulated in the liver while IL-12p40 and p35-deficient mice were susceptible to infection. Hence, the lack of induction of IL12p19 during infection support a role for IL12p70 and IL-12p40 homodimers rather than IL-23 in the observed enhanced susceptibility (101). In addition, blocking IL-12p40 or IL-18 with antibodies during *Salmonella* infection exaggerated the infection, led to uncontrolled bacterial growth, and less granuloma formation due to the diminished production of IFNγ (84-87).

TNFα and TNFα-related cytokines

TLR activation with bacterial constituents, chemicals and physical damage induce the production of TNFα, and the major source of TNFα is cells from the macrophage lineage. The earliest TNFα comes from pre-formed stores released by cleavage (102). The main function of TNFα is to induce multiple cytokines, chemokines and adhesion molecules that attract phagocytes and other leukocytes to the site of release. Furthermore, TNFα is of major importance in regulating the production other cytokines and enhances, for example, production of IL-1, IL-12 and IL-6 (102). TNFα and LTα bind to the same receptor, TNFR1. In addition, TNFα, LTα and IL-1 appear to have similar functions based on in vitro experiments (102).

During *Salmonella* infection, TNFα is important for regulating NADPH oxidase-mediated killing by macrophages, formation of granulomas to prevent bacterial spread, serum nitric oxide production and upregulating costimulatory molecules on DCs (28, 81, 93, 103-105). TNFα activation of the TNFR1 receptor mediates the fusion of NADPH oxidase with the vacuole containing *Salmonella*, thus making it possible for the cells to eliminate the intracellular bacteria with oxygen radicals (103). In the absence of TNFα, recruitment of neutrophils and development of lesions in spleen and liver progress as normal during first few days after infection, but after three days there is a failure in granuloma formation associated with reduced mononuclear cell infiltration and a proportional increase of neutrophils (103, 105). In addition, administration of anti-TNFα antibodies late during infection causes regression of already established granulomas while administration during a secondary infection results in less splenomegaly, no granulomas and no mononuclear infiltrate

(81, 105). Thus, in response to *Salmonella* infection, macrophage but not neutrophil accumulation in granulomas, as well as NADPH oxidase-mediated bacterial killing, are dependent on TNFα.

Monocytes

Monocytes are figuratively speaking "the cells in between", i.e. circulating cells that are in a transitional state between the progenitors in the bone marrow and the mature cells in the tissues. This heterogeneous cell population has an enormous plasticity, and their maturation status and the local microenvironment in the tissue will direct their development and function. A monocyte is, by definition, only a monocyte as long as it stays in the circulation. As soon as it arrives in a tissue it should be classified either as a macrophage or, in some cases, as a dendritic cell.

Monocyte subsets

Two major subsets of monocytes have been described in humans, mice and rats (106-108). These subsets differ in the level of maturation, chemokine receptor and adhesion molecule expression, differentiation potential and migration pattern (Table 1). The human $CD14^{low}CD16^{hi}CCR2^{low}$ monocytes behave similar to the murine $CX_3CR1^{hi}CCR2^{low}Gr\text{-}1^{low}$ cells, while the "classical" human $CD14^{hi}CD16^{low}CCR2^{hi}$ monocytes resemble murine $CX_3CR1^{low}CCR2^{hi}Gr\text{-}1^{hi}$ monocytes (106, 107). In addition, a third monocyte subset has been described in mice that expresses an intermediate level of Ly6C but a high level of CCR2 (109). A counterpart to the $Ly6C^{int}$ subset in mice has been described in humans (110). In humans, the $CD14^{hi}$ sub population constitutes 90-95% of total monocytes while in mice and rats they are decreased to 50 and 10-20%, respectively (110). The surface markers Gr-1 and Ly6C are used interchangeably to identify monocytes in the literature, a point that deserves clarification: The antibody Gr-1 recognizes Ly6G, a granulocyte surface marker, and also Ly6C (111, 112), which is expressed by monocytes and several other haematopoeitic cell populations (106, 113-115). Thus, several cell types are recognized by the Gr-1 mAb and distinguishing monocytes from neutrophils, for example, should make use of mAbs specific to Ly6C and Ly6G, as monocytes are $Ly6C^{+}Ly6G^{-}$ and neutrophils are $Ly6C^{+}Ly6G^{+}$ (112, 116) (see also Paper I).

In the first reports, murine Gr-1^{hi} monocytes were described as inflammatory since they, but not Gr-1^{low} monocytes, migrated to the inflammatory site during various inflammatory conditions (106, 117, 118). However, it was recently described that Gr-1^{low} cells were also recruited during infection (119). Gr-1^{low} monocytes also migrate to tissues such as the lungs, brain, and gut independently of inflammation (106). By eliminating blood monocytes with clodronate-loaded liposomes and examining their reappearance in the blood, it was shown that only Ly6C^{hi} monocytes repopulated the blood and subsequently down regulated their expression of Ly6C to become Ly6C^{low} monocytes (114, 120). Ly6C^{hi} (Gr-1^{hi}) blood or bone marrow monocytes were also found to shuttle back to the bone marrow and develop into Ly6C^{low} cells after i.v. transfer (120, 121). In addition, similar to data from mouse models, rat Gr-1^{+} monocyte equivalents converted to Gr-l^{-} monocytes without division (108). Together these data demonstrate that CCR2^{hi}Ly6C^{hi} monocytes are immature cells that develop into Ly6C^{low} monocytes, which will further differentiate to the final tissue cell type, macrophages or DCs.

Table 1. Cell surface antigen expression on the two principal monocyte subsets in mice and humans.

Cell surface markers	Murine Ly6C^{hi} monocytes	Murine Ly6C^{lo} monocytes	Human CD14^{hi} monocytes	Human CD14^{lo} monocytes
F4/80	+	+	ND	ND
CD11b	+	+	+	+ /-
Ly6C	++	-	ND	ND
CD115	+	+	ND	ND
CD14	ND	ND	++	+/-
CD16	ND	ND	-	+
CD11c	-	-	+	++
MHC-II	-	-	++	+
CD62L	+	-	+	-
CCR1	ND	ND	+	-
CCR2	+	-	+	-
CCR5	+/-	+/-	-	-
CX_3CR1	+	++	+	++

Adapted from reference (106, 114, 122). ND, not determined.

Macrophages

Macrophages are a very heterogeneous population of specialized cells that are widely distributed in the body. They develop distinct functions and display different patterns of surface molecules depending on what tissue they reside in and where in the tissue they are localized (107, 112, 123). Examples of specialized macrophages in the tissue include Kupfer cells in the liver, microglia in the central nervous system,

metallophilic and marginal zone macrophages in the spleen, as well as osteoclasts and alveolar macrophages (124). Macrophages have numerous functions both during homeostasis as well as during innate and adaptive immune responses. Hallmarks of their tasks are extensive phagocytosis, eradication of apoptotic bodies, destruction and clearance of pathogens, biosynthetic capacity and wound healing (125-127).

Emerging evidence suggests that some populations of macrophages are replenished by self-renewal during steady state while others are replaced by recruited monocytes, particularly after different types of trauma (121, 128-132). The classical pathway of macrophage activation is induced by IFNγ and mediates resistance against intracellular pathogens. However, an alternative pathway induced by IL-4 and IL-13 also exists (123). The latter activation pathway leads to a distinct macrophage phenotype involved in Th-2 responses during, for example, reactions against parasites and allergy. IFNγ-elicited macrophages have enhanced MHC class II expression and are primed to be fully activated by secondary stimuli such as bacterial constituents. IFNγ activated macrophages produce pro-inflammatory cytokines, nitric oxide, and kill intracellular bacteria and tumor cells (133). These functions, however, depend on which tissue the cells are localized in. Intestinal macrophages in humans are highly phagocytic and bactericidal but, unlike monocytes, do not produce proinflammatory cytokines upon stimulation with bacteria (132). Macrophages are also able to alter their phenotype in response to changes in the surrounding cytokine milieu (134).

Macrophages share many surface markers with neutrophils and dendritic cells (112, 129, 135). This makes it difficult to distinguish these cells unless multiple markers are simultaneously used, and assessing cell morphology and function should be considered to support phenotypic data. The antibodies F4/80 and CD68, which recognize EMR1 and the intracellular molecule macrosialin, respectively, are widely used to identify macrophages (135, 136). Caveats to using these antibodies, however, include that the molecules recognized by them are not expressed by all macrophages and their expression is not limited to macrophages. For example, the F4/80 antibody and anti-CD68 also react with DCs and monocytes (28, 112, 135, 136) (see also Paper I). Antibodies to CD11b, which is one chain of the CD11b/CD18 heterodimeric $\alpha_M\beta_2$ integrin, also known as complement receptor 3, are also used to identify subpopulations of macrophages. However, this molecule is not uniquely expressed by macrophages and is also found on neutrophils, monocytes, NK cells and DCs (137,

138). Thus, distinguishing macrophages from other myeloid lineage cells in particular, as well as identifying subpopulations of macrophages, requires thorough analysis of several characteristics including phenotype, morphology and function. Moreover, the tissue analyzed and the subpopulation of macrophage studied will influence the phenotypic markers that are most appropriate to use (135).

Dendritic cells

Dendritic cells were first identified and characterized in a series of studies examining mouse splenic adherent cells that were published in the 1970s by Steinman and Cohn (64, 65). Steinman and colleagues were the first to show that a distinguishing functional feature of DCs relative to the other "accessory cells", B cells and macrophages, is their superior capacity to stimulate T cells (139, 140), work that was extended to show that DCs are the antigen presenting cell type that stimulates antigen-specific naive CD4 and CD8 T cells (16, 66, 141). Since their discovery over three decades ago, work on DCs has greatly escalated, and a great deal of information regarding the phenotype and function of these cells is available. As the focus of this thesis is myeloid lineage cells other than DCs, particularly monocytes, the discussion of DCs presented here is limited to features relevant to the work in this thesis.

Several subpopulations of DCs have been identified and characterized (137). Conventional dendritic cells (cDCs) are spleen and lymph node resident $CD11c^{hi}MHC\text{-}II^{+}$ cells that can be further divided into subsets based on expression of myeloid and lymphoid lineage surface molecules. For example, $CD8\alpha^{+}CD11b^{-}$ $CD4^{-}$, $CD8\alpha^{-}CD11b^{+}CD4^{+}$, $CD8\alpha^{-}CD11b^{+}CD4^{-}$ DC subsets have been described in the spleen, peripheral lymph nodes and liver, except that the liver, MLN and PP lack DCs expressing CD4 (8, 28, 68, 71, 137, 142-144). Instead PP, MLN, and liver have a subset of double negative $CD8\alpha^{-}CD11b^{-}$ DC (8, 68, 145). Subsets of conventional DCs have been reported to be localized differently in lymphoid tissue, have differential expression of pathogen recognition receptors, and have functional specialization that can be influenced by the type of stimuli encountered (146). Distinct types of migratory DCs exist in the non-lymphoid organs and include Langerhans cells and dermal DCs in the skin (147). Interstitial DCs, which is a collective name for migratory DCs in various organs, also belong to this category (148). These migratory DCs are also detected in the lymph nodes, but not in spleen, since they act as sentinels

in peripheral tissues and traffic continually through the lymphatics to the T cell areas in the draining lymph nodes (143, 144).

After taking up antigens in peripheral tissues and receiving inflammatory signals, the DCs undergo a process called maturation where they upregulate surface MHC and costimulatory molecule expression and downregulate their capacity to internalize antigens. They also change their chemokine receptor pattern to upregulate CCR7, which mediates their emigration from the tissue to T cell areas in the lymph node and enables them to present the antigens to T cells (149). Signals important in initiating DC maturation include proinflammatory cytokines such as TNFα and IL-1β, which are made upon engagement of TLRs during an infection. Although these cytokines are capable of starting maturation and causing phenotypic changes in the DCs, the maturing DCs need additional stimuli, such as a direct TLR signal or CD40 engagement, to be fully immunogenic and activate effector T cells (150, 151). Instead, mature DCs that have not received additional stimuli will, similar to immature DCs, induce T cell tolerance (143, 152). The factors involved in inducing DC maturation during bacterial infection are many, and deciphering their contribution to this process in vivo is complex. For example, during *Salmonella* infection TNFα and IL-1β have overlapping roles in inducing the maturation of non-infected DCs in infected lymphoid organs (28). In addition, DCs directly associated with *Salmonella* can mature independently of TNFα and TLR signaling (28, 69).

There are also non-conventional DCs such as plasmacytoid DCs. These DCs are also localized in the lymphoid organs but are specialized to produce type I interferons and have an important role during viral infections (153). Plasmacytoid DCs express an intermediate level of CD11c, have low MHC and costimulatory molecule expression and a relatively low ability to stimulate T cells, properties that distinguish them from cDCs (153). Another type of cell that shares some features with DCs are cells that express an intermediate level of CD11c and have been reported in several different infection and inflammation models (28, 115, 124, 154-158). They are monocyte-derived cells and are discussed in more detail below.

What distinguishs a DC from a macrophage?

DCs and macrophages come from a common myeloid progenitor and share phenotypic and functional properties (113, 135, 159, 160), which makes

distinguishing these cell types complex. For example, both cell types are phagocytic and take up antigens. However, DCs and not macrophages are specialized antigen presenting cells and can prime naïve T cells. This was demonstrated by showing that splenic or lymph node-derived DCs efficiently activated T cells in vitro and in vivo while macrophages purified from spleen or peritoneum failed to induce a T cell response (139, 140, 161). Instead, high doses of either monocytes or macrophages inhibited DC-dependent cytotoxic T cell activation in vitro (140). In addition, ablation of DC in vivo abolished the activation of T cells against intracellular pathogens (16, 66).

Another difference between these two cell types is in their ability to degrade internalized antigen. Although both cell types are phagocytic, macrophages efficiently destroy engulfed antigen or debris while DCs in vivo have poor proteolytic activity in the lysosome. Thus, DCs, but not macrophages, degrade antigen poorly and can thus prolong the antigen presentation time frame (74, 162). Yet another distinguishing feature is their migratory ability in vivo, where macrophages are tissue-resident cells while DCs constitutively migrate to the lymph nodes (11). In conclusion, DCs are specialized for antigen presentation while macrophages, which are a heterogeneous population of adherent phagocytic cells, include a wide range of phenotypically different cells whose functions range from killing pathogens and producing pro inflammatory cytokines to down regulating the immune response by producting anti inflammatory cytokines (107, 123, 137).

Monocyte, DC and neutrophil progenitors

Hematopoietic stem cells in the bone marrow give rise to several different lineages of progenitors such as common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). B, T and NK cells are derived from CLPs while DCs can be derived both from CMPs and CLPs (163). CMPs are divided into granulocyte-monocyte progenitors and megakaryocyte-erythrocyte progenitors. Mast cells, eosinophils, basophils, monocyte-DC precursors and a neutrophil/monocyte precursor are separated from granulocyte-monocyte progenitors (163) (Fig. 2). Macrophage and neutrophil development requires PU.1 and C/EBPα, respectively, that regulate the differentiation into either population (164).

Mobilization from BM during inflammation

In response to infection or inflammation, there is an increased demand for myeloid cells, and neutrophils are quickly released into the blood followed by monocytes. The rapid release from the bone marrow precedes an increased production of neutrophils and monocytes in this compartment at the expense of lymphocyte production (165). This is regulated in a complex manner by a number of cytokines and growth factors including TNFα, IL-1β, CXCL12 and G-CSF (165, 166). During increased production of neutrophils in the bone marrow, they have a decreased maturation period before they exit, and both mature and immature neutrophils are released into the blood to provide more neutrophils (167, 168).

DC and macrophage progenitors

During steady state, Langerhans cells, dermal DCs and many macrophage populations, such as some types of spleen and lung macrophages, are long-lived and capable of self renewal (107, 128, 131, 169). In contrast, DCs resident in secondary lymphoid organs, lung DCs and some populations of macrophages, such as dermal macrophages, are short lived and are replenished by precursors from the bone marrow or spleen (129, 131, 170-172). In the bone marrow, a very small progenitor population (0.05%) named macrophage/DC precursor was discovered that had similarities to granulocyte-monocyte progenitors but had lower c-kit expression and higher CX_3CR1 expression (159) (Fig. 2). This progenitor gave rise to $CD8\alpha^+$ and $CD8\alpha^-$ DCs, but not plasmacytoid DCs, and to various types of macrophages including splenic marginal zone and marginal sinus macrophages. In contrast, another lineage-specific precursor in the bone marrow, called clonogenic common DC precursor, gave rise to all three DC populations ($CD8\alpha^+$, $CD8\alpha^-$ and plasmacytoid DCs) but not to monocytes/macrophages (173, 174) (Fig. 2). In comparison, very few $CD8\alpha^+$ and almost no $CD8\alpha^-$ DCs were generated from bone marrow monocytes (159). Furthermore, a direct precursor to all cDCs was found in spleen that did not give rise to plasmacytoid DCs or any other lineages (158). This may be a cell downstream of the macrophage/DC precursor and/or clonogenic common DC precursor originating in the bone marrow (Fig. 2). Furthermore, although very few $Ly6C^{low}$ and no $Ly6C^{hi}$ monocytes differentiated into cDC during steady state, $Ly6C^{hi}$ monocytes developed into a population of $CD11c^{int}CD11b^{hi}$ cells in spleen after inflammation (158).

Consistent with this, it was reported that the macrophage/DC precursor but not monocytes could differentiate into cDC in the spleen while monocytes could give rise to DCs and macrophages in non-lymphoid organs and to CD11c^{int} cells in spleen (121, 129). In conclusion, these reports support that DC/monocyte or DC precursors in the bone marrow and spleen can generate lymphoid organ resident cDCs while monocytes can not (121, 158, 159, 173, 174). The data also show that monocytes can differentiate to DCs in non-lymphoid organs.

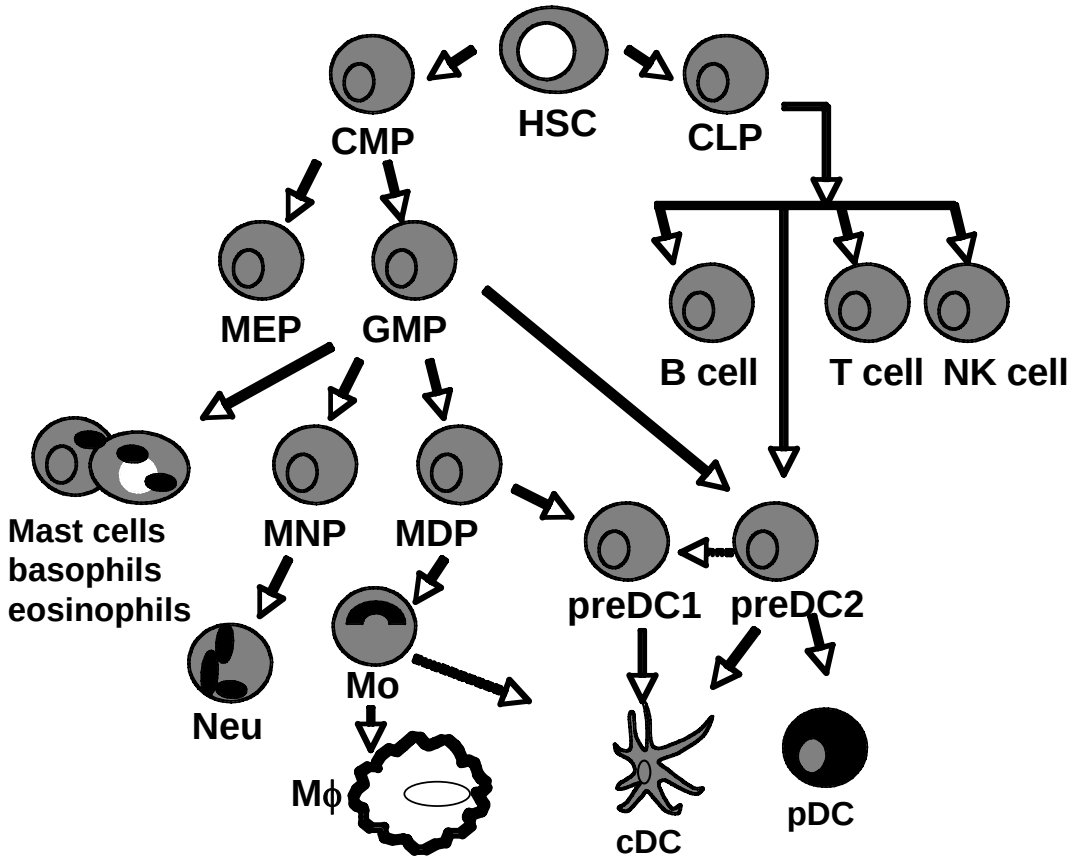

Figure 2. DC and monocyte ontogeny. Spleen and lymph node resident DCs can develop from separate lineages in the bone marrow. Monocytes are unlikely to give rise to resident DCs in the spleen. HSC, Hematopoietic stem cells; CMP, common myeloid progenitors; CLP, common lymphoid progenitors; MEP, megakaryocyte-erythrocyte progenitors; GMP, granulocyte-monocyte progenitors; MDP, macrophage/DC precursor; Neu, neutrophils; preDC1, direct bone marrow/spleen precursor; preDC2, clonogenic common DC precursor; Mo , monocytes; Mϕ, macrophages.

Differentiation of monocytes during steady state

As mentioned above, monocytes can differentiate into DCs in non-lymphoid organs and to macrophages during tissue homeostasis. In the intestine, rat Ly6C^{low} monocyte equivalents, which could be derived from Ly6C^{hi} monocytes, differentiate into

intestinal lymph dendritic cells during steady state in a model where monocytes are adoptively transferred and the pseodafferent lymph is collected (108) (Fig. 3). Moreover, adoptively transferred Ly6C^{hi} monocytes give rise to lamina propria CX_3CR1^+ DCs and CX_3CR1^- macrophages in mice deficient for lamina propria macrophages and DCs (121) (Fig. 3). In the lung, monocytes replenish two subsets of pulmonary DCs during steady state and convert to either DCs or macrophages during inflammation (128, 129, 175) (Fig. 3). While Ly6C^{hi} monocytes convert into Ly6C^{low} monocytes in the blood, there are distinct differentiation abilities between the monocyte subsets in the lung, where Ly6C^{hi} monocytes preferentially convert to CD11b^{low} lung DCs while both Ly6C^{hi} and Ly6C^{low} monocytes convert to CD11b^{hi} lung DCs (129, 175). These monocyte-derived DCs migrated to the draining lymph nodes, presented OVA and induced proliferation of naïve OVA-specific CD4$^+$ T cells (129). Monocytes do not convert into lung macrophages, which were characterized as autofluorescent CD11c^{hi}CD11b^{low} after transfer into untreated mice (129). However, after ablation of CD11c$^+$ cells in recipient mice, grafted Ly6C^{low} but not Ly6C^{hi} monocytes differentiate into parenchymal macrophages that further develop into alveolar macrophages (128). In addition, both macrophage populations in the lung have proliferative capacity. Thus, there is dual contribution to the macrophage population in the lung where macrophages contribute by self-renewal and monocytes maintain the macrophage number. However monocyte replacement is probably more important during inflammation than during steady state, since administration of LPS increases the number of monocyte-derived macrophages (128, 129). Hence, monocytes can develop into migratory DCs and macrophages in the lung and intestinal lamina propria (108, 121, 129).

Differentiation of monocytes during inflammation

TNFα and iNOS producing CD11c^{int} cells

In sharp contrast to steady state, conditions rapidly change during infection or inflammation induced by other means. For example, Langerhans cells and dermal DCs are replenished by local precursors in steady state while they can be derived from Ly6C^{hi}CCR2^{hi} monocytes after inflammation caused by UV-radiation (169, 171, 176). In the case of infection, a robust increase of Ly6C^{hi} monocytes in the blood and

increased emigration to the infected tissues is induced that results in an emerging population of differentiating cells in the tissue (106, 117, 155, 158). For example, a population of $CD11b^{hi}Ly6C^{hi}CD11c^{int}$ monocyte-derived cells producing iNOS and TNFα are found in the spleen and other lymphoid organs during infection with *Listeria, Salmonella* or after chemically-induced inflammation (28, 72, 115, 154, 156-158) (Fig. 3). Although these $CD11b^{hi}CD11c^{int}$ cells, which were called TipDCs when they were first described (115), express a high level of MHC-II and co-stimulatory molecules and induce a mixed lymphocyte reaction, the capacity of these cells to prime naïve antigen-specific T cells has not been directly assessed (28, 106, 115, 158). Thus, it remains to be experimentally shown whether these $CD11b^{hi}CD11c^{int}$ cells can process and present antigens to naïve T cells and whether they more resemble cDCs or are more related to macrophages. Interestingly, cells with the characteristics of TipDCs have recently been detected in the lamina propria and at a very low level in PP and MLN during homeostatic conditions. Here they are critical to induce IgA class switching of naïve B cells by producing iNOS (177). In contrast, other reports demonstrate that monocyte-derived cells exert a suppressive effect on adaptive immunity. For example, $CD11b^{hi}Gr\text{-}1^{hi}$ cells, which included cells resembling TipDCs, accumulated in inflamed tissues after chemotherapy, traumatic stress or helminth infection and produced nitric oxide that suppressed T cell proliferation (178-180). Moreover, recruited $CD11b^{hi}Ly6C^{hi}$ monocytes suppressed T cell proliferation by producing nitric oxide in the spleen, bone marrow and CNS during autoimmune encephalomyelitis (181). In a polymicrobial sepsis model, a heterogeneous population of $CD11b^{hi}Gr1^{hi}$ cells including neutrophils and monocytes were recruited to lymphoid organs and suppressed IFNγ production by CD8 T cells but did not affect CD4 T cell proliferation (182). Finally, the microenvironment can also direct $Ly6C^{hi}$ monocytes to convert to an anti-inflammatory phenotype. This was demonstrated during muscle injury, when recruited $Ly6C^{hi}$ moncytes that had phagocytosed cell debris proliferated and differentiated into macrophages that produced anti-inflammatory cytokines and promoted muscle repair (126). Hence, the microenvironment and cause of inflammation determines the fate and function of newly recruited monocytes.

Monocyte-derived DCs in tissues

A different situation occurs when monocytes are recruited to inflamed non-lymphoid organs instead of directly recruited to lymph nodes. In these situations it is suggested that some of the monocytes convert to migratory DCs and sequentially emigrate via lymphatics to the draining lymph node. This has been most extensively studied in the skin and peritoneum. One of the first studies of monocyte-derived DCs in the skin showed that Ly6C^{hi} monocytes are recruited to inflamed skin and engulf microspheres. While most of the microsphere^{+} monocytes differentiate into F4/80^{+} macrophages and remain at the injection site, some are found in the draining lymph node expressing the characteristic CD11c^{hi}MHCII^{hi} DC phenotype (109, 183) (Fig. 3).

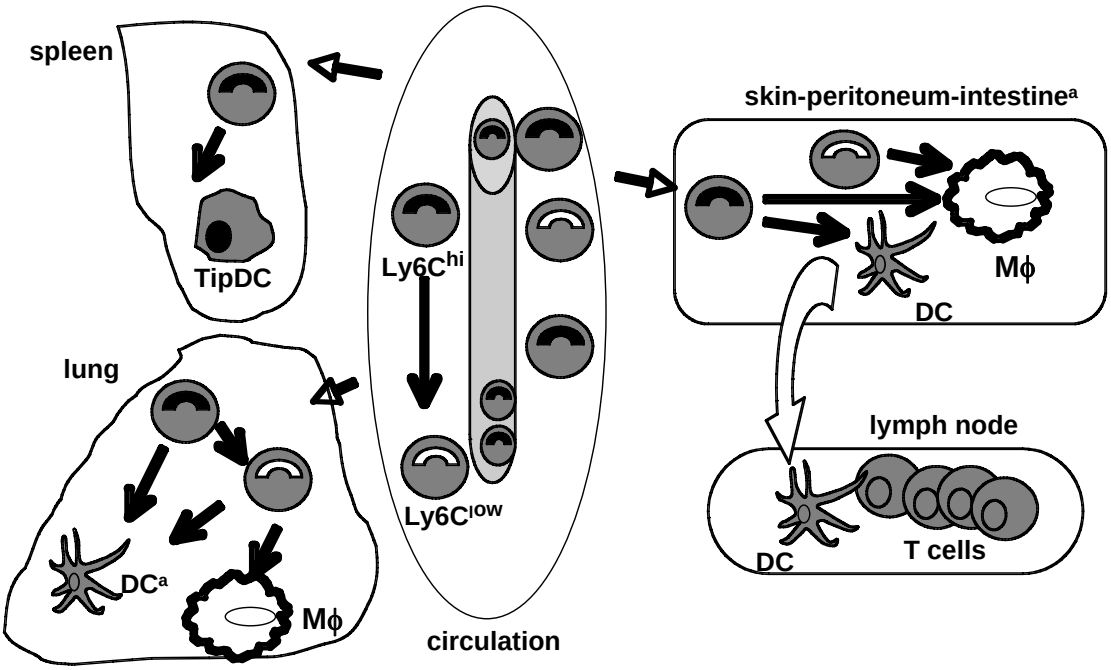

Figure 3. The fate of monocytes during inflammation. Monocytes differentiate into CD11c^{int} cells (TipDCs), macrophages or DCs depending on the specific tissue, the maturation stage, and the microenvironment. Ly6C^{hi} Mo, ”inflammatory” monocytes; Ly6C^{low} Mo, ”non-inflammatory” monocytes; Mϕ, macrophage. [a]detected also during steady state.

A similar phenomenon was detected during infection with the cutaneous parasite *Leishmania*, where monocytes recruited to the skin phagocytose amastigotes and some acquire a dermal DC phenotype (184). These cells with a DC phenotype are thought to subsequently migrate to the draining lymph node. Interestingly, these monocyte-derived dermal DCs found in the lymph node were far more effective at inducing IFNγ production by CD4 and CD8 T in vitro than the CD11b^{hi}CD11c^{int}

monocyte-derived cells recruited directly to the lymph node (184). However, some caution should be taken when interpreting this data since a rather unspecific method was used to separate the cells and some lymph node-resident DCs might have contaminated the assay (184). In another experimental system, monocytes were recruited to the peritoneum in response to an injection of aluminum hydroxide (alum) plus OVA and monocyte-derived DCs containing OVA were detected in the draining lymph node (185). The monocyte-derived cells in the peritoneum could not activate naïve T cells while transferred Ly6C^{hi} monocytes could partially restore proliferation of OVA-specific CD4 T cells in the draining lymph node after DC depletion (Fig. 3). In addition, after immunization with adjuvant plus OVA in the buccal mucosa, recruited Gr-1^{+} monocytes developed into MHC-II^{+} cells and were the only cells that cross primed CD8^{+} T cells in the draining lymph node (186). To summarize, these results suggest that monocytes recruited to non-lymphoid organs during inflammation can give rise to migratory DCs in addition to macrophages.

One possible mechanism behind the conversion of monocytes to DCs is that migration through a matrix may in itself influence the fate of monocytes. For instance, in a model of transendothelial migration, human monocytes migrated into the subendothelial matrix and some cells remained in the matrix and became macrophages while others reverse transmigrated and acquired a DC phenotype (187). The cells resembling DCs exerted strong stimulatory capacity in a mixed lymphocyte reaction, arguing for their conversion into DCs. Reverse transmigration was further enhanced by phagocytosis of foreign particles and mimicked the situation of DC migration from tissue into lymph in vivo (187, 188). Later studies detected a difference in the differentiation capacity between the human monocyte subsets, particularly the CD16^{hi} subset, which are equivalent to murine Ly6C^{low} monocytes. In these studies, CD16^{hi} monocytes transmigrated and differentiated into DCs although it was not excluded that the CD16^{low} subset also had this ability (188).

In contrast, other stimuli can block cell migration. For example, intradermal injection of either LPS or *Salmonella* together with latex beads blocks the conversion of monocytes into DCs and their migration to the lymph node (189, 190). Lung macrophages were also shown to prevent DC migration into the draining lymph node (191). This is also consistent with in vitro results demonstrating that LPS or cytokines including IL-6, IFNγ and IL-10 can block the differentiation of monocytes into DCs

while, in contrast, TNFα promotes differentiation to DCs (192-196). Thus, signals received during migration can skew monocytes towards a DC phenotype while stimulation with bacterial constituents and some cytokines can skew monocytes to become macrophages.

Recognition receptors

Several types of recognition receptors exist that are located on the cell surface or intracellularly in the cytoplasm or in vacuoles. They can be divided into phagocytic/endocytic receptors and pathogen recognition receptors that do not mediate phagocytosis but are important for sensing pathogens and alerting the immune system. Opsonin receptors such as complement (integrin) and Fc receptors belong to the first category. They require complement and antibody-opsonised elements, respectively, for phagocytosis/endocytosis. Scavenger receptors and C-type lectin-like receptors, including mannose and β-glucan receptors are also phagocytic/endocytic receptors (127, 197). The TLRs, NOD-like receptors and RIG-1 like receptors, which function as pathogen recognition receptors and are important sensors of bacteria, viruses, parasites and fungi, belong to the second type of recognition receptors. That is, they initiate effector functions upon activation but do not mediate phagocytosis/endocytosis. Each pathogen recognition receptor recognizes distinct conserved pathogen-associated molecular patterns (PAMPs) that only exist on microbes (197).

Toll Like Receptors

TLRs are well preserved throughout evolution and can be found both in vertebrates and mammals. Thus far 11 TLRs have been detected in humans and 13 in mice (198). TLRs are transmembrane glycoprotein receptors and TLR-1, -2, -4, -5, and -6 are expressed on the cell surface while TLR-3, -7, -8, and -9 are expressed intracellularly in endosomes/lysosomes (198). TLRs are not present in the cytoplasm but instead other pathogen recognition receptors, such as Nod-like receptors and RIG-1-like receptors, are present and will detect bacterial components and double stranded RNA (199). TLRs are expressed by many leukocytes including neutrophils, monocytes, DCs, and B-cells. Non-leukocytes including epithelial cells and fibroblasts also

express TLRs. Although the same cell expresses many TLRs, the combination of TLRs expressed depends on the cell type, location and state of activation (199). Moreover, the specific response depends on the site of infection.

The most important TLRs for sensing bacterial components are TLR2 in combination with TLR1 or TLR6, TLR4, TLR5 and TLR9 (Fig. 4). TLR4 recognizes lipopolysaccaride (LPS), the major constituent of the outer wall of Gram-negative bacteria but can also respond to other, less well-defined components. LPS needs to form a complex with LPS binding protein before it can bind to TLR4 with help of the co-receptors CD14 and MD2 at the cell membrane (200). TLR5 binds flagellin, the monomeric component of flagella, which are structures used by many bacteria to move. TLR9 resides intracellularly in endosomes and recognizes unmethylathed CpG (201). Complexes can be formed between TLR1/TLR2 and TLR2/TLR6 and together they recognize a wide range of lipoproteins and di- and tri-acylated lipopeptides. TLR2 is important during infections with Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (202-204)

Signaling pathways

To induce a downstream signaling cascade, activated TLRs need functional adaptor proteins. Five adaptor proteins have been described: MyD88, TRAM, MAL, TRIF and SARM (205). MyD88 is used by all TLRs except TLR3 while TRIF is specific for TLR4 and TLR3 signaling (Fig. 4). Thus, in contrast to other TLRs, activation of TLR4 induces both a MyD88-dependent and a MyD88-independent pathway. MAL is a bridging adaptor for MyD88 activation by TLR2 and TLR4 while TRAM associates with TRIF by TLR4 activation. In contrast, SARM is a negative regulator and acts on TRIF (205) (Fig. 4).

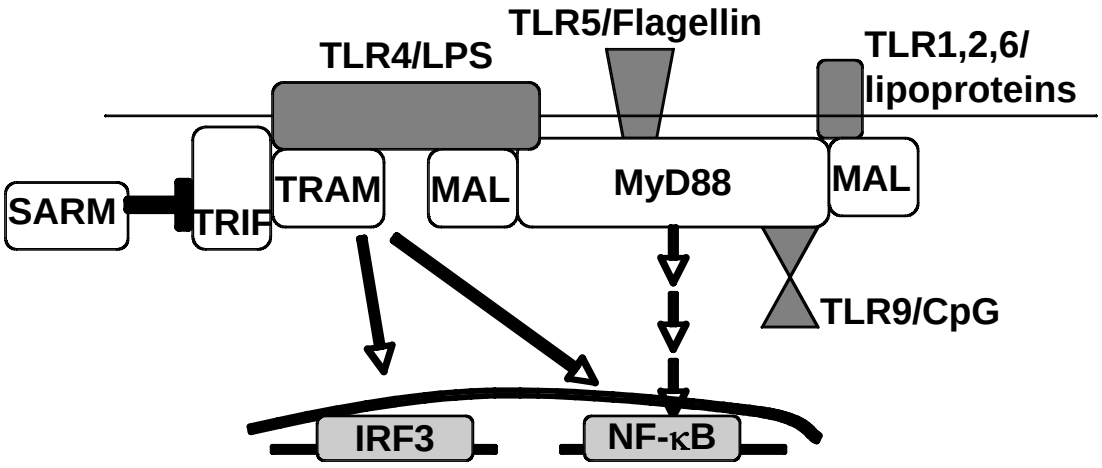

Figure 4. Toll like receptor signaling during bacterial infections. All TLRs relevant for bacterial infections signals via MyD88 and activate NF-κB that leads to the production of pro-inflammatory cytokines and chemokines. In addition, TLR4 signals via TRAM/TRIF, independent of MyD88, and activates IRF3 that initiates transcription of type I interferons and chemokines including CCL5,CCL12 and CXCL10.

Induction of the signaling cascade via MyD88 leads to NFκB translocation to the nucleus and activation of IRF-5 and MAP kinases, which results in transcription of pro-inflammatory cytokines and chemokines (198) (Fig. 4). The pro-inflammatory cytokines directly induced include TNFα, IL-6, IL-12 and IL-1β. On the other hand, MyD88-independent signaling via TRIF activates IRF3 that induces production of IFNβ and IFN inducible genes. In addition, after stimulation with LPS, NFkB and MAP kinases are induced with delayed kinetics by the TLR4 MyD88-independent pathway (206).

TLRs and cytokine/chemokine production

The importance of TLRs for host defense has been shown in humans as well as in mice. For example, patients deficient in IRAK4$^{-/-}$, which is downstream of MyD88, or NFκB are highly susceptible to Gram-positive bacteria and display a poor and delayed inflammatory response, and some patients die from infection (207). Similarly, MyD88-deficient mice are highly susceptible to a wide range of bacteria, fungi, protozoa and virus infections. These mice, for instance, have a higher bacterial load and succumb earlier to infection with *S. typhimurium*, *L. monocytogenes*, and *M. tuberculosis* (203, 208-210). However, the loss of a single TLR does not necessarily lead to increased susceptibility to infection, suggesting that the lack of one receptor

can be fully or partly replaced by another, although this is not always the case. For example, $TLR4^{-/-}$ mice or mice that have a functional defect in TLR4 are more sensitive to *Salmonella* infection while $TLR5^{-/-}$ mice have little if any defect in immunity to *Salmonella* (211-216). This may be due to the ability of flagellin to induce production of IL-1β and IL-18 via a TLR5-independent pathway in murine cells (217, 218). Interestingly, a polymorphism in human TLR5 that causes a non-functional receptor is associated with susceptibility to lung infection with *Legionella pneumophila* (219). Thus, genetic differences in the infected host, the route of infection and/or the pathogen can influence the relative importance of a given TLR or combination of TLRs.

Despite increased susceptibility of MyD88-deficient mice, several cytokines and chemokines are still produced, albeit at lower levels, upon infection with *M. tuberculosis*, *C. pneumoniae* or *L. monocytogenes* both in vivo and in vitro (156, 209, 220-224). The cytokines/chemokines that are induced via the MyD88 independent pathway depend on the infectious agent. For example IFNγ, TNFα, IL12p40 and CCL2 were induced by *Clamydia* while CCL2 but not IL12p40 was induced by *Listeria* (156, 223). Signaling via the TLR4 MyD88-independent pathway may play a role in the observed induction of effector proteins in the absence of MyD88. This pathway can, via the production of type I interferons or delayed activation of NFkB, induce production of MCP-5, CCL5, CXCL10 and upregulate CD86 and CD40 on dendritic cells (69, 206, 225). In contrast, it can not induce production of pro-inflammatory cytokines including IL1β, TNFα or IL-12 (226). Hence, in the absence of TLRs, other PRRs or pathways can induce production of inflammatory cytokines and chemokines, albeit at much lower levels.

Cell recruitment

Rolling, adhesion, and transmigration

Cell recruitment can be divided into several steps including mobilization from the bone marrow, rolling, arrest and adhesion to endothelial cells, and transmigration into the tissue or lymph node. In general, rolling of leukocytes is mediated by the interaction of selectins with their ligands. In most cases the first contact is between L-selectin (CD62L) and P-selectin glycoprotein ligand 1 (PSGL-1) on the cell surface with PNAd and P/E-selectins, respectively, on the endothelium (227). Integrins can

also form contacts during this stage. During rolling, chemokine receptors on the leukocytes recognize chemokines presented by glucosaminoglycans on the endothelium. This interaction leads to activation of integrins on the leukocytes, enabling engagement with counter receptors on the endothelium (227). The result of this is firm arrest of the leukocyte to the vessel wall. Next follow the additional steps of adhesion strengthening, spreading and crawling before the cells transmigrate through a paracellular or transcellular route (227). In reality these processes are intertwined and overlapping.

All secondary lymphoid organs except the spleen have high endothelial venules (HEVs) where leukocytes enter the lymph node from the blood. HEVs differs morphologically and functionally from normal venules. The characteristic appearance consists of tall and bulky endothelial cells covering a thick basal lamina and a prominent perivascular sheet (228). The composition of homing molecules differs between HEVs and normal venules, and it also varies between different HEVs.

In peripheral lymph nodes including the MLN, rolling is mediated by CD62L on the lymphocyte interacting with peripheral node addressins on the endothelium (229). In contrast, HEVs in PP do not express peripheral node addressins, and rolling mainly occurs by interaction of α4β7 and CD62L on the lymphocyte with MAdCAM-1 and MAdCAM-bearing specific carbohydrates, respectively, on the endothelial cells (230). MAdCAM-1 is expressed on the HEVs of intestinal tissue, including PP, MLN and the venules in the lamina propria, but not on HEVs of other peripheral lymph nodes (231). Similar to other HEVs, chemokine receptor activation on PP HEVs during rolling will induce adhesion. This occurs through triggering the integrin LFA1 to bind to ICAM1/ICAM2 on the vessel wall. In PP and MLN, however, MAdCAM-1 also mediates adhesion to the HEVs. Administration of anti MAdCAM-1 antibodies results in an almost complete block of lymphocyte migration to PP and a partial block to MLN (231). In addition, the absence of CD62L stops migration to peripheral lymph nodes while it delays, but does not prevent, lymphocyte homing to PP (232). This is because the MAdCAM-1-$\alpha_4\beta_7$ interaction can mediate selectin-independent rolling and thus be independent of CD62L.

HEVs present chemokines produced by endothelial cells, nearby cells or produced inside the lymph node and transported to the luminal surface of the HEVs (233). In addition, chemokines produced in tissues and transported to the draining

lymph node are presented on HEVs. For example, CCL2 is produced in inflamed skin and transported to the nearest lymph node where it induces adhesion of inflammatory monocytes on the HEVs (234). In another study, CXCL9 is presented on subsets of HEVs and recruits a small population of $CXCR3^{+}$ monocytes to the lymph node in a TNFα-dependent fashion (235). However, CXCL9 mRNA is present in the lymph nodes, indicating that it is locally produced. Hence, the combination of selectins, integrins and chemokines determines the selective migration for distinct cell populations to each tissue and lymph node.

Few $Ly6C^{hi}$ monocytes and neutrophils migrate to tissues during homeostasis but they are very quickly recruited during inflammation. Monocytes do not adhere to HEVs under non-inflammatory conditions, but LPS treatment induces monocyte rolling and adherence to PP HEVs (236). Rolling is preferentially dependent on P-selectin interacting with PSGL-1 on the monocyte, whereas adhesion is mediated mostly by VCAM-1, and somewhat by ICAM-1, expressed on the endothelial cells (236). This is consistent with the observed increased in P-selectin expression after trauma in PP (237). In mice infected with *Leishmania*, monocyte recruitment to the dermis was reduced by more than 75% when CD62L or PSGL-1 were blocked (238). In contrast, monocyte migration to the lymph nodes via HEVs was reduced by blocking CD62L but only marginally by blocking PSGL-1. In addition, monocyte recruitment to the inflamed peritoneum was dependent on CD62L (239). Thus, CD62L seems to be crucial for recruitment of monocytes while PSGL-1 can be replaced by other adhesion molecules during some conditions.

Neutrophils also express CD62L and PSGL-1 as well as the integrins LFA1, MAC1 and α4β1 (240, 241). Neutrophil rolling is mediated mainly by PSGL-1 and CD62L or integrin binding to the inflamed endothelium after infection, inflammation or trauma (232, 239, 240). In a peritonitis model, neutrophils used LFA1 and $\alpha_4\beta_1$ together with selectins during the rolling phase and MAC1 during transmigration (241).

Chemokines

Migration of cells to and within tissues is controlled by chemokines. The first chemokine was discovered 1988 and it was identified as a neutrophil-activating factor named IL-8 (242). Today, over 40 chemokines and 19 chemokine receptors have been characterized (243). Chemokines are small basic proteins that are named after the

position and number of cysteine residues, and are called C, CC, CXC or CX_3C chemokines. All chemokines except CX_3CL1 (fractalkine) and CXCL16 are secreted and bind to seven-transmembrane G protein-coupled receptors. There is a lot of redundancy within the chemokine family, as several chemokines bind the same receptor and the same chemokine is able to interact with more than one receptor (243) as shown in table 1.

Chemokine/chemokine receptor interactions direct several distinct sequential events during leukocyte recruitment including mobilization from the bone marrow, extravasation from the blood into lymph nodes and tissues, and finally, directional migration within the tissue. Chemokines bind readily to sulfated proteoglycans or glucosaminoglycans present on the cell surface or in the extracellular matrix. This serves to concentrate the chemokines locally in the tissue and prevent them from being flushed away by the blood when they are presented on endothelial cells (243, 244). In addition, they bind to distinct types of glucosaminoglycans with different affinities, thus adding an additional level of control (243, 244).

During inflammation or infection, pro-inflammatory cytokines and TLR ligands induce the production of chemokines that attract phagocytes as well as lymphocytes to the inflammatory site (244, 245). In addition, cytokines and TLR ligands direct the migration of the individual cell in the tissue by changing the repertoire of distinct chemokine receptors on the cell surface. For example, CXCR2 is down regulated on human neutrophils in response to CXCL2, LPS or TNF-α (246).

Chemokines and monocyte recruitment

$Ly6C^{hi}$ monocytes, T- and NK cells, but not $Ly6C^{low}$ monocytes, in the blood express CCR2 (106, 122). The most important and best-characterized ligand for CCR2 is CCL2, but CCL7, CCL8, CCL12 and CCL16 also bind the receptor (247). During infection, there is an increased egress of $Ly6C^{hi}$ monocytes from the bone marrow and this is dependent on CCR2. In the absence of CCR2, or the ligands CCL2 and CCL7 but not CCL8 or CCL12, monocytes are retained in the bone marrow during infection and also during non-inflammatory conditions (157, 248, 249). After monocytes are released into the blood, the recruitment to inflamed tissues is directed by CCR2 and its ligands during various inflammatory conditions. For example, defective monocyte recruitment in $CCR2^{-/-}$ mice has been reported during infection

with *M. tuberculosis* or *Listeria* and during inflammatory conditions such as atherosclerosis (115, 249-251).

$CCL2^{-/-}$ mice are more susceptible to *S. typhimurium* and *Listeria* (156, 252). However, one caveat is that these results may reflect the decreased egress of monocytes from the bone marrow and their accumulation in blood rather than the migration from the blood to the inflammatory site, since there are very few $Ly6C^{hi}$ monocytes in the blood of $CCR2^{-/-}$ mice compared to wild type mice (157). This was illustrated during *Listeria* infection when monocyte recruitment to inflamed organs was severely impaired in $CCR2^{-/-}$ mice but when $CCR2^{-/-}$ and $CCR2^{+/+}$ monocytes were injected into the blood they were recruited equally well to infected tissues (156, 157). Nevertheless, when the frequency of monocytes in blood was normalized, monocyte recruitment to peritoneum after thioglycollate injection was still dependent on CCR2 at early but not at later time points (157, 248). CCR2 was also shown to be required for monocyte accumulation in atherosclerotic plaques (249). In addition, the ligands to CCR2, particularly CCL2, are induced in many infected or inflamed organs (156, 252). This argues for a crucial role for CCR2 during monocyte recruitment to tissues in several inflammatory settings.

While the $Ly6C^{hi}$ monocyte subset expresses a high level of CCR2 and a low level of the fractalkine receptor CX_3CR1, the $Ly6C^{low}$ monocyte subset expresses the opposite pattern (106). CX_3CR1 mediates crawling of $Ly6C^{low}$ monocytes along the blood vessels and, in response to intraperitoneal injection of *Listeria*, they immediately transmigrate at the site of infection (119). In contrast, $Ly6C^{hi}$ but not $Ly6C^{low}$ monocytes use CX_3CR1 together with CCR2 and CCR5 to migrate into atherosclerotic lesions, while $Ly6C^{low}$ monocyte migration depends only on CCR5 (249, 253).

CCR5 is expressed by NK cells and by subpopulations of T cells but only very weakly by monocytes in murine blood (122). CCR5 but not CCR1 has been implicated in the migration of monocytes/macrophages to atherosclerotic lesions, as mentioned above (249, 254). In CCR5-deficient mice, the recruitment of monocytes/macrophages to the brain is impaired during West Nile virus infection, while normal recruitment of monocytes and clearance of bacteria are detected during *Listeria* infection (255, 256). CCL5 and CCL3 are ligands to both CCR5 and CCR1. CCL3 plays a critical role in monocyte/macrophage recruitment into wounds, but whether CCR5 and/or CCR1 mediated the recruitment was not investigated (257).

Furthermore, CCR1 and CCR5 expression increases when human monocytes differentiate into macrophages in vitro while CCR2 expressoin decreases (258). However, the role of these receptors for monocyte migration is not clear. It is likely that the chemokine receptors and their ligands have overlapping functions and are differently expressed in response to various inflammatory and infectious conditions.
Murine $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells but not human $Ly6C^{hi}$ monocytes express a low to intermediate level of CCR6 in the blood (106, 259). Monocyte/DC precursors probably do not rely on CCR6 for recruitment to inflamed organs since normal accumulation is detected in $CCR6^{-/-}$ mice during inflammation (171, 186, 260, 261). However, migration of monocyte-derived cells or DCs within the organs of $CCR6^{-/-}$ mice is severely impaired in inflamed buccosal mucosa and lung, respectively (186, 260). Moreover, both CCR2 and CCR6 are required for monocytes to replace lost Langerhans cell in inflamed skin (176, 262). Thus, CCR6 may have a more crucial role for migration within the organs than for the actual recruitment.

Chemokines and neutrophil recruitment

Human neutrophils express CXCR1 and CXCR2 and their most well known ligand is IL-8. The corresponding receptor-ligand interaction in mice is mainly represented by CXCR2 that binds with high affinity to CXCL2 and CXCL1 (263, 264). CXCR1, which so far is only known to bind CXCL6, is also expressed by murine neutrophils but has only a minor role in recruitment (263, 264).

Similar to CCR2 for monocytes, activation of CXCR2 in combination with G-CSFR is essential for rapid neutrophil mobilization from the bone marrow in response to inflammation (265). Neutrophil recruitment to infected tissues is totally or partly reliant on CXCR2 in numerous inflammatory conditions including peritonitis, arthritis, parasite infections and ulcerative colitis (265-270). Other chemokine receptors can also control neutrophil migration. For instance, neutrophils express CCR1 and migrate towards the corresponding ligand CCL3 after stimulation with neuropeptide substance P and after repeated challenges with OVA in CFA (271, 272). Neutrophil recruitment is also partly impaired in $CCR1^{-/-}$ mice at day one during *Toxoplasma* infection or in mice where the CCR1 receptor is blocked during chemical-induced inflammation (270, 273). Thus, CXCR1 and CXCR2 are the major

regulators of neutrophil recruitment, although additional chemokine receptors not mentioned here can direct the migration during certain conditions.

AIMS OF THE THESIS

The overall aims of the thesis were to examine the recruitment and function of monocytes and neutrophils during *Salmonella* infection, and the differentiation of monocytes into dendritic cells or macrophages.

The specific aims were:

I. To asses the accumulation and the effector functions of monocytes and neutrophils in the PP, MLN, and blood during oral *Salmonella* infection.

II. To identify which chemokines are important for the migration of monocytes and neutrophils to or within PP and MLN during oral *Salmonella* infection and to investigate the role of Toll like receptors for the cell recruitment.

III. To examine whether monocytes differentiate to DC during *Salmonella* infection and which underlying factors can inhibit this process.

RESULTS AND COMMENTS

Identification of monocytes and neutrophils (I-III)

One aim of my studies was to examine the extent of myeloid cell accumulation in PP and MLN and to further characterize these cells during *Salmonella* infection. The first step was to find suitable markers to identify monocytes and neutrophils to easily distinguish them from each other and from other cells. Many cells are activated during infection and start to express molecules that they normally do not express during steady state. In addition, immature myeloid cells with a broader expression of myeloid molecules are released to the blood. Thus, the similarity among different mature myeloid cells and heterogeneity of immature cells released in response to infection make finding markers that specifically recognize only neutrophils or monocytes impossible.

I initially used the intracellular marker CD68 and a gating strategy that included several other markers, such as Gr-1 and CD11c, to identify the cells. In the beginning of the studies, monocytes were identified as $CD68^{hi}Gr\text{-}1^{hi}$ cells, neutrophils as $CD68^{low}Gr\text{-}1^{hi}$ cells and cDC as $CD11c^{hi}$ cells. Although the populations were clearly identified with this strategy, a drawback was that staining for intracellular CD68 required fixation and permeabilization of the cells. This treatment, of course, killed the cells and negated the exclusion of dead cells using 7AAD. In addition, many cells were lost during the extensive staining and washing procedure. As discussed above, the Gr-1 antibody recognizes both Ly6G that is present on neutrophils but not monocytes, and Ly6C that is expressed by many cell types including inflammatory monocytes and neutrophils. It was thus a great improvement when an antibody specific for Ly6G became available, which greatly facilitated the identification of neutrophils versus monocytes. In addition, intracellular staining could be avoided and live monocytes could be sorted and used for further experiments. Thus, using this new strategy, monocytes were identified as $CD11b^{hi}Ly6C^{hi}Ly6G^{low}CD11c^{low\text{-}int}$ cells and neutrophils as $CD11b^{hi}Ly6C^{int}Ly6G^{hi}CD11c^{low}$ cells. Both cell types were gated negative for B, T, and NK cell markers. The specificity of the new gating strategy was confirmed by back-gating CD68 versus Gr-1. Moreover, the morphology of the cell types was

confirmed by performing cytospin on monocytes and neutrophils sorted using the new staining strategy. This revealed that monocytes and neutrophils had the typical morphology for these cells with a bean shaped and a segmented nucleus, respectively.

Accumulation and characterization of monocytes and neutrophils (I)

The first goal was to elucidate if these cells were recruited to the GALT during *Salmonella* infection and characterize their expression of various surface molecules to identify them phenotypically. I found that $CD68^{hi}Gr\text{-}1^{hi}$ monocytes and $CD68^{low}Gr\text{-}1^{hi}$ neutrophils increased in blood, MLN and PP as the infection progressed. A substantial increase was detected already day three post infection, with a continuous increase to day five. Similar numbers of monocytes and neutrophils were detected in the blood while monocytes outnumbered neutrophils in PP and MLN. Whether this depended on a higher influx of monocytes or a higher death rate of neutrophils recruited to the tissues is not clear.

Previous studies have identified two, and in some studies three, subsets of monocytes in mouse blood. These were identified as a more immature $Ly6C^{hi}CCR2^{hi}CX_3CR1^{low}$ subset, a more mature $Ly6C^{low}CCR2^{low}CX_3CR1^{hi}$ subset and a third minor subset with an intermediate level of Ly6C but otherwise similar to the more immature subset (106, 109). Consistently, we found that "our" $Gr\text{-}1^{int}$ monocytes expressed high levels of CCR2 and Ly6C and corresponded to the $Ly6C^{hi}CCR2^{hi}$ subset in the blood. In addition, the analysis of gated $CD68^{hi}Gr\text{-}1^{low}$ cells in the blood showed that they corresponded to the $Ly6C^{low}CCR2^{low}$ blood subset. These cells did not increase in blood during infection but remained at approximately the same frequency. Since there was no increase of $CD68^{hi}Gr\text{-}1^{low}$ cells in the tissue, and that many of these cells were found to be $CD11c^{hi}$ DCs, I did not continue to investigate the fate of these cells.

$Gr\text{-}1^{hi}$ monocytes in the blood expressed no or very low levels of CD11c while MHC-II, and to a minor degree CD80 and CD62L, were upregulated during infection. The $Gr\text{-}1^{hi}$ monocytes had a similar phenotype in PP and MLN except that the expression of CD80 in particular, but also CD86 and CD11c, increased while CD62L decreased. However, compared to DCs, the expression of CD11c, CD86 and MHC-II were still much lower on monocytes. Interestingly, CD80 expression reached a similar level on monocytes as seen on dendritic cells. As expected, neutrophils did not

express costimulatory molecules and downregulated the expression of CD62L and CXCR2 after entry into the GALT. This agrees with other studies showing that CD62L is shed from the surface after transmigration and CXCR2 is downregulated upon neutrophil activation (240, 246, 274).

Table 2. Phenotype of monocyte subsets, neutrophils and DC during *Salmonella* infection

Molecules	**Infected Blood**			**Infected MLN/PP**		
Surface/ intracellular	**Ly6C^{hi} monocytes**	**Ly6C^{low} monocytes**	**Ly6G^{hi} neutrophils**	**Ly6C^{hi} monocytes**	**Ly6G^{hi} neutrophils**	**CD11c^{hi} DC**
F4/80	+	+	-	+	-	-
CD11b	++	++	++	+ +	++	+/-
MHC-II	++	++	-	+ +	+/-	++
CD80	+/-	+/-	-	++	-	++
CD86	+/-	+/-	-	+	-	++
CD11c	-	-/+	-	+/-	-	++
CD62L	++	-	++	+	-	-
Ly6C	++	-	+	++	+	+/-
iNOS	-	-	-	++	+/-	+/-
TNFα	+	-	-	++	+/-	+/-
CCR2	++	+/-	-	++	-	-
CXCR2	-	-	++	-	+/-	-
CCR6	-	-	-	-	-	+

Monocytes produce proinflammatory cytokines and iNOS (I)

Having established that monocytes and neutrophils are recruited to PP and MLN during infection, I next set out to investigate their function during infection with *Salmonella*. First, I measured the production of TNFα and iNOS at day four after oral *Salmonella* infection directly ex vivo by intracellular staining and flow cytometry analysis. I found that monocytes were the main producers of TNFα and iNOS in spleen, MLN and PP. Approximately 20-30% of inflammatory monocytes expressed TNFα or iNOS while only about 5% expressed both molecules. In contrast, very few neutrophils or DCs stained positive for the molecules. Similar to our data, TNFα and iNOS producing monocyte-derived cells are also recruited to the spleen during *Listeria* infection (115, 154). Interestingly, despite that monocytes in the blood expressed MHC-II during *Salmonella* infection, very few expressed TNFα or iNOS until they reached the tissue. This in combination with the low expression of CD80 and CD11c of monocytes in the blood suggest that monocytes are further activated after arrival to the tissue. It may be that the cells require additional signals that are either not present or at high enough concentration in the blood, such as TLR

ligands/bacterial contact, cell/cell interactions or cytokines, to develop their effector functions.

These studies showed that monocytes in particular contained the intracellular effector molecules TNFα and iNOS while neutrophils harbor other toxic molecules in their granules. But I also wanted to establish that TNFα and other proinflammatory cytokines were secreted into the environment. I thus sorted cells from infected mice, put them in culture and measured cytokines in the supernatant by ELISA. After ex vivo culture of the sorted cells for 20-36 hours, monocytes produced TNFα, IL-1β and IL-6 while neutrophils produced particularly IL-1β but also some TNFα. In contrast, neither IL-12p40 was detected by direct ex vivo intracellular staining of monocytes at any time point examined (three and five days post infection) nor was IL-12p70 found in the supernatant after ex vivo culture. This was somewhat surprising since Gr-1^{hi}CD11b^{hi}CD11c^{int} monocytes produce IL-12p40 during *Listeria* infection (154). However, detecting IL-12 during *Salmonella* infection, particularly cellular sources of this cytokine in vivo, has been difficult. Indeed, IL-12p40 production by splenic DCs from *Salmonella*-infected mice ex vivo was not found in several studies despite that IL-12p40 production can be detected after in vitro stimulation with *Salmonella* (71, 142, 154). This is different than the situation during *Listeria* infection, where abundant IL-12p40^{+} DCs are detected directly ex vivo (154). The cellular source(s) of IL-12 during infection remain elusive despite that IL-12 has a role in host defense against *Salmonella* (78, 86, 87, 101).

Given the important role of IL-12 and IFNγ during *Salmonella* infection, I asked if TNFα, iNOS and MHC-II expression by monocytes was dependent on these cytokines. If so, this could partly explain the increased susceptibility of IFNγ$^{-/-}$ and IL-12p40$^{-/-}$ mice early during *Salmonella* infection. Indeed, iNOS and MHC-II expression by monocytes was dependent on IL-12 and IFNγ. In contrast, DCs expressed high levels of MHC-II even in the absence of IFNγ.

To summarize, monocytes produce the inflammatory cytokines TNFα, IL-1β and IL-6 and the antimicrobial molecule iNOS during *Salmonella* infection. In addition, IFNγ and IL-12 are required for iNOS and MHC-II expression by monocytes.

Bacterial uptake and killing capacity (I)

The next aim was to investigate if monocytes and neutrophils recruited to PP and MLN phagocytosed *Salmonella* after oral infection, the natural infection route, since one of the main tasks for these cells is to kill invading pathogens. To this end, mice were orally infected with eGFP-expressing *Salmonella* and the uptake of bacteria was detected four days post infection using flow cytometry. Between one to two percent of both monocytes and neutrophils were associated with bacteria and together they harbored >80% of all detected bacteria. The killing capacity of monocytes and neutrophils after bacterial uptake was also investigated using an in vitro approach. After a 2 h pulse of *Salmonella,* sorted monocytes or neutrophils phagocytosed bacteria although neutrophils were more efficient than monocytes. After 20 h both cell types had killed most bacteria, indicating that monocytes and neutrophils are efficient at killing *Salmonella*.

We next investigated the relationship between bacterial uptake and production of iNOS and TNFα. Most of the iNOS and/or TNFα-producing monocytes were not associated with bacteria. This was expected since 20-30% of the cells produce these effector molecules while only 1-2% are associated with bacteria. While many non-infected cells make effector molecules, 30-40% of the bacteria-containing cells expressed iNOS. This suggests that bacterial association may induce iNOS since the fraction of $iNOS^+$ cells is greater among bacteria-associated cells. However, most iNOS and TNFα are produced by bystander cells, which is consistent with observations in other infection models (115, 118).

Antigen presentation capacity of monocytes (I)

It has been suggested that both the $CCR2^{low}Ly6C^{low}$ and $CCR2^{hi}Ly6C^{hi}$ subsets of monocytes can differentiate to antigen presenting DCs during infection or inflammation (106, 115, 119, 121, 158, 183, 184). In addition, as previously discussed, we showed that $Ly6C^{hi}$ monocytes phagocytosed *Salmonella* and expressed MHC-II and costimulatory molecules.

We therefore asked if $Ly6C^{hi}$ monocytes in blood, spleen or MLN could process and present a model antigen encoded by *Salmonella* to primary antigen-specific T cells. Sorted DCs as well as $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes from blood were pulsed with *Salmonella* expressing OVA for 2 h. OVA-specific OT-II CD4 T cells were then added and their division was determined after 3.5 days. We found that

DCs, as expected, but not Ly6C^{hi} or Ly6C^{low} monocytes induced proliferation of the OT-II T cells. Ly6C^{hi} monocytes, sorted from MLN, behaved as their counterparts from blood. This suggested that monocytes were not capable of processing *Salmonella* for antigen presentation on MHC-II. As processing of bacteria for peptide presentation is a rather stringent system, I thus tested the antigen presentation capacity of monocytes using OVA peptide instead of *Salmonella* expressing OVA. In this assay, DCs and Ly6C^{low} monocytes induced extensive proliferation of naïve T cells while Ly6C^{hi} monocytes only induced a modest proliferation. Thus, when loaded with exogenous peptide, Ly6C^{low} "resident" monocytes can activate antigen-specific CD4 T cells while Ly6C^{hi} "inflammatory" monocytes do so only poorly. In contrast, neither monocyte subset can present an antigen expressed in *Salmonella*. As these antigen presentation assays represent the least stringent (exogenous peptide) and most stringent (bacterial processing) conditions, it would be interesting to test what happens if OVA protein is used.

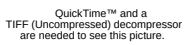

Figure 5. DC but not Ly6C^{hi} monocytes or Ly6C^{low} monocytes process and present a *Salmonella* antigen on MHC class II. Cells from the blood and spleen of mice infected 4 days earlier with χ8554 were pooled, depleted of B, T and NK cells and stained with anti-CD11b, CD11c, Ly6C, Ly6G and a mixture of CD19, NK1.1 and TCRβ. *A*, Cells were sorted into Ly6C^{hi} monocytes, Ly6C^{low} monocytes and CD11c^{hi} DC as shown. *B*, 1.5x10^5 cells/well were pulsed for 2 h with χ4550 expressing or not expressing OVA at a 5 to 1 bacteria to cell ratio. Alternatively, the cells were pulsed with 1 μg/ml OVA$_{(323-339)}$ peptide for 2 h. The cells were washed, resuspended in medium containing gentamicin and 2x10^5 MACS purified, CFSE labelled OT-II CD4$^+$ T cells were added to the wells. After 3.5 days, division of the CFSE labelled T cells was assessed by flow cytometry.

These data suggest that Ly6C^{hi} monocytes are relatively poor antigen presenting cells and perhaps resemble more a macrophage rather than a DC phenotype. Alternatively, they could need a certain time in the tissue to mature before they can convert into antigen presenting cells. Finally, it should be considered that down regulation of Ly6C and up regulation of CD11c is a sign of monocyte differentiation and maturation (114, 120, 156). While Ly6C^{hi} monocytes develop into Ly6C^{low} monocytes during homeostasis, it is not known if this also occur during infection or if environmental factors influence this step in monocyte development (114, 119, 120, 175). Thus, even though Ly6C^{low} monocytes in blood could not induce proliferation of T cells after a pulse with *Salmonella*, this does not exclude that Ly6C^{hi} cells develop to DCs in the tissue or that further maturation is required to give the Ly6C^{low} monocytes antigen processing and presentation capacity.

Do Monocytes differentiate into DCs during *Salmonella* infection? (III)

The previous results made us speculate that Ly6C^{hi} monocytes were too immature to process and present antigens, but with time some of these cells may become DCs. To follow the fate of the Ly6C^{hi} monocytes in vivo during oral *Salmonella* infection, I first tried two different methods to track the Ly6C^{hi} monocytes: adoptive transfer and a fluorescent bead approach. The adoptive transfer approach involved transferring CFSE-labeled monocytes into orally-infected recipent mice. However, two days after transfer too few monocytes were detected in MLN or PP for reliable flow cytometry analysis. I thus tried an approach published by Randolph et. al. (120) who described a method to specifically label Ly6C^{hi} monocytes by first injecting clodronate-loaded liposomes to remove all monocytes from the blood followed by injection of fluorescent beads. This led to an almost exclusive labeling of Ly6C^{hi} monocytes, which phagocytosed the beads. However, when this approach was used in *Salmonella*-infected mice, several cell populations including neutrophils, DCs and B cells also harbored fluorescent beads. Thus, neither of these methods worked out and we thus changed strategy to combine in vivo with in vitro methods to examine the differentiation fate of Ly6C^{hi} monocytes during *Salmonella* infection.

***Salmonella* inhibits monocyte differentiation into DCs (III)**

Given the difficulty with the experiments above, I next aimed to sort Ly6C^{hi} monocytes from mice, co-culture them with *Salmonella* ex vivo and analyze the phenotype and function of the potentially "differentiated" monocytes. To set up this system, I first needed to establish that Ly6C^{hi} monocytes sorted from infected mice had differentiation capacity. That is, can they differentiate into CD11c^{hi}MHC-II^{hi} DCs after culture in the presence of flt3L or GM-CSF as described in the literature for naïve murine bone marrow cultures (275, 276)? I found that Ly6C^{hi} monocytes sorted from the blood and spleen, but not the MLN, of *Salmonella*-infected mice differentiated into CD11c^{hi}MHC-II^{hi} DCs with either of the growth factors. In addition, the monocyte-derived DCs presented OVA-peptide and induced proliferation of OT-II CD4 T cells, although a bit less efficient than freshly isolated DCs. GM-CSF (+IL-4) or flt3L are commonly used to derive DCs from mouse bone marrow or from human blood cultures. Differences between DCs developed by the two systems have been reported in mice (277-279), but my results showed that GM-CSF or flt3L-monocyte-derived DCs were similar regarding their size and granularity, as well as their CD80, M-CSF, CD11c and Gr-1 expression.

It was intriguing to find that blood Ly6C^{hi} monocytes, but not Ly6C^{hi} monocytes from the MLN, survived in culture and differentiated to DCs. We hypothesized that the microenvironment of the MLN could have an inhibitory effect on monocyte differentiation by, for example, encountering bacteria or bacterial components. Indeed, Ly6C^{hi} monocytes from the blood as well as the bone marrow were totally or partly inhibited to convert into DCs after coincubation with heat-killed *Salmonella*. While no one has analyzed the direct effect on the differentiation of purified murine Ly6C^{hi} monocytes with bacteria before, this result is consistent with previous studies suggesting that *Salmonella* inoculated in the skin, or in vitro cultures of human or murine cells with LPS, skew monocyte differentiation towards macrophages instead of DCs (189, 195, 280).

We were next interested in examining if *Salmonella* inhibited the differentiation to DCs via TLR signaling. In contrast to monocytes from wild type mice, monocytes purified from MyD88$^{-/-}$ or MyD88$^{-/-}$TLR4$^{-/-}$ mice were not inhibited to differentiate into DCs in the presence of bacteria. However, when monocytes from wild type mice or either of the knock out mice were incubated together in the

presence of bacteria, monocytes from the knock out mice were also partly inhibited to convert into DCs. This result suggests that the block was induced by soluble factors such as cytokines released from the monocytes upon TLR activation. Indeed, monocytes produce several pro inflammatory cytokines such as IL-6, which could skew monocyte differentiation towards macrophages rather than DCs, as reported for human monocytes (193, 194). The cultures may also contain other cytokines, such as IL-10 and IL-12p40, which could have an effect on monocyte differentiation into DCs (189, 192). Although I did not measure IFNγ or IL12p40 in the culture supernatants, it is unlikely that IFNγ, which is made mainly by T cells, is present in large amounts while IL-12p40 could be present. However, monocytes make TNFα (Paper I), which can skew monocyte differentiation towards DCs, at least in the human system (196). Thus, the factor(s) inhibiting monocyte differentiation into DCs in the presence of *Salmonella* remain to be determined.

Recruitment of monocytes and neutrophils (I, II)

Having established in paper I that monocytes and neutrophils accumulate in PP and MLN, I set out to investigate the mechanisms behind the recruitment of these cells. Monocytes in blood express CD62L (Paper I), and others have shown that they express the α4 integrin (241, 281, 282), indicating that they could also express $\alpha_4\beta_7$. CD62L and $\alpha_4\beta_7$ are ligands for MAdCAM-1, which is expressed on high endothelial venules of PP and directs T cell homing to this organ (229, 283).

Although T cell recruitment to PP has been well studied, the mechanism of monocyte recruitment to PP, particularly during oral bacterial infection, was not known. I thus examined if, similar to T cell recruitment to PP, monocyte recruitment to this organ was abrogated when mAbs that block MAdCAM-1 were administered to mice orally infected with *Salmonella*. I found that monocyte recruitment was not impaired, despite that T cell homing was blocked. This suggests that monocytes can use other molecules to enter PP.

We next investigated the chemokine/chemokine receptor interactions that could mediate monocyte as well as neutrophil recruitment to PP. While neutrophils in the blood express CXCR2, it was down regulated on neutrophils in PP and MLN. In contrast, $Ly6C^{hi}$ monocytes in all three tissues expressed CCR2, a receptor

characterized to have a role in monocyte recruitment (106, 115, 122, 157, 248-251). I also found that CXCR3 was expressed by few monocytes and neutrophils while DCs in the MLN and PP expressed CCR6. Expression of other cytokine receptors tested, including CCR5 and CCR1 (preliminary data), was not detected on monocytes or neutrophils in the blood.

As detecting chemokine receptor expression on the cell surface is, at least for some receptors, hampered by the lack of good mAbs, not detecting the receptor does not necessarily mean it is not involved in recruitment. I thus took a complimentary approach and analyzed chemokine expression on total cells from PP and MLN of mice infected two or four days earlier with *Salmonella*. Chemokines that bind to CCR2, CXCR2 and CXCR3 were upregulated already day two post infection in PP and MLN while the expression of two chemokines binding to CCR1 and CCR5 were greatly increased first at day four. Based on these results, chemokines responding to CCR2 and CXCR2 were measured at the protein level with ELISA at day two post infection. Elevated amounts of the ligands for CCR2 and CXCR2, but particularly the ligand for CCR2, were found in PP, MLN and blood. Hence, these data suggest that CCR2 and CXCR2, and their corresponding chemokines, direct the recruitment of monocytes and neutrophils, respectively, to the GALT during *Salmonella* infection. This is supported by other studies showing that CCR2 and CXCR2 are extremely important for the monocyte and neutrophil egress from the bone marrow and for the accumulation of cells in inflamed tissues other than the GALT (157, 248, 265, 270). Interestingly, the delayed kinetics of gene expression for two of the chemokines (CCL3 and CCL4) in PP and MLN indicate that these may be expressed by newly recruited cells, such as monocytes and neutrophils, and further amplify cell recruitment.

Table 3. Analyzed chemokines and their corresponding receptors

Chemokine	Responding receptors
CCL2	CCR2
CCL3	CCR1, CCR5
CCL4	CCR5
CCL6	CCR1[a]
CCL9	CCR1
CCL20	CCR6
CXCL2	CXCR2
CXCL9	CXCR3

CX_3CL1	CX_3CR1

Modified from (284) (285) [a]

Migration within PP (II)

The next aim was to elucidate where recruited monocytes and neutrophils localize within the organs and what chemokines direct this movement. For these studies I focused on PP since it is the place where the host first encounters *Salmonella.* To achieve this I used three distinct approaches: 1) determining chemokine gene expression in different areas of PP; 2) localizing monocytes and neutrophils in the tissue by immunohistochemistry and fluorescence microscopy; and 3) measuring chemokine expression by monocytes and neutrophils purified from the PP of infected mice.

I first removed PP at day two after *Salmonella* infection and used laser capture microdissection technology to specifically cut out the different areas of PP. The goal was to examine chemokine gene expression by epithelial cells overlaying PP (FAE) and specific cell areas, particularly the SED and the B cell follicle, dissected from the PP of infected mice. The highest expression of the ligand to CXCR2, the receptor involved in neutrophil recruitment, was found in the FAE while the ligand to CCR2, which is involved in monocyte recruitment, was preferentially expressed in the B cell follicle. This differential expression suggested that neutrophils and monocytes might migrate to different areas of PP.

However, by using immunohistochemistry, very few recruited cells were detected at day two post infection while at day four, a large infiltrate of monocytes ($iNOS^+Ly6G^-$) and neutrophils ($iNOS^-Ly6G^+$) colocalized in inflammatory foci spread throughout PP were found. Thus, although the laser microdissection data supports differential localization of myeloid cells recruited in PP at day two post infection, the immunohistochemistry data particularly from day 4, did not. However, the first monocytes and neutrophils entering Peyer's patches might have distinct migration pattern that, with the progression of infection and concomitant altered chemokine environment, may change their distribution.

Since there is no single marker available that specifically recognizes monocytes, and antibodies that can recognize both neutrophils and monocytes were used in the immunohistochemistry experiments, the data could be misleading. We decided a better strategy would be to use an antibody against iNOS, which is produced by up to 35% of the monocytes but very few other cells in PP as shown in

paper I. The main advantage to this approach is that it specifically identifies monocytes, although the disadvantage is that we would not identify all monocytes. Hence, we cannot exclude that $iNOS^+$ monocytes migrate differently than $iNOS^-$ monocytes. This, however, seems unlikely since the cells otherwise have an identical phenotype regarding CCR2, CCR6, CD11c and Ly6C expression.

Resident cells produce chemokines early during infection, but it seems likely that recruited cells will add to the production of chemokines. This led us to investigate which chemokines were produced by recruited monocytes and neutrophils. The ligands to CCR2 and CXCR2, CCL2 and CXCL2, respectively, were produced by blood monocytes and neutrophils from infected mice. The highest production of the CXCR2 ligand was detected after restimulation with heat-killed *Salmonella*. Gene expression of the ligands to CCR1 and CCR5, was also upregulated by monocytes, and particularly by neutrophils, from the MLN of infected mice compared to their counterparts from the blood of naïve mice.

Together these results suggest that monocytes and neutrophils are first recruited to the GALT by chemokines released by resident cells. After organ entry, however, monocytes and neutrophils themselves produce chemokines to recruit more cells to the organ and organize themselves in the tissue to, for example, form granulomas.

TLRs and recruitment (II)

To gain further insight into the mechanism of chemokine production and accumulation of myeloid cells during *Salmonella* infection, we examined the role of TLRs in cell recruitment. Epithelial cells, stromal cells and several populations of leukocytes including monocytes and neutrophils express TLRs that are extremely important for activating the immune response during infection (199). This part of my thesis was aimed at understanding the role of specific TLRs and TLR signaling pathways in recruiting monocytes and neutrophils to PP and MLN in the early stage of oral *Salmonella* infection. To address this, cell recruitment was assessed in *Salmonella*-infected mice lacking one or more TLR pathway.

TLR4 recognizes LPS, and $TLR4^{-/-}$ mice are more susceptible to *Salmonella* infection (211, 215, 286, 287). In spite of this, the accumulation of monocytes and neutrophils in $TLR4^{-/-}$ mice were largely comparable to wild type mice at day two and

day four post infection. One exception was that neutrophil accumulation in the MLN of TLR4$^{-/-}$ mice was reduced compared to wild type mice. In addition, similar results were obtained when TLR4$^{-/-}$ mice were infected with non-flagellated *Salmonella*, which cannot be recognized by TLR5. Hence, myeloid cells are recruited independently of TLR4 and TLR5.

We continued to examine cell recruitment by using MyD88$^{-/-}$ mice since MyD88 is used by all TLR signaling pathways relevant for *Salmonella* although TLR4 also signals via a MyD88-independent pathway (199). The accumulation of myeloid cells was impaired in PP and MLN, but not in the blood, of infected MyD88$^{-/-}$ mice compared to wild type mice. One exception was the recruitment of neutrophils to PP, which was similar to wild type at day four post infection (199). Moreover, to eliminate all TLR signaling during *Salmonella* infection, we made MyD88$^{-/-}$TLR4$^{-/-}$ mice to remove the MyD88-independent pathway for TLR4. In infected MyD88$^{-/-}$ TLR4$^{-/-}$ mice, the accumulation of myeloid cells in PP, MLN as well as the blood was severely impaired. A difficulty with infection studies in immunocompromised mice such as the MyD88$^{-/-}$ and MyD88$^{-/-}$TLR4$^{-/-}$ strains is that they are more susceptible to *Salmonella* infection. To try to get mice with a similar bacterial burden for analysis, I therefore infected these two knockout strains with a two log lower dose of bacteria than wild type mice. Even though some of the knockout mice had a higher bacterial burden than wild type mice, lower numbers of myeloid cells were recovered from infected tissues.

To address the mechanism of MyD88-dependent cell recruitment during *Salmonella* infection, chemokine expression at day two and four post infection of MyD88$^{-/-}$TLR4$^{-/-}$ mice was assessed. As predicted, little upregulation of all chemokines tested, which includes the ligands for CCR1, CCR2, CCR5, CXCR2 and CXCR3, was observed. In summary, these data show that in the absence of signaling through TLR4 and TLR5 other TLRs, such as TLR9 and TLR2, mediate the recruitment. Moreover, TLR signaling via MyD88 is required for recruitment of myeloid cells to the GALT during oral *Salmonella* infection.

A schematic summary of the key findings in this thesis are shown in (Fig. 6).

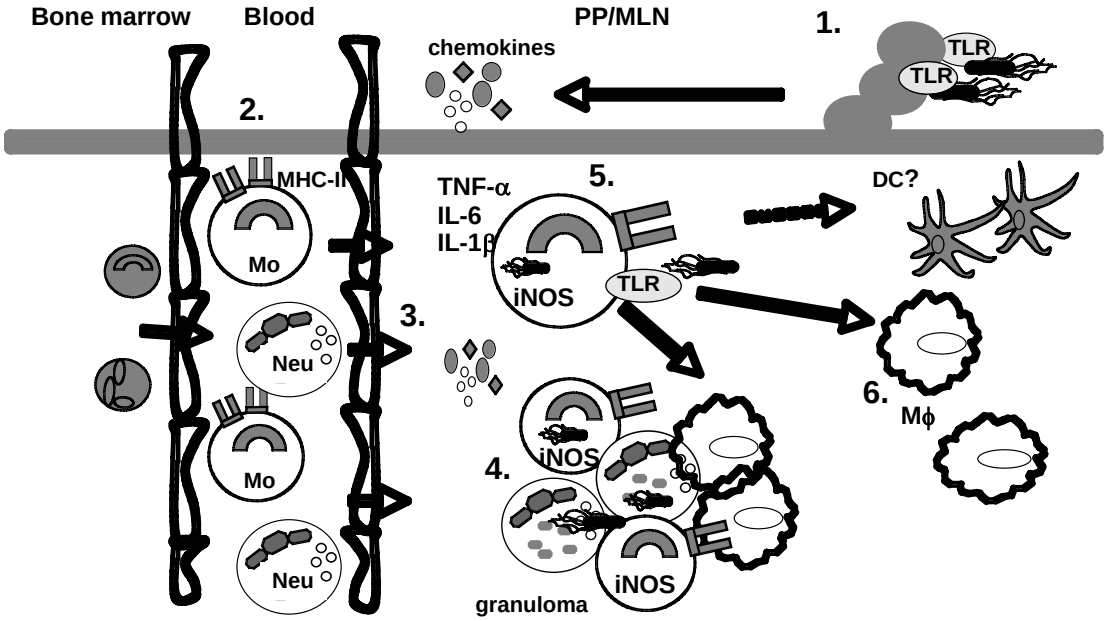

Figure 6. A scematic summary of the overall results in this thesis. After *Salmonella* encounter, first resident cells in PP and MLN and then recruited cells are activated via TLRs and produce chemokines (1). Release of chemokines leads to an release of neutrophils and monocytes in the blood from bone marrow (2) and migration into the infected PP and MLN (3). Monocytes and neutrophils phagocytose *Salmonella*, produce chemokines and establish inflammatory foci (4) Monocytes produce pro-inflammatory cytokines including TNFα, IL-1β and IL-6 and produce iNOS. The monocytes upregulate MHC-II, CD80 and some CD86 and CD11c (5). However, *Salmonella* skews monocyte differentiation towards macrophages during infection (6).

GENERAL DISCUSSION

In this thesis I have studied the recruitment and effector functions of monocytes and neutrophils in the PP, MLN and blood during infection with *Salmonella*. The natural route for *Salmonella* infection is via the intestine, and before *Salmonella* causes a systemic infection bacteria are detected in PP and the MLN. The architecture and regulation of the immune system differs in distinct parts of the body to cope with the types of challenges incurred in different immune compartments. Likewise, pathogens have also evolved strategies to survive in their special niche in the body. It is therefore very important to use as natural infection route as possible when examining the immune response to a given pathogen. In the case of *Salmonella* it is thus valuable to study the responses that occurs in the GALT as well as at systemic sites since the immune reaction is initiated in the gut and then spreads systemically.

Data demonstrating the importance of protective responses towards *Salmonella* beginning in the gut intestinal tissue showed that $CCR6^+$ DCs initiated the immune response by activating CD4 T cells in PP within hours after infection (16), and protective IgA was produced soon after *Salmonella* entry into PP (18). Moreover, the immune response initiated in the gut can further modulate the ensuing systemic response and the route of infection directs the seeding of organs. For instance, whether bacteria are administered intravenously or must cross the intestinal epithelium influences how *Salmonella* enters systemic organs, such that the kinetics, bacterial numbers and the extracellular versus intracellular niche of bacteria differ depending on the administration route. This, in turn, greatly influences the nature of the immune response. A better understanding of how the immune response is initiated and propagated in the gut is thus necessary in order to develop vaccines and drugs against orally acquired bacteria that are capable of establishing systemic infections.

The fate of monocytes during *Salmonella* infection

Monocytes are a currently a very active research area in the field of innate immunity, and a lot of new interesting findings describing the plasticity, differentiation and function of these cells during various conditions are constantly reported. Their impact during totally different conditions, such as infections, atherosclerosis, wound healing

and multiple sclerosis, and the diverse and sometimes contrasting effector functions makes them a very intriguing and essential cell to study (115, 126, 156, 181, 249).

My characterization of the accumulated Ly6C^{hi} monocytes during *Salmonella* infection pointed out phenotypic similarities with both DCs and macrophages (paper I). The differentiation fate of monocytes during homeostasis and during inflammation is often to macrophages (121, 126, 128, 131, 249). However, several groups have reported that monocytes also can develop into migratory DCs in non-lymphoid tissue during steady state and certain inflammatory conditions (108, 109, 121, 129, 175, 183-186, 189). Given this dichotomy and the different differentiation outcomes observed when various types of inflammation have been studied, we were interested in the fate of monocytes during *Salmonella* infection. In particular, we asked if monocytes have the ability to develop into DCs during infection with *Salmonella* given that lymphoid organs (PP, MLN and spleen) are the target tissues for the bacteria. In other words, do monocytes recruited to *Salmonella*-infected lymphoid tissues differentiate into DCs? Some data obtained suggest this is the case. For example, recruited monocytes upregulate MHC-II, CD80, CD86 and CD11c in the infected organs (paper I). Second, monocytes phagocytosed *Salmonella* in the PP and MLN during infection (paper I). Third, in the presence of flt3L or GM-CSF monocytes isolated from infected blood differentiate into DCs, defined by CD11c^{hi}MHCII^{hi} and the capacity to present peptide to CD4 T cells, in vitro (paper III).

However, as discussed above, these features are not unique to DCs, and could also support that the recruited monocytes differentiated into macrophages. Did monocytes recruited to *Salmonella*-infected lymphoid tissues differentiate into macrophages? There is also data to support this possibility. First, unlike cDCs, monocytes did not process and present *Salmonella* antigens to naïve CD4 T cells (paper I). Second, they expressed lower levels of MHC-II and CD86 than cDCs (paper I). Third, monocytes accumulated into granulomas, which is a typical behavior for macrophages (paper II). Fourth, recruited monocytes isolated from MLN did not convert to DCs since they did not express high levels of CD11c in combination with MHC-II, and bacteria inhibited blood monocytes to develop into DCs in vitro (paper III). Fifth, IFNγ is necessary for the induction of MHC-II on monocytes but not on DCs (paper I). These results suggest that, although Ly6C^{hi} monocytes upregulate

costimulatory molecules and have the capacity to differentiate to DCs in vitro, the microenvironment in lymphoid organs during *Salmonella* infection directs monocytes to convert into a poor antigen presenting cell distinct from DCs. Moreover, since all subpopulations of resident cDC in the spleen origin from a DC-specific precursor and some data indicates that also lymph node resident cDC are derived from the same precursor (121, 147, 158, 159, 172, 174), this further argues against monocyte differentiation to cDC in the lymphoid organs of *Salmonella*-infected mice.

Another question raised is whether monocytes recruited to *Salmonella*-infected tissues become $CD11c^{int}$ cells, particularly TipDCs. This is indeed possible. For example, the phenotype and function (TNFα and iNOS production) of the monocytes gated in my studies resemble that published for TipDCs. However, the expression of CD11c on my gated monocytes is not a tight population expressing an intermediate level of CD11c as reported to emerge in the spleen during *Listeria* infection and during other inflammatory conditions (28, 106, 115, 154, 156-158), but contains cells with little or low CD11c expression as well as a small fraction of cells with higher expression. It has been shown that recruited monocytes or $CD11c^{int}$ cells in other experimental systems can activate T cells in a mixed lymphocyte reaction (156, 158) and induce OT-I CD8 T cell proliferation after transfer into the inflamed peritoneum of a MHC-I-deficient host injected with OVA peptide (106). However, these results show that inflammatory monocytes can present a peptide and do not demonstrate that they can phagocytose, process and present an antigen, particularly one expressed inside bacteria, to naïve T cells. The question is whether these monocyte-derived cells can be defined as DCs before it has been proven that they can process exogenous antigen and subsequently activate naïve T cells? An additional factor to take into account is the production of iNOS by these cells, which leads to the production of nitric oxide that can cause suppression of adjacent lymphocytes (63, 91). Hence, monocyte-derived cells could instead inhibit T cell activation.

Yet another suggestion is that depending on the microenvironment in the lymph node or tissue, only a small number of monocytes encounter precisely the right mix of cytokines and antigen to direct their differentiation into DCs (183). This would agree with our in vitro findings that *Salmonella* signals via TLR and induce factors including IL-6 (paper III), which have been shown to skew monocyte differentiation into macrophages (194, 195). In an infected lymphoid organ, it is possible that

relatively few cells are present in a microenvironment lacking macrophage-skewing factors but containing DC-promoting cytokines. Interestingly, during *Listeria* infection, monocyte-derived $CD11c^{int}$ cells in the spleen could simultaneously produce TNFα and iNOS or TNFα and IL-12p40 but not iNOS and IL-12p40 as determined by double intracellular staining (154). Hypothetically this could mean that monocyte-derived cells producing IL12p40 are less immunosuppressive than iNOS producing monocytes and have a more immunostimulatory effect on T cells. However, further studies are needed to investigate whether the microenvironment directs monocytes to divide into distinct phenotypic and functional subpopulations in infected lymphoid organs.

Distinct populations of DCs present in non-lymphoid tissues in steady state include Langerhans cells and dermal DC in the skin. Interstitial DCs, which are the counterparts of dermal DC localized in all other peripheral tissues, are also included. All of these populations are classically described to migrate to the nearest lymph node via afferent lymph after antigen uptake to activate naïve T cells. Monocytes have been reported to contribute to these migrating DCs in vivo during steady state and inflammation (108, 109, 121, 129, 175, 183-186, 189). One theory to explain these observations, which is based on a transendothelial trafficking model, is that monocytes picking up antigens receive signals while egressing from the tissue into the lymph that turns the migrating cells into antigen presenting cells as they are migrating to the lymph node (183, 187). Thus, one possibility is that during *Salmonella* infection, some monocytes recruited to lamina propria develop into migrating DCs in the efferent lymph on their way to the MLN while monocytes directly recruited to MLN would not get these signals and instead become TNF and iNOS-producing cells. However, only two reports have used infection models to examine the fate of recruited monocytes (184, 189) and only one examine the natural niche for the pathogen (184). In this latter study, monocytes are recruited to the skin during cutaneus infection with *Leishmania* and differentiate into cells with a phenotype resembling dermal DCs. Identical cells are later found in the draining lymph nodes and can activate T cells ex vivo (184). However, a study that used an unnatural niche for the microbe, dermal infection of mice with *Salmonella,* found that the bacteria inhibited the differentiation of recruited monocytes into DCs and their migration to the draining lymph node (189). Similar results were found with dermal administration

of LPS instead of *Salmonella*. One drawback of this study is that *Salmonella* normally infects via the oral route, and what occurs in the skin may not reflect what happens in the gut. In support of this oral administration of a TLR7/8 ligand or i.v. administration of LPS results in substantial migration of DC from the intestinal lamina propria to the draining lymph node (288, 289). However, the observation that Gram negative bacteria or LPS inhibit monocyte differentiation into DCs is consistent with reports demonstrating that LPS and/or cytokines can block monocyte differentiation into DCs (192-195, 280) (paper III). Hence, bacterial induced block of monocyte differentiation to DCs but not of migration of DCs to draining lymph nodes are probably valid for most tissues in the host. To summarize, monocytes can convert to migratory DCs during inflammatory conditions, but additional studies are needed to elucidate if this also occurs during bacterial infection, particularly infections acquired by the natural route. In addition, a mission for future studies is to further evaluate the relationship between DCs in lymphoid organs and interstitial DCs and how these cells are related to monocytes and macrophages.

Finally, is monocyte conversion to DCs or macrophages most beneficial to the host during a bacterial infection? Differentiation into DCs could give a higher number of cells capable of activating T cells and initiating adaptive immunity, which is essential to survive infection with virulent *Salmonella* (75, 78). On the other hand this could lead to a higher risk of developing autoimmunity, since more cells present antigens. It could also result in faster replication and spread of bacteria, as DCs are less efficient at killing bacteria and their migration could be exploited to facilitate the spread of bacteria (290). Both macrophages and DCs produce cytokines that can regulate the immune response, so in this sense both cell types could be beneficial to the host. Macrophages are needed to efficiently kill bacteria, prevent bacterial spread by forming granulomas and provide help in the resolution of infection. Given these important functions of macrophages, particularly their efficiency in keeping the replication of bacteria in check, it could be more beneficial to the host to skew monocyte differentiation, or at least the majority of it, to macrophages during a bacterial infection.

The importance of TLR signaling for cell recruitment

Despite that flagella are a major bacterial constituent of *Salmonella*, TLR5 is dispensable for host defense to oral *Salmonella* infection (213). In contrast, $TLR4^{-/-}$ mice are much more susceptible to *Salmonella*, and these mice have a higher bacterial load in the organs (211, 286, 287). We speculated that the higher susceptibility of TLR4 deficient mice may be partly explained by delayed recruitment of myeloid cells to the GALT, and that this would be even more pronounced if signaling mediated by flagella was simultaneously abrogated. However, my data showed that, although $TLR4^{-/-}$ mice infected with a flagella-deficient strain are more susceptible to *Salmonella*, they had a comparable capacity to recruit neutrophils and monocytes as wild type mice (paper II). In contrast, almost no monocytes or neutrophils were recruited to the blood or GALT in infected $MyD88^{-/-}$ or $MyD88^{-/-}TLR4^{-/-}$ mice. Consistent with this, none of the examined chemokines were upregulated in $MyD88^{-/-}$ $TLR4^{-/-}$ mice, a situation where TLR signaling is abrogated during *Salmonella* infection. Thus, other TLRs must be able to replace TLR4/5 to induce chemokine production, and one potential candidate is TLR2. Although single TLR2 deficient mice are not susceptible to *Salmonella*, TLR2/4 double-deficient mice are more susceptible and have higher bacterial load compared to single $TLR4^{-/-}$ mice after oral or i.p infection (210, 211). Whether this is due to defective recruitment of myeloid cells in the double deficient mice is not known. However, it is likely that TLR2 can induce chemokine production in PP in response to *Salmonella*, since TLR2 is expressed by the FAE overlying PP and can promote DC migration (287). It should also be noted that $MyD88^{-/-}$ mice were even more susceptible to *Salmonella* than TLR2/4 double knock out mice, suggesting that TLR5 and/or TLR9 partly can replace TLR2/4 during the infection (210).

Another intriguing question is why the accumulation of myeloid cells is normal, which indicates normal chemokine levels, when there is a higher bacterial burden and increased susceptibility to *Salmonella* in TLR4-deficient mice. One explanation is that the bacterial killing capacity is initially impaired. This is indicated by a report showing that $TLR4^{-/-}$ macrophages have an early defect in killing *Salmonella* but later on the killing capacity was restored and comparable with $TLR2^{-/-}$ or wild type macrophages (210). In addition, there are less $iNOS^{+}$ monocytes in *Salmonella*-infected TLR4-deficient mice (preliminary data). Delayed activation of

the bactericidal capacity of host cells would result in uncontrolled bacterial growth and a higher bacterial burden, making it difficult for the host to clear the infection. Collectively, these data suggest that several TLRs can induce chemokine production and myeloid cell recruitment during *Salmonella* infection, but TLR4 is indispensable to control the infection.

How is a granuloma established?

I found that recruited monocytes and neutrophils form several inflammatory foci (granulomas) in PP during oral *Salmonella* infection (paper II), similar to what has been demonstrated in the spleen and liver (35, 38, 105). Granulomas are created to prevent the spread of bacteria, but they also function to prevent host tissue destruction by toxic molecules released by neutrophils and macrophages aimed at killing the pathogen, such as reactive nitrogen and oxygen species. For monocytes producing iNOS, localization within a granuloma would be good for two reasons. First, the concentration of bacteria/bacterial ligands and pro-inflammatory cytokines increases the chance for monocytes to get simultaneous stimulation with IFNγ and bacterial ligands that are needed to produce iNOS and its microbicidal products (58, 291). Second, although iNOS is necessary to clear the infection, it causes tissue destruction and immunosuppression. It is therefore advantageous for the host to limit the spread of iNOS-producing cells through the establishment of granulomas (44, 63, 91).

Little is known about the local response that induces granuloma formation or which chemokines direct the cells to the inflammatory focus. Interestingly, $CCR2^+$ monocytes and $CXCR2^+$ neutrophils produced chemokines recognized by CCR2 and CXCR2 (paper II). These cells could thus amplify their own recruitment to inflammatory foci. In addition, monocytes and particularly neutrophils, dissected from the MLN up regulated the expression of chemokines that bind to CCR1 and CCR5 (paper II). This suggests a role for these chemokines in granuloma formation as well. In addition, these chemokines were up regulated with delayed kinetics in PP and MLN, indicating that recruited cells, most likely neutrophils and monocytes produce them.

CCR2 could play a role in the formation of inflammatory foci. This is supported by studies of tuberculosis infection showing that granuloma formation is

delayed and the granulomas formed have another architecture in CCR2$^{-/-}$ mice (251, 292). However, these studies do not directly address if delayed granuloma formation depends on delayed recruitment of a small population of monocytes or if it is a direct effect on granuloma formation. Studies addressing the individual role of additional chemokines/chemokine receptors during infection with *M. tuberculosis* found no crucial function for CCR1, CCR5, CXCR2 or CXCR3 if absent alone. However, this is not surprising given the redundancy within the chemokine family (293).

On the other hand, it is known that production of TNFα and IFNγ are central for the induction and maintenance of granulomas during infection with *Salmonella* and *M. tuberculosis* (87, 105, 293). IFNγ is important in activating monocytes/macrophages to actively kill and prevent spread of bacteria from the granuloma. It is not, however, involved in the recruitment of myeloid cells, since recruitment of neutrophils or monocytes to the GALT was not impaired in IFNγ$^{-/-}$ mice (unpublished data). TNFα seems to be even more critical in the formation and maintenance of the granuloma, which may be due to its role in inducing and regulating local chemokine production. For example, TNFα can further enhance production of CCL3 by human neutrophils after stimulation with *Salmonella* (294).

Accumulation of monocytes was not significantly impaired in TNFR1$^{-/-}$ mice (unpublished data). However, with a less virulent strain or with a lower bacterial load in the organs, TNFα can be important to induce the recruitment of monocytes/monocyte-derived cells but not neutrophils to tissues (28, 105). Formation of granulomas is a crucial part of the host defense against intracellular bacteria, but *Salmonella* has the capacity to escape from already established granulomas to spread and develop new lesions elsewhere (38). Hence, further investigations are needed both to elucidate the role and the regulation of cytokines and chemokines in granuloma formation, and why granulomas sometimes fail to prevent spread of bacteria.

The studies in this thesis used a virulent strain that leads to an overwhelming infection where the host will die within a week. During these conditions, significant amounts of cytokines and bacteria are present in tissues that will influence the host's cells. Therefore, it would be interesting to study the clearance and resolution of infection using a less virulent strain, focusing on the phenotype and function of monocytes including cytokine production at a later stage. Macrophages can develop

into two distinct functional subsets of inflammatory and anti-inflammatory cells driven by a Th1 or Th2 immune response, respectively (123). Likewise, monocytes, which have a great deal of plasticity, likely change their functional phenotype in response to changes in the cytokine environment (125, 126, 134). The ability of these cells to adapt to environmental conditions would facilitate their ability to be beneficial to the host during the whole course of infection when opposing effector functions are needed. For example, during the early stage of infection, proinflammatory conditions would prevail while later, when the host is resolving the infection, anti-inflammatory conditions would likely predominate. With this in mind, it could be speculated that the iNOS/TNFα^+ monocytes recruited to *Salmonella*-infected tissues during the early stage of infection could readapt and promote tissue regeneration in response to changes in the microenvironment during the resolution stage of the infection.

The main focus of this thesis has been on monocytes, while neutrophils were studied in less depth. Nevertheless, neutrophils also exert a diverse array of effector functions such as eradication of bacteria and production of chemokines and cytokines (papers I and III), (295). However the caveat that contaminating inflammatory monocytes may account for the cytokines can not be excluded. It has also been reported that different subpopulations of neutrophils exist, similar to what has been reported for monocytes (296). Neutrophils increase their life span during an infection and this, in addition to their close proximity to monocytes in infected tissues, could lead to collaboration between these cells. Although not yet examined during *Salmonella* infection, it has been shown, for example, that neutrophils can be a source of antigen for cross-presentation in a *Listeria* infection model (297). In light of this data and the observation that macrophages killed by *Salmonella* infection can be a source of cross-presented antigen (74), examining cross-talk between myeloid cells in lymphoid tissue infected with *Salmonella* would be interesting to pursue.

In summary, the results in this thesis have revealed some mechanisms of monocyte and neutrophil recruitment, differentiation, and effector functions in the gut-associated lymphoid tissue during *Salmonella* infection. However, despite that some answers have been obtained, new intriguing questions have arisen: Which factors in the microenvironment skew monocytes to distinct effector cells during infection? Do monocytes alter their functions during different stages of the infection?

How is a granuloma established? Hopefully, the data in this thesis provide a good basis to continue to solve these questions.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Salmonella är en grupp av bakterier vars stammar orsakar allt ifrån en vanlig lokal infektion i tarmen till tyfoid feber som angriper de inre organen. Tyfoid feber finns främst i områden i världen som har dåliga hygienska förhållanden medan de stammar av *Salmonella* som finns i industraliserade länder enbart orsakar magsjuka som oftast läker ut av sig själv utan att kräva behandling. *Salmonella* sprids vid förtäring av smittad mat eller dryck och tar sig in i kroppen via lymforganen i tarmen, men bara bakteriestammarna som leder till tyfoid feber sprider sig vidare till blodet och de inre organen som lever och mjälte. Följden blir bland annat feber, huvudvärk samt förstorad lever och mjälte. Obehandlad leder ett fall av tio till döden men tyfoid feber kan oftast enkelt behandlas med antibiotika. Ett problem är dock att antibiotikaresistens har utvecklats mot flera stammar och innebär ett hot mot möjligheten att behandla tyfoid feber i framtiden. Därför är det viktigt att utveckla nya vacciner och läkemedel för att effektivt kunna bekämpa sjukdomen.

Kroppens immunförsvar kan delas upp i två delar: det medfödda och det specifika. Det medfödda immunförsvaret är viktigt för att förhindra att bakterier och virus tar sig in och sprider sig i kroppen, och om det ändå sker måste det hjälpa till att aktivera det specifika immunförsvaret. Det specifika immunförsvaret utvecklar antikroppar och producerar celler som känner igen den bakterie eller det virus som har tagit sig in i kroppen och hjälper till att döda inkräktaren.

Till det medfödda immunförsvaret hör celltyperna neutrofiler, monocyter, makrofager och dendritiska celler och en av deras viktigaste funktioner är att äta upp döda celler och bakterier. Varken monocyter eller neutrofiler finns i vanliga fall i någon högre grad ute i vävnaderna men i händelse av en infektion med bakterie eller inflammation lockas cellerna till det angripna organet.

I blodet är monocyterna omogna celler men när de kommer ut i vävnaden anpassar de sig efter signaler i omgivningen och omvandlas till makrofager eller dendritiska celler. Båda celltyperna äter upp bakterier, men medan makrofagerna är specialiserade på att döda och helt bryta ner bakterier så vandrar de dendritiska cellerna till lymfnoderna för att presentera bitar av bakterien för andra vita blodkroppar. På så sätt aktiveras det specifika immunförsvaret och reagerar specifikt mot inkräktaren. Mycket är okänt om vilka uppgifter monocyter och neutrofiler har

för att motarbeta en bakteriell infektion och viken slags celler monocyterna omvandlas till i infekterade lymfnoder.

Mitt mål med denna avhandling har varit att undersöka hur monocyter och neutrofiler lockas till tarmens lymfnoder där *Salmonella* först tränger in i kroppen och att undersöka på vilka sätt cellerna bidrar till att försöka utrota bakterierna. För att göra detta har jag använt en sjukdomsmodel i möss som liknar tyfoid feber i människa.

Jag upptäckte att monocyter och neutrofiler ökar i blodet och i tarmens lymfnoder tidigt under en *Salmonella* infektion. Genom att analysera uttrycket av vissa gener i tarmen upptäckte jag att signalmolekylerna (kemokinerna) CCL2 och CXCL2 producerades till följd av infektionen och att de receptorer på cellytan som känner igen dessa substanser fanns på monocyter respektive neutrofiler. På så sätt lockades cellerna att migrera från blodet in i de infekterade lymfnoderna i tarmen.
Efter att ha kommit fram till detta var nästa steg att undersöka vad som fick kemokinerna att börja produceras.

En familj av proteiner kallade Tollreceptorer som mestadels finns på ytan av vita blodkroppar kan upptäcka att bakterier har kommit in i kroppen och signalera fara. Genom att infektera möss som saknade en, flera eller alla Tollreceptorer (så kallade knock-out möss) upptäckte jag att neutrofiler och monocyterna migrerade som vanligt om en eller två receptorer saknades men om alla var borta avstannade migration nästan helt och hållet. Vidare fann jag att kemokinerna CCL2 och CXCL2 inte producerades i dessa möss till följd av att cellerna i immunförsvaret inte kunnat aktiverats via Tollreceptorena av *Salmonella* och detta förklarade varför inte monocyter och neutrofiler lockades till tarmen.

Härnäst undersökte jag vilken funktion monocyter och neutrofiler har i de infekterade lymforganen i tarmen. Jag använde en stam av självlysande bakterier och fann att det främst var monocyter och neutrofiler som åt upp bakterierna. Monocyter och i mindre grad neutrofiler producerade även själva vissa signalmolekyler som lockar fler celler till lymfnoderna samt speciella proteiner som kallas cytokiner som har till uppgift att aktivera immunförsvaret. Till sist undersökte jag om monocyterna omvandlades till dendritiska celler och på så sätt skulle kunna aktivera det specifika immunförsvaret genom att presentera bitar av S*almonella* för en viss slags vita blodkroppar. Men monocyterna kunde inte omvandlas till dendritiska celler och det

var i själva verket bakterien som hindrade detta genom att binda till Tollreceptorer på cellytan.

Dessa upptäcker är ett steg på vägen för att utreda vad som sker med monocyter och neutrofiler under en infektion. Sådan kunskap behövs för att kunna utveckla nya vacciner och läkemedel mot tarmbakterier men även för att förstå hur monocyter rekryteras till vävnader. Det senare är viktigt då man vill kunna stoppa rekryteringen av monocyter när de har en skadlig effekt, till exempel vid sjukdomar som åderförkalkning och multiple skleros.

REFERENCES

1. Crump, J. A., S. P. Luby, and E. D. Mintz. 2004. The global burden of typhoid fever. *Bull World Health Organ* 82:346-353.
2. Connor, B. A., and E. Schwartz. 2005. Typhoid and paratyphoid fever in travellers. *Lancet Infect Dis* 5:623-628.
3. Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams, and A. J. Baumler. 2001. Animal models of Salmonella infections: enteritis versus typhoid fever. *Microbes Infect* 3:1335-1344.
4. Vidal, S., M. L. Tremblay, G. Govoni, S. Gauthier, G. Sebastiani, D. Malo, E. Skamene, M. Olivier, S. Jothy, and P. Gros. 1995. The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. *J Exp Med* 182:655-666.
5. Plant, J., and A. A. Glynn. 1974. Natural resistance to Salmonella infection, delayed hypersensitivity and Ir genes in different strains of mice. *Nature* 248:345-347.
6. Hormaeche, C. E. 1979. The natural resistance of radiation chimeras to S. typhimurium C5. *Immunology* 37:329-332.
7. Jepson, M. A., and M. A. Clark. 2001. The role of M cells in Salmonella infection. *Microbes. Infect.* 3:1183-1190.
8. Iwasaki, A., and B. L. Kelsall. 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J. Exp. Med.* 191:1381-1394.
9. Kelsall, B. L., and W. Strober. 1996. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J. Exp. Med.* 183:237-247.
10. Zhao, X., A. Sato, C. S. Dela Cruz, M. Linehan, A. Luegering, T. Kucharzik, A. K. Shirakawa, G. Marquez, J. M. Farber, I. Williams, and A. Iwasaki. 2003. CCL9 is secreted by the follicle-associated epithelium and recruits dome region Peyer's patch CD11b+ dendritic cells. *J. Immunol.* 171:2797-2803.
11. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3:331-341.
12. Abbas, A. L., A. Pillai, S. 2007. *Cellular and Molecular Immunology*. Saunders, Philadelphia.
13. Carter, P. B., and F. M. Collins. 1974. The route of enteric infection in normal mice. *J Exp Med* 139:1189-1203.
14. Jones, B. D., and S. Falkow. 1996. Salmonellosis: host immune responses and bacterial virulence determinants. *Annu. Rev. Immunol.* 14:533-561.
15. Hopkins, S. A., F. Niedergang, I. E. Corthesy-Theulaz, and J. P. Kraehenbuhl. 2000. A recombinant Salmonella typhimurium vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell. Microbiol.* 2:59-68.
16. Salazar-Gonzalez, R. M., J. H. Niess, D. J. Zammit, R. Ravindran, A. Srinivasan, J. R. Maxwell, T. Stoklasek, R. Yadav, I. R. Williams, X. Gu, B. A. McCormick, M. A. Pazos, A. T. Vella, L. Lefrancois, H. C. Reinecker, and S. J. McSorley. 2006. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity* 24:623-632.

17. Shreedhar, V. K., B. L. Kelsall, and M. R. Neutra. 2003. Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches. *Infect. Immun.* 71:504-509.
18. Martinoli, C., A. Chiavelli, and M. Rescigno. 2007. Entry Route of Salmonella typhimurium Directs the Type of Induced Immune Response. *Immunity* 27:975-984.
19. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, D. R. Littman, and H. C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307:254-258.
20. Hohmann, A. W., G. Schmidt, and D. Rowley. 1978. Intestinal colonization and virulence of Salmonella in mice. *Infect. Immun.* 22:763-770.
21. Jang, M. H., M. N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P. D. Rennert, T. Hiroi, H. Tamagawa, H. Iijima, J. Kunisawa, Y. Yuki, and H. Kiyono. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc Natl Acad Sci U S A* 101:6110-6115.
22. Hapfelmeier, S., A. J. Muller, B. Stecher, P. Kaiser, M. Barthel, K. Endt, M. Eberhard, R. Robbiani, C. A. Jacobi, M. Heikenwalder, C. Kirschning, S. Jung, T. Stallmach, M. Kremer, and W. D. Hardt. 2008. Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltainvG S. Typhimurium colitis. *J Exp Med* 205:437-450.
23. Chieppa, M., M. Rescigno, A. Y. Huang, and R. N. Germain. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 203:2841-2852.
24. Rescigno, M., G. Rotta, B. Valzasina, and P. Ricciardi-Castagnoli. 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* 204:572-581.
25. Vallon-Eberhard, A., L. Landsman, N. Yogev, B. Verrier, and S. Jung. 2006. Transepithelial pathogen uptake into the small intestinal lamina propria. *J Immunol* 176:2465-2469.
26. Jones, B. D., N. Ghori, and S. Falkow. 1994. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* 180:15-23.
27. McSorley, S. J., S. Asch, M. Costalonga, R. L. Reinhardt, and M. K. Jenkins. 2002. Tracking *Salmonella*-specific CD4 T cells in vivo reveals a local mucosal response to a disseminated infection. *Immunity* 16:365-377.
28. Sundquist, M., and M. J. Wick. 2005. TNF-⟨-dependent and -independent maturation of dendritic cells and recruited $CD11c^{int}CD11b^{+}$ cells during oral *Salmonella* infection. *J. Immunol.* 175:3287-3298.
29. Cheminay, C., A. Mohlenbrink, and M. Hensel. 2005. Intracellular Salmonella inhibit antigen presentation by dendritic cells. *J Immunol* 174:2892-2899.
30. Mayrhofer, G., P. G. Holt, and J. M. Papadimitriou. 1986. Functional characteristics of the veiled cells in afferent lymph from the rat intestine. *Immunology* 58:379-387.
31. Milling, S. W., L. Cousins, and G. G. MacPherson. 2005. How do DCs interact with intestinal antigens? *Trends Immunol* 26:349-352.
32. Pullinger, G. D., S. M. Paulin, B. Charleston, P. R. Watson, A. J. Bowen, F. Dziva, E. Morgan, B. Villarreal-Ramos, T. S. Wallis, and M. P. Stevens. 2007. Systemic translocation of Salmonella enterica serovar Dublin in cattle occurs

predominantly via efferent lymphatics in a cell-free niche and requires type III secretion system 1 (T3SS-1) but not T3SS-2. *Infect Immun* 75:5191-5199.

33. Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang. 1999. Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature* 401:804-808.
34. Worley, M. J., G. S. Nieman, K. Geddes, and F. Heffron. 2006. Salmonella typhimurium disseminates within its host by manipulating the motility of infected cells. *Proc Natl Acad Sci U S A* 103:17915-17920.
35. Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay. 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J. Exp. Med.* 186:569-580.
36. Salcedo, S. P., M. Noursadeghi, J. Cohen, and D. W. Holden. 2001. Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo. *Cell. Microbiol.* 3:587-597.
37. Dunlap, N. E., W. H. Benjamin, Jr., R. D. McCall, Jr., A. B. Tilden, and D. E. Briles. 1991. A 'safe-site' for Salmonella typhimurium is within splenic cells during the early phase of infection in mice. *Microb Pathog* 10:297-310.
38. Sheppard, M., C. Webb, F. Heath, V. Mallows, R. Emilianus, D. Maskell, and P. Mastroeni. 2003. Dynamics of bacterial growth and distribution within the liver during *Salmonella* infection. *Cell. Microbiol.* 5:593-600.
39. Maskell, D. J., C. E. Hormaeche, K. A. Harrington, H. S. Joysey, and F. Y. Liew. 1987. The initial suppression of bacterial growth in a salmonella infection is mediated by a localized rather than a systemic response. *Microb Pathog* 2:295-305.
40. Meynell, G. G. 1957. The applicability of the hypothesis of independent action to fatal infections in mice given Salmonella typhimurium by mouth. *J. Gen. Microbiol.* 16:396-404.
41. Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62:379-433.
42. Galan, J. E. 1996. Molecular genetic bases of Salmonella entry into host cells. *Mol Microbiol* 20:263-271.
43. Abrahams, G. L., and M. Hensel. 2006. Manipulating cellular transport and immune responses: dynamic interactions between intracellular Salmonella enterica and its host cells. *Cell Microbiol* 8:728-737.
44. Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med.* 192:227-236.
45. Chakravortty, D., I. Hansen-Wester, and M. Hensel. 2002. Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. *J Exp Med* 195:1155-1166.
46. Tobar, J. A., L. J. Carreno, S. M. Bueno, P. A. Gonzalez, J. E. Mora, S. A. Quezada, and A. M. Kalergis. 2006. Virulent Salmonella enterica serovar typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. *Infect Immun* 74:6438-6448.

47. Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* 83:5189-5193.
48. Cummings, L. A., W. D. Wilkerson, T. Bergsbaken, and B. T. Cookson. 2006. In vivo, fliC expression by Salmonella enterica serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol Microbiol* 61:795-809.
49. O'Brien, A. D., I. Scher, and S. B. Formal. 1979. Effect of silica on the innate resistance of inbred mice to *Salmonella typhimurium* infection. *Infect. Immun.* 25:513-520.
50. Hormaeche, C. E., P. Mastroeni, A. Arena, J. Uddin, and H. S. Joysey. 1990. T cells do not mediate the initial suppression of a Salmonella infection in the RES. *Immunology* 70:247-250.
51. Conlan, J. W. 1997. Critical roles of neutrophils in host defense against experimental systemic infections of mice by *Listeria monocytogenes*, *Salmonella typhimurium*, and *Yersinia enterocolitica*. *Infect. Immun.* 65:630-635.
52. Cheminay, C., D. Chakravortty, and M. Hensel. 2004. Role of neutrophils in murine salmonellosis. *Infect. Immun.* 72:468-477.
53. Yrlid, U., M. Svensson, A. Hakansson, B. J. Chambers, H. G. Ljunggren, and M. J. Wick. 2001. In vivo activation of dendritic cells and T cells during *Salmonella enterica serovar Typhimurium* infection. *Infect. Immun.* 69:5726-5735.
54. Forbes, J. R., and P. Gros. 2001. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends Microbiol* 9:397-403.
55. Wyllie, S., P. Seu, and J. A. Goss. 2002. The natural resistance-associated macrophage protein 1 Slc11a1 (formerly Nramp1) and iron metabolism in macrophages. *Microbes Infect* 4:351-359.
56. Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J. Exp. Med.* 192:237-248.
57. Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* 10:29-38.
58. Eisenstein, T. K. 2001. Implications of Salmonella-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumor, immunosuppressive and immunoregulatory molecule. *Microbes. Infect.* 3:1223-1231.
59. Lu, L., C. A. Bonham, F. G. Chambers, S. C. Watkins, R. A. Hoffman, R. L. Simmons, and A. W. Thomson. 1996. Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *J. Immunol.* 157:3577-3586.
60. Eisenstein, T. K., L. M. Killar, B. A. Stocker, and B. M. Sultzer. 1984. Cellular immunity induced by avirulent Salmonella in LPS-defective C3H/HeJ mice. *J Immunol* 133:958-961.

61. Lee, J. C., C. W. Gibson, and T. K. Eisenstein. 1985. Macrophage-mediated mitogenic suppression induced in mice of the C3H lineage by a vaccine strain of Salmonella typhimurium. *Cell Immunol* 91:75-91.
62. al-Ramadi, B. K., M. A. Brodkin, D. M. Mosser, and T. K. Eisenstein. 1991. Immunosuppression induced by attenuated Salmonella. Evidence for mediation by macrophage precursors. *J Immunol* 146:2737-2746.
63. MacFarlane, A. S., M. G. Schwacha, and T. K. Eisenstein. 1999. In vivo blockage of nitric oxide with aminoguanidine inhibits immunosuppression induced by an attenuated strain of Salmonella typhimurium, potentiates Salmonella infection, and inhibits macrophage and polymorphonuclear leukocyte influx into the spleen. *Infect Immun* 67:891-898.
64. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142-1162.
65. Steinman, R. M., and Z. A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med* 139:380-397.
66. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman, and R. A. Lang. 2002. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 17:211-220.
67. Svensson, M., C. Johansson, and M. J. Wick. 2000. Salmonella enterica serovar typhimurium-induced maturation of bone marrow-derived dendritic cells. *Infect Immun* 68:6311-6320.
68. Johansson, C., and M. J. Wick. 2004. Liver dendritic cells present bacterial antigens and produce cytokines upon *Salmonella* encounter. *J. Immunol.* 172:2496-2503.
69. Tam, M. A., M. Sundquist, and M. J. Wick. 2008. MyD88 and IFN-alphabeta differentially control maturation of bystander but not Salmonella-associated dendritic cells or CD11c(int)CD11b(+) cells during infection. *Cell Microbiol*.
70. Yrlid, U., M. Svensson, C. Johansson, and M. J. Wick. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS. Immunol. Med. Microbiol.* 27:313-320.
71. Yrlid, U., and M. J. Wick. 2002. Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon *Salmonella* encounter. *J. Immunol.* 169:108-116.
72. Tam, M. A., and M. J. Wick. 2004. Dendritic cells and immunity to *Listeria*: TipDCs are a new recruit. *Trends. Immunol.* 25:335-339.
73. Svensson, M., B. Stockinger, and M. J. Wick. 1997. Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. *J Immunol* 158:4229-4236.
74. Yrlid, U., and M. J. Wick. 2000. *Salmonella*-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. *J. Exp. Med.* 191:613-624.
75. Sinha, K., P. Mastroeni, J. Harrison, R. D. de Hormaeche, and C. E. Hormaeche. 1997. Salmonella typhimurium aroA, htrA, and aroD htrA mutants cause progressive infections in athymic (nu/nu) BALB/c mice. *Infect Immun* 65:1566-1569.

76. McSorley, S. J., and M. K. Jenkins. 2000. Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. *Infect Immun* 68:3344-3348.
77. Mastroeni, P., C. Simmons, R. Fowler, C. E. Hormaeche, and G. Dougan. 2000. Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. *Infect Immun* 68:46-53.
78. Hess, J., C. Ladel, D. Miko, and S. H. Kaufmann. 1996. Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J. Immunol.* 156:3321-3326.
79. Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. *Infect Immun* 61:3981-3984.
80. Weintraub, B. C., L. Eckmann, S. Okamoto, M. Hense, S. M. Hedrick, and J. Fierer. 1997. Role of alphabeta and gammadelta T cells in the host response to Salmonella infection as demonstrated in T-cell-receptor-deficient mice of defined Ity genotypes. *Infect Immun* 65:2306-2312.
81. Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- Salmonella vaccines. *Microb Pathog* 13:477-491.
82. Mittrucker, H. W., B. Raupach, A. Kohler, and S. H. Kaufmann. 2000. Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. *J Immunol* 164:1648-1652.
83. Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. Interferon-gamma plays a critical role in intestinal immunity against Salmonella typhimurium infection. *Immunology* 99:464-472.
84. Dybing, J. K., N. Walters, and D. W. Pascual. 1999. Role of endogenous interleukin-18 in resolving wild-type and attenuated Salmonella typhimurium infections. *Infect. Immun.* 67:6242-6248.
85. Mastroeni, P., S. Clare, S. Khan, J. A. Harrison, C. E. Hormaeche, H. Okamura, M. Kurimoto, and G. Dougan. 1999. Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent Salmonella typhimurium. *Infect. Immun.* 67:478-483.
86. Mastroeni, P., J. A. Harrison, J. A. Chabalgoity, and C. E. Hormaeche. 1996. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. *Infect. Immun.* 64:189-196.
87. Mastroeni, P., J. A. Harrison, J. H. Robinson, S. Clare, S. Khan, D. J. Maskell, G. Dougan, and C. E. Hormaeche. 1998. Interleukin-12 is required for control of the growth of attenuated aromatic-compound-dependent salmonellae in BALB/c mice: role of gamma interferon and macrophage activation. *Infect. Immun.* 66:4767-4776.
88. Hirose, K., H. Nishimura, T. Matsuguchi, and Y. Yoshikai. 1999. Endogenous IL-15 might be responsible for early protection by natural killer cells against infection with an avirulent strain of Salmonella choleraesuis in mice. *J Leukoc Biol* 66:382-390.

89. Everest, P., M. Roberts, and G. Dougan. 1998. Susceptibility to Salmonella typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. *Infect. Immun.* 66:3355-3364.
90. Yamamoto, Y., T. W. Klein, and H. Friedman. 1996. Induction of cytokine granulocyte-macrophage colony-stimulating factor and chemokine macrophage inflammatory protein 2 mRNAs in macrophages by Legionella pneumophila or Salmonella typhimurium attachment requires different ligand-receptor systems. *Infect Immun* 64:3062-3068.
91. Schwacha, M. G., J. J. Meissler, Jr., and T. K. Eisenstein. 1998. Salmonella typhimurium infection in mice induces nitric oxide-mediated immunosuppression through a natural killer cell-dependent pathway. *Infect Immun* 66:5862-5866.
92. Ramarathinam, L., R. A. Shaban, D. W. et.al
Participation of macrophages in enhanced in vitro immune interferon (IFN gamma) production with mouse spleen cells. *Microb. Pathog.* 11:347-356.
93. Nauciel, C., and F. Espinasse-Maes. 1992. Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection. *Infect Immun* 60:450-454.
94. Berntman, E., J. Rolf, C. Johansson, P. Anderson, and S. L. Cardell. 2005. The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with *Salmonella typhimurium*. *Eur. J. Immunol.* 35:2100-2109.
95. Kirby, A. C., U. Yrlid, and M. J. Wick. 2002. The innate immune response differs in primary and secondary *Salmonella* infection. *J. Immunol.* 169:4450-4459.
96. Kagaya, K., K. Watanabe, and Y. Fukazawa. 1989. Capacity of recombinant gamma interferon to activate macrophages for Salmonella-killing activity. *Infect Immun* 57:609-615.
97. Goes, N., T. Sims, J. Urmson, D. Vincent, V. Ramassar, and P. F. Halloran. 1995. Disturbed MHC regulation in the IFN-gamma knockout mouse. Evidence for three states of MHC expression with distinct roles for IFN-gamma. *J. Immunol.* 155:4559-4566.
98. Eckman, L., and M. F. Kagnoff. 20001. Cytokines in host defense against *Salmonella*. *Microb. Infect.* 3:1191-1200.
99. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133-146.
100. Pflanz, S., J. C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W. M. Blumenschein, J. D. Mattson, J. L. Wagner, W. To, S. Zurawski, T. K. McClanahan, D. M. Gorman, J. F. Bazan, R. de Waal Malefyt, D. Rennick, and R. A. Kastelein. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* 16:779-790.
101. Lehmann, J., S. Bellmann, C. Werner, R. Schroder, N. Schutze, and G. Alber. 2001. IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against Salmonella enteritidis. *J Immunol* 167:5304-5315.
102. Feldmann, M., and R. N. Maini. 2001. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 19:163-196.
103. Vazquez-Torres, A., G. Fantuzzi, C. K. Edwards, 3rd, C. A. Dinarello, and F. C. Fang. 2001. Defective localization of the NADPH phagocyte oxidase to

Salmonella-containing phagosomes in tumor necrosis factor p55 receptor-deficient macrophages. *Proc Natl Acad Sci U S A* 98:2561-2565.

104. Mastroeni, P., A. Arena, G. B. Costa, M. C. Liberto, L. Bonina, and C. E. Hormaeche. 1991. Serum TNF alpha in mouse typhoid and enhancement of a Salmonella infection by anti-TNF alpha antibodies. *Microb Pathog* 11:33-38.
105. Mastroeni, P., J. N. Skepper, and C. E. Hormaeche. 1995. Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary Salmonella infections. *Infect Immun* 63:3674-3682.
106. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19:71-82.
107. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5:953-964.
108. Yrlid, U., C. D. Jenkins, and G. G. Macpherson. 2006. Relationships between distinct blood monocyte subsets and migrating intestinal lymph dendritic cells in vivo under steady-state conditions. *J. Immunol.* 176:4155-4162.
109. Qu, C., E. W. Edwards, F. Tacke, V. Angeli, J. Llodra, G. Sanchez-Schmitz, A. Garin, N. S. Haque, W. Peters, N. van Rooijen, C. Sanchez-Torres, J. Bromberg, I. F. Charo, S. Jung, S. A. Lira, and G. J. Randolph. 2004. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J. Exp. Med.* 200:1231-1241.
110. Tacke, F., and G. J. Randolph. 2006. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* 211:609-618.
111. Fleming, T. J., M. L. Fleming, and T. R. Malek. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* 151:2399-2408.
112. Taylor, P. R., G. D. Brown, A. B. Geldhof, L. Martinez-Pomares, and S. Gordon. 2003. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. *Eur. J. Immunol.* 33:2090-2097.
113. Lagasse, E., and I. L. Weissman. 1996. Flow cytometric identification of murine neutrophils and monocytes. *J. Immunol. Methods.* 197:139-150.
114. Sunderkötter, C., T. Nikolic, M. J. Dillon, N. Van Rooijen, M. Stehling, D. A. Drevets, and P. J. Leenen. 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* 172:4410-4417.
115. Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel, and E. G. Pamer. 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 19:59-70.
116. Daley, J. M., A. A. Thomay, M. D. Connolly, J. S. Reichner, and J. E. Albina. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 83:64-70.
117. Drevets, D. A., M. J. Dillon, J. S. Schawang, N. Van Rooijen, J. Ehrchen, C. Sunderkotter, and P. J. Leenen. 2004. The Ly-6C^{high} monocyte subpopulation transports *Listeria* monocytogenes into the brain during systemic infection of mice. *J. Immunol.* 172:4418-4424.
118. Mordue, D. G., and L. D. Sibley. 2003. A novel population of Gr-1^{+}-activated macrophages induced during acute toxoplasmosis. *J. Leukoc. Biol.* 74:1015-1025.

119. Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317:666-670.
120. Tacke, F., F. Ginhoux, C. Jakubzick, N. van Rooijen, M. Merad, and G. J. Randolph. 2006. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. *J. Exp. Med.* 203:583-597.
121. Varol, C., L. Landsman, D. K. Fogg, L. Greenshtein, B. Gildor, R. Margalit, V. Kalchenko, F. Geissmann, and S. Jung. 2007. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J. Exp. Med.* 204:171-180.
122. Mack, M., J. Cihak, C. Simonis, B. Luckow, A. E. Proudfoot, J. Plachy, H. Bruhl, M. Frink, H. J. Anders, V. Vielhauer, J. Pfirstinger, M. Stangassinger, and D. Schlondorff. 2001. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J. Immunol.* 166:4697-4704.
123. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.
124. Taylor, P. R., and S. Gordon. 2003. Monocyte heterogeneity and innate immunity. *Immunity* 19:2-4.
125. Nahrendorf, M., F. K. Swirski, E. Aikawa, L. Stangenberg, T. Wurdinger, J. L. Figueiredo, P. Libby, R. Weissleder, and M. J. Pittet. 2007. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 204:3037-3047.
126. Arnold, L., A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R. K. Gherardi, and B. Chazaud. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 204:1057-1069.
127. Gordon, S. 2007. The macrophage: past, present and future. *Eur J Immunol* 37 Suppl 1:S9-17.
128. Landsman, L., and S. Jung. 2007. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol* 179:3488-3494.
129. Landsman, L., C. Varol, and S. Jung. 2007. Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol* 178:2000-2007.
130. Van Furth, R., M. C. Diesselhoff-den Dulk, and H. Mattie. 1973. Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction. *J. Exp. Med.* 138:1314-1330.
131. van Furth, R., and M. M. Diesselhoff-den Dulk. 1984. Dual origin of mouse spleen macrophages. *J Exp Med* 160:1273-1283.
132. Smythies, L. E., M. Sellers, R. H. Clements, M. Mosteller-Barnum, G. Meng, W. H. Benjamin, J. M. Orenstein, and P. D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115:66-75.
133. Rutherford, M. S., A. Witsell, and L. B. Schook. 1993. Mechanisms generating functionally heterogeneous macrophages: chaos revisited. *J. Leukoc. Biol.* 53:602-618.
134. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 175:342-349.

135. Hume, D. A. 2006. The mononuclear phagocyte system. *Curr Opin Immunol* 18:49-53.
136. Holness, C. L., R. P. da Silva, J. Fawcett, S. Gordon, and D. L. Simmons. 1993. Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem* 268:9661-9666.
137. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2:151-161.
138. Ross, G. D., and V. Vetvicka. 1993. CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. *Clin Exp Immunol* 92:181-184.
139. Steinman, R. M., and M. D. Witmer. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* 75:5132-5136.
140. Nussenzweig, M. C., R. M. Steinman, B. Gutchinov, and Z. A. Cohn. 1980. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* 152:1070-1084.
141. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-258.
142. Kirby, A. C., U. Yrlid, M. Svensson, and M. J. Wick. 2001. Differential involvement of dendritic cell subsets during acute *Salmonella* infection. *J. Immunol.* 166:6802-6811.
143. Villadangos, J. A., and W. R. Heath. 2005. Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. *Semin Immunol* 17:262-272.
144. Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, and K. Shortman. 2001. The dendritic cell populations of mouse lymph nodes. *J Immunol* 167:741-748.
145. Iwasaki, A., and B. L. Kelsall. 2001. Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. *J. Immunol.* 166:4884-4890.
146. Pulendran, B. 2005. Variegation of the immune response with dendritic cells and pathogen recognition receptors. *J Immunol* 174:2457-2465.
147. Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7:19-30.
148. Wu, L., and Y. J. Liu. 2007. Development of dendritic-cell lineages. *Immunity* 26:741-750.
149. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.
150. Sporri, R., and C. Reis e Sousa. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* 6:163-170.
151. Fujii, S., K. Liu, C. Smith, A. J. Bonito, and R. M. Steinman. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 199:1607-1618.
152. Heath, W. R., and J. A. Villadangos. 2005. No driving without a license. *Nat Immunol* 6:125-126.
153. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5:1219-1226.

154. Tam, M. A., and M. J. Wick. 2006. Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with *Listeria* monocytogenes. *Cell. Microbiol.* 8:1172-1187.
155. Serbina, N. V., T. Jia, T. M. Hohl, and E. G. Pamer. 2008. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* 26:421-452.
156. Serbina, N. V., W. Kuziel, R. Flavell, S. Akira, B. Rollins, and E. G. Pamer. 2003. Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection. *Immunity* 19:891-901.
157. Serbina, N. V., and E. G. Pamer. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7:311-317.
158. Naik, S. H., D. Metcalf, A. van Nieuwenhuijze, I. Wicks, L. Wu, M. O'Keeffe, and K. Shortman. 2006. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol* 7:663-671.
159. Fogg, D. K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D. R. Littman, A. Cumano, and F. Geissmann. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311:83-87.
160. Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404:193-197.
161. Inaba, K., J. P. Metlay, M. T. Crowley, and R. M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J Exp Med* 172:631-640.
162. Delamarre, L., M. Pack, H. Chang, I. Mellman, and E. S. Trombetta. 2005. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307:1630-1634.
163. Iwasaki, H., and K. Akashi. 2007. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity* 26:726-740.
164. Laslo, P., C. J. Spooner, A. Warmflash, D. W. Lancki, H. J. Lee, R. Sciammas, B. N. Gantner, A. R. Dinner, and H. Singh. 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 126:755-766.
165. Ueda, Y., M. Kondo, and G. Kelsoe. 2005. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J. Exp. Med.* 201:1771-1780.
166. Lord, B. I., G. Molineux, Z. Pojda, L. M. Souza, J. J. Mermod, and T. M. Dexter. 1991. Myeloid cell kinetics in mice treated with recombinant interleukin-3, granulocyte colony-stimulating factor (CSF), or granulocyte-macrophage CSF in vivo. *Blood* 77:2154-2159.
167. Marsh, J. C., D. R. Boggs, G. E. Cartwright, and M. M. Wintrobe. 1967. Neutrophil kinetics in acute infection. *J Clin Invest* 46:1943-1953.
168. Terashima, T., B. Wiggs, D. English, J. C. Hogg, and S. F. van Eeden. 1996. Polymorphonuclear leukocyte transit times in bone marrow during streptococcal pneumonia. *Am J Physiol* 271:L587-592.
169. Merad, M., M. G. Manz, H. Karsunky, A. Wagers, W. Peters, I. Charo, I. L. Weissman, J. G. Cyster, and E. G. Engleman. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* 3:1135-1141.

170. Kamath, A. T., S. Henri, F. Battye, D. F. Tough, and K. Shortman. 2002. Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* 100:1734-1741.
171. Bogunovic, M., F. Ginhoux, A. Wagers, M. Loubeau, L. M. Isola, L. Lubrano, V. Najfeld, R. G. Phelps, C. Grosskreutz, E. Scigliano, P. S. Frenette, and M. Merad. 2006. Identification of a radio-resistant and cycling dermal dendritic cell population in mice and men. *J Exp Med* 203:2627-2638.
172. Liu, K., C. Waskow, X. Liu, K. Yao, J. Hoh, and M. Nussenzweig. 2007. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* 8:578-583.
173. Naik, S. H., P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, and K. Shortman. 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* 8:1217-1226.
174. Onai, N., A. Obata-Onai, M. A. Schmid, T. Ohteki, D. Jarrossay, and M. G. Manz. 2007. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol* 8:1207-1216.
175. Jakubzick, C., F. Tacke, F. Ginhoux, A. J. Wagers, N. van Rooijen, M. Mack, M. Merad, and G. J. Randolph. 2008. Blood Monocyte Subsets Differentially Give Rise to CD103+ and CD103- Pulmonary Dendritic Cell Populations. *J Immunol* 180:3019-3027.
176. Ginhoux, F., F. Tacke, V. Angeli, M. Bogunovic, M. Loubeau, X. M. Dai, E. R. Stanley, G. J. Randolph, and M. Merad. 2006. Langerhans cells arise from monocytes in vivo. *Nat. Immunol.* 7:265-273.
177. Tezuka, H., Y. Abe, M. Iwata, H. Takeuchi, H. Ishikawa, M. Matsushita, T. Shiohara, S. Akira, and T. Ohteki. 2007. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* 448:929-933.
178. Angulo, I., F. G. de las Heras, J. F. Garcia-Bustos, D. Gargallo, M. A. Munoz-Fernandez, and M. Fresno. 2000. Nitric oxide-producing CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood* 95:212-220.
179. Makarenkova, V. P., V. Bansal, B. M. Matta, L. A. Perez, and J. B. Ochoa. 2006. CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. *J Immunol* 176:2085-2094.
180. Brys, L., A. Beschin, G. Raes, G. H. Ghassabeh, W. Noel, J. Brandt, F. Brombacher, and P. De Baetselier. 2005. Reactive oxygen species and 12/15-lipoxygenase contribute to the antiproliferative capacity of alternatively activated myeloid cells elicited during helminth infection. *J Immunol* 174:6095-6104.
181. Zhu, B., Y. Bando, S. Xiao, K. Yang, A. C. Anderson, V. K. Kuchroo, and S. J. Khoury. 2007. CD11b+Ly-6Chi suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol* 179:5228-5237.
182. Delano, M. J., P. O. Scumpia, J. S. Weinstein, D. Coco, S. Nagaraj, K. M. Kelly-Scumpia, K. A. O'Malley, J. L. Wynn, S. Antonenko, S. Z. Al-Quran, R. Swan, C. S. Chung, M. A. Atkinson, R. Ramphal, D. I. Gabrilovich, W. H. Reeves, A. Ayala, J. Phillips, D. Laface, P. G. Heyworth, M. Clare-Salzler, and L. L. Moldawer. 2007. MyD88-dependent expansion of an immature GR-

1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med* 204:1463-1474.

183. Randolph, G. J., K. Inaba, D. F. Robbiani, R. M. Steinman, and W. A. Muller. 1999. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity* 11:753-761.
184. Leon, B., M. Lopez-Bravo, and C. Ardavin. 2007. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* 26:519-531.
185. Kool, M., T. Soullie, M. van Nimwegen, M. A. Willart, F. Muskens, S. Jung, H. C. Hoogsteden, H. Hammad, and B. N. Lambrecht. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*.
186. Le Borgne, M., N. Etchart, A. Goubier, S. A. Lira, J. C. Sirard, N. van Rooijen, C. Caux, S. Ait-Yahia, A. Vicari, D. Kaiserlian, and B. Dubois. 2006. Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity* 24:191-201.
187. Randolph, G. J., S. Beaulieu, S. Lebecque, R. M. Steinman, and W. A. Muller. 1998. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* 282:480-483.
188. Randolph, G. J., G. Sanchez-Schmitz, R. M. Liebman, and K. Schakel. 2002. The $CD16^{+}$ ($FcgRIII^{+}$) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J. Exp. Med.* 196:517-527.
189. Rotta, G., E. W. Edwards, S. Sangaletti, C. Bennett, S. Ronzoni, M. P. Colombo, R. M. Steinman, G. J. Randolph, and M. Rescigno. 2003. Lipopolysaccharide or whole bacteria block the conversion of inflammatory monocytes into dendritic cells in vivo. *J. Exp. Med.* 198:1253-1263.
190. Rotta, G., G. Matteoli, E. Mazzini, P. Nuciforo, M. P. Colombo, and M. Rescigno. 2008. Contrasting roles of SPARC-related granuloma in bacterial containment and in the induction of anti-Salmonella typhimurium immunity. *J Exp Med* 205:657-667.
191. Jakubzick, C., F. Tacke, J. Llodra, N. van Rooijen, and G. J. Randolph. 2006. Modulation of dendritic cell trafficking to and from the airways. *J Immunol* 176:3578-3584.
192. Allavena, P., L. Piemonti, D. Longoni, S. Bernasconi, A. Stoppacciaro, L. Ruco, and A. Mantovani. 1998. IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol* 28:359-369.
193. Delneste, Y., P. Charbonnier, N. Herbault, G. Magistrelli, G. Caron, J. Y. Bonnefoy, and P. Jeannin. 2003. Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood* 101:143-150.
194. Chomarat, P., J. Banchereau, J. Davoust, and A. K. Palucka. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat. Immunol.* 1:510-514.
195. Palucka, K. A., N. Taquet, F. Sanchez-Chapuis, and J. C. Gluckman. 1999. Lipopolysaccharide can block the potential of monocytes to differentiate into dendritic cells. *J Leukoc Biol* 65:232-240.
196. Chomarat, P., C. Dantin, L. Bennett, J. Banchereau, and A. K. Palucka. 2003. TNF skews monocyte differentiation from macrophages to dendritic cells. *J. Immunol.* 171:2262-2269.

197. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7:179-190.
198. Kawai, T., and S. Akira. 2007. TLR signaling. *Semin Immunol* 19:24-32.
199. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.
200. Takeda, K., and S. Akira. 2003. Toll receptors and pathogen resistance. *Cell Microbiol* 5:143-153.
201. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.
202. Takeda, K., O. Takeuchi, and S. Akira. 2002. Recognition of lipopeptides by Toll-like receptors. *J Endotoxin Res* 8:459-463.
203. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol* 165:5392-5396.
204. Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli, and R. Landmann. 2002. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis* 186:798-806.
205. Carpenter, S., and L. A. O'Neill. 2007. How important are Toll-like receptors for antimicrobial responses? *Cell Microbiol* 9:1891-1901.
206. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 167:5887-5894.
207. Ku, C. L., K. Yang, J. Bustamante, A. Puel, H. von Bernuth, O. F. Santos, T. Lawrence, H. H. Chang, H. Al-Mousa, C. Picard, and J. L. Casanova. 2005. Inherited disorders of human Toll-like receptor signaling: immunological implications. *Immunol Rev* 203:10-20.
208. Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J. Immunol.* 168:5997-6001.
209. Edelson, B. T., and E. R. Unanue. 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. *J Immunol* 169:3869-3875.
210. Weiss, D. S., B. Raupach, K. Takeda, S. Akira, and A. Zychlinsky. 2004. Toll-like receptors are temporally involved in host defense. *J. Immunol.* 172:4463-4469.
211. Lembo, A., C. Kalis, C. J. Kirschning, V. Mitolo, E. Jirillo, H. Wagner, C. Galanos, and M. A. Freudenberg. 2003. Differential contribution of Toll-like receptors 4 and 2 to the cytokine response to Salmonella enterica serovar Typhimurium and Staphylococcus aureus in mice. *Infect. Immun.* 71:6058-6062.
212. Totemeyer, S., N. Foster, P. Kaiser, D. J. Maskell, and C. E. Bryant. 2003. Toll-like receptor expression in C3H/HeN and C3H/HeJ mice during *Salmonella enterica serovar Typhimurium* infection. *Infect. Immun.* 71:6653-6657.

213. Vijay-Kumar, M., J. D. Aitken, A. Kumar, A. S. Neish, S. Uematsu, S. Akira, and A. T. Gewirtz. 2008. TLR5KO mice have dysregulated intestinal gene expression and non-specific resistance to Salmonella-induced typhoid-like disease. *Infect Immun*.
214. Vijay-Kumar, M., H. Wu, R. Jones, G. Grant, B. Babbin, T. P. King, D. Kelly, A. T. Gewirtz, and A. S. Neish. 2006. Flagellin suppresses epithelial apoptosis and limits disease during enteric infection. *Am J Pathol* 169:1686-1700.
215. O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1980. Genetic control of susceptibility to Salmonella typhimurium in mice: role of the LPS gene. *J Immunol* 124:20-24.
216. Uematsu, S., M. H. Jang, N. Chevrier, Z. Guo, Y. Kumagai, M. Yamamoto, H. Kato, N. Sougawa, H. Matsui, H. Kuwata, H. Hemmi, C. Coban, T. Kawai, K. J. Ishii, O. Takeuchi, M. Miyasaka, K. Takeda, and S. Akira. 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat Immunol* 7:868-874.
217. Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller, and A. Aderem. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol* 7:569-575.
218. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant, and G. Nunez. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol* 7:576-582.
219. Hawn, T. R., A. Verbon, K. D. Lettinga, L. P. Zhao, S. S. Li, R. J. Laws, S. J. Skerrett, B. Beutler, L. Schroeder, A. Nachman, A. Ozinsky, K. D. Smith, and A. Aderem. 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med* 198:1563-1572.
220. Scanga, C. A., A. Bafica, C. G. Feng, A. W. Cheever, S. Hieny, and A. Sher. 2004. MyD88-deficient mice display a profound loss in resistance to Mycobacterium tuberculosis associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect Immun* 72:2400-2404.
221. Fremond, C. M., V. Yeremeev, D. M. Nicolle, M. Jacobs, V. F. Quesniaux, and B. Ryffel. 2004. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J Clin Invest* 114:1790-1799.
222. Weighardt, H., S. Kaiser-Moore, R. M. Vabulas, C. J. Kirschning, H. Wagner, and B. Holzmann. 2002. Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. *J. Immunol.* 169:2823-2827.
223. Rodriguez, N., F. Fend, L. Jennen, M. Schiemann, N. Wantia, C. U. Prazeres da Costa, S. Durr, U. Heinzmann, H. Wagner, and T. Miethke. 2005. Polymorphonuclear neutrophils improve replication of *Chlamydia pneumoniae* in vivo upon MyD88-dependent attraction. *J. Immunol.* 174:4836-4844.
224. Shi, S., C. Nathan, D. Schnappinger, J. Drenkow, M. Fuortes, E. Block, A. Ding, T. R. Gingeras, G. Schoolnik, S. Akira, K. Takeda, S. Ehrt, S. Bekiranov, and T. Gaasterland. 2003. MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to *Mycobacterium tuberculosis*

Reprogramming of the macrophage transcriptome in response to interferon-gamma and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J. Exp. Med.* 198:987-997.

225. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* 198:1043-1055.
226. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.
227. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7:678-689.
228. Miyasaka, M., and T. Tanaka. 2004. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol* 4:360-370.
229. Streeter, P. R., B. T. Rouse, and E. C. Butcher. 1988. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol.* 107:1853-1862.
230. Berg, E. L., L. M. McEvoy, C. Berlin, R. F. Bargatze, and E. C. Butcher. 1993. L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature* 366:695-698.
231. Streeter, P. R., E. L. Berg, B. T. Rouse, R. F. Bargatze, and E. C. Butcher. 1988. A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* 331:41-46.
232. Arbones, M. L., D. C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D. J. Capon, and T. F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1:247-260.
233. von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* 3:867-878.
234. Palframan, R. T., S. Jung, G. Cheng, W. Weninger, Y. Luo, M. Dorf, D. R. Littman, B. J. Rollins, H. Zweerink, A. Rot, and U. H. von Andrian. 2001. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J. Exp. Med.* 194:1361-1373.
235. Janatpour, M. J., S. Hudak, M. Sathe, J. D. Sedgwick, and L. M. McEvoy. 2001. Tumor necrosis factor-dependent segmental control of MIG expression by high endothelial venules in inflamed lymph nodes regulates monocyte recruitment. *J. Exp. Med.* 194:1375-1384.
236. Ishii, N., Y. Tsuzuki, K. Matsuzaki, J. Miyazaki, Y. Okada, R. Hokari, A. Kawaguchi, S. Nagao, K. Itoh, and S. Miura. 2004. Endotoxin stimulates monocyte-endothelial cell interactions in mouse intestinal Peyer's patches and villus mucosa. *Clin Exp Immunol* 135:226-232.
237. Kunkel, E. J., C. L. Ramos, D. A. Steeber, W. Muller, N. Wagner, T. F. Tedder, and K. Ley. 1998. The roles of L-selectin, beta 7 integrins, and P-selectin in leukocyte rolling and adhesion in high endothelial venules of Peyer's patches. *J. Immunol.* 161:2449-2456.
238. Leon, B., and C. Ardavin. 2008. Monocyte migration to inflamed skin and lymph nodes is differentially controlled by L-selectin and PSGL-1. *Blood* 111:3126-3130.

239. Tedder, T. F., D. A. Steeber, and P. Pizcueta. 1995. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J Exp Med* 181:2259-2264.
240. Zarbock, A., and K. Ley. 2008. Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol* 172:1-7.
241. Henderson, R. B., L. H. Lim, P. A. Tessier, F. N. Gavins, M. Mathies, M. Perretti, and N. Hogg. 2001. The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. *J. Exp. Med.* 194:219-226.
242. Thelen, M. 2001. Dancing to the tune of chemokines. *Nat Immunol* 2:129-134.
243. Rot, A., and U. H. von Andrian. 2004. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* 22:891-928.
244. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.* 18:593-620.
245. Luster, A. D. 2002. The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol.* 14:129-135.
246. Khandaker, M. H., G. Mitchell, L. Xu, J. D. Andrews, R. Singh, H. Leung, J. Madrenas, S. S. Ferguson, R. D. Feldman, and D. J. Kelvin. 1999. Metalloproteinases are involved in lipopolysaccharide- and tumor necrosis factor-alpha-mediated regulation of CXCR1 and CXCR2 chemokine receptor expression. *Blood* 93:2173-2185.
247. Lukacs-Kornek, V., D. Engel, F. Tacke, and C. Kurts. 2008. The role of chemokines and their receptors in dendritic cell biology. *Front Biosci* 13:2238-2252.
248. Tsou, C. L., W. Peters, Y. Si, S. Slaymaker, A. M. Aslanian, S. P. Weisberg, M. Mack, and I. F. Charo. 2007. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* 117:902-909.
249. Tacke, F., D. Alvarez, T. J. Kaplan, C. Jakubzick, R. Spanbroek, J. Llodra, A. Garin, J. Liu, M. Mack, N. van Rooijen, S. A. Lira, A. J. Habenicht, and G. J. Randolph. 2007. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest* 117:185-194.
250. Peters, W., J. G. Cyster, M. Mack, D. Schlondorff, A. J. Wolf, J. D. Ernst, and I. F. Charo. 2004. CCR2-dependent trafficking of F4/80^{dim} macrophages and CD11c$^{dim/intermediate}$ dendritic cells is crucial for T cell recruitment to lungs infected with *Mycobacterium tuberculosis*. *J. Immunol.* 172:7647-7653.
251. Peters, W., H. M. Scott, H. F. Chambers, J. L. Flynn, I. F. Charo, and J. D. Ernst. 2001. Chemokine receptor 2 serves an early and essential role in resistance to *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA.* 98:7958-7963.
252. Depaolo, R. W., R. Lathan, B. J. Rollins, and W. J. Karpus. 2005. The chemokine CCL2 is required for control of murine gastric *Salmonella enterica* infection. *Infect. Immun.* 73:6514-6522.
253. Saederup, N., L. Chan, S. A. Lira, and I. F. Charo. 2007. Fractalkine Deficiency Markedly Reduces Macrophage Accumulation and Atherosclerotic

Lesion Formation in CCR2-/- Mice. Evidence for Independent Chemokine Functions in Atherogenesis. *Circulation*.

254. Zernecke, A., E. A. Liehn, J. L. Gao, W. A. Kuziel, P. M. Murphy, and C. Weber. 2006. Deficiency in CCR5 but not CCR1 protects against neointima formation in atherosclerosis-prone mice: involvement of IL-10. *Blood* 107:4240-4243.
255. Glass, W. G., J. K. Lim, R. Cholera, A. G. Pletnev, J. L. Gao, and P. M. Murphy. 2005. Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J Exp Med* 202:1087-1098.
256. Zhong, M. X., W. A. Kuziel, E. G. Pamer, and N. V. Serbina. 2004. Chemokine receptor 5 is dispensable for innate and adaptive immune responses to Listeria monocytogenes infection. *Infect Immun* 72:1057-1064.
257. DiPietro, L. A., M. Burdick, Q. E. Low, S. L. Kunkel, and R. M. Strieter. 1998. MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. *J Clin Invest* 101:1693-1698.
258. Kaufmann, A., R. Salentin, D. Gemsa, and H. Sprenger. 2001. Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 alpha during differentiation of human monocytes to macrophages. *J Leukoc Biol* 69:248-252.
259. Ravindran, R., L. Rusch, A. Itano, M. K. Jenkins, and S. J. McSorley. 2007. CCR6-dependent recruitment of blood phagocytes is necessary for rapid CD4 T cell responses to local bacterial infection. *Proc Natl Acad Sci U S A* 104:12075-12080.
260. Osterholzer, J. J., T. Ames, T. Polak, J. Sonstein, B. B. Moore, S. W. Chensue, G. B. Toews, and J. L. Curtis. 2005. CCR2 and CCR6, but not endothelial selectins, mediate the accumulation of immature dendritic cells within the lungs of mice in response to particulate antigen. *J. Immunol.* 175:874-883.
261. Robays, L. J., T. Maes, S. Lebecque, S. A. Lira, W. A. Kuziel, G. G. Brusselle, G. F. Joos, and K. V. Vermaelen. 2007. Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J Immunol* 178:5305-5311.
262. Merad, M., P. Hoffmann, E. Ranheim, S. Slaymaker, M. G. Manz, S. A. Lira, I. Charo, D. N. Cook, I. L. Weissman, S. Strober, and E. G. Engleman. 2004. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. *Nat Med* 10:510-517.
263. Lee, J., G. Cacalano, T. Camerato, K. Toy, M. W. Moore, and W. I. Wood. 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J. Immunol.* 155:2158-2164.
264. Fan, X., A. C. Patera, A. Pong-Kennedy, G. Deno, W. Gonsiorek, D. J. Manfra, G. Vassileva, M. Zeng, C. Jackson, L. Sullivan, W. Sharif-Rodriguez, G. Opdenakker, J. Van Damme, J. A. Hedrick, D. Lundell, S. A. Lira, and R. W. Hipkin. 2007. Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8. *J Biol Chem* 282:11658-11666.
265. Wengner, A. M., S. C. Pitchford, R. C. Furze, and S. M. Rankin. 2008. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood* 111:42-49.
266. Brown, C. R., V. A. Blaho, and C. M. Loiacono. 2003. Susceptibility to experimental Lyme arthritis correlates with KC and monocyte chemoattractant protein-1 production in joints and requires neutrophil recruitment via CXCR2. *J. Immunol.* 171:893-901.

267. Del Rio, L., S. Bennouna, J. Salinas, and E. Y. Denkers. 2001. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection. *J. Immunol.* 167:6503-6509.
268. Buanne, P., E. Di Carlo, L. Caputi, L. Brandolini, M. Mosca, F. Cattani, L. Pellegrini, L. Biordi, G. Coletti, C. Sorrentino, G. Fedele, F. Colotta, G. Melillo, and R. Bertini. 2007. Crucial pathophysiological role of CXCR2 in experimental ulcerative colitis in mice. *J Leukoc Biol* 82:1239-1246.
269. Ness, T. L., C. M. Hogaboam, R. M. Strieter, and S. L. Kunkel. 2003. Immunomodulatory role of CXCR2 during experimental septic peritonitis. *J. Immunol.* 171:3775-3784.
270. Garcia-Ramallo, E., T. Marques, N. Prats, J. Beleta, S. L. Kunkel, and N. Godessart. 2002. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J. Immunol.* 169:6467-6473.
271. Xue, M. L., A. Thakur, N. Cole, A. Lloyd, F. Stapleton, D. Wakefield, and M. D. Willcox. 2007. A critical role for CCL2 and CCL3 chemokines in the regulation of polymorphonuclear neutrophils recruitment during corneal infection in mice. *Immunol Cell Biol* 85:525-531.
272. Ramos, C. D., C. Canetti, J. T. Souto, J. S. Silva, C. M. Hogaboam, S. H. Ferreira, and F. Q. Cunha. 2005. MIP-1alpha[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-alpha and LTB4. *J Leukoc Biol* 78:167-177.
273. Khan, I. A., P. M. Murphy, L. Casciotti, J. D. Schwartzman, J. Collins, J. L. Gao, and G. R. Yeaman. 2001. Mice lacking the chemokine receptor CCR1 show increased susceptibility to Toxoplasma gondii infection. *J Immunol* 166:1930-1937.
274. Sabroe, I., L. R. Prince, E. C. Jones, M. J. Horsburgh, S. J. Foster, S. N. Vogel, S. K. Dower, and M. K. Whyte. 2003. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J. Immunol.* 170:5268-5275.
275. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693-1702.
276. Brasel, K., T. De Smedt, J. L. Smith, and C. R. Maliszewski. 2000. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 96:3029-3039.
277. Xu, Y., Y. Zhan, A. M. Lew, S. H. Naik, and M. H. Kershaw. 2007. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* 179:7577-7584.
278. Daro, E., B. Pulendran, K. Brasel, M. Teepe, D. Pettit, D. H. Lynch, D. Vremec, L. Robb, K. Shortman, H. J. McKenna, C. R. Maliszewski, and E. Maraskovsky. 2000. Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. *J Immunol* 165:49-58.
279. Mach, N., S. Gillessen, S. B. Wilson, C. Sheehan, M. Mihm, and G. Dranoff. 2000. Differences in dendritic cells stimulated in vivo by tumors engineered to

secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60:3239-3246.

280. Bartz, H., N. M. Avalos, A. Baetz, K. Heeg, and A. H. Dalpke. 2006. Involvement of suppressors of cytokine signaling in toll-like receptor-mediated block of dendritic cell differentiation. *Blood* 108:4102-4108.
281. Henderson, R. B., J. A. Hobbs, M. Mathies, and N. Hogg. 2003. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood* 102:328-335.
282. Fernekorn, U., E. C. Butcher, J. Behrends, S. Hartz, and A. Kruse. 2004. Functional involvement of P-selectin and MAdCAM-1 in the recruitment of alpha4beta7-integrin-expressing monocyte-like cells to the pregnant mouse uterus. *Eur. J. Immunol.* 34:3423-3433.
283. Hanninen, A., M. Salmi, O. Simell, D. Andrew, and S. Jalkanen. 1996. Recirculation and homing of lymphocyte subsets: dual homing specificity of beta 7-integrin(high)-lymphocytes in nonobese diabetic mice. *Blood* 88:934-944.
284. 2001. Chemokine/chemokine receptor nomenclature. *J Leukoc Biol* 70:465-466.
285. Berahovich, R. D., Z. Miao, Y. Wang, B. Premack, M. C. Howard, and T. J. Schall. 2005. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol* 174:7341-7351.
286. Bernheiden, M., J. M. Heinrich, G. Minigo, C. Schutt, F. Stelter, M. Freeman, D. Golenbock, and R. S. Jack. 2001. LBP, CD14, TLR4 and the murine innate immune response to a peritoneal Salmonella infection. *J. Endotoxin. Res.* 7:447-450.
287. Chabot, S., J. S. Wagner, S. Farrant, and M. R. Neutra. 2006. TLRs regulate the gatekeeping functions of the intestinal follicle-associated epithelium. *J Immunol* 176:4275-4283.
288. Yrlid, U., S. W. Milling, J. L. Miller, S. Cartland, C. D. Jenkins, and G. G. MacPherson. 2006. Regulation of intestinal dendritic cell migration and activation by plasmacytoid dendritic cells, TNF-alpha and type 1 IFNs after feeding a TLR7/8 ligand. *J Immunol* 176:5205-5212.
289. Turnbull, E. L., U. Yrlid, C. D. Jenkins, and G. G. Macpherson. 2005. Intestinal dendritic cell subsets: differential effects of systemic TLR4 stimulation on migratory fate and activation in vivo. *J Immunol* 174:1374-1384.
290. Alaniz, R. C., S. Sandall, E. K. Thomas, and C. B. Wilson. 2004. Increased dendritic cell numbers impair protective immunity to intracellular bacteria despite augmenting antigen-specific CD8+ T lymphocyte responses. *J Immunol* 172:3725-3735.
291. Cherayil, B. J., and D. Antos. 2001. Inducible nitric oxide synthase and Salmonella infection. *Microbes Infect* 3:771-776.
292. Scott, H. M., and J. L. Flynn. 2002. Mycobacterium tuberculosis in chemokine receptor 2-deficient mice: influence of dose on disease progression. *Infect Immun* 70:5946-5954.
293. Saunders, B. M., and W. J. Britton. 2007. Life and death in the granuloma: immunopathology of tuberculosis. *Immunol Cell Biol* 85:103-111.
294. Hachicha, M., P. Rathanaswami, P. H. Naccache, and S. R. McColl. 1998. Regulation of chemokine gene expression in human peripheral blood neutrophils phagocytosing microbial pathogens. *J Immunol* 160:449-454.

295. Theilgaard-Monch, K., B. T. Porse, and N. Borregaard. 2006. Systems biology of neutrophil differentiation and immune response. *Curr Opin Immunol* 18:54-60.
296. Tsuda, Y., H. Takahashi, M. Kobayashi, T. Hanafusa, D. N. Herndon, and F. Suzuki. 2004. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity* 21:215-226.
297. Tvinnereim, A. R., S. E. Hamilton, and J. T. Harty. 2004. Neutrophil involvement in cross-priming CD8+ T cell responses to bacterial antigens. *J Immunol* 173:1994-2002.