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The Role of Neutrophils upon Leishmania major Infection

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The Role of Neutrophils upon *Leishmania major* Infection

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Summary

Neutrophils constitute the first line of defense against a variety of pathogens. Following *Leihmania major* infection neutrophils migrate rapidly and massively to the site of parasite inoculation. They are important players in the orchestration of the anti-leishmania response through the release of a plethora of cytokines, chemokines and granular components. In addition, neutrophils interact with antigen-presenting cells such as dendritic cells (DCs) and thereby contribute to the development of the adaptive immune response. However, the impact of neutrophils on the activation of DCs and possible consequences on disease progression following *L. major* infection are poorly understood. Therefore, we first investigated the influence of neutrophils on DC activation *in vitro*. Next we analyzed the role of neutrophils on the activation of DCs present at the site of infection and in the draining lymph node (dLN) following inoculation of *L. major* in the ear pinna of C57BL/6 and BALB/c mice and analyzed the consequences of early neutrophil depletion on disease progression.

We could demonstrate that neutrophils had a negative impact on the activation of DCs exposed to *L. major in vitro* through mechanisms involving neutrophil-derived prostaglandins and sequestration of parasites. In BALB/c mice, depletion of neutrophils during the first days of infection with *L. major* in the ear dermis increased the expression of activation markers on dLN DCs but not on DCs present at the site of infection. In addition, BALB/c mice transiently depleted of neutrophils developed significantly smaller lesions, a decreased Th2 immune response and harbored fewer parasites at the site of infection compared to non-depleted littermates.

In C57BL/6, mice early depletion of neutrophils had no detectable impact on the activation of DCs both at the site of infection and in the dLN following *L. major* inoculation. Furthermore, depletion of neutrophils had no major effect on lesion evolution and parasite loads despite the development of a decreased Th2 immune response compared to non-depleted littermates.

In summary, at the onset of infection, neutrophils are detrimental for DC activation and the anti-leishmania response in BALB/c mice while their role appears to be less important in C57BL/6 mice. These findings could have important implications for the design of new vaccination strategies.

Résumé

Les neutrophiles constituent la première ligne de défense contre un grand nombre de pathogènes. Après infection avec *Leishmania major*, les neutrophiles migrent rapidement et massivement au site d'infection par le parasite. Les neutrophiles sont d'importants acteurs dans l'orchestration de la réponse anti-*Leishmania*, via la sécrétion de nombreuses cytokines, chimiokines et composés stockés dans leurs granules. De plus, les neutrophiles interagissent avec les cellules présentatrices d'antigènes, telles que les cellules dendritiques et en conséquences contribuent au développement de la réponse adaptative. A ce jour, l'impact des neutrophiles sur l'activation des cellules dendritiques et les possibles conséquences de l'interaction neutrophiles avec ces dernières sur l'évolution de la maladie suite à l'infection avec *L. major* reste peu connu. Ainsi, nous avons dans un premier temps investigué l'influence des neutrophiles sur l'activation des cellules dendritiques *in vitro*. Suite à cela, nous avons analysé le rôle des neutrophiles sur l'activation des cellules dendritiques présentes au site d'infection et dans les ganglions drainants *in vivo*, après inoculation intra-dermale de *L. major* dans le pavillon auriculaire de souris de souche C57BL/6 et BALB/c, ainsi que les conséquences de la déplétion des neutrophiles sur l'évolution de la maladie.

Nous avons pu démontrer que les neutrophiles ont un impact négatif sur l'activation des cellules dendritiques exposées à *L. major in vitro*, via des mécanismes impliquant la sécrétion de prostaglandines par les neutrophiles et la séquestration des parasites. La déplétion des neutrophiles dans les souris BALB/c durant les premiers jours après infection avec *L. major* dans le derme de l'oreille résulte en une augmentation de l'expression de marqueurs d'activation des cellules dendritiques présentes dans les ganglions drainants, mais pas au site d'infection. De plus, les souris BALB/c transitoirement déplétées en neutrophiles développent des lésions significativement plus petites, une réponse de type Th2 diminuée et une charge parasitaire plus faible au site d'infection que les souris non déplétées.

La déplétion des neutrophiles dans les souris C57BL/6 n'a pas d'influence détectable sur l'activation des cellules dendritiques que ce soit au niveau des ganglions drainants ou au site d'infection. La progression de la lésion et la charge parasitaire ne sont pas affectées par la déplétion des neutrophiles, malgré le développement d'une réponse Th2 diminuée par rapport aux souris non déplétées.

En résumé, dans les premières heures après infection, les neutrophiles ont un effet négatif sur l'activation des cellules dendritiques et sur la réponse anti-*Leishmania* dans les souris BALB/c tandis que leur rôle paraît moins important dans les souris C57BL/6. Ces résultats peuvent avoir d'importantes implications en terme de développement de nouveaux vaccins contre *Leishmania*.

Résumé pour un large public

Les neutrophiles représentent la première ligne de défense de l'organisme contre les agents infectieux et constituent la plus abondante population de globules blancs chez l'humain. Ils participent aussi à l'induction de la réponse immunitaire innée via la sécrétion de nombreuses cytokines, chimiokines, divers composants stockés dans leurs granules ainsi que par interaction avec d'autres cellules immunitaires telles que les cellules dendritiques. Les cellules dendritiques sont les principales cellules présentatrices d'antigène, font le lien entre le système inné et le système adaptatif et sont cruciales pour le développement d'une réponse protective. Les neutrophiles contribuent à l'attraction des cellules dendritiques au site d'infection et peuvent inhiber ou promouvoir leur activation selon le contexte.

Dans le modèle murin d'infection avec le parasite *Leishmania major,* les neutrophiles sont les premières cellules à arriver rapidement et massivement au site d'infection. Après infection avec *L. major*, la majorité des souches de souris, telles que les souris de la souche C57BL/6, développent une petite lésion qui se résorbe par elle-même et sont capables de contrôler la prolifération parasitaire. Ces souris développent une réponse immunitaire de type Th1et sont considérées comme *Leishmania*-résistantes. A contrario, un petit nombre de souches de souris, telles que les souris de souche BALB/c, succombent à l'infection avec *L. major* et sont référées comme *Leishmania*-susceptibles. Ces souris développent une importante lésion et sont incapables de contrôler la prolifération du parasite au site d'infection, des effets associés à une réponse immunitaire de type Th2. Le rôle des neutrophiles, rapidement recrutés au site d'infection des souris C57BL/6 et BALB/c, a été investigué par de nombreux groupes de recherche, mais avec des résultats controversés. Dans ce projet, nous avons ainsi voulu évaluer l'impact des neutrophiles sur l'activation des cellules dendritiques et sur l'évolution de la lésion après inoculation de *L. major* dans le pavillon auriculaire des souris de souche C57BL/6 et BALB/c.

Dans un premier temps, nous avons pu démontrer que les neutrophiles des souris BALB/c agissaient négativement sur l'activation des cellules dendritiques *in vitro*. Afin de caractériser l'influence des neutrophiles sur l'activation des cellules dendritiques *in vivo*, nous avons traité les souris avec un anticorps monoclonal qui dépléte les neutrophiles pendant les premiers jours après l'infection avec *L. major*. Nous avons observé que les neutrophiles ont une influence négative sur l'activation des cellules dendritiques dans les ganglions drainant le site d'infection, mais pas au site d'infection. De plus, les souris dont les neutrophiles sont déplétés avant l'infection développent une lésion plus petite, non-progressive et contenant moins de parasites que les souris non-déplétées. Les neutrophiles des souris *Leishmania*-

résistantes ont un impact moindre sur l'activation des cellules dendritiques *in vivo*. L'évolution de la lésion ainsi que la charge parasitaire ne sont pas altérées par la déplétion des neutrophiles.

En résumé, nous avons démontré dans ce projet que les neutrophiles des souris *Leishmania*-susceptibles de souche BALB/c ont un effet négatif sur la réponse anti-*Leishmania*, tandis que leur rôle dans les souris résistante de souche C57BL/6 semble moins important. Ces résultats peuvent avoir d'importantes implications en terme de développement de nouveaux vaccins contre *Leishmania*.

Abbreviations

Ab	antibody
APC	antigen presenting cell
BALF	bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
BM	bone marrow
cDC	conventional DC
CL	cutaneous leishmaniasis
CLR	C-type lectin receptor
CTL	cytotoxic T lymphocyte
DAMP	danger-associated molecular pattern
DC	dendritic cell
DC-SIGN	DC-specific ICAM-3-grabbing non-integrin
DCL	diffused cutaneous leishmaniasis
dLN	draining lymph node
fp	footpad
G-CSF	granulocyte-colony stimulating factor
Gfi-1	growth factor independent 1
GM-CSF	granulocyte macrophage colony-stimulating factor
HMGB1	high-mobility group protein B 1
iDC	immature DC
IFNγ	interferon gamma
IL	interleukin
iNOS	inducible NO synthase
iTreg	inducible regulatory T cell
LACK	Leishmania activated C-kinase
LCF	Leishmania chemotactic factor
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MCL	mucocutaneous leishmaniasis
MHC	major histocompatibility complex
MMP	matrix metalloproteinase

moDC	monocyte-derived DC
MPO	myeloperoxidase
NET	neutrophil extracellular trap
NK	natural killer
NLR	NOD-like receptor
NO	nitric oxide
p.i.	post infection
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
pDC	plasmacytoid DC
PG	prostaglandin
PRR	pattern recognition receptor
RLR	RIG-like receptors
ROI	reactive oxygen intermediate
ROS	reactive oxygen species
SLE	systemic lupus erythematosus
Stat	signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper
T_{FH}	T follicular helper cell
TGFβ	transforming growth factor beta
Tip DC	TNFα and iNOS producing DC
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TREM	triggering receptor expressed on myeloid cells
VL	visceral leishmaniasis

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Introduction

1. The immune system

Our body is constantly exposed to microorganisms present in our environment including bacteria, virus, fungi and parasites. To defend ourselves from those pathogens we possess three distinct lines of defense. The first line of defense consists of physical barriers such as the skin or mucosa to block the entry of pathogens into the body. If microorganisms succeed in crossing the epithelial barrier, the second and third lines of defense get activated – the innate and the adaptive immune system, respectively.

1.1 The innate immune system

The innate immune system is an evolutionary older form of host defense found in all classes of plant and animal life. It comprises cells and mechanisms that fight intruding pathogens in a fast but non-specific manner. Cells of the innate immune system such as neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, basophils and eosinophils express germline-encoded pattern recognition receptors (PRRs) to detect molecular motifs on invading pathogens called pathogen-associated molecular patterns (PAMPs) and endogenous danger signals indicating host injury termed danger-associated molecular patterns (DAMPs) [1]. PRRs are classified according to their structure, function and localization: Toll-like receptors (TLRs) [2], C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) [3] and NOD-like receptors (NLRs) [4]. After entering tissues, many pathogens are rapidly recognized and ingested by host cells. This leads to the secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and Tumor Necrosis Factor α (TNFα) as well as chemokines leading to the attraction of more innate immune cells such as DCs. After exposure to or phagocytosis of pathogens, DCs get activated, migrate to the draining lymph node and present antigen in the context of major histo compatibility complex (MHC) class II to naïve T cells, thereby initiating the adaptive immune response, our third line of defense [5, 6].

1.2 The adaptive immune response

The activation of the adaptive immune system is slower compared to the innate immune system. In return a much broader range of antigens can be recognized in a highly specific

manner. The main adaptive immune cells are B and T lymphocytes developing in the bone marrow and thymus, respectively. Each B cell expresses a B cell receptor (BCR) and each T cell a T cell receptor (TCR) recognizing only one single epitope. T cells are further subdivided in $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells instruct other cells via the secretion of cytokines and by interactions through costimulatory molecules. $CD8^+$ T cells, also called cytotoxic T cells (CTLs), can eliminate other cells infected with pathogens. B and T cells continuously circulate between secondary lymphoid organs such as spleen, lymph nodes (LN) or mucosa-associated lymphoid tissues. Once a B or T cell recognizes its cognate antigen presented by an antigen-presenting cell (APC) via their BCR or TCR, respectively, it gets activated. This recognition, together with additional costimulatory signals, leads to the differentiation into effector cells, proliferation and clonal expansion. Activated B cells differentiate into plasma cells secreting high-affinity antibodies. $CD4^+$ T cells can differentiate into various T helper subsets and secrete specific cytokines (T helper cell differentiation will be discussed more in detail in chapter 1.3). $CD8^+$ T cells differentiate into CTLs and eliminate infected cells. The development of the adaptive immune response leads, in most cases, to the efficient elimination of the agent of infection. Furthermore, some lymphocytes differentiate into memory cells during the adaptive immune response. Upon reinfection with the same pathogen, these memory cells respond faster and more efficiently. The development of memory is a hallmark of the adaptive immune response and most vaccines rely on the formation of good immunological memory and the generation of high titers of specific antibodies [7-9].

1.3 T helper differentiation

T helper cells play critical roles in orchestrating the adaptive immune response. After encountering their cognate antigen presented by APCs, naïve $CD4^+$ T cells differentiate into various subsets including Th1, Th2, Th17, follicular T helper (T_{FH}) and inducible regulatory T (iTreg) cells. Each T helper subset is characterized by a specific transcription factor, has a specific cytokine secretion pattern and exerts distinct functions (Figure 1). In this way an appropriate immune response against a variety of different pathogens can be induced and the pathogens be eliminated However, increasing data suggest that the different Th subsets are not necessarily terminally differentiated fixed lineages but that there is some plasticity among different Th subsets. Therefore, one Th subset can acquire characteristics of another Th subset under certain circumstances [10].

1.3.1 T helper 1 differentiation

Th1 cells induce cell-mediated immune responses against intracellular pathogens [11]. The differentiation of Th1 cells is driven by the cytokines IFNγ and IL-12 released by cells such as NK cells and DCs. IFNγ activates Stat1, which is the major transducer of IFNγ signaling, in the responding $CD4^+$ T cells leading to the upregulation of the Th1 master regulator T-bet [12, 13]. T-bet, in turn, induces the secretion of IFNγ by T cells and the up-regulation of the β2 chain of the IL12 receptor (IL-12Rβ2) which forms the heterodimeric IL-12 receptor together with the constitutively expressed IL-12Rβ1 chain. IL-12-signaling through Stat4 induces IFNγ production by activated T cells and sustains the expression of IL-12Rβ2 [14]. Thus IFNγ and IL-12 collaborate to induce full Th1 differentiation. At later stages IL-18 has been shown to increase IFNγ production by differentiated $CD4^+$ T cells [15]. IFNγ activates the microbicidal functions of innate cells such as macrophages resulting in the production of toxic compounds like nitric oxide which leads to the elimination of the pathogens [16]. Moreover, IFNγ triggers an isotype switch towards IgG2a and IgG3 antibodies secreted by B cells enhancing opsonization and phagocytosis of the pathogen. However, Th1 cells were also shown to be involved in chronic inflammation and tissue destruction illustrating the necessity of a tight regulation of Th1 cell differentiation.

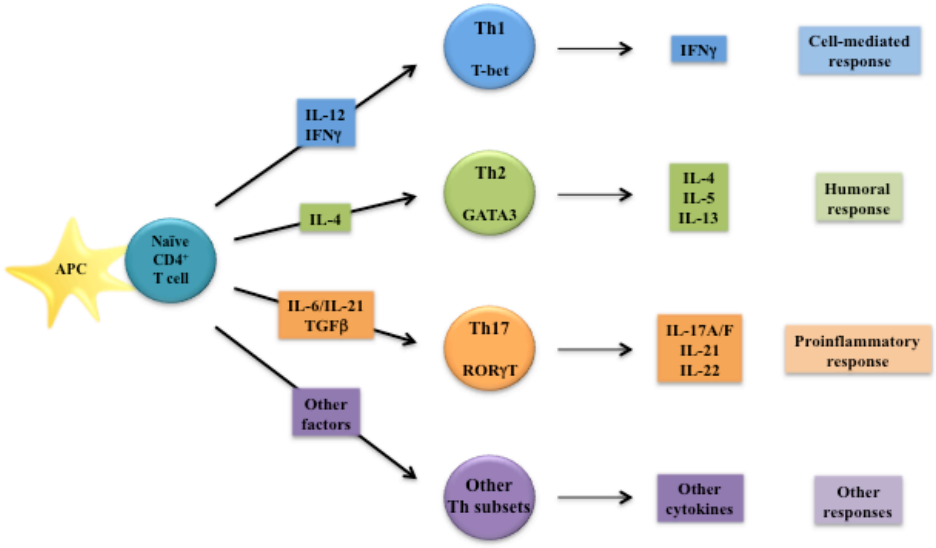

Figure 1: Schematic representation of Th cell differentiation in the mouse

1.3.2 T helper 2 differentiation

Th2 cells mediate humoral immune responses against extracellular parasitic infections such as helminth infections [11]. The differentiation of Th2 cells is mainly driven

by IL-4 which upregulates the expression of IL-4Rα and acts as positive feedback cytokine [17]. IL-4 activates the signal transducer Stat6 that in turn induces the expression of the master transcription factor GATA3 [18, 19]. However, accumulating *in vivo* studies indicate that IL-4 is not essential for Th2 differentiation under certain settings and other mechanisms might be involved in Th2 cell differentiation [20, 21]. The signature cytokines secreted by Th2 cells are IL-4, IL-5 and IL-13. These cytokines activate mast cells, eosinophils and induce a B cell isotype switch towards IgE and IgG1. This leads to the opsonization of pathogens and to the release of proinflammatory mediators such as cytokines, chemokines and histamins. However, excessive Th2 responses are often linked to the development of asthma or allergies and therefore need to be properly regulated.

1.3.3 T helper 17 differentiation

Th17 cells mediate host defense against extracellular bacteria and fungi [22]. Initially, Th17 cell differentiation was thought to be dependent on IL-6 and TGFβ which activate Stat3 leading to the expression of the Th17 master regulator RorγT [23]. However, an alternative Th17 cell differentiation model independent of TGFβ signaling was proposed recently. In this model IL-23 in combination with IL-1β led to differentiation of Th17 cells that are able to secrete IFNγ, termed pathogenic Th17 cells [24]. The signature cytokines of Th17 cells are IL-17A, IL17F, IL-21 and IL-22. IL-17A and IL17-F have different downstream targets leading to the secretion of pro-inflammatory cytokines and contributing to neutrophil recruitment [25, 26]. While IL-21 is thought to act as a positive feedback loop for Th17 cell differentiation [27], IL-22 induces antimicrobial agents in keratinocytes and is essential in the immune barrier function of epithelia [28, 29]. If not properly controlled exaggerated Th17 immune responses can cause multiple autoimmune diseases.

1.3.4 Other T helper subsets

Regulatory T cells

Treg cells play a critical role in maintaining self-tolerance as well as in regulating immune responses [30]. Two different types of Treg cells exist – natural Treg (nTreg) cells that derive directly from the thymus and inducible Treg (iTreg) cells that differentiate from naïve $CD4^+$ T cells in the presence of TGFβ. Both subtypes express the master regulator FoxP3 and the surface marker CD25 [31]. TGFβ, IL-2 and FoxP3 expression are required for the survival and for maintaining the suppressive function of Treg cells which is mainly exerted through the secretion of the immune-modulatory cytokines TGFβ and IL-10 [32, 33].

Increased Treg function may be beneficial in treating autoimmune diseases or preventing allograft rejections [34]. On the other hand, inhibiting Treg function could enhance immunity against tumors and chronic infectious agent. Thus, the differentiation of Treg cells needs also to be tightly regulated.

T follicular helper cells

T_{FH} cells provide help to B cells during immune responses. They are required for the formation of germinal centers and the generation of plasma and memory B cells secreting high affinity antibodies. T_{FH} cells are characterized by the expression of distinct surface marker such as CXCR5, PD1, ICOS, BTLA and CD40L. The master regulator of T_{FH} differentiation is Bcl-6. A recent report also demonstrated the involvement of Notch signaling in the differentiation of T_{FH} cells [35]. However, it is still unclear whether T_{FH} cells differentiate from naïve or already primed $CD4^+$ T cells [36, 37].

T helper 9 cells

Th9 cells have been recently described as IL-9 secreting cells. IL-9 secretion is induced by a combination of TGFβ and IL-4 and promotes mucus production from lung epithelial cells and pulmonary mastocytosis [38-40]. The transcription factor PU.1 is required for the development of IL-9-producing cells [41]. Th9 cells have been implicated to contribute to allergic diseases, however their exact role is still not well understood [42].

Tr1 cells

Type 1 regulatory (Tr1) cells are T cells playing an important role in promoting and maintaining tolerance. They secrete large amounts of IL-10 and minimal amounts of IL-4 and IL-17 which distinguish them from Th1 and Th17 cells. Tr1 cells express very little if any FoxP3 and are thus thought to be also distinct from Treg cells [43].

1.3.5 Role of Notch signaling in T helper differentiation

The Notch signaling pathway is an evolutionary highly conserved system involved in a variety of different processes such as embryonic development as well as adult tissue homeostasis and renewal. In mammals, four Notch receptors (Notch 1-4) and five Notch ligands (Delta-like ligand 1, 4, 5 and Jagged 1, 2) exist. Increasing data suggest that Notch signaling plays an important role both in the differentiation and function of different $CD4^+$ T helper subsets. However, the precise role of Notch in Th differentiation is not yet fully understood. Indeed, some studies show that Notch is involved in Th1 differentiation while others report a role for Notch in Th2 or T_{FH} differentiation. More work on the involvement of

Notch signaling in general and in particular on the contribution of single Notch receptors and ligands on T helper differentiation is needed to fully understand the contribution of Notch in this process [44, 45].

2. Neutrophils

Neutrophils constitute the most abundant population of white blood cells in humans. They are the first line of defense against infectious agents. If microorganisms have successfully entered tissues, neutrophils arrive massively and rapidly at the site of insult, fight the pathogens and participate in the activation and regulation of the adaptive immune response [46-49].

2.1 History

One of the first description about cellular components of the inflammatory response was made by William Addison in 1841. He compared colorless corpuscles in the blood with those of inflamed tissues and proposed that leukocytes enter tissues via diapedesis. Around 40 years later in 1880, Paul Ehrlich developed a staining technique using hematoxylin and eosin (H and E) that facilitated the identification of developing phagocytes in the bone marrow, blood and tissues. Using this technique he could distinguish neutrophils having a neutral and thus unstained cytoplasm from other cells such as basophils and eosinophils. In 1884, Metchnikov observed the phagocytic process under the microscope and is credited with the origin of the terms phagocyte and phagocytosis [50].

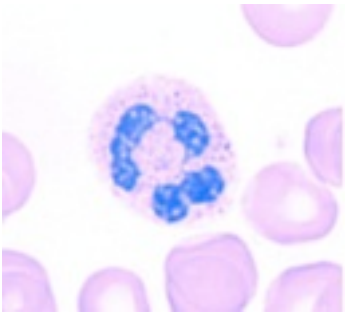

Figure 2: Neutrophil stained with the H and E method (adapted from http://medcell.med.yale.edu/histology/blood_bone_marrow_lab/neutrophil.php)

2.2 Development and homeostasis

Neutrophils develop in the bone marrow from hematopoietic stem cells (HSC) during a tightly regulated process termed granulopoiesis. The differentiation process takes about two weeks and is separated in five different morphological stages: myeloblast, promyelocyte, myelocyte and metamyelocyte, band cell, polymorphonuclear granulocyte [46]. The transcription factors CCAAT/enhancer binding protein a (C/EBPa), PU.1 and growth factor independent-1 (Gfi-1) are essential for terminal granulopoiesis [51-53]. The major cytokine for neutrophil differentiation and survival is granulocyte colony stimulating factor (G-CSF). However, G-CSF-deficient mice still possess 25% residual neutrophils and are able to generate fully mature neutrophils [54]. Thus, involvement of other cytokines such as GM-CSF, IL-6, IL-3, IL-17 and IL-22 in granulopoiesis has been suggested [55]. Once fully differentiated, matured neutrophils are retained in the bone marrow. Indeed, circulating neutrophils represent only 1-2% of morphologically mature neutrophils in the bone marrow [56]. The bone marrow reserve is critical for host defense, as these neutrophils can be rapidly mobilized in case of infection or injury. The retention of neutrophils in the BM is mainly regulated by the interaction of chemokine receptors such as CXCR4 (favoring retention) and CXCR2 (favoring release) and their ligands CXCL12 and KC and Groβ, respectively. The interaction of CXCR4 on neutrophils with CXCL12 secreted by BM stromal cells leads to retention of neutrophils in the BM. In case of an infection, G-CSF is released by BM macrophages leading to the downregulation of CXCR4 on neutrophils and to their release in the blood [57, 58]. Under homeostatic conditions, neutrophils are rather short-lived cells. The half-life of neutrophils in circulation was thought to be approximately 1.5 and 8 hours in mice and human, respectively. However recent studies challenged this concept proposing that the average circulatory lifespan of neutrophils is up to 12.5 hours in mice and 5.4 days in human [59, 60].

Granules are the hallmark of granulocytes and store proteins that can kill microbes and lead to tissue digestion. Neutrophil granules are classified into four different subsets based on the presence of characteristic granule proteins: primary or azurophilic granules, secondary or specific granules, tertiary or gelatinase granules and secretory vesicles. These granules harbor more than 300 different proteins. Granules are formed during granulocytic differentiation: primary granules at the early promyelocyte stage, secondary granules at the myelocyte-metamyelocyte stage, tertiary granules at the band cell stage and secretory vesicles in mature neutrophils [61, 62].

2.3 Functions

Once released into the blood stream neutrophils begin to seek signs of inflammation and infection leading to a series of events culminating in transendothelial migration to the site of insult. The neutrophil recruitment cascade involves the following steps: tethering, rolling, adhesion, crawling and transmigration. The first rolling step is mostly selectin-dependent. Chemokines presented on the luminal part of the endothelium activate neutrophils inducing conformational changes of neutrophil surface integrins. The next steps such as adhesion, crawling and transmigration depend on integrin interactions. Chemokine gradients along the endothelium guide neutrophils to the preferential site of transmigration. Two different models of transmigration exist: the paracellular model in which neutrophils transmigrate between endothelial cells and the transcellular model in which neutrophils transmigrate through endothelial cells [49].

2.3.1 Neutrophils in host defense

Neutrophils arrive rapidly and massively at the site of infection and contribute to pathogen elimination. Chemokines such as IL-8 in humans or KC and MIP2 in mice, complement C3 and possibly pathogen-derived factors contribute to neutrophil chemo-attraction [63, 64]. Neutrophils express a wide variety of PRRs such as TLRs, CLRs, RLRs and NLRs through which they recognize PAMPs and DAMPs [65]. This recognition leads to their activation and phagocytosis of the pathogen. Neutrophils possess different ways to eliminate pathogens such as the respiratory burst, nitric oxide (NO) production, the release of granular proteins and the formation of neutrophil extracellular traps (NETs).

Respiratory burst

The respiratory burst is mediated by the NADPH complex. In resting neutrophils, components of the NADPH complex are spatially separated into the cytosol and the membrane. Upon stimulation, the cytosolic part gets translocated to the membrane resulting in the activation of the NADPH complex [66]. Its activation leads to an increase in oxygen consumption and the generation of reactive oxygen intermediates (ROIs) such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO) then converts the relatively innocuous H_2O_2 in much more powerful antiseptics such as hypochlorus acid (HOCl) and chloramines [67]. Deficiencies of the NADPH complex were discovered in patients suffering from chronic granulomatous diseases (CGD) highlighting the importance of this complex.

NO production

NO production is mediated by the enzyme inducible nitric oxide synthase (iNOS). Phagocytes such as neutrophils and macrophages as well as TNFα and iNOS expressing (Tip) DCs express iNOS and are capable of producing NO. iNOS can be induced by stimuli such as IFNγ, LPS or TNFα leading to the synthesis of NO from L-arginine. NO is an antimicrobial agent which can inhibit or inactivate enzymes of the citric acid cycle and damage DNA. Furthermore, the reaction of NO with O_2^- leads to the generation of peroxynitrite ($ONOO^-$) capable of destroying proteins, lipids and nucleic acids from pathogens [68, 69].

Degranulation

Neutrophils possess four different types of granules involved in tissue degradation and pathogen killing. The first granules to be discharged are secretory vesicles facilitating the adhesion of neutrophils to the endothelium and their transmigration. The next granules to be released are tertiary and secondary granules containing an overlapping set of proteins including lactoferrin, lysozyme, LL37 and matrix-metalloproteases (MMPs). These proteins contribute to neutrophil recruitment and tissue breakdown [70]. The last granules to be emptied are the primary granules containing defensins and MPO. While primary granules fuse to the phagolysosome, secondary granules can also be secreted in the extracellular environment contributing to the elimination of pathogens [61, 70].

NET formation

NETs are composed of decondensed chromatin, histones and granular, antimicrobial peptides such as MPO, neutrophil elastase, lactoferrin and LL37. The release of NETs leads to the binding and killing of a variety of microbes including bacteria, fungi and parasites. Two different models of NET formation exist: direct neutrophil activation by microbes leads to nuclear decondensation and apoptosis resulting in NET formation. This process is rather slow taking between two to three hours. A faster process is mediated through indirect cell-mediated activation. In this process, lipopolysaccharide (LPS)-mediated activation of platelets induces their binding to neutrophils which is rapidly (10 minutes) followed by NET release. Activation of the NADPH oxidase and ROS production play a central role in initiating the program, however the exact molecular basis of NET formation is still poorly understood [71, 72].

2.3.2 Neutrophils in the orchestration of adaptive immunity

Initially, neutrophils were thought to be short-lived cells that migrate to the site of infection, participate in pathogen killing and finally undergo apoptosis. However, this view has been changed over the last years. Neutrophils secrete numerous cytokines and chemokines (Figure 3) thereby interacting with numerous other cell populations such as DCs, NK cells, T cells and B cells. As a result it is now clear that neutrophils participate in the initiation of the adaptive immune response [47-49].

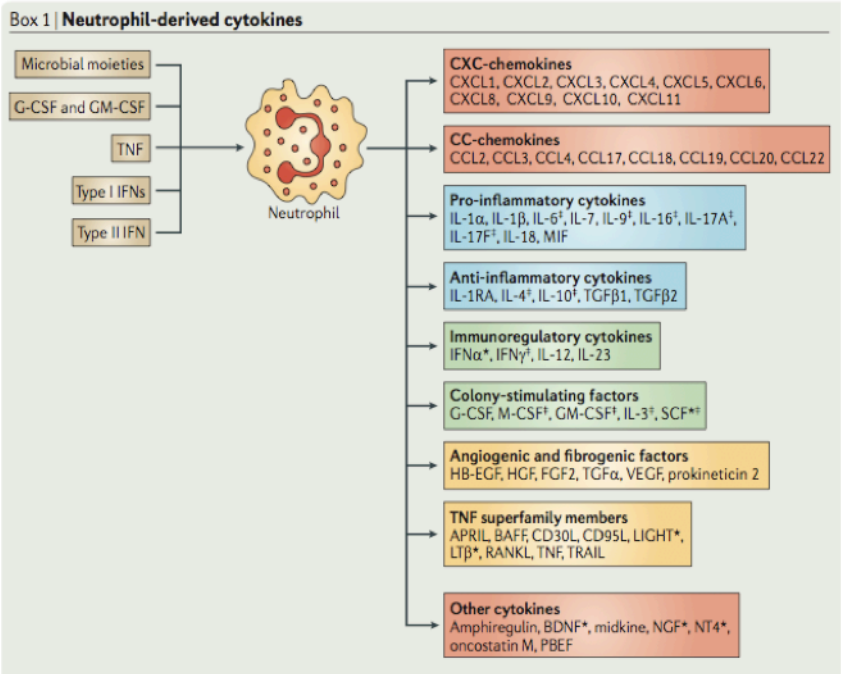

Figure 3: Cytokine secretion pattern of human neutrophils [47]

It was shown in an infection model of *Legionella pneumophilia* that depletion of neutrophils attenuated host defense against *Legionella* and blocked IFNγ production by NK cells. Indeed, IL-18 released by neutrophils induced IFNγ secretion by NK cells in response to *Legionella* infection suggesting that interactions between neutrophils and NK cells are of functional importance [73]. In 2012, Jaeger and colleagues provided novel and unexpected evidence that neutrophils regulate NK cell maturation, function and homeostasis. NK cells from mice lacking neutrophils, either due a genetic mutation or antibody-mediated neutrophil depletion, displayed hyperproliferation, poor survival and were blocked at an immature stage associated with hyporesponsiveness. The role of neutrophils regulating NK cell function was also shown in neutropenic patients [74]. Thus neutrophils are important regulators of NK cell function and homeostasis both in human and mice.

Neutrophils can also interact with lymphocytes. A study by Puga and colleagues demonstrated that neutrophils stimulated the diversification and production of immunoglobulin in the marginal zone of the spleen to T cell-independent antigens. Splenic neutrophils induced immunoglobulin class switching and antibody production by mechanisms involving the cytokines BAFF, APRIL and IL-21. The authors further showed that neutropenic patients had fewer marginal zone B cells and a lower abundance of antibodies to T cell-independent antigens [75].

Neutrophils are also involved in the regulation of T cell responses. Following injection of antigens in adjuvants neutrophils migrate to the dLNs where they control the magnitude and the spread of T cell responses through a mechanism involving the eicosanoid thromboxane A_2 [76]. Furthermore, neutrophils can attract Th1 and Th17 cells to sites of inflammation via the secretion of the chemokines CCL2 and CCL9 or CCL2 and CCL20, respectively [77]. On the other side, Th17 but not Th1 cells secrete CXCL8 leading to neutrophil attraction. Moreover, activated $CD4^+$ and $CD8^+$ T cells, including Th17 cells, produce cytokines including IFNγ, GM-CSF and TNFα that promote neutrophil survival and activation [78, 79].

In summary, these data demonstrate that neutrophils are not just short-lived cells that kill pathogens and undergo apoptosis. They crosstalk with a variety of different cell populations and as a consequence participate in shaping both the innate and the adaptive immune response. Another important cellular crosstalk – between neutrophils and DCs – will be discussed in chapter 4.

2.4 Role of neutrophils in different diseases

Patients with congenital neutrophil deficiencies suffer from severe infections that are often fatal. However, neutrophil products can also be both targets and mediators of autoimmunity. MPO and proteinase 3 (PR3) for instance are main targets for anti-neutrophil cytoplasmic antibodies (ANCA), autoantibodies directed against antigens present in the cytoplasm of neutrophils and involved in diseases such as Wegener's granulomatosis or SLE [80].

Given the importance of neutrophils in humans their role has been studied in several models of infection in mice using neutrophil-depleting antibodies. In most infection models caused by pathogens such as *Mycobacterium, Toxoplasma, Listeria, Legionella, Candida* and *Aspergillus* the presence of neutrophils was associated with resistance to infection [81-86]. In the infection models of *Candida* and *Legionella* susceptibility following neutrophil depletion was associated with a change in the immune response from Th1 to Th2. In *Mycobacterium* and *Toxoplasma* infection the depletion of neutrophils led to a decreased Th1 response

without initiating the development of a Th2 response. In the infection model of *Plasmodium* neutrophils have a deleterious role and depletion of neutrophils prevents infected mice from mortality [87].

These data demonstrate that neutrophils play an important role in both innate and adaptive immunity. However, their activation needs to be tightly regulated to avoid possible adverse effects. The role of neutrophils during infections with *Leishmania* will be discussed in chapter 5.5.

3. Dendritic cells

DCs were first described by Ralph Steinman in 1973. They are the main APCs and essential mediators of immunity and tolerance. DCs are specialized for the capture, processing and presentation of antigens to T cells and constitute the link between the innate and the adaptive immune response. Whether DCs differentiate from a single or multiple precursor cells is still a matter of debate [88].

3.1 DC subsets in mice

DCs are heterogeneous and can be classified in different subsets according to their phenotype, location, immunological function and dependence on infections or inflammatory stimuli for their generation. DCs can be divided into three main subsets as conventional DCs, that can be further subdivided into migratory and lymphoid-tissue resident DCs, inflammatory DCs and plasmacytoid DCs (pDCs) (Figure 4) [88-90]. All DC subsets express the marker CD11c on their surface.

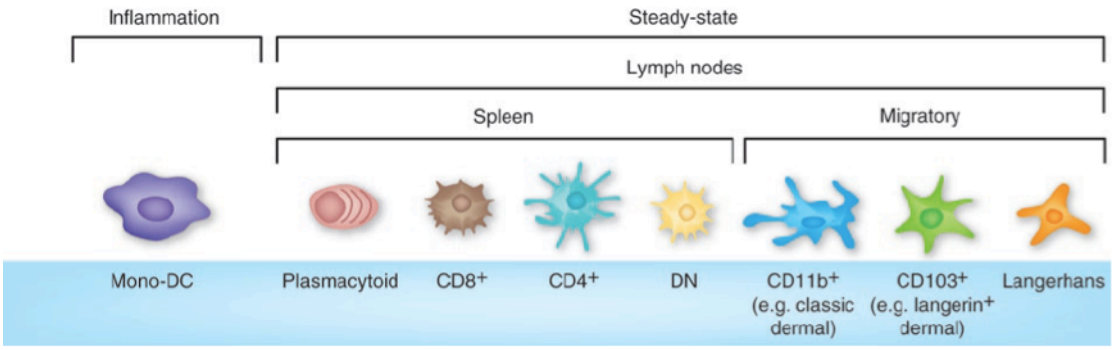

Figure 4: Murine DC subsets during steady state and inflammation [89]

cDCs already have a dendritic form and exhibit DC functions in steady state. Dependent on their location they can be further divided into migratory and lymphoid resident DCs.

Migratory DCs act as sentinels in peripheral tissues such as the skin and are classified in three groups. Langerhans cells (LCs) are localized in the epidermis and express high levels of langerin. The two other DC types are found in the dermis and can be distinguished according to the expression of langerin as langerin$^+$ (also called CD103$^+$) and langerin$^-$ (also called CD11b$^+$) dermal DCs (dDCs) [91].

Lymphoid-tissue resident DCs collect and present antigens in the lymphoid organ itself. They can be further subdivided dependent on the expression of CD8 and CD4 as CD8$^+$, CD4$^+$ or double negative cDCs [92].

Inflammatory DCs are not present in steady state. Their differentiation requires a microbial or inflammatory stimulus. Both inflammatory and non-inflammatory monocytes are thought to be able to differentiate into inflammatory DCs such as monocyte-derived DCs (moDCs) or Tip-DCs [88]. moDCs express the surface marker CD11b and Ly6C while Tip-DCs stain positive for iNOS.

pDCs are also known as natural interferon-producing cells and found as pre-DC in steady state. After stimulation by viral or microbial infection this pre-DC transforms into a pDC secreting large amounts of type I interferons [93]. pDCs express B220 on their surface.

3.2 Activation and function of DCs

DCs are sentinels of the immune system and enriched in non-lymphoid tissues at the interface with the environment and in lymphoid tissues. DCs express numerous PRRs on their surface allowing the sensing of a wide range of PAMPs and DAMPs. However, the expression profile of PRRs varies from one DC subset to another indicating differences in function of these subsets [90].

Immature DCs (iDCs) have a high endocytic activity and constantly sample for antigens. While migratory DCs collect antigens in surface-exposed tissues, lymphoid tissue resident DCs filter antigens arriving from the blood or afferent lymphatic vessels that drain non-lymphoid tissues [94]. Upon recognition and phagocytosis of a foreign antigen, iDCs undergo a maturation process involving phenotypic and functional changes. This process includes the downregulation of their endocytic activity and the upregulation of the chemokine

receptor CCR7 allowing iDCs to home to the dLN following a CCL19 and CCL21 gradient. Furthermore, activated DCs increase the expression of the costimulatory molecules CD80 (B7.1), CD86 (B7.2) and CD40 as well as MHC II. In addition, activated DCs secrete polarizing cytokines such as IL-12, IL-6 and TNFα. The process of maturation and migration to the dLN allows DCs to present antigens to antigen-specific, naïve T cells. Furthermore, DCs deliver costimulatory and activating signals to T cells leading to their activation and proliferation thereby initiating the adaptive immune response [90].

Distinct DC subsets have different although partially overlapping functions. Lymphoid tissue $CD8^+$ DCs are the main producer of IL-12 [95]. Both $CD8^+$ and $CD103^+$ cDC are able to cross-present exogenously acquired antigens on MHC I molecules. $CD11b^+$ cDCs express higher levels of genes encoding proteins involved in the MHC II antigenic pathway compared to $CD8^+$ DCs and are thus thought to have a predominant role in MHC II presentation [96]. The function of LCs *in vivo* is still a matter of debate and might depend on the type of infection or inflammatory signal they are subjected. In this line, different studies using mouse depletion models for LCs have shown that LCs are alternatively dispensable, required or redundant with dermal $CD103^+$ DCs for induction of contact hypersensitivity [97]. Inflammatory DCs such as moDCs are able to carry antigen to the dLNs and induce potent Th1 responses [98]. More work is needed to decipher the exact contribution of each DC subset to the initiation of the adaptive immune response. The role of different DC subsets during infections with *Leishmania* will be discussed in chapter 5.6.

4. Crosstalk between neutrophils and DCs

The crosstalk between neutrophils and DCs has been recently reviewed by Prof. Tacchini-Cottier and me [99]. On the following pages (p. 29 - 32) excerpts of this review are presented.

Neutrophils and DCs are both important members of the innate immune system. Under steady-state condition, these cells are localized in different compartments of the body. However, upon inflammation or infections, neutrophils and DCs colocalize at the site of insult and neutrophils were shown to participate in recruiting DCs and to either activate or inhibit their functions (Figure 5) [99]. The crosstalk of neutrophils and DCs during *L. major* infections will be discussed in a separate chapter 5.7.

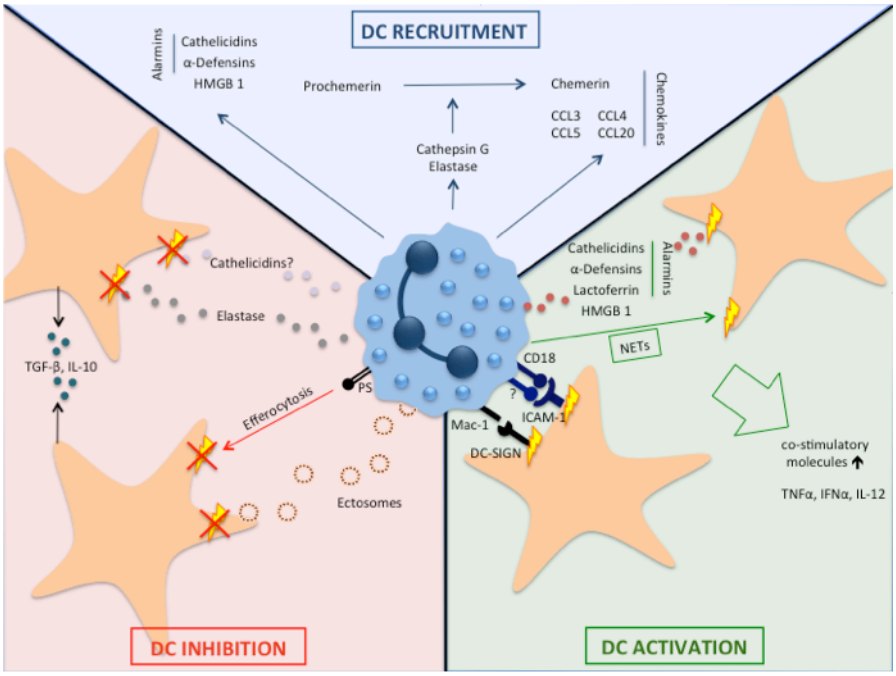

Figure 5: The crosstalk between neutrophils and DCs [99]

4.1 Neutrophils contribute to DC migration to sites of infection and inflammation

It has been known for more than a decade that human and mouse neutrophils exposed *in vitro* to microorganisms (or parts of them) are able to secrete chemokines that contribute to leukocyte accumulation [100]. During neutrophil degranulation, alarmins are also released which contribute to the chemoattraction of iDCs, either by a direct action [α-defensins or the alarmin-like high mobility group protein B1 (HMGB1)] or indirectly through their induction of CCL3 and CCL5 release [101]. In addition, neutrophils release proteases such as cathepsin G and elastase that can process inactive non-classical chemokines such as prochemerin into chemoattractive molecules [102].

Exposure to protozoan parasites such as *Toxoplasma gondii* triggers the neutrophil release of CCL3, CCL4, CCL5 and CCL20 iDC-attracting chemokines and supernatants of *T. gondii* tachyzoite-exposed neutrophils was shown to attract immature cDCs *in vitro* [103]. Mycobacteria were also reported to induce the release of DC-attracting chemokines by neutrophils, and depletion of neutrophils in infected mice delayed DC migration to the dLNs. Interestingly, ingestion of *M. tuberculosis* by neutrophils influenced DC migration: DCs that acquired *M. tuberculosis* through the ingestion of infected neutrophils migrated better to dLNs than DCs that acquired the bacteria directly [104].

Collectively, these studies reveal an important role for neutrophils in promoting DC migration during infection, a property that may vary depending on the host or/and the type of pathogen.

4.2 Conditions where neutrophils induce DC activation

Neutrophils can contribute to the mobilization of DCs to sites of infection, thus an obvious question is to determine how the colocalization of neutrophils and DCs can modulate DC activation. Most of the studies describing neutrophil-induced DC activation were performed *in vitro*, with a few exceptions. In response to several pathogens, neutrophils can release DC-maturing cytokines such as TNFα. Pioneer work from the group of E. Denkers demonstrated that *T. gondii*-exposed mouse neutrophil supernatants induced cDC maturation *in vitro*, as defined by the upregulation of co-stimulatory markers on DCs and their release of TNFα [103, 105]. A role in the activation of cDCs was also reported for BCG-infected neutrophils. Contact-dependent DC activation was characterized by the release of IL-12 by mouse cDCs, and by the secretion of decreased levels of IL-10 in human DCs compared to cDCs exposed to BCG alone [106]. Moreover, mouse neutrophils exposed *in vitro* to *Aspergillus* germ tubes induced the activation of cDCs, also by a contact-dependent mechanism involving DC-specific ICAM3-grabbing non-integrin (DC-SIGN) on DCs [107]. Activated human neutrophils were shown to induce the maturation of human moDCs by cell-cell contact-dependent mechanisms, where Mac-1 (CD11b/CD18) on neutrophils interacts with DC-SIGN inducing the secretion of TNFα. Interestingly, interactions of neutrophils and DCs were visualized in colonic mucosa of patients with Crohn's disease, suggesting that interactions between neutrophils and DCs could occur *in vivo*, however DC activation by neutrophils was not investigated in that study [108]. Contact-dependent activation of DCs by human neutrophils was also reported in response to LPS and IL-2 or IL-15/18 stimulation; neutrophils were shown to potentiate the activity of slanDCs, a subset of DCs present only in humans. Activation was also contact-dependent, but involved CD18/ICAM-1 interactions. In this system, neutrophil-activated DCs released IL-12p70 which induced the secretion of IFNγ by NK cells, potentiating DC-neutrophil interaction and contributing to the launching of the adaptive immune response [109].

Upon degranulation, neutrophils release numerous granule components that can also influence DC maturation. In addition to cytokines such as TNFα, they release alarmins that can activate nearby cells including DCs [110]. Among alarmins, α-defensins, cathelicidins, lactoferrin as well as HMGB-1 that has alarmin-like properties have been reported in most cases to induce the activation of pDCs, either directly or indirectly through their presence in

neutrophil extracellular traps (NETs). The presence of LL-37 on NETs was recently described to activate pDCs, a subset of DCs specialized in sensing nucleic acid danger signals and which upon activation secrete large amounts of IFNα. The activation of pDCs by NETs contributes to the pathogenesis of systemic lupus erythematosus (SLE), an autoimmune disease. Activation of pDCs by NETs resulted in the secretion of TNFα, IL-6 and IFNα, a TLR9-mediated process that involved the neutrophil granule peptides LL-37 and the human neutrophil peptide (HNP) [111, 112]. In psoriasis, secretory leukocyte proteinase inhibitor (SLPI), another component of NETs, was also shown to stimulate pDCs in a TLR9-dependent manner [113]. These studies demonstrate that NETs can modulate the immune response through the activation of pDCs.

Collectively, these studies convincingly show that neutrophils have the potential to activate DCs *in vitro*. However, more studies performed in both humans and mice will need to be conducted to determine the *in vivo* conditions where neutrophils activate DCs, if this activation is restricted to specific DC subsets, and to define how these interactions impact the development of the adaptive immune response.

4.3 Conditions where neutrophils can prevent DC activation

In contrast to their DC-activating role discussed above, in some circumstances, neutrophils can prevent DC activation. Phagocytosis of apoptotic cells (efferocytosis) leads to the resolution of inflammation [114]. In this line, the uptake of apoptotic or necrotic neutrophils was shown to inhibit the induction of co-stimulatory molecules on DCs, affecting T cell responses [115]. On the contrary, the presence of high numbers of apoptotic neutrophils has been reported to induce DC maturation [116] and DCs can acquire antigens through the phagocytosis of microorganism-containing apoptotic neutrophils. Delaying neutrophil apoptosis will thus have a direct effect on the availability of antigens for DCs and the development of the adaptive immune response. In this line, phagocytosis of *M. tuberculosis* by neutrophils was shown to inhibit neutrophil apoptosis, limiting antigen uptake by DCs, a process delaying the onset of the immune response [117]. Collectively, accumulating data suggest that the effect of apoptotic neutrophils on DC maturation may vary and be context-dependent.

Neutrophils can also release cell surface-derived vesicles called ectosomes that expose phosphatidyl serine on their surface, an “eat me” signal for DCs. Ectosomes can prevent the LPS-induced maturation of moDCs *in vitro*. Indeed, human neutrophil ectosomes modified the phagocytic properties of moDCs, impaired the expression of co-stimulatory molecules and the secretion of cytokines including TNFα and IL-12 while they increased the release of TGFβ, an

anti-inflammatory cytokine [118]. Upon neutrophil degranulation, the release of elastase was also shown to downmodulate DC activation, inducing DC secretion of TGFβ [119].

Neutrophils can migrate rapidly and transiently to dLNs. The presence of neutrophils in the dLNs may also influence DC activation, a process that is just beginning to be investigated. Following injection of protein antigens in adjuvants, neutrophils that rapidly migrated to dLNs made brief contact with DCs. Interestingly the brief presence of neutrophils in the dLNs had a major negative impact on the development of $CD4^+$ T cell priming. This negative effect appeared to occur only with the first wave of neutrophils that reaches the dLNs during the first days after injection [120]. It will be interesting to characterize in more details the importance of the early migration of neutrophils to the dLNs in several models of infection, and to better characterize the type of DC involved in the impaired activation and if the direct effect on T cells may also contribute to the neutrophil inhibitory impact.

In summary, neutrophils contribute to the migration of DCs to the site of infection. In addition, they are able to activate DCs or inhibit DC activation in a contact-dependent manner or by the release of soluble factors and NETs. The knowledge gained on the impact of neutrophils on DC function could have important implications in vaccination.

5. Leishmaniasis

5.1 Epidemiology

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus Leishmania. The parasites belong to the order Trypanosomatidae and are spread by the bites of infected sandflies of the genus Phlebotomus or Lutzomyia (figure 6). Leishmaniasis is endemic in 88 countries of the world with a majority of the cases occurring in developing countries mostly in tropical and subtropical regions. Currently, about 12 million people are infected and 350 million people are living in endemic areas. An estimated 2 million new cases (1.5 million cases of cutaneous and 0.5 million of visceral leishmaniasis) occur each year. There are more than 20 different species of leishmania parasites that can infect mammals including humans. In humans, infections with leishmania causes a wide range of clinical symptoms that can be classified in 4 different forms:

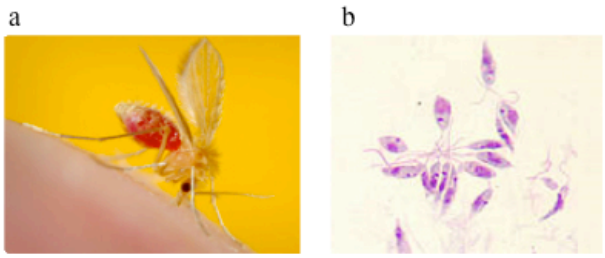

Figure 6: Example of a sand fly vector and leishmania promastigotes in culture (adapted from http://www.cdc.gov/parasites/leishmaniasis)

Cutaneous leishmaniasis (CL)

CL is the most common form characterized by local, ulcerative lesions that are in most cases self-healing. Most cases of CL in the Old World (Africa, Asia and Europe) occur in Afghanistan, Iran, Saudi Arabia, Syria, Ethiopia and Sudan caused by *L. major*, *L. tropica* and *L. aethiopica*. In the New World (North and South America) most cases occur in Brazil and Peru due to infections with *L. mexicana* and *L. amazonensis*.

Diffused cutaneous leishmaniasis (DCL)

DCL is characterized by the presence of disseminated and chronic skin lesions resembling those of lepromatous leprosy. DCL is difficult to treat.

Mucocutaneous leishmaniasis (MCL)

In MCL, parasites disseminate from the skin via the lymphatic system to the naso-oropharyngeal mucosa. This can lead to the destruction of mucosal tissues of the mouth, nose and throat. MCL mostly occurs in Brazil, Bolivia and Peru due to infections with *L. braziliensis.*

Visceral leishmaniasis (VL)

VL, also called *Kala-azar*, is the most severe form of leishmaniasis and can be fatal if left untreated. In VL, the parasites visceralize to inner organs such as the spleen, liver and bone marrow. Manifestations of VL include fever, weight loss, hepatosplenomegaly and pancytopenia. The majority of VL cases occur in just six countries – Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan caused by *L. donovani* in East Africa and the Indian subcontinent and by *L. infantum* (also known as *L. chagasi*) in Europe, North Africa and Latin America.

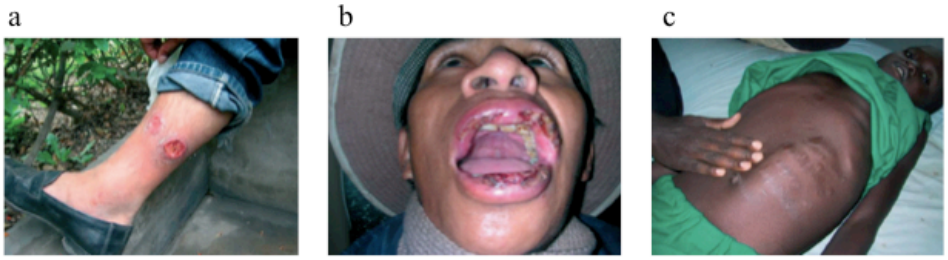

Figure 7: Different clinical forms of Leishmaniasis: Cutaneous (a), mucocutaneous (b) and visceral (c) leishmaniasis (adapted from [121])

5.2 Life-cycle of the parasite

All leishmania spp share a similar life cycle. They are transmitted by the bite of a female sand fly of the genus *Phlebotomus* or *Lutzomyia*. The parasites are found in the salivary glands of the sand fly as metacyclic promastigotes which is the infectious form of the parasite. This form is characterized by an elongated shape and the presence of a flagellum. Once transmitted into the skin of the host, neutrophils are the first cells that arrive at the site of infection and that are in contact with the parasite. Next monocytes and macrophages, the latter being the definite host cells of the parasites, enter the site of infection and take up the parasites. However, it is still not clear whether macrophages take up the parasites directly or via the phagocytosis of infected cells. Within the phagolysosome of macrophages, the metacyclic promastigotes transform into amastigotes. This form is the replicative form characterized by a round shape without flagellum. The amastigotes replicate within the macrophages until the cells burst. Free amastigotes are taken up by surrounding cells or, depending on the species, disseminate to other tissues or organs. The cycle continues when another sand fly is taking a blood meal on an infected host thereby taking up free parasites or infected cells. The parasites are released in the midgut of the sand fly and transform into procyclic promastigotes. This form of the parasite has a low virulence but replicates rapidly by binary fission. Finally, the parasites transform into metacyclic promastigotes, migrate to the salivary gland of the sand fly and the cycle continues when this sand fly takes another blood meal (Figure 8).

Depending on the Leishmania strain and the region, parasites can be transmitted in an anthropontic or a zoonotic manner. Anthroponotic Leishmaniasis is transmitted from human to vector to human while zoonotic Leishmaniasis is transmitted from animal to vector to human.

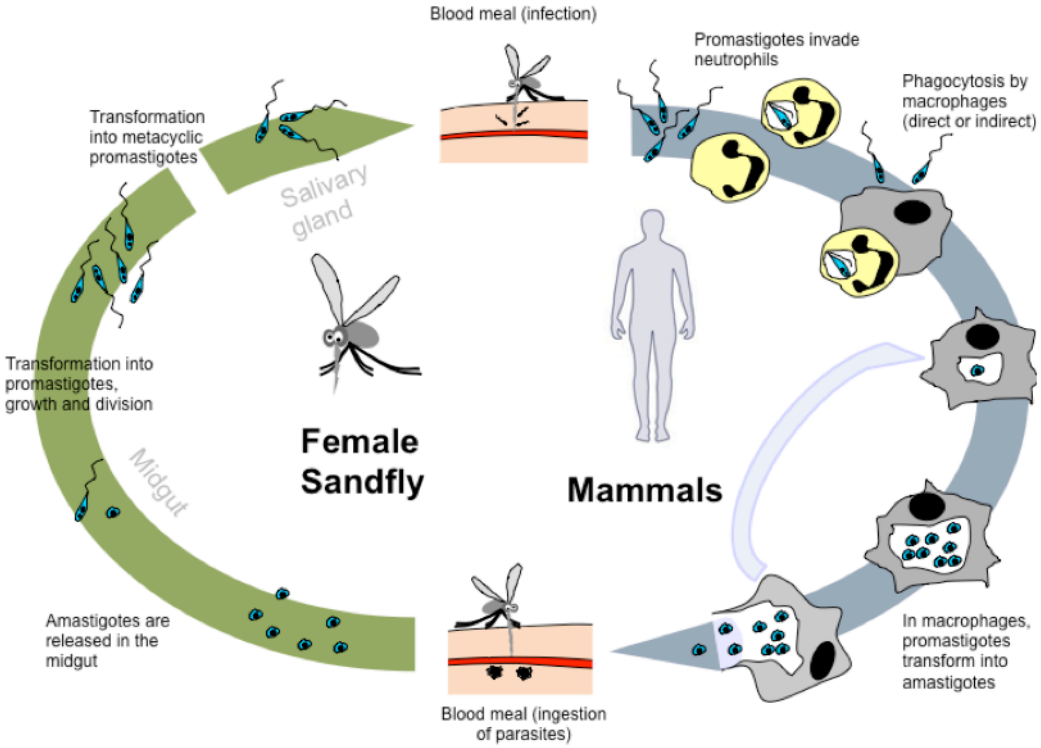

Figure 8: The leishmania life cycle

5.3 Treatment and vaccines

The treatment of leishmaniasis is difficult since infections with different strains of parasites cause different manifestations of the disease. Thus, the treatment approach depends in part on parasite and host factors and the area of the world the patient is located. The first choice among drugs used for the treatment of leishmaniasis are still pentavalent antimonials such as sodium stibogluconate (Pentostam, manufactured by Wellcome Foundation) and meglumine antimoniate (Glucantime, manufactured by Rhone Poulenc). Other chemotherapeutic drugs that are currently used for the therapy of leishmaniasis are amphotericin B, pentamidine, miltefosine and paramomycin. In addition, azoles, allopurinol, sitamaquine and antiretroviral drugs are in clinical use as well. A more recent drug is liposomal amphotericin B for which the elevated prices were reduced by 90% thanks to the WHO's advocacy campaign. However, the cost of these compounds is still high and thus a major issue. Other problems of these drugs are their high toxicity and the numerous adverse side effects leading patients to withdraw from treatment favoring the emergence of resistant strains. Therefore new approaches are needed. One such approach is the combination therapy combining different drugs in order to shorten the duration of drug administration and to prevent the emergence of resistant strains [122, 123].

The most of effective protection against leishmaniasis would be a vaccine. Following resolution of cutaneous lesions, a long-lasting immunity usually develops in both human and mice. This observation let to the assumption that the prevention of the disease by vaccination should be feasible. Great efforts have been taken to develop a protective vaccine against leishmaniasis but to date no effective vaccine against the disease in humans is available yet. The first vaccination strategy against leishmaniasis was the injection of infective and virulent Leishmania from skin lesion exudates called Leishmanization. However, the health threat for patients, especially for immune-compromised patients, treated this way was too high and first generation vaccines consisting of killed whole parasite and live attenuated parasite vaccines were developed. Other vaccination strategies that have been or are currently being evaluated are DC-based vaccines, salivary antigen-based vaccines and DNA vaccines [124-126]. More work and a better understanding of the factors regulating parasite persistence and maintaining immunological memory are needed, both in mice and especially in humans. These insights might be critical for the design and the development of an effective anti-*Leishmania* vaccine.

5.4 The murine model of *L. major* infection

The experimental murine model of *L. major* infection led to the first observations that revealed the involvement of different T helper subsets in disease outcome. Since then it has been an important tool in deciphering T helper cell differentiation *in vivo*. Initially, a high dose of stationary phase parasites (1 to 3 million) was injected subcutaneously in the hind footpad. Recently, another model injecting a lower dose (10 – 1000) of metacyclic promastigotes in the ear dermis was developed which better mimics the number of parasites transmitted during the bite of a sand fly [127].

Following the transmission of the parasite either by needle inoculation or the bite of an infected sand fly, most mouse strains such as C57BL/6, C3H, SV129 or B10.D2 develop a small lesion which heals over time. These mice develop a Th1 response characterized by the secretion of high levels of IFNγ by $CD4^+$ T cells. IFNγ leads to iNOS induction in macrophages and subsequent NO production. This results in the killing of the parasites and control of infection. In addition, these mice are resistant to a second parasite challenge and thus called *Leishmania*-resistant mice.

Few mouse strains however, such as BALB/c mice, develop an inflammatory non-healing lesion. These mice develop a Th2 immune response and secrete high levels of IL-4, IL-13 and IL-5. These cytokines lead to alternative macrophage activation and to proliferation and dissemination of the parasites. As a result these mice succumb to infection and are called *Leishmania*-susceptible mice (Figure 9).

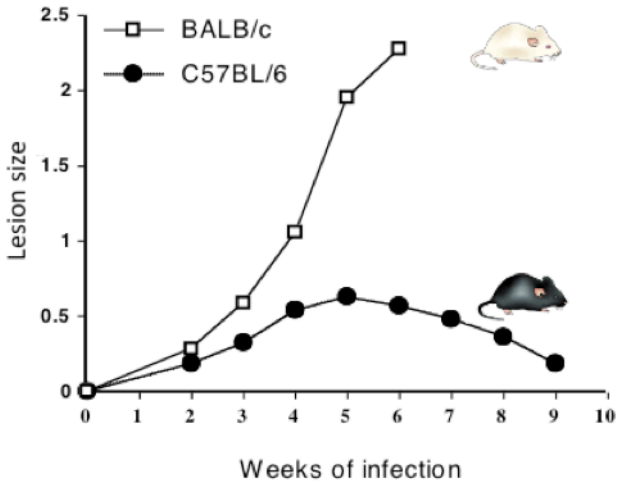

Figure 9: The murine model of *L. major* infection

The murine model of *L. major* infection has been studied extensively to decipher the mechanisms leading to resistance or susceptibility to infection. The mechanisms include host genetic factors as well as the developing immune response [128-130]. Studies on mice of the *leishmania*-resistant C57BL/6 background showed the importance of IL-12 and IFNγ in resistance to a high dose *L. major* infection. IL-12p70 is a heterodimer formed by the subunits IL-12p40 and IL-12p35. Following *L. major* infection mice lacking IL-12p40, IL-12p35 or the IL-12-singaling transducer Stat4 develop non-healing lesions and show exacerbated parasite growth [131-133]. Mice deficient for IFNγ default to the Th2 pathway and are susceptible to *L. major* infection [134]. Mice lacking the IFNγ receptor are also unable to control parasite growth and lesion size despite the development of a polarized Th1 immune response [135]. IFNγ induces iNOS expression in macrophages leading to NO-production and parasite killing. The importance of iNOS was shown when resistant mice treated with iNOS inhibitor failed to control infection [136].

The mechanisms leading to susceptibility to *L. major* infection were mainly studied using mice on the leishmania-susceptible genetic background BALB/c. One important cytokine for the induction of Th2 immune responses is IL-4. Following *L. major* infection, a $CD4^+$ T cell subset expressing the Vβ4Vα8 T cell receptor recognizes the immunodominant epitope *Leishmania* activated C kinase (LACK) resulting in an early burst of IL-4 [137]. This early IL-4 is thought to induce IL-12 unresponsiveness and may thus be important for the induction of a Th2 immune response [138, 139]. In 1996, Kopf and coworkers published that IL-4-deficient mice on a BALB/c background were resistant to infection following a high dose inoculation of *L. major* (strain FEBNI) [140]. However, in the same year the group of Muller showed that BALB/c mice deficient for IL-4 were still susceptible to a high dose infection of *L. major* (strain LV39) infection indicating that $IL\text{-}4^{-/-}$ BALB/c mice reveal

differences in susceptibility to *L .major* substrains and that other factors may be involved in susceptibility to *L. major* infection [141]. In 1999, Noben-Trauth and colleagues showed that IL-4$^{-/-}$ BALB/c mice were susceptible to infections with *L. major* LV39 but partially controlled infections with *L. major* IR173. Several factors including the arginase activity of different *L. major* isolates as well as the age of infected mice were shown to contribute to the *L. major* strain-specific disease outcome in IL-4$^{-/-}$ BALB/c mice [142]. It has been further demonstrated that BALB/c mice deficient for the IL-4 receptor α chain (IL-4Rα$^{-/-}$) were susceptible to infections with *L. major* LV39 while IL-4Rα$^{-/-}$ mice efficiently controlled infections with *L. major* IR173 [143]. The fact that IL-4Rα$^{-/-}$ mice were more resistant to *L. major* IR173 infection than IL-4$^{-/-}$ mice suggested a role for IL-13 since IL-4Rα is a component of both the IL-4R and the IL-13R. Indeed, mice deficient for IL-13 are resistant to *L. major* infection [144]. However, blocking IL-13 signaling in IL-4$^{-/-}$ mice did not change the course of infection and these mice were still susceptible to *L. major* infection [145]. These findings suggest that other factors than just IL-4 and IL-13 are involved in susceptibility to *L. major* infection.

Other factors involved in resistance or susceptibility to *L. major* infection are IL-17, IL-10, TNFα, arginase activity and Treg cells. Following *L. major* (strain Friedlin) injection BALB/c mice deficient for IL-17A developed smaller lesions and showed decreased parasite loads but still mount a Th2 immune response. Improved disease outcome was associated with a reduced neutrophilic infiltrate into lesions of IL-17$^{-/-}$ mice [146]. BALB/c mice lacking IL-10 or treated with an anti-IL-10R mAb were partially resistant to *L. major* LV39 infection. Lesion sizes and parasite loads are further reduced in BALB/c IL-4Rα x IL-10 double knockouts [147]. In C57Bl/6 mice IL-10 plays an essential role in parasite persistence since sterile cure is achieved in IL-10$^{-/-}$ mice and in mice treated with an anti-IL-10R mAb during the chronic phase of *L. major* infection [148]. An important role for TNF has been shown using TNF$^{-/-}$ and membrane TNF-knock-in (mTNF) mice that express functional mTNF but do not release soluble TNF. While TNF$^{-/-}$ mice developed unhealing lesions mTNF mice controlled lesion development and were able to clear neutrophils from the site of infection showing that the membrane bound form of TNF is sufficient to mediate protection [149]. Another factor involved in susceptibility to *L. major* infection is the enzyme arginase which is a hallmark of alternatively activated macrophages and induced by Th2 cytokines such as IL-4. Arginase activity promotes the growth of *Leishmania* parasites by affecting the polyamine synthesis in macrophages and promotes pathology during *L. major* infection [150] Besides soluble and cell-intrinsic factors, different cell populations such as Treg cells are also involved in promoting resistance or susceptibility to *L. major* infection. Indeed, depletion of Treg cells in C57BL/6 mice enhances the development of a Th1 response but renders them

susceptible to reinfection [151]. In BALB/c mice however, depletion of Treg cells enhances the development of a Th2 response and these mice harbor more parasites and increased lesions [152].

In summary, the mechanisms leading to resistance or susceptibility to *L. major* infection involve multiple mechanisms and are a combination of genetic and cellular factors as well as cytokines. The murine model of *L. major* infection is an invaluable tool to further study these mechanisms and new insights can be used for novel vaccine designs.

5.5 The role of neutrophils in *L. major* infection

Neutrophils are the first cells that are rapidly and massively recruited to the site of *L. major* infection. Numerous studies have investigated the role of neutrophils in response to *L. major*, both *in vitro* and *in vivo*. However, the impact of neutrophils in promoting resistance or susceptibility is still not fully understood. One reason for that is the heterogeneity of these studies using different parasite strains, doses, different mouse strains and treatments to deplete neutrophils. Furthermore, different ways of parasite transmission had been used such as needle inoculation in the footpad or the ear or infection of ears by bites of infected sand flies [153-155].

First studies by Beil *et al*. described the predominant neutrophilic infiltrate 3h post injection of a high dose of *L. major* in the footpad of C57BL/6 and BALB/c mice [156]. Since then these findings have been confirmed and extended in both mouse strains to the ear pinna injection site by needle inoculations as well as by the bite of infected sand flies in C57BL/6 mice [157-161]. Chemokines such as IL-8 in humans and CXCL1 (KC), CXCL2 (MIP-2) and CXCL6 (GCP-2) in mice contribute to neutrophil recruitment [162]. Following *L. major* inoculation in the skin of mice KC mRNA expression has been reported to be rapidly upregulated [157]. Furthermore, the parasites themselves produce the *leishmania* chemotactic factor (LCF) inducing IL-8 secretion by human neutrophils promoting their own recruitment [163]. Other factors contributing to neutrophil recruitment to the site of infection are complement C3 as well as components of the salivary glands of *Lutzomyia intermedia* and *Lutzomyia longipalpis* [64, 164, 165]. IL-17 contributes to neutrophil recruitment in later phases of infection in BALB/c mice following *L. major* inoculation [146]. Thus, various factors derived from the host, the parasite and the vector contribute to neutrophil recruitment to the site of *L. major* infection.

Once at the site of infection neutrophils secrete numerous cytokines, chemokines, granular components and interact with other cells and participate in shaping the immune response. However, *L. major* induces distinct neutrophil phenotypes in mice resistant or susceptible to *L. major* infection [166]. The expression levels of TLR2, TLR7 and TLR9 in

response to *L. major in vitro* are significantly higher on neutrophils from C57BL/6 mice. Furthermore, only neutrophils from C57BL/6 mice secrete IL-10 and biologically active IL-12 while those from BALB/c mice secrete IL-12p40 homodimers that block IL-12 signaling [166]. In addition, neutrophils from C57BL/6 mice release two to three times more neutrophil elastase than neutrophils from BALB/c mice [167]. Moreover, the outcome of co-cultures of apoptotic non-infected neutrophils with macrophages infected with *L. major in vitro* is dependent on the genetic background of the donor mice. The engulfment of apoptotic neutrophils by macrophages from C57BL/6 mice led to parasite killing, a process which was dependent on TNF, neutrophil elastase and TLR4 expression by macrophages. In contrast, using cells from BALB/c mice resulted in exacerbated parasite growth through the production of PGE_2 and TGFβ [167, 168]. Moreover it was shown that co-cultures of neutrophils with an excess of macrophages significantly increased neutrophil apoptosis. This finding was even more enhanced when *L. major* parasites were added to the cultures [169].

The interactions of neutrophils with macrophages *in vivo* are not clear. It was demonstrated that *L. major* parasites are able to inhibit the oxidative burst and thus survive in neutrophils [170]. In 2008, Laskay and colleagues reported that macrophages phagocytose apoptotic neutrophils containing live *L. major* and through this process silently take up the parasites in the absence of activation signals induced via direct engulfment of the parasites. This process was termed the Trojan Horse infection model [171]. In the same year, Peters and colleagues used two-photon intra-vital microscopy to visualize the events occurring early post *L. major* inoculation in the ear dermis and could not observe the uptake of infected neutrophils by macrophages within the analyzed time frame [158]. Thus, macrophages take up the parasite either via the Trojan Horse model or by phagocytosis of viable parasites released by apoptotic neutrophils. In both models, the anti-inflammatory effect of the efferocytosis of dying neutrophils by macrophages leads to the silent entry of the parasite into macrophages.

The role of neutrophils in the development of the immune response and disease progression following *L. major* infection *in vivo* was mainly studied by treating mice with neutrophil-depleting monoclonal antibodies (mAbs) [158, 161, 168, 172, 173]. Initial studies were carried out using the mAb RB6-8C5 which recognizes the Gr1 epitope present on Ly6G and Ly6C [174]. Thus this mAb does not only deplete neutrophils but also other cells expressing Gr1 including inflammatory monocytes, eosinophils and subsets of DCs. Another mAb used to deplete neutrophils is the NIMP-R14 [175]. This mAb efficiently depletes neutrophils, however the exact epitope and possible cross-reactivity with other cells are not completely known. The third neutrophil-depleting mAb is the 1A8 [176]. This mAb is neutrophil-specific and does not deplete other cell populations. However, the depletion of

neutrophils is incomplete and does not last as long as with the other two mAbs. Depending on the mAb used and the site and route of parasite injection the outcomes vary.

Mouse strain	Site and route of infection	Parasite strain	Antibody	Outcome	Ref.
C57BL/6	Footpad (sc)	Bokkara	RB6-8C5	Increased parasite load in dLN 6h, 24h, 16d and 29d p.i.	[172]
C57BL/6	Footpad (sc)	LV39 (MRHO/Sv/59/P)	NIMP-R14	Increased parasite load in fp 35d p.i; no difference 65d p.i.	[161]
C57BL/6	Footpad (sc)	LV39 (MRHO/Sv/59/P)	RB6-8C5	Increased parasite load in dLN 10d p.i.	[168]
C57BL/6	Ear Sand fly	Friedlin (MHOM/IL/80)	RB6-8C5	Decreased parasite loads in ear 2 and 4 weeks p.i.	[158]
BALB/c	Footpad (sc)	Bokkara	RB6-8C5	No difference in dLN 24h, 16d and 29d p.i.	[172]
BALB/c	Footpad (sc)	LV39 (MRHO/Sv/59/P)	NIMP-R14	Decreased parasite load in fp 35d p.i.	[161]
BALB/c	Footpad (sc)	LV39 (MRHO/Sv/59/P)	RB6-8C5	Decreased parasite load in dLN 10d p.i.	[168]
BALB/c	Footpad (sc)	MHOM/SU/73/ 5ASKH	RB6-8C5	Increased parasite loads in fp 11 weeks p.i.	[173]

Table 1: Outcomes of Leishmania infection following neutrophil depletion *in vivo* (adapted from [155])

Studies carried out in our lab using the mAb NIMP-R14 showed that transient depletion of neutrophils prior to infection with *L. major* resulted in a transient increased susceptibility to infection in C57BL/6 mice. However, the final disease outcome was not affected by early neutrophil depletion [161]. In BALB/c mice however, depletion of neutrophils prevented the early burst of IL-4 mRNA otherwise occurring in the dLN of BALB/c mice. Furthermore, $CD4^+$ T cells remained responsive to IL-12 signaling and decreased Th2 immune responses were observed. This resulted in the development of non-progressive, chronic lesions and decreased parasite loads [161]. These results indicate an important and deleterious role for neutrophils in susceptible BALB/c mice while their role might be less important in resistant C57BL/6 mice. An overview of results from different studies on the outcome of *L. major* infection following neutrophil depletion *in vivo* is shown in table 1.

Following *L. major* infection, neutrophils are the first cells to arrive at the site of insult. Although many studies have been performed to analyze their role during *L. major* infection, it is still not clear if and how neutrophils promote or suppress disease progression. More work and better tools are needed to be able to understand the impact of neutrophils during infections with *L. major*.

5.6 The role of DC subsets during *L. major* infection

DCs are the main APCs of the body and constitute the link between the innate and adaptive immune system. During infections with *L. major*, DCs were shown to be indispensable to launch a protective immune response. Phagocytosis of *L. major* by DCs leads to their activation and IL-12 secretion [177]. However, it is still controversial which DC subset is presenting *L. major* antigens in the dLN *in vivo*. In 2004, Ritter *et al* showed that following high dose *L. major* infection $CD11b^+$ LN-resident DCs and $CD8a^-$ $langerin^-$ dermal DCs were infected and responsible for leishmania antigen presentation in the dLN [178]. Two years later in 2006, Iezzi *et al*. demonstrated that LN-resident rather than skin-derived DCs initiated specific T cell responses after a high dose injection *L. major* IR173 or Friedlin [179]. In 2007, Leon *et al.* showed that moDCs formed at the site of infection control the induction of protective Th1 responses against *L. major* [98]. The role of LCs in *L. major* infection is controversial. In 2004, Ritter and colleagues showed that LCs are dispensable following a high dose of *L. major* FEBNI infection. However, in 2011, Kautz-Neu and coworkers demonstrated that LCs have a negative effect on disease progression since the selective depletion of LCs resulted in reduced Treg cell immigration to the site of infection and an enhanced Th1 response resulting in attenuated disease following inoculation of a low dose of metacyclic *L. major* Friedlin parasites [180]. Thus it is still not clear which DC subset is initiating a protective immune response but it might be possible that different DC subsets have distinct functions for the initiation and the maintenance of the immune response.

5.7 The crosstalk of neutrophils and DCs during *L. major* infection

Both neutrophils and DCs have important functions during *L. major* infections. However, only little is known about their crosstalk during infection. Our lab has demonstrated that following *L. major* inoculation, neutrophil-derived CCL3 is essential for the recruitment of moDCs to the site of infection in C57BL/6 but not BALB/c mice. Depletion of neutrophils abolished recruitment of this DC subset to the site of infection. Furthermore, *L. major*-infected $CCL3^{-/-}$ mice adoptively reconstituted with WT neutrophils were able to attract DCs to the site of parasite inoculation, demonstrating that neutrophil-derived CCL3 is contributing

to the early DC recruitment following *L. major* infection [159]. Moreover neutrophils were shown to inhibit DC activation in the skin early after *L. major* infection. Infected neutrophils in the skin expressed elevated levels of apoptotic markers and were preferentially captured by dermal DCs. Depletion of neutrophils prior to *L. major* infection led to increased DC activation, enhanced the priming of *L. major* specific $CD4^+$ T cells and promoted the early anti-leishmania response [160]. Of note, both of the above mentioned studies were performed using resistant C57BL/6 mice. The impact of neutrophils on the recruitment and activation of DCs in susceptible mice is less clear. Also, the role of neutrophils on DC function in the dLN following *L. major* infection is not well understood.

6. Triggering receptor expressed on myeloid cells 1

Triggering receptors expressed on myeloid cells (TREMs) are evolutionary conserved innate immune receptors which have been described over the last years. In human, the TREM family consists of TREM receptors 1 and 2 as well as TREM-like receptors 1 to 4. TREM3 exists only as pseudogene in human. In mice, this family consists of TREM receptors 1 to 5 as well as TREM-like receptors 1, 2, 4, 5 and 6 and the recently characterized TREM family member PDC-TREM [181]. TREM-like genes and pseudogenes have been predicted by computational analysis of the TREM genomic region [182].

TREM1 is a transmembrane glycoprotein that consists of a single extracellular immunoglobulin-like domain of the V-type, a transmembrane region with a charged lysine residue and a short cytoplasmic tail. It associates with the adaptor molecule DNAX activation protein 12 (DAP12) for signaling and function [183, 184]. In human, TREM1 is expressed on blood neutrophils, $CD14^{hi}$ monocytes and macrophage subsets, in particular on lymph node, peritoneal and alveolar macrophages. In mice, neutrophils, monocytes, macrophages and BMDCs but not bone-marrow derived macrophages express TREM1 [181]. Additionally to expression on the cell surface, TREM1 is also found as soluble form (sTREM1) in serum and bronchoalveolar lavage fluid (BALF) in patients with infections. Increased concentrations of sTREM1 in the BALF have been shown to represent a highly precise marker for the presence of bacterial or fungal infections in pneumonia patients [185, 186]. The functions of sTREM1 as well as the ligand for TREM1 are still unknown.

TREM1 is expressed on both monocytes and neutrophils. On neutrophils TREM1 ligation in synergy with LPS stimulation leads to secretion of IL-8 and myeloperoxidase [183, 187]. Moreover it has been shown that ligation of TREM1 induced rapid degranulation of neutrophilic granules, respiratory burst and, to a lesser extent, phagocytosis, the major effector functions for clearance of pathogens. The activation of effector functions occurred

synergistically with various TLR ligands, e.g. LPS (TLR4), Pam_3Cys (TLR2) or R-848 (TLR7/8). However, TREM1 accelerates apoptosis in the TLR-stimulated neutrophil population [188]. Thus TREM1 plays a regulatory role not only in activating effector function, but also in limiting neutrophils survival and, along with this, an ongoing inflammatory response by the induction of apoptosis.

TREM1 is also expressed on mononuclear phagocytes and administration of mononuclear phagocytes with an agonistic TREM1 antibody followed by activation of PRR such as those of the TLR family, results in greater increase in cytokine and chemokine secretion such as TNFα, GM-CSF, IL-1β, IL-8 and MCP-1, compared to either stimulus alone. Treatment of monocytes with an agonistic TREM1 antibody was also shown to increase expression of molecules related to antigen presentation and T cell activation such as CD86 and MHC class II, which correlated with a greater ability of these cells to activate T cells *in vitro*. Moreover engagement of TREM1 led to a strong up-regulation of surface expression of the adhesion molecules CD29, CD11c and CD49e [183, 184, 187, 189, 190]. Crucial functions for TREM1 signaling *in vivo* have also been reported. In a mouse model of LPS-induced endotoxemia it was shown that blocking TREM1 signaling either by a TREM1-Fc fusion protein or by an antagonistic peptide (LP17) was sufficient to reduce circulating inflammatory cytokines to sublethal levels and thus protect the experimental animals from septic shock and death [183, 190]. Furthermore, TREM1 was shown to be involved in human inflammatory bowel disease and in models of experimental colitis [191, 192]. In summary, TREM1 is thought to be a positive regulator of inflammatory responses.

Material and Methods

1. Mice

Female C57BL/6, BALB/c and DBA mice were purchased from Charles River (Lyon, France). C57BL/6 Trem-1-deficient mice were given by Prof. C. Muller (University of Bern, Switzerland). All mice were kept in the pathogen-free facility at the CIIL center in Epalinges and used between 6 to 8 weeks of age. All animal experimental models were approved by the veterinary office regulations of the State of Vaud, Switzerland, authorization 1266.5 and 1266.6 to FTC.

2. Parasites, injections and limiting dilution assay

L. major LV 39 parasites (MRHO/Sv/59/P strain) expressing the red fluorescent protein mcherry were a gift from Prof. Jeremy Mottram (University of Glasgow, Scotland). The parasites were maintained *in vivo* in DBA mice and grown *in vitro* in M199 media (GIBCO) supplemented with 10% FCS (PAA Laboratories), 4% HEPES (Amimed), 1% antibiotics (penicillin, streptomycin, neomycin, GIBCO) and 50 μg/ml hygromycin B (PAA Laboraties). For ear infections, $2x10^5$ stationary phase or metacyclic parasites in 10 μl incomplete DMEM (GIBCO) were injected i.d. in the ears. Lesion development was measured weekly using a Vernier caliper. For *in vitro* experiments, metacyclic parasites were isolated from stationary growth cultures using a ficoll (Sigma) gradient. Briefly, a three phase gradient was established by underlying the parasites first with 10% ficoll followed by 20% ficoll. The falcon was spun for 15 min at room temperature without break and the top two phases containing the metacyclic parasites collected. The parasites were washed and resuspended in IMDM medium (Gibco) containing 10% FCS (Brunschwig), 10 mM HEPES, 50 μM β-mercaptoethanol and 50 U/ml antiobiotics (penicillin, streptomycin, Gibco) (further referred to as complete IMDM). To determine parasite loads in infected ears, ears were digested (see chapter 6) and limiting dilution assays using ten-fold serial dilutions plating $1x10^5$ cells in 100 μl in the first column/dilution were performed [193]. The numbers of parasites per ear were calculated using the program Estimfree.

3. *in vitro* experiments

3.1 CD8α$^+$ MutuDC line

The CD8α$^+$ MutuDC line H-2^d [194] was given by Prof. Hans Acha-Orbea and kept *in vitro* in complete IMDM at 37C with 5% CO_2.When required DCs were incubated for 10 min in 1X sterile PBS supplemented with 5 mM EDTA and 20 mM HEPES and harvested. The cells were washed, resuspended in complete IMDM and used for the various experiments.

3.2 Purification of inflammatory neutrophils

BALB/c mice were injected with 500 µl sterile PBS containing 2% starch (Merck). Twelve hours later, mice were sacrificed and peritoneal washes in a final volume of 7 ml cold PBS performed. Peritoneal exudate cells (PECs) were washed, resuspended in PBS containing 0.5 % BSA (AppliChem) and 2 mM EDTA and filtered through a 40 µm cell strainer. PECs were stained with an FITC-anti-mouse Ly6G mAb and purified by magnetic activated cell sorting (MACS) using anti-FITC beads (Milteny Biotech) according to the manufactures instructions. Inflammatory neutrophils were resuspended in complete IMDM and purity of neutrophils was at least 96% as analyzed by FACS staining. For conditions where apoptotic neutrophils were used, neutrophils were exposed to UV light for 10 min. After this treatment around 75% of cells were apoptotic as analyzed by Annexin V staining. In some experiments, neutrophils were incubated with 0.2 mg/ml indomethacin (Sigma) for 15 min at 37C, washed and added to the cultures.

3.3 Setup of *in vitro* cultures

For *in vitro* experiments, 2.5x10^5 CD8α$^+$ Mutu DCs were plated in 600 µl complete IMDM in a 24-well plate. Twelve hours later, 2.5x10^6 neutrophils were added to the appropriate wells in a volume of 200 µl. At the same time, 1.25x10^6 metacyclic *L. major* parasites (ratio DC : parasites = 1 : 5) were added to the appropriate wells. As positive controls, DCs or neutrophils were stimulated with 0.1 µM CpG (Trilink), 5 µg/ml poly I:C (Invivogen) and 10 ng/ml IFNγ (eBioscience). All wells were added up with complete IMDM to a final volume of 1 ml. After 18 hours of co-cultures supernatants were taken and cells harvested as described above. Levels of IL-12p70 (BD Bioscience), IL-12p40 and TNFα (both eBioscience) in supernatants were analyzed by ELISA according to the manufacturer's

instruction. Expression of activation markers on DCs and apoptosis of neutrophils were analyzed by FACS (see chapter 4).

4. Flow cytometry

4.1 Analysis of cell surface molecules

For analysis of surface molecules, cells were incubated with the mAb 24G2 on ice to block FcRs and then stained with antibodies in 50 µl FACS buffer (PBS containing 3% FCS) for 20 min on ice. After one washing step the cells were incubated with secondary reagents in 50 µl FACS buffer on ice for 20 min. Cells were washed again and resuspended in 100 µl for flow cytometrical analysis. In some experiments DAPI (20 µg/ml) was added to the samples right before acquisition to exclude dead cells. All antibodies and secondary reagents used are listed in table 2.

4.2 Intracellular staining

For intracellular cytokine staining, $1x10^6$ dLN cells were stimulated with PMA (50 ng/ml, Sigma), ionomycin (500 ng/ml, Sigma) and Brefeldin A (1 µg/ml, BD Pharmingen) for 4h at 37°C. Cells were then harvested, washed, blocked and stained with the cell surface marker AF700-anti-mouse CD4 and APC-anti-mouse CD8. Cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 2% FCS and 0.5% saponin (Sigma) over night. Intracellular cytokines were stained using PE- anti mouse IFNγ, FITC-anti mouse IL-4 or the isotype controls PE- IgG2a or FITC- IgG1.

Target	Species	Clone	Conjugate	Supplier
Ly6G	Rat	1A8	FITC	BD Pharmingen
Ly6G	Rat	1A8	APC-Cy7	BioLegend
Ly6G	Rat	NIMP-R14	FITC	Hybridoma
CD11c	Ar hamster	N418	PE-Cy7	eBioscience
F4/80	Rat	BM8	APC	eBioscience
F4/80	Rat	BM8	Biotin	BioLegend
CD11b	Rat	M1/70	eFluor 450	eBioscience
CD80	Ar hamster	16-10A1	FITC	BD Pharmingen
CD80	Ar hamster	16-10A1	APC	eBioscience
CD86	Rat	GL1	PE	BD Pharmingen
MHC II	Rat	M5/114.15.2	AF700	BioLegend
CD40	Ar hamster	HM40-3	Biotin	eBioscience
Ly6C	Rat	HK1.4	FITC	BioLegend
Ly6C	Rat	HK1.4	APC	eBioscience
B220	Rat	RA3-6B2	PE	eBioscience
Gr-1	Rat	RB6-8C5	PE	BD Pharmingen
Gr-1	Rat	RB6-8C5	APC	eBioscience
CD4	Rat	GK1.5	AF700	BioLegend
CD8	Rat	53-6.7	APC	BioLegend
CD45	Rat	30-F11	PerCP-Cy5.5	BD Bioscience
TcRβ	Ar hamster	H57-597	PE-Cy5	eBioscience
CD19	Rat	6D5	APC	BioLegend
IFNγ	Rat	XMG1.2	PE	eBioscience
IL-4	Rat	BVD6-24G2	FITC	eBioscience
IgG1	Rat	eBRG1	FITC	eBioscience
IgG2k	Ar hamster	B81-3	FITC	BD Pharmingen
IgG1	Rat	eBRG1	PE	eBioscience
IgG2a	Rat	eBR2a	PE	eBioscience
IgG2b	Rat	RTK4530	AF700	BioLegend
Streptavidin			PE-Cy5	BD Bioscience
Annexin V			FITC	BioLegend
Annexin V			PE	BD Pharmingen

Table 2: Reagents used for flow cytometry

4.3 Staining for apoptosis

For analysis of apoptosis, cells were first blocked and stained with primary mAbs as described above. Then cells were washed twice with PBS and resuspended in 100 µl Annexin V binding buffer (BD Pharmingen). Five minutes before acquisition 2.5 µl of Annexin per sample were added. Right before acquisition 1 µl of DAPI (20 µg/ml) was added to the samples. Cells negative for both Annexin V and DAPI were considered viable. Annexin V single positive cells were considered as early apoptotic and double positive cells as late apoptotic or necrotic.

4.5 Analysis

All cell events were acquired on a LSR II or LSR II SORP flow cytometer (BD Bioscience). Data were analyzed using FlowJo software (Tree Star).

5. Neutrophil depletion

For transient neutrophil depletion mAbs were used. Six hours prior to and 1.5 days post infections, mice were inoculated i.p. with 100 µg of the mAb NIMP-R14 (Adipogen, Epalinges, Switzerland), a rat IgG2b mAb recognizing selectively Ly6G [175]. As control, mice were inoculated i.p. with 100 µg of the mAb RR3-16 recognizing the V alpha 3.2 chain of the T cell receptor in mouse strains bearing the b haplotype such as C57BL/6 mice (gift from R. McDonald, Ludwig Institute of Cancer Research, Epalinges, Switzerland). Of note, the V alpha 3.2 chain is absent in mice with the d haplotype including BALB/c mice.

6. Digestion of ears and dLNs

For analysis of cellular content and expression of activation markers on DCs, infected ears were taken, the dorsal and ventral sheets separated with forceps, cut into small pieces and incubated in 1 ml DMEM medium (GIBCO) containing 0.2 mg/ml Liberase TL (Roche) at 37°C. After two hours the reaction was stopped by adding 2 ml of DMEM medium supplemented with 5% FCS, 2% HEPES, 2% antibiotics (penicillin, neomycin, streptomycin) and 1% β-mercaptoethanol (further referred to as complete DMEM). Cells were filtered through a 40 µm cell strainer in a final volume of 10 ml, washed and counted. dLNs were

perfused with 1 ml DMEM medium containing 1 mg/ml collagenase D (Roche) and 40 µg/ml DNase (Roche) using a syringe and incubated for 15 minutes at 37°C. The reaction was stopped by adding 2 ml of complete DMEM. Lymph nodes were then homogenized in a final volume of three ml using a glass homogenizer, washed and counted.

7. Cytokine detection

Mice infected with *L. major* were sacrificed at the indicated time points. dLNs were taken and digested as described above. Cells were incubated at a concentration of $5x10^6$ cells/ml in complete DMEM alone, on plate-bound anti-CD3 (0.5 µg/ml) or in the presence of UV-irradiated *L. major* stationary phase parasites ($1x10^6$ parasites/ml) for 72 hours at 37°C. Cell-free supernatants were taken and cytokine contents were analyzed by ELISA according to the manufacturer's instructions: IL-4, IL-10 (both BD Bioscience) and IL-17 (eBioscience). IFN-γ was detected by an in-house ELISA. Briefly, ELISA plates (F96 Maxisorb NUNC immune plates) were coated with an anti-IFNγ mAb (clone 01E70 3B2) in PBS over night at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (AppliChem) and standard and sample dilutions incubated for two hours. The plates were washed again and bound IFNγ detected with a biotinylated anti-IFNγ mAb (clone AN-18.14.24) diluted in PBS containing 1% BSA for two hours. After another washing step tetramethylbenzidine solution (BD TMB substrate reagent set) was added to the wells and the reaction was stopped with 2N H_2SO_4. The optical density of 450 nm was determined with a plate reader (Dynamica LEDETECT 96).

9. Statistics

Results represent means +/- SEM. Data was analyzed using GraphPad Prism 5 software. Statistical significance was determined by an unpaired two-tailed Student's *t*-test. P values were considered significant when $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

Aim of the thesis

Neutrophils arrive rapidly and massively at the site of *Leishmania major* inoculation in the skin. They participate in shaping the immune response by the secretion of numerous factors including cytokines and chemokines and by interactions with antigen-presenting cells such as dendritic cells (DC). More specifically, neutrophils participate in the attraction of DCs to the site of infection and can either promote or inhibit their activation. However, the influence of neutrophils on the activation of DCs following *L. major* inoculation is poorly understood.

In this study, we firstly aimed to investigate if neutrophils have an impact on the activation of DCs *in vitro* and to decipher the mechanisms involved in their regulation of DC activation. To this end we set up co-cultures of $CD8\alpha^+$ DCs, *L. major* parasites and inflammatory neutrophils. As readout we analyzed the expression of activation markers on DCs and analyzed the cytokine content in the supernatants.

Furthermore we assessed the role of neutrophils on the activation of DCs *in vivo* at the site of infection and in the dLN three days following *L. major* inoculation in the ear pinna of *Leishmania*-resistant C57BL/6 and susceptible BALB/c mice. To this end, we made use of the neutrophil-depleting mAb NIMP-R14. When injected prior to infection, neutrophils are absent during the first three days of infection. The expression of activation markers on DCs present at the site of infection or in the dLN from mice depleted or not of neutrophils was analyzed three days post infection. We followed lesion development over time and analyzed parasite loads as well as the impact of the absence of neutrophils during the first three days of infection on the development of the adaptive immune response.

Results

1. The role of neutrophils in BALB/c mice upon *L. major* infection

Neutrophils are the first line of defense against microbial agents and participate in the elimination of pathogens. Furthermore, it has been shown that neutrophils interact with various other cell types and participate in the orchestration of the adaptive immune response. Following *L. major* infection, neutrophils migrate massively and rapidly to the site of insult. Previous studies demonstrated the deleterious role of the early neutrophilic infiltrate in BALB/c mice using mAbs to transiently deplete neutrophils. Mice depleted transiently of neutrophils at the onset of infection developed significantly smaller and non-progressive lesions compared to non-depleted littermates. However, the impact of neutrophils on the activation of DCs early after infection has not been investigated in BALB/c mice.

1.1 The impact of neutrophils on DC activation *in vitro*

1.1.1 Neutrophils down-regulate DC activation *in vitro*

We first analyzed whether neutrophils have an impact on the activation of DCs *in vitro*. Since we were interested in the role of neutrophils on DC activation in the dLN, we used CD8α^+ DCs which are the main IL-12-producing DC subset for these experiments . The MutuDC CD8α^+ cell line H-2^d retained all major features of freshly isolated *ex vivo* CD8α^+ DCs such as expression of activation marker and cytokine secretion [194]. We set up co-cultures of CD8α^+ DCs, inflammatory neutrophils and metacyclic *L. major* parasites and analyzed the expression of the DC activation markers CD80, CD86, CD40 and MHC II. Cytokine secretion was also analyzed in the culture media. The incubation of DCs with *L. major* parasites led to increased expression of all four activation markers on DCs compared to when DCs are cultured alone. However, the addition of inflammatory neutrophils together with *L. major* to DCs significantly decreased the expression of CD86 and to a lesser extend the expression of CD80, CD40 and MHC II on DCs (Figure 1A).

Next we determined the levels of IL-12p40, IL-12p70 and TNFα in the supernatants. In cultures of DCs only, neutrophils in presence or absence of *L. major*, or neutrophils and DCs, the levels of IL-12p40, IL-12p70 and TNFα detected 18 hours after culture onset were below detection limit. Incubation of DCs with *L. major* induced a robust secretion of IL-12p40, p70 and TNFα. However, the addition of neutrophils and *L. major* to DCs led to

significantly decreased levels of IL-12p70 and TNFα, and to a smaller degree to decreased IL-12p40 levels, compared to those observed in cultures of DCs with *L. major* only (Figure 1B).

In the same experiment the rate of infected DCs in the different co-cultures was analyzed. When DCs were incubated with *L. major*, around 30% of the DCs were infected after 18 hours. The addition of neutrophils decreased the infection rate of DCs almost three fold (to 11%) (Figure 1C). Thus, neutrophils appear to sequester *L. major* and downregulate the activation of DCs *in vitro*.

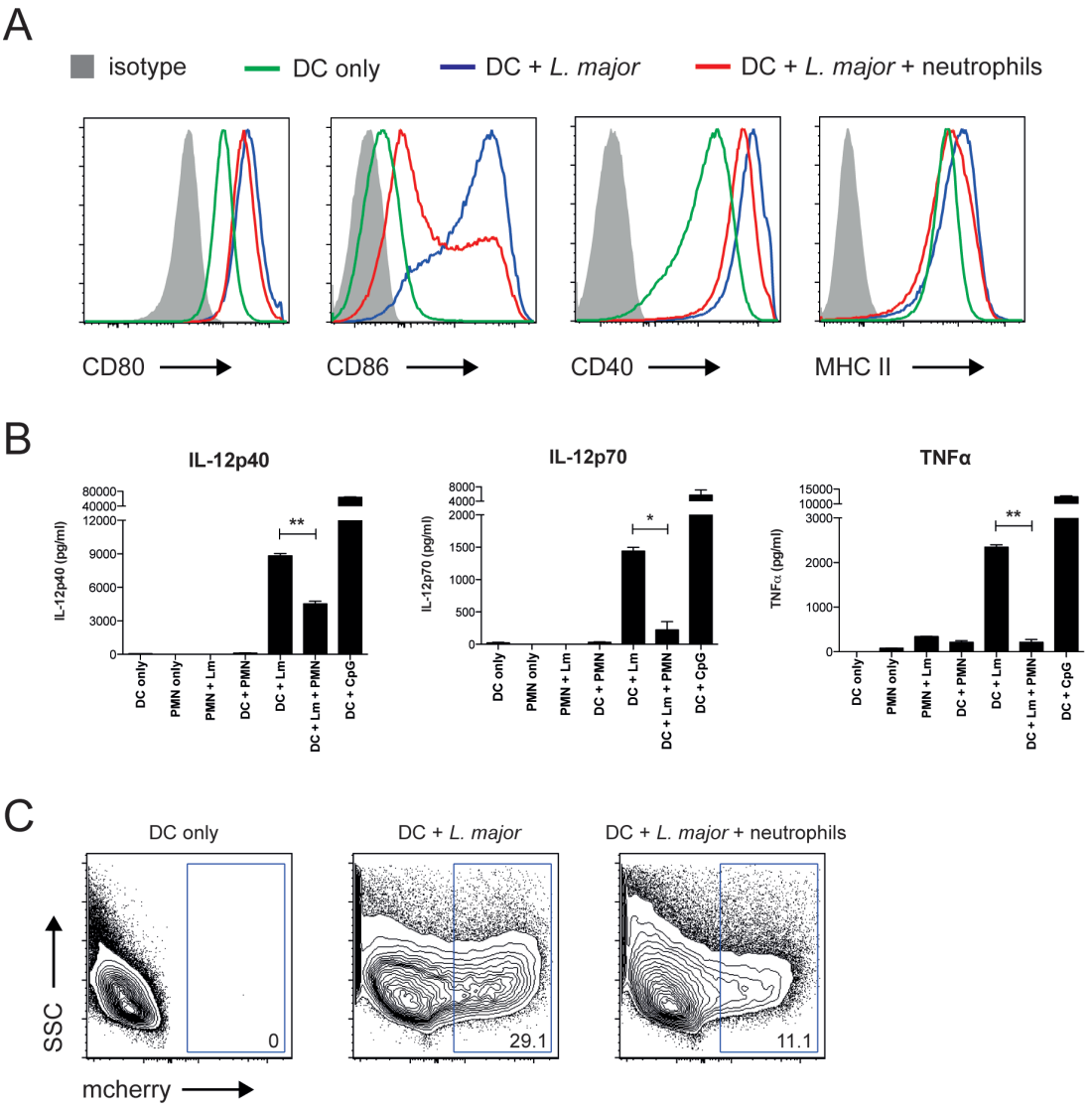

Figure 1: Neutrophils have a negative impact on DC activation *in vitro*
Co-cultures of DCs, metacyclic *L. major* (Lm) parasites and inflammatory neutrophils (PMN) were set-up *in vitro* as indicated. After 18 hours, cells were collected and (A) the expression of activation markers CD80, CD86, CD40 and MHC II gated on DCs was analyzed by FACS. (B) Cytokine content in the supernatants was determined by ELISA. (C) In the same experiment, the percentages of infected mcherry-*L. major*-positive DCs were analyzed in the different co-cultures. Numbers indicated in the gates represent the percentage of mcherry-*L. major*-positive DCs. The data shown are from one experiment representative of three individual experiments ((*, $p<0.05$; **, $p<0.01$).

1.1.2 The negative effect of neutrophils on DC activation is not due to efferocytosis

The phagocytosis of apoptotic cells (efferocytosis) by DCs is known to downregulate their activation. We reasoned that the apoptotic state of neutrophils could contribute to their negative effects on DC activation. To this end we first analyzed if the infection of neutrophils with *L. major* induced apoptosis. We incubated neutrophils with *L. major* and analyzed apoptosis in infected and non-infected neutrophils. No difference in the expression of the apoptotic marker phosphatidylserine detected by Annexin V staining as well as uptake of DAPI was seen between infected and non-infected neutrophils (Figure 2A).

In order to test whether the apoptotic state of neutrophils has an effect on DC activation, DCs were incubated with *L. major* and viable or UV-treated apoptotic/necrotic neutrophils were added to the culture. After UV-treatment, 75% of neutrophils were apoptotic/necrotic (Annexin V^+) as measured by the expression of Annexin V and DAPI by FACS (Figure 2B). The addition of viable neutrophils to DCs and *L. major* decreased the expression of all four activation markers analyzed on DCs compared to cultures without neutrophils. However, when apoptotic neutrophils were added to cultures of DCs and *L. major*, the negative impact on the expression of activation markers on DCs was lower than that observed when viable neutrophils were added. (Figure 2C).

Next we analyzed the levels of IL-12p40 and TNFα in the supernatants of the co-cultures. When apoptotic neutrophils were added to DC and *L. major*, the negative impact on cytokine secretion observed when live neutrophils were added was reduced and similar levels of IL12-p40 and TNFα were measured (Figure 2D). Thus, the negative effect of neutrophils on DC activation does not appear to result from exposure to apoptotic neutrophils On the contrary, viable neutrophils have a stronger, negative impact on DC activation than apoptotic neutrophils *in vitro*.

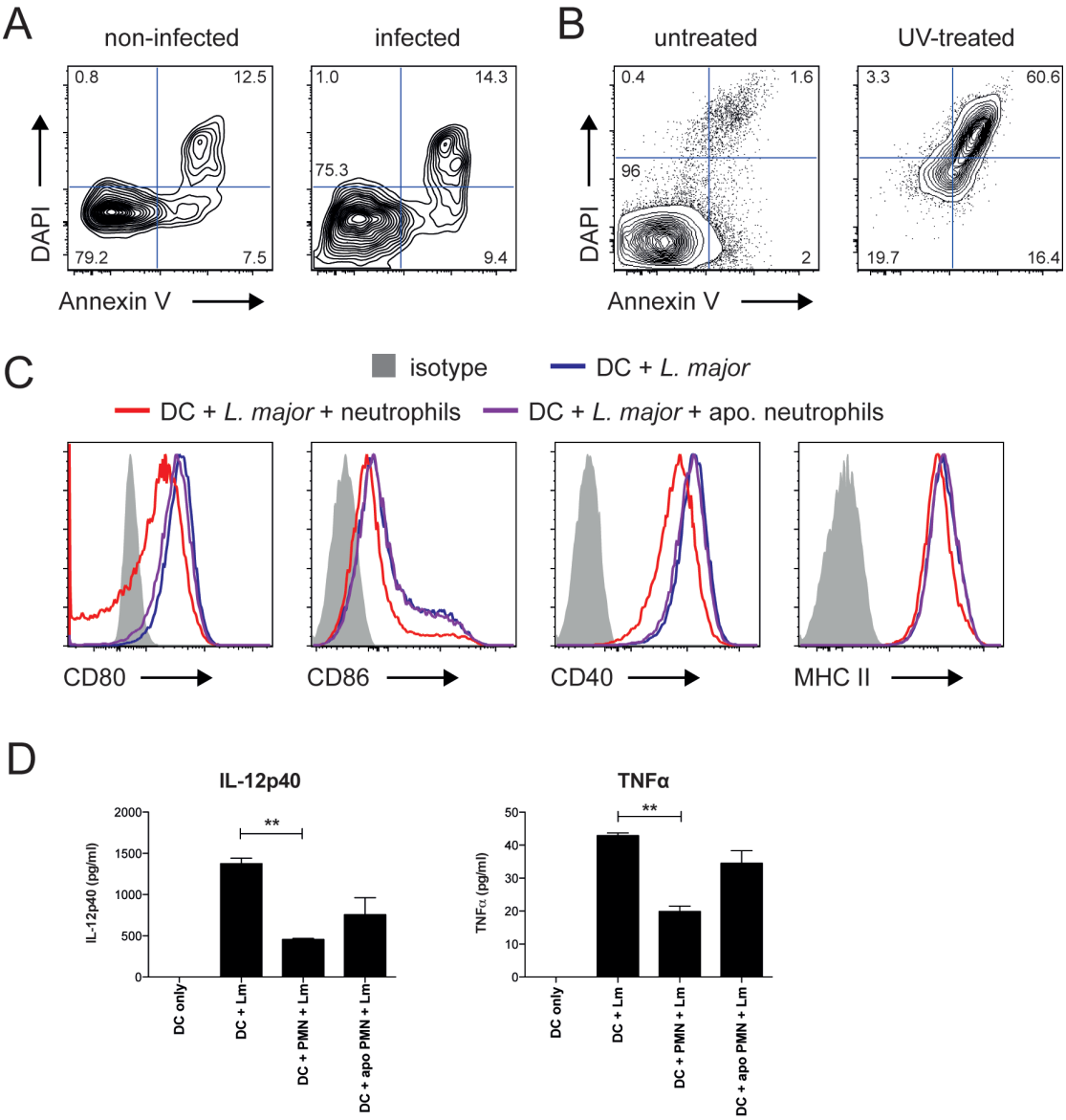

Figure 2: Impact of neutrophil apoptosis on DC activation

(A) Inflammatory neutrophils were incubated with metacyclic *L. major* parasites for 18 hours and the expression of phosphatidylserine detected by Annexin V and the uptake of DAPI analyzed by flow cytometry on non-infected and *L. major*-infected neutrophils (B) Co-cultures of DCs, metacyclic *L. major* (Lm) parasites and inflammatory viable or UV-treated apoptotic (apo) inflammatory neutrophils (PMN) were set-up *in vitro* as indicated. Apoptosis was analyzed by staining for Annexin V and DAPI gated on $Ly6G^{+}$ neutrophils. Numbers in the quadrants represent the percentages in the respective quadrants. (C) Co-cultures were incubated for 18 hours, cells were collected and the expression of activation markers CD80, CD86, CD40 and MHC II gated on DCs analyzed by FACS and (D) cytokine content in the supernatants determined by ELISA. The data shown are from one preliminary experiment (**, $p<0.01$).

1.1.3 Neutrophil-derived prostaglandins contribute to the downregulation of DC activation in the presence of *L. major*

A recent report by the group of Unanue showed that following injection of antigen in adjuvants prostaglandins secreted by neutrophils in the dLN control the magnitude and spread of the immune response [76]. For this reason we wanted to analyze whether neutrophil-derived prostaglandins have an effect on DC activation in the presence of *L. major*.

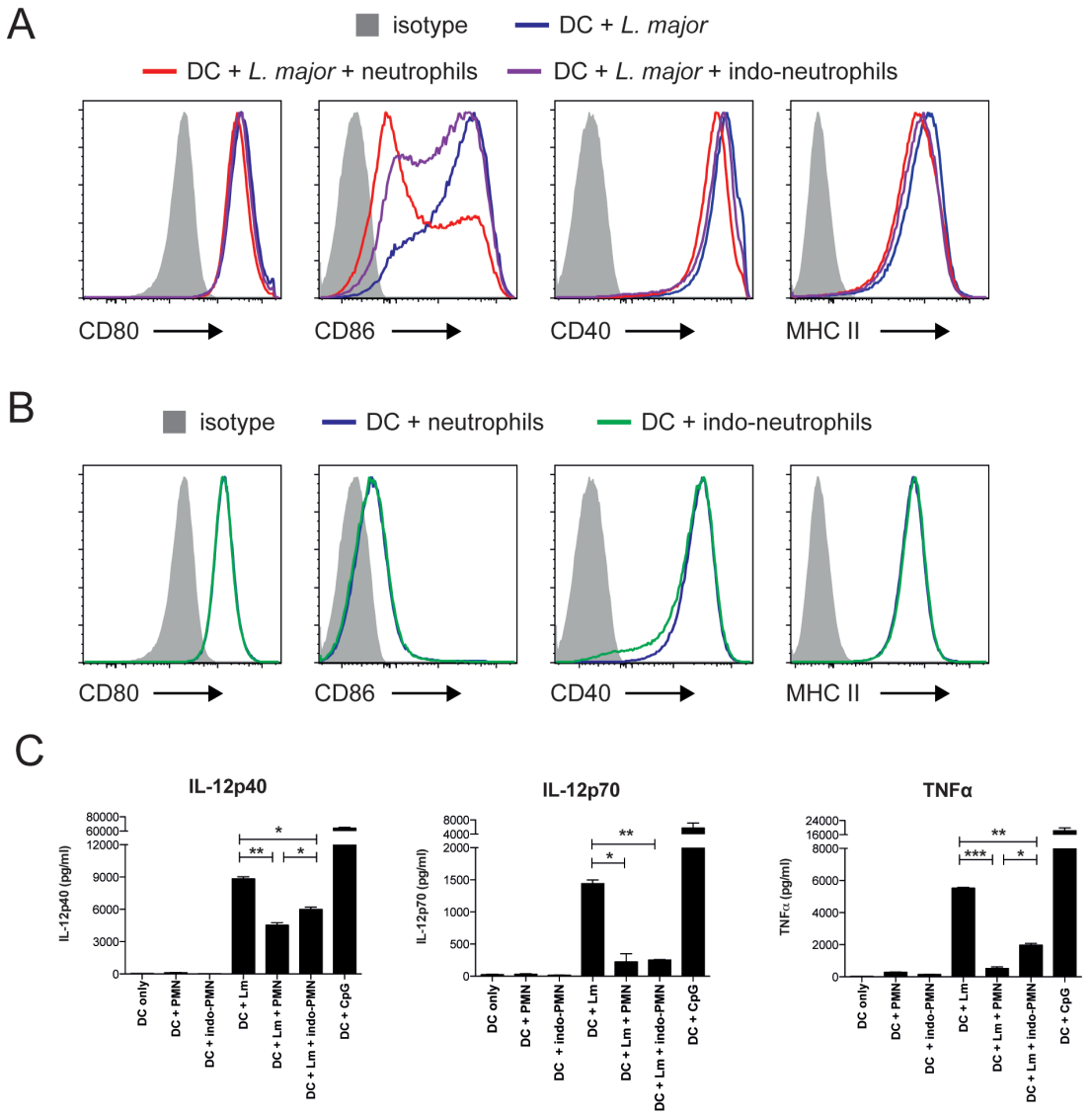

Figure 3: Neutrophil-derived prostaglandins contribute to the downregulation of DC activation
Co-cultures of DCs, metacyclic *L. major* (Lm) parasites and inflammatory non- or indomethacin-treated (indo) neutrophils (PMN) were set-up *in vitro* as indicated. (A, B) Co-cultures were incubated for 18 hours, cells were collected and the expression of activation markers CD80, CD86, CD40 and MHC II gated on DCs analyzed by FACS. (C).Cytokine content in the supernatants of co-cultures was determined by ELISA The data shown are from one preliminary experiment (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

To this end, we incubated DCs and *L. major* with non- or indomethacin-treated neutrophils and analyzed the expression of activation markers on DCs. Indomethacin is an inhibitor of the enzymes cyclooxygenase (COX) 1 and 2 that participate in the synthesis of prostaglandins from arachidonic acid. As seen previously, the addition of neutrophils to cultures of DCs and *L. major* resulted in decreased expression of CD86 and to a lesser extent CD80, CD40 and MHC II on DCs. However, treatment of neutrophils with indomethacin partially blocked this effect resulting in higher expression of CD86 and to a smaller degree of CD40 on DCs compared to conditions where non-treated neutrophils were added (Figure 3A). Of note, treatment of neutrophils with indomethacin had no effect on the viability of neutrophils (data not shown.) Furthermore, there was no difference in the expression of activation markers on DCs when non- or indomethacin-treated neutrophils were incubated with DCs without addition of *L. major* (Figure 3B).

We next assessed the content of IL12-p40, IL12-p70 and TNFα in the supernatants of these co-cultures. As seen previously, the addition of neutrophils to DCs and *L. major* significantly decreased the levels of all three cytokines in the supernatants. Comparing the cytokine content between cocultures of DCs, *L. major* and non-treated neutrophils with cocultures of DCs, *L. major* and indomethacin-treated neutrophils, the levels of IL12-p40 and TNFα were significantly higher in the latter cultures. However, cytokine levels from these latter cultures were still significantly lower compared to cultures of DCs with *L. major* alone (Figure 3C). Thus, neutrophil-derived prostaglandins contribute partially to the negative impact on DC activation *in vitro*.

1.2 The impact of neutrophils on DC activation *in vivo*

1.2.1 Characterization of the mAb NIMP-R14

Having seen that neutrophils down-regulate DC activation *in vitro*, we next wanted to investigate if neutrophils have an impact on DC activation both at the site of infection and in the dLN following *L. major* infection *in vivo*. To this end we made use of the neutrophil-depleting mAb NIMP-R14 that has been shown to efficiently deplete neutrophils for three days [161]. However, the exact epitope and thus the specificity of this mAb is still unknown.

In order to further characterize this mAb we injected i.p. 100 µg of the mAbs NIMP-R14 or a control mAb (RR3-16) six hours prior and 1.5 days post infection of *L. major* in the ear pinna. At different time points the numbers of $Ly6C^{int}Gr\text{-}1^{hi}$ neutrophils (gate 1), $Ly6C^{int}Gr\text{-}1^{-}$ cells including resident monocytes (gate 2), $Ly6C^{hi}Gr\text{-}1^{int}$ cells including

inflammatory monocytes (gate 3), $CD11c^+$ DCs (gate 4) and $CD11c^+Ly6C^+CD11b^+$ moDCs (gate 5) were analyzed at the site of infection and in the dLNs since these are the sites where DC activation will be studied. Analysis of the different populations is shown in Figure 4A. Neutrophils were efficiently depleted for three days in NIMP-R14 treated animals that were infected with *L. major* 6 hours after the first mAb injection both at the site of infection (Figure 4B) and in the dLN (Figure 4C). The injection of the mAb NIMP-R14 did not affect $Ly6C^{int}$ cells, $Ly6C^{hi}$ cells, DCs and moDCs both at the site of infection and in the dLN. A small increase (two fold) in the number of $Ly6C^{int}$ cells in the dLNs 72 hours post infection was observed (Figure 4C). Consequently, the mAb NIMP-R14 (from now on referred to as anti-PMN) was a good tool and further used to study the impact of neutrophils on the activation of DCs at the site of infection and in the dLNs.

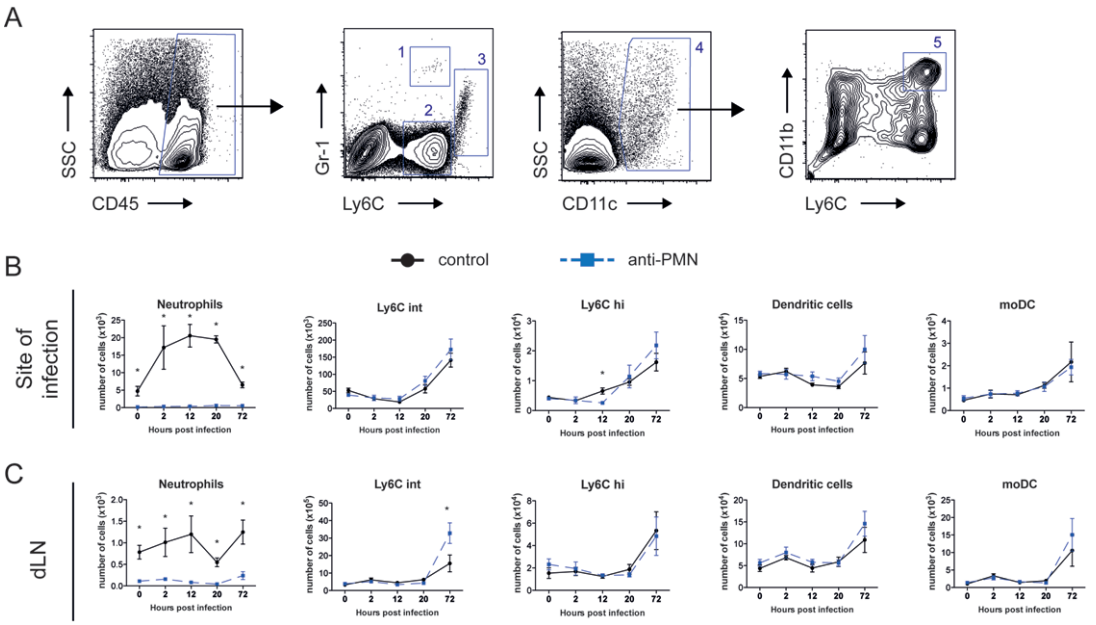

Figure 4: The mAb NIMP-R14 selectively depletes neutrophils at the site of infection and in the dLN. BALB/c mice were injected i.p. with 100 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to and 1.5 days post inoculation of *L. major* parasites in the ear dermis. At 2, 12, 20 and 72 hours post parasite inoculation, infected ears and dLNs were harvested, digested and cellular content analyzed by FACS. (A) A representative gating strategy of $Ly6C^{int}Gr\text{-}1^{hi}$ neutrophils (gate 1), $Ly6C^{int}Gr\text{-}1^{-}$ cells (gate 2), $Ly6C^{hi}Gr\text{-}1^{int}$ cells (gate 3), $CD11c^+$ DCs (gate 4) and $CD11c^+Ly6C^+CD11b^+$ moDCs (gate 5) gated on $CD45^+$ cells is shown. (B) The values of four individual ears (site of infection) and (C) dLNs for each time point are expressed as mean +/- SEM. The data shown are pooled from two individual experiments per time point. (*, $p<0.05$)

1.2.2 Depletion of neutrophils has no detectable impact on DC activation at the site of infection

In order to study the impact of neutrophils on the activation of DCs *in vivo*, BALB/c mice were treated with an anti-PMN or control mAb and inoculated with *L. major* parasites in the ear dermis. Three days post infection the cellularity of infected ears and the expression of activation markers on DCs were analyzed.

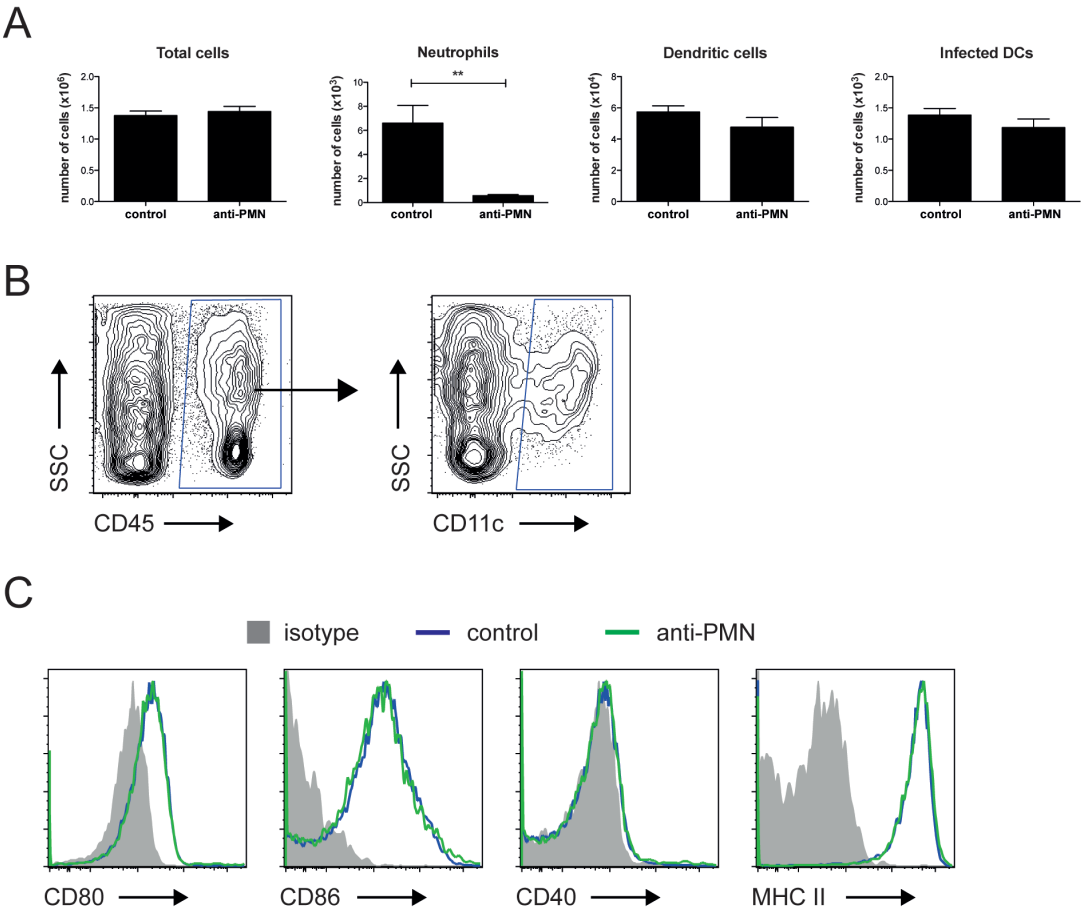

Figure 5: Depletion of neutrophils has no detectable impact on DC activation at the site of infection three days post *L. major* inoculation BALB/c mice were injected i.p. with 100 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to and 1.5 days post inoculation of *L. major* parasites in the ear dermis. Three days post parasite inoculation, infected ears (n = 6 ears per group) were harvested, digested and cellular content analyzed by flow cytometry. (A) Bar graphs express the mean +/- SEM of numbers of total cells, $Ly6C^{int}Gr\text{-}1^{hi}$ neutrophils, $CD11c^{+}$ DCs and $mcherry^{+}CD11c^{+}$ infected DCs per ear. (B) A representative gating strategy for $CD11c^{+}$ DCs gated on $CD45^{+}$ cells is shown. (C) Histograms of CD80, CD86, CD40 and MHC II expression gated on $CD11c^{+}$ DCs from concatenated samples are shown. The data shown are from one experiment and representative of three experiments (**, $p<0.01$).

Neutrophils were efficiently depleted in anti-PMN treated mice while no differences in the number of total cells, DCs and infected DCs were observed (Figure 5A). Furthermore we could not detect any major differences in the expression of the activation markers CD80,

CD86, CD40 and MHC II on total CD11c$^+$ DCs between neutrophil-depleted and non-depleted mice (Figure 5B, C). Thus neutrophils do not appear to have a major impact on DC activation at the site of infection three days post *L. major* inoculation.

1.2.3 Neutrophils decrease DC activation in the dLN

It has been shown that neutrophils migrate to the dLN following *L. major* infection ([161] and Figure 4). Thus, we next wanted to examine the effect of neutrophil-depletion on the cellularity and DC activation in the dLN.

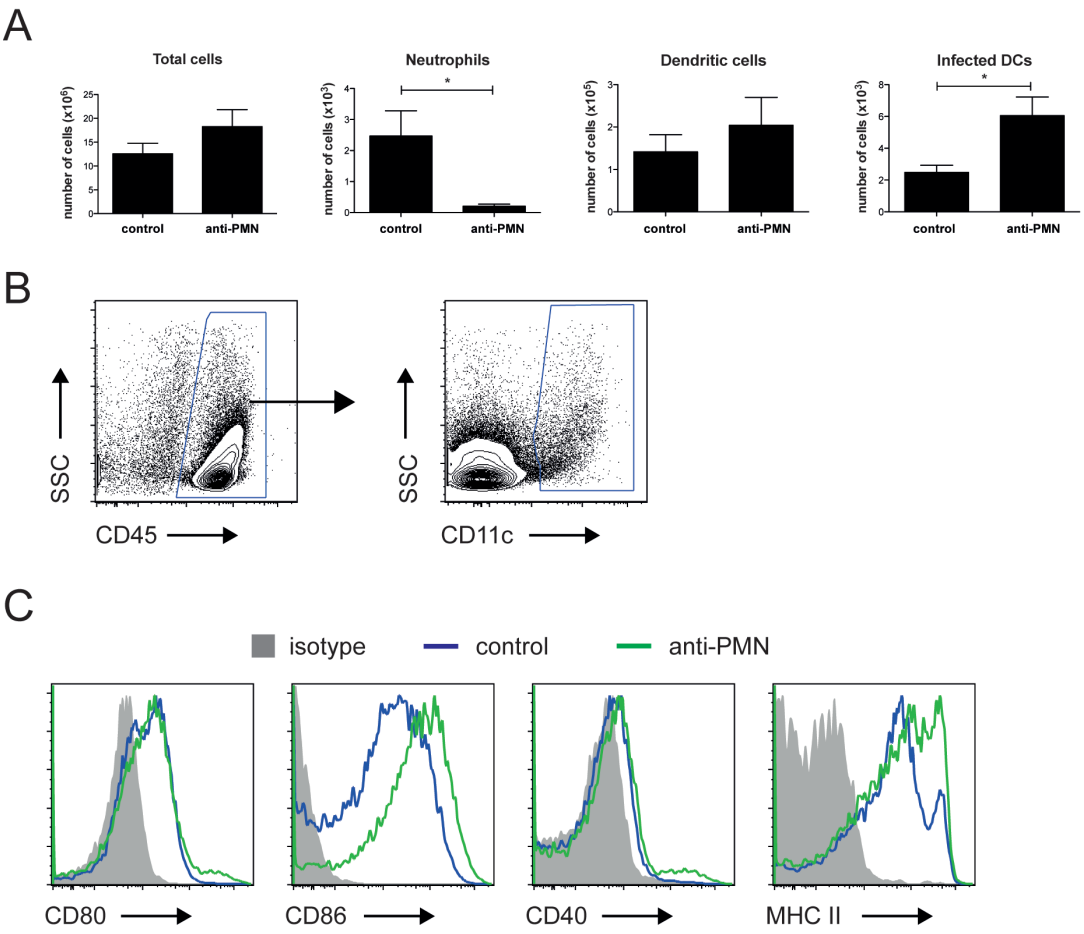

Figure 6: Depletion of neutrophils increases DC activation in the dLN three days post *L. major* inoculation BALB/c mice were injected i.p. with 100 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to and 1.5 days post inoculation of *L. major* parasites in the ear dermis. (A) Three days post parasite inoculation, dLNs (n= 6 per group) were harvested, digested and cellular content analyzed by flow cytometry. Bar graphs express the mean +/- SEM of numbers of total cells, Ly6C^{int}Gr-1^{hi} neutrophils, CD11c$^+$ DCs and mcherry$^+$CD11c$^+$ infected DCs per ear. (B) A representative gating strategy for CD11c$^+$ DCs gated on CD45$^+$ cells is shown. (C) Histograms of CD80, CD86, CD40 and MHC II expression gated on CD11c$^+$ DCs from concatenated samples are shown. The data shown are from one experiment and representative of three experiments (*, $p<0.05$).

Injection of the anti-PMN mAb efficiently depleted neutrophils while we did not observe any differences in the numbers of total cells and DCs. However, the number of *L.*

major-infected DCs was significantly higher in neutrophil-depleted mice (Figure 6A). When we analyzed the expression of activation marker on total $CD11c^+$ DCs, the expression of CD86 and MHC II was significantly increased in neutrophil-depleted mice compared to non-depleted littermates. The expression of CD80 and CD40 was slightly increased (Figure 6C). Taken together, depletion of neutrophils led to increased numbers of infected DCs and enhanced DC activation in the dLN three days post *L. major* infection.

1.3 The consequences of early neutrophil depletion on lesion development, parasite load and the developing immune response

Neutrophils have a negative impact on DC activation both *in vitro* and in the dLN three days post infection *in vivo*. We next analyzed the effects of the early and transient neutrophil depletion on lesion evolution, parasite loads and the developing immune response.

1.3.1 Development of a novel scoring method to monitor lesion development following intra-dermal injection of *L. major* in the ear

Initially, most studies using the murine model of *L. major* infection were carried out inoculating parasites in the hind footpad. In recent years, new protocols have been adapted injecting the parasites intradermally into the ears. However, given the different anatomy of the ear compared to the footpad, monitoring lesion development in the ear is technically more difficult and until now there is no consensus on how to monitor lesion size development in this anatomic localization. For this reason we developed a novel scoring method to follow lesion evolution. This system takes into account each step of lesion evolution occurring during the infection: from the appearance of first signs of inflammation such as redness to development of necrosis with potential tissue destruction (Figure 7).

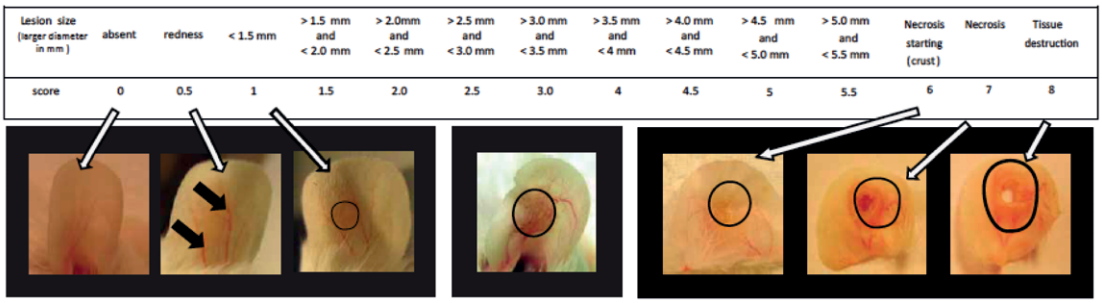

Figure 7: A new scoring method to monitor lesion evolution in the ear following *L. major* infection in the ear dermis. BALB/c mice were inoculated with $1x10^5$ *L. major* parasites in the ear dermis. Lesion size was measured weekly using a Vernier caliper. A table describing the attribution of different scores depending on the appearance and size of the lesion is shown. Pictures of infected ears representing different scores as indicated in the figure are shown.

Lesion scoring is comprised between 0 and 8. At the onset of infection, observations of inflammatory signs (redness, swelling of blood vessels) are assigned a score of 0.5. The appearance of swelling or a small non-measurable papule receives a score of 1. As lesions become quantifiable the length and width of the lesion is measured using a caliper. The larger dimension is used to assign a score. For example, if the higher diameter of the lesion is between 2.00 mm and 2.49 mm a score of 2 is attributed, from 2.50 to 2.99 a score of 2.5 and so on until a diameter higher than 5.50 mm (score 5.5). A score of 6 is assigned when first signs of necrosis are observed at the site of infection. Severe necrotic lesions without tissue destructions receive a score of 7. Once tissue destruction at the site of infection appears a score of 8 is attributed (Figure 7). Consequently, this scoring system represents the evolution of lesion development as observed visually in an adequate way. Importantly, fine and precise lesion modulations, especially at the beginning of lesion formation and at later time points when tissue modifications and necrosis occur, are properly represented.

1.3.2 Early and transient depletion of neutrophils resulted in the development of non-progressive lesions and decreased parasite loads

In order to investigate the role of the early neutrophilic infiltrate, BALB/c mice were treated with an anti-PMN or control mAb prior to injection of *L. major* parasites in the ear dermis. Lesion evolution was monitored using the novel scoring method described above.

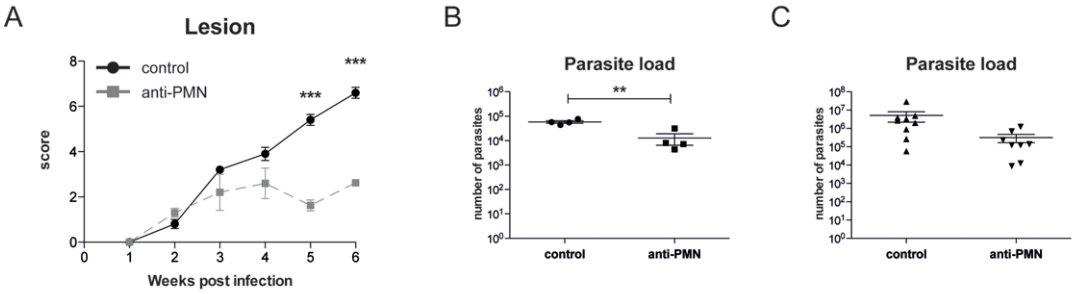

Figure 8: Depletion of neutrophils leads to the development of non-progressive lesion following *L. major* infection BALB/c mice were injected i.p. with 250 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to inoculation of *L. major* parasites in the ear dermis. Lesion development was assessed by measuring lesion size weekly for six weeks. (A) Values represent the mean lesion score +/- SEM of 5 mice per group. (B) At 14 days and (C) six weeks post infection, numbers of parasites in ear lesions were quantified using limiting dilution assay. Parasite loads of individual ears are shown as mean +/- SEM. Results are pooled from two independent experiments (C) or representative of two independent experiments (A, B) (**, $p<0.01$; ***, $p<0.001$).

BALB/c mice that received the anti-PMN mAb developed significantly smaller, non-progressive lesions compared to control-injected littermates (Figure 8A). Furthermore the

number of parasites in the infected ears was quantified 14 days and 6 weeks post infection. Depletion of neutrophils led to a significant reduction in parasite loads 14 days and 6 weeks post infection at the site of infection. (Figure 8B, C). Thus, early and transient depletion of neutrophils led to the development of non-progressive lesions and decreased parasite loads in infected ears.

1.3.3 Depletion of neutrophils leads to decreased secretion of IL-4 and IL-17 six weeks post *L. major* infection

To determine if the depletion of neutrophils had an impact on the developing immune response, we harvested dLNs from control and anti-PMN-treated mice two and six weeks post *L. major* infection and analyzed cytokine secretion of restimulated dLN cells by ELISA. We observed no significant difference in the levels of IFNγ in the supernatants both at two and six weeks post infection. However, IL-4 content in the supernatants was decreased in cell cultures from neutrophil-depleted compared to non-depleted mice at both time points analyzed (Figure 9). Of note, results obtained for IFNγ and IL-4 by ELISA were confirmed by intracellular FACS staining (data not shown). The levels of IL-10 in the supernatants were comparable between neutrophil-depleted and non-deleted mice, both at two and six weeks post infection. Analyzing IL-17 secretion, we found increased levels in cell cultures from neutrophil-depleted mice at two weeks post *L. major* infection but decreased levels at six weeks post infection (Figure 9).

Consequently, transient depletion of neutrophils decreased IL-4 secretion at two and six weeks post *L. major* infection while it led to a transient increased IL-17 secretion at two weeks while IL-17 secretion was significantly decreased at six weeks post *L. major* infection.

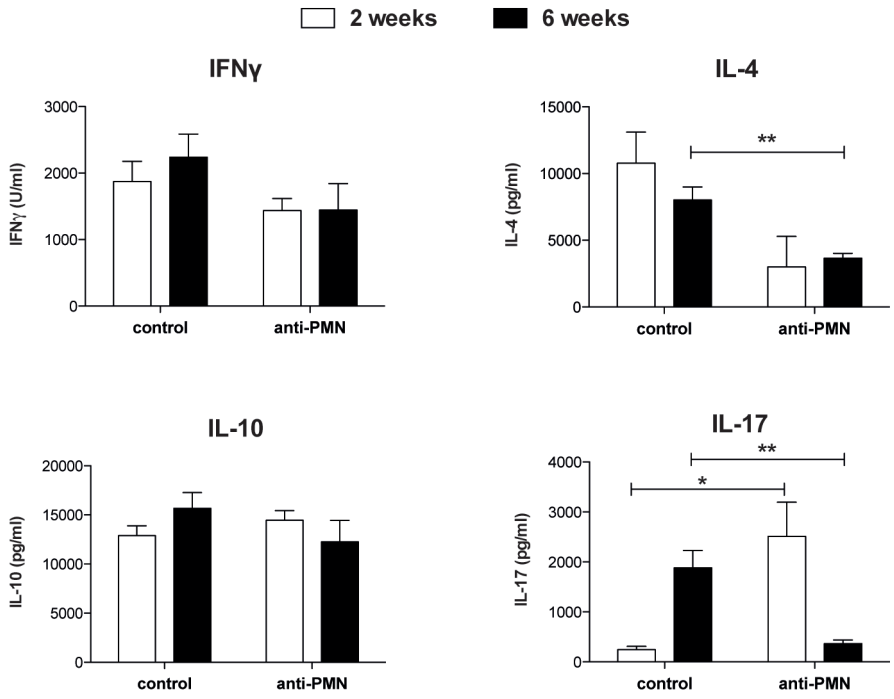

Figure 9: Depletion of neutrophils leads to decreased secretion of IL-4 and IL-17 six weeks post *L. major* infection. BALB/c mice were injected i.p. with 250 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to inoculation of *L. major* parasites in the ear dermis. Two (white bars) and six (black bars) weeks post infection, dLN cells were isolated and restimulated with UV-treated *L. major* parasites for 72 hours. Cell-free supernatants were collected and cytokine levels of IFNγ, IL-4, IL-17 and IL-10 analyzed by ELISA. Values represent the mean +/- SEM of 5 mice per group and are representative of two experiments (*, $p<0.05$).

2. The role of neutrophils in C57BL/6 mice upon *L. major* infection

2.1 The impact of neutrophils on DC activation *in vivo*

In *Leishmania*-susceptible BALB/c mice, we observed that neutrophils have a negative impact on DC activation early after parasite inoculation. Next we wanted to investigate the impact of neutrophils on DC activation following *L. major* infection in *Leishmania*-resistant C57BL/6 mice, both at the site of infection and in the dLN.

2.1.1 Depletion of neutrophils has no detectable impact on DC activation at the site of infection three days post *L. major* inoculation

In order to analyze the impact of neutrophils on DC activation at the site of infection, C57BL/6 mice were treated with a control- or anti-PMN mAb prior to *L. major* inoculation in the ear dermis. Three days post infection, the cellularity of infected ears and the expression of activation markers on DCs were analyzed. In anti-PMN-treated mice, neutrophils were efficiently depleted while there was no significant difference in the numbers of total cells, DCs and infected DCs compared to control-treated mice (Figure 10A). Analyzing the expression of the activation markers CD80, CD86, CD40 and MHC II on total $CD11c^+$ DCs, there was no significant difference between control- and anti-PMN-treated mice (Figure 10B). Thus, we could not detect any impact on DC activation at the site of infection three days post *L. major* inoculation.

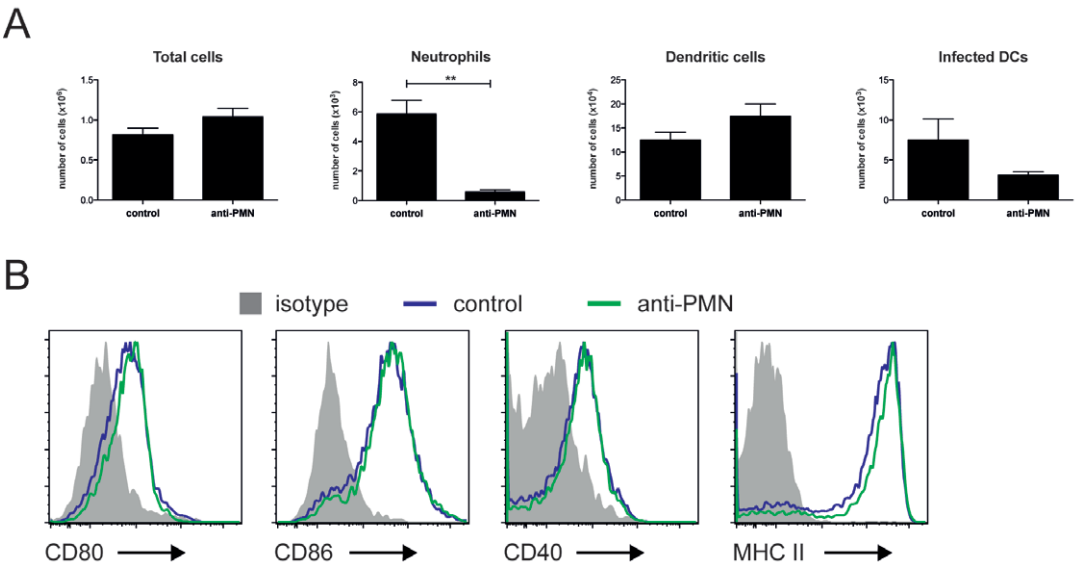

Figure 10: Depletion of neutrophils has no detectable impact on DC activation at the site of infection three days post *L. major* inoculation C57BL/6 mice were injected i.p. with 100 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to and 1.5 days post inoculation of *L. major* parasites in the ear dermis. (A) Three days post parasite inoculation, infected ears (n = 6 ears per group) were harvested, digested and cellular content analyzed by flow cytometry. Bar graphs express the mean +/- SEM of numbers of total cells, $Ly6C^{int}Gr\text{-}1^{hi}$ neutrophils, $CD11c^+$ DCs and $mcherry^+CD11c^+$ infected DCs per ear. (B) Histograms of CD80, CD86, CD40 and MHC II expression gated on $CD11c^+$ DCs from concatenated samples are shown. The data shown are from one experiment and representative of three experiments (**, $p<0.01$).

2.1.2 Depletion of neutrophils has no detectable impact on DC activation in the dLN three days post *L. major* infection

Having seen that neutrophils have a negative impact on DC activation in the dLN of BALB/c mice, we next wanted to investigate the role of neutrophils on the activation of DCs in the dLNs of C57BL/6 mice. To this end, C57BL/6 mice were treated as described above and the cellularity and expression of activation markers on DCs in the dLNs analyzed three days post *L. major* infection in the ear dermis. Injection of the anti-PMN mAb led to an efficient depletion of neutrophils while numbers of total cells, DCs and infected DCs were comparable to control-injected mice (Figure 11A). Furthermore, depletion of neutrophils had no major impact on the expression of CD80, CD86, CD40 and MHC II on total dLN $CD11c^+$ DCs (Figure 11B). Consequently, neutrophils have no detectable impact on DC activation in the dLN three days post *L. major* infection.

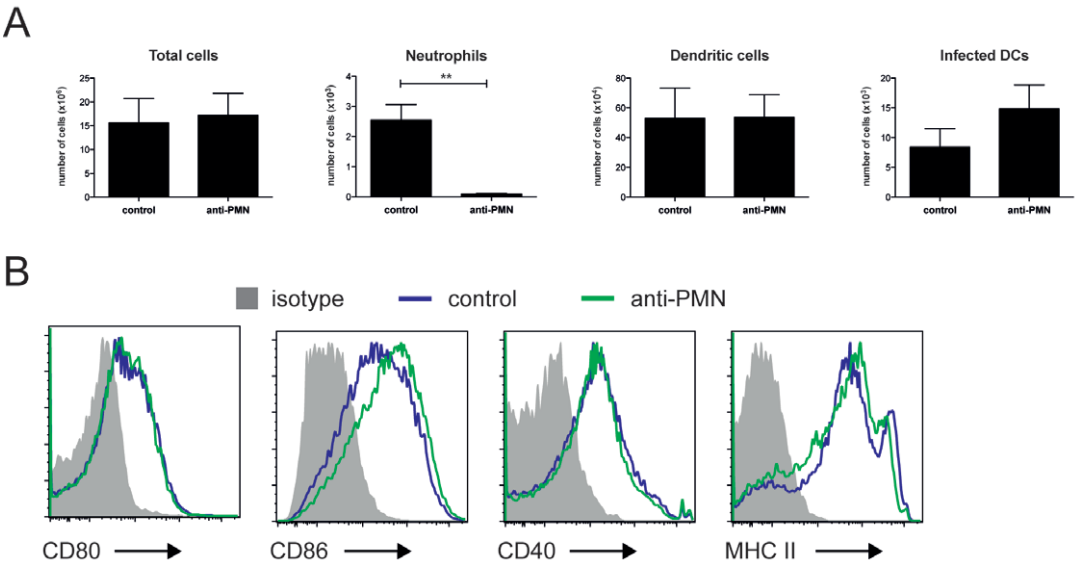

Figure 11: Depletion of neutrophils has no detectable impact on DC activation in the dLN three days post *L. major* inoculation C57BL/6 mice were injected i.p. with 100 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to and 1.5 days post inoculation of *L. major* parasites in the ear dermis. (A) Three days post parasite inoculation, dLNs (n = 6 dLNs per group) were harvested, digested and cellular content analyzed by flow cytometry. Bar graphs express the mean +/- SEM of numbers of total cells, $Ly6C^{int}Gr\text{-}1^{hi}$ neutrophils, $CD11c^+$ DCs and $mcherry^+CD11c^+$ infected DCs per ear. (B) Histograms of CD80, CD86, CD40 and MHC II expression gated on $CD11c^+$ DCs from concatenated samples are shown. The data shown are from one experiment and representative of three experiments (**, $p<0.01$).

2.2 The consequences of early neutrophil depletion on lesion development, parasite loads and the developing immune response

2.2.1 Early and transient depletion of neutrophils has no major effect on lesion development and parasite loads

In order to investigate the role of the early neutrophilic infiltrate on lesion development and parasite loads in infected ears, C57BL/6 mice were treated with a control- or anti-PMN mAb prior to inoculation of *L. major* in the ear dermis. Early depletion of neutrophils had no effect on lesion evolution compared to control-treated littermates (Figure 12A). Furthermore, infected ears from neutrophil-depleted and non-depleted mice harbored similar numbers of parasites six weeks post parasite inoculation (Figure 12B).

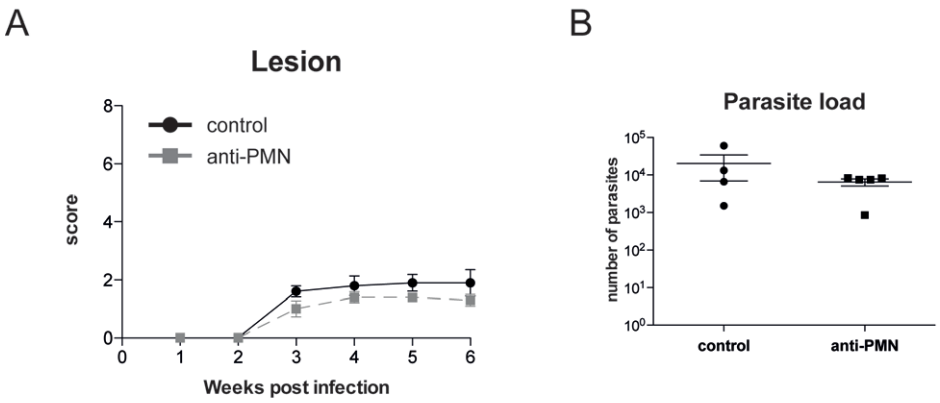

Figure 12: Depletion of neutrophils has no effect on lesion development in C57BL/6 mice following *L. major* infection. C57BL/6 mice were injected i.p. with 250 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to inoculation of *L. major* parasites in the ear dermis. (A) Lesion development was assessed by measuring lesion size weekly for six weeks. Values represent the mean lesion score +/- SEM of 5 mice per group. (B) Six weeks post infection, numbers of parasites in ear lesions were quantified using limiting dilution assay. Parasite loads of individual ears are shown as mean +/- SEM. Results are representative of two independent experiments.

2.2.2 Early and transient depletion of neutrophils has no effect on the Th1 cell immune response following *L. major* infection

Next we wanted to analyze whether the depletion of neutrophils affected the immune response 14 days and 6 weeks post *L. major* infection. To this end, C57BL/6 mice were treated and infected as described above. At the indicated time points, dLNs were harvested and cytokine production by restimulated dLN cell cultures determined by ELISA. Depletion of neutrophils had no effect on the production of IFNγ, IL-4, IL-17 and IL-10 14 days post

infection (Figure 13A). However, at six weeks post infection, depletion of neutrophils resulted in decreased levels of IL-4 while there where no differences for IFNγ, IL-17 and IL-10 (Figure 13B). Thus, early and transient neutrophil depletion did not impact on Th1 cell response but decreased the Th2 immune response six weeks post infection.

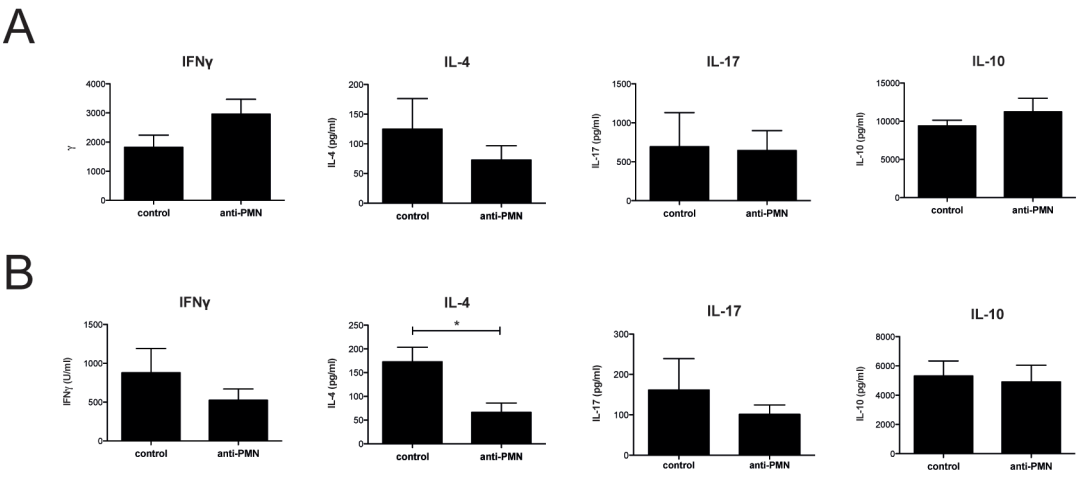

Figure 13: Depletion of neutrophils does not change Th1 cell differentiation but leads to decreased IL-4 secretion by dLN cells six weeks post *L. major* infection. C57BL/6 mice were injected i.p. with 250 µg of the mAbs RR3-16 (control) or NIMP-R14 (anti-PMN) 6 hours prior to inoculation of *L. major* parasites in the ear dermis. (A) Fourteen days and (B) six weeks post infection, dLN cells were restimulated with UV-treated *L. major* parasites for 72 hours. Cell-free supernatants were collected and cytokine levels of IFNγ, IL-4, IL-17 and IL-10 analyzed by ELISA. Values represent the mean +/- SEM of 5 mice per group and are representative of two experiments (*, $p<0.05$).

Discussion

1. The role of neutrophils upon *L. major* infection in BALB/c mice

1.1 The role of neutrophils on DC activation *in vitro*

Neutrophils and DCs are both important cells of the immune system. Under homeostatic conditions, these cells are localized in different compartments of the body. However, neutrophils and DCs colocalize at sites of insult as well as in the dLN following infection or inflammation. It has been shown that neutrophils contribute to the recruitment of DCs and either promote or inhibit their activation. Neutrophils can promote DC activation by the release of soluble factors such as cytokines and granular components as well as in a contact-dependent manner. In addition, the release of NETs can lead to DC activation. However, neutrophils are also able to inhibit DC activation under certain circumstances. The efferocytosis of apoptotic neutrophils by DCs can downregulate DC activation. Furthermore, the release of neutrophil granular components can prevent DC activation (reviewed in [99]). In the context of *L. major* infection, only little is known on the role of neutrophils on DC function. Our lab has previously demonstrated that the neutrophil-derived CCL3 is essential for the recruitment of DCs to the site of infection in C57BL/6 mice [159]. Furthermore, neutrophils were shown to inhibit DC activation in the skin early after inoculation of a high dose of metacyclic *L. major* Friedlin parasites in the ear pinna of C57BL/6 mice [160]. However, both of the above mentioned studies have been performed in *Leishmania*-resistant C57BL/6 mice. The impact of neutrophils on DC activation in *Leishmania*-susceptible BALB/c mice is less clear. Especially the role of neutrophils on DC activation in the dLN following *L. major* infection is poorly understood.

In order to investigate the influence of neutrophils on DC activation, we first performed different experiments *in vitro*. Since we were especially interested in the role of neutrophils on DC activation in the dLN, we wanted to use DCs present in LNs. However, the isolation of DCs *ex vivo* poses a number of technical limitations. Firstly the number of DCs that can be recovered *ex vivo* and used for experiments is very limited. Secondly, the isolation process can induce significant DC activation and isolated DCs undergo rapid apoptosis in culture [195]. For these reasons, we made use of the CD8α^+ Mutu DC line originating from splenic BALB/c CD8α^+ cDC [194]. These DCs have retained all major features compared to wild type CD8α^+ DCs including the expression of surface markers such as Clec9A, DEC205 and CD24, positive responses to TLR3 and TLR9 stimuli, secretion of cytokines and

chemokines as well as the upregulation of activation markers upon stimulation (Devika Ashok et al, manuscript in preparation and [194]). Thus, the CD8α$^+$ Mutu DCs were a good tool for us to study the impact of neutrophils on DC activation *in vitro*.

We showed that the incubation of CD8α$^+$ DCs with metacyclic *L. major* parasites activated DCs characterized by increased expression of the activation markers CD80, CD86, CD40 and MHC II and a robust secretion of IL-12p40, IL-12p70 and TNFα. These findings are in line with previously published results using skin DCs [177, 196]. However, the addition of inflammatory neutrophils together with *L. major* parasites to DCs led to a lower expression of CD86 and to a smaller degree of CD80, CD40 and MHC II on DCs. In addition, a corresponding decreased secretion of IL-12p40, IL-12p70 and TNFα *in vitro* was observed, compared to cultures of DCs with *L. major* only.

Neutrophils are short-lived cells that become rapidly apoptotic and it has been demonstrated that the uptake of apoptotic neutrophils by DCs decreased the expression of activation markers on DCs [115]. In addition, a recent report showed that *M. tuberculosis* promoted its replication through the suppression of neutrophil apoptosis thereby limiting pathogen uptake by DCs and delaying the onset of the adaptive immune response [117]. Thus we reasoned that neutrophil apoptosis and the availability of parasite antigens could influence DC activation. The impact of *L. major* in promoting neutrophil apoptosis is controversial. While some studies showed that *L. major* delays neutrophil apoptosis *in vitro* [153, 197, 198], another report demonstrated that infected neutrophils recovered form the ear dermis were more apoptotic than non-infected cells [160]. When we compared neutrophil apoptosis between infected and non-infected neutrophils *in vitro*, we could not detect any major differences. Possible explanations for the different results obtained in different studies could be due to the different *L. major* strains, the neutrophil to parasite ratios, incubation times of neutrophils and *L. major* used, as well as the origin of neutrophils (dermal versus BM-derived or peritoneally induced (p.i.) inflammatory neutrophils). Analyzing neutrophil-apoptosis in the co-cultures of DCs, *L. major* and p.i. inflammatory neutrophils, the majority of neutrophils were non-apoptotic and did not stain for Annexin V and DAPI after 18 hours in culture. The addition of UV-treated, apoptotic/necrotic neutrophils to DCs and *L. major* did not further decrease DC activation compared to the addition of viable neutrophils. In contrast, the negative effect of apoptotic/necrotic neutrophils on DC activation in the presence of *L. major* was less strong. than the effect of viable neutrophils. Thus, it seems that in our *in vitro* studies, neutrophil apoptosis does not play a major role in the downregulation of DC activation and other factors might be involved in preventing DC activation.

Neutrophils migrate rapidly to the site of *L. major* infection or inoculation with a high dose of parasites. They are the major infected cells early after infection. A recent report

demonstrated that following immunization with antigen in adjuvants neutrophils competed with APCs for antigens resulting in a decreased T cell response [120]. Thus we thought that the sequestration of antigens by neutrophils could have a negative impact on DC activation *in vitro* considering that DCs get activated when exposed to *L. major* [177, 196]. When we analyzed the percentage of infected DCs in co-cultures of DCs and *L. major*, almost 30% of DCs were infected after 18 hours. However, when neutrophils together with *L. major* were added to the DCs, the percentage of infected DCs dropped to 11% while 20% of neutrophils were infected. These results suggest that the sequestration of parasites by neutrophils might contribute to their negative impact on DC activation.

Following injection of antigen in adjuvants in the footpads of mice, neutrophils migrated rapidly to dLNs and neutrophil-derived prostanoids were responsible for the control of the magnitude and spread of the T cell response [76]. The enzymes COX-1 and COX-2 convert arachidonic acid into prostanoids. It was shown that upon PMA-stimulation of neutrophils the expression of the genes Ptgs-1 and Ptgs-2 encoding COX-1 and COX-2, respectively, was upregulated. Increased levels of the prostanoid metabolites PGE_2, PGD_2, PGF_2 and thromboxane were detected by mass spectrometry in cultures of stimulated compared to non-stimulated neutrophils [76]. The release of prostanoids by neutrophils exposed to *L. major* has not yet been investigated to our knowledge. However, incubation of neutrophils with *L. chagasi* and *Lutzomyia longipalpis* salivary gland sonicate (SGS) induced PGE_2 release by neutrophils [199]. Furthermore, the ingestion of apoptotic neutrophils by macrophages stimulated the release of TGF-β and PGE_2 resulting in a downregulation of macrophage activation [168, 198]. Stimulation of DCs with phlebotomine SGS induced the production of PGE_2 and IL-10. These factors acted in an autocrine manner on DCs leading to the decreased expression of MHC II and CD86 [200]. Consistently, the addition of PGE_2 to DCs induced IL-10 production that suppressed IL-12 release and decreased MHC II expression [201]. This negative effect of PGE_2 on DC function was mediated through the PGE_2 receptors EP_2 and EP_4 [202]. Since prostanoids can have a negative effect on DC activation, we reasoned that neutrophil-derived prostanoids could contribute to the observed negative effect of neutrophils on DC activation *in vitro*. Indeed, when we pre-treated neutrophils with the COX-inhibitor indomethacin before we added them to the cocultures of DCs and *L. major*, we observed a significant increase in CD86 expression on DCs as well as elevated secretion of IL12-p40 and TNFα compared to when non-treated neutrophils were added to the cocultures. However, the addition of indomethacin-treated neutrophils together with *L. major* to DCs resulted in significantly decreased levels of IL-12p40, IL12-p70 and TNFα compared to cocultures of DCs with *L. major* alone. Thus, neutrophil-derived prostanoids contribute to the negative effect of neutrophils on DC activation but there are also

other factors involved in this process. Since indomethacin is a general inhibitor of COX-1 and COX-2 other neutrophil-derived prostanoids than PGE_2 could mediate the negative effect on DC activation. Furthermore we cannot exclude that the neutrophil-derived prostanoids trigger the release of other cytokines such as IL-10 which could also contribute to decreasing DC activation.

Collectively, in this study we demonstrated that neutrophils have a negative impact on DC activation *in vitro*. This negative effect of neutrophils is mediated, at least in part, through the sequestration of parasite antigens as well as the secretion of prostanoids.

1.2 The role of neutrophils on DC activation *in vivo*

The analysis of the role of neutrophils *in vivo* has been difficult due to the lack of good neutropenic mouse models. Mice deficient for G-CSF or the G-CSF receptor have markedly reduced numbers of circulating neutrophils that have an increased susceptibility to apoptosis. Nevertheless, these mice still produce mature neutrophils [54, 203]. A recently described mouse strain termed *Genista* is neutropenic and offers an interesting model to study neutrophils *in vivo*. These mice have a point mutation in the transcriptional repressor growth factor independent 1 that causes a block in terminal granulopoiesis. *Genista* mice do not have mature neutrophils but they have a small number of atypical $CD11b^{+}Ly6G^{int}$ neutrophils [204]. However, to date these mice are only available on a C57BL/6 genetic background. Thus, in order to study the role of neutrophils in BALB/c mice we made use of neutrophil-depleting antibodies. Three different antibodies are currently used to deplete neutrophils *in vivo* – the RB6-8C5, 1A8 and NIMP-R14 mAbs. However, these mAbs differ in their specificity and efficacy. The mAb RB6-8C5 recognizes the epitope Gr-1 present on Ly6G and Ly6C [174]. Thus besides neutrophils this mAb also depletes other Gr-1 expressing cells such as inflammatory monocytes, eosinophils and different DC subsets. Another mAb used to deplete neutrophils is the 1A8 [176]. This mAb specifically recognizes neutrophils, however the depletion is incomplete and does not last as long as depletions using the other two mAbs. The third neutrophil-depleting mAb is the NIMP-R14 [175]. This mAb efficiently depletes neutrophils, however the exact epitope and possible cross-reactivity with other cells are not completely known. For this reason we further characterized the NIMP-R14 mAb. Since we intended to study the role of neutrophils on DC activation both at the site of infection and in the dLN, we analyzed these two sites at different time points after inoculation of *L. major* in the ear pinna and administration of the mAb NIMP-R14 or a control mAb 6 hours prior infection. At both sites neutrophils were efficiently depleted for three days in NIMP-treated mice while we could not detect major differences in the numbers of $Ly6C^{int}$ cells, $Ly6C^{hi}$

cells, DCs and moDCs compared to control injected littermates. However, we cannot exclude that other cell subsets such as eosinophils or other organs such as the spleen are affected by the mAb NIMP-R14. Nevertheless, for the purpose of our study the mAb NIMP-R14 was a good tool to study the impact of neutrophils on DC activation and was used for further experiments.

Our *in vitro* data suggested that neutrophils have a negative impact on DC activation through mechanisms involving the sequestration of antigens and the secretion of prostanoids. A recent report demonstrated that neutrophils have a negative impact on DC activation at the site of infection three days following inoculation of $2x10^6$ metacyclic *L. major* Friedlin in the ear pinna of C57BL/6 mice. The phagocytosis of *L. major* by neutrophils was shown to induce apoptosis and dermal DCs acquired the parasites mainly by phagocytosing apoptotic, infected neutrophils. The authors suggested that the efferocytosis of apoptotic neutrophils decreased DC activation [160]. In our study, depletion of neutrophils did not have any detectable impact on DC activation at the site of infection three days post inoculation of $2x10^5$ stationary phase or metacyclic *L. major* LV39. Thus, it is likely that the different strains of parasites and the genetic background of the hosts account for the different results obtained concerning the role of neutrophils on DC activation in the ear dermis. Whether in our *in vivo* studies the phagocytosis of *L. major* LV39 by neutrophils induces neutrophil apoptosis and whether DCs acquire the parasites via efferocytosis of infected neutrophils will need to be investigated *in vivo*.

Neutrophils are the first cells that arrive at the site of parasite infection or inoculation of a high dose of *L. major*, and they rapidly migrate to the dLNs. A negative effect of neutrophil-recruitment to the dLNs on the developing immune response has been recently reported [76, 117, 120]. Following immunizations with antigens in adjuvants, neutrophils migrated rapidly to the dLNs and competed for antigens with APCs resulting in attenuated T and B cell responses [120]. Furthermore neutrophils controlled the magnitude and the spread of T cell responses to distal LNs, a process that was dependent on neutrophil-derived prostanoids [76]. Another report demonstrated that during infections with *M. tuberculosis*, neutrophils limited the pathogen uptake by DCs thereby delaying the immune response [117]. In our study, depletion of neutrophils prior to inoculation of *L. major* resulted in an enhanced expression of activation markers on dLN DCs compared to non-depleted littermates. In addition, the number of infected DCs was significantly higher in dLNs of neutrophil-depleted compared to non-depleted mice. These data suggest that one of the mechanism involved in the negative effect on DC activation observed following *L. major* inoculation may occur through the sequestration of parasite antigens by neutrophils. These data are in line with results obtained *in vitro*. Direct uptake of the parasites by DCs could enhance DC activation through the recognition of parasite derived PAMPs by PRRs such as TLRs expressed on DCs

and parasite antigens could be more accessible to the MHC class I and II processing machinery. In our *in vitro* experiments neutrophil-derived prostanoids contributed to the negative impact of neutrophils on DC activation. The role of neutrophil-derived prostanoids in hampering DC activation *in vivo* following *L. major* inoculation still needs to be determined.

The contribution of distinct DC subsets in the initiation of the *L. major* protective immune response is still not clear. Different studies demonstrated the involvement of $CD11b^+$ LN-resident, $CD8\alpha^-$langerin$^-$ dermal or moDC formed at the site of infection in antigen presentation and initiation of the adaptive immune response following *L. major* infection [98, 178, 179]. In our studies we analyzed total $CD11c^+$ DCs present in the dLN three days post *L. major* infection. This includes LN-resident DC subsets such as $CD8\alpha^+$ DCs and DCs that migrated from the site of infection to the dLN such as dermal DCs, LCs and moDCs. The effect of neutrophils on the activation of distinct DC subsets *in vivo* needs further investigation.

We demonstrated that following needle inoculation of a high dose of *L. major* in the ear dermis, neutrophils that migrated in the dLNs have a negative effect on DC activation three days post infection. During natural transmission of the parasites by the bite of an infected sand fly, the initial neutrophilic infiltrate is more massive, localized and sustained compared to needle inoculation of the parasite. Thus, the negative effect of neutrophils on DC activation might be even stronger following natural sand fly transmission of the parasites thereby playing an important role on the establishment and progression of the disease.

1.3 The consequences of early neutrophil depletion on disease progression

The role of neutrophils in promoting or suppressing the anti-leishmania response in leishmania-susceptible BALB/c mice *in vivo* was primarily addressed by treating mice with neutrophil-depleting antibodies. Depending on the mAb used to deplete neutrophils and the *L. major* strain the outcomes of the studies varied. In 1998, Lima *et al*. demonstrated that in BALB/c mice the depletion of neutrophils using the mAb RB6-8C5 prior to *L. major* inoculation led to increased parasite spreading and footpad swelling compared to non-treated littermates [172]. Our lab showed in 2000 that the early and transient depletion of neutrophils prior to injection of *L. major* LV39 resulted in the development of significantly decreased lesions compared to non-depleted littermates. Furthermore, neutrophil-depleted mice had lower parasite loads and developed decreased Th2 immune responses [161]. Then, the group of Dos Reis found in 2004 that neutrophil-depleted mice (mAb RB6-8C5) had decreased

parasite loads in the dLN 10 days post inoculation of *L. major* LV39 [168]. Using the RB6 neutrophil-depleting mAb, Chen *et al*. showed in 2005 that depletion of neutrophils prior to inoculation of *L. major* (MHOM/SU/73/5ASKH) resulted in the development of larger lesions that harbored more parasites [173]. Thus, different studies have addressed the role of neutrophils upon *L. major* infection in BALB/c mice and found different results depending on the *L. major* strain used to infect mice and the mAb administered to deplete neutrophils. However, all of the above mentioned studies used s.c. inoculations of the parasites in the footpads. The impact of neutrophils on the anti-leishmania response following intra dermal needle injection of *L. major* in the ear of BALB/c mice has not been investigated yet.

We demonstrated that neutrophil-depleted mice developed significantly smaller, non-progressive lesions compared to control-treated mice. Furthermore, the numbers of parasites in infected ears from neutrophil-depleted mice were decreased at the time points analyzed (two and six weeks post infection). Depletion of neutrophils significantly decreased Th2 responses while Th1 responses were not altered. These results are in line with previous data obtained in our lab using s.c. inoculation in the footpad [161]. In the former study dLN $CD4^+$ T cells of neutrophil-depleted BALB/c mice did not downregulate the expression of the IL-12R β2-chain and remained responsive to IL-12 signaling unlike those of control-treated littermates. Neutralization of IL-12 reversed the protective effect of neutrophil-depletion [161]. Since here we observed a similar decrease in Th2 immune responses it is likely that $CD4^+$ T cells from BALB/c mice depleted of neutrophils remain responsive to IL-12 signaling. Having observed a strong negative effect of neutrophils on DCs in terms of expression of activation markers and secretion of the Th1-inducing cytokine IL-12p70 and TNF *in vitro*, the increased activation of dLN DCs *in vivo* could contribute to promoting a protective anti-leishmania immune response in BALB/c mice.

IL-17 was shown to promote progression of cutaneous leishmaniasis in susceptible BALB/c mice. Indeed, IL-17-deficient BALB/c mice developed significantly smaller, non-progressive lesions. Improved disease outcome was associated with an impaired recruitment of neutrophils to the site of infection due to a decreased accumulation of CXCL2 in the lesion [146]. We could demonstrate that early and transient depletion of neutrophils prior to *L. major* inoculation resulted in significantly increased production of IL-17 by dLN cells two weeks post infection. However, when we analyzed IL-17 secretion by dLN cells six weeks post *L. major* inoculation, we found significantly lower levels in anti-PMN-treated mice. In addition to the decreased levels of IL-17, the numbers of neutrophils present in the lesions of anti-PMN treated mice was significantly reduced six weeks post *L. major* inoculation compared to control-treated littermates (data not shown). One possible explanation for this discrepancy in IL-17 production two and six weeks post infection could be that the early depletion of neutrophils induced the secretion of Th17-promoting factors such as IL-6, TGFβ

or IL-1β resulting in the development of Th17 cells early after parasite inoculation. Furthermore it has been suggested that IL-23 production by *L. major* infected DCs maintains Th17 cells [146]. Early after infection in anti-PMN-treated mice a higher number of infected DCs and higher IL-17 levels were observed. In contrast, in at later stages we observed decreased parasite numbers and lower IL-17 levels. Another report demonstrated that following *Mycobacteria* infection neutrophil-derived IL-10 specifically shut down IL-17 production by Th17 cells but did not affect IFNγ production by Th1 cells due to the Th17 CD4 cell-restricted expression of the IL-10R [205].

Collectively, we could demonstrate here that neutrophils have a negative impact on DC activation *in vitro* as well as *in vivo* and that they promote progression of cutaneous leishmaniasis in susceptible BALB/c mice following needle inoculation of high doses of *L. major*. Following natural transmission of the parasites by the bite of an infected sand fly the neutrophilic infiltrate is more massive and sustained compared to needle inoculation of the parasites. It has been demonstrated that mice vaccinated with a killed *L. major* vaccine were protected against needle inoculation of the parasites but not against the bite of an infected sand fly. Only the sand fly but not the needle challenge resulted in a sustained presence of neutrophils at the inoculation site and depletion of neutrophils following sand fly transmission of the parasites promoted the efficacy of the killed vaccine [206]. Thus, the deleterious effect of neutrophils, especially following the bite of an infected sand fly, should be considered for new vaccination strategies.

2. The role of neutrophils upon *L. major* infection in C57BL/6 mice

2.1 The impact of neutrophils on DC activation *in vivo*

Our lab has previously demonstrated that the early infiltrate of neutrophils to the site of *L. major* inoculation is comparable between C57BL/6 and BALB/c mice. However, only neutrophils from C57BL/6 mice secreted significant levels of the DC-attracting chemokine CCL3 upon exposure to *L. major* resulting in the accumulation of LCs, dermal and moDCs at the site of parasite inoculation and depletion of neutrophils prior to *L. major* inoculation significantly decreased DC numbers compared to control-treated littermates [159]. When we analyzed the numbers of DCs and infected DCs present at the site of infection we did not observe any significant differences between control- and anti-PMN-treated mice. However, while the previous study analyzed DCs migrating out of the ear dermis *ex vivo* 24 hours post parasite inoculation, enzymatic digestion to recover total DCs from ears three days post *L.*

major infection was used in the present study. Thus different isolation procedures could explain the differences observed concerning the impact of neutrophil-depletion on DC recruitment.

A recent report has shown that neutrophil depletion prior to inoculation of *L. major* Friedlin in the ear dermis induced increased expression of activation markers on dermal DCs [160]. However, we did not observe any detectable differences in the expression of the activation markers CD80, CD86, CD40 and MHC II on total $CD11c^+$ DCs recovered from ears of control- or anti-PMN-treated C57BL/6 mice three days post *L. major* LV39 inoculation,. Factors that might account for the different results are the use of different *L. major* strains, different inoculum doses and the use of distinct neutrophil-depleting mAbs. Our results suggest that neutrophils have no detectable impact on the activation of DCs present at the site of infection three days post *L. major* LV39 inoculation.

We found similar numbers of neutrophils migrating to the dLNs of BALB/c and C57BL/6 mice following *L. major* inoculation in the ear dermis. However, depletion of neutrophils in C57BL/6 mice had no major impact on the expression of activation markers on dLN DCs. Furthermore we did not see any differences in the numbers of infected DCs in the dLNs of control- or anti-PMN-treated C57BL/6 mice while neutrophil-depletion in BALB/c mice resulted in higher numbers of infected DCs in the dLNs compared to control-treated littermates. Thus, the differential sequestration of parasites by neutrophils from C57BL/6 and BALB/c mice could account for the different outcomes on DC activation. Furthermore *L. major* induces distinct phenotypes in C57BL/6 or BALB/c neutrophils. Only neutrophils from C57BL/6 mice secrete IL-10 and biologically active IL-12 while those from BALB/c mice secrete IL-12p40 homodimers and TGF-β [166, 169]. In addition, the expression of levels of TLR2, TLR7 and TLR9 as well as secretion of elastase in response to *L. major in vitro* was significantly higher in neutrophils from C57BL/6 mice [166, 167]. Other neutrophil-derived factors that have been shown to negatively influence DC activation are prostanoids and MPO [76, 207]. Whether these factors are involved in hampering DC activation in response to *L. major* infection *in vivo* and whether their expression is differentially regulated by neutrophils from C57BL/6 or BALB/c mice still needs to be investigated. In summary, in the present study the depletion of neutrophils in C57BL/6 mice had no detectable impact on the activation of DCs both at the site of infection and in the dLN following *L. major* inoculation. This may be linked to the distinct neutrophil phenotypes observed in *L. major* exposed in C57BL/6 and BALB/c neutrophils.

2.2 The consequences of early neutrophil depletion on disease progression

Several reports have demonstrated that in C57BL/6 mice depletion of neutrophils prior to inoculation of *L. major* s.c. in the footpads led to increased parasites loads in the dLNs at early stages of infection (from 6 hours to 29 days post infection) [161, 168, 172]. However, following natural infection involving *L. major* transmission by the bite of infected sand flies, parasite loads were substantially reduced at the site of infection at one and four weeks post parasite transmission in anti-PMN treated C57BL/6 mice [158]. Previous studies from our lab have shown that early and transient depletion of neutrophils prior to infection of *L. major* in the footpad led to a transient increase in lesion growth. Yet, neutrophil-depleted C57BL/6 eventually controlled lesion growth and parasite replication as efficiently as control-treated littermates [161]. The role of neutrophils on disease progression following needle inoculation of high dose of *L. major* in the ear pinna has not been investigated yet. We could demonstrate that depletion of neutrophils had no major influence on lesion development and parasite loads in infected ears following *L. major* inoculation. Furthermore depletion of neutrophils did not affect the production of IFNγ, IL-4, IL-17 and IL-10 two weeks post infection. At six weeks post parasite inoculation IL-4 levels were reduced in anti-PMN-treated C57BL/6 mice. However, both control- and anti-PMN-treated mice developed a dominant Th1 immune response and secreted IL-4 levels were very low. Consequently, the decreased IL-4 levels in anti-PMN-treated mice did not further improve disease outcome. Thus, the impact of neutrophils on the anti-leishmania response in C57BL/6 mice depends on the site and route of infection and the *L. major* parasite strain.

Besides the differences in phenotypes of neutrophils from C57BL/6 and BALB/c mice exposed to *L. major*, their crosstalk with other cells also varies. In this line, the engulfment of apoptotic neutrophils by macrophages led to parasite killing using cells derived from C57BL/6 mice. In contrast, co-cultures of apoptotic neutrophils and macrophages from BALB/c mice resulted in exacerbated parasite growth and the production of PGE_2 and TGFβ [167, 168]. Thus, different effects of *L. major* on neutrophils from C57BL/6 and BALB/c mice and consequently the influence of neutrophils on other cells such as macrophages and DCs might account for the distinct roles of neutrophils on disease progression in C57BL/6 and BALB/c mice.

In summary, we have demonstrated that neutrophils have a negative impact on DC activation both *in vitro* and *in vivo* and play a deleterious role during infections with *L. major* in susceptible BALB/c mice. These results suggest that *L. major* parasites exploit neutrophils to impair the innate response of DCs and macrophages in order to delay the onset of the adaptive immune response Taking into consideration that the neutrophilic infiltrate is more

massive and sustained following natural transmission of *L. major* by the bite of an infected sand fly, the harmful role of neutrophils could be even more enhanced upon natural transmission. In this line it has been demonstrated that a killed *Leishmania* vaccine promoted protection against needle inoculation of *L. major* parasites but failed to protect against infected sand fly challenge. This failure in protection was linked to a sustained presence of neutrophils at the site of infection following the bite of an infected sand fly and depletion of neutrophils following vector transmission promoted the efficacy of the killed vaccine and protected mice against the sand fly challenge [206]. Thus, the impact of neutrophils on the anti-leishmania response should be considered for the development of new therapeutic approaches as well as for the design of new vaccination strategies. ,

Perspectives

We have demonstrated that neutrophils have a negative impact on DC activation both *in vitro* and *in vivo* in BALB/c mice following *L. major* infection. Furthermore we have shown that the early neutrophilic infiltrate following *L. major* inoculation is detrimental in BALB/c mice while its role in C57BL/6 mice appears to be less important. However, there are still open questions that need further investigation:

1. Do neutrophils exposed to *L. major* secrete prostanoids and are there differences between neutrophils from C57BL/6 and BALB/c mice?

Our studies have demonstrated that neutrophils from C57BL/6 and BALB/c mice have a different impact on DC activation. Moreover, neutrophil-derived prostanoids are involved in the downregulation of DC activation *in vitro*. However, which prostanoids are secreted by neutrophils in response to *L. major* and if there are differences between neutrophils from C57BL/6 and BALB/c mice is not known. To test this neutrophils from C57BL/6 or BALB/c mice could be incubated with *L. major* parasites and the secretion of different prostanoids such PGE_2, PGF_2, PGD_2 and thromboxane A analyzed in the culture supernatants by ELISA. These results would allow us to further define the mechanisms by which neutrophils downregulate DC activation and could explain, at least in part, the differences observed in C57BL/6 and BALB/c mice.

2. How do neutrophils downregulate DC activation *in vitro*

Depending on the results obtained from the phenotypical characterization of neutrophils we will use specific prostanoid-inhibitors to analyze the contribution of distinct prostanoids on DC activation. Furthermore it has been demonstrated that the addition of PGE_2 to DCs induced IL-10 secretion that suppresses IL-12 release and decreases MHC II expression on DCs. Thus the impact of IL-10 on the decreased DC activation in the co-culture system could be analyzed.

In addition to the release of soluble factors, neutrophils can also affect DC activation by cell-contact dependent mechanisms. In order to test if direct cell contact is involved in the negative impact of neutrophils on DC activation, transwell assays in which neutrophils are

separated from DCs by a membrane that allows the trafficking of soluble mediators but inhibits direct cell contact could be used. Depending on the results further use of mAbs blocking specific molecules such as DC SIGN or Mac-1 on neutrophils could be used to identify the surface molecules that could influence DC activation by a cell contact dependent mechanism.

So far we have used $CD8\alpha^+$ Mutu DCs and starch-elicited, inflammatory neutrophils in our *in vitro* co-cultures. It will be interesting to analyze the impact of neutrophils on different *ex vivo* FACS-sorted DC subsets.

3. Do mechanisms found *in vitro* also translate to studies *in vivo*?

Experiments performed *in vitro* will provide mechanisms indicating how neutrophils can downregulate DC activation. As a next step we will expand these findings to studies *in vivo*. Depending on the results obtained *in vitro* mice will be treated with specific pharmaceutical inhibitors or mAbs to block soluble factors or surface molecules and analyze DC activation both at the site of infection and in the dLN following *L. major* inoculation. To analyze if such factors are neutrophil-specific, we could deplete mice of neutrophils and reinject in the same mice neutrophils that have been treated with specific inhibitors or that are deficient for the production of certain factors. Mice treated this way could be compared to neutrophil-depleted mice reinjected with untreated wild type neutrophils in terms of expression of activation markers on DCs.

Furthermore selected DC subsets from mice depleted or not of neutrophils could be analyzed for their expression of activation markers.

4. Does the enhanced DC activation observed in neutrophil depleted BALB/c mice lead to increased T cell proliferation and function?

We have demonstrated that in BALB/c mice depletion of neutrophils led to increased expression of activation markers on DCs in the dLN. In order to investigate whether this increased DC activation has an impact on T cell proliferation and function, DCs from control- and anti-PMN-treated BALB/c mice isolated from the dLN three days post *L. major* inoculation by FACS will be incubated with purified CFSE-labeled $CD4^+$ T cells from three weeks *L. major* infected mice for five days. As read out of T cell function T cell proliferation in terms of CFSE dilution will be analyzed and the cytokine content in the supernatants measured.

5. What is the impact of neutrophils during *L. major* infection in neutropenic mice

Due to the lack of good neutropenic mouse models, the role of neutrophils on the anti-leishmania response has been mainly studied using neutrophil-depleting mAbs. However, each of the currently used mAbs has certain disadvantages and even more importantly, neutrophils can be depleted only transiently for a short period of time. Depending on the mAb used to deplete neutrophils, the outcome of different studies varied. Recently, a new neutropenic mouse strain termed *Genista* on the leishmania-resistant C57BL/6 genetic background has been generated. It will be interesting to use those mice to study the role of neutrophils during infections with *L. major* in the absence of possible side effects caused by the injection of different mAbs. Results obtained using Genista mice could solve some of the discrepancies on the role of neutrophils following *L. major* infection between distinct reports that had used different mAbs to deplete neutrophils.

Since we have demonstrated that while in C57BL/6 the early neutrophilic infiltrate seems to be less important, it is harmful in BALB/c mice following *L. major* inoculation. Thus it would be interesting to backcross *Genista* mice on the *Leishmania*-susceptible BALB/c background and analyze the anti-*Leishmania* response in those mice in the absence of mature neutrophils.

Data from all these experiments could provide insight in the mechanisms how neutrophils can affect DC activation in the context of *L. major* infection and might be important for the development of new potential therapies and vaccines against Leishmania.

Other contributions

1. Deficiency in TREM-1 attenuates disease severity without affecting pathogen clearance

TREM-1 is an immune receptor expressed by myeloid cells such as neutrophils that has the capability to greatly amplify proinflammatory responses. The role of TREM-1 *in vivo* has been mainly studied by injecting agonistic or antagonistic proteins or peptides. However, interactions of these agents with the yet unknown TREM-1 ligand could also effect signaling through other receptors and controversial results have been obtained concerning the involvement of TREM-1 for microbial control. In order to explicitly study the role of TREM-1 during homeostasis and disease, we have generated Trem-1 deficient mice.

Trem-1$^{-/-}$ mice were viable, fertile and showed no alterations in the hematopoietic compartment. Following experimentally-induced intestinal inflammation or challenge with *L. major*, *influenza* virus or *L. pneumophilia*, Trem-1$^{-/-}$ mice showed substantially reduced immune-associated disease while still being able to control infections. These results suggest a pathogenic role for TREM-1 during parasitic as well as viral infections. Thus, therapeutic blocking of TREM-1 might be beneficial in various inflammatory disorders by hampering excessive inflammation while preserving the capacity for microbial control.

My contribution to this study was to investigate the role of Trem-1 in infections with *L. major*. I performed and analyzed experiments for Figure 6 and contributed to the Figure design.

Deficiency in TREM-1 attenuates disease severity without affecting pathogen clearance

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Abstract

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a potent amplifier of pro-inflammatory innate immune reactions. While TREM-1-amplified responses likely aid an improved detection and elimination of pathogens, excessive production of cytokines and oxygen radicals can also severely harm the host. Studies addressing the pathogenic role of TREM-1 during endotoxin-induced shock or microbial sepsis have so far mostly relied on the administration of TREM-1 fusion proteins or peptides representing part of the extracellular domain of TREM-1. However, binding of these agents to the yet unidentified TREM-1 ligand could also impact signaling through alternative receptors. More importantly, controversial results have been obtained regarding the requirement of TREM-1 for microbial control. To unambiguously investigate the role of TREM-1 in homeostasis and disease, we have generated mice deficient in *Trem1*. *Trem1*$^{-/-}$ mice are viable, fertile and show no altered hematopoietic compartment. In CD4^{+} T cell- and dextran sodium sulfate-induced models of colitis, *Trem1*$^{-/-}$ mice displayed significantly attenuated disease that was associated with reduced inflammatory infiltrates and diminished expression of pro-inflammatory cytokines. *Trem1*$^{-/-}$ mice also exhibited reduced neutrophilic infiltration and decreased lesion size upon infection with *Leishmania major*. Furthermore, reduced morbidity was observed for influenza virus-infected *Trem1*$^{-/-}$ mice. Importantly, while immune-associated pathologies were significantly reduced, *Trem1*$^{-/-}$ mice were equally capable of controlling infections with *L. major*, influenza virus, but also *Legionella pneumophila* as *Trem1*$^{+/+}$ controls. Our results not only demonstrate an unanticipated pathogenic impact of TREM-1 during a viral and parasitic infection, but also indicate that therapeutic blocking of TREM-1 in various inflammatory disorders holds considerable promise by blunting excessive inflammation while preserving the capacity for microbial control.

Author Summary

Triggering receptor expressed on myeloid cells-1 (TREM-1) is an immune receptor expressed by myeloid cell subsets that has the capacity to greatly augment pro-inflammatory responses in the context of a microbial infection. While a TREM-1-amplified response likely serves the efficient clearance of pathogens, it also bears the potential to cause substantial tissue damage or even death. Hence, TREM-1 appears a possible therapeutic target for tempering deleterious host-pathogen interactions. However, in models of bacterial sepsis controversial findings have been obtained regarding the requirement of TREM-1 for bacterial control - depending on the overall degree of the TREM-1 blockade that was achieved. In order to conclusively investigate harmful versus essential functions of TREM-1 *in vivo*, we have generated mice deficient in *Trem1*. *Trem1*$^{-/-}$ mice were subjected to experimentally-induced intestinal inflammation (as a model of a non-infectious, yet microbial-driven disease) and also analysed following infections with *Leishmania major*, *influenza virus* and *Legionella pneumophila*. Across all models analysed, *Trem1*$^{-/-}$ mice showed substantially reduced immune-associated disease. We thus describe a previously unanticipated pathogenic role for TREM-1 also during a parasitic and viral infection. More importantly, our data suggest that effective microbial control can be achieved in the context of blunted inflammation in the absence of TREM-1.

Introduction

Innate immune cells express several cell surface receptors and intracellular sensing molecules that allow for autonomous recognition of pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) and initiation of pro-inflammatory anti-microbial reponses. Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors, which recognize a diverse group of highly conserved microbial structures, represent only two examples of large innate immune receptors families with activating functions. Over the last decade, an additional family of evolutionary conserved innate immune receptor has been identified and characterized, the so-called triggering receptors expressed on myeloid cells (TREMs). TREMs belong to the immunoglobulin (Ig) superfamily of receptors and contain both inhibitory and activating receptors [1,2,3]. In contrast to the fairly ubiquitously expressed TLRs and NOD-like receptors, expression of TREMs is restricted to cells of the myeloid lineage [4]. Moreover, based on their capacity to integrate and potently modulate TLR- and NOD-induced signals, TREMs appear to mainly act as fine-tuners rather than initiators of inflammatory responses [3,5]. While TREM-1, TREM-2, TREM-3 (in the mouse) receptors [4,6], and the TREM-1 like transcripts TLT-1 and TLT-2 have been described [7,8], TREM-1 is the first identified and best characterized receptor of the TREM family with activating functions. TREM-1 consists of an ectodomain, composed of a single Ig V-type domain, a transmembrane region and a short cytoplasmic tail that recruits DAP12 for signaling [4]. TREM-1 is constitutively expressed on neutrophils and on subsets of monocytes and macrophages, and TREM-1 expression is further upregulated upon exposure of cells to microbial products [4]. Whereas crosslinking of TREM-1 with agonistic antibodies alone induces only modest cellular activation, TREM-1 potently synergizes with distinct TLR ligands for a substantial amplification of oxidative burst and production of pro-inflammatory mediators such as TNF, IL-1β, IL-6, IL-8, MCP-1 and Mip-1α [4,9,10].

In vivo, the role of TREM-1 has been mostly characterized in experimental models of endotoxin-induced shock or microbial sepsis where blockade of TREM-1 signaling conferred significant protection [9,11,12]. The detection of TREM-1 in inflammatory lesions caused by bacterial or fungal agents, but not in psoriasis or immune-mediated vasculitis [9], has further led to the general concept that TREM-1 is primarily

involved in microbial diseases, particularly, since elevated levels of the serum soluble form of the shed TREM-1 surface receptor (sTREM-1) also appear to associate with bacterial infections in patients with pneumonia or suspected sepsis [13,14].

However, increasing evidence is now emerging that TREM-1 may additionally play a role in non-infectious inflammatory conditions. Thus, expression of TREM-1 can also be induced by the non-microbial agent monosodium urate monohydrate crystals (MSU) or by hypoxic cell culture conditions *in vitro* [15,16]. Augmented sTREM-1 levels have been reported for patients with rheumatoid arthritis, acute pancreatitis, chronic obstructive pulmonary disease and cardiac arrest [17,18,19,20]. Furthermore, we have previously described an involvement of TREM-1 in human inflammatory bowel diseases (IBD) and in models of experimental colitis [21,22,23].

Investigations on the precise function of TREM-1 in distinct diseases have so far been complicated by the still unidentified ligand(s) for TREM-1. Putative ligands for TREM-1 have been described on the surface of human platelets and on murine granulocytes during experimental peritonitis and endotoxaemia [12,24,25]. In addition, necrotic cell lysates also appear to stimulate pro-inflammatory responses in a TREM-1-dependent manner, which may relate to association of TREM-1 with the High Mobility Group Box 1 (HMGB1) protein [26,27]. Hence, it can be speculated that not only PAMPs but also DAMPs induce signaling *via* TREM-1 and that several ligands for TREM-1 may exist.

In the absence of clearly defined ligands for TREM-1, studies addressing the impact of TREM-1 in disease have so far mostly relied on the use of TREM-1/Ig fusion proteins or synthetic peptides mimicking part of the extracellular domain of TREM-1. Although by the use of these agents substantial protection from endotoxin-induced shock, microbial sepsis or experimental colitis could be conferred [9,11,12,22], several aspects regarding the true biological role of TREM-1 remain unclear. First, considering the redundancy of innate immune receptor-ligand interactions, the possibility exists that in these previous studies not only signaling through TREM-1 but through additional, potentially more relevant receptors was prevented. Second, controversial findings have been obtained with respect to the impact of impaired TREM-1 signaling on microbial control [9,28,29,30].

In order to unambigously investigate the role of TREM-1 in homeostasis and disease, we have generated a TREM-1-deficient (*Trem1*$^{-/-}$) mouse by targeted deletion of exon 2. Here we show, employing distinct inflammation and infection models ranging from

experimental colitis to infections with *Leishmania major*, influenza virus and *Legionella pneumophila*, that complete absence of TREM-1 significantly attenuates morbidity and immune-mediated pathologies while microbial control remains unimpaired. These findings not only demonstrate an unanticipated clear role for TREM-1 in chronic inflammatory disorders, parasitic and viral infections, but also illustrate the potential for a novel therapeutic intervention in various disease settings.

Results

Deletion of *Trem1* has no apparent impact under homeostatic conditions

To account for potential embryonically lethal effects of a total deletion of the *Trem1* gene and leave open the possibility for a cell-specific ablation of TREM-1 expression, a targeting vector was designed for conditional deletion of exon 2 (Fig. S1). Exon 2 encodes the extracellular domain of TREM-1 and also contains the putative ligand binding site [31]. Breeding of $Trem1^{+/flox}$ chimeric offspring mice with deleter mice that expressed Cre ubiquitously yielded viable $Trem1^{+/-}$ *x* $Cre^{+/-}$ offspring. Moreover, interbreeding of $Trem1^{+/-}$ mice gave rise to $Trem1^{-/-}$ mice at the expected Mendelian frequencies, and $Trem1^{-/-}$ mice were equal in size, weight and fertility to littermate $Trem1^{+/+}$ controls. We thus continued to characterize these $Trem1^{-/-}$ mice with a ubiquitously deleted *Trem1* gene by elementary flow cytometry analyses. Deletion of *Trem1* indeed resulted in a gene-dose-dependent loss of TREM-1 surface expression by peripheral blood neutrophils and $Ly6C^{lo}$ monocytes (Fig. 1). Accordingly, TREM-1 was still expressed at ~2-fold reduced levels in $Trem1^{+/-}$ mice, while surface TREM-1 expression was absent on myeloid cells in $Trem1^{-/-}$ mice (Fig. 1). Absence of *Trem1* did not appear to affect the composition of various immune compartments, since almost identical numbers of distinct myeloid and lymphoid cell subsets could be retrieved from the peripheral blood, bone marrow (BM) and spleen of $Trem1^{-/-}$ mice compared to age- and sex-matched $Trem1^{+/+}$ controls (Fig. S2). However, to formally exclude a potential effect of TREM-1 on hematopoiesis, the BM of $Trem1^{-/-}$ and $Trem1^{+/+}$ mice was analysed in more depth with respect to hematopoietic stem cell and myeloid progenitor numbers following lineage depletion and depletion of lymphoid progenitors (Fig. 2). Stem cell-enriched cells were identified by their $lineage^{-}$ (lin^{-}) $Sca\text{-}1^{+}$ $c\text{-}kit^{hi}$ phenotype (LSK cells) while common myeloid progenitors (CMP), granulocyte/macrophage progenitors (GMP) and

megakaryocyte/erythrocyte precursors (MEP) were discriminated within the Sca-1^{-} c-kit^{hi} population according to their differential expression of FcγR and CD34, respectively (Fig. 2a). Compared to *Trem1*$^{+/+}$ mice, *Trem1*$^{-/-}$ mice exhibited equal numbers of LSK cells, CMP, GMP and MEP (Fig. 2b). Moreover, similar numbers of colony forming units could be observed in lineage-depleted (lin^{-}) BM cells isolated from *Trem1*$^{-/-}$ mice (Fig. 2b). Although these analyses indicated again that TREM-1 was unlikely to play a substantial role in hematopoietic processes, we were intrigued by the selective expression of surface TREM-1 by GMP, but not by CMP (Fig. 2a). As a final measure, we therefore established mixed bone marrow chimeras with either *Trem1*$^{-/-}$ (*x GFP*$^{-/-}$) and *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ BM cells or, as a control, *Trem1*$^{+/+}$ (*x GFP*$^{-/-}$) and *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ BM cells. Analysis of chimeric mice at 10 and 31 weeks post reconstitution and calculation of the respective ratios of GFP^{-} to GFP^{+} peripheral blood neutrophil, Ly6C^{hi} or Ly6C^{lo} monocyte numbers, respectively, demonstrated an equal capacity of *Trem1*$^{-/-}$ BM to give rise to distinct myeloid subsets as *Trem1*$^{+/+}$ BM. Thus, while the potential role of TREM-1 expression by GMP still remains to be explored, deficiency in *Trem1* does not appear to affect hematopoietic processes under homeostatic conditions.

We next addressed whether absence of *Trem1* could affect other receptors that use DAP12 for signaling, either by the potential presence of increased levels of intracellularly available DAP12 or by the lack of counterregulatory signals conferred by TREM-1. Indeed, the hyperresponsive phenotype of DAP12-deficient macrophages is largely ascribed to a lack of inhibitory signals by TREM-2 which also employs DAP12 [32]. Due to the important role of TREM-2 in osteoclast formation and function [33,34], we reasoned that lack of TREM-1 expression in *Trem1*$^{-/-}$ mice could possibly manifest in altered osteoclastogenesis. However, as determined by Xray and MicroCT analyses, no differences in bone density could be detected between *Trem1*$^{-/-}$ mice and their age- and sex-matched *Trem1*$^{+/+}$ controls (Fig. S3).

Taken together, these analyses revealed no apparent phenotype of *Trem1*$^{-/-}$ mice under homeostatic conditions.

Trem1$^{-/-}$ *x Rag2*$^{-/-}$ mice are largely protected from a CD4 T cell-induced colitis

We have previously demonstrated a substantial accumulation of TREM-1 expressing macrophages in the inflamed, but not uninflamed, intestinal mucosa of patients with

IBD and of mice with experimental colitis [22,23]. Hence, one of our major interests in the characterization of the *Trem1*$^{-/-}$ mouse was to unambigously investigate the role of *Trem1* in the pathogenesis of IBD. To this end, CD4^{+} CD25^{-} CD45RB^{hi} T cells were adoptively transferred into Helicobacter-positive *Trem1*$^{+/+}$ x *Rag2*$^{-/-}$ and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ recipient mice and mice were monitored regularly for clinical signs of colitis. Compared to *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ mice, which had lost ~20% of their initial body weight at the end of the observation period, weight loss in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice was minimal and only transient (Fig. 3a). Furthermore, shortening of the colon was substantially attenuated in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice (Fig. 3a). While some of the *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice still exhibited moderate histopathological signs of intestinal inflammation, the overall histopathological score was significantly reduced (Fig. 3b).

In order to gain insight in the potential underlying mechanism of the highly attenuated colitis in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice, colonic lamina propria cells that were isolated from both groups of mice in the absence of an adoptive CD4^{+} T cell transfer (healthy colon) or 12-13 days post colitis induction were analysed by FACS. As depicted in the representative FACS plots shown in Fig. 4a, the colonic lamina propria of healthy *Trem1*$^{+/+}$ *x Rag2*$^{+/+}$ mice and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice contained a similar proportion of CD11b^{+} MHCII^{hi} cells and Gr1^{+} cells were virtually absent. In contrast, Gr1^{+} cells, representing infiltrating Ly6C^{hi} Gr1^{int} monocytes and Ly6C^{int} Gr1^{hi} neutrophils, were readily detected in *Trem1*$^{+/+}$ *x Rag2*$^{+/+}$ mice and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice at 12-13 days post colitis induction (Fig. 4a). Notably, the relative frequency of Gr1^{+} cells among CD45^{+} CD11b^{+} colonic LP cells was ~5-fold lower in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice (Fig. 4a). In further contrast to the colonic LP of healthy mice, among LP MHCII^{+} Gr1^{-} cells of colitic mice two populations of MHCII^{hi} Ly6C^{lo} and MHCII^{int} Ly6C^{hi} cells could be discriminated, likely representing intestinal macrophages and monocytes in the process of differentiation (Fig. 4a) [35,36]. Also within this gate of MHCII^{+} Gr1^{-} cells, substantial differences could be detected between the two groups of mice. Accordingly, the relative frequency of MHCII^{int} Ly6C^{hi} cells was increased in colitic *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ mice whereas *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice exhibited a larger proportion of MHCII^{hi} Ly6C^{lo} macrophages (Fig. 4a).

When colonic LP cells of n=9 mice of both groups were systematically analysed at 12-13 days post colitis induction, substantially reduced numbers of various cell subsets could be seen in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice (Fig. 4b). Thus, *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice not only exhibited reduced infiltrating CD4^{+} T cells but also significantly

decreased numbers of neutrophils, Ly6C^{hi} monocytes and MHCII^{int} Ly6C^{hi} cells (Fig. 4b). These differences were not apparent for the colonic LP of healthy *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice which mainly contained MHCII^{hi} Ly6C^{lo} cells anyway (Fig. 4a and 4c).

To gain more insight which myeloid TREM-1-expressing cell subset could potentially be involved in driving intestinal inflammation in *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ mice, TREM-1 surface expression was analysed on colonic LP neutrophils, Ly6C^{hi} monocytes as well as CD11b^{+} Ly6C^{+} Gr1^{-} and CD11b^{+} Ly6C^{-} Gr1^{-} cells. As reported previously [23,37], in the healthy colonic LP TREM-1 expression was hardly detectable owing to the absence of infiltrating neutrophils and Ly6C^{+} cells (Fig. 4a and 4d). In colitic *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ mice, TREM-1 expression was observed on neutrophils, Ly6C^{hi} monocytes and CD11b^{+} Ly6C^{+} Gr1^{-} cells (Fig. 4d). Moreover, CD11b^{+} Ly6C^{-} Gr1^{-} cells, likely representing intestinal macrophages, that were isolated from colitic *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ mice exhibited a ~3-fold upregulated expression of surface TREM-1 (Fig. 4d).

In line with the reduced infiltrating cell numbers, expression of mRNA for several innate and adaptive pro-inflammatory chemokine and cytokine was significantly decreased in the lamina propria of *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ compared to *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice (Fig. 4e).

Trem1$^{-/-}$ mice display an attenuated form of an acute T cell independent dextran sodium sulfate (DSS)-induced colitis

TNFα produced by nonlymphoid cells plays a non-redundant role in the CD4^{+} T cell transfer modell of colitis as *Tnf*$^{-/-}$ *x Rag2*$^{-/-}$ mice are completely protected from colitis induction [38]. However, in experimental models of acute intestinal inflammation such as the T cell-independent DSS-induced colitis, *Tnf*$^{-/-}$ mice exhibit aggrevated disease [39,40], presumably, because early anti-microbial responses are fundamentally impaired. Due to the central function of TREM-1 in amplifying pro-inflammatory cytokine production and oxidative burst, we hypothesized that during acute intestinal inflammation complete absence of *Trem1* could also prove detrimental. Intriguingly, although upon administration of 3% DSS *Trem1*$^{-/-}$ mice initially lost weight to a similar extent as *Trem1*$^{+/+}$ mice, weight loss was considerably attenuated at 7 days post colitis induction and at 9 days *Trem1*$^{-/-}$ mice

had already improved again (Fig. 5a). Moreover, in *Trem1*$^{-/-}$ mice shortening of the colon was markedly attenuated and histopathological colitis scores were significantly decreased (Fig. 5b). Thus, while exhibiting reduced immune-mediated pathologies, *Trem1*$^{-/-}$ mice still appeared capable of dealing with the microbial translocation that ensues following DSS-induced breaching of the intestinal epithelial barrier.

Infection with *Leishmania major* leads to smaller inflammatory lesions with decreased neutrophilic cellular infiltrates in *Trem1*$^{-/-}$ mice

The observations made in the acute DSS model of colitis raised our interest whether *Trem1*$^{-/-}$ mice would also be able to control *bona fide* microbial infections, in particular, since maximal silencing of TREM-1 by a siRNA approach had proven deleterious in a fecal peritonitis model [29]. Since the rapid kinetics of this model hardly allowed to simultaneously look at beneficial effects of the *Trem1* deficiency on immune-mediated tissue damage or to assess potential adverse consequences for the priming of adaptive immune responses, we chose the *Leishmania major* infection model. Following infection with *L. major*, C57BL/6 mice develop local cutaneous lesions that spontaneously resolve within 4-8 weeks. Central to the resolution is the TNFα-mediated control of the early inflammatory response or the clearance of neutrophils, respectively, and the later IFNγ-mediated and Th1-driven elimination of the parasite by the infected macrophages [41,42].

Upon injection of $3x10^6$ *L. major* promastigotes s.c. in the footpad of *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice, an attenuation in lesion development was apparent in *Trem1*$^{-/-}$ mice already at 14 days post infection. From thereof, *Trem1*$^{-/-}$ mice showed a significantly decreased lesion size (Fig. 6a). Notably, however, parasite counts did not differ between *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ mice (Fig. 6b). We further analysed the cellular composition of infected footpads from *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ mice at 21 days post infection. While the overall cell counts were comparable, the cellular infiltrate in *Trem1*$^{-/-}$ mice exhibited ~ 3-fold reduced neutrophil numbers (Fig. 6c). To look at the potential impact of the *Trem1*-deficiency on the priming of Th1 responses, expression of IFNγ was analysed in cells isolated from the draining lymph node. The frequency of IFNγ-secreting $CD4^+$ T cells was similar in *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ mice (Fig. 6d). In addition, comparable levels of IFNγ were detected in cells of both groups of mice upon re-stimulation in vitro with the parasite (Fig. 6d). These data are in line with the

the comparable parasite killing that was observed for *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ mice. Thus, in the *L. major* infection model, absence of *Trem1* does not appear to have adverse consequences on the priming of adaptive immune responses and on parasite control while neutrophil-mediated inflammatory lesion development is substantially reduced.

Stimulation *via* TREM-1 induces TNF secretion and resistance to apoptosis in SCF-^{cond}Hoxb8 progenitor-derived neutrophils

The reduced neutrophil numbers at *L. major*-infected sites and the decreased lesion size in *Trem1*$^{-/-}$ mice agree with the notion that neutrophils play a central role in inflammatory lesion development. Indeed, the presence of non-healing lesions in *L. major* susceptible BALB/c strains is associated with elevated numbers of neutrophils [43]. Since neutrophils constitutively express high levels of TREM-1 (Fig. 1b, 4d), we aimed to investigate the consequences of TREM-1-mediated stimulation on their functional responses in more detail. Analysis of mouse neutrophils has so far been complicated by the limited numbers of cells that can be retrieved, their short life span and their distinct differentiation stages. Hence, we made use of a recently described system by which neutrophils can be differentiated *ex vivo* in large numbers using conditional Hoxb8 [44]. Using a slightly modified protocol, BM-derived progenitors were lentivirally transduced with conditional Hoxb8 in the presence of SCF, resulting in immortalized myeloid progenitor lines, termed SCF-^{cond}Hoxb8. Upon shutdown of Hoxb8 expression by withdrawal of 4-OHT cells differentiate into mature neutrophils in the presence of SCF. As shown in Figure 7a and 7b, withdrawal of 4-OHT in fact induced the appearance of cells bearing the characteristic phenotype of mouse neutrophils after *in vitro* differentiation for 5 days. Moreover, *Trem1*$^{+/+}$ SCF-^{cond}Hoxb8-derived mature neutrophils also expressed distinct levels of surface TREM-1 (Fig. 7b). TREM-1-mediated stimulation of *Trem1*$^{+/+}$, but not of *Trem1*$^{-/-}$, SCF-^{cond}Hoxb8-derived mature neutrophils resulted in pronounced expression of TNF and iNOS (Fig. 7c and 7d). Since neutrophil survival versus apoptosis could represent a deciding factor in the control of inflammation not only in the *L. major* infection model but also in the pathogenesis of experimental colitis [45], we compared the susceptibility of *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ neutrophils to spontaneous apoptosis. Following prolonged culture of fully differentiated SCF-^{cond}Hoxb8-derived

neutrophils *in vitro*, an increasing and comparable frequency of AnnexinV^{+}DAPI^{+} cells was detected for *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ neutrophils (Fig. 7e). However, in the presence of additional TREM-1-mediated stimulation by an agonistic antibody, a reduced apoptosis rate as based on diminished Caspase 3/7 activity could be observed for *Trem1*$^{+/+}$ neutrophils (Fig. 7f). Thus, TREM-1-mediated activation of mouse neutrophil appears to significantly contribute to survival.

Influenza virus-infected *Trem1*$^{-/-}$ mice exhibit reduced morbidity but an equal capacity for viral clearance

Intrigued by the substantially diminished inflammatory lesions, yet intact parasite clearance in *L. major*-infected *Trem1*$^{-/-}$ mice, we aimed to substantiate these findings in an altogether different infection model. Since high expression of TREM-1 by alveolar macrophages and previously published data [28,30,46,47] suggest a potential role for TREM-1 in lung inflammatory responses, we infected *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice intratracheally with 50 PFU influenza A virus PR8. Hypothermia and body weight loss, which are characteristically associated with experimental influenza virus infection, were observed in *Trem1*$^{+/+}$ mice at 7 days post infection (Fig. 8a and 8b). While the body temperature also dropped in *Trem1*$^{-/-}$ mice, a quicker recovery from hypothermia was seen in the *Trem1*$^{-/-}$ group (Fig. 8a). Moreover, weight loss in *Trem1*$^{-/-}$ mice was significantly attenuated and *Trem1*$^{-/-}$ mice further exhibited reduced levels of IL-6 in bronchoalveolar lavage fluid (BALF) at 10 days post infection (Fig. 8b and 8c). Notably, in spite of the reduced morbidity observed, *Trem1*$^{-/-}$ mice were equally capable of clearing the influenza virus infection as *Trem1*$^{+/+}$ controls (Fig. 8d).

***Trem1*$^{-/-}$ mice are equally capable of clearing *L. pneumophila* as *Trem1*$^{+/+}$ controls**

After having established that deficiency in TREM-1 attenuates disease but does not impair pathogen control during a parasitic and viral infection, respectively, we last sought to address the significance of TREM-1 in a bacterial infection model. Indeed, controversial results have been obtained with respect to the importance of TREM-1 in microbial control following infection of experimental animals with *Pseudomonas aeruginosa* [28,30]. Here, we employed a *Legionella pneumophila* infection model which also causes severe upper airway inflammation in permissive mice and critically depends on neutrophil-mediated microbial control. As shown in Figure 9, 3 days after

infection with 5 x 10^6 CFU of *L. pneumophila*, CFU and neutrophil numbers in the BALF did not significantly differ between *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice. Furthermore, no differences in CFU could be detected when *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were analysed at 5 days post infection (data not shown).

Discussion

The significance of TREM-1 as a central amplifier of acute pro-inflammatory responses during endotoxin-induced shock and microbial sepsis is well established. However, increasing evidence, including the recently reported association of TREM-1 with the DAMP protein HMGB1 [26,27], also suggests a potential role for TREM-1 during non-infectious and chronic inflammatory conditions. In line with this notion, we have previously described a crucial involvement of TREM-1 in IBD as based on the significant amelioration of experimental colitis upon blockade of TREM-1 with the antagonistic LP17 peptide. Blocking TREM-1 signaling by daily administration of TREM-1-Ig fusion proteins or synthetic analogues in chronic disease models is not only costly and straining but also fails to cover for the possibility that the yet unidentified TREM-1 ligand may signal through alternative receptors.

Here, we have generated a *Trem1*$^{-/-}$ mouse to unambiguously investigate the impact of a complete TREM-1-deficiency on the pathogenesis of experimental colitis but also of two other distinct sub-acute disease settings where the role of TREM-1 has so far not been addressed *in vivo*, i.e. inflammation induced by a parasitic and viral infection. Our findings demonstrate that *Trem1*$^{-/-}$ mice not only show a highly attenuated CD4$^+$ T cell- and DSS-induced colitis but also display significantly reduced lesion size and diminished morbidity during infections with *L. major* and influenza virus, respectively.

The substantial attenuation of illness and immune-mediated pathologies in *Trem1*$^{-/-}$ mice across these distinct models suggests a common mechanism by which TREM-1 signaling promotes inflammation irrespective of the original trigger. Several non-exclusive scenarios can be considered that may account for the attenuated disease in *Trem1*$^{-/-}$ mice: *A priori* reduced chemotactic recruitment of *Trem1*$^{-/-}$ neutrophils and monocytes, diminished pro-inflammatory activities, reduced life-span, and/or altered hematopoietic generation of myeloid cell subsets. Although we observed significantly decreased numbers of distinct myeloid cell subsets in the LP of colitic *Trem1*$^{-/-}$ *x*

Rag2$^{-/-}$ mice and at *L. major*-infected sites in *Trem1*$^{-/-}$ mice, respectively, we consider it unlikely that deficiency in TREM-1 causes an intrinsic primary defect in chemotaxis. When we analysed *L. major* infected sites at an early time-point, i.e. 3 days post infection, no differences in cellularity were detected between *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice (data not shown). Moreover, a recent study clearly demonstrated that TREM-1/3 proteins are not required for transendothelial migration of neutrophils [30]. Nonetheless, the markedly decreased expression of monocyte-, granulocyte-, but also T cell-specific chemokine mRNAs in the LP of *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice will in a secondary manner certainly have contributed to the decreased accumulation of inflammatory cells. Besides the diminished expression of chemotactic mediators, the colonic LP of colitic *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice also exhibited substantially reduced mRNA levels of several innate cytokines, including IL-1β, IL-6 and TNF. While it was beyond the scope of our analyses to determine the most relevant cellular sources for these mediators, and their reduced expression in the entire colonic LP in *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice certainly also reflects the decreased cellular infiltration, we consider it likely that TREM-1-amplified production of pro-inflammatory cytokines by distinct infiltrating myeloid cell subsets represents a key and early pathogenic event that will ultimately determine the later disease course and may largely account for the attenuated disease in *Trem1*$^{-/-}$ mice. In this respect, it is noteworthy that the colonic LP of colitic *Trem1*$^{-/-}$ mice contained markedly fewer MHCII^{int} Ly6C^{hi} cells or inflammatory macrophages with the capacity for expression of pro-inflammatory mediators [35,36].

As we have employed a CD4$^+$ T cell-dependent colitis model and indeed observed considerably reduced CD4$^+$ T cell numbers and correspondingly also mRNA levels for IFNγ and IL-17 in the colonic LP of transferred *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice, the question arises whether deficiency in TREM-1 may directly impact the priming of adaptive immune responses. Whereas in the colitis model we have not analysed CD4$^+$ T cell responses in more detail, CD4$^+$ T cells isolated from *L. major*-infected and CD8$^+$ T cells retrieved from influenza virus-infected *Trem1*$^{-/-}$ mice, respectively, exhibited an unimpaired capacity for IFNγ production compared to T cells from *Trem1*$^{+/+}$ mice. Moreover, we have also addressed the impact of a complete TREM-1-deficiency in an asthma model, hypothesizing that in the absence of TREM-1 induction of Th2 responses may be favoured. However, *Trem1*$^{-/-}$ mice showed no

evidence for increased airway hyperresponsiveness, decreased Th1 or increased Th2 responses (data not shown). Last, we have directly compared *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ bone-marrow derived dendritic cells (which unlike *bona fide* DC do express TREM-1 due to the presence of GM-CSF in the differentiation medium) in their T cell priming capacity *in vitro*. Besides reduced production of TNF, IL-1β and IL-6, *Trem1*$^{-/-}$ BMDC showed lower production of IL-2, IL-10 and IL-23 in response to stimulation with LPS and agonistic TREM-1 mAbs (data not shown). Nevertheless, proliferation as well as IL-17 and IFNγ production did not differ between CD4 T cells co-cultured with TREM-1-stimulated *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ BMDC, respectively (data not shown).

Another cytokine that was expressed at distinctly lower levels in the colonic LP of colitic *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice was GM-CSF that has recently been ascribed an important pathogenic role in experimental colitis by promoting the accumulation of bone marrow but also peripheral granulocyte-monocyte progenitors (GMPs) [48]. It is therefore plausible that the attenuated disease and decreased myeloid infiltrates observed in *Trem1*$^{-/-}$ mice may to some extent also relate to diminished stimulation of upstream and extramedullary hematopoietic processes due to overall lower levels of inflammatory cytokines and translocated microbial products. Importantly, a positive feedback loop appears to exist between TREM-1 and GM-CSF: On one hand, TREM-1 expression can be induced in the presence of GM-CSF [25], on the other hand, engagement of TREM-1 in the presence of TLR agonists potently increases production of GM-CSF [49,50]. Although under homeostatic conditions we found no evidence for altered myelopoiesis in *Trem1*$^{-/-}$ mice, the expression of TREM-1 by GMP, but not CMP, is intriguing and we are currently comparing hematopoietic processes in *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ mice that have been exposed to inflammatory stimuli.

The deciding role of neutrophils in the *L. major* infection model [42] and the substantially decreased lesion size in *Trem1*$^{-/-}$ mice have prompted us to investigate the impact of TREM-1 on neutrophil-mediated functions in more detail. In particular, we were interested in the potential modulatory effect of TREM-1 ligation on neutrophil survival as delayed neutrophil apoptosis could also represent a deciding pathogenic factor in intestinal inflammation [45]. In agreement with a previous report [30], deficiency in TREM-1 caused no intrinsic predisposition for increased

spontaneous neutrophil apoptosis. However, agonistic TREM-1 stimulation significantly promoted survival of SCF-^{cond}Hoxb8 progenitor-derived neutrophils. Hence, in chronic disease settings TREM-1 could indeed prolong inflammation by negatively regulating neutrophil apoptosis.

Considering the various effector cell types and mechanisms by which TREM-1-mediated stimulation could contribute to disease, it may appear intriguing that deficiency in TREM-1 did not completely protect from disease. Accordingly, the degree of protection from colitis was not much higher in *Trem1*$^{-/-}$ mice compared to mice that were treated with the antagonistic LP17 peptide in our previous study [22]. We hypothesize that the absence of complete protection in *Trem1*$^{-/-}$ mice may primarily relate to the role of TREM-1 as an amplifier but not inducer of pro-inflammatory reactions [3,5]. While we cannot rule out a potential participation of TREM-3, we believe that the protective effects seen in *Trem1*$^{-/-}$ mice are too substantial for a major involvement of TREM-3 in the inflammatory models analysed.

One of the most striking findings of the present study was the observation that microbial control was apparently not impaired in *Trem1*$^{-/-}$ mice in spite of the blunted inflammatory responses. Hence, while *Tnf*$^{-/-}$ or anti-TNF-treated mice exhibit an aggrevated acute DSS-induced colitis [39,40] and also show enhanced parasite and bacterial burdens upon infection with *L. major* and *L. pneumophila*, respectively [41,51], *Trem1*$^{-/-}$ mice appeared equally capable of controlling a parasitic, viral and bacterial infection as *Trem1*$^{+/+}$ controls. This observation is in line with the main function of TREM-1 as an inflammatory fine-tuner which still allows for pro-inflammatory TLR or NOD-like receptor-induced reactions in its absence. Moreover, TREM-1 does not appear to be involved in phagocytic or direct antimicrobial activity of myeloid cells [30,49,52]. Still, conflicting data on the effect of a TREM-1 blockade on microbial control have been reported from various bacterial challenge models. Injection of a TREM-1/IgG fusion protein allowed for sufficient control of an *E. coli*-induced peritoneal infection and conferred protection [9] whereas maximal but not half-dose siRNA silencing of TREM-1 increased mortality in a fecal peritonitis model [29]. Similarly, administration of the antagonistic LP17 peptide protected rats from a *P. aeruginosa*-induced pneumonia [28], whereas complete deficiency in TREM-1/3 led to markedly increased mortality in *Pseudomonas aeruginosa*-challenged mice due to defective transepithelial migration of neutrophils [30]. It has been proposed that the degree of TREM-1 blockade was a likely critical parameter to account for these

disparant findings [29,30]. However, our findings demonstrate that microbial control must not necessarily be impaired in mice with a complete deficiency in TREM-1, even when employing a *L. pneumophila* infection model where early neutrophil accumulation is also crucial for bacterial clearance [53]. Thus, we speculate that possibly the infection dose, the nature of the microbial agent and/or the kinetics of the infection may be critical parameters regarding the requirement for TREM-1.

In summary, while the impact of TREM-1 on microbial control still needs further investigations across different experimental models, our extensive characterisation of *Trem1*$^{-/-}$ mice shows an unanticipated prominent role for TREM-1 in parasitic and viral infections. Our findings thus suggest that therapeutic targeting of TREM-1 holds considerable promise for various non-infectious and infectious inflammatory disorders and may bypass the increased risk for impaired microbial control which is associated with the general targeting of TNF.

Materials and Methods

Mice

Breedings and cohort maintenance were performed under SPF conditions in isolated ventilated cages in the central animal facility of the Medical School, University of Bern.

Ethics statement

All animal experiments were approved by the Veterinary Offices of the Cantons of Bern, Lausanne and Zurich and performed in compliance with Swiss laws for animal protection.

Generation of *Trem1*-deficient mice.

The generation of *Trem1*-deficient mice was designed and carried out in collaboration with the TaconicArtemis GmbH (Köln, Germany). To account for potential lethal effects of a total deletion of the *Trem1* gene and to allow for a possible cell-specific ablation of *Trem1* expression, a targeting vector was designed for conditional deletion of exon 2, which encodes the extracellular part of *Trem1* and also contains the putative ligand binding site [31]. As illustrated in the supplemental material Fig. S1, the targeting vector was constructed on the basis of the cloning vector KS loxP ftr Neo BS to flank exon 2 with loxP sites, to comprise additional restriction sites (AseI and AvaI) and to contain PuroR (flanked by F3 sites) and Neomycin (flanked by FRT sites) positive selection marker cassettes to control for homologous recombination upstream and downstream of exon 2, respectively. For counterselection, a Tk cassette was included downstream of exon 4. As a template for the PCR reactions a BAC-based plasmid containing the entire genomic mouse *Trem1* locus (RP23-32N8) was obtained from BACPAC Resources Center BPRC (Oakland, USA). The targeting vector was electroporated into a C57BL/6N.tac embryonic stem cell line (TaconicArtemis). On day 2, cells were selected with Puromycin and G418 and on day 8 counterselection with Gancyclovir was initiated. Isolated and expanded ES clones were screened for complete integration of the targeted allele by standard Southern blotting analyses with probes located upstream of exon 1 (5e2) or exon 3 (ila1) (Fig. S1). Primer sequences for generation of the 5e2 Southern probes were: CGGATTTGACCAGGAATGACAG(sense) and CTTCCAGTTCATTCATGGACAGC (antisense) and for the ila1 Southern probe: AGCTCCTCTTGTCTGCCATTCAAGGC (sense) and GGCTACAACCTTGTTCTGCAG (antisense). Eight positive clones could be identified and the ES clone A-A5 was subsequently injected into Balb/c derived blastocytes which were then transferred to pseudopregnant NMRI females. Chimeric offspring were bred to C57BL/6 female mice *(C57BL/6-Tg(ACTB-Flpe)2Arte,* TaconicArtemis) transgenic for *Flp* recombinase to achieve deletion of the FRT and F3 flanked selection cassettes PuroR and Neomycin, respectively.

Germline transmission of the targeted *Trem1* allele was identified by coat color contribution and by PCR using oligo 1_sense (GTGCTCAGAGAATGTCTTTGTATCC) and oligo 4_antisense (CCCTGGTCAGACCATTTACC) which either yield a 1.3 kb fragment for the wildtype (WT) allele or a 1.6 kb fragment for the conditional *Trem1*^{flox} allele. (Fig. S1). Cycling conditions were: 5' at 95°C followed by 35 cycles consisting of 30'' at 95°C, 30'' at 60°C, 1' at 72°, followed by 10' at 72°C. Thus identified *Trem1*$^{+/flox}$ mice (*C57BL/6-TREM-*$^{1tm1821\_33.1Arte}$) were mated with male mice carrying the Cre recombinase under the control of the ROSA26 locus (C57BL/6-Gt(ROSA)26Sor$^{tm16(Cre)arte}$, TaconicArtemis) to obtain systemic deletion of one *Trem1* allele (*Trem1*$^{+/-}$). *Trem1*$^{+/-}$ *x Cre*$^{+/-}$ mice were interbred to achieve deletion of *Cre* and to obtain wildtype controls (*Trem1*$^{+/+}$) and heterozygous (*Trem1*$^{+/-}$) and homozygous (*Trem1*$^{-/-}$) *Trem1*-deficient mice. Deletion of exon 2 in *Trem1*$^{+/-}$ and *Trem1*$^{-/-}$ mice was assessed by the genotyping PCR strategy described above and depicted in Fig. S1. *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were subsequently expanded for experiments. For the CD4 adoptive transfer model of colitis, *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice were generated by crossing *Trem1*$^{-/-}$ mice with Helicobacter^{+} *Rag2*$^{-/-}$ mice and interbreeding of the F1 offspring. The Helicobacter^{+} status of the *Trem-1*$^{-/-}$ *x Rag2*$^{-/-}$ offspring was confirmed by PCR testing (MicrobioS GmbH, Reinach, Switzerland).

Flow cytometry (FACS)

The following mAbs were used: anti-mouse CD11b-Pacific Blue (M1/70), CD45-Pacific Blue (30-F11), CD45-Brilliant Violet570 (30-F11), CD4-APC-Cy7 (RM4-5), Gr1-PE (RB6-8C5), NK1.1-PE-Cy7 (PK136), Ly6G-APC-Cy7 (1A8) and F4/80-biotin (BM8) IL-7Rα-biotin (A7R34), CD3ε-biotin (145-2C11) CD19-biotin (6D11), Gr1-biotin (RB6-8C5) and Ter119-biotin (TER119), all purchased from Biolegend; anti-mouse CD115-PE (AFS98), Gr1-APC (RB6-8C5), CD45-eFluor605 (30-F11), CD45.1-PE (A20), MHCII-APC (M5/114.15.2), CD11c-PE (N418), CD11b-eFluor450 (M1/70), Streptavidin-PE-Cy7, were purchased from eBioscience (San Diego, USA); anti-mouse Ly6C-FITC (AL-21) from BD Pharmingen (San Diego, USA) and anti-mouse TREM-1-APC (174031) from R&D Systems. DAPI (Invitrogen) was used in a final concentration of 0.5 µg/ml to exclude dead cells. Prior to FACS stainings, Fc receptors were blocked using supernatant from the hybridoma 2.4G2. Cells were acquired on a LSRII SORP (BD Biosciences, San Diego, USA) and analysed using FlowJo cytometric analysis program (Tree Star, Ashland, USA).

Impact of TREM-1 on hematopoiesis

Analysis of hematopoietic stem cells and progenitors

For FACS analysis of stem cell enriched LSK cells and myeloid progenitors, and also for determination of colony-forming units, a prior lineage depletion was performed using biotinylated Abs against red blood cell precursors (α-Ter119), B cells (α-CD19), T cells (α-CD3ε), myeloid cells (α-Gr1), MACS α-biotin beads, and LS columns (Miltenyi Biotec). Lymphoid progenitors were further removed by adding anti-IL-7Rα-biotin.

Determination of colony forming units

3.33×10^3 lin^- cells were transferred into methocult base medium (M3134; Stemcell Technologies) supplemented with 15% FCS, 20% BIT (50 mg/ml BSA in IMDM, 1.44 U/ml recombinant-human (rh) insulin (Actrapid, Novo Nordisk) and 250 ng/ml human holo transferrin [Prospec]), 100 µM 2-β-mercaptoethanol, 2 mM l-glutamine, penicillin/streptomycin, and 50 ng/ml recombinant-mouse SCF (rmSCF), 10 ng/ml rm–IL-3, 10 ng/ml rh-IL-6 and 50 ng/ml rm-Flt3-ligand (all from Prospec). Colonies and cells were enumerated after 7 days (≥30 cells/colony).

Generation of mixed bone marrow chimeras

Congenic ($CD45.1^+$) recipient mice were irradiated in two split doses with 650 cGy in a 4 h interval in a Gammacell 40 exactor (Best Theratronics). Total donor bone marrow (BM) was collected from *Trem1*$^{-/-}$, *Trem1*$^{+/+}$ and *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ mice as described below. Donor BM was mixed 1:1 and 15 x 10^6 total cells of either mixed *Trem1*$^{-/-}$ and *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ BM cells or mixed *Trem1*$^{+/+}$ and *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ BM cells were transferred in 200 µl PBS i.v. into the irradiated recipient mice. After transfer, the recipients were treated with antibiotics (Baitryl and Bactrim) in the drinking water for two weeks. After 10 weeks, the grade of chimerism was controlled in the peripheral blood by calculating the $CD45.2^+$ / $CD45.1^+$ ratio. In all chimeras, the grade of chimerism among the circulating myeloid cells was at least 99%.

Generation and analysis of SCF-dependent conditional Hoxb8-immortalised progenitor cells and neutrophils

Generation of Hoxb8 progenitor lines and differentiation of neutrophils

The protocol was adapted from the method described by Wang et al. [44] with the major modification of using a different inducible expression system [54]. In brief, bone marrow-derived haematopoietic progenitors were isolated from *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice by magnetic bead-based lineage depletion (BD IMag™ mouse hematopoietic progenitor cell enrichment set-DM, BD Biosciences) following the manufacturer instructions. 2-5 x 10^5 $lineage^-$ cells were incubated for 36 hours in complete RPMI Medium (RPMI 1640/Glutamax

supplemented with 10% FCS, 1% penicillin/streptomycin solution and 50 µM 2-mercaptoethanol). Cells were then transduced with pF-5xUAS-Hoxb8(mm)-SV40-puro-GEV16 lentiviral particles by spin-infection (1000 rpm, 2 hours, 30°C) in presence of 8 µg/ml polybrene. Cells were subsequently cultured in complete RPMI medium supplemented with SCF (added as 10% of CHO/SCF(mm)-conditioned supernatant) and Hoxb8 expression was induced by addition of 100 nM 4-OHT. Transduced cells were positively selected with 1.0 µg/ml puromycin for a minimum of one month. The resulting immortalized cell lines were termed SCF-^{cond}Hoxb8. For *in vitro* differentiation of SCF-^{cond}Hoxb8 cells into mature neutrophils, cells were washed twice in PBS to remove all traces of 4-OHT and were then replated at 20'000 cells / ml in complete RPMI medium containing SCF (but no 4-OHT). Mature neutrophils were thus obtained after 5 - 6 days as judged by morphology and surface expression of CD11b and Gr-1, as well as loss of c-kit (CD117) as determined by flow cytometry.

Apoptosis measurements

Spontaneous apoptosis of mature *Trem1*$^{+/+}$ versus *Trem1*$^{-/-}$ neutrophils was assessed at 5 days post differentiation in SCF-containing medium and 8 h, 24 h, and 48 h later, respectively. Immediately upon removal from plates, neutrophils were washed in cold PBS and AnnexinV Binding Buffer and stained with FITC-conjugated AnnexinV (BD Pharmingen) according to the manufacturer's instructions. DAPI (Sigma-Aldrich) was added immediately prior to aquisition by flow cytometry. Only AnnexinV and DAPI double-positive cells (AnnexinV$^+$ DAPI$^+$) were considered in the analysis of apoptotic cells. For determination of TREM-1-mediated effects on apoptosis induction, 5 days differentiated neutrophils were stimulated *in vitro* in 96-well U-bottom plates at 2 x 10^5 cells / well in complete RPMI medium lacking SCF in the presence of 10 µg/ml plate-bound anti-TREM-1 mAb (MAB1187; R&D) or a respective isotype control (RTK2758, Biolegend) for 24 h. Since the relative stickiness of neutrophils activated by plate-bound anti-TREM-1 did not allow for removal from the plates and FACS-based analysis of AnnexinV$^+$ DAPI$^+$ cells without causing a substantial bias by the selective analysis of non-adherent cells, Caspase 3/7 activity was determined by the Apo-Tox-Glo^{TM} assay (Promega) according to manufacturer's instructions.

Experimental animal models

CD4 T cell adoptive transfer model of colitis

Colitis was induced in (Helicobacter-positive) *Rag2*$^{-/-}$ and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice by adoptive transfer of 2 x 10^5 CD4$^+$ CD25$^-$ CD45RB^{hi} FACS-sorted T cells as described previously [55]. Mice were sacrificed at 12-13 d post CD4 T cell transfer at the onset of clinical signs of

colitis (diarrhea, weight loss, symptoms of abdominal pain) in *Trem1*$^{+/+}$ mice.

Dextran sodium-sulfate (DSS)-induced colitis

Colitis was induced in (Helicobacter-negative) *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice by administration of 3% 36'000 – 50'000 MW DSS (MP Biomedicals, Solon, USA) in the drinking water for 5 d followed by 4 d of regular tap water and euthanization at d 9.

***Leishmania major* (*L. major*) infections**

L. major LV39 (MRHO/Sv/59/P strain) parasites were maintained *in vivo* in DBA/2J mice and further cultured *in vitro* in M199 medium supplemented with 10% FCS, 4% HEPES and 2% antibiotics (penicillin, streptomycin, neomycin). Mice were infected with $3x10^6$ parasites s.c. in the hind footpad in a final volume of 50 µl as previously described [41]. Footpad lesion size was measured with a Vernier caliper. The number of parasites in lesions were evaluated by limiting dilution analysis [56].

Influenza virus infections

Influenza virus strain PR8 (A/Puerto Rico/34 H1N1) was originally provided by J. Pavlovic (University of Zurich, Switzerland). For infections, mice were anaesthetized and inoculated intratracheally with 50 PFU influenza virus in 50 µl endotoxin-free PBS. For collection of bronchoalveolar lavage (BAL) fluid, lungs were flushed with 3x 400 µl PBS. To determine influenza viral titers in the lungs, lungs were collected at the indicated time-points, homogenized and serially diluted with MDCK cells as previously described [57].

Legionella pneumophila infections

L. pneumophila strain JR32 (Philadelphia-1; sg1) (reference: PMID: 8225610) was grown for 3 days on charcoal yeast extract (CYE) agar plates at 37°C and resuspended in PBS prior to infection. Mice were anesthetized by i.p. injection of 5 µg xylazine/100 µg ketamine per gram body weight and infected intranasally with 5×10^6 *L. pneumophila*. Three days post infection, mice were sacrificed and perfused and bronchoalveolar lavage fluid (BALF) was extracted with 1 ml PBS, 5mM EDTA. CFU were quantified by plating serial dilutions on CYE agar plates and counted after 3 days incubation at 37°C. BALF neutrophils were quantified by flow-cytometry.

Cell isolations

Spleen and peripheral blood

Blood was collected by tail vain incision or by cardiac puncture into heparinised PBS. Spleen cells were released by homogenization of spleens between the frosted ends of two glass slides

into PBS containing 5% horse serum. Erythrocytes were depleted by brief incubation with ACK lysis buffer.

Bone marrow

Femurs and tibiae were removed and placed in ice-cold PBS. Remaining flesh was removed and bones were rinsed with sterile PBS. After opening of the bones, the BM was flushed with a 26 GA 3/8 needle into RPMI 10% FCS. Erythrocyte-depleted BM cells were either used for FACS-based characterizations with or without prior lineage depletion or for the generation of mixed BM chimeras.

Colonic lamina propria

Colons were opened longitudinally and cut into small pieces. The epithelium was removed by incubation in HBSS/HEPES containing 5% horse serum, 5 mM EDTA and 2 mM DTT at 37°C for 3 x 30 min under magnetic stirring. Lamina propria (LP) cells were obtained by subsequent digestion with 200 U/ml collagenase (Type IV; Sigma-Aldrich) and 50 U/ml DNase (Type I, grade II; Roche) for 2 x 45 min. The LP fraction was filtered through a 40 μM cell strainer, counted by Trypan Blue staining and further characterized by FACS.

Footpads

Footpads were digested using 1 mg/mL collagenase D in HBSS.

Histopathological analysis of mouse intestinal tissue sections

To assess the presence of histopathological alterations on formalin-fixed, paraffin-embedded and hematoxylin-eosin-stained colonic tissue sections, a scoring system ranging from 0 (no alterations) to 15 (most severe signs of colitis) was established, including the following parameters: cellular infiltration (0-3), loss of goblet cells (0-3), crypt abscesses (0-3), epithelial erosions (0-1), hyperemia (0-2), thickness of the colonic mucosa (0-3). Histological scoring was performed by a pathologist (V.G.) blinded to sample identity.

RNA extractions and quantitative RT-PCR analyses

RNA was isolated using RNA isolation reagent (Tri-Reagent, Molecular Research Center). DNA was digested using DNase I (Ambion), and cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression of genes was analysed using Qiagen Quantitect Primer Assays on a 7500 Real-time PCR System (AB Biosystems). The house keeping gene GAPDH was used for normalization of gene expression.

Analysis of cytokines in supernatants and bronchoalveolar lavage (BAL)

Cell-free supernatants derived from SCF^{-cond} Hoxb8 neutrophils and BAL fluid from influenza virus infected mice were analysed by ELISA (Biolegend).

Analysis of bone density

X-ray

High resolution X-ray analyses on anesthetized mice were performed with the MX-20 Faxitron (X-ray Corporation, Edimex, LePlessis, France).

MicroCT

For high resolution microcomputed tomography (MicroCT) tissues were fixed in 4% paraformaldehyde in PBS for 24 h and subsequently transferred to 70% EtOH for μCT analysis (MicroCT40, Scanco, Bruettisellen, Switzerland).

Statistical analyses

All data were analysed with GraphPad Prism software using the Student's t-test.

Online supplemental material

Fig. S1 provides a schematic illustration of the wildtype *Trem1* locus, the targeting vector and the conditional and constitutive knockout alleles. In addition, Southern blot and PCR analyses for detection of the targeted allele in embryonic stem cells and detection of wildtype, conditional and constitutive knockout alleles in offspring mice, respectively, are presented. Fig. S2 shows a comparison of the composition of peripheral blood, bone marrow and spleen immune compartments in $Trem1^{+/+}$ versus $Trem1^{-/-}$ mice. Fig. S3 shows X-ray and MicroCT analyses that were carried out to determine bone density in $Trem1^{+/+}$ versus $Trem1^{-/-}$ mice.

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Figure legends

Figure 1. TREM-1 surface expression by peripheral blood myeloid cell subsets from wildtype versus *Trem1*-deficient mice. Peripheral blood cells obtained from wildtype (*Trem1*$^{+/+}$) and *Trem1*-deficient (*Trem1*$^{+/-}$ and *Trem1*$^{-/-}$) mice (n=2 mice for each group) were stained for surface expression of TREM-1 and analysed by FACS. (A) Representative gating strategy to identify neutrophils and LyC6^{lo} and Ly6C^{hi} monocytes. (B) Representative histograms showing TREM-1 surface expression (lines) versus isotype controls (filled histograms). (C) Mean fluorescence intensity (MFI) of TREM-1 surface expression. Mean values of n=2 mice analyzed are shown with error bars indicating the range.

Figure 2. Unimpaired hematopoiesis in *Trem1*$^{-/-}$ mice. (A) Representative dot plots show the FACS-based identification of lineage-depleted (lin$^-$) Sca1$^+$ c-kit^{hi} (LSK) cells and lin$^-$ Sca1$^-$ c-Kit^{hi} myeloid progenitors in *Trem1*$^{+/+}$ (top panels) and *Trem1*$^{-/-}$ (bottom panels) bone marrow (BM) following lineage depletion and depletion of lymphoid progenitors by MACS. Common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP) were further discriminated according to their expression of FcγR and CD34. Filled histograms show TREM-1 surface expression by LSK cells, CMP, GMP and MEP progenitors from *Trem1*$^{-/-}$mice in comparison to *Trem1*$^{+/+}$ mice (lines). (B) Absolute cell numbers of total BM cells, lin$^-$ BM cells, lin$^-$ Sca1$^-$ c-kit^{hi} myeloid progenitors, LSK cells, CMP, GMP and MEP and colony forming units (CFU) of hematopoietic precursors isolated from the BM of *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were determined as described in the Materials and Methods section. Mean values of n=2 mice analysed are shown with error bars indicating the range. (C) Mixed BM chimeras were generated by i.v. transfer of 1:1 mixed *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ and *Trem1*$^{-/-}$ *x GFP*$^{-/-}$ BM cells (white circles, dotted lines) into irradiated recipient mice. As control, and to account for potential interfering effects of the GFP expression, mixed BM from *Trem1*$^{+/+}$ *x GFP*$^{+/+}$ and *Trem1*$^{+/+}$ *x GFP*$^{-/-}$ mice (black circles and lines) was transferred into additional recipient mice. BM chimeras were analyzed after 10 and 31 weeks of chimerism. Neutrophils, Ly6C^{hi} and Ly6C^{lo} monocytes were identified in the peripheral blood according to the depicted gating strategy and the GFP$^-$: GFP$^+$ cell ratio in the respective cell subsets was determined. Mean values of n=4-5 mice

analyzed per group are shown with error bars indicating the SEM. ns, no statistically significant difference.

Figure 3. *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice are protected from CD4^{+} T cell-induced colitis. Colitis was induced in *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ (filled circles) and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice (white circles) by i.p. injection of 2 x 10^5 CD4^{+} CD45RB^{hi} T cells. (A) Weight loss relative to the initial body weight. Mean values of n=9 mice analysed per group are shown with error bars indicating the SEM. Colon lenghts were determined in individual mice (symbols). Lines show mean values for each group of mice. (B) Photographs (magnification: 10x) of representative H&E-stained colonic tissue sections of a *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ (histopathological score: 14) and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mouse (histopathological score: 2). Histopathological scores were determined for individual mice by a pathologist according to criteria defined in the Materials and Methods section. Symbols show scores for individual mice and lines indicate the mean value for each group of mice. ***, $p < 0.001$; **, $p < 0.01$.

Figure 4. Upon colitis induction, *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice exhibit substantially reduced inflammatory infiltrates and diminished expression of pro-inflammatory mediators. (A-C) Lamina propria cells were isolated from the colon of *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$ and *Trem1*$^{-/-}$ *x Rag2*$^{-/-}$ mice 12-13 days post adoptive transfer of colitogenic CD4 T cells or from untransferred mice (healthy colons) and analysed by FACS. (A) After exclusion of doublets and dead cells, CD11b^{+} cells were discriminated from CD4^{+} T cells and further subgated into MHC^{lo} Gr1^{+} (gate 1) and MHC^{hi} Gr1^{-} (gate 2) cells. In gate 1, monocytes and neutrophils were identified according to their Ly6C^{hi} Gr1^{int} and Ly6C^{int} Gr1^{hi} phenotype, respectively. In gate 2, MHCII^{+} cells were further subdivided into two populations of MHCII^{int} Ly6C^{hi} and MHCII^{hi} Ly6C^{lo} cells. (B, C) Absolute numbers of total cells recovered from individual mice (symbols; lines indicate mean values per group) and mean values ± SEM for CD45^{+} cells, CD4^{+} T cells, CD11b^{+} cells and subsets defined within the CD11b^{+} gate as illustrated in (A). Per group, n=9 mice adoptively transferred with CD4 T cells (B) and n=4 untransferred (C) mice were analysed. (D) TREM-1 surface expression by neutrophils (Ly6C^{int} Gr1^{hi}), monocytes (Ly6C^{hi} Gr1^{int}) and CD11b^{+} Gr1^{-} Ly6C^{+} versus Ly6C^{-} subsets identified in the lamina propria (according to the gating strategy depicted in D) of colitic (n=9) versus healthy (n=4) *Trem1*$^{+/+}$ *x Rag2*$^{-/-}$

mice. (E) Colonic tissues were assessed for the expression of pro-inflammatory mediators by qRT-PCR. Bars show mean values ± SEM for n=9 mice. (E). ***, $p < 0.001$; **, $p < 0.01$. N.D. = not determined due to insufficient cell numbers.

Figure 5. Attenuated dextran sodium-sulfate (DSS)-induced colitis in *Trem1*$^{-/-}$ mice. Colitis was induced in *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice by administration of 3% DSS in the drinking water for 5 days, followed by 4 days on regular tap water. (A) Weight loss relative to the initial body weight. Mean values of n=17 (*Trem-1*$^{+/+}$) and n=16 (*Trem1*$^{-/-}$) mice are shown with error bars indicating the SEM. Colon lenghts were determined in individual mice (symbols). Lines show mean values for each group of mice. (B) Photographs (magnification: 10x) of representative H&E-stained colonic tissue sections of a *Trem1*$^{+/+}$ (histopathological score: 13) and *Trem1*$^{-/-}$ mouse (histopathological score: 5.5). Histopathological scores were determined for individual mice by a pathologist. Symbols show scores for individual mice and lines indicate the mean value for each group of mice. ***, $p < 0.001$; **, $p < 0.01$.

Figure 6. *Trem1*$^{-/-}$ mice develop smaller inflammatory lesions and show decreased cellular infiltrates at *L. major* infection sites. (A) *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were inoculated with $3x10^6$ *L. major* promastigotes s.c. in the footpad and lesion development was measured over time. Each data point represents the mean lesion size ± SEM of n=5 mice analysed per group. (B) Parasite load was assessed at 35 days post infection (p.i.) by limiting dilution analysis. (C) Infected footpads from *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice (n=4-5 mice per group) were isolated 21 days p.i., digested and the cellular content was analysed by flow-cytometry. Data show mean values ± SEM of cells and are representative of two independent experiments. (D) Draining lymph node cells from *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice (n=4 mice per group) were isolated 35 days p.i.; the frequency of $CD4^+$ $IFN\gamma^+$ T cells was analysed by intracellular FACS staining or cells were re-stimulated with UV-treated *L. major* parasites and IFNγ levels in the supernatants were assessed by ELISA. Data show mean values ± SEM of triplicate measurements. Representative data from one out of three independent experiments are shown. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Figure 7. TREM-1 mediates TNFα secretion and resistance to apoptosis in SCF-^{cond}Hoxb8 progenitor-derived neutrophils. SCF-^{cond}Hoxb8 *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ progenitor lines were generated by lentiviral transduction of Hoxb8 into BM-derived hematopoietic cells obtained from the respective mice and culture in the presence of SCF and 4-hydroxytamoxifen (4-OHT). For *in vitro* neutrophil differentiation, SCF-^{cond} Hoxb8 cells were cultured for additional 5-6 days in the absence of 4-OHT. (A) H&E stained cytospins of *Trem1*$^{+/+}$ SCF-^{cond}Hoxb8 progenitor cells (left) and differentiated neutrophils (right). (B) FACS-based characterization of *Trem1*$^{+/+}$ (top panels) and *Trem1*$^{-/-}$ (bottom panels) SCF-^{cond}Hoxb8 progenitor cells (left) and differentiated neutrophils (right). (C) TNFα and iNOS mRNA expression by *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ SCF-^{cond}Hoxb8 differentiated neutrophils following 2 h stimulation with plate-bound agonistic anti-TREM-1 mAb or an isotype control antibody was determined by qRT-PCR. (D) TNFα secretion by *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ SCF-^{cond}Hoxb8 differentiated neutrophils in response to stimulation with an agonistic anti-TREM-1 mAb was assessed by ELISA. (E) Spontaneous apoptosis of *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ SCF-^{cond}Hoxb8 neutrophils *in vitro* was analysed at 5 days post differentiation with SCF (0 h) and the indicated time-points beyond by FACS-based determination of AnnexinV and DAPI double-positive cells. Bars show mean values ± SEM for n=3 *in vitro* replicates from one representative experiment out of three independent experiments. (F) Caspase 3/7 activity was assessed upon 24 h stimulation of differentiated *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ SCF-^{cond}Hoxb8 neutrophils with plate-bound agonistic anti-TREM-1 mAb or an isotype control antibody. Bars show mean values ± SEM for n=3 *in vitro* replicates from one representative experiment out of three independent experiments. **, $p < 0.01$.

Figure 8. Reduced morbidity but intact viral clearance in influenza virus-infected *Trem1*$^{-/-}$ mice. *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were infected intratracheally with 50 PFU influenza A virus PR8. (A, B) Body temperature and weight loss relative to the initial body weight following infection. Graphs show mean values ± SEM of n=10 mice per group (C, D) Mice were sacrificed at the indicated time-points post infection. Bars show mean values ± SEM of n=4 (day 6) and n=6 (day 10) mice per group. (C) Lung viral titers were determined by plaque assay on MDCK cells. (D) IL-6 and IL-12 levels in BAL fluid were assessed by ELISA. **, $p < 0.01$; *, $p < 0.05$.

Figure 9. *Trem1*$^{-/-}$ mice are equally capable of clearing *L. pneumophila* as *Trem1*$^{+/+}$ controls. *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice were infected intranasally with 5 x 10^6 CFU *L. pneumophila*. 3 days post infection CFU (A) and neutrophils (B) were quantified in the BALF. ns, no statistically significant difference.

Supplemental Figure S1. Generation of *Trem1*-deficient mice.

Trem1-deficient mice were generated as described in detail in the Materials and Methods section. In brief and as depicted in (A), a targeting vector was designed for conditional deletion of exon 2 (coding for the extracellular V-type Ig-like domain) by flanking of exon 2 with loxP sites. The targeting vector was further constructed to contain additional restriction sites (AseI and AvaI), the positive selection markers PuroR flanked by F3 sites and Neo flanked by FRT sites and the counterselection cassette Tk. The vector was electroporated into a C57BL/N.tac embryonic stem (ES) cell line. Genomic DNA of selected ES clones was subjected to enzymatic digestion with either AseI or AvaI and standard Southern blotting analyses with probes located upstream of exon 1 (5e2) or exon 3 (ila1) to identify successful recombination or presence of the correctly targeted allele, respectively. Balb/c-derived blastocytes injected with the so identified targeted ES clone A-A5 were then transferred to pseudopregnant NMRI females and chimerism in the offsprings was assessed by coat colour contribution (white/black). Chimeric offspring were bred to C57BL/6 females transgenic for Flp to achieve Flp-mediated removal of the F3 and FRT flanked PuroR and Neo selection markers, respectively. Germline transmission was identified by the presence of black C57BL/6 offspring, representing heterozygous floxed *Trem1* ($^{+/flox}$) mice that possessed the conditional knockout allele following Flp recombination. *Trem1*$^{+/flox}$ female mice were mated with heterozygous Cre-transgenic (Cre$^{+/-}$) "deleter" males to generate *Trem1*$^{+/-}$ mice with a heterozygous constitutive knockout allele. *Trem1*$^{+/-}$ *x Cre*$^{+/-}$ mice were interbred to generate fully *Trem1*-deficient (*Trem1*$^{-/-}$) mice. (A) Schematic presentation of the *Trem1* wildtype allele, the targeting vector, the targeted allele before and after Flp recombination *in vivo* and the final constitutive knockout allele after Cre recombination *in vivo*. (B) Southern blot analyses of the ES clone A-A5 demonstrating presence of the targeted allele (TA) following either digestion of genomic DNA with AseI and hybridization with 5e2 probe or digestion with AvaI and hybridization with ila1 probe. Restriction enzyme

and Southern blot hybridization sites are indicated in (A). (C) A PCR-based genotyping strategy was developed to identify presence of the wildtype allele, the conditional knockout allele in *Trem1*$^{+/flox}$ mice and the knockout allele in *Trem1*$^{+/-}$ mice. PCR primer sites are indicated in (A).

Supplemental Figure S2. Composition of immune compartments in *Trem1*$^{+/+}$ and *Trem1*$^{-/-}$ mice. Peripheral blood (A), bone marrow (B) and spleen cells (C) from 16 weeks old age- and sex-matched *Trem1*$^{+/+}$ (n=3) and *Trem1*$^{-/-}$ mice (n=3) were characterized by FACS. Representative dot plots show the gating strategies for identification of the respective cell subsets and graphs show the mean values ± SEM of total cell counts of n=3 mice per group.

Supplemental Figure S3. Comparison of bone density in *Trem1*$^{+/+}$ versus *Trem1*$^{-/-}$ mice. Bone density in 40 and 56 weeks old female *Trem1*$^{+/+}$ (n=5) and *Trem1*$^{-/-}$ (n=5) mice was determined by X-ray analysis. Two 56 weeks old mice per group were additionally analysed by MicroCT. Analysis of vertebral bodies is shown.

FIGURE 1

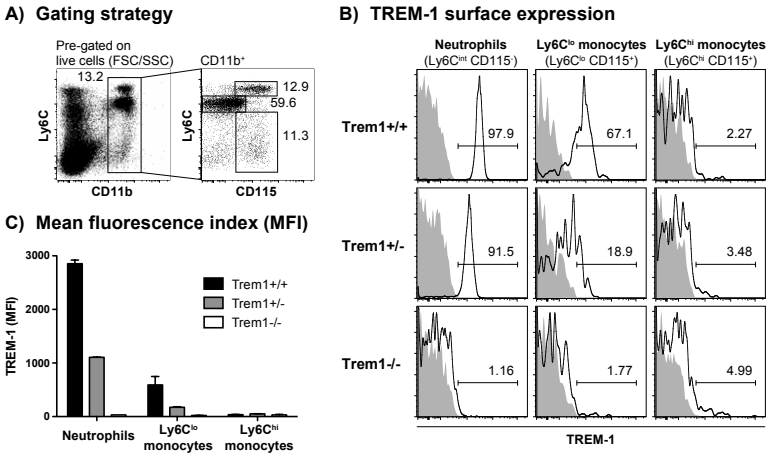
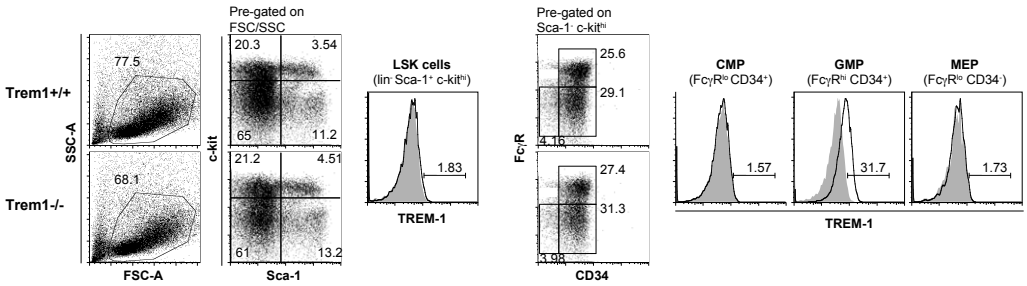
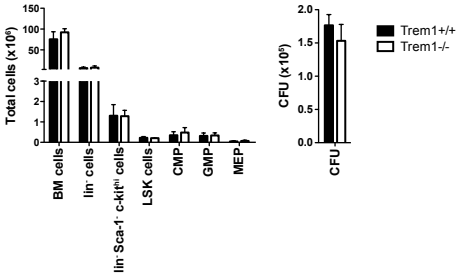

A) Gating strategy
Pre-gated on live cells (FSC/SSC)
CD11b+
13.2
12.9
59.6
11.3
Ly6C
CD11b
CD115
B) TREM-1 surface expression
Neutrophils (Ly6Cint CD115-)
Ly6Clo monocytes (Ly6Clo CD115+)
Ly6Chi monocytes (Ly6Chi CD115+)
Trem1+/+
Trem1+/-
Trem1-/-
97.9
67.1
2.27
91.5
18.9
3.48
1.16
1.77
4.99
TREM-1
C) Mean fluorescence index (MFI)
TREM-1 (MFI)
3000
2000
1000
0
Trem1+/+
Trem1+/-
Trem1-/-
Neutrophils
Ly6Clo monocytes
Ly6Chi monocytes

FIGURE 2

A) Hematopoietic stem cells and progenitors (relative population sizes)



B) Absolute cell numbers and colony forming units (CFU)



C) Mixed bone marrow chimeras

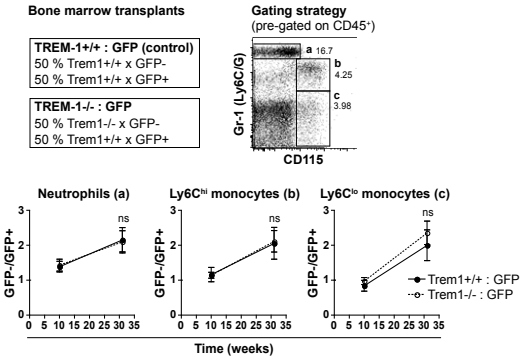


FIGURE 3

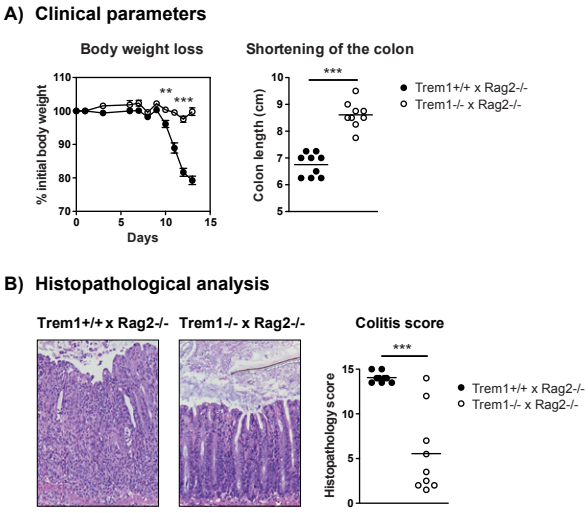

A) Clinical parameters
Body weight loss
Shortening of the colon
% initial body weight
Days
Colon length (cm)
Trem1+/+ x Rag2-/-
Trem1-/- x Rag2-/-
B) Histopathological analysis
Trem1+/+ x Rag2-/-
Trem1-/- x Rag2-/-
Colitis score
Histopathology score

FIGURE 4

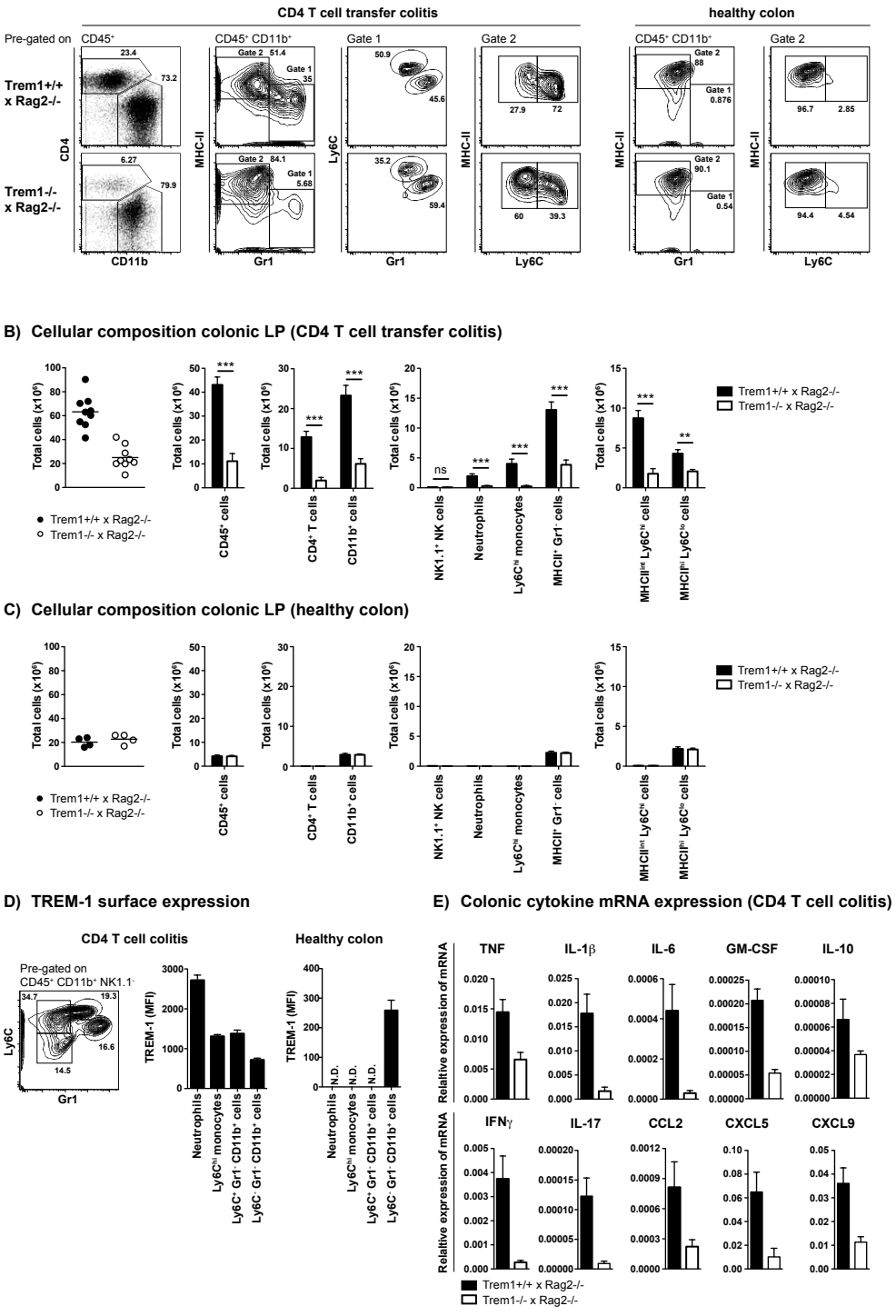
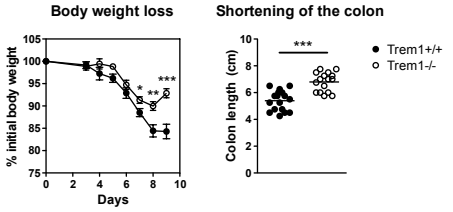

A) Representative FACS stainings colonic lamina propria
CD4 T cell transfer colitis
healthy colon
Pre-gated on
CD45+
CD45+ CD11b+
Gate 1
Gate 2
Trem1+/+ x Rag2-/-
Trem1-/- x Rag2-/-
CD4
CD11b
MHC-II
Gr1
Ly6C
B) Cellular composition colonic LP (CD4 T cell transfer colitis)
C) Cellular composition colonic LP (healthy colon)
Total cells (x10⁶)
Trem1+/+ x Rag2-/-
Trem1-/- x Rag2-/-
CD45+ cells
CD4+ T cells
CD11b+ cells
NK1.1+ NK cells
Neutrophils
Ly6Chi monocytes
MHCII+ Gr1- cells
MHCIIint Ly6Chi cells
MHCIIhi Ly6Clo cells
ns
D) TREM-1 surface expression
CD4 T cell colitis
Healthy colon
Pre-gated on
CD45+ CD11b+ NK1.1-
TREM-1 (MFI)
N.D.
Ly6Chi monocytes
Ly6C- Gr1+ CD11b+ cells
Ly6C- Gr1- CD11b+ cells
E) Colonic cytokine mRNA expression (CD4 T cell colitis)
Relative expression of mRNA
TNF
IL-1β
IL-6
GM-CSF
IL-10
IFNγ
IL-17
CCL2
CXCL5
CXCL9

FIGURE 5

A) Clinical parameters



B) Histopathological analysis

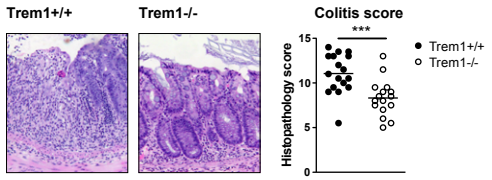


FIGURE 6

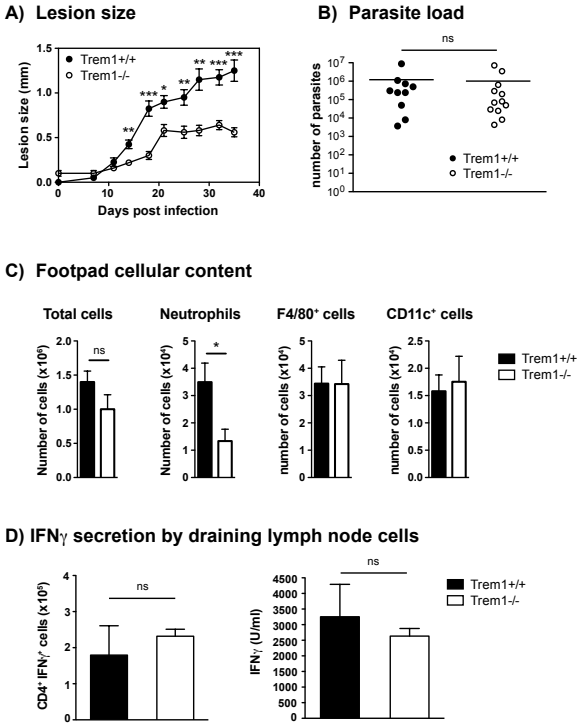
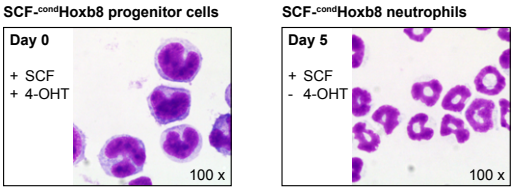
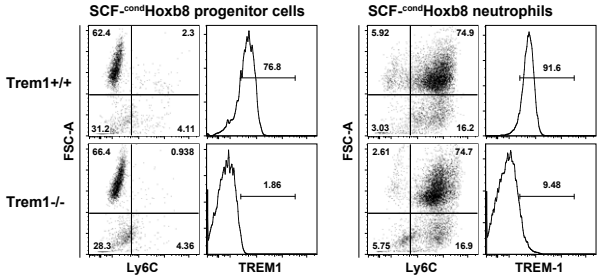

A) Lesion size
Trem1+/+
Trem1-/-
Lesion size (mm)
Days post infection
B) Parasite load
ns
number of parasites
Trem1+/+
Trem1-/-
C) Footpad cellular content
Total cells
Neutrophils
F4/80+ cells
CD11c+ cells
Number of cells (x10⁶)
number of cells (x10⁴)
ns
*
Trem1+/+
Trem1-/-
D) IFNγ secretion by draining lymph node cells
CD4+IFNγ+ cells (x10⁶)
IFNγ (U/ml)
ns
Trem1+/+
Trem1-/-

FIGURE 7

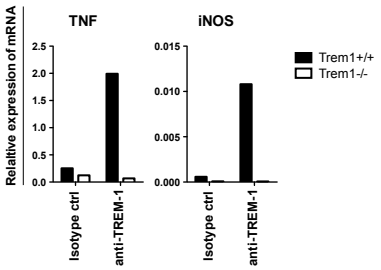
A) SCF-^{cond}Hoxb8 cell differentiation into mature neutrophils



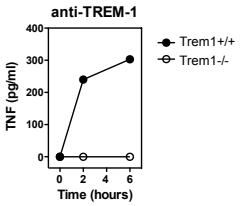
B) Trem1+/+ vs. Trem1-/- SCF-^{cond}Hoxb8 progenitors and neutrophils



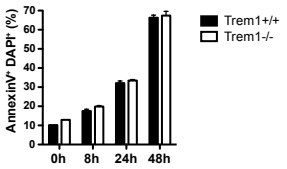
C) Real-time RT-PCR analysis



D) ELISA



E) Spontaneous apoptosis



F) Caspase 3/7 activity

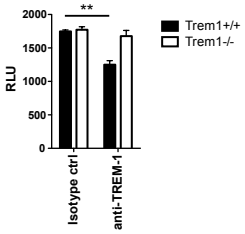


FIGURE 8

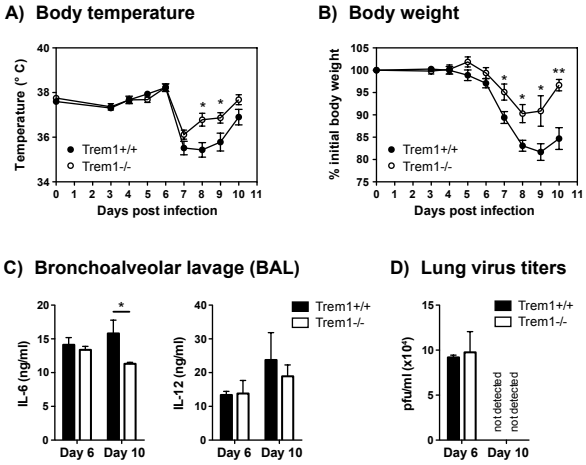
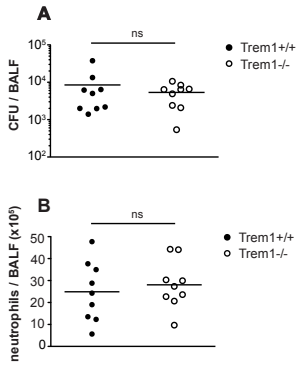
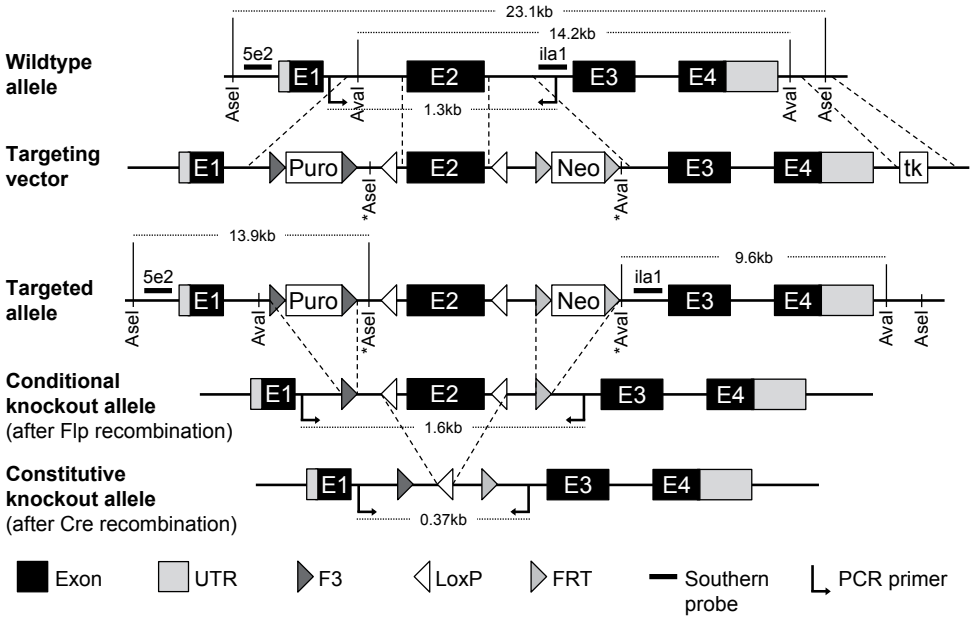

A) Body temperature
Temperature (° C)
Trem1+/+
Trem1-/-
Days post infection
B) Body weight
% initial body weight
Trem1+/+
Trem1-/-
Days post infection
C) Bronchoalveolar lavage (BAL)
IL-6 (ng/ml)
Day 6
Day 10
IL-12 (ng/ml)
Day 6
Day 10
Trem1+/+
Trem1-/-
D) Lung virus titers
pfu/ml (x10⁶)
not detected
not detected
Day 6
Day 10
Trem1+/+
Trem1-/-

FIGURE 9

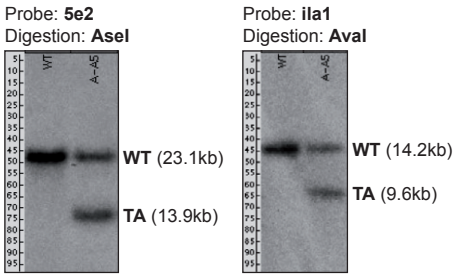

A
ns
Trem1+/+
Trem1-/-
CFU / BALF
10^5
10^4
10^3
10^2
B
ns
Trem1+/+
Trem1-/-
neutrophils / BALF (x10^6)
50
40
30
20
10
0

Supplemental FIGURE S1

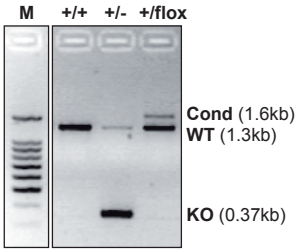
A) ***Trem1*** **knockout strategy**



B) **Southern blot analysis**

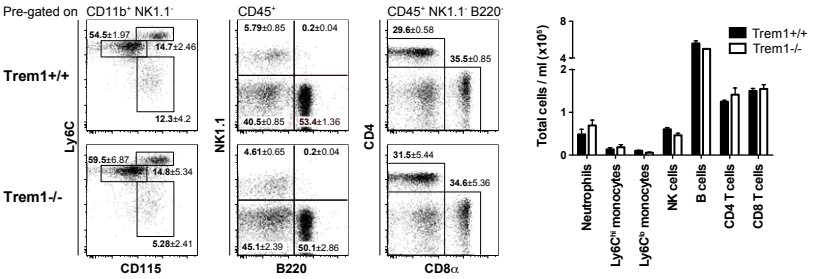


C) **Genotyping PCR**

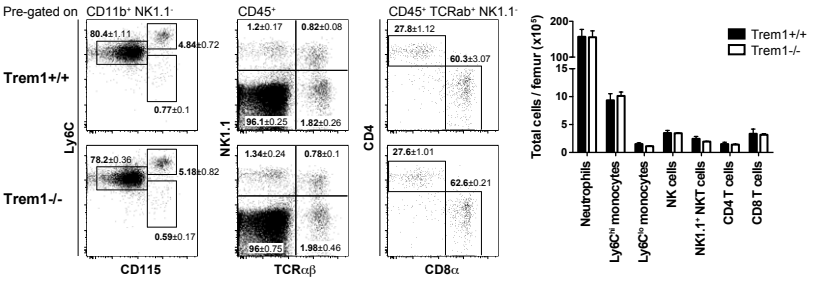


Supplemental FIGURE S2

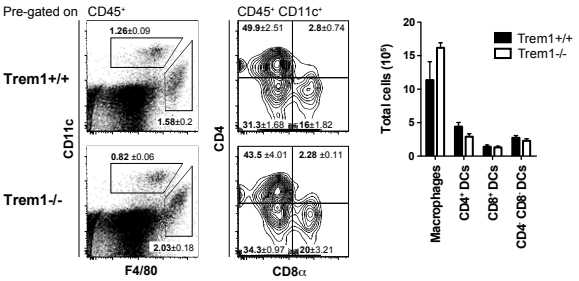
A) Peripheral blood



B) Bone marrow

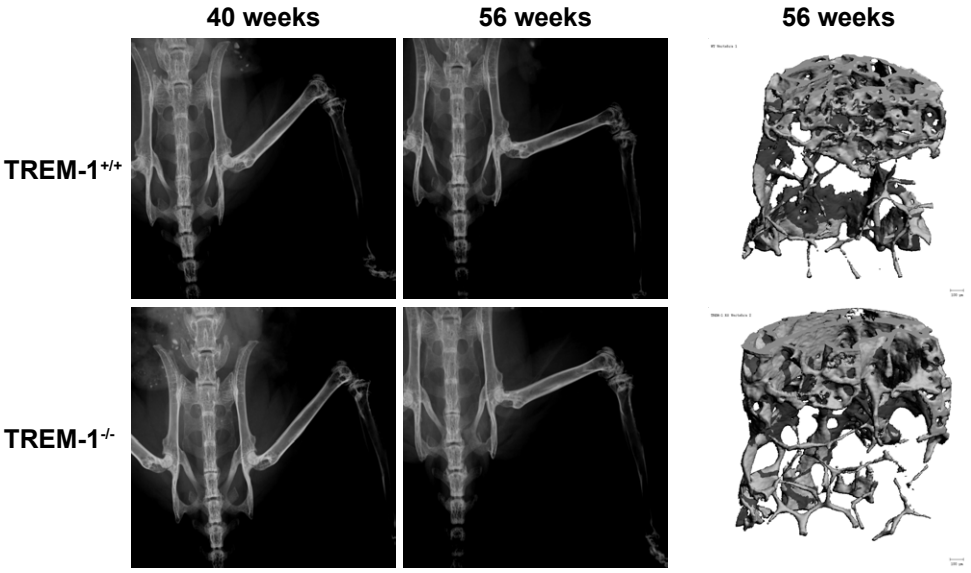


C) Spleen



A) X-ray

B) Micro-CT (L4)



C) Micro-CT measurements (L3-L5)

	L3					L4					L5				
	TV	BV/TV	Tb.N	Tb.Th	Tb.Sp	TV	BV/TV	Tb.N	Tb.Th	Tb.Sp	TV	BV/TV	Tb.N	Tb.Th	Tb.Sp
TREM-1+/+_1	1.860	**0.100**	2.422	0.044	0.422	2.090	**0.106**	2.542	0.041	0.408	2.236	**0.141**	2.709	0.049	0.380
TREM-1+/+_2	1.917	**0.121**	2.705	0.045	0.368	1.889	**0.109**	2.413	0.045	0.417	2.322	**0.128**	1.980	0.046	0.530
TREM-1-/-_1	1.537	**0.106**	2.629	0.045	0.392	1.928	**0.108**	2.585	0.048	0.396	2.054	**0.110**	2.431	0.045	0.427
TREM-1-/-_2	2.035	**0.095**	2.245	0.041	0.462	2.313	**0.108**	2.657	0.044	0.389	2.495	**0.112**	1.938	0.042	0.533

TV: Total Volume (mm3)
BV/TV: Bone Volume/Total Volume
Tb.N: Trabecular Number (1/mm)
Tb.Th: Trabecular Thickness (mm)
Tb.Sp: Trabecular Space (mm)

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JLB

Mini-Review

Crosstalk between neutrophils and dendritic cells: a context-dependent process

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ABSTRACT

Neutrophils are massively and rapidly recruited following infection. They migrate to the site of acute infection and also transiently to dLNs. In addition to their well-established role as microbial killers, accumulating evidence shows that neutrophils can play an immunoregulatory role. Neutrophils were recently shown to influence the activation of different leukocyte types including NK cells, B cells, and DCs. DCs are professional APCs playing a key role to the launching and regulation of the immune response; thus, crosstalk between neutrophils and resident or newly recruited DCs may have a direct impact on the development of the antigen-specific immune response and thereby, on the outcome of infection. Neutrophils may regulate DC recruitment and/or activation. We will review here recent progress in the field, including those presented during the first international symposium on "Neutrophil in Immunity", held in Québec, Canada, in June 2012, and discuss how neutrophil regulatory action on DCs may differ depending on the type of invading microorganism and local host factors. *J. Leukoc. Biol.* 93: 000–000; 2013.

Introduction

Neutrophils are among the first cells recruited to sites of infection, and they contribute significantly to acute inflammation. In addition to their well-described antimicrobial innate immune function, their transient presence significantly affects the microenvironment through their secretion of soluble mediators. Among these, cytokines, chemokines, and alarmins (endogenous molecules that are constitutively available, including antimicrobial peptides) may affect hematopoietic and nonhematopoietic cells and contribute to the initiation of the adaptive immune response [1].

Neutrophils that are recruited within tissues can interact locally with several leukocyte subsets, including DCs. Neutrophils were shown to participate in the recruitment and activation of macrophages [2], and their role as key regulators of NK cell maturation and function was reported in humans and mice [3, 4]. In addition, Cerutti and coworkers [5] recently described a population of splenic neutrophils that interacts with marginal zone B cells, contributing to the regulation of T cell-independent antibody responses. We will focus here on the crosstalk between neutrophils and DCs, as DCs are key players in the initiation of the antigen-specific immune response, bridging innate and adaptive immunity (reviewed in ref. [6]). DCs can detect and acquire antigens in peripheral tissues, and under inflammatory conditions, they up-regulate the expression of several surface molecules, including chemokine receptors that promote their migration through the afferent lymphatic vessels to the dLNs. During this process, DCs lose their antigen uptake property and acquire antigen-presentation capacities, increasing their secretion of cytokines, up-regulating the expression of costimulatory and MHC molecules, and allowing the presentation of processed antigens to the few antigen-specific, naïve $CD4^+$ or $CD8^+$ T cells present in the dLNs (reviewed in ref. [6]). In contrast, during steady state, DCs will induce tolerance to the presented antigen. There exist two main subsets of DCs in humans and mice, classified as cDCs and pDCs (for more details, see refs. [7, 8]). Here, we will briefly review how neutrophils contribute to the regulation of DC recruitment and activation at the site of infection and in the dLNs and discuss the various factors determining whether neutrophils contribute to DC activation or inhibition, a decision that will have a major impact on the developing T cell immune response.

NEUTROPHILS CONTRIBUTE TO DC MIGRATION TO SITES OF INFECTION AND INFLAMMATION

It has been known for more than one decade that human and mouse neutrophils exposed in vitro to microorganisms (or parts of them) are able to secrete chemokines that contribute to leukocyte accumulation [9]. Human neutrophils found at sites of inflammation were shown to change their chemokine expression pattern, suggesting a role for neutrophil-secreted chemokines in leukocyte recruitment [10]. During neutrophil

Abbreviations: BCG=Bacillus Calmette-Guérin, cDC=classical DC, DC-SIGN=DC-specific ICAM-3-grabbing nonintegrin, dLN=draining LN, HMGB1=high-mobility group protein B1, iDC=immature DC, Mac-1=macrophage antigen-1, Mo-DC=monocyte-derived DC, MRSA=methicillin-resistant Staphylococcus aureus, NET=neutrophil extracellular trap, pDC=plasmacytoid DC

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degranulation, alarmins are also released [11]. Alarmins contribute to the chemoattraction of iDCs, either by a direct action (α-defensins or the alarmin-like HMGB1) or indirectly through their induction of CCL3 and CCL5 release, two chemokines involved in the recruitment of iDCs. In addition, neutrophils release proteases, such as cathepsin G and elastase, that can process inactive, nonclassical chemokines, such as prochemerin, into chemoattractive molecules [12].

Exposure to protozoan parasites, such as *Toxoplasma gondii*, triggers the neutrophil release of CCL3, CCL4, CCL5, and CCL20 iDC-attracting chemokines, and supernatants of *T. gondii* tachyzoite-exposed neutrophils were shown to attract immature cDCs in vitro [13]. We reported that exposure to *Leishmania major*, another protozoan parasite, induced the release of CCL3 by neutrophils of a *L. major*-resistant mouse strain, whereas only very low levels of this chemokine were released by neutrophils of a *L. major*-susceptible mouse strain. We then showed that following *L. major* inoculation, neutrophil-derived CCL3 is a chemokine essential for the recruitment of Mo-DCs to the site of infection. Depletion of neutrophils using the mAb NIMP-R14, specific for Ly6G, abolished recruitment of this DC subset to the site of infection. Furthermore, *L. major*-infected $CCL3^{-/-}$ mice, adoptively reconstituted with WT neutrophils, were able to chemoattract DCs to the site of parasite inoculation, demonstrating that neutrophil-derived CCL3 is contributing to the early DC recruitment following *L. major* infection [14]. Neutrophils from MRSA-resistant or MRSA-susceptible mice secreted distinct DC-attracting chemokines, with the selective release of CCL3 by neutrophils from MRSA-resistant mice, but DC migration was not tested in this in vitro study [15]. Mycobacteria were also reported to induce the release of DC-attracting chemokines by neutrophils, and depletion of neutrophils in infected mice delayed DC migration to the dLNs. Interestingly, ingestion of *Mycobacterium tuberculosis* by neutrophils influenced DC migration: DCs that acquired *M. tuberculosis*, through the ingestion of infected neutrophils, migrated better to dLNs than DCs that acquired the bacteria directly [16]. Collectively, these studies reveal an important role for neutrophils in promoting DC migration during infection, a property that may vary depending on the host or/and the type of pathogen.

CONDITIONS WHERE NEUTROPHILS INDUCE DC ACTIVATION

Neutrophils can contribute to the mobilization of DCs to sites of infection; thus, an obvious question is to determine how the colocalization of neutrophils and DCs can modulate DC activation. Most of the studies describing neutrophil-induced DC activation were performed in vitro, with a few exceptions. To evaluate the impact of neutrophils on DC activation, it is essential to consider the neutrophil status, determining if they are alive or if they are dying, and in that case, which type of cell death (apoptosis, necrosis, NETosis) they are undergoing, as this will critically influence the neutrophil impact on DC activation. Ingestion of apoptotic cells by DCs mostly impairs DC activation, leading to tolerance [17]. In contrast, live neutrophils may activate DCs. GM-CSF-activated, live human neutrophils were shown to induce cDC activation in vitro by contact-dependent and -independent, undefined mechanisms [18]. In addition, in response to several pathogens, neutrophils can release DC-maturing cytokines, such as TNF-α. Pioneer work from Denkers and coworkers [13, 19] demonstrated that *T. gondii*-exposed mouse neutrophil supernatants induced cDC maturation in vitro, as defined by the upregulation of costimulatory markers on DCs and their release of TNF-α. The exact impact of neutrophil-derived chemokines on DC activation in vivo remains to be determined using a system that selectively depletes neutrophils. A role in the activation of cDCs was also reported for BCG-infected neutrophils. Contact-dependent DC activation was characterized by the release of IL-12 by mouse cDCs and by the secretion of decreased levels of IL-10 in human DCs compared with cDCs exposed to BCG alone [20]. Similarly, live human neutrophils infected with *M. tuberculosis* were shown to activate cDCs [21]. However, as discussed below, the impact of neutrophils on DC activation during this infection is still not clear and may also delay the onset of the immune response. Activated human neutrophils were shown to induce the maturation of human Mo-DCs by cell-cell, contact-dependent mechanisms, where CD11b (CD11b/CD18, Mac-1) on neutrophils interacts with DC-SIGN, inducing the secretion of TNF-α. Interestingly, interactions of neutrophils and DCs were visualized in colonic mucosa of patients with Crohn's disease, suggesting that interactions between neutrophils and DCs could occur in vivo; however, DC activation by neutrophils was not investigated in that study [22]. Mouse neutrophils exposed in vitro to *Aspergillus* germ tubes induced the activation of cDCs, also by a contact-dependent mechanism involving DC-SIGN on DCs [23]. Contact-dependent activation of DCs by human neutrophils was also reported in response to LPS and IL-2 or IL-15/18 stimulation; neutrophils were shown to potentiate the activity of 6-sulfo LacNAc $(SLAN)^+$ DCs, a subset of DCs present only in humans. Activation was also contact-dependent but involved CD18/ICAM-1 interactions. In this system, neutrophil-activated DCs released IL-12p70, which induced the secretion of IFN-γ by NK cells, potentiating DC-neutrophil interaction and contributing to the launching of the adaptive immune response [24].

Upon degranulation, neutrophils release numerous granule components that can also influence DC maturation. In addition to cytokines, such as TNF-α, they release alarmins that can activate nearby cells, including DCs [25]. Among alarmins, α-defensins, cathelicidins, lactoferrin, as well as HMGB1, which has alarmin-like properties, have been reported, in most cases, to induce the activation of pDCs, directly or indirectly through their presence in NETs. Monocytes that are differentiated into DCs in the presence of the human cathelicidin LL-37 showed increased activation by LPS [26], whereas LL-37 added at the same time as LPS decreased activation of Mo-DCs [27], showing distinct immunomodulation roles by neutrophils. The presence of LL-37 on NETs was described recently to activate pDCs, a subset of DCs specialized in sensing nucleic acid danger signals, and which upon activation, secretes large amounts of IFN-α. NETs are composed of neutrophil DNA and a range of antibacterial proteins. The formation of NETs helps to contain and eliminate pathogens [28]. Gilliet and coworkers [29] presented recent data, demonstrating that activation of pDCs by NETs contributes to the pathogenesis of systemic lupus erythematosus, an autoimmune disease. Activation of pDCs by NETs resulted in the secretion of TNF-α, IL-6, and IFN-α, a TLR9-mediated process that involved the neutrophil granule peptides LL-37 and the human neutrophil peptide [30]. In psoriasis, secretory leukocyte proteinase inhibitor, another component

of NETs, was also shown to stimulate pDCs in a TLR9-dependent manner [31]. These studies demonstrate that NETs can modulate the immune response through the activation of pDCs.

Collectively, these studies convincingly show that neutrophils have the potential to activate DCs in vitro. However, more studies performed in humans and mice will need to be conducted to determine the in vivo conditions where neutrophils activate DCs, if this activation is restricted to specific DC subsets, and to define how these interactions impact the development of the adaptive immune response.

CONDITIONS WHERE NEUTROPHILS CAN PREVENT DC ACTIVATION

In contrast to their DC-activating role discussed above, in some circumstances, neutrophils can prevent DC activation. Phagocytosis of apoptotic cells (efferocytosis) leads to the resolution of inflammation [32]. Internalization of apoptotic neutrophils (containing or not microorganisms or their antigens) by DCs may also lead to the prevention of DC activation. In this line, the uptake of apoptotic or necrotic neutrophils was shown to inhibit the induction of costimulatory molecules on DCs, affecting T cell responses [33]. On the contrary, the presence of high numbers of apoptotic neutrophils has been reported to induce DC maturation [34], and DCs can acquire antigens through the phagocytosis of microorganism-containing apoptotic neutrophils. Delaying neutrophil apoptosis will thus have a direct effect on the availability of antigens for DCs and the development of the adaptive immune response. In this line, phagocytosis of *M. tuberculosis* by neutrophils was shown to inhibit neutrophil apoptosis, limiting antigen uptake by DCs, a process delaying the onset of the immune response [35]. The parasite *L. major* has also been shown to delay neutrophil apoptosis in vitro [36]; however, it remains controversial whether this also occurs in vivo. In contrast, ingestion of *L. major* by neutrophils in parasite-inoculated mice was shown to increase neutrophil apoptosis compared with neutrophils that did not engulf parasites. This favored the capture of apoptotic neutrophils by DCs, preventing the activation of infected DCs in the skin [37]. Collectively, accumulating data suggest that the effect of apoptotic neutrophils on DC maturation may vary and be context-dependent.

Neutrophils can also release cell surface-derived vesicles, called ectosomes, that expose phosphatidyl serine on their surface, an "eat me" signal for DCs. Ectosomes can prevent the LPS-induced maturation of Mo-DCs in vitro. Indeed, human neutrophil ectosomes modified the phagocytic properties of Mo-DCs and impaired the expression of costimulatory molecules and the secretion of cytokines, including TNF-α and IL-12, whereas they increased the release of TGF-β, an anti-inflammatory cytokine [38]. These data suggest that neutrophil ectosomes can induce DC tolerance in vitro; however, these findings still need to be confirmed in vitro and in vivo. Upon neutrophil degranulation, the release of elastase was also shown to down-modulate DC activation, inducing DC secretion of TGF-β [39].

Neutrophils can migrate rapidly and transiently to dLNs. The presence of neutrophils in the dLNs may also influence DC activation, a process that is just beginning to be investigated. Following injection of protein antigens in adjuvants, neutrophils that rapidly migrated to dLNs made brief contact with DCs. Interestingly the brief presence of neutrophils in the dLNs had a major negative impact on the development of $CD4^+$ T cell priming. This negative effect appeared to occur only with the first wave of neutrophils that reaches the dLNs, during the first days after injection [40]. Of note, neutrophils also transiently migrated to the dLNs following inoculation with *L. major*, and depletion of neutrophils during the first days of infection resulted in a major change in the development of the T cell immune response, whereas depletion of neutrophils occurring at a later time did not have an effect [41]. It will be interesting to characterize in more details the importance of the early migration of neutrophils to the dLNs in several models of infection and to better characterize the type of DC involved in the impaired activation and if the direct effect on T cells may also contribute to the neutrophil inhibitory impact. The knowledge gained could have important implications in vaccination.

CONCLUDING REMARKS

As discussed in this review, neutrophils are capable of recruiting iDCs and can activate or inhibit DCs (**Fig. 1**). However, when examining the increasing data reported on the crosstalk between neutrophils and DCs, some caution must be exerted. First, one needs to consider the differences that exist between human and mouse neutrophils. Neutrophil numbers differ significantly between the two species: they comprise 50–70% of leukocytes of peripheral blood in humans and only 10–25% in that of mice [42]. Moreover, human and mouse neutrophils also differ qualitatively in the factors they release with a direct impact on DCs. For instance, as presented by Cassatella and coworkers [43], the IL-10 cytokine is secreted by mouse- but not by human-activated neutrophils. In addition, α-defensins are present selectively in human but not mouse neutrophils [44, 45]. Some of the mechanisms of neutrophil-DC crosstalk may thus differ between humans and mice. Secondly, most findings concerning neutrophils have been obtained in vitro and still need to be confirmed in vivo. The majority of mouse studies has been performed using neutrophil-depleting mAb. The mAb RB6-8C5 was widely used to deplete neutrophils, but it recognizes not only the molecule Ly6G present on neutrophils but also Ly6C, a molecule also present on other cell types, such as pDCs and monocytes. The two other mAb available to deplete neutrophils are the 1A8, an IgG2a mAb [46], and the NIMP-R14, an IgG2b mAb [47], which recognize Ly6G selectively. However, injection of the 1A8 mAb leads to incomplete depletion of neutrophils in vivo, and relatively high doses of mAb are needed. The NIMP-R14 mAb has a better efficiency in neutrophil depletion in vivo; however, the exact epitope recognized by this mAb is not well defined [48]. As reported during the meeting by Nigrovic and colleagues [49], injection of small amounts of the 1A8 mAb was shown to block neutrophil recruitment, and the antibody decreases the expression and function of β2 integrins, an effect that will need to be taken into account when using antibodies directed against Ly6G. Methods of neutrophil depletion that do not rely on the use of antibodies are therefore needed to exclude potential artifacts as a result of antibody presence and possible indirect effects not related to the absence of neutrophils. The newly described neutropenic *Genista* mice offer an interesting model to study the role of neutro-

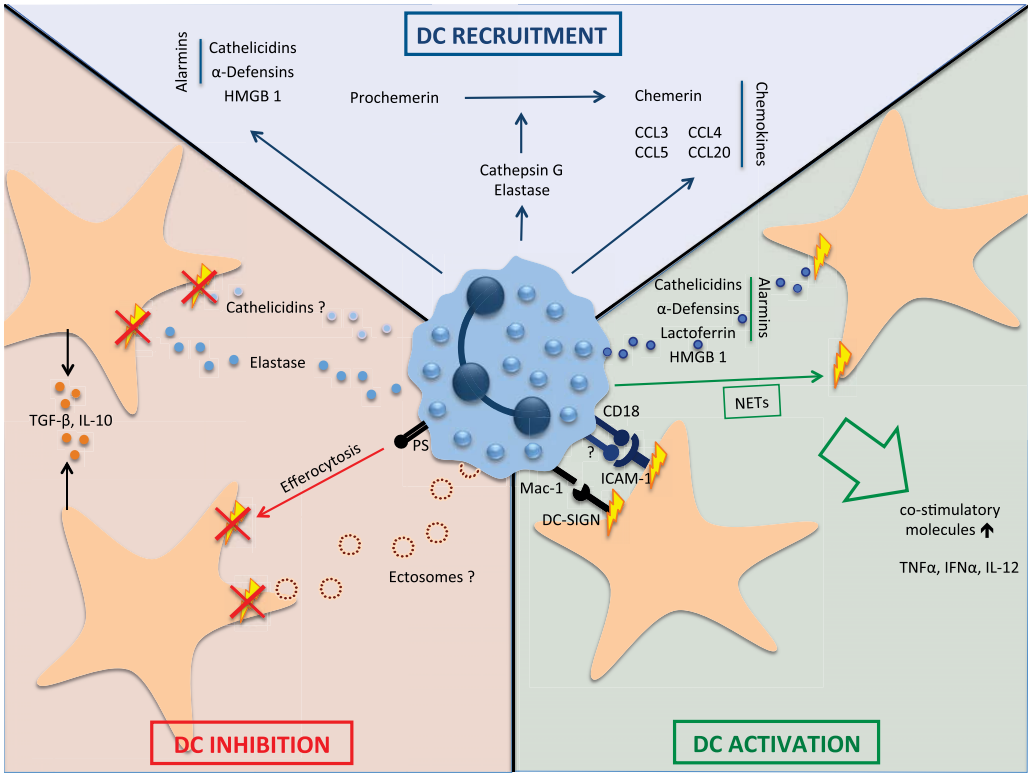

Figure 1. Crosstalk between neutrophils and DCs. Recruitment: neutrophils secrete alarmins, including cathelicidins, α-defensins, and HMGB1, as well as chemokines, such as CCL3, CCL4, CCL5, and CCL20, which chemoattract iDCs. The proteases cathepsin G and elastase can convert prochemerin to active chemerin, a potent chemoattractant for DCs. Activation: neutrophils can activate DCs in a cell contact-dependent manner involving Mac-1 (CD11b/CD18) or CD18, together with a yet-unknown receptor on neutrophils and DC-SIGN or ICAM-1 on DCs, respectively. The release of NETs stimulates pDCs to secrete inflammatory cytokines. Neutrophils release alarmins that also contribute to DC activation. Inhibition: neutrophils can also inhibit DC function. Phagocytosis of apoptotic neutrophils [phosphatidyl-serine$^+$ (PS)] may inhibit DC function, and neutrophil-derived ectosomes can inhibit DC maturation. Furthermore, neutrophils release soluble factors, such as elastase and cathelicidins, that can suppress DC activation.

phils. These mice have neutropenia, resulting from a point mutation in the transcriptional repressor growth factor independence 1, which causes a block in terminal granulopoiesis [4, 50]. *Genista* mice do not have mature neutrophils, but they have a small number of atypical CD11b$^+$ Ly6G^{int} neutrophils. The availability of new tools together with new advances in imaging will improve the study of the immunoregulatory role of neutrophils in vivo and should allow a better characterization of the importance of DC-neutrophil crosstalk in different diseases.

AUTHORSHIP

F.T-C. wrote the review. S.S. and B.H. contributed to the writing and figure design.

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Other work performed not presented in this thesis

1. Redundant Notch1 and Notch2 signaling is necessary for IFNγ secretion by T helper 1 cells during infection with Leishmania major. Auderset F, **Schuster S,** Coutaz M, Koch U, Desgranges F, Merck E, MacDonald HR, Radtke F, Tacchini-Cottier F. PLoS Pathog. 2012;8(3):e1002560. doi: 10.1371/journal.ppat.1002560. Epub 2012 Mar 1.

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