Neutrophils as Key Players in the Outcome of Cutaneous Leishmaniasis

Regli Ivo Beat

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département de Biochimie

Neutrophils as Key Players in the Outcome of Cutaneous Leishmaniasis

Thèse de doctorat en médecine et ès sciences

MD-PhD

Présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Ivo Beat REGLI

Médecin diplômé de la Confédération Helvétique

Jury

Prof. Daniel Speiser, Président et Répondant MD-PhD

Prof. Fabienne Tacchini-Cottier, Directrice de Thèse

Prof. Tamás Laskay, Expert

Prof. Hans Acha-Orbea, Expert

Prof. Blaise Genton, Expert

Lausanne 2018

Dedication

To my father, **Beat Regli-Quevedo,** for his support, love and for teaching me the important values in life

To my brother, **Pius Regli**, for his friendship and companionship during my childhood and adolescence

To my grandfather, **Pedro Quevedo Ahon**, Professor at the National University of San Marcos in Lima, Peru, for he was an inspiration and awakened my interest in science already in my childhood

1. Table of Contents

2. Summary

Cutaneous leishmaniasis is a neglected tropical disease with a prevalence of roughly 12 million people worldwide. It is caused by protozoan parasites of the genus *Leishmania* and transmitted by the bite of female sandflies of the subfamily *Phelbotominae*. Its clinical presentation is characterized by the development of an ulcerative lesion but can be highly variable. Neutrophils play an important role in the immunity against leishmaniasis. Upon infection they are massively recruited and infected. Furthermore, they can act as "Trojan Horses", when apoptotic neutrophils that are phagocytized by macrophages silently transmit parasites into the latter.

Here, we focused on specific roles of neutrophils in leishmaniasis. We show that an effective neutrophil response against *Leishmania major (L. major)* depends on Toll-like receptor 7 (TLR7) signaling. The absence of this receptor in neutrophils leads to chronic leishmaniasis despite the development of a strong Th1 response in *Tlr7*-deficient mice. In contrast, activation of TLR7 at the time of infection leads to better disease control. In addition, we were able to show that the absence of TLR7 leads to a differentially regulated gene expression in *L. major* infected neutrophils.

Furthermore, we demonstrate that the drug susceptibility phenotype of another *Leishmania* sp, *Lesihamania (Viannia) panamensis (L. (V.) p.),* modulates neutrophils main effector functions including neutrophil extracellular trap formation, reactive oxygen species production and the expression of extravasation and degranulation markers. In addition, drug resistant *L. (V.) p.* lines are more able to resist neutrophil mediated parasite killing.

Taken together our data demonstrate the utmost importance of neutrophil response in the control of leishmaniasis and indicates the significance of continued investigation in this field to further improve patient care.

3. Résumé

La leishmanisose cutanée est une maladie tropicale négligée qui a une prévalence mondiale d'environ 12 millions de personnes. Elle est occasionnée par des parasites protozoaires du genre *Leishmania* et transmise par la piqûre de phlébotomes femelles. La manifestation clinique de la maladie est très variée. Souvent, elle est caractérisée par l'apparition d'une lésion cutanée ulcéreuse. Les neutrophiles jouent un rôle important dans la réponse immunitaire contre la leishmaniose. Ils sont massivement recrutés et infectés lors de la transmission du parasite. De plus, la phagocytose des neutrophiles infectés par des macrophages est une façon pour le parasite d'entrer les macrophages sans activer la réponse anti-inflammatoire connue comme mécanisme du «cheval de Troie». Dans la thèse présente, l'intérêt est mis sur certains rôles spécifiques des neutrophiles dans la leishmaniose.

Premièrement, l'importance du signalement du récepteur de type Toll 7 (TLR7) dans la réponse des neutrophiles contre *Leishmania major* (*L. major*) a été démontrée. L'absence du TLR7 dans les neutrophiles cause une chronicisation de la leishmaniose. La stimulation du récepteur, au moment de l'infection avec *L. major*, résulte en un meilleur contrôle de la maladie. Ceci se produit chez les souris déficientes en TLR7 en dépit du développement d'une réponse de type 1 des cellules T auxiliaires, normalement protective. De plus, les neutrophiles infectés avec *L. major* ont montré des différences importantes dans l'expression génétique globale en l'absence de TLR7. Dans une deuxième partie, nous avons démontré l'implication du phénotype de la susceptibilité médicamenteuse de *Leishmania (Viannia) panamensis* dans la régulation des fonctions principales des neutrophiles. De plus, les parasites résistant aux médicaments ont été démontrés plus résistants à l'élimination par les neutrophiles. Ensemble, nos données démontrent l'importance d'une réponse immunitaire adéquate des neutrophiles pour le contrôle de la leishmaniose et indiquent l'importance de la poursuite des investigations dans ce domaine pour améliorer les soins aux patients.

4. Introduction

4.1 The Immune System

4.1.1 The History of Immunology

The first recorded accounts of immunological concepts were documented around 2000 B.C. in the Babylonian epic of Gilgamesh. Even though people living in these times believed that diseases were brought upon them by deities as punishment of unfavorable behavior, they noted that people who survived a particular disease had a certain protection against it in the future. This observation was also made during the plague outbreak in Athens 430 B.C. as reported by the Greek historian Thucydidies[1]. Later, in 900 A.D. the Persian physician Abü Bakr Muhammad ibn Zakariyyā al-Rāzī described the concept of acquired immunity in the context of his research about smallpox and measles[2]. It has been reported that from around the year 1000 on, people in China used crusts of pustules from smallpox victims as an inoculum in order to immunize people against this disease. In 1796, the English physician Edward Jenner inoculated James Phipps, the son of his gardener, with cowpox, based upon the observation that milkmaids that were infected with cowpox tended not to get infected with smallpox. After the inoculation with cowpox, James Phipps was exposed to smallpox against which he then was immune[3]. This was the beginning of the small pox vaccine that led to the eradication of smallpox in 1980[4]. Jenner called this inoculum Vaccinia, derived from the Latin word “vacca” which means cow.

In 1843 the French physician Gabriel Andral and the English physician William Addison described leukocytes for the first time[5,6]. The Russian biologist Ilya Ilyich Mechnikov published his “phagocyte theory” in 1883 which contained the first description of phagocytes and their role in host protection[7]. During the 20th century the field of immunology gained a lot of momentum, research efforts were intensified and a large series of discoveries were made, some

of which were awarded with the Nobel Prize in Medicine and Physiology and are listed in the table below **(Figure 1)**.

Year	Name(s)	Country(s)	Research
1901	E.A. Von Behring	Germany	Defined the concept of serum therapy by showing that diphtheria and tetanus exotoxins can be used to raise 'antitoxins' that could be passively transferred, protecting against disease
1905	R. Koch	Germany	Investigations concerning tuberculosis
1908	E. Mechnikov	Russia	Developed the first 'cellular theory' of immunity, including work on phagocytosis
	P. Ehrlich	Germany	Developed the first theory of humoral immunity and of natural self-tolerance. 'Side-chain receptor' theory anticipates Burnet's clonal selection theory by half a century
1913	C.R. Richet	France	First experimental demonstration of anaphylaxis; showed that injection of dead or attenuated microbes leads to specific immunity and that subsequent re-exposure provokes severe illness or death
1919	J. Bordet	Belgium	Discovered the basis of immune haemolysis of foreign red blood cells, including the involvement of separate complement and antibody components
1930	K. Landsteiner	Austria, USA	Discovery of human blood groups
1951	M. Theiler	South Africa	Discoveries and developments concerning the universally successful yellow fever vaccine
1957	D. Bovet	Italy, Switzerland	Discoveries related to histamine and compounds that inhibit the action of histamine and other substances of the vascular system and the skeleton muscles
1960	Sir F. McFarlane Burnet	Australia	Developed theories of clonal selection of antibody production and for applying this to the concept of acquired immunological tolerance
	Sir P.B. Medawar	UK	Experimentally confirms Burnet's theory; shows that graft rejection is due to an immunological reaction and that tolerance can be built up by injections into embryos
1972	G.M. Edelman, R.R. Porter	USA, UK	Discovery concerning the chemical structure of antibodies, showing that immunoglobulins are composed of two heavy and two light chains covalently bonded
1980	B. Benacerraf, J. Dausset, G.D. Snell	USA, France, USA	Discoveries concerning genetically determined structures on the cell surface (MHC – H-2 and HLA) that regulate immune reactions
1984	N.K. Jerne	Denmark, Switzerland	Theories concerning the specificity in development (lymphocyte clonality) and control of the immune system
	G.J.F. Köhler, C. Milstein	Germany, Switzerland, Argentina, UK	Discovery of the principle for production of monoclonal antibodies
1987	S. Tonegawa	Japan, USA	Discovery of the genetic principle for generation of antibody diversity, thus overturning the 'one gene, one protein' paradigm
1990	J.E. Murray, E.D. Thomas	USA	Discovery concerning organ and cell transplantation in the treatment of human disease
1996	P.C. Doherty, R.M. Zinkernagel	Australia, USA, Switzerland	Discoveries concerning the specificity of the cell-mediated immune defense

Figure 1: Noble Prizes awarded to immunologist in the 20th century (Doherty and Robertson, 2004)

In the 21th century the field of immunology continues to thrive and a lot of outstanding discoveries are made. In 2002, the group of Jürg Tschopp first described the concept of inflammasome, an intracellular protein-complex that induces inflammation upon sensing of danger signals[8]. In 2004, a group of scientist including Arturo Zychlinky discovered that neutrophils can form extracellular traps made of chromatin and granule proteins. In the first 18 years of this century the knowledge of immunology broadens while the fields of study become

more specific. In 2011 Bruce A. Beutler and Jules A. Hoffmann received the Nobel price in medicine and physiology for their discoveries concerning the activation of innate immunity, including Toll-like receptor signaling . The laboratory of Fabienne Tacchini-Cottier, in which this thesis was carried out, dedicates itself to research in immunity against *Leishmania* protozoan parasites. A lot of cutting-edge research was and is conducted in this laboratory, notably in the interaction of neutrophils with *Leishmania*[9-15].

4.1.2 The Immune System in Health and Disease

The human body is constantly exposed to potential dangers present in the environment such as pathogens, toxins, foreign bodies and trauma but also to dangers arising from its own tissues and cells, i.e. cancer[16]. The immune system confers a certain protection against such dangers and consists of a complex interplay between organs, tissues, cells and molecules. It can be thought to be a system with various layers of defense: physical barriers such as skin or mucosae, chemical barriers such as stomach acid, the innate immune system and the adaptive immune system[17].

In order to function properly, the immune system has to discriminate between what is part of the healthy human body and what is not part of the healthy human body. A failure of the discrimination between "self" and "non-self" leads to the targeting of nonpathological cells and tissues by the immune system and which is known as autoimmunity and can lead to a wide variety of diseases, for example systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis or psoriasis[18].

4.1.3 Innate Immunity

After the physical and chemical barriers of the immune system, innate immunity is the next line of defense against dangers. It consists of soluble components such as natural antibodies, proteins (e.g. C-reactive protein), the complement system and cellular components. The cellular

components are phagocytic cells such as neutrophils, antigen presenting cells such as dendritic cells and cytotoxic cells such as natural killer cells (NK-cells)[19].

The cellular components of the innate immune system can be divided into myeloid cells and lymphoid cells, depending on the form which common progenitor cells they arose. The lymphoid cells of the innate immune system include NK-cells and some subtypes of B- and T-cells (e.g. NKT-cells, γδ-T-cells, B-1 B-cells)[19,20]. The myeloid cells consist of granulocytes, such as neutrophils, eosinophils and basophils, monocytes, macrophages and mast cells. Dendritic cells (DCs), important cellular components of the innate immune system, can arise from either the lymphoid or myeloid cell precursors[21-23].

4.1.3.1 Myeloid Cells

Macrophages, whose name derives from the Greek words "makrós" meaning big and "phageín" meaning to eat, are cells that are specialized in phagocytosis, a process in which cells engulf and internalize foreign structures for the neutralization of potential dangers such as debris, pathogens or foreign bodies. Generally speaking there are two different types of macrophages. Tissue resident macrophages are nonmigratory cells that are found in specific tissues under physiological conditions (e.g. Kupffer cells in the liver, or microglia in the central nervous system) and are responsible for immune surveillance. Monocyte derived macrophages are recruited to the site of inflammation upon the sensing of chemotactic factors[24].

Monocytes are cells found in the blood circulation under homeostatic conditions. They make up about 10% of all circulating blood leukocytes with a half-life of about 3 days. Upon sensing of danger signals, monocytes are recruited to the site of infection and differentiate into either macrophages or DCs[25].

Dendritic cells are antigen presenting cells that can be divided into various subpopulations. The two main categories of DCs are classical DCs (cDCs) and plasmacytoid DCs (pDCs). pDCs are cells that are specialized in the production of type I interferons (IFN) upon activation. They are

found in lymphoid organs, the blood circulation and also in different tissues[26]. cDCs can then be further subdivided into lymphoid organ resident DCs and circulating tissue DCs. While lymphoid organ resident DCs have an immature phenotype and are not found outside the lymphoid organs, circulating DCs patrol between the different tissues in the body and the lymphoid organs, presenting antibodies to T-cells. Upon inflammation, another subset of cDCs, the inflammatory DCs, differentiates from monocytes and are also capable to present antigens to lymphocytes[27].

Mast cells are tissue resident effector cells that can be activated by different means. They are best known for their degranulation of several inflammatory and vasoactive mediators (e.g. Histamine, IL-6)[28] after activation. This is the underlying mechanism of immediate onset hypersensitivity reactions[29]. They can be activated by IgE, the complex system, aggregated IgG, physical stimuli, cytokines and neuropeptides.

Granulocytes play an important role in the rapid clearing of pathogens and in the regulation of immune responses. Their name derives from their characteristic granules in their cytoplasm that they can release upon activation. Under homeostatic conditions, they are found circulating in the bloodstream. Upon sensing of danger signals, they are readily recruited to the site of inflammation. There are three major types of granulocytes. Basophil granulocytes express IgE-antibodies on the cell membrane and release high levels of interleukin-4 (IL-4) upon activation[30]. Eosinophil granulocytes are known to store a wide variety of cytokines in their granules and thus influence the immune response to a given stimuli. They furthermore play a role in immunity against pathogens by a variety of mechanisms including phagocytosis and reactive oxygen species (ROS) formation[31]. Neutrophil granulocytes are the most abundant leukocytes in the human blood circulation and are key players in immunity.

4.1.3.1.1 Neutrophils

Neutrophils, also known as polymorphonuclears (PMN), have a characteristic lobulated nucleus and are important phagocytic cells of a variety of pathogens[32]. They have a diameter of about 7-10 μm[33], a life span of about 24 hours in the circulation[34] which can be increased upon inflammation[35]. Neutropenia, a condition characterized by a low number of neutrophils in the blood circulation, that can be congenital or acquired, leads to an increased susceptibility to infection[36,37].

4.1.3.1.1.1 Development

Most neutrophils originate from hematopoietic stem cells (HSC) in the bone marrow (BM) in a process called granulopoiesis. A healthy adult produces around $2x10^{11}$ neutrophils per day. Granulopoiesis is a multistep process in which various cytokine and transcription factors such as granulocyte colony stimulating factor (G-CSF) and the transcriptional suppressor growth factor independent 1 (Gfi1)[38]. Hematopoietic stem cells differentiate and proliferate into myeloblasts and then into promyelocytes. During this time the azurophilic granules of the neutrophils are formed. Then, they differentiate into myelocytes and further proliferate. Myelocytes are the last proliferating form in granulopoiesis that in a next step differentiate into metamyelocytes. During this step the specific granules of the neutrophil are formed. Metamyelocytes differentiate in band cells and the gelatinase granules are formed. Band cells are a first immature functional form in neutrophils that can be found in the blood circulation under inflammatory conditions. Under homeostatic conditions band cells form and turn into mature neutrophils which are then released into the blood circulation[39]. The last granule type called secretory vesicles are formed after the neutrophils exit the bone marrow[40] **(Figure 2)**. However, neutrophils can also differentiate from circulating hematopoietic stem and progenitor cells (HSPC) under inflammatory conditions[41]. It has been reported that *Candida albicans* drives HSPC to differentiate into mature neutrophils and macrophages[42,43]. Furthermore, HSPC

have been shown to traffic to *Stahpylococcus aureus* infected skin injuries and differentiate into mature neutrophils[44].

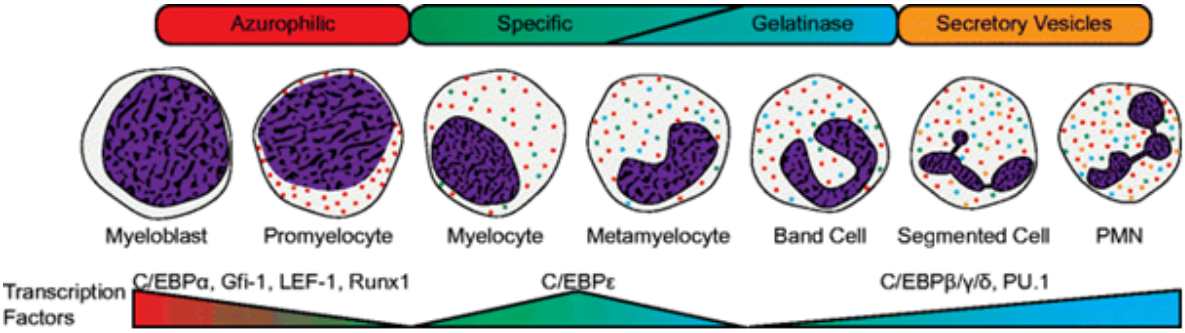

Figure 2: Schematic illustration of granulopoiesis (Yin et al., 2018)

4.1.3.1.1.2 Migration and Extravasation

Upon sensing of danger signals by the immune system, circulating neutrophils are quickly recruited to the site of inflammation by chemotactic factors such as IL-8[45]. This migration involves neutrophils exiting the blood vessels by a process called extravasation. Upon the sensing of danger signals by tissue resident immune cells, chemotactic factors are released and the endothelium is activated in order to recruit leukocytes, which are in the beginning mostly neutrophils. The endothelium starts to express structures that can interact with neutrophil surface molecules. The neutrophils begin to roll along the blood vessel walls upon, which together with cytokines, a signaling cascade is activated which leads to the integrin mediated tight junction of neutrophils to the endothelium and subsequently to the migration through the endothelium to the inflammation site[46].

4.1.3.1.1.3 Neutrophil Effector Functions

After migration to the site of inflammation, neutrophils exert different effector functions. Neutrophil possess three main effector strategies: neutrophil extracellular trap (NET) formation, phagocytosis and subsequent reactive oxygen species (ROS) production and degranulation of granules that contain microbicidal proteins[47] **(Figure 3)**. To kill engulfed

microbes, neutrophils produce different ROS which are synthesized by the enzymatic activity of NADPH-synthase[48].

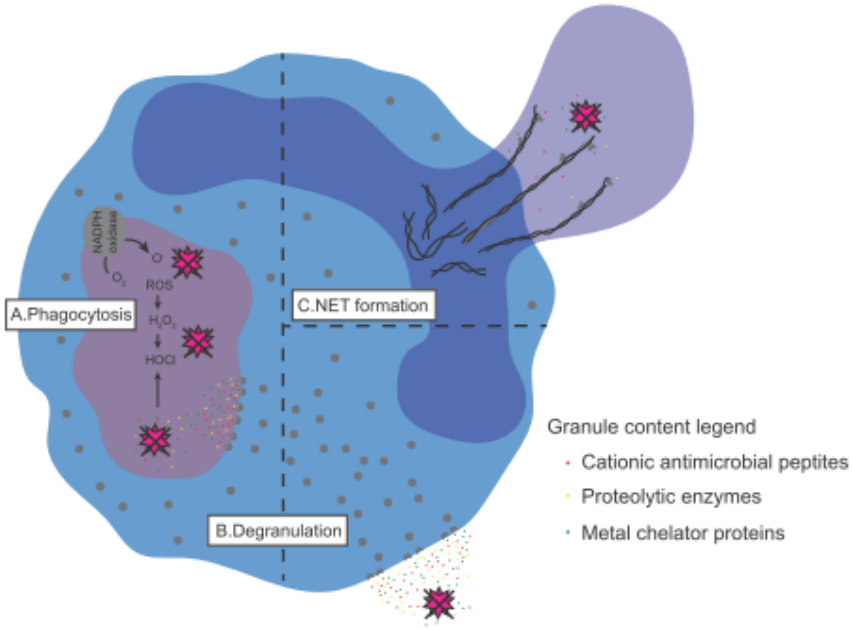

Figure 3: Overview of neutrophil effector functions

ROS are highly oxidative molecules that are microbicidal in a lot of contexts and are also involved in NET formation and signaling pathways[49]. During the process of degranulation, neutrophils granules can either fuse with pathogen containing phagosomes or be released into the extracellular space[50]. Three of the different classes of granules are formed during different stages of granulopoiesis: Azurophilic granules, specific granules and gelatinase granules. The fourth form of granules, secretory vesicles, are formed after the neutrophil exits the bone marrow[40]. All of them contain an arsenal of different microbicidal proteins[46].

Neutrophils are able to form NETs, fibrous structures that are composed of dsDNA coated with a variety of different microbicidal proteins. The process of NET formation is characterized by a cessation of cytoskeletal dynamics, cell membrane depolarization and subsequent expulsion of nuclear as well as in some cases mitochondrial DNA[49].

4.1.3.1.1.4 Immune Modulatory Functions

PMNs are not only short-lived effector cells but they are also involved in the shaping of the subsequent innate and adaptive immunity. In addition, they are important mediators of inflammatory processes. Upon activation, neutrophils are capable of secreting a great number of different cytokines and chemokines and they can interact with different types of leukocytes **(Figure 3)**. Neutrophils have also been shown to be able to migrate to the draining lymph-node. Furthermore, it has been reported that neutrophils are able to attract T-cells to the site of inflammation, that they can modulate DC and B-cell function and that they are crucial in the modulation NK-cell function[51,52]. Finally, apoptotic neutrophils that are phagocytized by macrophages shape the functional phenotype of the latter[53].

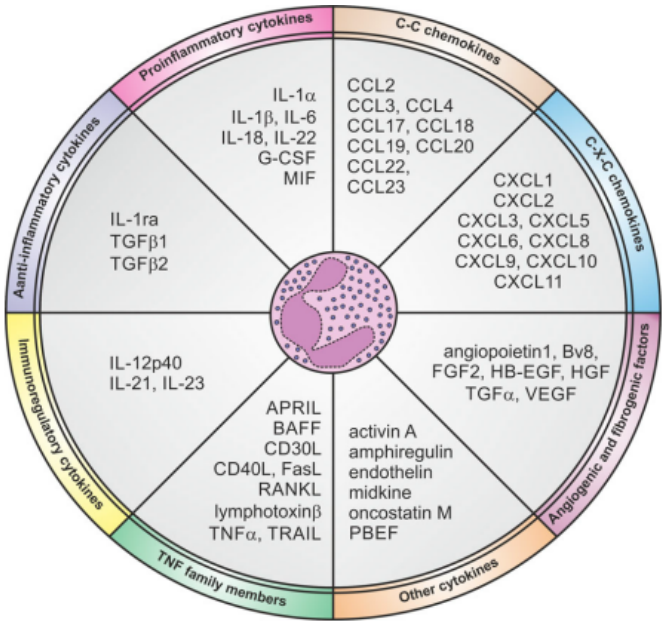

Figure 4: Overview of cytokines and chemokines that can be produced by neutrophils

(Tamassia et al., 2018)

4.1.3.2 Pattern Recognition Receptors

Leukocytes possess different classes of receptors that can detect a wide array of danger signals. The most important types of receptors are: The C-type lectin receptors that recognize glycan structures on pathogens[54], the NOD-like receptors that sense intracellular danger signals and

the Toll like receptors that recognize pathogen associated molecular patterns (PAMPs) on the cell membrane and in the endosomal compartment[55]. PAMPs are structures or motifs that are invariant in pathogens of a given class and that are not present in the host, which makes them well suited for immune recognition[56].

4.1.3.2.1 Toll-Like Receptors

Toll, a gene that was previously known to be involved in the embryonic development *Drosophila melanogaster,* was shown to be crucial in the antifungal immune response in these flies[57]. After that discovery, a series of similar receptors named Toll-like receptors (TLRs) that recognize pathogen associated structures were described in humans and other organisms[58]. TLRs are composed from a leucin-rich extracellular domain that recognizes pathogen associated proteins, glycans or nucleic acids and an intracellular domain that induces a signaling cascade over a Toll/interleukin-1 receptor (TIR)-domain upon receptor-activation. Signaling from the TIR-domains depends on different adaptor proteins like the myeloid differentiation primary-response protein 88 (MyD88). This protein is essential for all TLR signaling except in TLR3 and endosomal TLR4 which are able to signal through TIR-domain-containing adaptor protein inducing interferon-β (TRIF) dependent pathway[59] **(Figure 5)**. TLR signaling leads to the activation of transcription factor such as nuclear factor-κB (NF-κB) or interferon (IFN)-regulatory factors (IRF) and eventually leads to the transcription of pro-inflammatory cytokines and interferons[60]. The 10 human TLRs (TLR1-10) and the 12 murine TLRs (TLR1-9 and TLR11-13) can be subdivided into two groups. TLR1, TLR2, TLR 4, TLR5, TLR6 and TLR10 are expressed on the cell membrane. TLR3, TLR4, TLR7, TLR8, TLR9, TLR 11, TLR12 and TLR13 are expressed on the endosomal membranes[61]. Phagocytized particles and pathogens like *Leishmania* are found within endosomes and can potentially induce TLR7 signaling as it was shown for RNA from group B streptococci in cDCs[62].

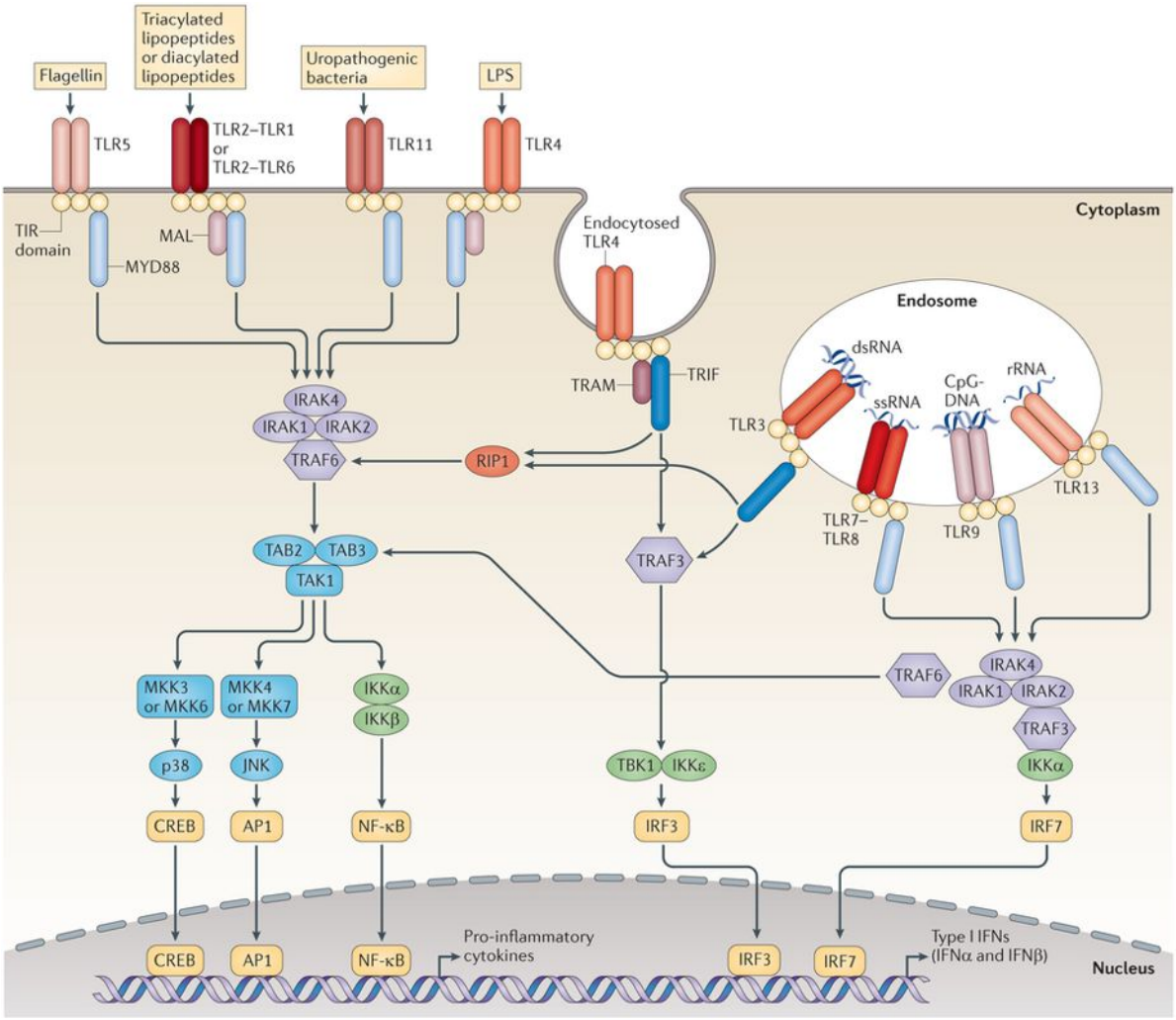

Figure 5: Overview of TLR location, signaling pathways and potential ligands (O'Neill, 2013)

4.1.3.2.1.1 Toll-Like Receptor 7

TLR7 is an endosomal Toll-like receptor that is known to recognize foreign single stranded RNA (ssRNA) and also responds to imidazoquinoline compounds such as imiquimod or resiquimod. It is closely related to TLR8 which in humans also recognizes ssRNA. TLR7 has been shown to have a high affinity to guanosine and uridine moieties in ssRNA[63]. In humans, TLR7 has been shown to be expressed on monocytes, macrophages DCs, B-cells, T-cells and NK-cells[64-70], while in mice TLR7 has been shown to be expressed in DCs, macrophages, neutrophils, eosinophils and B-cells[71,72].

4.1.3 Adaptive Immunity

The adaptive immune response is the more specific line of defense of the immune system. There are two main branches of the adaptive immune system: the humoral response in which B-cells are the most important players and the cellular immune response which relies mainly on T-cells. The adaptive immune system differs from the innate immune system in various aspects. While the innate immune system has receptors that recognize a fixed set of epitopes which are mostly conserved molecular patterns, the adaptive immune system can recognize a nearly infinite numbers of epitopes. This is because its receptors are not fixed in the genome but are composed of gene segments that are genetically recombined in a process called gene rearrangement. However, while the effector structures of the innate immune system are readily available, the adaptive immune system has to clonally expand the cells with the required specificity which makes it slower in the response. Finally, unlike the innate immune system, the adaptive immune system is able to establish immunological memory, the underlying mechanism of vaccines[73].

4.1.3.1 Humoral Immunity

The hallmarks of humoral immunity are serum antibodies that are produced by long lived plasma cells that arise from B-cells. Antibodies interact with pathogens by interfering with the functions of their surface structures by increasing the phagocytosis of antibody marked structures and by activating the complement system. Antibodies can be categorized into different classes. IgD act mainly as B-cell receptor on resting B-cells. IgM is expressed on and secreted by B-cells in the early phases of an infection. IgG is secreted by plasma cells and are responsible for long term humoral immunity and can further be subdivided in their isotypes IgG1, IgG2, IgG3 and IgG4, which differ in their non-antigen-specific regions[74]. IgA and IgE are also produced by plasma cells. IgA is predominantly found in mucosal tissues while IgE is found in low concentration in the blood and is involved in atopic reactions[75]. Resting B-cells

are found in the lymph-node and are constantly exposed to a wide variety of antigens in the circulating lymph[76]. Upon binding of a B-cell receptor to its specific antigen and upon receiving costimulatory signals from T-helper cells, B-cells get activated. Subsequently, they proliferate and finally differentiate into different B-cell subsets such as plasma cells and memory B-cells[77]. Plasma cells are responsible for the maintenance of antibodies specific to the encountered antigen. Memory B-cells are resting cells that get reactivated upon reencounter of their specific antigen. Both cell types are very important in the maintenance of the antibody mediated immunity against already encountered antigens[78].

4.1.3.2 Cellular Adaptive Immune Response

The main effector cells of cellular adaptive immunity are T-cells. Naïve T-cells are found in lymph nodes and express an antigen specific T-cell receptor. Antigen presenting cells such as dendritic cells present antigens to the T-cells on major histocompatibility complexes by interacting with its antigen specific T-cell receptor on T-cells. Besides antigen presentation, engagement of costimulatory molecules and cytokine signaling is necessary for T-cell activation[79,80]. There are different subtypes of T-cells such as T-helper cells (Th), cytotoxic T-cells (CTL) or regulatory T-cells (Tregs). CTL express CD8 and are capable to kill targets that are recognized to be a threat to the organism. The direct cytotoxic effector functions of CTL are exerted either through the binding of the Fas ligand that is expressed on CTL with the Fas receptor on the target cell inducing apoptosis or through the action of perforins and granzymes which permeabilize the cell membrane of the target cell. Furthermore, these cells can secrete cytokines that are involved in the elimination of possible threats[81]. T-helper cells express CD4 and have a wide variety of functions which are defined by the set of cytokines that they secret upon expression of distinct transcription factors. Several subtypes of T-helper (Th) cells have been characterized[82]. Tregs are immune suppressive cells that regulate the immune response.

They are important in preventing an excess of inflammation and preserve the immunological unresponsiveness to self-antigens[83].

4.1.3.2.1 T-helper Responses

$CD4^+$ T-helper cells can be categorized in different subsets: Th1, Th2, Tfh, Th17, Th22 and Th9. Th1 are known to play an important role in phagocyte activation and CTL proliferation and are known to be crucial in immunity against intracellular pathogens. The differentiation of naïve T-cells to Th1 cells is driven by the activation of the transcription factor T-bet through IL-12 and IFN-γ in a STAT-1 and STAT-4 dependent manner. Th1 cells are known to produce high levels of IFN-γ. Th2 cells are known to confer protection against extracellular pathogens such as helminths and to be involved in the pathogenesis of allergic diseases such as asthma or atopic dermatitis[84]. Th2 differentiation is driven by IL-4 which activates the transcription factor GATA3 through STAT-6. Tfh are important in the formation of germinal centers in secondary lymphoid organs where they provide T-cell help to B-cells. There play an important role in the induction of high affinity antibody production by B-cells and the formation of memory B-cells[85]. Th17 cells are important for the clearance of extracellular pathogens through their ability to recruit and activate neutrophils. The differentiation of naïve Th cells into Th17 cells differs among species. In humans the differentiation is driven by TGF-β and IL-21 which leads to the activation of the transcription factor RORc. In mice TGF-β and IL-6 drive the Th differentiation through the transcription factor RORγt. In both species Th17 cells are known to produce high amounts of IL-17 but also IL-21 and IL-22[86]. Another Th subset was characterized that produces IL-22 without producing IL-17 and which was enriched in the human caecum. This subset is thought to play a role in mucosal immunity and is termed Th22. Naïve T-helper cells that are differentiated in presence of IL-4 and TGF- β were found to secrete IL-9 and were thus named Th9 cells. They are thought to be involved in autoimmunity and atopy[87,88].

4.2 Leishmaniasis

Leishmaniasis are a group of diseases caused by the protozoan parasites of the genus *Leishmania*. It is transmitted the bite of female phlebotomine sandflies of the genera *Phlebotomus* and *Lutzomyia.*

4.2.1 The History of Leishmaniasis

The first evidence for *Leishmania* was found in a sand fly conserved in 100 million old Burmese amber from the Hukawng valley. The *Leishmania*-like fossil species that was found in this sand fly, that also contained remnants of reptile blood, was assigned to a new *genus* and *species* and was called *Paleoleishmania proterus*[89]. In the Americas, another fossil species was found in a 20-30 million old Dominican amber and was named *Paleoleishmania neotropicum*[90].

Leishmania donovani (*L. donovani*) mitochondrial DNA was found on four Egyptian mummies, dating from around 2000 B.C., that were found in a tomb in what was in the ancient city of Thebes[91]. In Peru, *Leishmania* infected macrophages in mummies from 800 B.C. were identified by immunohistochemistry[92]. In 930 A.D. the Persian physician Abubakr Muhammad ibn Zakariyya described cutaneous lesions reminiscent of leishmaniasis in patients of the Baghdad region[93]. In ceramics of the Moche culture, found in Peru and dating from the 5th century, depictions of facial conditions reminiscent of mucocutaneous leishmaniais (MCL) were found[94]. In 1571 the Spanish chronicler Pedro Pizzaro described cases of coca farmers in Peru who suffered from a disease that destroyed their nose and lips and was indicative of MCL. In 1756 the Scottish physician Alexander Russel made the first detailed description of the clinical picture of cutaneous leishmaniasis (CL) in patients from Aleppo[95]. In 1900, the Scottish physician and officer in the Royal Army Medical Corps William Boog Leishman described ovoid bodies in cells on smears taken form spleens of soldiers stationed in Dum Dum, India and that died from a disease causing splenomegaly. In 1903, he published his findings and

hypothesized that these ovoid bodies were degenerated trypanosomes and called the disease "Dum Dum fever"[96]. The Irish physician Charles Donovan published a paper about the finding of similar bodies in native Indian patients from Madras the same year [97]. Also in the same year, the British physician Ronald Ross published two articles in which he concluded that the ovoid bodies were actually a novel form of protozoan parasite which he named *Leishmania donovani*[98,99]. Also in 1903, the U.S. pathologist James Homer Wright described organisms found in a CL lesion of an Armenian patient which would be eventually by named *Leishmania tropica*[100]. In 1973, Bray et al. proposed to classify *L. tropica* as two species based on the difference of size, *Leishmania tropica* and *Leishmania major*[101]. In the Americas, the first description of *Leishmania* parasites was done independently by the Brazilian physician Adolpho Carlos Lindenberg and the Italian physician Antonio Carini in skin lesions of patients from the State of Sao Paolo in 1909. In the beginning it was thought that the isolated parasites were identical to *L. tropica,* but in 1911 Brazilian physician Gaspar de Oliveira Vianna noted morphological differences from *L. tropica* in parasites isolated form a patient from São João de Além Paraiba and concluded that it was a different species which was named *Leishmania braziliensis* and would later, like other species found in the Americas, be part of the subgenus *Viannia*. In 1913 *Leishmania peruviana* was described for the first time. In 1930 the first cases of VL in the Americas were reported. In 1937, the Brazilian physicians Aristides Marques da Cunha and Evandro Serafim Lobo Chagas reported to have discovered a new species which they named *Leishmania chagasi.* However, more recent analysis techniques showed that *L. chagasi* and *L. infantum* were indistinguishable. The other *Leishmania* species that are known today were subsequently discovered from 1953 (*Leishmania mexicana)* until just recently in 2015 (*Leishmania waltoni*)[95], including *L. martiniquensis* that was first described in 1995 and was latter assigned to a third subgenus called *Mundinia*[102].

4.2.2 Epidemiology

Leishmaniasis is considered a highly neglected tropical disease which predominantly affects people of low socioeconomic status and is considered the second most deadly vector transmitted disease just after malaria[103,104]. It has been reported to be endemic in 98 countries and three territories. The worldwide incidence of CL is estimated to be 690'000 to 1'200'000 cases per year and that of VL is estimated to be 200'000 to 400'000 cases per year[105]. The cumulative worldwide prevalence is 12 million people[106]. There is an estimate that 20'000 to 50'000 people per year succumb due to leishmaniasis[105,106].

4.2.3 Clinical Manifestation and Diagnosis

4.2.3.1 Cutaneous Leishmaniasis

Leishmania parasites can cause a wide spectrum of cutaneous disease. In many cases, the infection with *Leishmania* remains clinically silent. In the clinically apparent cases, a papule appears after a sand fly bite that gradually develops and increases in size. It can develop into an ulcer, become a plaque or turn into a hyperkeratotic or wart-like lesion over the subsequent weeks and months[107]. This clinical picture is called localized cutaneous leishmaniasis (LCL) and varies widely in clinical appearance. Most cases of LCL are self-healing within two month to two years and results in cutaneous scaring and some degree of protection against the disease[108]. In some cases the parasites disseminate from the initial site of infection though the lymphatic system and create multiple pleomorphic nodules in two or more noncontiguous body areas, a condition called disseminated leishmaniasis (DL)[109]. In diffuse cutaneous leishmaniasis (DCL) the clinical picture is characterized by widespread multiple papulonodular or infiltrative cutaneous lesions[110]. In some other cases new papular lesions appear around healed LCL, a condition known as leishmaniasis recidivans (LR)[111]. Parasites can also disseminate to mucosal tissues which is known as mucocutaneous leishmaniasis (MCL). MCL begins often with an

erythema that develops into ulceration of the mucosal tissues of the oro-naso-pharyngeal area. It is associated with facial disfigurement and can end deadly due to secondary infections and the impairment of functions of the affected organs[109].

4.2.3.2 Visceral Leishmaniasis

In visceral leishmaniasis (VL) *Leishmania* parasites infect secondary lymphoid organs and the liver. The clinically manifestations of VL can be hepatomegaly, splenomegaly, fever, anorexia and nocturnal hyperhidrosis and pallor. The patients often suffer from anemia, leukopenia, thrombocytopenia and hypergammaglobulinaemia. With disease progression patients suffer from pronounced cachexia, bleeding due to a decrease in coagulation capacity, secondary infections due to immune insufficiency and eventually succumb to the disease. However, also clinically silent or oligosymptomatic disease progressions have been reported[107,112].

4.2.4 *Leishmania*

There many different species of the genus *Leishmania* described, 19 of which are known to cause disease in humans[95]. The human pathological *Leishmania* spp. can be divided into three subgenera: *Leishmania Leishmania, Leishmania Viannia* and *Leishmania Mundinia*[102]. Moreover, there is the subgenus *Sauroleishmania* that is only infective to lizards. Species from the subgenus *Leishmania Leishmania* are found in tropical regions worldwide and can cause all the clinical forms of leishmaniasis. *Leishmania Viannia* are found only in the tropical regions of Latin America and can cause CL or MCL. Members of the subgenus *Leishmania Mundinia* can cause CL or VL and are found in Martinique and Thailand[113,114] **(Figure 6)**. *Leishmania* species are also categorized as Old World species and New World species depending on whether they are endemic in Europe, Asia and Africa or the Americas[115].

Subgenus	Species	Old/New World	Clinical disease	Distribution
Leishmania	*L. aethiopica*	OW	LCL, DCL	East Africa (Ethiopia, Kenya)
	L. amazonensis	NW	LCL, DCL, MCL	South America (Brazil, Venezuela, Bolivia)
	L. donovani	OW	VL, PKDL	Central Africa, South Asia, Middle East, India, China
	L. infantum (syn. *L. chagasi*)	OW, NW	VL, CL	Mediterranean countries (North Africa and Europe), Southeast Europe, Middle East, Central Asia, North, Central and South America (Mexico, Venezuela, Brazil, Bolivia)
	L. major	OW	CL	North and Central Africa, Middle East, Central Asia
	L. mexicana (syn. *L. pifanoi*)	NW	LCL, DCL	USA, Ecuador, Venezuela, Peru
	L. tropica	OW	LCL, VL	North and Central Africa, Middle East, Central Asia, India
	L. venezuelensis	NW	LCL	Northern South America, Venezuela
	L. waltoni	NW	DCL	Dominican Republic
Viannia	*L. braziliensis*	NW	LCL, MCL	Western Amazon Basin, South America (Guatemala, Venezuela, Brazil, Bolivia, Peru)
	L. guyanensis	NW	LCL, MCL	Northern South America (French Guinea, Suriname, Brazil, Bolivia)
	L. lainsoni	NW	LCL	Brazil, Bolivia, Peru
	L. lindenbergi	NW	LCL	Brazil
	L. naiffi	NW	LCL	Brazil, French Guinea
	L. panamensis	NW	LCL, MCL	Central and South America (Panama, Columbia, Venezuela, Brazil)
	L. peruviana	NW	LCL, MCL	Peru, Bolivia
	L. shawi	NW	LCL	Brazil
Mundinia	*L. martiniquensis*	NW, OW	LCL, VL	Martinique, Thailand

Figure 6: Overview of the most important human pathological *Leishmania* species (Steverding, 2017)

4.2.4.1 *Leishmania* Life Cycle

The *Leishmania* life cycle is similar among the different *Leishmania* subgenera and species. Upon the bite of a host by a female sand fly, metacyclic promastigotes are regurgitated in the dermis together with sand fly saliva which contains neutrophils chemotactic factors[116-120]. This leads to a rapid recruitment of neutrophils to the site of infection[9]. Subsequently, neutrophils phagocytize the parasites or trap them in NETs[14,121]. In addition, neutrophils phagocytize other already infected cells which down-regulates neutrophil effector functions and is a mean of silent entry of *Leishmania* into neutrophils[122]. During and after the first wave of neutrophil recruitment, parasites will get taken up by macrophages which are their definitive host. This happens by the phagocytosis of apoptotic neutrophils that are infected with *Leishmania* by macrophages, a mechanism which is named "Trojan Horse" and again provides a mode of silent entry into the host cell[123]. Apoptotic neutrophil may also release parasites and the free parasites which can then get phagocytized by macrophages[120]. Inside the macrophages the parasites

reside within an acidic parasitophorus vacuoles (PV) where they transform due to the change in pH from their promastigote to their amastigote stage, i.e. their ovoid shaped replicative form[124]. Upon a blood-meal, another sandfly can take up the amastigotes which will transform into proliferating procyclic promastigotes and finally into infective metacyclic promastigotes[125].

4.2.4.2 *Leishmania* Vectors

Region/country	Vector spp.	Disease type
Argentina/Mexico	*Lutzomyia longipalpis*	Cutaneous/visceral
Belize/Mexico	*L. olmeca*	Cutaneous
Panama	*L. panamensis*	Cutaneous
Brazil	*L. whitmani/intermedia*	Cutaneous
Columbia	*L. evansi, gomezi*	Urban
Venezuela	*L. vallesi, gomezi*	Cutaneous
Sudan	*Phlebotomus langeroni orientalis*	Visceral
Kenya/Ethiopia	*P. martini*	Visceral
Palestinian W. Bank	*P. papatasi*	Cutaneous
	P. sergentii	Cutaneous
	P. syriacus	Visceral
N. W. Africa	*P. dubosqi*	Cutaneous
Greece	*P. neglectus*	Visceral
India	*P. papatasi*	Cutaneous
India	*P. argentipes*	Visceral
Saudi Arabia	*P. papatasi*	Cutaneous
Monaco	*P. perniciosus*	Visceral
	P. ariasi	Visceral
Egypt	*P. langeroni*	Visceral
China	*P. alexandri*	Visceral
	P. chinensis	Visceral
	P. longiductus,	Visceral

Figure 7: Overview of different vectors of leishmaniasis (Claborn, 2010)

Over 500 sand-fly species have been described that belong to three genera: *Phlebotomus, Sergentomyia* and *Lutzomyia.* While *Phlebotomus and Sergentomyia* are found in the old world, *Lutzomyia* is found in the new world. Only a small proportion of the sand-fly species are known to be vectors of *Leishmania* in humans[126] **(Figure 7)**.

4.2.5. Treatment of leishmaniasis

Even though there would be a need for efficient drugs against leishmaniasis there are only few compounds available, which have a lot of adverse effects. There is no efficient vaccine for humans on the market neither[127]. The most commonly used drugs against leishmaniasis are listed in the following table **(Figure 8)**.

Drugs	Administration route	Dosage	Efficacy	Toxicity
Pentavalent antimonials	IM, IV, or IL	20 mg/kg/day (28–30 days)	35–95% (depending on area)	Severe cardiotoxicity, pancreatitis, nephrotoxicity, hepatotoxicity
Amphotericin B	IV	0.75–1 mg/kg/day (15–20 days, daily or alternately)	>90%	Severe nephrotoxicity, infusion-related reactions, hypokalemia, high fever
Liposomal amphotericin B	IV	10–30 mg/kg total dose (single dose 3–5 mg/kg/dose)	>97%	Mild rigors and chills during infusion Mild nephrotoxicity (infrequent and mild)
Miltefosine	Oral	100–150 mg/day (28 days)	Asia: 94% (India); Africa: 60%–93%	Vomiting and diarrhoea, nephrotoxicity, hepatotoxicity, teratogenicity
Paromomycin	IM (VL) or topic (CL)	15 mg/day (21 days) or 20 mg/kg (17 days)	94% (India) 46–85% (Africa)	Severe nephrotoxicity, ototoxicity, hepatotoxicity
Pentamidine	IM	3 mg/kg/day IM every other day for 4 injections	35–96% (depending on *Leishmania* species)	High rate of hyperglycemia, as a result of pancreatic damage; hypotension, tachycardia, and electrocardiographic changes

IV: intravenous administration; IM: intramuscular administration; IL: intralymphatic administration.

Figure 8: Overview of different antileishmanial drugs (Bezerra de Menezes at al., 2015)

4.2.5.1 Antimonny

Pentavalent antimony (SbV) is the most ancient and the most commonly used compound in antileishmanial drug treatment. Currently, there are two different pentavalent antimony formulations available on the market: Sodium Stibogluconate (SSG) and meglumine antimoniate (MA). These compounds are usually used at dosage of 20mg/kg/day for 4 weeks. The use of SbV is associated with a wide variety of secondary adverse effects including cardio-, hepato-, and renotoxicity[128,129]. Even though SbV has been the mainstay therapy against leishmaniasis for decades, its exact mode of action is still debated. Antimony is thought to kill *Leishmania* spp. by a variety of mechanisms including induction of cell death by a process involving DNA fragmentation[130-132]. Furthermore, antimony has been shown to inhibit the

enzymes trypanothione reductase and glutathione synthetase[133] and to induce the efflux of antioxidant substances such as thiols and glutathione[134,135], thereby inducing an increase of oxidative stress within the parasite. In recent years a marked increase in antimony treatment failure has been observed. SbV has already been eliminated as antileishmanial drug treatment against VL in India due to the resistance situation. Also in other countries there is an increasing number of reports about SbV resistance in leishmaniasis, for instance in Colombia 20% of *Leishmania Viannia panamensis (L. (V.) p.)* strains isolated from patients were resistant to meglumine antimony[136].

4.2.5.2 Miltefosine

Miltefosine (MIL) was originally developed as antineoplastic drug but has since then been introduced as an antileishmanial drugs in various countries in the early 2000s. MIL is the first and thus far only antileishmanial drug that does not have to be administered parenterally but can be given per os. Usually, it is administered at an dosage of 100-150 mg/day for four weeks[136]. As SbV, miltefosine has a wide variety of secondary effects such as hepato- and renotoxicity[137]. Even though the mechanisms of action of miltefosine are not precisely understood, evidence suggests that it kills the parasite by inducing mitochondrial depolarization, decreasing ATP levels[138] and interfering with the lipid metabolism[139]. The half-life of miltefosine is approximately 120 hours. This renders it vulnerable to the emergence of drug resistance in *Leishmania* spp.*,* because the parasite is exposed sub-therapeutic drug levels after completion of therapy[140].

4.2.5.3 Other Drugs

Amphotericin B was developed as antifungal agent which has to be administered IV. It can be used in its plain or its liposomal form. The latter is usually better tolerated by the patients since

it has a similar efficacy but less side effects[141]. It is thought that its mechanism of action is the formation of pores in the parasite cell membrane through sterol binding[142].

Paromomycin is a broad spectrum antibiotic which can be also act against certain protozoan parasites by binding to their 30S ribosomal subunit and thus interfering with their protein synthesis. It can be administered topically or intramuscularly and can also cause severe side effects such as oto-, nephron- or hepatotoxicity [143].

Pentamidine is another drug with antileishmanial properties. It is given in an intramuscular manner and its efficacy highly depends on the geographic region[144,145].

4.3 The Immune Response against Leishmaniasis

4.3.1 The Innate Immune Response against Leishmaniasis

When an infected sand fly bites a host and regurgitates *Leishmania* parasites, in addition immune modulatory molecules from the salivary glands are deposited at the site of infection[116-120]. Together with the inflammation due to the injury of the skin and the skin capillaries, this results in rapid neutrophil recruitment[9]. The parasites are phagocytized by neutrophils, which eventually become apoptotic and the parasites are transferred eventually to the macrophages where they transform into amastigotes, their replicative form[124]. The role of neutrophils during these first hours of infection is of utmost importance. In the following two reviews "Different *Leishmania* Species Drive Distinct Neutrophil Functions" and "Survival Mechanisms Used by some *Leishmania* Species to Escape Neutrophil Killing" different aspects of the role of neutrophils in leishmaniasis are discussed.

4.3.1.1 Review 1: Different *Leishmania* Species Drive Distinct Neutrophil Functions

Trends in Parasitology

CellPress

Review

Different *Leishmania* Species Drive Distinct Neutrophil Functions

Benjamin P. Hurrell,[1] Ivo B. Regli,[1] and Fabienne Tacchini-Cottier[1,*]

Leishmaniases are vector-borne diseases of serious public health importance. During a sand fly blood meal, *Leishmania* parasites are deposited in the host dermis where neutrophils are rapidly recruited. Neutrophils are the first line of defense and can kill pathogens by an array of mechanisms. They can also form web-like structures called neutrophil extracellular traps (NETs) that can trap and/or kill microbes. The function of neutrophils in leishmaniasis was reported to be either beneficial by contributing to parasite killing or detrimental by impairing immune response development and control of parasite load. Here we review recent data showing that different *Leishmania* species elicit distinct neutrophil functions thereby influencing disease outcomes. Emerging evidence suggests that neutrophils should be considered important modulators of leishmaniasis.

Leishmaniases

Leishmania (L.) are protozoan parasites causing leishmaniases, a spectrum of vector-borne neglected diseases affecting over 150 million people worldwide with 350 million at risk in about 98 countries or territories [1]. There is an incidence of over 2 million new cases per year, with an estimate of 0.5 million cases of visceral leishmaniasis (VL) and 1.5 million of cutaneous leishmaniasis (CL). The parasites are transmitted to mammals upon the blood meal of infected female phlebotomine sand flies. The species of *Leishmania* infecting the host broadly determines the type of disease that will evolve. An estimated 20 different *Leishmania* spp. cause the three main clinical disease manifestations comprising the cutaneous, mucocutaneous, and visceral forms. Some *Leishmania* species cause CL, the most common form of the disease. CL is characterized by the presence of localized skin ulcers that, in immunocompetent individuals, are self-healing, leaving disfiguring scars over months to years depending on the infecting *Leishmania* species. Several years following infection with *Leishmania* species of the *Vianna* subgenus, mucocutaneous leishmaniasis (MCL) can develop. MCL is characterized by the migration of parasites to mucosal tissues of the mouth and upper respiratory tract, leading to partial or total tissue destruction. Infection with *Leishmania donovani* or *Leishmania infantum* causes VL, which is the least common form of the disease but can be fatal if left untreated. In VL, the parasites migrate and replicate in lymphoid organs including the spleen, liver, and bone marrow, eventually leading to organ malfunction. No satisfactory treatment that is affordable, efficacious, easy to administer, and with low toxicity is currently available. Resistance against each of the drugs presently in use has been reported (reviewed in [2]). In addition, no vaccine is currently available; thus better understanding of the immune response that occurs following infection with the various *Leishmania* species is required to better target immunopreventive approaches.

Trends

Neutrophils are massively recruited following infection with *Leishmania* parasites. Neutrophils are well known for their microbicidal properties; however, some *Leishmania* parasites are able to escape killing.

The impact of neutrophils on leishmaniases remains not well understood. Emerging data suggest that neutrophils play a crucial role in the development of the different forms of the disease.

Studies in animal models revealed that neutrophils may play protective or deleterious roles at the onset of *Leishmania* infection. Recent models of neutropenic mice offer new tools to investigate the mechanisms involved in these processes.

Parasite and host factors appear to direct neutrophil effector function as well as neutrophil immunomodulatory function.

[1]Department of Biochemistry, WHO-IRTC, University of Lausanne, Lausanne, Switzerland

*Correspondence: Fabienne.Tacchini-Cottier@unil.ch (F. Tacchini-Cottier).

The early events occurring at the site of infection are thought to be critical in the development of an efficient protective immune response against *Leishmania* infection. Special attention has been given to neutrophils, as these cells are among the first cells massively recruited to the site of infection [3–5]. In this review we discuss recent developments in the field with an emphasis on recent findings demonstrating that distinct *Leishmania* spp. specifically impact neutrophil function, with direct consequences on disease development.

Study of the Crosstalk between Neutrophils, Parasites, and Neighboring Cells

Neutrophils are innate immune cells mobilized from the bone marrow that migrate via the blood to areas of trauma or infection. They play a crucial role in the elimination of many invading pathogens [6,7]. Neutrophils can kill microbes using several mechanisms including the release of their toxic granule content in the local environment or in the **phagosome** (see Glossary) and via the formation of fibrous structures called **NETs**. NETs can trap pathogens and sometimes kill them through the toxicity of **histones** and/or antimicrobial granule-derived proteins associated with these structures [8]. Neutrophil effector functions need to be tightly regulated, as excessive activity can lead to tissue destruction and ultimately chronic inflammation [9]. In addition to these killing functions, increasing evidence supports a role for neutrophils in the modulation of innate and adaptive immune responses. This can be through the release of cytokines [10] or of other factors such as **eicosanoids** (thromboxane A2) that lead to dampening of the immune response by acting on T cells [11]. Crosstalk with other cells present at the site of infection or within the draining lymph nodes, including dendritic cells (reviewed in [12,13]), natural killer (NK) cells [14], and B cells [15], as well as sequestration of antigens [16,17] may also modulate the immune response in positive or negative ways.

Most of our current understanding of the role of neutrophils in leishmaniasis has been obtained by work performed in experimental mouse models. Although results from these studies provide important insights, they cannot always be extrapolated to humans as there are significant differences between human and murine neutrophils. While in human blood 50–70% of all leukocytes are neutrophils, they represent only 10–25% of leukocytes in murine blood [18,19]. Furthermore, the antimicrobial molecules present in human and mouse neutrophil granules differ. For instance, murine neutrophils do not express **defensins** or **azurocidin**, while these proteins are abundantly present in human neutrophils (reviewed in [20]). Assessing the function of human neutrophils in *Leishmania* infection has been difficult for several reasons. First, most experiments performed with human neutrophils are conducted using peripheral blood cells, as these cells are the most accessible in patients. However, blood neutrophils may not behave in the same way as neutrophils present at the site of infection (reviewed in [21]). For instance, no difference in **arginase** activity was observed in neutrophils isolated from the peripheral blood of healthy or infected individuals. By contrast, the arginase activity of neutrophils isolated from skin biopsies was found to be significantly increased in CL patients compared with that observed in biopsies of healthy patients [22]. Second, it is well established that host factors play important roles in disease development in leishmaniasis. The impact of *Leishmania major* LV39 on neutrophils from mice of the C57BL/6 or BALB/c genetic background was shown to differ dramatically regarding cytokine release and disease outcome [5,23,24]. The comparability of data obtained from samples from different patients may thus be complicated by genetic background, age, sex, lifestyle, and comorbidities [25], a point also valid for other diseases. This complexity stresses the need to obtain more insights on the role of neutrophils during human leishmaniasis.

Recruitment of Neutrophils to the Site of *Leishmania* Infection

The presence of neutrophils throughout the *Leishmania* life cycle is shown in Figure 1 (Key Figure). Rapid and massive neutrophil recruitment following needle inoculation of a high

Glossary

Amastigote: non-motile intracellular stage of *Leishmania* spp. that replicates within macrophages of the infected mammalian host.
Arginase: an enzyme that catalyzes the hydrolysis of L-arginine to ornithine and urea. It limits the L-arginine that is available for the synthesis of nitric oxide and contributes to immune regulation.
Azurocidin: multifunctional inflammatory mediator present in human neutrophil granules. It has bactericidal functions.
CD11b: surface molecule expressed on myeloid cells.
Defensin: small, cysteine-rich proteins found in animals and some plants with bactericidal and fungicidal properties. In humans they can be found in the azurophilic granules of neutrophils, in the Paneth cells of the small intestine, and in various types of epithelial cells.
Eicosanoid: molecules derived from 20-carbon fatty acids that have immune-modulatory effects.
GP63: metalloprotease that is abundantly expressed on the surface of promastigotes of many *Leishmania* spp. and is considered a virulence factor.
Granulopoiesis: formation of granulocytes in blood-forming tissues, which under physiological conditions are located in the bone marrow. The emergence of granulocytes occurs via several precursors, such as myeloblasts, promyelocytes, myelocytes, and metamyelocytes.
Granzmye B: important component of granules in cytotoxic lymphocytes and NK cells that induces cell death on entrance into target cells by caspase activation and mitochondrial permeabilization.
Histone: protein found in eukaryotic cells that envelopes and structures DNA. It is the main component of chromatin.
Human neutrophil elastase quantitation: measurement of the concentration of human neutrophil elastase in supernatants of cell suspensions by the addition of a fluorogenic substrate and measurement of the resulting fluorescence intensity.
Lipophosphoglycan (LPG): a surface molecule and virulence factor abundantly present on the surface of *Leishmania* spp.

Leishmania inoculum was observed in experimental models of CL following *L. major* [3,5,26,27], *Leishmania amazonensis* [27,28], *Leishmania braziliensis* [29], or *Leishmania mexicana* [30] infection and of VL following *L. infantum* [31] or *L. donovani* infection [32]. Using LysM-eGFP fluorescent reporter mice [33], allowing neutrophil visualization *in vivo*, **two-photon microscopy** confirmed rapid neutrophil recruitment following natural infection with *L. major* through sand fly bites [4] and intradermal needle inoculation of a high dose of *L. major* [4] or *L. mexicana* [30] (Figure 1, step 1). Most sand flies transmit fewer than 600 *L. major* parasites per blood meal, with a quarter transmitting more than 1000 and reaching 10^5 parasites in rare cases [34]. Tissue damage to the skin caused by infected or uninfected sand fly bites, or by the needle itself on injection, is sufficient to induce neutrophil recruitment within the first 90 min, showing that initial neutrophil recruitment appears to be parasite independent [4]. Accordingly, needle injection of $\geq 10^5$ *Leishmania* parasites is required to observe parasite-dependent neutrophil recruitment to the site of inoculation in mice at later times (6–24 h post-infection) [30,31]. Following natural infection, factors derived from the sand fly also contribute to neutrophil recruitment in the infected dermis. In line with this, injection of salivary gland extracts from sand flies [35,36] or of **promastigote** secretory gel (PSG) [37] – a gel secreted by the parasites in the fly midgut and regurgitated on blood meal – was shown to recruit neutrophils in mice. Furthermore, the site of *L. major* inoculation was recently shown to have an impact on neutrophil recruitment following needle but not sand fly inoculation [38]. In this study, intradermal needle inoculation of the parasite (compared with subcutaneous or intraperitoneal injection) best mimicked the neutrophil recruitment observed on natural infection. Collectively, all of these studies show that various factors, summarized in Table 1, contribute to neutrophil recruitment following *Leishmania* infection. They can come from the host, the sand fly, or even the parasite itself.

The lifespan of neutrophils is relatively short in the blood, ranging from 1.5 to 8 h in mouse and humans respectively, but can be increased several fold during inflammation [39]; this is also observed in inflammatory tissues [40–42]. However, neutrophil lifespan remains uncertain due, among others things, to technical limitations in tracing neutrophils *in vivo* [43,44]. Following needle injection of most *Leishmania* parasites in mice, the neutrophil peak occurs between 12 and 24 h post-infection, decreasing to basal low numbers at 48–72 h after infection [26,29–31]. Interestingly, mice vaccinated with killed *L. major* + CpG and subsequently infected via sand fly bites showed a prolonged neutrophil presence of up to 8 days post-infection [45]. This suggests that, in addition to sustained recruitment, sand fly-derived factors that together with CpG contribute to the inflammatory environment may extend neutrophil lifespan. This, however, needs to be further investigated.

To understand the role of neutrophils in experimental leishmaniasis, the impact of transient or sustained **neutropenia** (low neutrophil count) on the development of disease can be induced by neutrophil-depleting antibodies following *Leishmania* infection. This approach, however, has limitations that have been extensively discussed [38,46–48]. Recently, *Genista* mice, which have a point mutation in the growth factor-independent 1 transcription repressor (*Gfi1*) gene, were obtained by *N*-ethyl-*N*-nitrosourea-induced mutagenesis [49] leading to a block in terminal **granulopoiesis**. These mice thus do not have any mature neutrophils and have a normal myeloid and lymphoid population. A small number of **CD11b**$^+$ atypical neutrophils expressing only intermediate levels of **lymphocyte antigen 6G (Ly6G)** is observed in the periphery. *Genista* mice thus provide a good model to study the impact of neutropenia throughout infection. Comparing short-term **monoclonal antibody (mAb)** depletion of neutrophils at the onset of *L. mexicana* infection with that of sustained neutropenia observed in *Genista* mice recently allowed us to demonstrate the importance of neutrophils in the early hours of *L. mexicana* infection. We showed that neutrophils recruited to the site of infection following *L. mexicana* infection ingested the parasites and formed NETs without major impact on parasite survival. Additionally, mice transiently depleted of neutrophils at the onset of infection and neutropenic

Lymphocyte antigen 6G (Ly6G): glycosylphosphatidylinositol (GPI)-anchored protein that is predominantly expressed on murine neutrophils.
Monoclonal antibody (mAb): an antibody against a single specific epitope of interest that is produced by cell clones derived from a single antibody-producing B lymphocyte.
Myeloperoxidase (MPO): a lysosomal protein present in neutrophils that can be released from azurophilic granules during degranulation.
Neutropenia: pathologically low concentration of neutrophils in the blood. Clinically often defined as a value under 1500/μl.
Neutrophil extracellular traps (NETs): made of extracellular fibers comprising DNA and granular contents released by neutrophils on stimulation.
Phagosome: organelle containing phagocytosed particles and organisms.
Picogreen assay: used for the quantitation of dsDNA through the specific binding of a fluorochrome that has an excitation maximum at 480 nm and an emission peak at 520 nm.
Promastigote: motile, flagellated stage of *Leishmania* spp. It is found in the midgut of the sand fly and is transmitted to the host during a sand fly blood meal.
Two-photon imaging: fluorescence microscopy technique using near-infrared excitation wavelengths, which are less absorbed by biological specimens than light of greater wavelength. It offers good tissue penetration and is therefore often used for *in vivo* imaging.

Key Figure

The Role of Neutrophils in the Mammalian *Leishmania* Life Cycle

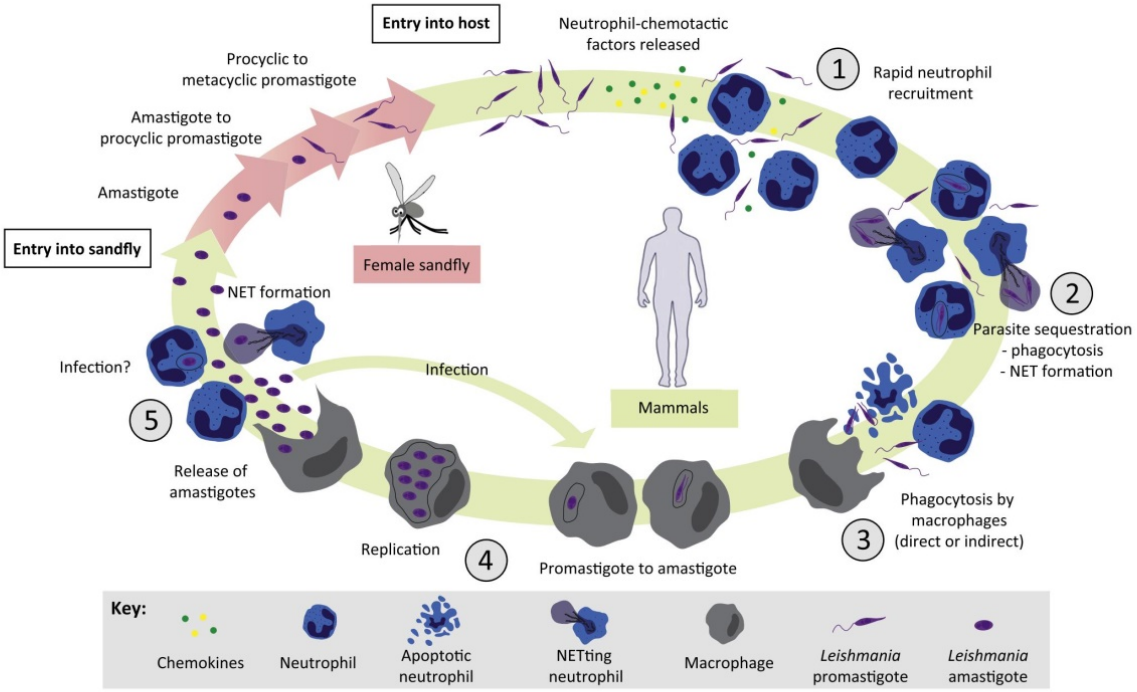

Figure 1. (1) Female sand flies transmit infectious metacyclic promastigotes into human or vertebrate hosts on blood meal. Parasite presence and vascular damage lead to the release of chemotactic factors derived from the host, the parasite itself, and/or the sand fly. Neutrophils are rapidly and transiently recruited to the infection site. There, (2) parasites are phagocytosed by neutrophils and/or induce the formation of neutrophil extracellular traps (NETs). *Leishmania* promastigotes directly or indirectly enter macrophages (3), their final host cells. Within macrophages, promastigotes transform into amastigotes and replicate by simple division (4). Days or weeks after the initial infection, a second wave of neutrophils is recruited to the infection site where their presence can be observed within chronic inflammatory lesions. Amastigotes are released from bursting macrophages. They will be recaptured by macrophages and/or possibly by neutrophils (5). This latter point needs to be further investigated. In addition, intralesional parasites can induce NET formation. On a blood meal, uninfected female sand flies take up amastigotes, which transform within their midgut following a multistep process into infectious metacyclic promastigotes that will eventually be transmitted to other hosts.

Genista mice similarly infected with *L. mexicana* were fully able to resolve their lesion size and control parasite load. These data demonstrate that early recruitment of neutrophils induced by *L. mexicana* has a major impact on disease pathology, impairing the control of lesion development and blocking the subsequent induction of a protective immune response [30].

Induction of NET Formation in the Early Hours of Infection

NETs can trap pathogens, limiting their spread and concentrating antimicrobial molecules that are in most cases detrimental to the invading pathogen. Although bacteria-induced NET release is well documented since the breakthrough study by Brinkmann *et al.* in 2004 [8], its involvement in response to *Leishmania* infection is only now emerging (Figure 1, step 2). Guimarães *et al.* first

Table 1. Neutrophil Chemotactic Factors Following *Leishmania* Infection

Chemotractant	Source	Description	Refs
IL-8 (human) CXCL1 and CXCL2 (mouse)	Host	IL-8 in humans is released by host cells including neutrophils themselves; CXCL1 and CXCL2 mRNA upregulated at the site of infection in mice	[67,68]
Complement C3	Host	C3 cleavage is required for neutrophil recruitment to the site of infection	[69]
IL-17	Host	Not involved in early neutrophil recruitment but involved weeks after initial infection in BALB/c mice	[70]
GCP-2 (CXCL6)	Host	GCP-2 (CXCL6) leads to rapid neutrophil mobilization after *Leishmania major* infection	[71]
Leishmania chemotactic factor (LCF)	Parasite	LCF is released by *L. major*, *Leishmania aethiopica*, and *Leishmania donovani* and is chemotactic for neutrophils *in vitro*	[68]
PSG	Parasite	PSG is capable of recruiting neutrophils on its own	[37]
Saliva	Sand fly	Saliva and salivary gland sonicate induce strong neutrophil recruitment	[36,37]

showed that exposure of neutrophils to *L. amazonensis*, *L. major*, or *L. infantum* promastigotes *in vitro* triggers NET formation in human blood neutrophils [50]. An increasing number of studies are now reporting *Leishmania*-induced NET formation by human or mouse neutrophils *in vitro*, as detailed in Table 2.

Although various *Leishmania* parasites were shown to induce NETs *in vitro*, the outcome on parasite survival within NETs differed between *Leishmania* species. *L. amazonensis* was shown to trigger NETs that killed the parasites, a process depending on promastigote surface **lipophosphoglycan (LPG)**, whereas parasite killing was partially reverted using antihistones mAbs [50]. By contrast, several other *Leishmania* spp. were able to escape NET killing through various mechanisms. Gabriel *et al.* showed that *L. donovani* promastigotes survived within NETs *in vitro*. LPG was not necessary for the formation of NETs but was instead involved in parasite resistance to killing [51]. Recently, Guimarães *et al.* demonstrated that the parasite-specific nuclease 3′-nucleotidase/nuclease favored the survival of *L. infantum* promastigotes within NETs [52] and we recently showed that NETs failed to kill *L. mexicana* promastigotes *in vitro* [30]. Interestingly, saliva of the *Leishmania* vector *Lutzomyia longipalpis* was shown to contain a 'NET-destroying' endonuclease (Lundep) capable of releasing parasites from NETs *in vitro* [53]. These findings suggest that the outcome of NETs on parasite survival is *Leishmania* species specific, at least *in vitro*.

In addition to differences in virulence factors between distinct *Leishmania* spp., factors involving either the host or the vector may also contribute to the final NET microbicidal outcome. A recent report suggests that human NETs may be directly involved in modulating the activation of cells in the microenvironment, suggesting that these structures may have multiple functions during the course of infection [54]. Regardless, all studies so far suggest that neutrophils form NETs in response to promastigote encounter, both *in vitro* and *in vivo* (Figure 1, step 2).

Leishmania is Present First in Neutrophils and Then in Macrophages

Neutrophils account for over 80% of the total infected cells observed in the ear dermis of C57BL/6 mice 12 and 24 h following either *L. major* [26] or *L. mexicana* [30] infection. Most neutrophils at this time point harbor at least one viable promastigote [55] and, strikingly, *L. major*

Table 2. *Leishmania*-Induced NET Formation and Microbicidal Function

Leishmania spp.	Parasite Stage	Host	Model	NET Readout	Observation	Refs
Leishmania amazonensis	Promastigote	Human	*in vitro/in vivo*	Confocal and electron microscopy, **picogreen assay**	Parasites induce NET formation through surface LPG and are killed in a histone-dependent manner	[50,52,72]
L. amazonensis	Amastigote	Human	*in vitro*	Picogreen assay	Stimulation leads to dose-dependent release of DNA; no information on parasite survival	[50]
Leishmania major Fn	Promastigote	Human	*in vitro*	Picogreen assay	Stimulation leads to DNA release in medium; no information on parasite survival	[50]
Leishmania chagasi	Promastigote	Human	*in vitro*	Picogreen assay	Stimulation leads to DNA release in medium; no information on parasite survival	[50]
Leishmania donovani 1S	Promastigote	Human	*in vitro*	Confocal microscopy, picogreen assay	Parasites trapped by released NETs but are not killed; neither LPG or **GP63** nor superoxide production is responsible for NET formation; LPG-deficient promastigotes are susceptible to killing	[51]
L. donovani LV9	Promastigote	Human	*in vitro*	Picogreen assay	Dose-dependent release of DNA in medium; no information on parasite survival	[51]
L. major LV39	Promastigote	Human	*in vitro*	Picogreen assay	Stimulation leads to DNA release in medium; no information on parasite survival	[51]
L. major WR 2885	Promastigote	Human	*in vitro*	Confocal microscopy, **human neutrophil elastase quantitation**	Neutrophils release NETs but are destroyed by Lundep, a secreted salivary endonuclease; treatment with recombinant Lundep increases long-term mouse lesion development, implying that NETs favor parasite killing	[53]
Leishmania infantum	Promastigote	Human	*in vitro*	Picogreen assay, fluorescence microscopy	Parasites trigger NET release and are killed but high parasite-specific nuclease activity enables them to escape from NETs and thus killing	[52]
Leishmania mexicana	Promastigote	Mouse	*in vitro/in vivo*	Confocal microscopy	Parasites trigger NET release but are not killed	[30]
ATL	Amastigote	Human	*in vivo*	Immunohistochemistry, confocal microscopy	NETs and amastigotes are found in close proximity in human lesions; dynamic process involved in the control of parasite burden	[60]

was shown to survive within neutrophils hours following *in vitro* infection [56]. After residing within neutrophils for an as-yet unknown time period after infection, *Leishmania* spp. will invade macrophages (Figure 1, step 3). Two potential mechanisms of *Leishmania* transmission have been proposed. First, the 'Trojan Horse' model is based on silent transmission of the parasite from neutrophil to macrophage through phagocytosis of *Leishmania*-containing apoptotic neutrophils by macrophages [57]. This model agrees with the reported ability of some *Leishmania* species, such as *L. major* [26] and *L. braziliensis* [29], to induce neutrophil apoptosis. However, the impact of *Leishmania* spp. on neutrophil apoptosis appears to vary depending on the origin of the neutrophils (blood, peritoneal, dermis). For instance, *L. major* was reported to delay neutrophil apoptosis in peritoneally induced mouse neutrophils [48] and human blood-derived neutrophils [58] but not in mouse dermal neutrophils [26]. Second, two-photon imaging of the ear dermis of *L. major*-infected mice revealed that parasites released from neutrophils may also infect macrophages [4]. *Leishmania* transmission from neutrophil to macrophage may also depend on the *Leishmania* species. We recently demonstrated that, unlike *L. major* [26], *L. mexicana* does not induce rapid apoptosis of dermal neutrophils *in vivo* [30]. Even if the parasite is inducing a delay in neutrophil apoptosis, one has to consider that eventually neutrophils will become apoptotic; thus, it is possible that parasites enter macrophages both following their release from neutrophils and via phagocytosis of apoptotic neutrophils. *Leishmania* parasites will then transform into the **amastigote** stage and will replicate within macrophages (Figure 1, step 4).

Neutrophil–*Leishmania* Interactions within Unhealing Cutaneous Lesions

The presence of neutrophils was reported in human inflammatory cutaneous lesions [59,60] and in unhealing lesions of experimental leishmaniasis by flow cytometry analysis and/or histology [47,61–63]. However, neutrophil involvement at this stage of the disease is not well defined (Figure 1, step 5). In mice, a second wave of neutrophils independent of the first early wave of neutrophils that is observed within the first days of infection is recruited to the site of *L. major* infection, correlating with the appearance of a lesion [26]. More recently, Crosby *et al.* showed a positive correlation between the presence of neutrophils and lesion size in chronic lesions of mice coinfected with *L. major* and lymphocytic choriomeningitis virus (LCMV). This increased pathology was mediated by CD8 T cells and the authors hypothesized that dermal neutrophils may be the target of CD8 T cell-mediated killing, contributing to inflammation and pathology [61]. Chronic lesions of mice infected with *L. major* Seidman (Sd), a parasite strain that results in the development of non-healing cutaneous lesions in C57BL/6 mice [64], were also shown to contain high numbers of neutrophils associated with increased lesion development and amastigote proliferation [63]. To investigate the role of neutrophils in the chronic phase of infection, the neutropenic *Genista* mice were needle inoculated intradermally (i.d.) with 1000 *L. major* Sd. Strikingly, these mice were fully able to cure their lesion and clear parasites from the infection site, unlike similarly infected control C57BL/6 mice. Furthermore, in this recent study the deleterious role of neutrophils observed in response to *L. major* Sd correlated with the activation of the nucleotide-binding and oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome [63]. These results demonstrate that, as observed following injection of a high dose of *L. mexicana* [30], neutrophils also contribute negatively to the pathology observed following infection with this particular strain of *L. major*.

L. amazonensis amastigotes were reported to be poor inducers of murine neutrophil activation and to resist neutrophil microbicidal activity *in vitro* [65]. By contrast, *L. braziliensis* amastigotes, which are better internalized by neutrophils than *L. amazonensis* amastigotes, are killed by neutrophils *in vitro* [66]. Although these data will need to be validated *in vivo*, they suggest that amastigotes from different *Leishmania* spp. may also have distinct impacts on neutrophil activation and microbicidal function.

Although NET formation is well documented in response to promastigote exposure in *vitro* and also *ex vivo* as described above, only a few studies have investigated NET formation in response to amastigotes within unhealing cutaneous lesions. Guimarães *et al.* were the first to identify elastase-positive fibrous extracellular material in the skin lesions of patients with CL, showing that NETs were present in chronic leishmaniasis lesions [50]. Furthermore, the authors showed that human neutrophils stimulated *in vitro* with *L. amazonensis* amastigotes released DNA in the extracellular milieu. NETs were also described in the lesions of patients with American tegumentary leishmaniasis (ATL), with a strong correlation observed between the presence of NETs and that of amastigotes, suggesting a role for amastigotes in NET formation [60]. In the latter study, the authors proposed that the presence of NETs impaired parasite dissemination, clearing extracellular amastigotes in a dynamic process. Further analysis would be required to characterize this process and its outcome on parasite survival. Nevertheless, these studies suggest that NETs not only play a role in the early stages of infection but may also be involved at later stages of lesion development. Additional studies are warranted to better characterize the interaction between neutrophils and the amastigote stage of the parasite.

Concluding Remarks

Neutrophils are rapidly and massively recruited following infection with *Leishmania* spp. These cells are increasingly emerging as important players in the control of the infection. Interestingly, distinct *Leishmania* spp. appear to differ regarding their impact on neutrophil function, with direct consequences on the disease. Many questions emerge, as outlined in the Outstanding Questions. The impact of sand fly-derived factors on neutrophil activity and NET formation during the first days of infection should be further investigated. What is the contribution of NET formation to the pathology and what are the mechanisms involved in the differences observed between distinct *Leishmania* spp.? It will also be important to investigate whether early transient neutrophil migration in draining lymph nodes is observed following infection with different *Leishmania* spp. and how and whether this impacts the development of a protective immune response. Furthermore, as neutrophils are present within unhealing cutaneous lesions it will be interesting to analyze potential differences in NET induction and microbicidal function following infection with different *Leishmania* spp. that cause unhealing infection. A better understanding of the role of neutrophils in visceral forms of the disease would also warrant further investigation.

More studies performed in experimental models and in humans are required to understand the mechanisms determining how neutrophils play either a protective or a deleterious role in leishmaniasis. This should contribute to the design of either preventive or curative strategies against the different forms of leishmaniases.

Outstanding Questions

Is neutrophil lifespan influenced by sand fly-derived or host tissue-specific factors?

How do sand fly and host factors influence NET formation/persistence early in infection?

Is there any role for NET formation during visceral leishmaniasis *in vivo*?

Neutrophils can transiently migrate to lymph node draining sites following infection with some *Leishmania* species. What is the neutrophil function in the infected draining lymph node?

Are amastigotes phagocytosed by neutrophils in cutaneous lesions?

If amastigotes are internalized within neutrophils, are they able to replicate within these cells? This would imply that neutrophil lifespan is increased within the inflammatory lesions.

Acknowledgments

The authors thank Slavica Masina for editing of the manuscript and the Swiss National Foundation (310030.146187/1 and IZLSZ3_149026/1 to F.T-C.) for grant support.

References

1. Alvar, J. *et al.* (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* 7, e35671
2. Sundar, S. *et al.* (2015) Strategies to overcome antileishmanial drugs unresponsiveness. *J. Trop. Med.* 2014, 646932
3. Beil, W.J. *et al.* (1992) Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *J. Leukoc. Biol.* 52, 135–142
4. Peters, N.C. *et al.* (2008) *In vivo* imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* 321, 970–974
5. Tacchini-Cottier, F. *et al.* (2000) An immunomodulatory function for neutrophils during the induction of a CD4$^+$ Th2 response in BALB/c mice infected with *Leishmania major*. *J. Immunol.* 165, 2628–2636
6. Mantovani, A. *et al.* (2011) Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* 11, 519–531
7. Mocsai, A. (2013) Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J. Exp. Med.* 210, 1283–1299
8. Brinkmann, V. *et al.* (2004) Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535
9. Amulic, B. *et al.* (2012) Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* 30, 459–489

10. Cassatella, M.A. (1999) Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* 73, 369–509
11. Yang, C.W. and Unanue, E.R. (2013) Neutrophils control the magnitude and spread of the immune response in a thromboxane A2-mediated process. *J. Exp. Med.* 210, 375–387
12. Scapini, P. and Cassatella, M.A. (2014) Social networking of human neutrophils within the immune system. *Blood* 124, 710–719
13. Schuster, S. *et al.* (2013) Crosstalk between neutrophils and dendritic cells: a context-dependent process. *J. Leukoc. Biol.* 94, 671–675
14. Jaeger, B.N. *et al.* (2012) Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *J. Exp. Med.* 209, 565–580
15. Puga, I. *et al.* (2012) B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat. Immunol.* 13, 170–180
16. Yang, C.W. *et al.* (2010) Neutrophils influence the level of antigen presentation during the immune response to protein antigens in adjuvants. *J. Immunol.* 185, 2927–2934
17. Abadie, V. *et al.* (2005) Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood* 106, 1843–1850
18. Mestas, J. and Hughes, C.C. (2004) Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172, 2731–2738
19. Doeing, D.C. *et al.* (2003) Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin. Pathol.* 3, 3
20. Kolaczkowska, E. and Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–1775
21. Nauseef, W.M. and Borregaard, N. (2014) Neutrophils at work. *Nat. Immunol.* 15, 602–611
22. Abebe, T. *et al.* (2012) Local increase of arginase activity in lesions of patients with cutaneous leishmaniasis in Ethiopia. *PLoS Negl. Trop. Dis.* 6, e1684
23. Charmoy, M. *et al.* (2007) *Leishmania major* induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. *J. Leukoc. Biol.* 82, 288–299
24. Ribeiro-Gomes, F.L. *et al.* (2007) Neutrophils activate macrophages for intracellular killing of *Leishmania major* through recruitment of TLR4 by neutrophil elastase. *J. Immunol.* 179, 3988–3994
25. Blackwell, J.M. (1996) Genetic susceptibility to leishmanial infections: studies in mice and man. *Parasitology* 112 (Suppl.), S67–S74
26. Ribeiro-Gomes, F.L. *et al.* (2012) Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-*Leishmania* response. *PLoS Pathog.* 8, e1002536
27. Xin, L. *et al.* (2010) Type I IFN receptor regulates neutrophil functions and innate immunity to *Leishmania* parasites. *J. Immunol.* 184, 7047–7056
28. Sousa, L.M. *et al.* (2014) Neutrophils have a protective role during early stages of *Leishmania amazonensis* infection in BALB/c mice. *Parasite Immunol.* 36, 13–31
29. Falcao, S.A. *et al.* (2015) Exposure to *Leishmania braziliensis* triggers neutrophil activation and apoptosis. *PLoS Negl. Trop. Dis.* 9, e0003601
30. Hurrell, B.P. *et al.* (2015) Rapid sequestration of *Leishmania mexicana* by neutrophils contributes to the development of chronic lesion. *PLoS Pathog.* 11, e1004929
31. Thalhofer, C.J. *et al.* (2011) Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan *Leishmania infantum chagasi*. *Infect. Immun.* 79, 108–117
32. McFarlane, E. *et al.* (2008) Neutrophils contribute to development of a protective immune response during onset of infection with *Leishmania donovani*. *Infect. Immun.* 76, 532–541
33. Faust, N. *et al.* (2000) Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood* 96, 719–726
34. Kimblin, N. *et al.* (2008) Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10125–10130
35. Teixeira, C.R. *et al.* (2005) Saliva from *Lutzomyia longipalpis* induces CC chemokine ligand 2/monocyte chemoattractant protein-1 expression and macrophage recruitment. *J. Immunol.* 175, 8346–8353
36. de Moura, T.R. *et al.* (2010) Immunity to *Lutzomyia intermedia* saliva modulates the inflammatory environment induced by *Leishmania braziliensis*. *PLoS Negl. Trop. Dis.* 4, e712
37. Rogers, M. *et al.* (2009) Proteophosophoglycans regurgitated by *Leishmania*-infected sand flies target the L-arginine metabolism of host macrophages to promote parasite survival. *PLoS Pathog.* 5, e1000555
38. Ribeiro-Gomes, F.L. *et al.* (2014) Site-dependent recruitment of inflammatory cells determines the effective dose of *Leishmania major*. *Infect. Immun.* 82, 2713–2727
39. Pillay, J. *et al.* (2010) *In vivo* labeling with $^{2}H_{2}O$ reveals a human neutrophil lifespan of 5.4 days. *Blood* 116, 625–627
40. Kim, M.H. *et al.* (2011) Neutrophil survival and c-kit^{+}-progenitor proliferation in *Staphylococcus aureus*-infected skin wounds promote resolution. *Blood* 117, 3343–3352
41. Stasulli, N.M. *et al.* (2015) Spatially distinct neutrophil responses within the inflammatory lesions of pneumonic plague. *MBio* 6, e01530–e1615
42. Summers, C. *et al.* (2010) Neutrophil kinetics in health and disease. *Trends Immunol.* 31, 318–324
43. Tak, T. *et al.* (2013) What's your age again? Determination of human neutrophil half-lives revisited. *J. Leukoc. Biol.* 94, 595–601
44. Tofts, P.S. *et al.* (2011) Doubts concerning the recently reported human neutrophil lifespan of 5.4 days. *Blood* 117, 6050–6052 author reply 6053–6054
45. Peters, N.C. *et al.* (2009) Vector transmission of *Leishmania* abrogates vaccine-induced protective immunity. *PLoS Pathog.* 5, e1000484
46. Ribeiro-Gomes, F.L. and Sacks, D. (2012) The influence of early neutrophil–*Leishmania* interactions on the host immune response to infection. *Front. Cell. Infect. Microbiol.* 2, 59
47. Carlsen, E.D. *et al.* (2015) Permissive and protective roles for neutrophils in leishmaniasis. *Clin. Exp. Immunol.* 182, 109–118
48. Charmoy, M. *et al.* (2010) The prominent role of neutrophils during the initial phase of infection by *Leishmania* parasites. *J. Biomed. Biotechnol.* 2010, 719361
49. Ordonez-Rueda, D. *et al.* (2012) A hypomorphic mutation in the Gfi1 transcriptional repressor results in a novel form of neutropenia. *Eur. J. Immunol.* 42, 2395–2408
50. Guimaraes-Costa, A.B. *et al.* (2009) *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6748–6753
51. Gabriel, C. *et al.* (2010) *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *J. Immunol.* 185, 4319–4327
52. Guimaraes-Costa, A.B. *et al.* (2014) 3′-Nucleotidase/nuclease activity allows *Leishmania* parasites to escape killing by neutrophil extracellular traps. *Infect. Immun.* 82, 1732–1740
53. Chagas, A.C. *et al.* (2014) Lundep, a sand fly salivary endonuclease increases *Leishmania* parasite survival in neutrophils and inhibits XIIa contact activation in human plasma. *PLoS Pathog.* 10, e1003923
54. Barrientos, L. *et al.* (2014) Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells. *J. Immunol.* 193, 5689–5698
55. van Zandbergen, G. *et al.* (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J. Immunol.* 173, 6521–6525
56. Laufs, H. *et al.* (2002) Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect. Immun.* 70, 826–835
57. Laskay, T. *et al.* (2008) Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology* 213, 183–191
58. Aga, E. *et al.* (2002) Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J. Immunol.* 169, 898–905

59. Abebe, T. *et al.* (2013) Arginase activity – a marker of disease status in patients with visceral leishmaniasis in Ethiopia. *PLoS Negl. Trop. Dis.* 7, e2134

60. Morgado, F.N. *et al.* (2015) Are neutrophil extracellular traps playing a role in the parasite control in active American tegumentary leishmaniasis lesions? *PLoS ONE* 10, e0133063

61. Crosby, E.J. *et al.* (2015) Lymphocytic choriomeningitis virus expands a population of NKG2D^{+}CD8^{+} T cells that exacerbates disease in mice coinfected with *Leishmania major*. *J. Immunol.* 195, 3301–3310

62. Novais, F.O. *et al.* (2009) Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. *J. Immunol.* 183, 8088–8098

63. Charmoy, M. *et al.* (2015) The Nlrp3 inflammasome, IL-1β, and neutrophil recruitment are required for susceptibility to a non-healing strain of *Leishmania major* in C57BL/6 mice. *Eur. J. Immunol.* Published online December 22, 2015. http://dx.doi.org/10.1002/eji.201546015

64. Anderson, C.F. *et al.* (2005) Nonhealing infection despite Th1 polarization produced by a strain of *Leishmania major* in C57BL/6 mice. *J. Immunol.* 174, 2934–2941

65. Carlsen, E.D. *et al.* (2013) *Leishmania amazonensis* amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms. *Infect. Immun.* 81, 3966–3974

66. Carlsen, E.D. *et al.* (2015) Interactions between neutrophils and *Leishmania braziliensis* amastigotes facilitate cell activation and parasite clearance. *J. Innate Immun.* 7, 354–363

67. Muller, K. *et al.* (2001) Chemokines, natural killer cells and granulocytes in the early course of *Leishmania major* infection in mice. *Med. Microbiol. Immunol.* 190, 73–76

68. van Zandbergen, G. *et al.* (2002) *Leishmania* promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. *Infect. Immun.* 70, 4177–4184

69. Jacobs, T. *et al.* (2005) Complement C3 is required for the progression of cutaneous lesions and neutrophil attraction in *Leishmania major* infection. *Med. Microbiol. Immunol.* 194, 143–149

70. Lopez Kostka, S. *et al.* (2009) IL-17 promotes progression of cutaneous leishmaniasis in susceptible mice. *J. Immunol.* 182, 3039–3046

71. Uyttenhove, C. *et al.* (2011) Amine-reactive OVA multimers for auto-vaccination against cytokines and other mediators: perspectives illustrated for GCP-2 in *L. major* infection. *J. Leukoc. Biol.* 89, 1001–1007

72. Rochael, N.C. *et al.* (2015) Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by *Leishmania* parasites. *Sci. Rep.* 5, 18302

4.3.1.2 Review 2: Survival Mechanisms Used by some *Leishmania* Species to Escape Neutrophil Killing

frontiers in Immunology

MINI REVIEW
published: 16 November 2017
doi: 10.3389/fimmu.2017.01558

Survival Mechanisms Used by Some *Leishmania* Species to Escape Neutrophil Killing

*Ivo B. Regli, Katiuska Passelli, Benjamin P. Hurrell and Fabienne Tacchini-Cottier**

Department of Biochemistry, WHO Immunology Research and Training Collaborative Center, University of Lausanne, Lausanne, Switzerland

OPEN ACCESS

Edited by:
Celio Geraldo Freire De Lima, Universidade Federal do Rio de Janeiro, Brazil

Reviewed by:
Hira Nakhasi, Center for Biologics Evaluation and Research (FDA), United States
Marise Pinheiro Nunes, Fundação Oswaldo Cruz (Fiocruz), Brazil

***Correspondence:**
Fabienne Tacchini-Cottier
fabienne.tacchini-cottier@unil.ch

Summary sentence: In this mini-review, we discuss the dual function of neutrophils that may efficiently kill or in contrast, serve as a safe transient shelter for Leishmania spp. allowing in some cases their replication.

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *10 October 2017*
Accepted: *31 October 2017*
Published: *16 November 2017*

Citation:
Regli IB, Passelli K, Hurrell BP and Tacchini-Cottier F (2017) Survival Mechanisms Used by Some Leishmania Species to Escape Neutrophil Killing. Front. Immunol. 8:1558. doi: 10.3389/fimmu.2017.01558

Neutrophils are the most abundant leukocytes in human blood. Upon microbial infection, they are massively and rapidly recruited from the circulation to sites of infection where they efficiently kill pathogens. To this end, neutrophils possess a variety of weapons that can be mobilized and become effective within hours following infection. However, several microbes including some *Leishmania* spp. have evolved a variety of mechanisms to escape neutrophil killing using these cells as a basis to better invade the host. In addition, neutrophils are also present in unhealing cutaneous lesions where their role remains to be defined. Here, we will review recent progress in the field and discuss the different strategies applied by some *Leishmania* parasites to escape from being killed by neutrophils and as recently described for *Leishmania mexicana*, even replicate within these cells. Subversion of neutrophil killing functions by *Leishmania* is a strategy that allows parasite spreading in the host with a consequent deleterious impact, transforming the primary protective role of neutrophils into a deleterious one.

Keywords: *Leishmania*, neutrophils, *Leishmania* survival, neutrophil extracellular traps, reactive oxygen species, neutrophil granules, *Leishmania* replication

NEUTROPHILS AND *Leishmania*: A MULTIFACETED STORY

Neglected parasitic diseases are affecting more than one million people worldwide. Amongst them, leishmaniases are a complex of diseases that affects 2 million people per year across 98 countries. The *Leishmania* protozoan parasites are transmitted by blood-sucking sand flies that deposit the parasites in the mammalian skin during their blood meal. There are more than 20 different *Leishmania* species worldwide. The infecting species together with host factors determine the various clinical manifestations leishmaniasis can have as well as the outcome of the disease. Cutaneous leishmaniasis is the most predominant form of the diseases. Following infection, an ulcerative lesion usually appears near the insect bite site. In mucocutaneous leishmaniasis, the disease affects the mucocuatenous tissues of the oro-naso-pharyngeal areas and often leads to local tissue destruction and death due to secondary infections if left untreated. Visceral leishmaniasis is characterized by hepatosplenomegaly and impeded bone marrow function due to the proliferation of parasites in macrophages within these organs. If not treated, visceral leishmaniasis patients develop cachexia, pancytopenia, subsequent immunosuppression and they eventually succumb to their disease (1). There are several treatments available against leishmaniasis of which pentavalent antimonials have been the standard of care for decades. However, these drugs have many adverse effects and the emergence of drug-resistant parasites is increasing worldwide. As the increase in drug resistance renders the available therapeutics less efficient, the need of efficient vaccines and a better understanding of the diseases is crucial to fight leishmaniases (2, 3).

Neutrophils are massively and rapidly recruited to sites of injury and microbial infections. They are the most abundant leukocytes in human blood. Neutrophils play very important roles in innate immunity and in the regulation of adaptive immune response (4, 5). They are well known for their antimicrobial functions, playing a decisive role in innate host defense against a variety of pathogens, including bacteria and fungi. To kill microbes, neutrophils possess an arsenal of weapons that include phagocytosis and subsequent microbe degradation within phagolysosomes, where granules fuse to rapidly release their microbicidal agents. Neutrophils can degranulate their granule content also in the local microenvironment and they can also kill pathogens through the production of reactive oxygen species (ROS). In addition, neutrophils can extrude neutrophil extracellular traps (NETs) that consist of a DNA backbone associated with microbicidal proteins. NETs allow entrapping of the pathogens, preventing their spread, and in some cases killing them (6). Cytokines and chemokines released by neutrophils are involved in the activation and/or recruitment of other innate cells thereby contributing to the shaping and development of an adaptive immune response (7, 8). The relevance of the role played by neutrophils in the fight against many infections is underlined by the susceptibility to repeated life-threatening bacterial and fungal infections observed in patients suffering from genetically inherited or acquired neutropenia or who have neutrophils with functional defects (9). The important role of neutrophils in regulating defense against parasites and some viruses has more recently emerged (10, 11) and increasing evidence points out to a crucial role for neutrophils in leishmaniasis disease outcome (10–13).

In contrast to their well-described protective roles in many infections, neutrophils may play a detrimental role in leishmaniasis disease development, at least in some instances. In addition to their early recruitment following infection, neutrophils were reported to infiltrate damaged tissues of human mucosal leishmaniasis (14) and to be present in the chronic form of the disease in human and animals (14–20). Following experimental infection with most *Leishmania* spp. neutrophils are rapidly and massively recruited to the site of parasite inoculation where they rapidly phagocytose most of the parasites present. Several groups have used genetically neutropenic mice or mice rendered neutropenic by injection of anti-neutrophil antibodies to show the importance of this early wave of neutrophil on disease outcome. Collectively, most of these studies reported that neutropenic mice had a better disease outcome, indicating a negative role for neutrophils in some forms of cutaneous leishmaniasis (2, 21–24). In contrast, neutrophils may facilitate parasite clearance as observed for *Leishmania braziliensis* and *Leishmania amazonensis* (25–30) and for *Leishmania donovani* (31). However, *L. amazonensis* killing appeared to be parasite stage-dependent as promastigotes, the infecting form of the parasites, but not amastigotes, the intracellular replicative forms of the parasite, were killed *in vitro* by neutrophils (32).

One of the immune evasion strategies used by *Leishmania* parasites may be linked to the status of neutrophil apoptosis as phagocytosis of apoptotic neutrophils has been shown to impair dendritic cells (DCs) maturation and the development of an efficient adaptive immune response [reviewed in Ref. (7)]. Indeed, internalization of apoptotic *Leishmania major*-infected neutrophils by DCs impaired development of *Leishmania*-specific immune response (33, 34). Interaction of apoptotic neutrophils with macrophages also has a negative impact on the disease (35). Following *Leishmania* delivery by sand fly bite or needle inoculation, parasites were reported to induce, delay or have no impact on neutrophil apoptosis, depending on the *Leishmania* spp. or the origin of neutrophils. *Leishmania mexicana* did not influence dermal neutrophil survival *ex vivo* (36) and *L. infantum* did not induce neutrophil apoptosis *in vitro* (37). In contrast, *L. brasiliensis* induced neutrophil apoptosis, at least *in vitro* (30). *L. major* infection induced murine neutrophil apoptosis in the dermis (22, 34) while it delayed human blood-derived neutrophil apoptosis (22, 34, 38, 39). These results suggest that the effect of *Leishmania* on neutrophil apoptosis may differ between murine and human neutrophils, or the difference observed may come from the diverse *Leishmania* spp. or neutrophil origins.

Recent data reported that a subset of low density neutrophils expressing HLA-DR express high levels of PDL1 in human CL and VL patients (19, 40), a marker promoting T cell exhaustion. These data suggest a novel negative role for this neutrophil subset in leishmaniasis.

THE DISTINCT MECHANISMS USED BY *Leishmania* spp. TO ESCAPE KILLING BY NEUTROPHILS

Leishmania are using neutrophils transiently to finally be ingested by macrophages, their final host. The parasites may be released by dying neutrophils and/or infected apoptotic neutrophils may be phagocytosed by macrophages. This latter process referred to as the "Trojan horse" entry in macrophages, confers a silent entry for the parasites in these cells (41). We will now discuss the several mechanisms used by some *Leishmania* spp. to escape neutrophil killing and even in some cases how the parasites can use these cells to replicate, collectively resulting in a negative impact on disease outcome.

Using *in vivo* two-photon imaging, intact and live parasites have been detected in neutrophils during the first days of *L. major* and *L. mexicana* infections, revealing that a good proportion of parasites can resist neutrophil microbicidal functions (22, 36). Several strategies used by *Leishmania* parasites to escape killing by neutrophils have been described. During neutrophil development there is a continuity of granule formation, including azurophil granules (primary or peroxidase-positive granules), specific (secondary granules), and gelatinase granules (tertiary granules). Secretory granules are formed last (42). During the maturation of myeloblasts into neutrophils, more than 300 different proteins are stored into granules. One of the ways parasites may survive in neutrophils is through interference in the process of granule fusion with the *Leishmania* containing phagosome. *In vitro* studies showed that *L. major* and *L. donovani* promastigotes regulate granule fusion with phagosomes, allowing azurophil but preventing specific and

gelatinase granule fusion with parasite-containing phagosomes (43). This prevents their destruction by neutrophils microbicidal granule contents (**Figure 1A**). In addition, *L. donovani* was shown to traffic to non-lytic compartments within neutrophils (44), establishing yet another strategy to escape the neutrophil killing machinery (**Figure 1B**).

In addition to the release of antimicrobial molecules, the assembly of a functional NADPH oxidase (NOX2) is playing a crucial role for neutrophil microbicidal function (45). NOX2 assembly is inducing the generation of reactive oxygen species (ROS), a process called oxidative burst. Interference with oxidative burst increases pathogen survival within neutrophils. It has been shown that *L. major* does not elicit the generation of ROS upon phagocytosis by human neutrophils (**Figure 1C**) (43). However, *L. braziliensis* induce high levels of ROS production upon infection of human and murine neutrophils but ROS generation in human neutrophils did not affect parasite survival (31, 45). In addition to its major role in neutrophil intracellular killing functions, NOX2-mediated generation of ROS has also been reported to be crucial for classical (NADPH-dependent) NET formation. This is exemplified by the lack of NET formation in patients with chronic granulomatous disease (46, 47) and restoration of NET formation in these patients upon reintroduction of NOX2 by genetic engineering (48). Moreover, there also exists ROS-independent NET release. *L. amazonensis* promastigotes were shown to elicit both types of NETs and be killed by them (29). Thus, the impact of parasites on ROS formation is also *Leishmania* spp. dependent.

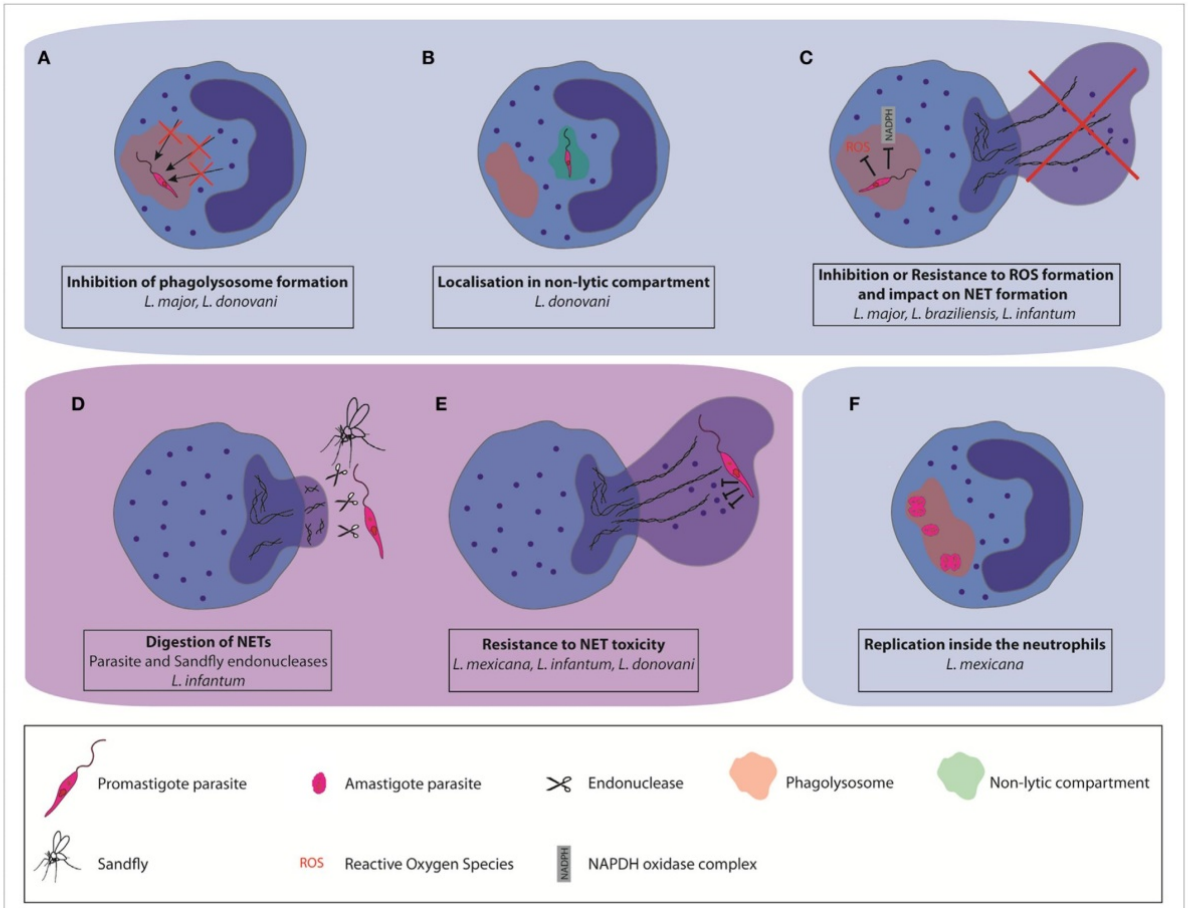

FIGURE 1 | Different mechanisms used by some *Leishmania* spp. to escape neutrophil killing. *Leishmania* can impair parasite destruction by neutrophils **(A)** by affecting the formation of mature phagolysosomes and their fusion with neutrophil granules, **(B)** by localization in non-lytic compartments, and **(C)** by resisting to the toxicity associated with reactive oxygen species production. Some *Leishmania* spp. can also resist to the microbicity associated with neutrophil extracellular trap (NET) formation **(D)** by directly inhibiting NET formation, or by digestion of the NET scaffold using pathogen-or vector-derived endonucleases **(E).** They can also resist NET antimicrobial factors through the expression of protease-resistant surface molecules. **(F)** A subset of *L. mexicana* amastigotes was shown to replicate in neutrophils.

PATHOGENS ESCAPE FROM NETs

Upon activation, neutrophils can form NETs that can entrap and often kill pathogens, reviewed in Ref. (49). However, several microbes including some *Leishmania* spp. have developed various mechanisms to escape NET trapping and/or killing. Whether parasites are killed or not by NETs depends on the involved *Leishmania* spp. For instance, in humans, *L. amazonensis* was shown to induce NET formation and to be killed by them (50). In contrast, NETs failed to kill (36) *L. infantum* (51) *and L. donovani* (52) parasites. Furthermore, murine NETs were not able to kill *L. mexicana* (36).

A very efficient strategy used by *Leishmania infantum* (51) is to prevent NET formation by suppressing or inducing decreased efficiency of the oxidative burst (**Figure 1C**).

As another strategy to avoid NET killing, several microbes express nucleases that degrade the NET DNA backbone. For example, surface DNAse and wall anchored nuclease expression were reported in Gram-positive bacteria (53–56) and for several Gram-negative bacteria (53, 57–59). NET degrading endonucleases have also been reported in Gram-negative bacteria (60–62). Expression of the enzyme 3′nucleotidase/nuclease by *Leishmania* also contributes to protection from the microbicidal activity of NETs as shown for *L. infantum* (51). In addition, the parasite sand fly vector may interfere with NET formation. The saliva of the New World *Leishmania* vector, *Lutzomyia longipalpis,* was shown to contain an endonuclease capable of degrading NETs (63). As salivary gland proteins are deposited by the sandfly in the host during the insect blood meal its endonucleases may indirectly influence the role of NETs in the disease pathogenesis (**Figure 1D**).

Microbes may also avoid NET killing through the synthesis of cell surface components rendering them resistant to NET-associated protease activity (**Figure 1E**). This has been observed for *L. amazonensis* and *L. donovani. Leishmania* surface coat is densely packed with lipophosphoglycan (LPG), a glycoconjugate that is polymorphic among *Leishmania* spp. and which is differentially expressed in the infective promastigote form compared to the replicative amastigote form (64). In *L. amazonensis*, LPG was shown to induce NET formation, and confer resistance to NET-mediated killing by forming a thick glycocalyx that protects the parasite from microbicidal agents (50). In contrast, LPG of *L. donovani,* was shown not to induce NET formation, although it also conferred protection against NET mediated parasite killing (52). Peripheral blood neutrophils from active VL patients were unable to release NETs despite an active phenotype (65), showing that the replicating amastigote stage of the parasites also has an impact on neutrophil functions, contributing to the pathology of the disease.

NEUTROPHILS AS A PLACE TO REPLICATE

Neutrophils are short-lived non-dividing cells that become rapidly apoptotic in the circulation. However, during inflammation and infection, the neutrophil lifespan can be extended to several days (66), although it still remains difficult to estimate neutrophil lifespan in tissues, mostly due to technical issues. For some *Leishmania* spp. transient inhibition or delay of neutrophil apoptosis is an obvious strategy to allow prolongation of their presence within these cells. The PI3K/AKT, ERK1/2 p28MAPK pathways which maintain expression of the antiapoptotic Mcl1 protein were shown to contribute to prolonged neutrophil lifespan in *L. major* infection (67).

The induction of delayed neutrophil apoptosis together with the inhibition of neutrophil killing machinery elicited by some *Leishmania* spp. suggested that the parasite could use these cells to replicate. *Leishmania* parasites have two life cycle stages, the infective flagellated promastigote form which is elongated with a size comprised between 6 and 12 μm, not including the flagellum length, and the replicative, non-flagellated amastigote form, which is intracellular and of smaller size (3–5 μm). The sand fly is depositing in the skin metacyclic promastigotes, a process inducing rapid recruitment of neutrophils. It is therefore not surprising that most studies investigating interactions between neutrophils and *Leishmania* have been performed with the promastigote form of the parasite, reviewed in Ref. (12, 13, 24). In addition, neutrophils have been detected in smears of unhealing cutaneous lesions of *L. braziliensis* patients, at a time when the parasite is in its intracellular amastigote form. The presence of neutrophil-attracting chemokine mRNA was observed in biopsies of patients with chronic lesions due to *L. panamensis* and *L. braziliensis,* suggesting neutrophil presence in the lesion. Also, neutrophils were observed in biopsies of tegumentary leishmaniasis patients (14, 68–71). Furthermore, neutrophil presence was also observed in unhealing lesions of experimental cutaneous leishmaniasis following *L. major* (18, 21) and *L. mexicana* infection (36). Very few studies have investigated the interactions between neutrophils and the amastigote form of the parasite. The group of Soong was the first to show that neutrophils internalized *in vitro L. amazonensis* and *L. braziliensis* amastigotes. While *L. amazonensis* amastigotes survived in neutrophils, *L. braziliensis* amastigotes were efficiently killed (28, 32). We recently reported that *L. mexicana* amastigotes are also internalized and survive in neutrophils *in vitro.* After overnight incubation, we observed an average of one amastigote per neutrophils. In contrast, the majority of lesion-derived neutrophils harbored >2 intact amastigotes per neutrophil. Imaging of the lesion-derived neutrophils showed the presence of several aligned amastigotes within neutrophils, suggesting possible parasite replication in these cells. Parasite uptake by neutrophils was relatively neutral, eliciting low level of apoptosis or neutrophil activation in infected neutrophils (20). To measure parasite replication, we generated transgenic parasites expressing a photoconvertible GFP mKikume gene (72). These *L. mex*$^{\text{SWITCH}}$ parasites express constitutively green fluorescence that can be converted to red fluorescence upon exposure to a pulse of violet light. Upon cell division, the photoconverted red proteins are diluted as *de novo* green protein in synthesized, and the fluorescence recovery after conversion (FRAC) is measured in dividing cells. Analysis of FRAC by imaging flow cytometry and time-lapse microscopy revealed that, 48 h after photoconversion, a subset of highly infected neutrophils containing more than 4 amastigotes per cell

showed high replication (**Figure 1F**). Amastigotes were found in large vesicular acidic compartment. In macrophages, *Leishmania* amastigotes reside in phagolysosome-like compartments called parasitophorous vacuoles (PVs) where they multiply. For most *Leishmania* spp. one amastigote is enclosed within these PVs with little vacuole space. However, *L. mexicana* and *L. amazonensis* form upon division communal large PVs containing numerous amastigotes, a process diluting toxic components and directly linked to parasite evasion to host immune responses (73, 74). We observed larger Lysosensor-positive vacuoles in *L. mexicana* infected neutrophils (20), suggesting the formation of communal PVs in neutrophils. It remains to be determined whether the replication of amastigotes in neutrophils is linked to the presence of these large PVs.

The majority of parasite replication is taking place in macrophages, and most lesional parasites divide at a slow rate even if, as observed *in vitro*, there is likely variability in the growth rates of parasites in unhealing cutaneous lesions (75). Indeed, in a recent study a small subset of parasites that appeared to divide rapidly was reported. These parasites could use neutrophils as a safe transient place to replicate.

The demonstration that a subset of *L. mexicana* parasites is able to replicate within neutrophils revealed a novel role of neutrophils that can act as a niche for parasite replication during the chronic phase of infection. However, there very likely exist differences in the ability of the invading *Leishmania* spp. to replicate in neutrophils. These could originate from parasite factors but also from host factors.

CONCLUDING REMARKS

The primary function of neutrophils in innate immunity resides in killing invading microorganisms. It is therefore not surprising that some pathogens have evolved several ways to escape elimination by these cells, allowing their silent entry in the host and even sometimes their replication in these cells. Caution in the interpretation of some of these studies should be taken as most human studies are performed with peripheral blood neutrophils that functionally differ from extravasated neutrophils present in inflamed tissues. To better understand the relevance of neutrophil functions *in vivo*, experimental murine models are used. However, it should be kept in mind that functional differences exist between mouse and human neutrophils as well, including differences in the antimicrobial repertoire and number of circulating neutrophils (76). That being said, the generation of new tools such as two-photon microscopy imaging (77) and the use of photo-switchable pathogens (78) for probing pathogen biology during infections should allow finer investigation of the mechanisms used by pathogens to promote their own survival in neutrophils *in vivo*. Furthermore, neutrophils appear to be a more heterogeneous cell population than previously anticipated (79) and new markers defining mature from immature circulating neutrophils are emerging (80). It will thus be interesting to assess whether selective *Leishmania* spp. transient survival and/or replication occur in a specific neutrophil subset, while *Leishmania* killing would take place in other subsets.

Survival of pathogens in neutrophils is not specific to *Leishmania*, indeed several bacteria, fungi or viruses are also able to escape neutrophil killing and use these cells to propagate in the host, reviewed in Ref. (81). For instance, intracellular bacteria including *Francisella tularensis* (82), *Neisseira gonorrhoae* (83), *Chlamydia pneumonia* (84); and more recently, *Yersina* spp. (85) have been shown to replicate *in vitro* in human or murine neutrophils, suggesting that not only *Leishmania* parasites but also other pathogens are diverting the primary neutrophil killing function to their own benefit and dissemination in the invaded host. Finer understanding of the mechanisms used by some *Leishmania* spp. to block neutrophil effector functions will be important in the design of prophylactic or therapeutic measures taken against leishmaniasis.

AUTHOR CONTRIBUTIONS

IR and FTC wrote the review. BH, KP, and IR contributed to the figures. All authors provided input to the review.

FUNDING

We acknowledge the financial support of the Swiss National Foundation for Scientific Research (310030_166651/1 to FC).

REFERENCES

1. Herwaldt BL. Leishmaniasis. *Lancet* (1999) 354(9185):1191–9. doi:10.1016/S0140-6736(98)10178-2
2. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* (2011) 9(8):604–15. doi:10.1038/nrmicro2608
3. McGwire BS, Satoskar AR. Leishmaniasis: clinical syndromes and treatment. *QJM* (2014) 107(1):7–14. doi:10.1093/qjmed/hct116
4. Mocsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J Exp Med* (2013) 210(7):1283–99. doi:10.1084/jem.20122220
5. Nauseef WM, Borregaard N. Neutrophils at work. *Nat Immunol* (2014) 15(7):602–11. doi:10.1038/ni.2921
6. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* (2004) 303(5663):1532–5. doi:10.1126/science.1092385
7. Schuster S, Hurrell B, Tacchini-Cottier F. Crosstalk between neutrophils and dendritic cells: a context-dependent process. *J Leukoc Biol* (2013) 94(4):671–5. doi:10.1189/jlb.1012540
8. Scapini P, Cassatella MA. Social networking of human neutrophils within the immune system. *Blood* (2014) 124(5):710–9. doi:10.1182/blood-2014-03-453217
9. Klein C. Genetic defects in severe congenital neutropenia: emerging insights into life and death of human neutrophil granulocytes. *Annu Rev Immunol* (2011) 29:399–413. doi:10.1146/annurev-immunol-030409-101259
10. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* (2013) 13(3):159–75. doi:10.1038/nri3399
11. Drescher B, Bai F. Neutrophil in viral infections, friend or foe? *Virus Res* (2013) 171(1):1–7. doi:10.1016/j.virusres.2012.11.002
12. Carlsen ED, Liang Y, Shelite TR, Walker DH, Melby PC, Soong L. Permissive and protective roles for neutrophils in leishmaniasis. *Clin Exp Immunol* (2015) 182(2):109–18. doi:10.1111/cei.12674
13. Hurrell BP, Regli IB, Tacchini-Cottier F. Different *Leishmania* species drive distinct neutrophil functions. *Trends Parasitol* (2016) 32(5):392–401. doi:10.1016/j.pt.2016.02.003
14. Boaventura VS, Santos CS, Cardoso CR, de Andrade J, Dos Santos WL, Clarencio J, et al. Human mucosal leishmaniasis: neutrophils infiltrate areas

of tissue damage that express high levels of Th17-related cytokines. *Eur J Immunol* (2010) 40(10):2830–6. doi:10.1002/eji.200940115
15. Morgado FN, Schubach A, Rosalino CM, Quintella LP, Santos G, Salgueiro M, et al. Is the in situ inflammatory reaction an important tool to understand the cellular immune response in American tegumentary leishmaniasis? *Br J Dermatol* (2008) 158(1):50–8. doi:10.1111/j.1365-2133.2007.08255.x
16. Vercosa BL, Melo MN, Puerto HL, Mendonca IL, Vasconcelos AC. Apoptosis, inflammatory response and parasite load in skin of *Leishmania* (*Leishmania) chagasi* naturally infected dogs: a histomorphometric analysis. *Vet Parasitol* (2012) 189(2–4):162–70. doi:10.1016/j.vetpar.2012.04.035
17. Dantas ML, Oliveira JM, Carvalho L, Passos ST, Queiroz A, Guimaraes LH, et al. Comparative analysis of the tissue inflammatory response in human cutaneous and disseminated leishmaniasis. *Mem Inst Oswaldo Cruz* (2014) 109(2):202–9. doi:10.1590/0074-0276130312
18. Charmoy M, Hurrell BP, Romano A, Lee SH, Ribeiro-Gomes F, Riteau N, et al. The Nlrp3 inflammasome, IL-1beta, and neutrophil recruitment are required for susceptibility to a nonhealing strain of *Leishmania major* in C57BL/6 mice. *Eur J Immunol* (2016) 46(4):897–911. doi:10.1002/eji.201546015
19. Davis RE, Sharma S, Conceicao J, Carneiro P, Novais F, Scott P, et al. Phenotypic and functional characteristics of HLA-DR+ neutrophils in Brazilians with cutaneous leishmaniasis. *J Leukoc Biol* (2017) 101(3):739–49. doi:10.1189/jlb.4A0915-442RR
20. Hurrell BP, Beaumann M, Heyde S, Regli IB, Muller AJ, Tacchini-Cottier F. Frontline science: *Leishmania mexicana* amastigotes can replicate within neutrophils. *J Leukoc Biol* (2017). doi:10.1189/jlb.4HI0417-158R
21. Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, et al. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J Immunol* (2000) 165(5):2628–36. doi:10.4049/jimmunol.165.5.2628
22. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* (2008) 321(5891):970–4. doi:10.1126/science.1159194
23. Charmoy M, Auderset F, Allenbach C, Tacchini-Cottier F. The prominent role of neutrophils during the initial phase of infection by *Leishmania parasites*. *J Biomed Biotechnol* (2010) 2010:719361. doi:10.1155/2010/719361
24. Ribeiro-Gomes FL, Sacks D. The influence of early neutrophil-*Leishmania* interactions on the host immune response to infection. *Front Cell Infect Microbiol* (2012) 2:59. doi:10.3389/fcimb.2012.00059
25. Souza-Lemos C, de-Campos SN, Teva A, Corte-Real S, Fonseca EC, Porrozzi R, et al. Dynamics of immune granuloma formation in a *Leishmania braziliensis*-induced self-limiting cutaneous infection in the primate *Macaca mulatta*. *J Pathol* (2008) 216(3):375–86. doi:10.1002/path.2403
26. Novais FO, Santiago RC, Bafica A, Khouri R, Afonso L, Borges VM, et al. Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. *J Immunol* (2009) 183(12):8088–98. doi:10.4049/jimmunol.0803720
27. Tavares NM, Araujo-Santos T, Afonso L, Nogueira PM, Lopes UG, Soares RP, et al. Understanding the mechanisms controlling *Leishmania amazonensis* infection in vitro: the role of LTB4 derived from human neutrophils. *J Infect Dis* (2014) 210(4):656–66. doi:10.1093/infdis/jiu158
28. Carlsen ED, Jie Z, Liang Y, Henard CA, Hay C, Sun J, et al. Interactions between neutrophils and *Leishmania braziliensis* amastigotes facilitate cell activation and parasite clearance. *J Innate Immun* (2015) 7(4):354–63. doi:10.1159/000373923
29. Rochael NC, Guimaraes-Costa AB, Nascimento MT, DeSouza-Vieira TS, Oliveira MP, Garcia e Souza LF, et al. Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by *Leishmania parasites*. *Sci Rep* (2015) 5:18302. doi:10.1038/srep18302
30. Falcao SA, Weinkopff T, Hurrell BP, Celes FS, Curvelo RP, Prates DB, et al. Exposure to *Leishmania braziliensis* triggers neutrophil activation and apoptosis. *PLoS Negl Trop Dis* (2015) 9(3):e0003601. doi:10.1371/journal.pntd.0003601
31. McFarlane E, Perez C, Charmoy M, Allenbach C, Carter KC, Alexander J, et al. Neutrophils contribute to development of a protective immune response during onset of infection with *Leishmania donovani*. *Infect Immun* (2008) 76(2):532–41. doi:10.1128/IAI.01388-07
32. Carlsen ED, Hay C, Henard CA, Popov V, Garg NJ, Soong L. *Leishmania amazonensis* amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms. *Infect Immun* (2013) 81(11):3966–74. doi:10.1128/IAI.00770-13
33. Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL. Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-*Leishmania* response. *PLoS Pathog* (2012) 8(2):e1002536. doi:10.1371/journal.ppat.1002536
34. Ribeiro-Gomes FL, Romano A, Lee S, Roffe E, Peters NC, Debrabant A, et al. Apoptotic cell clearance of *Leishmania major*-infected neutrophils by dendritic cells inhibits CD8(+) T-cell priming in vitro by Mer tyrosine kinase-dependent signaling. *Cell Death Dis* (2015) 6:e2018. doi:10.1038/cddis.2015.351
35. Afonso L, Borges VM, Cruz H, Ribeiro-Gomes FL, DosReis GA, Dutra AN, et al. Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*. *J Leukoc Biol* (2008) 84(2):389–96. doi:10.1189/jlb.0108018
36. Hurrell BP, Schuster S, Grun E, Coutaz M, Williams RA, Held W, et al. Rapid sequestration of *Leishmania mexicana* by neutrophils contributes to the development of chronic lesion. *PLoS Pathog* (2015) 11(5):e1004929. doi:10.1371/journal.ppat.1004929
37. Marques CS, Passero LF, Vale-Gato I, Rodrigues A, Rodrigues OR, Martins C, et al. New insights into neutrophil and *Leishmania infantum* in vitro immune interactions. *Comp Immunol Microbiol Infect Dis* (2015) 40:19–29. doi:10.1016/j.cimid.2015.03.003
38. Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Muller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* (2002) 169(2):898–905. doi:10.4049/jimmunol.169.2.898
39. Sarkar A, Aga E, Bussmeyer U, Bhattacharyya A, Moller S, Hellberg L, et al. Infection of neutrophil granulocytes with *Leishmania major* activates ERK 1/2 and modulates multiple apoptotic pathways to inhibit apoptosis. *Med Microbiol Immunol* (2013) 202(1):25–35. doi:10.1007/s00430-012-0246-1
40. Sharma S, Davis RE, Srivastva S, Nylen S, Sundar S, Wilson ME. A subset of neutrophils expressing markers of antigen-presenting cells in human visceral leishmaniasis. *J Infect Dis* (2016) 214(10):1531–8. doi:10.1093/infdis/jiw394
41. van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, et al. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J Immunol* (2004) 173(11):6521–5. doi:10.4049/jimmunol.173.11.6521
42. Borregaard N, Sorensen OE, Theilgaard-Monch K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* (2007) 28(8):340–5. doi:10.1016/j.it.2007.06.002
43. Mollinedo F, Janssen H, de la Iglesia-Vicente J, Villa-Pulgarin JA, Calafat J. Selective fusion of azurophilic granules with *Leishmania*-containing phagosomes in human neutrophils. *J Biol Chem* (2010) 285(45):34528–36. doi:10.1074/jbc.M110.125302
44. Gueirard P, Laplante A, Rondeau C, Milon G, Desjardins M. Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cell Microbiol* (2008) 10(1):100–11. doi:10.1111/j.1462-5822.2007.01018.x
45. Nauseef WM. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* (2008) 283(25):16961–5. doi:10.1074/jbc.R700045200
46. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* (2007) 176(2):231–41. doi:10.1083/jcb.200606027
47. von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, et al. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* (2008) 111(6):3070–80. doi:10.1182/blood-2007-07-104018
48. Bianchi M, Hakkim A, Brinkmann V, Siler U, Seger RA, Zychlinsky A, et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* (2009) 114(13):2619–22. doi:10.1182/blood-2009-05-221606

49. Sorensen OE, Borregaard N. Neutrophil extracellular traps – the dark side of neutrophils. *J Clin Invest* (2016) 126(5):1612–20. doi:10.1172/jci84538
50. Guimaraes-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao-Silva F, et al. *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* (2009) 106(16):6748–53. doi:10.1073/pnas.0900226106
51. Guimaraes-Costa AB, DeSouza-Vieira TS, Paletta-Silva R, Freitas-Mesquita AL, Meyer-Fernandes JR, Saraiva EM. 3'-Nucleotidase/nuclease activity allows *Leishmania* parasites to escape killing by neutrophil extracellular traps. *Infect Immun* (2014) 82(4):1732–40. doi:10.1128/iai.01232-13
52. Gabriel C, McMaster WR, Girard D, Descoteaux A. *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *J Immunol* (2010) 185(7):4319–27. doi:10.4049/jimmunol.1000893
53. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, et al. DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* (2006) 16(4):396–400. doi:10.1016/j.cub.2005.12.039
54. Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, Henriques-Normark B. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol* (2006) 16(4):401–7. doi:10.1016/j.cub.2006.01.056
55. Morita C, Sumioka R, Nakata M, Okahashi N, Wada S, Yamashiro T, et al. Cell wall-anchored nuclease of *Streptococcus sanguinis* contributes to escape from neutrophil extracellular trap-mediated bacteriocidal activity. *PLoS One* (2014) 9(8):e103125. doi:10.1371/journal.pone.0103125
56. Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Kockritz-Blickwede M. Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. *J Innate Immun* (2010) 2(6):576–86. doi:10.1159/000319909
57. Sumby P, Barbian KD, Gardner DJ, Whitney AR, Welty DM, Long RD, et al. Extracellular deoxyribonuclease made by group A *Streptococcus* assists pathogenesis by enhancing evasion of the innate immune response. *Proc Natl Acad Sci U S A* (2005) 102(5):1679–84. doi:10.1073/pnas.0406641102
58. Derre-Bobillot A, Cortes-Perez NG, Yamamoto Y, Kharrat P, Couve E, Da Cunha V, et al. Nuclease A (Gbs0661), an extracellular nuclease of *Streptococcus agalactiae*, attacks the neutrophil extracellular traps and is needed for full virulence. *Mol Microbiol* (2013) 89(3):518–31. doi:10.1111/mmi.12295
59. de Buhr N, Neumann A, Jerjomiceva N, von Kockritz-Blickwede M, Baums CG. *Streptococcus suis* DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity. *Microbiology* (2014) 160(Pt 2):385–95. doi:10.1099/mic.0.072199-0
60. Seper A, Hosseinzadeh A, Gorkiewicz G, Lichtenegger S, Roier S, Leitner DR, et al. *Vibrio cholerae* evades neutrophil extracellular traps by the activity of two extracellular nucleases. *PLoS Pathog* (2013) 9(9):e1003614. doi:10.1371/journal.ppat.1003614
61. Mollerherm H, Neumann A, Schilcher K, Blodkamp S, Zeitouni NE, Dersch P, et al. *Yersinia enterocolitica*-mediated degradation of neutrophil extracellular traps (NETs). *FEMS Microbiol Lett* (2015) 362(23):fnv192. doi:10.1093/femsle/fnv192
62. Juneau RA, Stevens JS, Apicella MA, Criss AK. A thermonuclease of *Neisseria gonorrhoeae* enhances bacterial escape from killing by neutrophil extracellular traps. *J Infect Dis* (2015) 212(2):316–24. doi:10.1093/infdis/jiv031
63. Chagas AC, Oliveira F, Debrabant A, Valenzuela JG, Ribeiro JM, Calvo E. Lundep, a sand fly salivary endonuclease increases *Leishmania* parasite survival in neutrophils and inhibits XIIa contact activation in human plasma. *PLoS Pathog* (2014) 10(2):e1003923. doi:10.1371/journal.ppat.1003923
64. Franco LH, Beverley SM, Zamboni DS. Innate immune activation and subversion of mammalian functions by *Leishmania* lipophosphoglycan. *J Parasitol Res* (2012) 2012:165126. doi:10.1155/2012/165126
65. Yizengaw E, Getahun M, Tajebe F, Cruz Cervera E, Adem E, Mesfin G, et al. Visceral leishmaniasis patients display altered composition and maturity of neutrophils as well as impaired neutrophil effector functions. *Front Immunol* (2016) 7:517. doi:10.3389/fimmu.2016.00517
66. Pillay J, den Braber I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JA, et al. In vivo labeling with $2H_2O$ reveals a human neutrophil lifespan of 5.4 days. *Blood* (2010) 116(4):625–7. doi:10.1182/blood-2010-01-259028
67. Ruhland A, Leal N, Kima PE. *Leishmania* promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. *Cell Microbiol* (2007) 9(1):84–96. doi:10.1111/j.1462-5822.2006.00769.x
68. Navas A, Vargas DA, Freudzon M, McMahon-Pratt D, Saravia NG, Gomez MA. Chronicity of dermal leishmaniasis caused by *Leishmania panamensis* is associated with parasite-mediated induction of chemokine gene expression. *Infect Immun* (2014) 82(7):2872–80. doi:10.1128/IAI.01133-13
69. Morgado FN, Nascimento MT, Saraiva EM, de Oliveira-Ribeiro C, Madeira Mde F, da Costa-Santos M, et al. Are neutrophil extracellular traps playing a role in the parasite control in active American tegumentary leishmaniasis lesions? *PLoS One* (2015) 10(7):e0133063. doi:10.1371/journal.pone.0133063
70. Novais FO, Carvalho LP, Passos S, Roos DS, Carvalho EM, Scott P, et al. Genomic profiling of human *Leishmania braziliensis* lesions identifies transcriptional modules associated with cutaneous immunopathology. *J Invest Dermatol* (2015) 135(1):94–101. doi:10.1038/jid.2014.305
71. Conceicao J, Davis R, Carneiro PP, Giudice A, Muniz AC, Wilson ME, et al. Characterization of neutrophil function in human cutaneous leishmaniasis caused by *Leishmania braziliensis*. *PLoS Negl Trop Dis* (2016) 10(5):e0004715. doi:10.1371/journal.pntd.0004715
72. Habuchi S, Tsutsui H, Kochaniak AB, Miyawaki A, van Oijen AM. mKikGR, a monomeric photoswitchable fluorescent protein. *PLoS One* (2008) 3(12):e3944. doi:10.1371/journal.pone.0003944
73. Wilson J, Huynh C, Kennedy KA, Ward DM, Kaplan J, Aderem A, et al. Control of parasitophorous vacuole expansion by LYST/Beige restricts the intracellular growth of *Leishmania amazonensis*. *PLoS Pathog* (2008) 4(10):e1000179. doi:10.1371/journal.ppat.1000179
74. Real F, Mortara RA. The diverse and dynamic nature of *Leishmania parasitophorous* vacuoles studied by multidimensional imaging. *PLoS Negl Trop Dis* (2012) 6(2):e1518. doi:10.1371/journal.pntd.0001518
75. Kloehn J, Saunders EC, O'Callaghan S, Dagley MJ, McConville MJ. Characterization of metabolically quiescent *Leishmania* parasites in murine lesions using heavy water labeling. *PLoS Pathog* (2015) 11(2):e1004683. doi:10.1371/journal.ppat.1004683
76. Bruijnzeel PL, Uddin M, Koenderman L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? *J Leukoc Biol* (2015) 98(4):549–56. doi:10.1189/jlb.3VMR1214-600RR
77. Carneiro MB, Hohman LS, Egen JG, Peters NC. Use of two-photon microscopy to study *Leishmania major* infection of the skin. *Methods* (2017) 127:45–52. doi:10.1016/j.ymeth.2017.04.012
78. Muller AJ, Aeschlimann S, Olekhnovitch R, Dacher M, Spath GF, Bousso P. Photoconvertible pathogen labeling reveals nitric oxide control of *Leishmania major* infection in vivo via dampening of parasite metabolism. *Cell Host Microbe* (2013) 14(4):460–7. doi:10.1016/j.chom.2013.09.008
79. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* (2012) 122(1):327–36. doi:10.1172/jci57990
80. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature CD10+ and immature CD10- neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood* (2017) 129(10):1343–56. doi:10.1182/blood-2016-04-713206
81. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* (2012) 30:459–89. doi:10.1146/annurev-immunol-020711-074942
82. McCaffrey RL, Allen LA. *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J Leukoc Biol* (2006) 80(6):1224–30. doi:10.1189/jlb.0406287
83. Simons MP, Nauseef WM, Apicella MA. Interactions of *Neisseria gonorrhoeae* with adherent polymorphonuclear leukocytes. *Infect Immun* (2005) 73(4):1971–7. doi:10.1128/IAI.73.4.1971-1977.2005
84. van Zandbergen G, Gieffers J, Kothe H, Rupp J, Bollinger A, Aga E, et al. *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol* (2004) 172(3):1768–76. doi:10.4049/jimmunol.172.3.1768
85. Spinner JL, Winfree S, Starr T, Shannon JG, Nair V, Steele-Mortimer O, et al. *Yersinia pestis* survival and replication within human neutrophil

phagosomes and uptake of infected neutrophils by macrophages. *J Leukoc Biol* (2014) 95(3):389–98. doi:10.1189/jlb.1112551

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.3.1.3 Other Innate Immune Cells in Leishmaniasis

The *Leishmania*-neutrophil interaction is very important during the first hours of infection. However, the parasite will be also taken up by other phagocytic cells. Dermal DCs and macrophages are known to take up parasites shortly after infection and dermal DCs migrate to the draining lymph node (dLN) for antigen (Ag) presentation[120,146-148]. Neutrophils recruited early after infection are known to recruit and activate macrophages to the site of infection in an MIP-1β dependent manner[149,150]. Furthermore, neutrophils recruit dendritic cells thorough CCL3 secretion to the infection site[151]. Also, inflammatory monocytes are recruited to the site of infection by a mechanism involving the secretion of platelet-derived growth factor by activated platelets which in turns leads to CCL2 secretion by leukocytes at the site of infection[152]. Once recruited, macrophages phagocytize apoptotic infected neutrophils as well as free parasites and become the main host for parasite replication. At the beginning of infection neutrophils are the predominant infected cell type but over time neutrophil numbers decrease and macrophages become the most infected cell population[120]. Macrophages are important effector cells in the destruction of intracellular parasites when activated by IFN-γ[153]. The antigen presenting moDCs phagocytize parasites and are crucial in the induction of adaptive immunity as they up-regulate MHC-II, migrate to the dLN for Ag presentation and are able to secrete IL-12 in order to induce a protective adaptive immune response[154]. Inflammatory monocytes take up parasites and are efficient in the killing of *Leishmania* parasites. $CCR2^{-/-}$ mice infected with *L. major* develop nonhealing lesions. Neutropenic *Genista* mice the increased resistance to *L. mexicana* infection correlated with the recruited inflammatory monocyte number, indicating an important role of these cells in immunity against leishmaniasis[155].

4.3.1.4 Toll-like Receptors in Leishmaniasis

The importance of Toll-like receptors in leishmaniasis is known since it has been described that mice lacking MyD88 are more susceptible to *L. major* infection than wild type mice[156]. Furthermore, it has been shown that the *Leishmania* surface structure lipophosphoglycan (LPG) is able to induce MyD88/TLR-signaling thorugh TLR2[157]. Also, it has been shown that *Tlr2*$^{-/-}$ mice infected with either *L. major* or *L. mexicana* subcutanoeusly in the rump developed bigger lesions and a higher parasite burden than their wild type counterparts[158]. Moreover, it is known that LPG-induced TLR2 siganling can lead to a inducation of NO production, an inhibition of phagosome maturation and a downregulation of TLR9 in macrophages[159-161]. Furthermore, it has been recently shown that TLR2 expressing nonhematopoietic cells are important in the neutrophil recruitment after *L. major* infection (Passelli, K. et al., 2018, manuscript submitted). LPG-TLR2-NK interactions have also been described to induce leishmanicidal functions in NK-cells[160]. Interesstingly, *Tlr2*$^{-/-}$ were able to better control *L. donovani* infection. Also, TLR4 has been shown to be important in the control of *L. major* infection[162]. *Tlr4*$^{-/-}$ mice developed significantly bigger lesions than their wild type counterparts[163]. Futhermore, neutrophils were reported to induce macrophages mediated killing of *L. major* through neutrophil elastase in a TLR4 dependent manner[164]. The *L. donovani* derived glycoprotein gp29 was shown to be a TLR4 ligand and to induce IL-12 and NO production in *L. donovani* infected macrophages. *Tlr3/7/9* tripple knock-out mice infected subcutaneously in the footpad with *L. major* were shown to be more susceptible to infection. These mice showed bigger lesions and a higher parasite burden with increased IL-10 and decreased IFN-γ levels. The *Tlr3*$^{-/-}$ showed no difference and the *Tlr7*$^{-/-}$ and *Tlr9*$^{-/-}$ only a very small difference in lesion size compared to the WT mice. However, it has been shown that the vaccination with soluble leishmania antigen (SLA) can be rendered successful by using TLR7 and TLR8 agonists as adjuvants[165]. Tlr*9*$^{-/-}$ mice infected with a high parasite inoculum have been shown to develop transiently bigger

lesion and higher parasite burden. It has further been shown, that TLR9 is important in the early NK cell activation[166].

4.3.2 The Adaptive Immune Response against Leishmaniasis

Mouse models of *L. major* infection have shown that different mouse strains develop different disease phenotypes in response to infection. C57BL/6 mice that are infected with *L. major* LV39 develop small, self-healing lesions that heal over 8-12 weeks wherase BALB/c mice infected with the same parasite strain develop progressive nonhealing disease **(Figure 9)**. The self-healing phenotype is associated with a strong Th1 response which leads to IFN-γ induced parasite killing through NO formation. The nonhealing phenotype is linked to a Th2 response that is characterised by a IL-4 mediated upregulation of agrinase prodcution that favors parasite survival in macrophages[167-169].

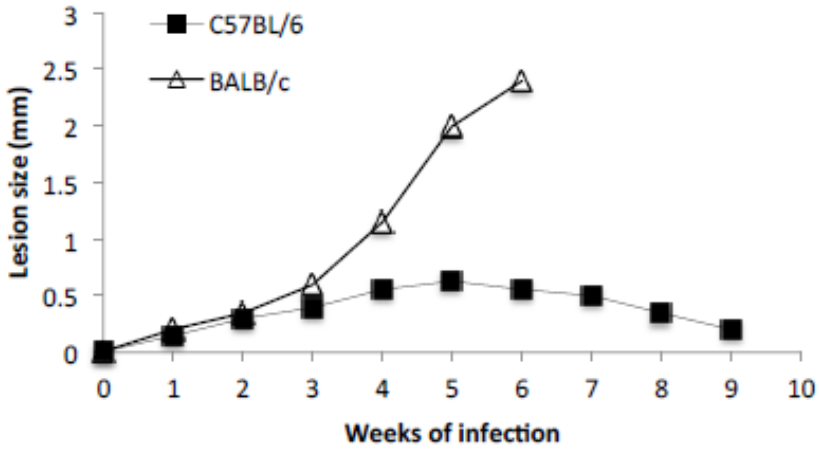

Figure 9: Lesion development of mice infected subcutaneously in the footpad with *L. major*

Once an infection is established, antigen presenting cells such as moDCs will phagocyte parasites, migrate to the lymph node and interact with T-cells, which then get activated and proliferate. In C57BL/6 mice, the antigen presenting cells are triggered to produce IL-12 which

leads to the induction of a Th1 response. In BALB/c mice the lack of IL-12 by antigen presenting cells leads to a Th2 response **(Figure 9)**.

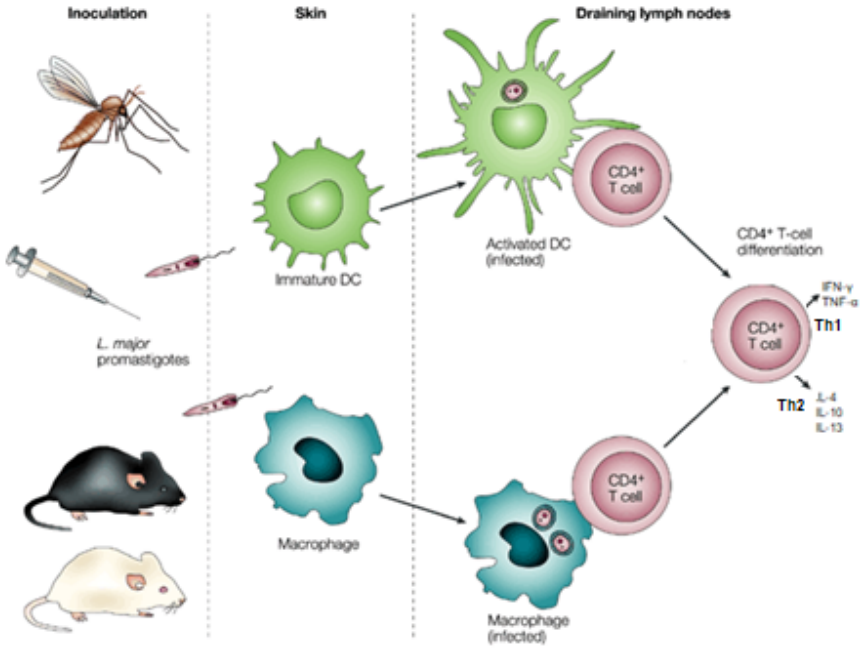

Figure 9: Overview of the induction of adaptive immunity by *L. major* in mice (Sacks at al., 2002)

The development of a Th1 response against *Leishmania*, the subsequent production of IFN-γ and TNF-α and the NO mediated killing of intracellular parasites in macrophages is also crucial in human disease. On the other hand, Th2 and Treg associated cytokines such as TGFβ or IL-10 correlate with exacerbation of disease[170-172]. In many CL patients, the usual Th response ranges from a strong Th1 to a mixed Th1/Th2 response and the clinical presentation is a result from the balance or pro- and anti-inflammatory factors[173-180]. While a Th1 response is beneficial for disease development a too vigorous Th1 response to *Leishmania* infection can lead to tissue damage and an increased lesion development. Indeed, the lesion size of LCL patients correlates with the increase of IFN-γ and TNF-α secretion by $CD4^+$ T-cells[181,182]. During the resolution of disease the intralesional IL-10 and TGF-β transcripts increase[183,184]. Tregs are also thought to be involved in the pathogenesis of LCL[185,186]. In MCL the levels of IFN-γ and TNF-α are even

higher than those observed in LCL patients. In addition, there are only few parasites found at the lesion site[175]. Furthermore, increased IL-17 expression found in lesion is suggestive of a role of Th17 response MCL pathogenesis[187,188]. This deregulation of pro- and anti-inflammatory cytokines is thought to be one of the major driver of tissue destruction of MCL. In DL patients, the peripheral blood mononuclear cells (PBMCs) produce lower levels of Th1 cytokines, however, the immune response at the site of infection is very similar and even more vigorous than in LCL[189]. In DCL patients, reduced numbers of IFN-γ producing cells and subsequently less NO production is found at the site of infection[190]. This disease manifestation is thought to be the result of the lack of an effective adaptive immune response and a predominance of anti-inflammatory cytokines **(Figure 9)**.

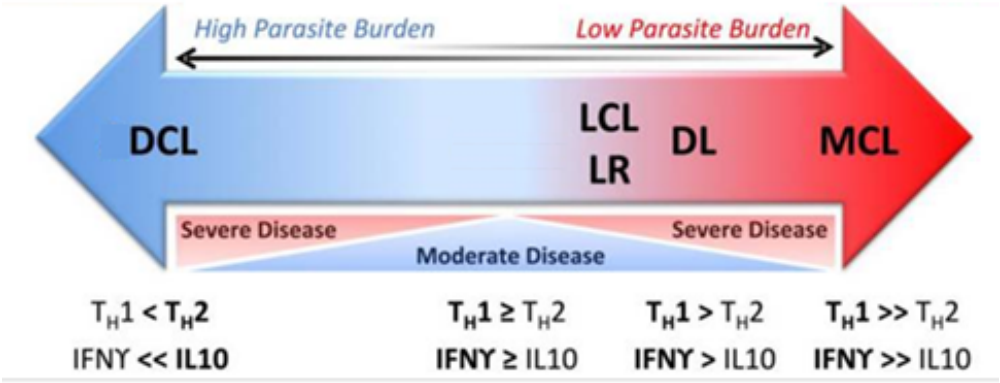

Figure 9: Spectrum of clinical presentations of the human cutaneous leishmaniasis (Scorza et al., 2017)

5. Material and Methods

The material and methods used in this thesis are included in the two manuscripts that are presented in part “6. Results”.

6. Aims

The general aim of this thesis was the **investigation of role of innate immunity in cutaneous leishmaniasis.** This was done in two projects with separate foci.

The focus of the first project was the **assessment of the role or TLR7 in *Leishmania major* infection** which was done aiming to investigating the following:

1. Identification of cells expressing TLR7 upon *L. major* infection
2. Characterization of the role of TLR7 in the adaptive immune response
3. Influence of TLR7 in neutrophil recruitment, infection frequency and functionality
4. Assessment of the mechanisms responsible for the differences in neutrophil activation
5. Evaluation of the effect of early TLR7 signaling in neutrophils on disease outcome

The focus of the second project was the **investigation of the influence of *Leishmania Viannia panamensis* drug susceptibility phenotype on the neutrophil-parasite interactions** aiming to elucidate the following:

1. Assessment of the effect of *L. (V.) p.* drug susceptibility phenotype on the induction of neutrophil effector functions in human and mouse neutrophils
2. Evaluation of the capability of *L. (V.) p.* that are resistant or susceptible to antileishmanial drugs to survive neutrophil mediated killing

7. Results

The results of this thesis are presented in two manuscripts. In the first manuscript entitled "Early sensing of *Leishmania major* by Toll-like receptor 7 in neutrophils is essential for the control of cutaneous leishmaniasis" we investigated the role of TLR7 in murine *L. major* infection. We explored the role of TLR7 following injection of *L. major* at different site of infection. The results demonstrate the important role of TLR7 signaling in neutrophils early after infection and it impact on disease outcome. The second manuscript entitled "Resistance of *Leishmania (Viannia) panamensis* to meglumine antimoniate or miltefosine Modulates Neutrophil Effector Functions" investigates the influence of *L. (V.) p.* drug susceptibility phenotype on human and murine neutrophil activation and function. From a common drug susceptible strain, a meglumine antimony and a miltefosine resistant *L. (V.) p.* line were derived and the interaction of these strains with neutrophils was assessed. Our data shows that drug resistant and drug susceptible parasites modulate neutrophil effector functions differently and that drug resistant parasites are more resistant to neutrophil mediated killing.

7.1 Manuscript 1

Early Sensing of *Leishmania major* by Toll-like Receptor 7 in Neutrophils is Essential for the Control of Cutaneous Leishmaniasis

Ivo Beat Regli, Berenice Martínez-Salazar, Katiuska Passelli, Benjamin P. Hurrell and Fabienne Tacchini-Cottier[1]*

[1] Department of Biochemistry, WHO-Immunology Research and Training Center, University of Lausanne, Epalinges, Vaud, 1066,Switzerland

* Corresponding author: Fabienne.Tacchini-Cottier@unil.ch

Summary

Leishmania major (L. major) are protozoan parasites that cause cutaneous leishmaniasis. Upon infection with *L. major*, neutrophils are the predominant cells recruited locally and the first cells internalizing the parasites prior to their transfer into macrophages. In this study we investigated the importance of endosomal TLR7 signaling in neutrophils early after infection and its impact on disease outcome. In contrast to C57BL/6 mice that were able to heal their lesion and control parasite burden, *L. major* infected *Tlr7*$^{-/-}$ mice developed a chronic nonhealing lesion with partial parasite burden control, despite the development of a Th1 response similar to that observed in C57BL/6 mice. Twenty-four hours after infection, a higher frequency of infected neutrophils that contained a greater number of parasites per cell was observed in *Tlr7*$^{-/-}$ mice compared to C57BL/6 mice. Transcriptome analysis of infected dermal neutrophils at that time point revealed that *L. major* infection induced the upregulation of genes associated with immunity against leishmaniasis in C57BL/6 but not in *Tlr7*$^{-/-}$ neutrophils. The gene expression pattern observed in infected *Tlr7*$^{-/-}$ neutrophils suggested impaired neutrophil activation. In this line, *L. major* infected *Tlr7*$^{-/-}$ neutrophils showed impaired effector functions including decreased release of reactive oxygen species and neutrophil extracellular trap formation. Injection of *Tlr7*$^{-/-}$ neutrophils in neutropenic mice led to the development of chronic nonhealing lesion with partial parasite control. In contrast, injection of C57BL/6 neutrophils at the time of infection in neutropenic mice allowed subsequent lesion healing and parasite burden control. Conversely, C57BL/6 mice treated topically with a TLR7 agonist for the first three days of infection developed significantly smaller lesions than untreated mice. Collectively, our data show that early triggering of TLR7 signaling in neutrophils is playing an essential role in shaping the local microenvironment at the site of infection, impacting subsequent control of lesion development.

Keywords

Leishmania, Toll-like receptor 7, neutrophils, NETs, ROS, replication, TLR-agonists, *Genista* mice.

Introduction

Cutaneous leishmaniasis is an important public health problem affecting 98 countries worldwide. Approximately 350 million people live in endemic areas and over 2 million new cases are reported annually (Alvar et al., 2012). *Leishmania major* is a causative agent of cutaneous leishmaniasis that is endemic in Africa, Asia and Europe. In most mouse strains such as C57BL/6, *L. major* causes the development of a spontaneously healing cutaneous lesion, which is associated with the development of a strong T-helper 1 response. These cells secrete IFN-γ, which activates macrophage microbicidal mechanisms leading to the elimination of the intracellular parasites. In contrast, a few strains such as BALB/c mice develop nonhealing progressive disease which is associated with the induction of a T-helper 2 response, which is characterized by IL-4 secretion, which impairs macrophage activation and results in parasite proliferation in these cells (Sacks and Noben-Trauth, 2002).

L. major parasites expresse pathogen-associated molecular patterns (PAMPs) that can be recognized by pattern recognition receptors (PRR) expressed by leukocytes such as Toll-like receptors (TLR). TLRs recognize different PAMPs on the cell surface and in endosomes. They consist of leucin-rich repeats, a transmembrane region and a cytoplasmic signaling domain (Chandel et al., 2014, Takeuchi and Akira, 2010). Upon TLR activation, a signaling cascade is triggered that ultimately leads to the development of an immune response against the recognized pathogen, fostering innate immune cell activation and the secretion of pro-inflammatory cytokines (Ashour, 2015). Mice lacking the myeloid differentiation factor 88 (MyD88), showed a much higher susceptibility towards *L. major* than C57BL/6 wild type mice demonstrating an important role of the TLR receptors in leishmaniasis (Muraille et al., 2003;

Revaz-Breton et al., 2010). TLR signaling depends in most cases on the adaptor protein MyD88 except in the case of TLR3 which signals in a MyD88-independent but TRIF-dependent manner and TLR4 that can trigger MyD88-dependent and TRIF-dependent signaling (Takeuchi and Akira, 2010). Most TLRs are expressed at the cell surface, however, TLR3, TLR7, TLR8 and TLR9 are exclusively expressed in the endosomal compartment. *Tlr*3/7/9 triple knock-out mice infected subcutaneously in the footpad with *L. major* were more susceptible to *L. major* infection than C57BL/6 mice demonstrating the importance of endosomal TLR signaling in *L. major* infection (Schamber-Reis et al., 2013). In addition, *Tlr9*$^{-/-}$ mice infected s.c. with *L. major* were shown to be transiently more susceptible to infection than C57BL/6 mice (Abou Fakher et al., 2009; Liese et al., 2007). TLR7 recognizes single-stranded RNA (ssRNA) and also responds to imidazoquinoline compounds such as imiquimod or resiquimod (Akira, 2006; Vasilakos and Tomai, 2013). Vaccination with soluble *Leishmania* antigen (SLA) could be improved using TLR7 agonists as adjuvants (Zhang and Matlashewski, 2008) and *L. major* was shown to induce *Tlr7* mRNA expression in inflammatory C57BL/6 neutrophils (Charmoy et al., 2007).

Neutrophils are the most abundant leukocyte in human circulation (Nauseef and Borregaard, 2014). They possess three major effector killing strategies: Phagocytosis with the subsequent production of reactive oxygen species (ROS), the release of granules that contain microbicidal proteins and the formation of neutrophil extracellular traps (NETs) (Segal, 2005). NETs are fibrous structures composed of dsDNA coated with a variety of different microbicidal proteins that can trap and kill a variety of pathogens (Brinkmann et al., 2004). Upon phagocytosis of pathogens, neutrophils produce different reactive oxygen species that are synthesized by the enzymatic activity of NADPH-synthase (Roos et al., 2003). Neutrophil granules can either fuse with pathogen containing phagosomes or be released into the extracellular space (Mayadas et al., 2014). Following *L. major* infection, neutrophils are rapidly recruited to the site of infection in great numbers (Charmoy et al., 2007; Charmoy et al., 2016; Lee et al., 2018; Peters et al.,

2008; Ribeiro-Gomes et al., 2014; Tacchini-Cottier et al., 2000). In many infections, neutrophils have a protective role, in leishmaniasis however, they can have a detrimental role following infection with some *Leishmania* spp. (Hurrell et al., 2016). In both mouse and human neutrophils, *L. major* parasites have been shown not only to survive but also to induce the release of chemokines that attract dendritic cells (DCs) (Charmoy et al., 2010). Furthermore, *L. major* can use neutrophils as "Trojan horses" to enter silently into the host macrophages (Laskay et al., 2003; van Zandbergen et al. 2004).

In the present study, we aimed to establish the early role of TLR7 signaling in neutrophils during the first days of *L. major* infection. We showed that in absence of TLR7 neutrophil functions were impaired, changing parasite load and the microenvironment during the first days of infection, with consequences on the outcome of the disease.

Material and Methods

Ethics statement

Animal experimentation protocols were approved by the veterinary office of the Canton of Vaud (Authorization 1266.6-7 to F.T.C.) and were done in accordance to cantonal and federal legislation as well as the principles of the declaration of Basel.

Mice

C57BL/6 mice were purchased from Envigo (Cambridgeshire, United Kingdom) and bred under specified pathogen-free conditions at the animal facility of the University of Lausanne in Epalinges. *Tlr7*$^{-/-}$ mice on a C57BL/6 background (backcrossed more than eight generations) were a gift from Prof. Shizuo Akira (University of Osaka, Japan). *Genista* mice (Ordonez-Rueda et al., 2012) were a gift from Prof. Bernard Malissen (Centre d'Immunologie de Marseille-Luminy). 5 to 10 weeks-old mice were used in the experiments.

Leishmania major parasites

Leishmania major LV39 parasites (MRHO/Sv/59/P strain) and *L. major*-mCherry (*Passelli et al,* submitted) were cultured in M199 medium with 10% fetal bovine serum (PAA Laboratories), 4% HEPES (Amimed) and 2% antibiotics (penicillin, streptomycin, neomycin, Invitrogen) at 26°C. Hygromycin B (PAA Laboratories) at a concentration of 50 µg/mL was added to the medium for the culture of *L. major* LV39 parasites (MRHO/Sv/59/P strain) expressing the mCherry red fluorescent protein. Metacyclic *L. major* parasites were isolated using a Ficoll (Sigma) density gradient. Parasites were re-suspended in M199 medium and layered on 10% and 20% Ficoll-medium suspension phases. After centrifugation at 1000 xg for 15 minutes, metacyclic *L. major* parasites were isolated in the 10% Ficoll phase. The parasites were washed and counted and adjusted to a suitable concentration.

Isolation of tissue cells

Ears were recovered and processed into a single cell suspension. The two dermal layers were split and cut into small pieces, then digested in DMEM (Gibco) containing 0.2 mg/ml Liberase TL (Roche) for 2 hours at 37°C. Digestion was stopped by adding DMEM containing 5% FCS, 1% HEPES Buffer, 1% PSN and 0.5% beta-mercaptoethanol. Subsequently, the tissue was mashed through a 40 µm filters (Falcon) to obtain a single cell suspension in medium. Footpads were recovered and processed into a single cell suspension. The skin was removed and the footpad tissue was separated from the bones. The recovered tissue was digested for 1 hour at 37°C in HBSS (Invitrogen) containing 1 mg/ml CollagenaseD (Sigma). The digestion was stopped by the adding of DMEM containing 5% FCS, 1% HEPES Buffer, 1% PSN and 0.5% beta-mercaptoethanol and the tissue was filtered with 40 µm filters (Falcon). Lymph nodes were recovered, and cells were processed into a single cell suspension by mechanical homogenization

Neutrophil isolation

Neutrophils were derived of bone marrow from femora and tibia of C57BL/6 mice and were isolated by negative MACS using the neutrophils isolation kit (Miltenyi Biotec) according to the manufacturer's indications. Neutrophil purity was assessed using CytoSpin and Quick-Fix staining and was established to be >95%.

Reactive oxygen species formation

ROS production was measured using a luminol based chemiluminescence assay. Neutrophils were incubated in X-Vivo 15 Medium (Lonza) with either *L. major,* PMA or without stimulus in white, opaque 96-well plates (Perkin Elmer). Luminol (Carbosynth) was added at a final concentration of 20 μg/mL. ROS induced chemiluminescence was measured at a wavelength range from 400nm to 650nm. with a plate reader (Molecular Devices, SpectraMax MiniMax 300) every 5 minutes for 1 hour. *In vivo* ROS production was measured with flow cytometry using the fluorescent probe DHR123 (Thermo Fisher). Whole ear cells were incubated with the probe for 30 minutes at 37°C. Then, the cells were stained with CD11b, Ly6G and with the Live/Dead fixable Aqua Dead Cell Stain Kit (Invitrogen) and run through the BD LSR-Fortessa (Becton Dickinson). Data was analyzed with the software FlowJo (Tree Star).

Cytokine secretion

Draining lymph node cells were incubated with UV treated (5 min) parasites, anti-CD3 antibody or without stimulus for 72 hours in DMEM with 5% FCS, 1% HEPES Buffer, 1% PSN and 0.5% beta-mercaptoethanol. After incubation, cells were centrifuged at 500 xg for 5 minutes and supernatants were collected. IFN-γ and IL-4 production was assessed using enzyme linked immunosorbent assays (ELISA) according to the manufacturer's instructions (BD Biosciences; R&D).

Analysis of parasite burden

Single cell suspensions of infected tissues in biphasic medium (50 µL NNN medium containing 20% of defibrinated rabbit blood and overlaid with 100 µL M199) were serially diluted in 96-well plates and cultured for 7 days at 26°C in 8-fold replicates (Titus et al., 1985). The parasite number was determined from the lowest cell concentration from which promastigotes could be grown using the ESTIMFRE software which is based on the Poisson limit theorem as previously described.

Flow cytometry

Fluorescent parasites, stained parasites or stained murine cells were analyzed using Flow Cytometry analyzer of either the BD LSR II or the BD LSR-Fortessa series (Becton Dickinson) and analyzed with FlowJo software (Tree Star). The following antibodies were used for the identification of mouse antigen: anti-CD45-PerCPCy5, anti-CD8-APC, anti-CD4-AF700, anti-Ly6G-APC, anti-CD11c-PECy7, anti-CD11b-PB, anti-CD62L-PE, anti-F4/80-APC, anti-Ly6C-FITC, anti-Ly6G-APCCy7, anti-IFNγ-PECy7, anti-IFNγ-PE and anti-IL-4-FITC. For the assessment of cell viability: Live/Dead fixable Aqua Dead Cell Stain Kit (Invitrogen).

Imaging flow cytometery

An ImageStream cytometer (Amnis; Millipore Sigma, Billerica, MA, USA) at low speed and highest magnification (603) and the IDEA software was used to analyze samples. Internalized vs. noninternalized parasites were defined using the internalization of the bright parasite spots within the membrane marker mask. The following reagents were used for the identification of mouse antigens: Anti-Ly6G-APC-Cy7, anti-CD11b-FITC, anti-CD11b-PE-Cy5, anti-CD45-

PerCP-Cy5.5 (eBioscience). The cell viability was assessed with DAPI (Sigma-Aldrich).

Measurement of antioxidant capacity

A redox sensor system was used (EDEL-for-life) to assess the antioxidant capacity in homogenized ears in PBS. This electrochemical-based method responds to all water soluble compounds with reducing potential in a given liquid sample (Liu et al., 2005; Liu et al., 2006). The reductive agents are oxidized within a defined potential range and the resulting current is measured and expressed in an arbitrary unit (EDEL). For low molecular weight antioxidants the relationship between potential and current within the defined potential range is linear (Sauvain et al., 2011).

NET formation

Neutrophils were seeded on Poly-L-Lysine coated coverslips and exposed for 4 hours with either *L. (V.) p.,* PMA (Phorbol-12-myristate-13-acetate, Sigma), PMA + DNase (Sigma) or without stimulus. Subsequently, cells were fixed with 4% PFA (Paraformaldehyde, Sigma) and stained with rabbit anti-human MPO (Dako) primary antibody and Alexa Fluor-488 goat anti-rabbit secondary antibody (Life Technologies). Coverslips were mounted on glass slides using a DAPI containing mounting medium (Molecular Probes) and analyzed by confocal microscopy (ZEISS LSM 510).

NET formation was also assessed through the measurement of dsDNA in the supernatant using the Quant-iT PicoGreen kit (Thermo Fisher) by adapting a technique previously described (Amini et al., 2016). Neutrophils were primed with 25 ng/mL for 25 minutes at 37°C. $2x10^6$ Neutrophils were incubated for 4 hours in X-vivo medium (Lonza) with either *L. (V.) p.*, PMA or PMA + DNase or without stimulus. After incubation, the cell suspensions were centrifuged, and the supernatants were collected and transferred into black 96-well plates (Perkin Elmer). The picogreen dye was added and fluorescence was measured in using a plate reader (Molecular

Devices, SpectraMax MiniMax 300) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Transcriptome analysis

Mice were infected with 10^6 metacyclic promastigotes of mCherry expressing *L. major* intradermally in the ear. Control mice were injected with 10µL PBS. Mice were put down, the ear excised and homogenized in PBS. The resulting cell suspension was stained with anti-Ly6G-APCCy7. anti-CD11b-FITC and DAPI. Infected neutrophils and noninfected neutrophils from *L. major* infected mice and total neutrophils from PBS injected mice were FACS-sorted. The transcriptome was analyzed by RNA-sequencing carried out by Genewiz Inc. Data analysis was done with the support of Swiss Institute of Bioinformatics.

Results

Intradermal *L. major* infection induces early upregulation of *Tlr7* expression in the infected skin, a process correlating with neutrophil presence.

In order to see whether TLR7 plays a role in the context of *L. major* infection, we infected C57BL/6 mice intradermally in the ear with 10^6 parasites or PBS. After 24 hours, the infected ears were collected and *Tlr7* mRNA expression was analyzed in the ear cells. *Tlr7* expression was more than two-fold upregulated in *L. major* infected ear compared to the levels measured in mice injected with PBS **(Figure 1A)**. To determine if neutrophils contributed to *Tlr7* expression, we depleted neutrophils 4 hours before infection with *L. major*. Neutrophil-depleted mice expressed about three times less *Tlr7* at the site of infection than WT mice injected with a control mAb **(Figure 1A)**. These results indicate that most of the *Tlr7* mRNA expression detected in the infected ear dermis 24 hours post infection is associated with the presence of neutrophils. Neutrophils are massively and rapidly recruited to the site of infection, a process that is more profound after intradermal injection compared to subcutaneous infection (Ribeiro-

Gomes et al., 2014). As the early presence of neutrophils was shown to modulate the development of *L. major* immune response (Charmoy et al., 2007; Charmoy et al., 2016; Lee et al., 2018; Peters et al., 2008; Tacchini-Cottier et al., 2000), we analyzed if TLR7 signaling could influence neutrophil recruitment 24 hours post infection. To this end, mice genetically deficient in the *Tlr7* gene (*Tlr7*$^{-/-}$) and C57BL/6 mice were infected with 10^6 *L. major* and the composition of myeloid cells assessed in the infected tissues.

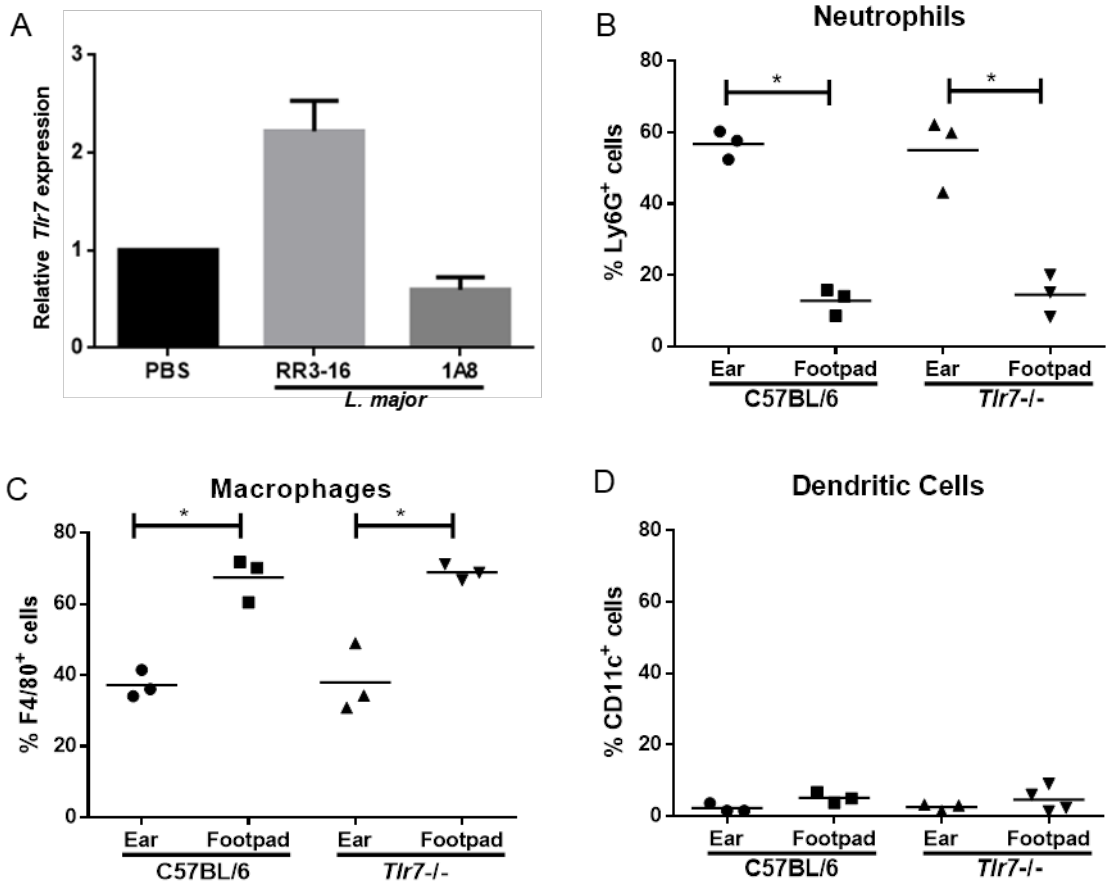

Figure 1. *Tlr7* expression in the ear and frequency of myeloid cells at the site of infection 24 hours after i.d. or s.c. *L. major* infection. A) Relative *Tlr7* mRNA expression in the ear of C57BL/6 mice that were either injected with *L. major* or PBS. Mice were either depleted of neutrophils with the 1A8 antibody or injected with the RR3-16 control antibody. (**B-D)** Myeloid cell recruitment in mice that were injected with 10^6 metacyclic *L. major* promastigotes either i.d.in the ear or s.c in the footpad. After 24 hours, infected tissues were processed and analyzed by flow cytometry. The frequency of **(A)** $CD45^+$ $CD11b^+$ $Ly6G^+$ cells neutrophils **(B)** $CD45^+$ $CD11b^+$ $F4/80^+$ macrophages and **(C)** $CD45^+$ $CD11b^+$ $CD11c^+$ DCs out of total $CD11b^+$ cells present in the infected ear or footpad cells is shown . n≥3/group, * p-value<0.05. These are representative of two independent experiments.

Marked differences in the type of myeloid cell recruited were observed depending on the site of *L. major* infection. Following intradermal infection, the frequency of recruited neutrophils

was two times higher than that observed following subcutaneous infection, while the frequency of macrophages was two times lower than that observed following subcutaneous infection **(Figure 1B-D)**. These data are in line a previous report (Ribeiro-Gomes et al., 2014). However, both *Tlr7*$^{-/-}$ and C57BL/6 mice showed a similar frequency of myeloid cell recruitment to the site of infection**,** suggesting that TLR7 signaling does not impact early myeloid recruitment to the site of *L. major* infection.

TLR7 is essential for the control of the disease following intradermal but not subcutaneous *L. major* infection

The genetic absence of *Tlr7* was reported to have only a minor impact on lesion development and parasite control following s.c. infection with *L. major* (Schamber-Reis et al., 2013). In line with these results, we did not observe any impact on lesion development, parasite load and immune response following s.c. infection with $5x10^5$ *L. major* (**Figure S1**). Here we showed that *Tlr7* mRNA expression correlated with neutrophil presence*,* and that a significantly higher frequency of neutrophils was recruited to the site of infection following i.d. infection. We therefore postulated that if TLR7 was playing a role in neutrophils following *L. major* infection, this could be better observed following i.d. infection. Thus, *Tlr7*$^{-/-}$ and C57BL/6 mice were infected with 10^5 *L. major* in the ear dermis. In addition, *Myd88*$^{-/-}$ mice were included in the experiments as a control of nonhealing lesion development. *L. major*-infected *Tlr7*$^{-/-}$ mice developed chronic nonhealing lesion that did not necrose in contrast to C57BL/6 mice that healed their lesion. *Myd88*$^{-/-}$ mice developed faster progressive nonhealing lesion **(Figure 2A-B)** that necrosed 10 weeks post infection. Analysis of the parasite burden at the site of infection as well as in the dLNs 10 weeks post infection revealed that *Tlr7*$^{-/-}$ mice had a ten times higher parasite burden than C57BL/6 mice. Nevertheless, *Tlr7*$^{-/-}$ mice controlled their parasite load better than *Myd88*$^{-/-}$ mice **(Figure 2C)**.

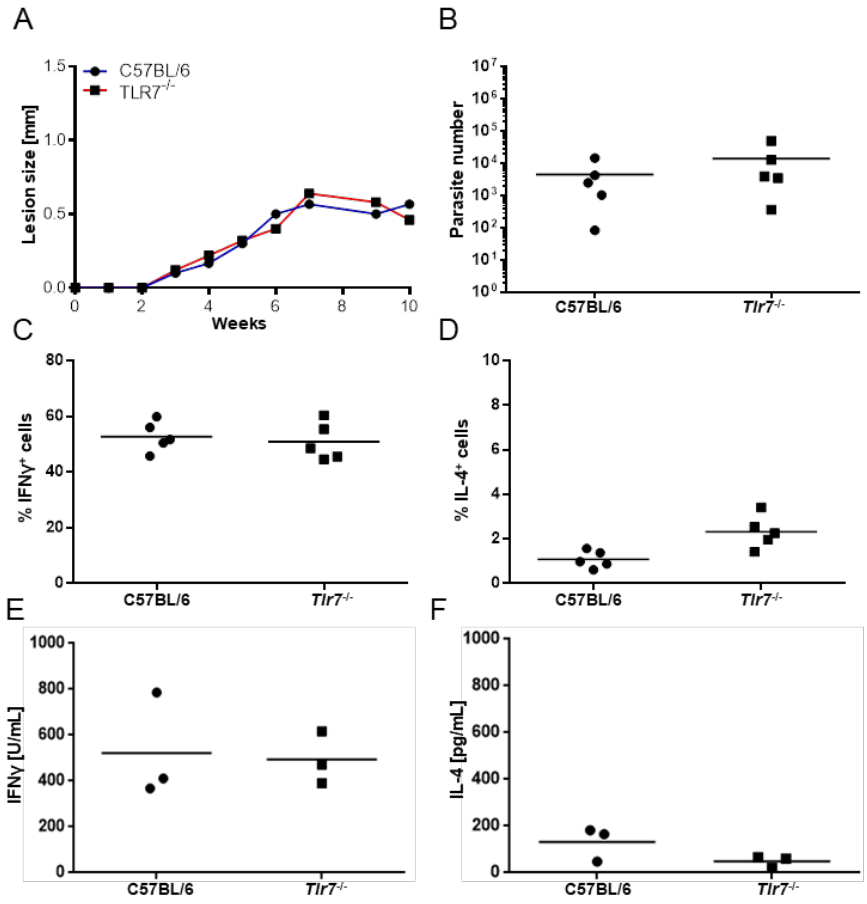

Figure S1. Lesion Development, parasite burden and cytokine secretion after *L, major* infection of C57Bl/6 WT and *Tlr7*$^{-/-}$ mice. Mice were injected with 5x10^5 *L. major* metacyclic promastigotes s.c.in the footpad A) Lesion development. **B)** Parasite burden in the footpad 10 weeks after infection. **C)** IFN-γ and **D)** IL-4 expression in CD45$^+$ CD4$^+$ T cells in the footpad 10 weeks post infection **E)** IFN-γ and **F)** IL-4 secretion of dLN cells restimulated with UV-treated L. major. Data representative of 2 independent experiments, n≥3/group, * p-value<0.05, ** p-value<0.01.

Next, we analyzed the development of T-helper cells, analyzing the production of IFN-γ and IL-4 in T-cells at the site of infection by flow cytometry. A high frequency of CD4$^+$ IFNγ$^+$ T cells was observed at the site of infection of *L. major* infected *Tlr7*$^{-/-}$ and C57BL/6 mice and no difference was observed between both groups **(Figure 2D)**. In addition, no differences in the level of IFN-γ secretion was observed in dLN cells of C57BL/6 and *Tlr7*$^{-/-}$ infected mice, as analyzed by ELISA following antigen restimulation in vitro **(Figure 2F).** The frequency of CD4$^+$IL-4$^+$ producing cells and IL-4 secreted levels at the site of infection and in dLN cells as very low in both groups of mice **(Figure 2D-G)**. These data demonstrate that *Tlr7*$^{-/-}$ mice are fully able to develop a Th1 type of immune response following infection.

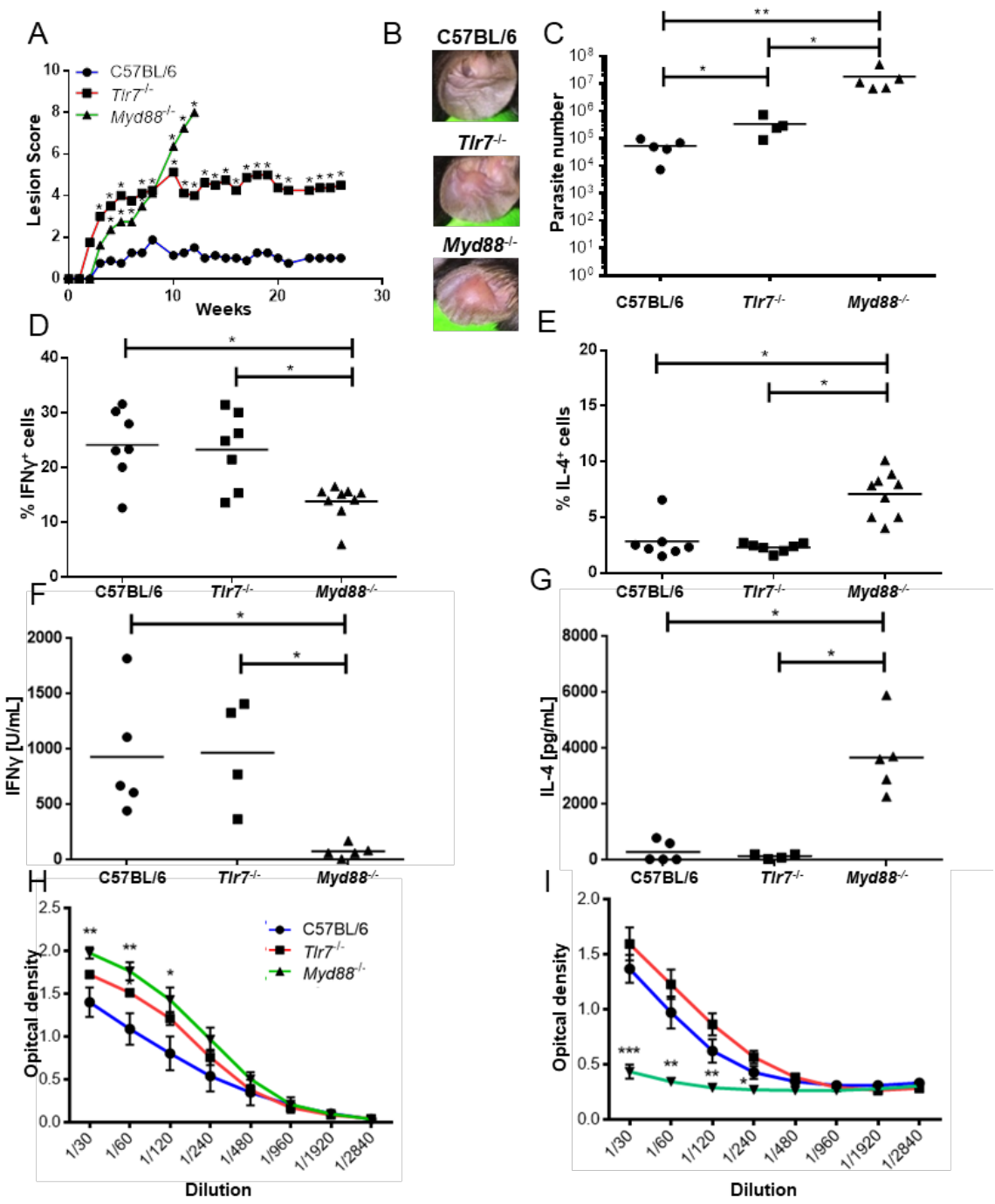

Figure 2. Lesion Development, parasite burden and cytokine secretion after L, major infection of C57Bl/6 WT and Tlr7-/- mice. Mice were injected with 10^5 L. major metacyclic promastigotes intradermally in the ear **A)** Lesion development **B)** Representative pictures of lesions in the ear 8 weeks after infection. **C)** Parasite burden in the ear 10 weeks after infection. **D)** IFN-γ expression in $CD45^+$ $CD4^+$ cells in the ear 10 weeks post infection. **E)** IL-4 expression in $CD45^+$ $CD4^+$ cells in the ear 10 weeks post infection **F)** IFN-γ secretion of dLN cells restimulated with UV-treated L. major. **G)** IL-4 secretion of dLN cells restimulated with UV-treated L. major. **H)** Relative amount of IgG1 found in the sera of mice **I)** Relative amount of IgG2a found in the sera of mice. Parasite burden was measured with limiting dilution analysis. For cytokine expression measurements cells were stained with fluorochrome coupled antibodies and the frequency $CD45^+$ $CD4^+$ cells expressing either IFNγ or IL-4 was measured by flow cytometry. IFNγ and IL-4 secretion as well and IgG concentrations were measured with ELISA. Data representative of 6 individual experiments, n≥5/group, * p-value<0.05, ** p-value<0.01.

In contrast, *Myd88*$^{-/-}$ mice showed markedly decreased IFN-γ and an increased IL-4 levels at the site of infection or after restimulation of dLN cells, indicative of the development of a Th2 response **(Figure 2D-G)**. In line with these data, low levels of IgG1 indicating low IL-4 and IL-13 presence and high levels of IgG2c indicative of high levels of IFN-γ presence were observed in the serum of infected C57BL/6 and *Tlr7*$^{-/-}$ mice while elevated levels of IgG1 and low levels of IgG2c were observed *Myd88*$^{-/-}$ mice **(Figure 2H-I)**. Collectively, these data show that despite the development of nonhealing chronic lesion, *L. major* infection induces the development of Th1 cells in *Tlr7*$^{-/-}$ mice.

Absence of TLR7 results in higher parasite frequency in neutrophils and more elevated parasite number per neutrophils

As the adaptive immune type 1 immune response developed normally in *Tlr7*$^{-/-}$ mice, we next investigated the impact of TLR7 absence on early parasite burden in neutrophils. To this end, *Tlr7*$^{-/-}$ and C57BL/6 mice were infected i.d. with 10^6 metacyclic *L. major*-mCherry parasites in the ear. The frequency of infected neutrophils in the infected ear dermis was determined by flow cytometry 24 hours later. The frequency of recruited neutrophils was similar between both groups of mice as shown before. However, the frequency of *L. major* infected neutrophils was three times higher in *Tlr7*$^{-/-}$ cells than in C57BL/6 neutrophils **(Figure 3A-B)**. Further analysis of infected neutrophils by imaging flow cytometry additionally showed that *Tlr7*$^{-/-}$ neutrophils harbored more parasites per cell than C57BL/6 neutrophils 24 hours after infection **(Figure 3C-D)**. To further estimate the number of live parasites presented in the ear 24 hours post infection, we performed limiting dilution analysis (LDA). The number of parasites observed in the ears of infected *Tlr7*$^{-/-}$ mice was significantly higher than that observed in ears of C57BL/6 mice **(Figure 3.E)**. Similarly, a higher number of parasites survived in *Tlr7*$^{-/-}$ neutrophils compared to to C57BL/6 neutrophils *in vitro* **(Figure 3F)**.

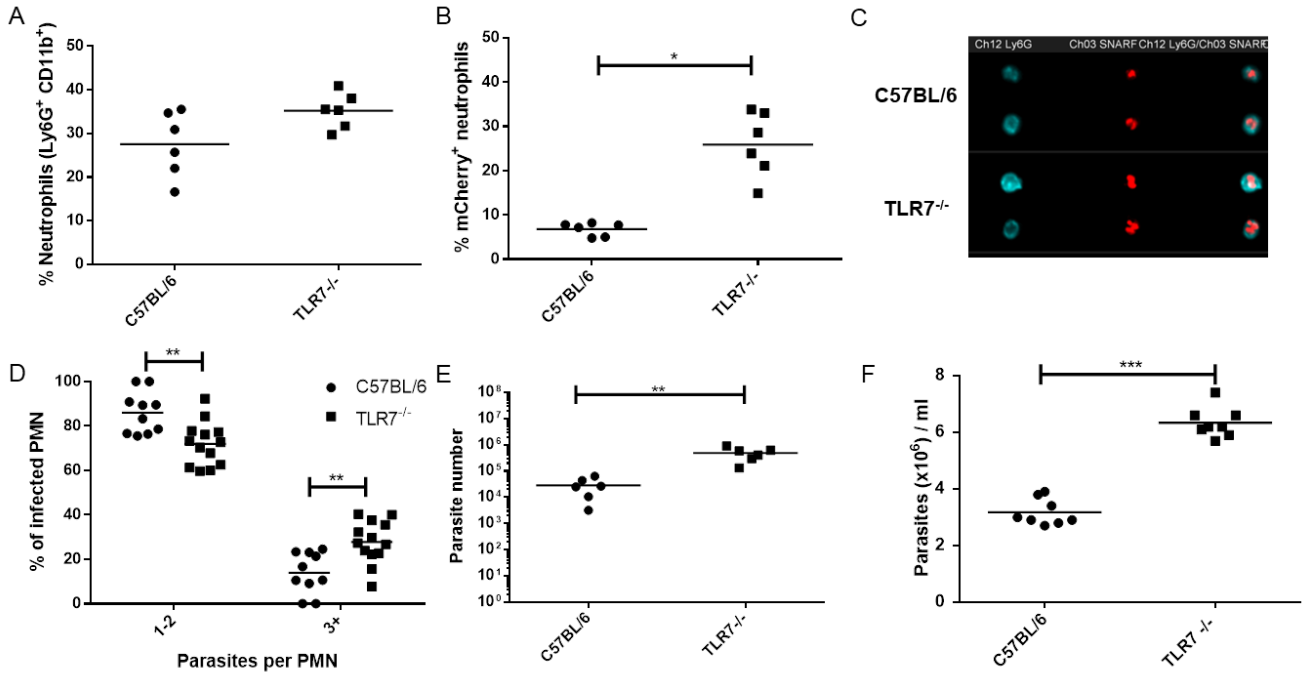

Figure 3. Neutrophil recruitment and infection 24 hours post infection in the ear. Mice were injected with 10^6 mCherry expressing metacyclic *L. major* promastigotes. After 24 hours mice were sacrificed, the infected ears were excised and homogenized, digested and the cell content was analyzed by flow cytometry. **A)** $CD45^+$ $CD11b^+$ $Ly6G^+$ neutrophils frequency out of total $CD45^+$ $CD11b^+$ cells at the site of infection. **B)** Infected *L. major*-mCherry$^+$$CD45^+$ $CD11b^+$ $Ly6G^+$ neutrophil frequency out of total neutrophils at the site of infection **C)** Representative images taken by imaging flow cytometry showing infected neutrophils. **D)** Number of parasites per infected neutrophils **E)** Parasite burden in the ear analyzed by LDA **F)** Number of *L. major*-mCherry infected parasites after *in vitro* coincubation of *L. major* and neutrophils. Results of ≥2 representative experiments, n≥6/group, * p-value<0.05.

L. major-infected dermal neutrophils induce upregulation of genes associated to an antileishmanial immune response in C57BL/6 mice but not in *Tlr7*$^{-/-}$ mice.

To further investigate the role of TLR7 in neutrophils at the onset of *L. major* infection, we FACS sorted infected dermal neutrophils from the ears of *Tlr7*$^{-/-}$ and C57BL/6 mice infected with 10^6 *L. major* metacyclic promastigotes for 24 hours. As controls, ear neutrophils recruited following injection with PBS were similarly isolated. We sequenced the transcriptome of these neutrophils and analyzed the gene expression of C57BL/6 and *Tlr7*$^{-/-}$ infected neutrophils and compared them to that obtained in neutrophils from C57BL/6 and *Tlr7*$^{-/-}$ mice injected with PBS. Gene-ontology (GO) analysis revealed that genes associated with IFN-γ synthesis, cellular response to nitric oxide, positive regulation of macrophage migration, granulocyte differentiation, IL-2 synthesis and the defense response to protozoan were upregulated in C57BL/6 mice. All these processes are linked to an effective immune response to *L. major*.

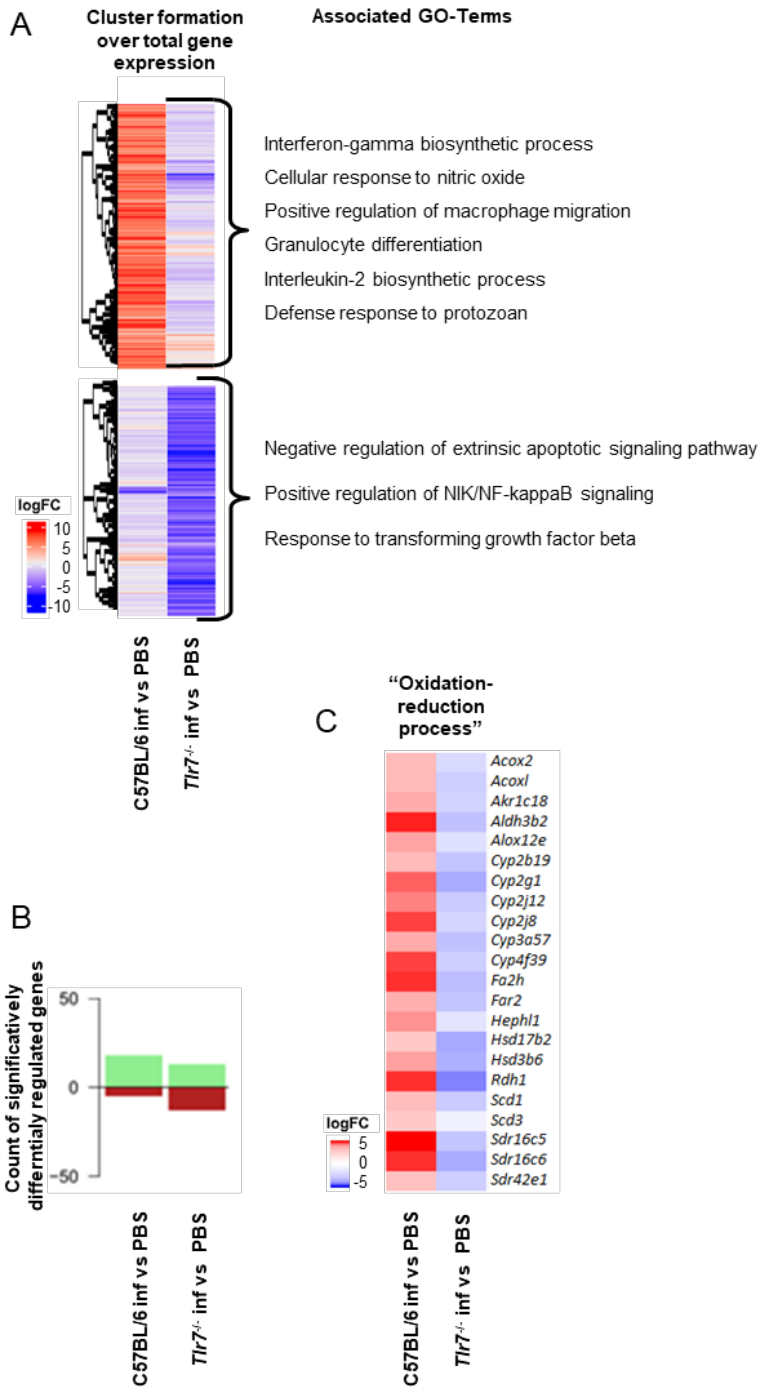

Figure 4. Transcriptome analysis of C57BL/6 and *Tlr7*$^{-/-}$ dermal neutrophils infected or not with *L. major*. C57BL/6 and *Tlr7*$^{-/-}$ mice were injected with 10^6 metacyclic *L. major* promastigotes or 10µl PBS intradermally in the ear. The mRNAs of FACS sorted dermal neutrophils from infected mice were compared to that from dermal neutrophils of mice injected with PBS. **A)** Representation of manually selected clusters with their associated GO-Terms **B)** Number of significantly differentially up- and downregulated genes between C57BL/6 and *Tlr7*$^{-/-}$ *L. major* infected or PBS recruited neutrophils **C)** Differences of gene expression of 22 selected genes associated with the GO-Term "Oxidation-reduction process" between *L. major* infected and PBS recruited neutrophils in C57BL/6 and *Tlr7*$^{-/-}$ mice.

In contrast, in infected *Tlr7*$^{-/-}$ a different gene expression pattern was observed, with downregulation of genes associated with the negative regulation of the extrinsic apoptotic signaling pathway, the positive regulation of NF-κB signaling and the response to transforming growth factor beta. This gene expression pattern suggested decreased neutrophil activation **(Figure 4A).** Further analysis of significantly differentially regulated genes in neutrophils of both mouse strains revealed that there are more genes upregulated in infected C57BL/6 neutrophils compared to infected *Tlr7*$^{-/-}$ neutrophils, further suggesting a lower neutrophil activation in *Tlr7*$^{-/-}$ neutrophils **(Figure 4B)**. Genes associated to oxidation/reduction processes that are important in the innate immune response against *L. major* were upregulated in infected C57BL/6 neutrophils but were downregulated in infected *Tlr7*$^{-/-}$ neutrophils **(Figure 4C)**. Taken together, these data show that the absence of TLR7 differentially modulates the transcriptional neutrophil response against *L. major* suggesting an impact on neutrophil effector functions.

L. major triggering of TLR7 expression contributes to neutrophil effector functions

The transcriptome analysis performed in *L. major* infected or noninfected neutrophils suggested that the absence of TLR7 would negatively modulate neutrophil effector functions at the onset of infection. To confirm this, *Tlr7*$^{-/-}$ and C57BL/6 bone marrow-derived neutrophils were infected with metacyclic *L. major* parasites at a multiplicity of infection (MOI) of 5 and the chemiluminescence induced by the reaction of luminol with ROS analyzed over 80 minutes. ROS production by C57BL/6 neutrophils was significantly more elevated than that measured in *Tlr7*$^{-/-}$ neutrophils. High levels of ROS were observed following PMA stimulation, and no difference were observed between *Tlr7*$^{-/-}$ and C57BL/6 neutrophils suggesting that the deficient ROS production observed in *Tlr7*$^{-/-}$ neutrophils is *L. major* specific **(Figure 5A).** To analyze if impaired ROS production would similarly be observed in vivo, C57BL/6 and *Tlr7*$^{-/-}$ mice were infected with *L. major* and 24 hours later the fluorescent oxidative species dye DHR123 was added to the isolated ear cell suspension to analyze *in vivo* ROS production by flow cytometry.

In line with the *in vitro* data, the frequency of *Tlr7*$^{-/-}$ neutrophils producing ROS was markedly lower than that observed in C57BL/6 neutrophils **(Figure 5B-C)**.

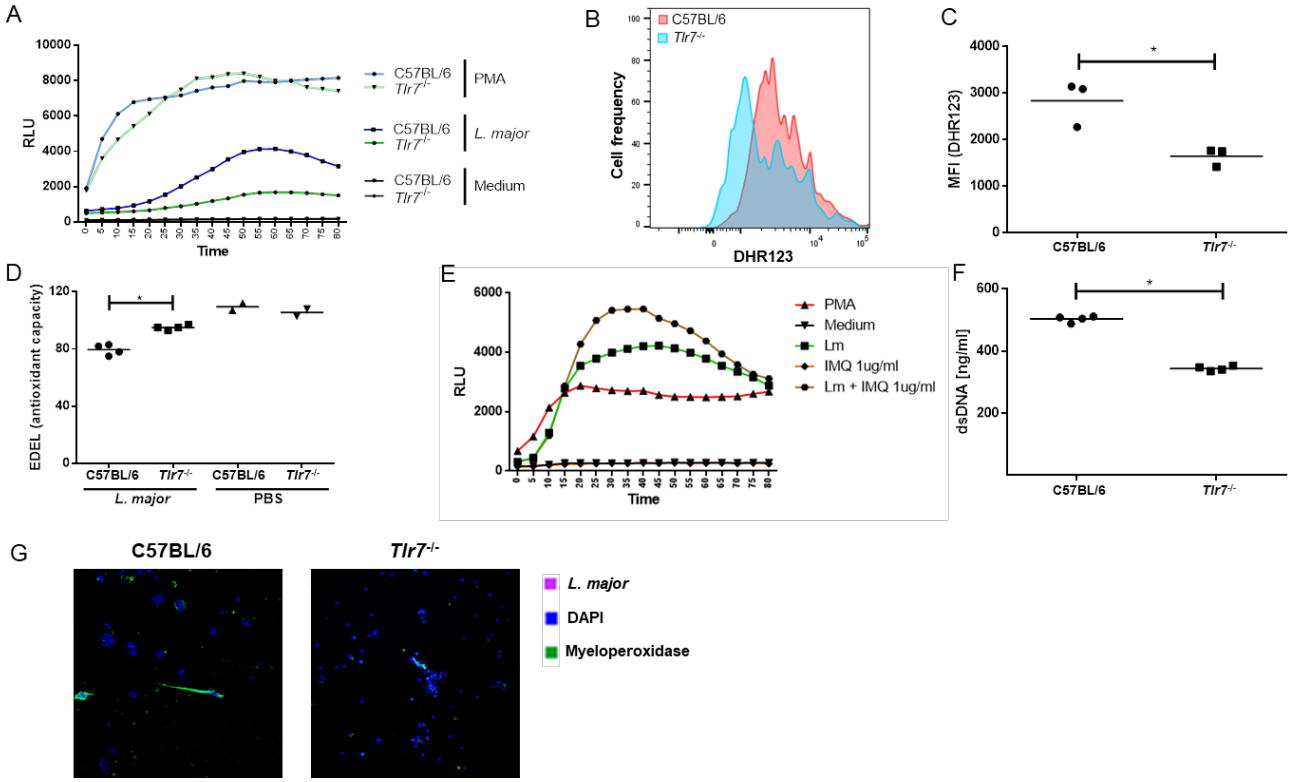

Figure 5. Induction of ROS and NET formation in *Tlr7*$^{-/-}$ and C57BL/6 neutrophils following *L. major* infection A-D) Sorted bone marrow derived neutrophils from *Tlr7*$^{-/-}$ and C57BL/6 mice were kept in luminol containing medium and exposed to either metacyclic *L. major* promastigotes at a MOI of 5 **(A)** or the TLR7 agonist imiquimod **(E)** ROS formation was assessed by measuring luminol chemiluminescence. Data are shown as an arbitrary scale, RLU = relative light units.. **B+C)** Mice were injected with 10^6 metacyclic *L. major* mCherry promastigotes. After 24 hours mice were put down, the infected ears excised, homogenized, digested and ROS formation was measured with DHR123 and analyzed by flow cytometry. One representative histogram **(B)** and MFI of one representative experiment are shown **(C)**. **D)** The antioxidant capacity of homogenized ears was measured by measuring the conduction characteristics. Measurements indicated on an arbitrary scale (EDEL). **F)** Measurement of NET formation by quantification of dsDNA released by murine neutrophils upon exposure to *L. major* promastigotes for 4 hours at a MOI of 5. **G)** Representative confocal microscopy pictures of neutrophils forming NETs upon challenge with *L. major*. One representative experiment out of ≥3 is shown in each graph. n≥3/group, * p-value<0.05

The antioxidant capacity of homogenized ears of both mouse strains 24 hours post infection was analyzed. Conversely, we could show that *Tlr7*$^{-/-}$ mouse ears had a higher antioxidant capacity than C57BL/6 mouse ears and thus had been less subject to oxidative stress **(Figure 5D).**

Given that the absence of TLR7 leads to a decreased *L. major* induced ROS production, we next measured if the stimulation of C57BL/6 neutrophils with a TLR7 agonist, imiquimod, would induce ROS production. Stimulation with imiquimod alone did not induce ROS production, however, the co-stimulation of these neutrophils with *L. major* and imiquimod led to increased ROS production compared to that obtained following stimulation with *L. major* alone **(Figure 5E)**.

To then assess NET formation in response to *L. major,* we measured the release of dsDNA by neutrophils challenged with *L. major in vitro* using the picogreen fluorescence assay. A significant decrease in dsDNA correlating with NET release was observed in *Tlr7*$^{-/-}$ neutrophils compared to C57BL/6 neutrophils **(Figure 5F)**. Furthermore, these data were confirmed by confocal microscopy analysis of NET forming neutrophils staining DNA with DAPI and the NET associated myeloperoxidase (MPO) with a mAb against MPO **(Figure 5G).** Altogether, these data show that *Tlr7*$^{-/-}$ mice have impaired neutrophil effector functions and therefore are likely less able to control infection during the first 24 hours.

TLR7 activation on neutrophils early after infection determines disease progression over time

To establish the importance of TLR7 signaling early after infection we applied imiquimod on the ears of C57BL/6 mice one day before as well as one and three days after infection in order to stimulate TLR7 signaling. Mice treated with imiquimod at the onset of infection developed smaller lesions than untreated C57BL/6 mice and had a lower parasite burden 12 weeks post infection **(Figure 6A-C)**. A similar elevated frequency of $CD4^+$ IFN-γ^+ T cells and a low frequency of $CD4^+$ IL-4^+ T cells was observed in both treated or untreated group of mice **(Figure 6D-E)**. These data demonstrate that early TLR7 activation has an influence on the development of the inflammatory lesion but not on the development of Th1 cells. This is in line

with the increased parasite numbers observed in *Tlr7*$^{-/-}$ mice compared to C57BL/6 mice 24 hours after infection **(Figure 3E)**.

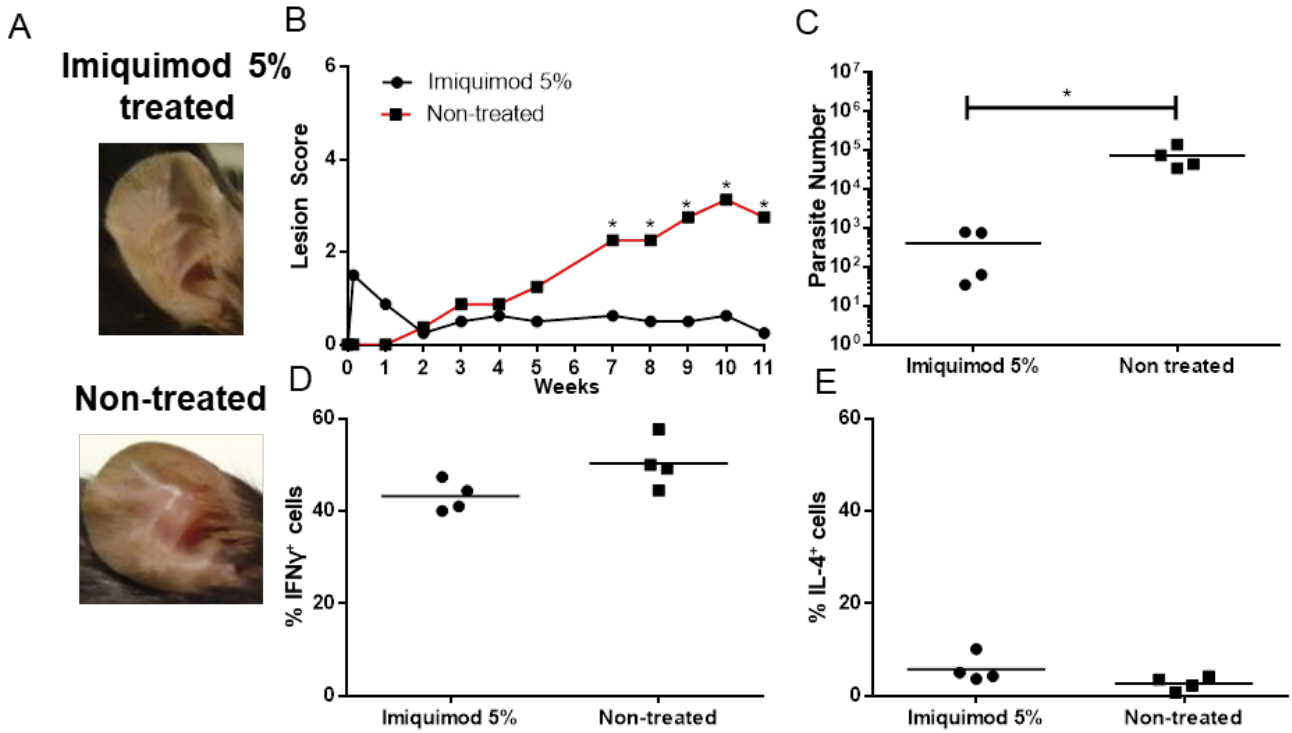

Figure 6. Disease evolution after infection of C57BL/6 with 10^5 *L. major* metacyclic promastigotes intradermally in the ear and topical treatment with 5% imiquimod cream at the onset of infection. A) Representative pictures of ear lesions 11 weeks after infection of C57BL/6 mice treated or not with 5% imiquimod cream at the onset of infection **B)** Lesion development in mice treated or not with 5% imiquimod cream. **C)** Parasite burden in the ear 11 weeks after infection **D)** The frequency of $CD4^+CD45^+IFN\text{-}\gamma^+$ and **E)** of $CD4^+CD45^+IL\text{-}4^+$ T cells was analyzed by flow cytometry at the site of infection 11 weeks post infection. This is a representative experiment out of three, n≥4/group, * p-value<0.05

In order to further assess the importance of neutrophil function during the first 24 hours in disease phenotype we infected neutropenic *Genista* mice with 10^5 *L. major* together with either 10^6 *Tlr7*$^{-/-}$ or 10^6 C57BL/6 neutrophils. Mice injected with *Tlr7*$^{-/-}$ neutrophils at the onset of infection developed nonhealing chronic lesion that were very similar to those observed following infection of *Tlr7*$^{-/-}$ mice **(Figure 7A-B)** and a higher parasite burden was observed in the infected ears 12 weeks post infection compared to similarly infected mice injected with C57BL/6 neutrophils at the onset of infection **(Figure 7C)**.

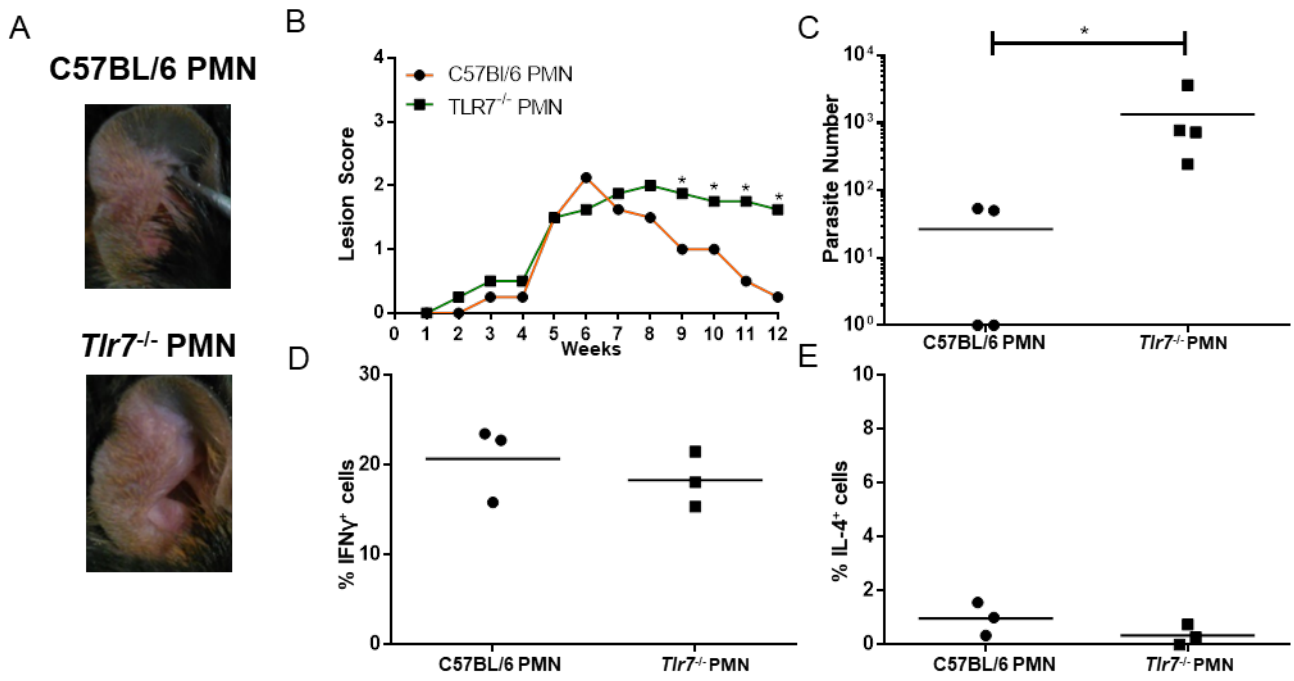

Figure 7. Diseases outcome following transfer of C57BL/6 or *Tlr7*$^{-/-}$ neutrophils in neutropenic *Genista* mice at the onset of infection. A) Representative pictures of lesion observed in the ear 12weeks after infection. **B)** Lesion development in mice transferred with C57BL/6 or *Tlr7*$^{-/-}$ neutrophils at the onset of infection **C)** Parasite burden in the ear of the indicated groups 10 weeks after infection **D)** Frequency of $CD4^+$ $CD45^+$ $IFN\gamma^+$and **E)** $CD4^+$ $CD45^+$ $IL\text{-}4^+$ T cells in infected ears 12 weeks post infection. Data are representative of three experiments n≥3/group, * p-value<0.05

An equally strong Th1 response developed in both groups of mice, as determined by the high frequency of $CD45^+$ $CD4^+$ IFN-γ^+ T cells present at the site of infection **(Figure 7D-E)**. These data demonstrate a predominant role of TLR7 signaling in neutrophils at the onset of infection which has consequences on the outcome of infection.

Discussion

In this study we show that the early triggering of TLR7 signaling in neutrophils plays an important role in the protective defense against *L. major* infection. The importance of Toll-like receptors in leishmaniasis has been established since early studies describing that mice lacking MyD88, a common signaling molecule on the pathway for the most of TLRs, are more susceptible to *L. major* infection than C57BL/6 mice (de Veer et al. 2003; Muraille et al., 2003).

Here, we show that activation of the endosomal TLR7 in neutrophils early after infection is a major player in the control of the cutaneous lesion and parasite load.

Of note, we show that the absence of TLR7 influences the diesease development of intradermal but not subcuteaneous *L. major* infection. Human disease is established parasites are deposited into the dermal and epidermal layers by a sand fly bite. We and others have shown that myeloid recuitment of cells differs between subcutaneous injections and intradermal injection. Thus, intradermal needle inoculation of parasites is thought to be clinically more relevant (Ribeiro-Gomes et al., 2014). Nevertheless in a majority of cases, sand flies deposit less than 1000 *L. major* in the dermis (Kimblin et al., 2008). In nature, sand fly derived factors have been shown to contribute to neutrophil recruitment to the site of infection (de Moura et al., 2010; Peters et al. 2008; Silva al. 2005; Teixeira et al., 2005). Due to the lack of those factors, we infected mice with 10^6 parasitse looking at neutrophil recruitment. When assessing lesion development, we infected mice with 10^5 *L. major* since we have observed that a lower inoculum did not change the disease phenotype but only delayed the disease onset (data not shown). To assure a high enough parasite inoculum we infected $5x10^5$ parasites when looking at development of subcutaneous infection.

Neutrophils have been shown to have a deleterious function in infection with a variety of *Leishmania* spp. but to be able to efficiently kill parasites in a few *Leishmania* spp. such as *L. amazonensis* (Hurrell et al., 2016). *Genista* mice are neutropenic mice due to a point mutation in the transcrption al repressor Growth Factor Independence 1 (Gfi1). Here we show that *Genista* mice infected with *L. major* and simultaneously injected with *Tlr7*$^{-/-}$ neutrophils developed chronic nonhealing lesion that resembles those observed following infection of *Tlr7*$^{-/-}$ mice. In contrast, similarly infected mice injected with wild type neutrophils were able to resolve their lesion size and control their paraite load. These data suggest that transit through neutrophils and the initial parasite load in these cells influences the microenvironment and

parasite load early in infection, with consequences on the subsequent control of lesion development, a process controlled by TLR7 signaling.

The decrease observed in neutrophil effector functions and the resulting higher parasite load observed in these cells during the first days of infection suggested decreased neutrophil killing functions. However, neutrophils were reported to act as shelter for the silent entry of *L. major* parasites into macrophages. Despite ROS production and NET formation, *L. major* was reported to survive in neutrophils (Aga et al., 2002; Laskay et al., 2003; Laufs et al., 2002; van Zandbergen et al., 2004). The results presented here suggest either that a small proportion of parasites are killed in C57BL/6 neutrophils, a process dependent on TLR7 and/or that *L. major* may survive better in *Tlr7*$^{-/-}$. In absence of TLR7, *L. major* may even be able to replilcate in neutrophils as was previously reported for *L. mexicana* (Hurrell 2017), an hypothesis that needs to be further tested.

The development of Th1 cells was not impacted in absence of TLR7 and despite high levels of IFN-γ that were similar to those observed in draining lymph nodes and infected ears of C57BL/6 mice, the parasite load observed up to 30 weeks after infection was roughly 10 times higher in infected ears of *Tlr7*$^{-/-}$ mice than in infected ears of C57BL/6 mice. The frequency of C57BL/6 and *Tlr7*$^{-/-}$ infected macrophages was similar during the first day of infection. *Tlr7*$^{-/-}$ BM-derived macrophages infected with *L. major* had the same infection frequency than C57BL/6 macrophages. However, one cannot exclude a possible role of TLR7 signaling in macrophages later in infection, contributing to the increased parasite burden observed in *Tlr7*$^{-/-}$ mice. Preferential targetting of the parasites to distinct subset of dermal macrophages was recently reported to allow the development of inflammatory lesion despite the elevated levels of IFN-γ at the site of infection (Lee 2018). In absence of TLR7 signaling, it is possible that *L. major* LV39 targetting of dermal macrophages is modified, a process that will need further investigation.

We report here that a lack of TLR7 signaling leads to an increase in lesion size. However, the development of nonhealing lesion did not coincide with a altered T-helper response. Collectively these data demonstrate that while Th1 cells and IFN-γ production are important in immunity against *L. major* infection, they are not the only determinant of disease outcome. These data are in line with previous reports showing that an other *L. major* clinical strain isolated from a chronic LCL patient, *L major* Seidmann (*L.m.*Sd*.)*, induces nonhealing lesions in C57BL/6 mice despite the induction of a strong Th1 response (Charmoy et al., 2016).

Here, RNA-sequencing was performed for the first time in *L. major* infected neutrophils. We compared infected and noninfected neutrophils of *L. major* infected C57BL/6 and *Tlr7*$^{-/-}$ mice. Furthermore, we compared those neutrophils to neutrophils recruited in mice injected with PBS. Interestingly, we did find very similar gene expression patterns when comparing infected with noninfected neutrophils derived from the same infected mice. This indicates that gene expression is mainly driven by the lesion environment and that *L. major* uptake per se has not a big influence on gene expression. Therefore, we focused on the comparison of infected neutrophils of *L. major* infected mice with neutrophils from mice injected with PBS. We could show that upon infection of C57BL/6 mice, in neutrophils a series of genes associated to immunity against *L. major* are positively regulated. In contrast, in *Tlr7*$^{-/-}$ we saw a down-regulation of another series of genes indicative of a lack of neutrophil activation. Thus, the neutrophil response against *L. major* infection differs on a transcriptional level between *Tlr7*$^{-/-}$ or C57BL/6 neutrophils. Here, we have analyzed the gene expression of selected genes that are assigned to the GO-term “oxidation-reduction processes”. We focused on that term because it is known that ROS and NO production are important oxidative processes that are involved in the antileishmanial immune response. Assessing further the biological relevance of the pathways indicated by the gene ontology analysis would be important to further understand the exact function of *L. major* has in neutrophil biology.

Here, we are showing for the first time the importance of TLR7 signaling in neutrophils in the context of leishmaniasis. TLR7 and TLR8 are closely related endosomal TLRs that recognize ssRNA. Human neutrophils express TLR8, however, TLR7 expression in human neurophils is controversial. In contrast, in mice, TLR7 is expressed in mouse neutrophils. Moreover, in contrast to human neutrophils, murine TLR8 has been reported not to be functional (Liu et al., 2010). It is thought that murine TLR7 and human TLR8 have some common functions. For instance, it was reported that HIV-1 derived guanosine and uridine rich ssRNA oligonucleotides are recognized by murine TLR7 but human TLR8[191]. Intracellular human neutrophil stimulation with ssRNA and TLR8 agonists was previoulsy reported to lead to increased ROS production and neutrophil degranulation (Janke et al., 2009). Furthermore, stimultion of TLR8 led to priming of the NADPH oxidase activation in human neutrophils (Makni-Maalej et al., 2015). Here, we show that the stimulation of murine neutrophils with the TLR7 agonist imiquimod did not lead to increased in ROS formation. However, *in vitro* stimulation with *L. major* together with this agonist led to an increase in ROS formation compared to *L. major* stimulation alone. Together with the decrease of ROS production observed in *Tlr7*$^{-/-}$ neutrophils compared to wild type neutrophils follwoing exposure to *L. major*, our results indicate that TLR7 plays an important role in neutrophil activation in murine neutrophils. In human nuetrophils, TLR8 is likely to have similar functions than TLR7 in murine neutrophils. Furthermore, we show here that early activation of TLR7 by topical treatment prior and during the first day after *L. major* infection leads to an important decrease of lesion size, further stressing the importance of TLR7 signaling early after infection. In this sense, topical application of imiquimod in combination with meglumine antimony was reported to be beneficial to disease outcome in leishmaniasis patients (Arevalo et al., 2007; Miranda-Verastegui et al., 2005; Miranda-Verastegui et al., 2009). Even though imiquimod was applied at different time points than in our study and the study was done in Peru, thus involved New World species, these findings still indicate a therapeutic potential of TLR7/TLR8 activation human lesihmaniasis. However, imiquimod has

not yet been shown to be beneficial in the treatment of leishmaniasis caused by Old World species. (Al-Mutairi et al., 2009; Firooz et al., 2006; Seeberger et al., 2003). Nonetheless, it would be concivable that TLR7/TLR8 signaling in cutaneous leishmaniasis patients could exert neutrophil activating properties and lead to a beneficial disease outcome since it is known that neutrophils are found in significant numbers in chronic experimental cutaneous leishmaniasis lesions (Hurrell et al., 2016). Moreover, imiquimod used as an adjuvant vaccine administered toghether with soluble leishmanial antigen was able to induce IFN-γ in spleen cells of BALB/c mice (Zhang and Matlashewski, 2008).

We show here that *Tlr7*$^{-/-}$ and *Myd88*$^{-/-}$ mice developed bigger lesions than C57BL/6 mice, but *Tlr7*$^{-/-}$ mice were better able to control lesion size and parasite burden than *Myd88*$^{-/-}$ mice. *Myd88*$^{-/-}$ mice are not able to signal after TLR-ligand engagement, except following TLR3 and partially TLR4 triggering (Kawai and Akira, 2011). TLR2 signaling can lead to an inhibition of phagosome maturation and a downregulation of TLR9 in macrophages supporting the survival of the parasites (Kavoosi et al., 2010; Srivastava et al., 2013). Furthermore, it has been reported that TLR2 expressing nonhematopoietic cells are important in the neutrophil recruitment after *L. major* infection (Passelli, K. et al. 2018, submitted). On the other hand, TLR9 was reported to enhance phagocytic function in neutrophils (Prince et al., 2011). All these process should still take place in *Tlr7*$^{-/-}$ and could at least partially explain the differences in disease progression observed between *Tlr7*$^{-/-}$ and *Myd88*$^{-/-}$ mice.

In conclusion, our results demonstrate the critical importance of TLR7 signaling in neutrophils early in infection, with an impact on subsequent resolution and control of *L. major* infection. Furthermore our data highlight the importance of innate immune response early after infection in leishmaniasis disease progression.

Declaration of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

We thank Jordi Mollà Mas, Yazmin Hauyon-La Torre, the Flow Cytometry Facility and the Cellular Imaging Facility of the University of Lausanne, Switzerland for technical assistance.

Authors' Contributions

IR and FTC conceived and designed the experiments. IR, BMS, KP, BH performed the experiments and analyzed the data. IR and FTC wrote the manuscript, BMS critically reviewed the manuscript.

Funding

This work was supported by financing of the Swiss National Science Fondation.

References:

Abou Fakher, F.H., Rachinel, N., Klimczak, M., Louis, J., and Doyen, N. (2009). TLR9-dependent activation of dendritic cells by DNA from Leishmania major favors Th1 cell development and the resolution of lesions. Journal of immunology. 182(3), 1386-1396.

Aga, E., Katschinski, D.M., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., Solbach, W., and Laskay, T. (2002). Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. Journal of immunology. 169(2), 898-905.

Akira, S. (2006). TLR signaling. Current topics in microbiology and immunology. 311, 1-16.

Al-Mutairi, N., Alshiltawy, M., El Khalawany, M., Joshi, A., Eassa, B.I., Manchanda, Y., Gomaa, S., Darwish, I., and Rijhwani, M. (2009). Tropical medicine rounds: Treatment of Old World cutaneous leishmaniasis with dapsone, itraconazole, cryotherapy, and imiquimod, alone and in combination. International journal of dermatology. 48(8), 862-869.Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., and Team, W.H.O.L.C. (2012). Leishmaniasis worldwide and global estimates of its incidence. PloS one. 7(5), e35671. DOI: 10.1371/journal.pone.0035671.

Amini, P., Stojkov, D., Wang, X., Wicki, S., Kaufmann, T., Wong, W.W., Simon, H.U., and Yousefi, S. (2016). NET formation can occur independently of RIPK3 and MLKL signaling. European journal of immunology. 46(1), 178-184. DOI: 10.1002/eji.201545615.

Arevalo, I., Tulliano, G., Quispe, A., Spaeth, G., Matlashewski, G., Llanos-Cuentas, A., and Pollack, H. (2007). Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 44(12), 1549-1554. DOI: 10.1086/518172.

Ashour, D.S. (2015). Toll-like receptor signaling in parasitic infections. Expert review of clinical immunology. 11(6), 771-780. DOI: 10.1586/1744666X.2015.1037286.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science. 303(5663), 1532-1535. DOI: 10.1126/science.1092385.

Chandel, H.S., Pandey, S.P., Roy, S., Doyen, N., Saha, B. (2014). TLR-CD40 Cross-Talk in Anti-Leishmanial Immune Response. Frontiers in Immunology. 6(2), 16;5:220. DOI: 10.3389/fimmu.2014.00220.

Charmoy M, Hurrell BP, Romano A, Lee SH, Ribeiro-Gomes F, Riteau N, Mayer-Barber K, Tacchini-Cottier F, Sacks DL.. (2016). The Nlrp3 inflammasome, IL-1β, and neutrophil recruitment are required for susceptibility to a nonhealing strain of Leishmania major in C57BL/6 mice. European journal of immunology. 46(4):897-911. DOI: 10.1002/eji.201546015.

Charmoy, M., Megnekou, R., Allenbach, C., Zweifel, C., Perez, C., Monnat, K., Breton, M., Ronet, C., Launois, P., and Tacchini-Cottier, F. (2007). Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. Journal of leukocyte biology. 82(2), 288-299. DOI: 10.1189/jlb.0706440.

de Veer, M.J., Curtis, J.M., Baldwin, T.M., DiDonato, J.A., Sexton, A., McConville, M.J., Handman, E., and Schofield, L. (2003). MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. European journal of immunology. 33(10), 2822-2831. DOI: 10.1002/eji.200324128.

de Moura TR, Oliveira F, Rodrigues GC, Carneiro MW, Fukutani KF, Novais FO, Miranda JC, Barral-Netto M, Brodskyn C, Barral A, de Oliveira CI., (2010). Immunity to Lutzomyia intermedia saliva modulates the inflammatory environment induced by Leishmania braziliensis. PLOS neglected tropical diseases. 15;4(6):e712. DOI: 10.1371/journal.pntd.0000712.

Firooz, A., Khamesipour, A., Ghoorchi, M.H., Nassiri-Kashani, M., Eskandari, S.E., Khatami, A., Hooshmand, B., Gorouhi, F., Rashighi-Firoozabadi, M., and Dowlati, Y. (2006). Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: a randomized assessor-blind controlled trial. Archives of dermatology. 142(12), 1575-1579. DOI: 10.1001/archderm.142.12.1575.

Hachiya, O., Takeda, Y., Miyata, H., Watanabe, H., Yamashita, T., and Sendo, F. (1995). Inhibition by bacterial lipopolysaccharide of spontaneous and TNF-alpha-induced human neutrophil apoptosis in vitro. Microbiology and immunology. 39(9), 715-723.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 303(5663), 1526-1529. DOI: 10.1126/science.1093620.

Hurrell, B.P., Beaumann, M., Heyde, S., Regli, I.B., Muller, A.J., and Tacchini-Cottier, F. (2017). Frontline Science: Leishmania mexicana amastigotes can replicate within neutrophils. Journal of leukocyte biology. 102(5), 1187-1198. DOI: 10.1189/jlb.4HI0417-158R.

Hurrell, B.P., Regli, I.B., and Tacchini-Cottier, F. (2016). Different Leishmania Species Drive Distinct Neutrophil Functions. Trends in parasitology. 32(5), 392-401. DOI: 10.1016/j.pt.2016.02.003.

Janke, M., Poth, J., Wimmenauer, V., Giese, T., Coch, C., Barchet, W., Schlee, M., and Hartmann, G. (2009). Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. The Journal of allergy and clinical immunology. 123(5), 1026-1033. DOI: 10.1016/j.jaci.2009.02.015.

Kavoosi, G., Ardestani, S.K., Kariminia, A., and Alimohammadian, M.H. (2010). Leishmania major lipophosphoglycan: discrepancy in Toll-like receptor signaling. Experimental parasitology. 124(2), 214-218. DOI: 10.1016/j.exppara.2009.09.017.

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 34(5), 637-650. DOI: 10.1016/j.immuni.2011.05.006.

Kaye, P., and Scott, P. (2011). Leishmaniasis: complexity at the host-pathogen interface. Nature reviews. Microbiology. 9(8), 604-615. DOI: 10.1038/nrmicro2608.

Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, Fay MP, Kamhawi S, Sacks D. (2008). Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. Proceedings of the national academy of sciences. 105(29):10125-30 DOI: 10.1073/pnas.0802331105.

Laufs, H., Muller, K., Fleischer, J., Reiling, N., Jahnke, N., Jensenius, J.C., Solbach, W., and Laskay, T. (2002). Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. Infection and immunity. 70(2), 826-835.

Laskay, T., van Zandbergen, G., and Solbach, W. (2003). Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? Trends in microbiology. 11(5), 210-214.

Lee, S.H., Charmoy, M., Romano, A., Paun, A., Chaves, M.M., Cope, F.O., Ralph, D.A., and Sacks, D.L. (2018). Mannose receptor high, M2 dermal macrophages mediate nonhealing Leishmania major infection in a Th1 immune environment. The Journal of experimental medicine. 215(1), 357-375. DOI: 10.1084/jem.20171389.

Liese, J., Schleicher, U., and Bogdan, C. (2007). TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. European journal of immunology. 37(12), 3424-3434. DOI: 10.1002/eji.200737182.

Liu, J., Roussel, C., Lagger, G., Tacchini, P., and Girault, H.H. (2005). Antioxidant sensors based on DNA-modified electrodes. Analytical chemistry. 77(23), 7687-7694. DOI: 10.1021/ac0509298.

Liu, J., Su, B., Lagger, G., Tacchini, P., and Girault, H.H. (2006). Antioxidant redox sensors based on DNA modified carbon screen-printed electrodes. Analytical chemistry. 78(19), 6879-6884. DOI: 10.1021/ac0608624.

Liu, J., Xu, C., Hsu, L.C., Luo, Y., Xiang, R., and Chuang, T.H. (2010). A five-amino-acid motif in the undefined region of the TLR8 ectodomain is required for species-specific ligand recognition. Molecular immunology. 47(5), 1083-1090. DOI: 10.1016/j.molimm.2009.11.003.

Makni-Maalej, K., Marzaioli, V., Boussetta, T., Belambri, S.A., Gougerot-Pocidalo, M.A., Hurtado-Nedelec, M., Dang, P.M., and El-Benna, J. (2015). TLR8, but not TLR7, induces the priming of the NADPH oxidase activation in human neutrophils. Journal of leukocyte biology. 97(6), 1081-1087. DOI: 10.1189/jlb.2A1214-623R.

Mayadas, T.N., Cullere, X., and Lowell, C.A. (2014). The multifaceted functions of neutrophils. Annual review of pathology. 9, 181-218. DOI: 10.1146/annurev-pathol-020712-164023.

Miranda-Verastegui, C., Llanos-Cuentas, A., Arevalo, I., Ward, B.J., and Matlashewski, G. (2005). Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antimoniate in the treatment of cutaneous leishmaniasis in Peru. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 40(10), 1395-1403. DOI: 10.1086/429238.

Miranda-Verastegui, C., Tulliano, G., Gyorkos, T.W., Calderon, W., Rahme, E., Ward, B., Cruz, M., Llanos-Cuentas, A., and Matlashewski, G. (2009). First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiquimod in combination with pentavalent antimony. PLoS neglected tropical diseases. 3(7), e491. DOI: 10.1371/journal.pntd.0000491.

Muraille, E., De Trez, C., Brait, M., De Baetselier, P., Leo, O., and Carlier, Y. (2003). Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. Journal of immunology. 170(8), 4237-4241.

Nauseef, W.M., and Borregaard, N. (2014). Neutrophils at work. Nature immunology. 15(7), 602-611. DOI: 10.1038/ni.2921.

Ordonez-Rueda, D., Jonsson, F., Mancardi, D.A., Zhao, W., Malzac, A., Liang, Y., Bertosio, E., Grenot, P., Blanquet, V., Sabrautzki, S., et al. (2012). A hypomorphic mutation in the Gfi1 transcriptional repressor results in a novel form of neutropenia. European journal of immunology. 42(9), 2395-2408. DOI: 10.1002/eji.201242589.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M.P., Germain, R.N., and Sacks, D. (2008). In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science. 321(5891), 970-974. DOI: 10.1126/science.1159194.

Prince, L.R., Whyte, M.K., Sabroe, I., and Parker, L.C. (2011). The role of TLRs in neutrophil activation. Current opinion in pharmacology. 11(4), 397-403. DOI: 10.1016/j.coph.2011.06.007.

Revaz-Breton, M., Ronet, C., Ives, A., Torre, Y.H., Masina, S., Tacchini-Cottier, F., and Launois, P. (2010). The MyD88 protein 88 pathway is differently involved in immune responses induced by distinct substrains of Leishmania major. European journal of immunology. 40(6), 1697-1707. DOI: 10.1002/eji.200939821.

Ribeiro-Gomes, F.L., Roma, E.H., Carneiro, M.B., Doria, N.A., Sacks, D.L., and Peters, N.C. (2014). Site-dependent recruitment of inflammatory cells determines the effective dose of Leishmania major. Infection and immunity. 82(7), 2713-2727. DOI: 10.1128/IAI.01600-13.

Roos, D., van Bruggen, R., and Meischl, C. (2003). Oxidative killing of microbes by neutrophils. Microbes and infection. 5(14), 1307-1315.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nature reviews. Immunology. 2(11), 845-858. DOI: 10.1038/nri933.

Sauvain, J.J., Setyan, A., Wild, P., Tacchini, P., Lagger, G., Storti, F., Deslarzes, S., Guillemin, M., Rossi, M.J., and Riediker, M. (2011). Biomarkers of oxidative stress and its association with the urinary reducing capacity in bus maintenance workers. Journal of occupational medicine and toxicology. 6(1), 18. DOI: 10.1186/1745-6673-6-18.

Schamber-Reis, B.L., Petritus, P.M., Caetano, B.C., Martinez, E.R., Okuda, K., Golenbock, D., Scott, P., and Gazzinelli, R.T. (2013). UNC93B1 and nucleic acid-sensing Toll-like receptors mediate host resistance to infection with Leishmania major. The Journal of biological chemistry. 288(10), 7127-7136. DOI: 10.1074/jbc.M112.407684.

Seeberger, J., Daoud, S., and Pammer, J. (2003). Transient effect of topical treatment of cutaneous leishmaniasis with imiquimod. International journal of dermatology. 42(7), 576-579.

Segal, A.W. (2005). How neutrophils kill microbes. Annual review of immunology. 23, 197-223. DOI: 10.1146/annurev.immunol.23.021704.115653.

Silva F, Gomes R, Prates D, Miranda JC, Andrade B, Barral-Netto M, Barral A. (2005). Inflammatory cell infiltration and high antibody production in BALB/c mice caused by natural exposure to Lutzomyia longipalpis bites. The american journal of tropical medicine and hygiene. 72(1), 2005, pp. 94–98.

Srivastava, S., Pandey, S.P., Jha, M.K., Chandel, H.S., and Saha, B. (2013). Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clinical and experimental immunology. 172(3), 403-409. DOI: 10.1111/cei.12074.

Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., Mukankundiye, C., Vasei, M., Launois, P., Milon, G., and Louis, J.A. (2000). An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major. Journal of immunology. 165(5), 2628-2636.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell. 140(6), 805-820. DOI: 10.1016/j.cell.2010.01.022.

Teixeira CR, Teixeira MJ, Gomes RB, Santos CS, Andrade BB, Raffaele-Netto I, Silva JS, Guglielmotti A, Miranda JC, Barral A, Brodskyn C, Barral-Netto M.. (2005) Saliva from Lutzomyia longipalpis induces CC chemokine ligand 2/monocyte chemoattractant protein-1 expression and macrophage recruitment. The journal of immunology. 175 (12) 8346-8353; DOI: https://doi.org/10.4049/jimmunol.175.12.8346.

Titus RG, Marchand M, Boon T, Louis JA. (1985) A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite immunology. 7(5):545-55.

van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., and Laskay, T. (2004). Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. Journal of immunology. 173(11), 6521-6525.

Vasilakos, J.P., and Tomai, M.A. (2013). The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert review of vaccines. 12(7), 809-819. DOI: 10.1586/14760584.2013.811208.

Zhang, W.W., and Matlashewski, G. (2008). Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against Leishmania major in BALB/c mice. Infection and immunity. 76(8), 3777-3783. DOI: 10.1128/IAI.01527-07.

7.2 Manuscript 2

Resistance of *Leishmania (Viannia) Panamensis* to Meglumine Antimoniate or Miltefosine Modulates Neutrophil Effector Functions[1]

Ivo B. Regli[1], Olga Lucía Fernández[2,3], Berenice Martínez-Salazar[1], Maria Adelaida Gómez[2,3], Nancy Gore Saravia[2,3] and Fabienne Tacchini-Cottier[1]*

[1]Department of Biochemistry, WHO-Immunology Research and Training Center, University of Lausanne, Epalinges, VD, Switzerland

[2]Centro Internacional de Entrenamiento e Investigaciones Médicas, Cali, Colombia

[3]Universidad ICESI, Cali, Colombia

- **Correspondence:**
  Prof. Fabienne Tacchni-Cottier
  Fabienne.Tacchini-Cottier@unil.ch

Keywords: *Leishmania*, neutrophils, drug resistance, miltefosine, antimony, NETs.

[1]Regli IB, Fernández OL, Martínez-Salazar B, Gómez MA, Saravia NG, Tacchini-Cottier F. Resistance of *Leishmania (Viannia) Panamensis* to Meglumine Antimoniate or Miltefosine Modulates Neutrophil Effector Functions. Front Immunol. 2018 Dec 21;9:3040. doi: 10.3389/fimmu.2018.03040. eCollection 2018

Abstract

Leishmania (Viannia) panamensis (L. (V.) p.) is the main causative agent of cutaneous leishmaniasis in Colombia and is usually treated with either meglumine antimoniate (MA) or miltefosine (MIL). In recent years, there has been increasing evidence of the emergence of drug resistance against these compounds. Neutrophils are known to play an important role in immunity against *Leishmania*. These cells are rapidly recruited upon infection and are also present in chronic lesions. However, their involvement in the outcome of infection with drug resistant *Leishmania* has not been examined. In this study, human and murine neutrophils were infected *in vitro* with MA or MIL drug resistant *L. (V.) p.* lines derived from a parental *L. (V.) p.* drug susceptible strain. Neutrophil effector functions were assessed analyzing the production of reactive oxygen species (ROS), the formation of neutrophil extracellular trap (NET) and the expression of cell surface activation markers. Parasite killing by neutrophils was assessed using *L. (V.) p.* transfected with a luciferase reporter. We show here that MA and MIL resistant *L. (V.) p.* lines elicited significantly increased NET formation and MA resistant *L. (V.) p.* elicited significantly increased ROS production in both murine and human neutrophils, compared to infections with the parental MIL and MA susceptible strain. Furthermore, neutrophils exposed to drug resistant lines showed increased activation, as revealed by decreased expression of CD62L and increased expression of CD66b in human neutrophils. In contrast, *L. (V.) p.* lines resistant to either MA or MIL presented higher survival within neutrophils than the drug susceptible strain.

These results provide evidence that parasite drug susceptibility influences neutrophil activation and function as well as parasite survival within neutrophils. Further studies on the impact of drug susceptibility on neutrophil effector function should contribute to better understanding of the factors involved in susceptibility to antileishmanial drugs.

Introduction

Cutaneous leishmaniasis is an important public health problem affecting 98 countries worldwide. Approximately 350 million people live in endemic areas and over 2 million new cases are reported annually (Alvar et al., 2012). In Colombia, *L. (V.) p.* is responsible for the majority of reported cases of cutaneous leishmaniasis (Ramirez et al., 2016) though *L. (V) braziliensis* may be more frequent among occupationally exposed military personnel (Patino et al., 2017). The first line of treatment in Latin America (Organización Panamericana de la Salud, 2013) and most of the world, consists of parenteral administration of meglumine antimoniate (MA) (Mitropoulos et al., 2010). Oral miltefosine (MIL) is generally administered as the second line treatment. In recent years, there has been an increasing number of reports of treatment failure and loss of susceptibility of clinical *Leishmania* strains to antimonial drugs, and association of resistance in some cases of therapeutic failure in leishmaniasis patients. The reported frequency of MA and MIL tolerance ranges from 10 to 70% and varies among *Leishmania* species and by geographic origin. The underlying mechanisms contributing to antileishmanial treatment failure in Colombia and the rest of the world are poorly understood (Goyeneche-Patino et al., 2008; Rojas et al., 2006; Obonaga et al., 2014).

It is well established that the outcome of antileishmanial treatment is not solely dependent on parasite drug susceptibility, but also on intrinsic host factors. The interplay between parasites, the host and the immune response is also a factor determining the clinical outcome of *Leishmania* infection and treatment (Croft et al., 2006; Uliana et al., 2018). There is currently a lack of information on the role of neutrophils in *Leishmania* infection and its impact on drug susceptibility. Recently, high levels of transcriptional signatures associated with chemokines promoting neutrophil recruitment were observed in *L. (V.) p.* infected primary macrophages and in chronic cutaneous lesions of CL patients, suggesting a potential role for neutrophils in the chronicity of infection (Navas et al., 2014). Neutrophils are essential phagocytes of the innate immune system and the most abundant leukocytes in human circulation (Nauseef and

Borregaard, 2014). They usually are the first cells recruited to the site of infection upon pathogen entry into a host (Branzk and Papayannopoulos, 2013). Neutrophils possess three major effector killing strategies: phagocytosis and subsequent production of reactive oxygen species (ROS), the release of granules that contain microbicidal proteins (Segal, 2005) and the formation of neutrophil extracellular traps (NETs). NETs are fibrous structures composed of dsDNA coated with a variety of different microbicidal proteins that can trap and kill a variety of pathogens (Brinkmann et al., 2004). Upon phagocytosis of pathogens, neutrophils produce different reactive oxygen species that are synthesized by the enzymatic activity of NADPH-synthase (Roos et al., 2003). Indeed, a lack of NADPH-synthase function manifests itself in higher susceptibility to a variety of infections and is known as chronic granulomatous disease (Thomas, 2018). Also a lack of Myeloperoxidase (MPO), an enzyme that catalyzes the synthesis of highly microbicidal hypochlorous acid, is thought to lead to increased susceptibility to infection (Winterbourn et al., 2016). Neutrophil granules can either fuse with pathogen containing phagosomes or be released into the extracellular space (Mayadas et al., 2014).

Increasing evidence points towards a crucial role of neutrophils in leishmaniasis (Ribeiro-Gomes and Sacks, 2012; Hurrell et al., 2016). It has been shown that neutrophils are massively recruited to the site of infection upon infection with *L. major* (Lee et al., 2018; Peters et al., 2008; Ribeiro-Gomes et al., 2014; Tacchini-Cottier et al., 2000), *L. amazonensis* (Sousa et al., 2014), *L. braziliensis* (Falcao et al., 2015), *L. mexicana* (Hurrell et al., 2015), *L. infantum* (Thalhofer et al., 2011) and *L. donovani* (Dey et al., 2018; McFarlane et al., 2011) and that a subset of *L. mexicana* can even use these cells to replicate (Hurrell et al., 2017). While neutrophils are well known to have a protective role in many infections they can have either beneficial or detrimental roles in leishmaniasis depending on the *Leishmania* spp. involved[192]. In most studies performed in murine models, the lack of neutrophils was beneficial to disease outcome in *Leishmania* infection. Furthermore, parasites are known to use these cells as "Trojan horses" to enter silently into the host macrophages (Laskay et al., 2003). In contrast, neutrophil

presence has been reported to positively affect disease outcome in the case of *L. braziliensis* (Souza-Lemos et al., 2008; Novais et al., 2009; Carlsen et al., 2015; Falcao et al., 2015), *L. amazonensis* (Tavares et al., 2014) and *L. donovani* infection (McFarlane et al., 2008).

Here, using human and mouse neutrophils, we have investigated *in vitro* the role of these cells in the context of antileishmanial drug resistance following *L. (V.) p.* infection. We found that *L. (V.) p.* lines with different drug susceptibility have different effects on neutrophil phenotype and functionality. Our data show that neutrophils are differentially activated by drug resistant and drug susceptible parasites and that drug resistant parasites are less affected by neutrophil killing than drug susceptible parasites. These differences in the elicitation of neutrophil function and parasite killing may influence antileishmanial drug therapy.

Materials and Methods

Mice

C57BL/6 mice (5-10 weeks old) were purchased from Envigo (Cambridgeshire, United Kingdom) and bred under specified pathogen-free conditions at the animal facility of the University of Lausanne in Epalinges.

Ethics statement

All procedures involving human blood samples were approved by the Ethical Committee of the Canton of Vaud (CER-VD 2017-00182). Written informed consent was obtained from the healthy blood donors participating in this study. The study was conducted in compliance with the legislation of the Canton of Vaud and the Swiss Confederation as well as the declaration of Helsinki. Animal experimentation protocols were approved by the veterinary office of the Canton of Vaud (Authorization 1266.6-7 to F.T-C.) and were done in accordance to cantonal and federal legislation as well as the principles of the declaration of Basel. This study and the use of patient derived *Leishmania* strains and lines was approved by the Ethics Committees of

CIDEIM- Colombia, in accordance with the national and international guidelines for Good Clinical Practice.

Leishmania (Viannia) panamensis parasites

The *L. (V.) p.* strain (MHOM/COL/86/1166LUC) WT-control was used to generate the strains resistant to potassium antimony (III) tartrate hydrate and miltefosine as described previously (Brochu et al., 2003). Briefly, *L. (V.) p.* promastigotes were cultured in media containing increasing concentrations of drugs, either up to 2885 μM Sb or up to 60 μM of miltefosine. Upon establishment of drug resistance, the strains were cultured in RPMI (Gibco) with 10% FCS and 1% PSN at 26°C. Antimony resistant parasites were cultured in medium containing 1 mM Sb. Miltefosine resistant strains were cultured in medium containing 60 μM miltefosine. Luciferase expressing strains were grown in medium containing 60 μM G418 (Sigma)

SNARF-1 staining of *Leishmania (Viannia) panamensis parasites*

Parasites were stained with the intracellular dye SNARF-1 (Thermofisher) to render them fluorescent. Parasites were incubated in 6 μM solution of SNARF-1 in PBS for 30 minutes and subsequently washed twice. SNARF-1 stained fluorescent parasites were detected at an excitation wavelength of 488 nm and an emission wavelength of 610 nm.

Drug susceptibility screening

Drug susceptibility of parasites was measured as a reduction of intracellular *L. (V.) p.* amastigote burden in macrophages derived from a U-937 cell line as described previously (Fernandez et al., 2012). Briefly, 1.2×10^5 U-937 cells were treated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma) to differentiate them into macrophages. Cells were cocultured with *L. (V.) p.* promastigotes opsonized with 10% AB positive human serum at a MOI of 5:1 and incubated for 24 hours at 34°C. Subsequently, the culture medium was replaced

with RPMI containing either 16 µM miltefosine or 32 µg/ml Sb(V) and the cells were incubated for another 48 hours. The Sb-containing medium was replaced after 24 hours whereas the miltefosine-containing medium was not replaced during the 48 hours of incubation. Parasite burden was assessed by light microscopy in four replicates by 2 microscopists in a blinded manner. The cut-offs defining sensitive respectively resistant strains were based on a previously published analysis (Fernandez et al., 2012).

Isolation metacyclic *Leishmania (Viannia) panamensis* parasites

Metacyclic *L. (V.) p.* parasites were isolated using a Percoll (GE Healthcare) density gradient. Parasites were resuspended in 45% Percoll-medium suspension and layered onto 60% and 90% Percoll-medium suspension phases. After centrifugation without brakes at 4300 xg for 45 minutes, metacyclic parasites were isolated from the 45%/60% Percoll-medium suspension interphase (Castilho et al., 2010). The parasites were washed twice and counted using a Neubauer chamber.

Human neutrophil isolation

Peripheral blood neutrophils were isolated from venous blood of healthy volunteers. Density gradient centrifugation using PolymorphPrep (Progen) was performed and the enriched neutrophils were isolated according to the manufacturer's instructions. The remaining red blood cells were lysed using ACK-Buffer. Neutrophil purity was assessed using Cyto-Spin and Quick-Fix staining and was established to be ≥ 95%.

Murine neutrophil isolation

Bone marrow of the femora and tibia of C57BL/6 mice were flushed with RPMI. Erythrocytes were lysed with ACK buffer, the remaining leukocytes were washed and the neutrophils were then isolated by negative MACS using the neutrophil isolation kit (Miltenyi Biotec) according

to the manufacturer's indications.

Assessment of NET formation

$3x10^5$ Neutrophils were seeded on poly-L-Lysine coated coverslips, primed with 25ng/mL GM-CSF and exposed for 4 hours with either *L. (V.) p., L. major,* PMA (Phorbol-12-myristate-13-acetate, Sigma), PMA + DNase (Sigma) or without a stimulus. Subsequently, cells were fixed with 4% PFA (Paraformaldehyde, Sigma) and stained with rabbit anti-human MPO (Dako) primary antibody and Alexa Fluor-488 goat anti-rabbit secondary antibody (Life Technologies). Coverslips were mounted on glass slides using a DAPI containing mounting medium (Molecular Probes) and analyzed by confocal microscopy (ZEISS LSM 510). To quantify NET formation, NETs were counted using fluorescence microscopy in a blinded manner based, at least in three replicates. NET formation was also assessed through the measurement of dsDNA in the supernatant using the Quant-iT PicoGreen kit (Thermo Fisher) by adapting a technique previously described (Amini et al., 2016). $2x10^6$ neutrophils were incubated for 4 hours in X-vivo medium (Lonza) with either *L. (V.) p.*, PMA or PMA + DNase or without stimulus. After incubation, the cell suspensions were centrifuged, and the supernatants were collected and transferred into black 96-well plates (Perkin Elmer). The picogreen dye was added and fluorescence was measured in using a plate reader (Molecular Devices, SpectraMax MiniMax 300) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Measurement of reactive oxygen species production

ROS production was measured using a luminol-based chemiluminescence assay. $5x10^5$ neutrophils were incubated with either *L. (V.) p.*, PMA or without stimulus in white, opaque 96-well plates (Perkin Elmer). Luminol (Carbosynth) was added at a final concentration of 20 µg/mL. ROS induced chemiluminescence was measured at all wavelengths with a plate reader

at 5 minutes intervals during 1 hour.

Parasite viability measurement with luciferase assay

$5x10^5$ neutrophils were infected with luciferase-expressing *L. (V.) p.* strains with different drug susceptibility phenotypes during 2 hours at 34°C at a MOI of 5, washed and subsequently incubated for 24 hours. Parasite viability after incubation was measured using a luciferase assay system (Promega) according to the manufacturer's instructions. Chemiluminescence was measured in white, opaque 96-well plates in a plate reader (Molecular Devices, SpectraMax MiniMax 300) at all wavelengths.

Identification of neutrophil cell surface markers and measurement of cell viability by flow cytometry

Surface activation markers and cell viability markers in blood derived human neutrophils were analyzed using Flow Cytometry analyzer of either the BD LSR II or the BD LSR Fortessa series (Becton Dickinson) and analyzed with FlowJo software (Tree Star). The following antibodies were used: Anti-human: CD15-APC, CD62L-PerCP-efluor710, CD66b-PE-Cy7. Anti-mouse: Ly6G(1A8)-APC-Cy7, CD62L-PE, CD11b-PE-Cy7 (all from e-Bioscience). Cell viability: Live/Dead fixable Aqua Dead Cell Stain Kit (Invitrogen).

Results

Increased reactive oxygen species production by murine and human neutrophils challenged with miltefosine resistant *Leishmania (Viannia) panamensis* strains

To investigate the contribution of neutrophils in the intracellular survival of *Leishmania* during drug exposure, we first investigated whether *L. (V.) p*. strains susceptible or resistant to MA or

MIL would induce different phenotype and function in neutrophils. The production of ROS by neutrophils is known to be an important defense mechanism against pathogens (Nguyen et al., 2017) and also contribute to NET formation (Papayannopoulos, 2015).

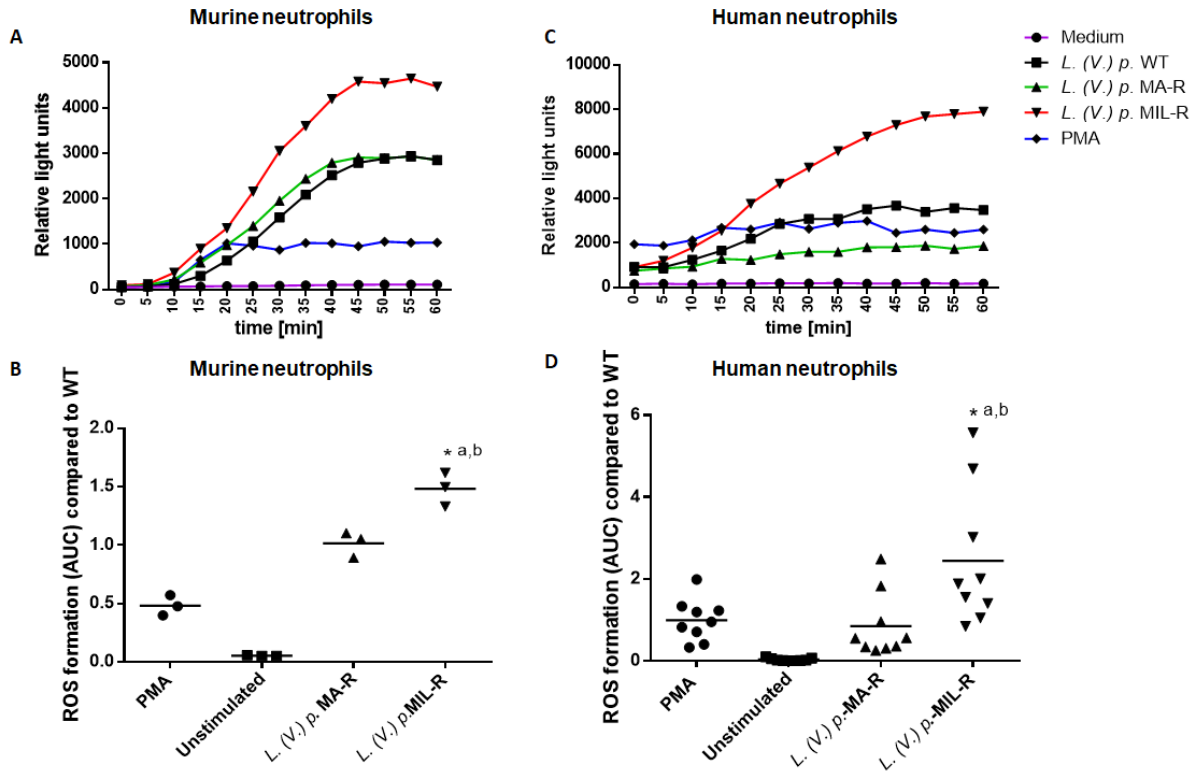

Figure 1. MIL resistant *L. (V.) p.* parasites induce more ROS production in murine and human neutrophils than the MA resistant or drug susceptible strains. ROS production was measured using a luminol based chemiluminescence assay. Bone marrow derived murine neutrophils and blood-derived human neutrophils from healthy donors were incubated with either MIL resistant, MA resistant or susceptible *L. (V.) p.*, PMA or without stimulus. Luminol was added and ROS induced chemiluminescence was measured with a plate reader every 5 minutes during 1 hour. **A,C)** One representative curve of ROS-induced luminol chemiluminescence values in murine (**A**) and human (**C**) neutrophils. **B,D)** The corresponding area under the curve (AUC) of 60 minutes of measurement for murine (**B**) and human (**D**) neutrophils. * $p<0.05$, *a MIL-R compared to WT, *b MIL-R compared to MA-R, WT: Wild type, MA-R: Resistant to meglumine antimoniate, MIL-R: Resistant to miltefosine, the data are pooled out of three (human) or two (mouse) independent experiments.

Therefore, we analyzed ROS production during the first hour of incubation of neutrophils with the different *L. (V.) p.* populations. Parasites that are resistant to miltefosine induced 1.5 times more ROS production compared to drug susceptible parasites in BM-derived murine neutrophils (**Figure 1 A,B**) and more than two times as much ROS production than drug susceptible parasites in blood-derived human neutrophils from healthy donors (**Figure 1 C,D**). In contrast, the induction of ROS by antimony resistant parasites and drug susceptible *L. (V.)*

p. strains in neutrophils did not differ from that of drug susceptible parasites. Even though all *L. (V.) p.* strains induced ROS in neutrophils, these results show that the drug susceptibility phenotype, specifically MIL resistance, influences neutrophil ROS production.

Drug resistant *Leishmania (Viannia) panamensis* induce more neutrophil extracellular trap formation than drug susceptible strains in murine and human neutrophils

Next, to visualize NETs, neutrophils were infected with *L. (V.) p.* of different drug susceptibility and NET formation analyzed by confocal microscopy. NETs were stained with DAPI to detect DNA filaments and an MPO mAb to detect the MPO associated with it. All parasite populations tested induced NET formation in both murine and human neutrophils as observed by DNA-MPO colocalization (**Figure 2A**). MIL and MA resistant *L. (V.) p.* induced substantially more NETs (two to three times) in murine and human neutrophils compared to drug susceptible parasites (**Figure 2 B-C**).

We observed parasites in association with the NETs, a process more easily detectable in human NETs. To confirm these results, we used the picogreen assay that measures the amount of dsDNA released by neutrophils into the supernatant (Amini et al., 2016). We again observed that drug resistant *L. (V.) p.* parasites induced a significantly higher amount of dsDNA release compared to drug susceptible parasites in human and murine neutrophils (**Figure 3**). Collectively, these results demonstrate that MIL and MA resistant *L. (V.) p* lines induce more NET formation than the parental drug susceptible strain.

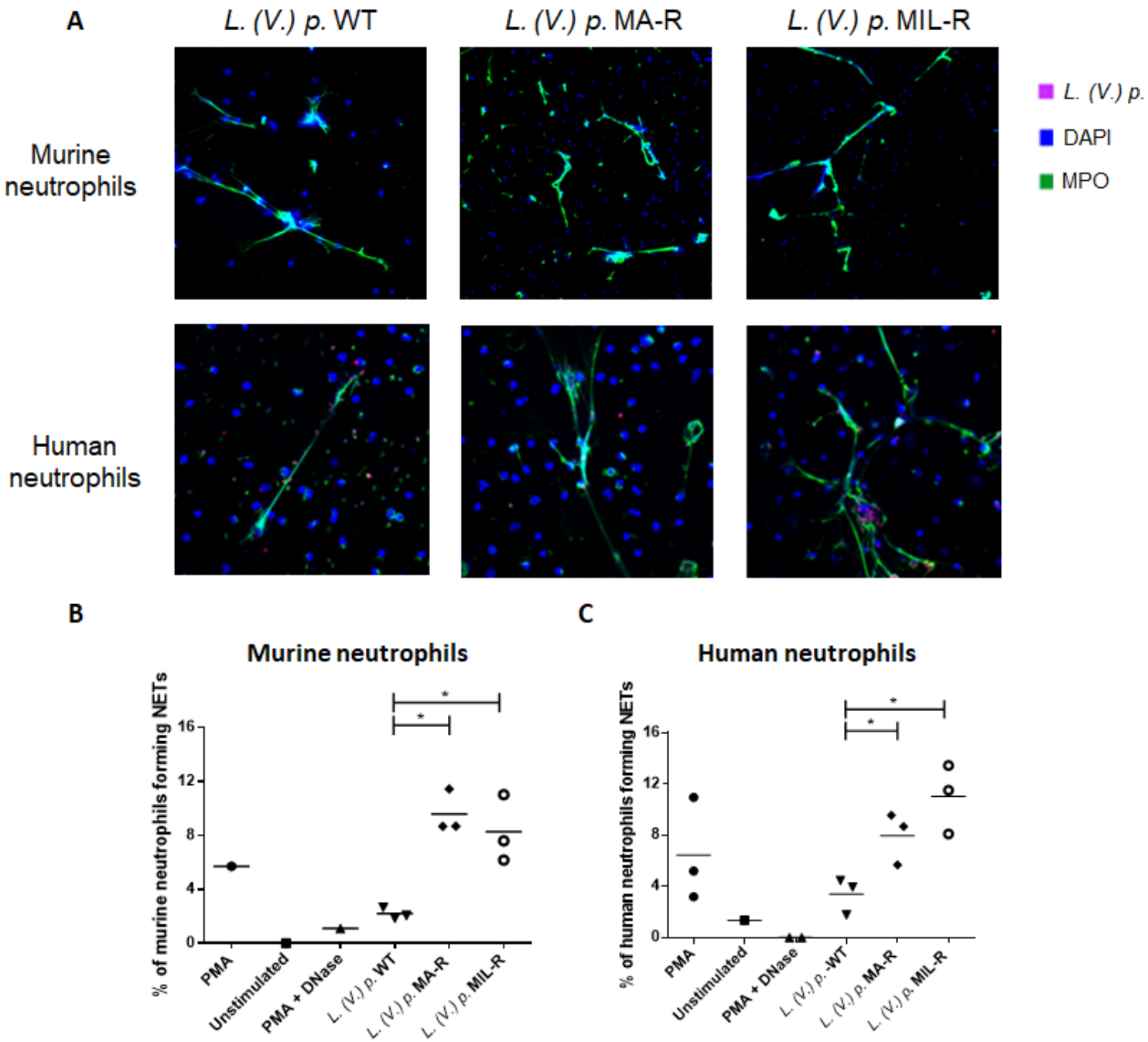

Figure 2. Murine and human neutrophils form more NETs when exposed to *L. (V) p.* strains that are resistant to either MA or MIL. Neutrophils were seeded on poly-L-Lysine coated coverslips and exposed for 4 hours with either *L. (V.) p.* of the indicated drug susceptibility, PMA, PMA + DNase or without stimulus. Subsequently, cells were fixed, stained with anti-human MPO and DAPI **A)** Confocal microscopy images of murine or human neutrophils exposed to SNARF-1 labeled *L. (V.) p.* promastigotes lines that were either resistant or susceptible to MA or MIL at parasite-cell MOI of 5. **B-C).** The frequency of murine (**B**) or human (**C**) neutrophils forming NETs was counted with a fluorescence microscope. * $p<0.05$, each point corresponds to one healthy blood donor or one mouse. One representative experiment out of four is shown.

Modulation of neutrophil cell surface activation markers by *Leishmania (Viannia) panamensis* of different drug susceptibility phenotypes

We next investigated if exposure of neutrophils to *L. (V.) p*. of different drug susceptibility would result in distinct modulations of cell surface markers. Using flow cytometry, we observed that BM derived murine neutrophils exposed to MIL and MA drug resistant *L. (V.) p* lines expressed more CD11b (Mac-1) and CD62L (L-selectin) on their surface than neutrophils exposed to drug susceptible *L. (V.) p.* (**Figure 4**).

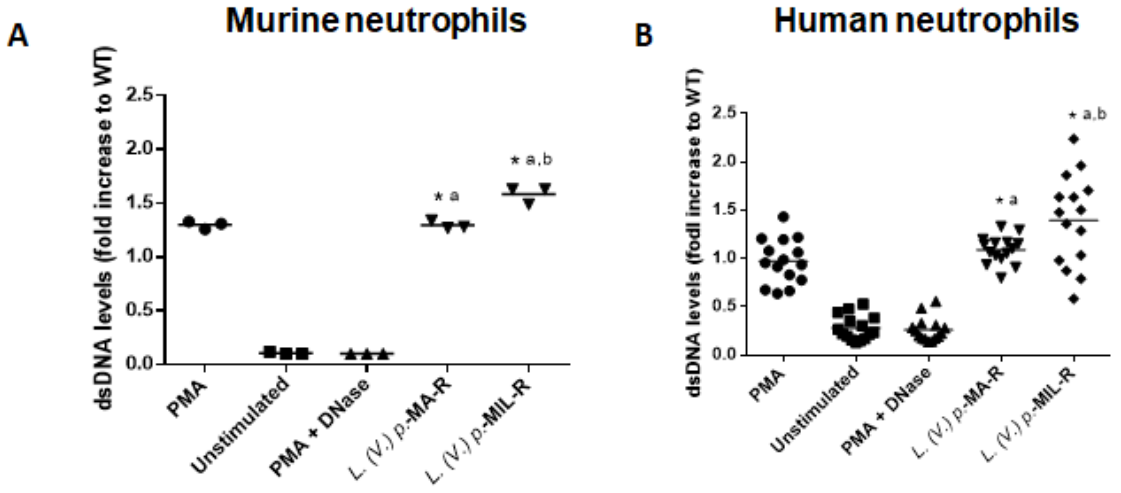

Figure 3. Murine and human neutrophils release more dsDNA when exposed to *L. (V) p.* strains that are resistant to either MA or MIL. Bone marrow derived murine neutrophils **(A)** and blood derived human neutrophils from healthy donors (**B**) were exposed to *L. (V.) p.* of the indicated susceptibility at a MOI of 5. As controls, incubations with PMA, PMA + DNase or without stimulus were also performed. Four hours later, NET formation was quantified by measuring the levels of double stranded DNA (dsDNA) released in the supernatant using the PicoGreen fluorescent dye assay. * $p<0.05$, *a MA-R or MIL-R compared to WT, *b MIL-R compared to MA-R, Each point corresponds to one healthy blood donor or one mouse. One representative experiment out of four (human) and three (mice) independent experiments is shown.

CD62L and CD66b (exocytosis of secondary granules) expression by human neutrophils derived from the blood of healthy individuals exposed to drug susceptible and resistant *L. (V.) p* revealed that MA and MIL resistant parasites elicited a decrease in expression of CD62L and an increase in expression of CD66b (**Figure 5**). These data show that both human and murine neutrophils exposed to drug resistant *L. (V.) p.* lines are activated to a greater extent and have a phenotype that is more associated with extravasation, compared to neutrophils challenged with drug susceptible *L. (V.) p.*

Drug resistant *Leishmania (Viannia) panamensis* strains are more resistant to neutrophil parasite killing 24 hours after infection

As we observed differences in ROS production, NET formation and neutrophil activation in neutrophils exposed to *L. (V.) p.* of different drug susceptibility phenotypes, we assessed whether this would have an impact on parasite killing.

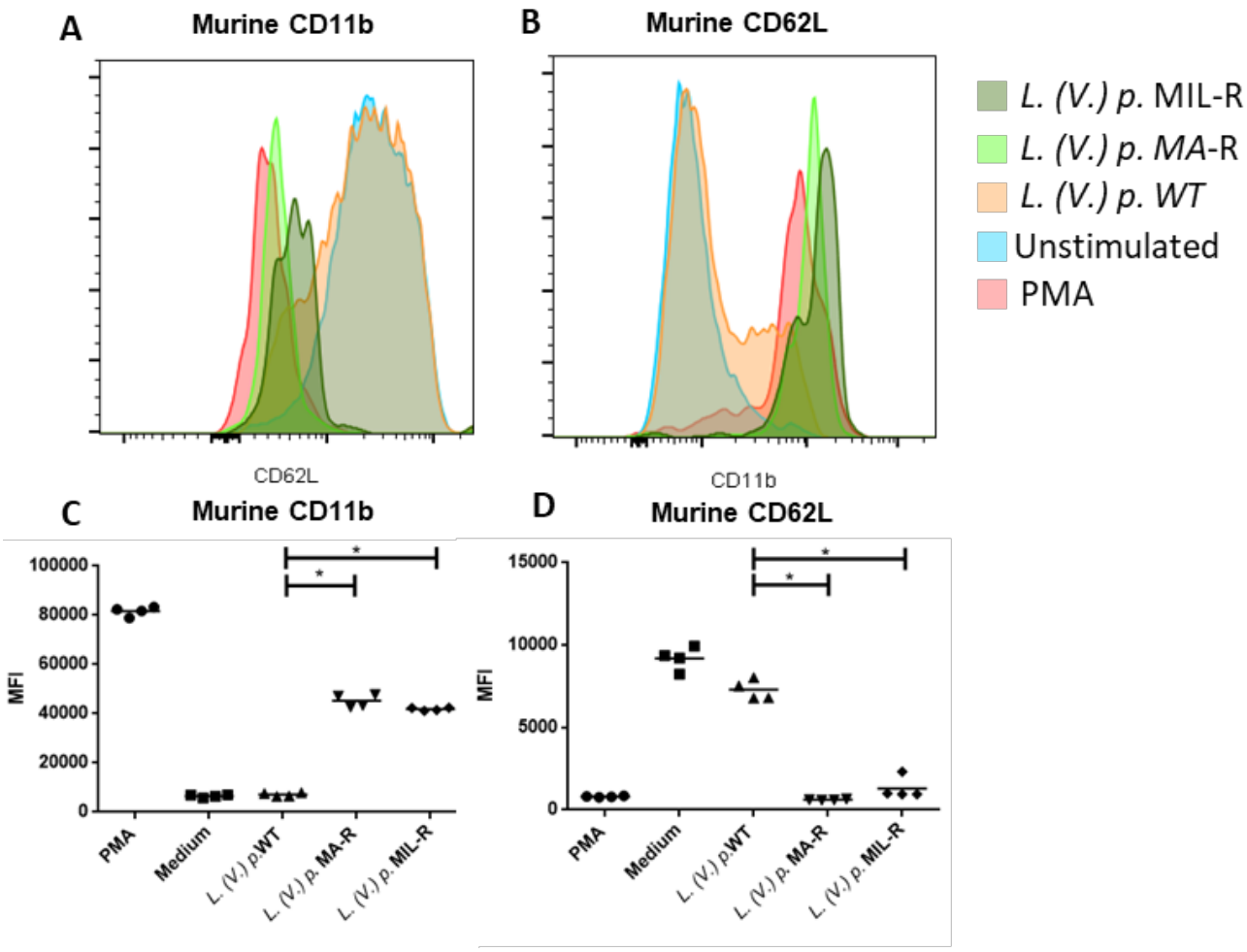

Figure 4. Expression of cell surface activation markers of murine neutrophils in response to exposure to *L. (V.) p.* promastigotes with different drug susceptibility phenotypes. Bone marrow derived murine neutrophils were exposed to the indicated *L. (V.) p.* lines at a MOI of 10 or incubated without stimulus for 90 minutes. Incubation with PMA was used as a positive control. Neutrophils were then stained with the CD11b and CD62L mAbs and surface expression was analyzed by flow cytometry. **A-B)** A representative flow cytometry plot of normalized fluorescence values of murine neutrophils stained with antibodies against CD11b and CD62L is shown. **C-D)** MFI values of murine neutrophils stained with antibodies against CD62L and CD11b. WT: Wild type, MA-R: Resistant to meglumine antimoniate, MIL-R: Resistant to miltefosine. The data is representative of three experiments. * $p<0.05$

Luciferase expressing *L. (V.) p.* lines of the indicated drug susceptibility phenotypes were exposed to murine or human neutrophils for 24 hours and parasite viability was analyzed by measuring luciferase activity.

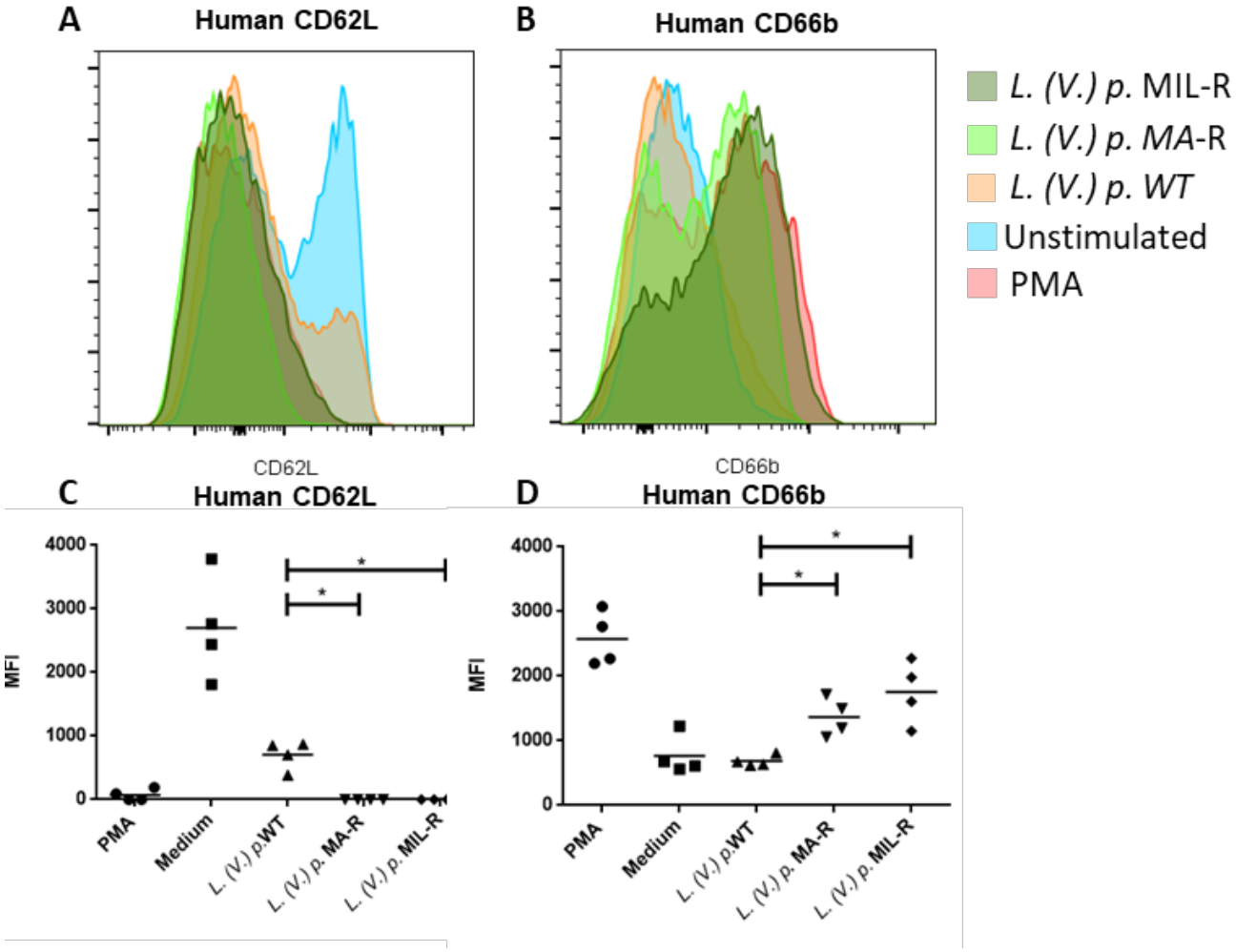

Figure 5. Expression of surface activation markers of human neutrophils in response to exposure to *L. (V.) p.* metacyclic promastigotes of different drug susceptibility phenotypes. Blood-derived neutrophils from healthy donors were exposed to the indicated *L. (V.) p.* lines at a MOI of 10 or incubated without stimulus for 90 minutes. Incubation with PMA was used as a positive control. Neutrophils were then stained with the CD62L and CD66b mAbs and surface expression was analyzed by flow cytometry. **A-B)** One representative flow cytometry plot of normalized fluorescence values of human neutrophils stained with antibodies against CD62L and CD66b is shown **C-D)** MFI values of human neutrophils stained with antibodies against CD62L and CD11b. WT: Wild type, MA-R: Resistant to meglumine antimoniate, MIL-R: Resistant to miltefosine. The data is representative of a total of five experiments. * $p<0.05$

Drug resistant parasites were significantly more resistant to neutrophil killing by murine and human neutrophils than the drug susceptible strain (**Figure 6**). Taken together, our data

demonstrate that, in both murine and human neutrophils, drug resistant *L. (V.) p.* lines induce more mechanisms known to kill pathogens than the drug susceptible parasites, yet are better able to resist neutrophil killing.

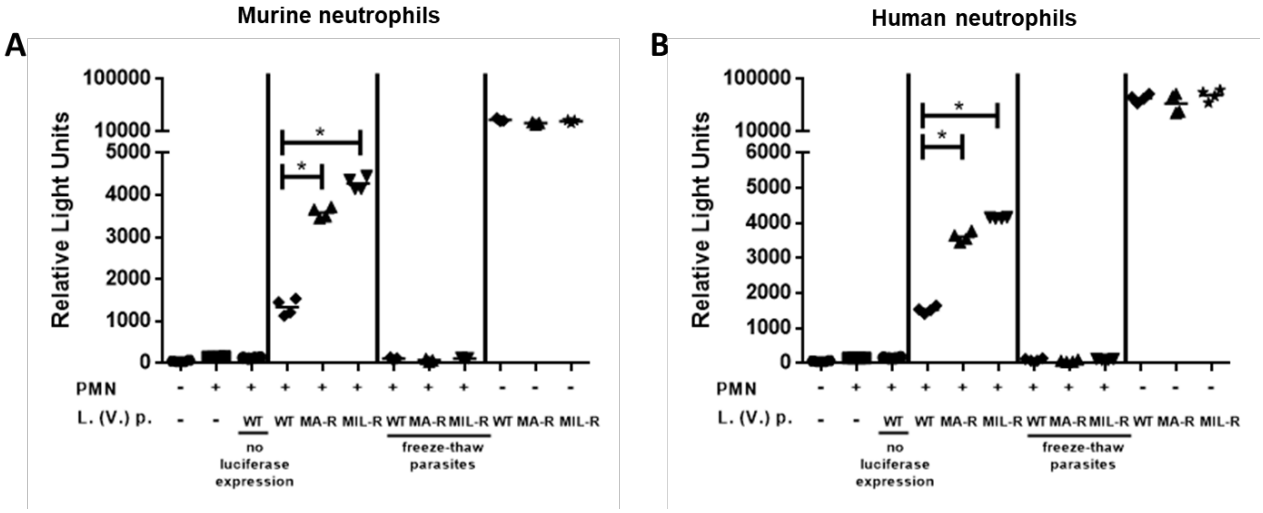

Figure 6. Killing of *L. (V.) p.* of different drug susceptibilities by murine and human neutrophils. Neutrophils were infected with luciferase expressing *L. (V.) p.* lines with different drug susceptibility phenotypes for 2 hours at 34°C, washed and further incubated for 24 hours prior to analysis. Parasite viability after incubation was measured using a luciferase assay system. Murine (**A**) and human (**B**) neutrophils were exposed to luciferase expressing *L. (V.) p.* of the indicated drug susceptibility at a MOI of 5. 24 hours later chemiluminescence was measured represented as relative light units and parasite killing was assessed MIL-R: Resistant to miltefosine, MA-R: Resistant to meglumine antimoniate, freeze-thaw parasites were killed by 10 cycles of freezing and subsqequent thawing, PMN: Neutrophils, One representative experiment is shown out of four.

Discussion

Antileishmanial drug resistance is an increasing concern in Colombia, but also worldwide (Azeredo-Coutinho et al., 2007; Bilbao-Ramos et al., 2017; Fernandez et al., 2014). In India, miltefosine has replaced antimony as the first line of treatment against visceral leishmaniasis due to widespread evidence of antimony resistance (Vanaerschot et al., 2011). However, the efficacy of miltefosine has also dropped considerably during the first ten years following its introduction (Rijal et al., 2013; Sundar et al., 2013; Sundar and Chakravarty, 2012). It is crucial to consider that parasite drug resistance is not equal to clinical treatment failure. Therapeutic response is also determined by host factors, such as previous infection, comorbidities, nutritional status, pharmacokinetics etc. (Castro et al., 2017).

Nevertheless, drug resistance is a very important determinant of treatment failure, thus understanding how drug resistance phenotype of *L. (V.) p.* impacts the antileishmanial immune response is relevant to the understanding of the potential role of neutrophils in therapeutic outcome.

Here, we have demonstrated for the first time that *L. (V.) p.* parasites having different drug susceptibility phenotypes elicit distinct neutrophil effector functions, shown by a higher induction of ROS production, NET formation and the expression of surface markers characteristic of neutrophil activation. ROS production is a crucial microbicidal mechanism of neutrophils, but the impact of ROS on *Leishmania* survival is species and host dependent (Regli et al., 2017). Here, we showed that *L. (V.) p.* induces ROS production in murine and human neutrophils and that ROS production is increased when murine and human neutrophils are infected with the miltefosine resistant *L (V.) p.* compared to the drug susceptible parasites. It has been reported that ROS production is critical for the induction of ROS-dependent NET formation (Branzk et al., 2014). Activation and nuclear translocation of neutrophil elastase are crucial in ROS-dependent NET formation, reviewed in (Papayannopoulos, 2018) and (Brinkmann, 2018). However, there is also an NOX-independent pathway of NET formation that does not depend on ROS (Manfredi et al., 2018). *L. amazonensis* has been reported to induce ROS-independent and ROS-dependent NET formation. In our study, we show that MA resistant and MIL resistant *L. (V.) p.* induce more NET formation than drugg susceptible strain, however only MIL resistant *L. (V.) p.* induced an increased ROS production. These data suggests that the NET formation induced by MA resistant *L. (V.) p.* may be partly ROS-independent.

Neutrophil effector functions contribute to microbial destruction in most infections, however, their role in leishmaniasis varies, depending on host factors and the *Leishmania* spp. involved (Hurrell et al., 2016; Regli et al., 2017). We observed *L. (V.) p.* parasites associated with the filamentous structures of NETs, which substantiates parasite trapping by these structures. Most

Leishmania spp. are trapped by NETs. Some, such as *L. mexicana* (Hurrell et al., 2015), *L. donovani* (Gabriel et al., 2010) *or L. infantum* (Guimaraes-Costa et al., 2014) survive NET exposure, while others such as *L. amazonensis* (Guimaraes-Costa et al., 2009; Guimaraes-Costa et al., 2014; Rochael et al., 2015) are killed by NETs, at least in their promastigote stage. The higher survival of drug resistant parasites despite induction of greater ROS and NET response suggests that these *L. (V.) p.* lines resist better to neutrophil killing than *L. (V.) p*. susceptible strains. This is in line with studies performed on macrophages, reporting that MA as well as MIL resistant *Leishmania donovani* strains were more resistant to ROS mediated killing due to increased intracellular thiol-levels. (Das et al., 2013; Deep et al., 2017 ; Mandal et al., 2007; Mishra and Singh, 2013 ; Mittal et al., 2007). Neutrophil recruitment and increased neutrophil activation are known to play a key role in many inflammatory diseases (Delgado-Rizo et al., 2017; Yang et al., 2016). Our results demonstrate that there is an increase in neutrophil activation upon stimulation by drug resistant *L. (V.) p.* compared to drug susceptible parasites. It is therefore conceivable that treatment failure may result at least in part due to the increased resistance of *L. (V.) p.* strains to neutrophil effector functions. An increase in parasite survival would then lead to an increase of inflammation and in turn would lead to more neutrophil recruitment and activation, and potentially constitute a self-sustaining circle.

In this study, we used laboratory-derived *L. (V.) p.* drug resistant lines. Since there are differences in the drug resistance mechanisms of parasites that develop drug resistance *in vivo* compared to those experimentally selected for drug resistance *in vitro* (Ashutosh et al., 2007; Goyeneche-Patino et al., 2008) the assessment of the interaction of neutrophils and *L. (V.) p.* with different drug susceptibility phenotypes that have been isolated from patients in a clinical setting should further elucidate of the role of the interaction of *L. (V.) p.* and neutrophils in antileishmanial therapy. Also, the study of the *in vivo* immune response, notably of neutrophils, against those parasites would contribute to better understanding of the role the immune system plays in antileishmanial treatment outcome.

Further understanding of the impact of drug resistant parasites on neutrophil function, including the analysis ROS production and NET formation *ex vivo* (e.g. from lesion biopsies or patient blood samples), should be further investigated to assess if this neutrophil activation could constitute be used a surrogate marker for predicting the expected treatment outcome success in a given patient.

Declaration of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

IR and FTC conceived the experiments. NGS, OF and MAG contributed to the design and interpretation of the experiments and provided and authenticated the *L. (V.) p.* strains and lines of defined drug susceptibility phenotype. IR, OF and BMS performed the experiments and analyzed the data. IR wrote the manuscript, FTC, OF, BMS, MAG and NGS contributed to and critically reviewed the manuscript.

Acknowledgements

We thank Yazmin Hauyon-La Torre and Maryori Vidarte for technical assistance, Dr. Benjamin Hurrell for discussion, CIDEIM personnel in Calí, Colombia, the Cellular Imaging Facility and the Flow Cytometry Facility of the University of Lausanne, Switzerland.

Funding

This work was supported by a bilateral research collaboration between Switzerland and Colombia (Seed money grant and Merger grant) from the leading house for the Latin American Region, and in part by NIH/NIAID Tropical Medicine Research Centers (TMRC) grant U19AI129910.

References

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al. (2012).Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7(5), e35671. doi: 10.1371/journal.pone.0035671.

Amini, P., Stojkov, D., Wang, X., Wicki, S., Kaufmann, T., Wong, W.W., et al. (2016). NET formation can occur independently of RIPK3 and MLKL signaling. Eur J Immunol 46(1), 178-184. doi: 10.1002/eji.201545615.

Ashutosh, Sundar, S., and Goyal, N. (2007). Molecular mechanisms of antimony resistance in Leishmania. J Med Microbiol 56(Pt 2), 143-153. doi: 10.1099/jmm.0.46841-0.

Azeredo-Coutinho, R.B., Mendonca, S.C., Callahan, H., Portal, A.C., and Max, G. (2007). Sensitivity of Leishmania braziliensis promastigotes to meglumine antimoniate (glucantime) is higher than that of other Leishmania species and correlates with response to therapy in American tegumentary leishmaniasis. J Parasitol 93(3), 688-693. doi: 10.1645/GE-1031R.1.

Bilbao-Ramos, P., Dea-Ayuela, M.A., Cardenas-Alegria, O., Salamanca, E., Santalla-Vargas, J.A., Benito, C., et al. (2017). Leishmaniasis in the major endemic region of Plurinational State of Bolivia: Species identification, phylogeography and drug susceptibility implications. Acta Trop 176, 150-161. doi: 10.1016/j.actatropica.2017.07.026.

Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., et al. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol 15(11), 1017-1025. doi: 10.1038/ni.2987.

Branzk, N., and Papayannopoulos, V. (2013). Molecular mechanisms regulating NETosis in infection and disease. Semin Immunopathol 35(4), 513-530. doi: 10.1007/s00281-013-0384-6.

Brinkmann, V. (2018). Neutrophil Extracellular Traps in the Second Decade. J Innate Immun, 1-8. doi: 10.1159/000489829.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., et al. (2004). Neutrophil extracellular traps kill bacteria. Science 303(5663), 1532-1535. doi: 10.1126/science.1092385.

Brochu, C., Wang, J., Roy, G., Messier, N., Wang, X.Y., Saravia, N.G., et al. (2003). Antimony uptake systems in the protozoan parasite Leishmania and accumulation differences in antimony resistant parasites. Antimicrob Agents Chemother 47(10), 3073-3079.

Carlsen, E.D., Jie, Z., Liang, Y., Henard, C.A., Hay, C., Sun, J., et al. (2015). Interactions between Neutrophils and Leishmania braziliensis Amastigotes Facilitate Cell Activation and Parasite Clearance. J Innate Immun 7(4), 354-363. doi: 10.1159/000373923.

Castilho, T.M., Goldsmith-Pestana, K., Lozano, C., Valderrama, L., Saravia, N.G., and McMahon-Pratt, D. (2010). Murine model of chronic L. (Viannia) panamensis infection: role of IL-13 in disease. Eur J Immunol 40(10), 2816-2829. doi: 10.1002/eji.201040384.

Castro, M.D.M., Cossio, A., Velasco, C., and Osorio, L. (2017). Risk factors for therapeutic failure to meglumine antimoniate and miltefosine in adults and children with cutaneous leishmaniasis in Colombia: A cohort study. PLoS Negl Trop Dis 11(4), e0005515. doi: 10.1371/journal.pntd.0005515.

Croft, S.L., Sundar, S., and Fairlamb, A.H. (2006). Drug resistance in leishmaniasis. Clin Microbiol Rev 19(1), 111-126. doi: 10.1128/CMR.19.1.111-126.2006.

Delgado-Rizo, V., Martinez-Guzman, M.A., Iniguez-Gutierrez, L., Garcia-Orozco, A., Alvarado-Navarro, A., and Fafutis-Morris, M. (2017). Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. Front Immunol 8, 81. doi: 10.3389/fimmu.2017.00081.

Dey, R., Joshi, A.B., Oliveira, F., Pereira, L., Guimaraes-Costa, A.B., Serafim, T.D., et al. (2018). Gut Microbes Egested during Bites of Infected Sand Flies Augment Severity of Leishmaniasis via Inflammasome-Derived IL-1beta. Cell Host Microbe 23(1), 134-143 e136. doi: 10.1016/j.chom.2017.12.002.

Falcao, S.A., Weinkopff, T., Hurrell, B.P., Celes, F.S., Curvelo, R.P., Prates, D.B., et al. (2015). Exposure to Leishmania braziliensis triggers neutrophil activation and apoptosis. PLoS Negl Trop Dis 9(3), e0003601. doi: 10.1371/journal.pntd.0003601.

Fernandez, O., Diaz-Toro, Y., Valderrama, L., Ovalle, C., Valderrama, M., Castillo, H., et al. (2012). Novel approach to in vitro drug susceptibility assessment of clinical strains of Leishmania spp. J Clin Microbiol 50(7), 2207-2211. doi: 10.1128/JCM.00216-12.

Fernandez, O.L., Diaz-Toro, Y., Ovalle, C., Valderrama, L., Muvdi, S., Rodriguez, I., et al. (2014). Miltefosine and antimonial drug susceptibility of Leishmania Viannia species and populations in regions of high transmission in Colombia. PLoS Negl Trop Dis 8(5), e2871. doi: 10.1371/journal.pntd.0002871.

Gabriel, C., McMaster, W.R., Girard, D., and Descoteaux, A. (2010). Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol 185(7), 4319-4327. doi: 10.4049/jimmunol.1000893.

Goyeneche-Patino, D.A., Valderrama, L., Walker, J., and Saravia, N.G. (2008). Antimony resistance and trypanothione in experimentally selected and clinical strains of Leishmania panamensis. Antimicrob Agents Chemother 52(12), 4503-4506. doi: 10.1128/AAC.01075-08.

Guimaraes-Costa, A.B., DeSouza-Vieira, T.S., Paletta-Silva, R., Freitas-Mesquita, A.L., Meyer-Fernandes, J.R., and Saraiva, E.M. (2014). 3'-nucleotidase/nuclease activity allows Leishmania parasites to escape killing by neutrophil extracellular traps. Infect Immun 82(4), 1732-1740. doi: 10.1128/IAI.01232-13.

Guimaraes-Costa, A.B., Nascimento, M.T., Froment, G.S., Soares, R.P., Morgado, F.N., Conceicao-Silva, F., et al. (2009). Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A 106(16), 6748-6753. doi: 10.1073/pnas.0900226106.

Hurrell, B.P., Beaumann, M., Heyde, S., Regli, I.B., Muller, A.J., and Tacchini-Cottier, F. (2017). Frontline Science: Leishmania mexicana amastigotes can replicate within neutrophils. J Leukoc Biol 102(5), 1187-1198. doi: 10.1189/jlb.4HI0417-158R.

Hurrell, B.P., Regli, I.B., and Tacchini-Cottier, F. (2016). Different Leishmania Species Drive Distinct Neutrophil Functions. Trends Parasitol 32(5), 392-401. doi: 10.1016/j.pt.2016.02.003.

Hurrell, B.P., Schuster, S., Grun, E., Coutaz, M., Williams, R.A., Held, W., et al. (2015). Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. PLoS Pathog 11(5), e1004929. doi: 10.1371/journal.ppat.1004929.

Laskay, T., van Zandbergen, G., and Solbach, W. (2003). Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? Trends Microbiol 11(5), 210-214.

Lee, S.H., Charmoy, M., Romano, A., Paun, A., Chaves, M.M., Cope, F.O., et al. (2018). Mannose receptor high, M2 dermal macrophages mediate nonhealing Leishmania major infection in a Th1 immune environment. J Exp Med 215(1), 357-375. doi: 10.1084/jem.20171389.

Manfredi, A.A., Ramirez, G.A., Rovere-Querini, P., and Maugeri, N. (2018). The Neutrophil's Choice: Phagocytose vs Make Neutrophil Extracellular Traps. Front Immunol 9, 288. doi: 10.3389/fimmu.2018.00288.

Mayadas, T.N., Cullere, X., and Lowell, C.A. (2014). The multifaceted functions of neutrophils. Annu Rev Pathol 9, 181-218. doi: 10.1146/annurev-pathol-020712-164023.

McFarlane, E., Carter, K.C., McKenzie, A.N., Kaye, P.M., Brombacher, F., and Alexander, J. (2011). Endogenous IL-13 plays a crucial role in liver granuloma maturation during Leishmania donovani infection, independent of IL-4Ralpha-responsive macrophages and neutrophils. J Infect Dis 204(1), 36-43. doi: 10.1093/infdis/jir080.

McFarlane, E., Perez, C., Charmoy, M., Allenbach, C., Carter, K.C., Alexander, J., et al. (2008). Neutrophils contribute to development of a protective immune response during onset of infection with Leishmania donovani. Infect Immun 76(2), 532-541. doi: 10.1128/IAI.01388-07.

Mitropoulos, P., Konidas, P., and Durkin-Konidas, M. (2010). New World cutaneous leishmaniasis: updated review of current and future diagnosis and treatment. J Am Acad Dermatol 63(2), 309-322. doi: 10.1016/j.jaad.2009.06.088.

Nauseef, W.M., and Borregaard, N. (2014). Neutrophils at work. Nat Immunol 15(7), 602-611. doi: 10.1038/ni.2921.

Navas, A., Vargas, D.A., Freudzon, M., McMahon-Pratt, D., Saravia, N.G., and Gomez, M.A. (2014). Chronicity of dermal leishmaniasis caused by Leishmania panamensis is associated with parasite-mediated induction of chemokine gene expression. Infect Immun 82(7), 2872-2880. doi: 10.1128/IAI.01133-13.

Nguyen, G.T., Green, E.R., and Mecsas, J. (2017). Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. Front Cell Infect Microbiol 7, 373. doi: 10.3389/fcimb.2017.00373.

Novais, F.O., Santiago, R.C., Bafica, A., Khouri, R., Afonso, L., Borges, V.M., et al. (2009). Neutrophils and macrophages cooperate in host resistance against Leishmania braziliensis infection. J Immunol 183(12), 8088-8098. doi: 10.4049/jimmunol.0803720.

Obonaga, R., Fernandez, O.L., Valderrama, L., Rubiano, L.C., Castro Mdel, M., Barrera, M.C., et al. (2014). Treatment failure and miltefosine susceptibility in dermal leishmaniasis caused by Leishmania subgenus Viannia species. Antimicrob Agents Chemother 58(1), 144-152. doi: 10.1128/AAC.01023-13.

Organización Panamericana de la Salud (2013). Leishmaniasis en las Américas: recomendaciones para el tratamiento.

Papayannopoulos, V. (2015). Sweet NETs, Bitter Wounds. Immunity 43(2), 223-225. doi: 10.1016/j.immuni.2015.08.002.

Papayannopoulos, V. (2018). Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol 18(2), 134-147. doi: 10.1038/nri.2017.105.

Patino, L.H., Mendez, C., Rodriguez, O., Romero, Y., Velandia, D., Alvarado, M., et al. (2017). Spatial distribution, Leishmania species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis 11(8), e0005876. doi: 10.1371/journal.pntd.0005876.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., et al. (2008). In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321(5891), 970-974. doi: 10.1126/science.1159194.

Ramirez, J.D., Hernandez, C., Leon, C.M., Ayala, M.S., Florez, C., and Gonzalez, C. (2016). Taxonomy, diversity, temporal and geographical distribution of Cutaneous Leishmaniasis in Colombia: A retrospective study. Sci Rep 6, 28266. doi: 10.1038/srep28266.

Regli, I.B., Passelli, K., Hurrell, B.P., and Tacchini-Cottier, F. (2017). Survival Mechanisms Used by Some Leishmania Species to Escape Neutrophil Killing. Front Immunol 8, 1558. doi: 10.3389/fimmu.2017.01558.

Ribeiro-Gomes, F.L., Roma, E.H., Carneiro, M.B., Doria, N.A., Sacks, D.L., and Peters, N.C. (2014). Site-dependent recruitment of inflammatory cells determines the effective dose of Leishmania major. Infect Immun 82(7), 2713-2727. doi: 10.1128/IAI.01600-13.

Ribeiro-Gomes, F.L., and Sacks, D. (2012). The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front Cell Infect Microbiol 2, 59. doi: 10.3389/fcimb.2012.00059.

Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N.R., Dorlo, T.P., et al. (2013). Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. Clin Infect Dis 56(11), 1530-1538. doi: 10.1093/cid/cit102.

Rochael, N.C., Guimaraes-Costa, A.B., Nascimento, M.T., DeSouza-Vieira, T.S., Oliveira, M.P., Garcia e Souza, L.F., et al. (2015). Classical ROS-dependent and early/rapid ROS-independent release of Neutrophil Extracellular Traps triggered by Leishmania parasites. Sci Rep 5, 18302. doi: 10.1038/srep18302.

Rojas, R., Valderrama, L., Valderrama, M., Varona, M.X., Ouellette, M., and Saravia, N.G. (2006). Resistance to antimony and treatment failure in human Leishmania (Viannia) infection. J Infect Dis 193(10), 1375-1383. doi: 10.1086/503371.

Roos, D., van Bruggen, R., and Meischl, C. (2003). Oxidative killing of microbes by neutrophils. Microbes Infect 5(14), 1307-1315.

Segal, A.W. (2005). How neutrophils kill microbes. Annu Rev Immunol 23, 197-223. doi: 10.1146/annurev.immunol.23.021704.115653.

Sousa, L.M., Carneiro, M.B., Resende, M.E., Martins, L.S., Dos Santos, L.M., Vaz, L.G., et al. (2014). Neutrophils have a protective role during early stages of Leishmania amazonensis infection in BALB/c mice. Parasite Immunol 36(1), 13-31. doi: 10.1111/pim.12078.

Souza-Lemos, C., de-Campos, S.N., Teva, A., Corte-Real, S., Fonseca, E.C., Porrozzi, R., et al. (2008). Dynamics of immune granuloma formation in a Leishmania braziliensis-induced self-limiting cutaneous infection in the primate Macaca mulatta. J Pathol 216(3), 375-386. doi: 10.1002/path.2403.

Sundar, S., and Chakravarty, J. (2012). Recent advances in the diagnosis and treatment of kala-azar. Natl Med J India 25(2), 85-89.

Sundar, S., Sinha, P., Jha, T.K., Chakravarty, J., Rai, M., Kumar, N., et al. (2013). Oral miltefosine for Indian post-kala-azar dermal leishmaniasis: a randomised trial. Trop Med Int Health 18(1), 96-100. doi: 10.1111/tmi.12015.

Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., Mukankundiye, C., Vasei, M., Launois, P., et al. (2000). An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major. J Immunol 165(5), 2628-2636.

Tavares, N.M., Araujo-Santos, T., Afonso, L., Nogueira, P.M., Lopes, U.G., Soares, R.P., et al. (2014). Understanding the mechanisms controlling Leishmania amazonensis infection in vitro: the role of LTB4 derived from human neutrophils. J Infect Dis 210(4), 656-666. doi: 10.1093/infdis/jiu158.

Thalhofer, C.J., Chen, Y., Sudan, B., Love-Homan, L., and Wilson, M.E. (2011). Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan Leishmania infantum chagasi. Infect Immun 79(1), 108-117. doi: 10.1128/IAI.00338-10.

Thomas, D.C. (2018). How the phagocyte NADPH oxidase regulates innate immunity. Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2018.06.011.

Uliana, S.R.B., Trinconi, C.T., and Coelho, A.C. (2018). Chemotherapy of leishmaniasis: present challenges. Parasitology 145(4), 464-480. doi: 10.1017/S0031182016002523.

Vanaerschot, M., De Doncker, S., Rijal, S., Maes, L., Dujardin, J.C., and Decuypere, S. (2011). Antimonial resistance in Leishmania donovani is associated with increased in vivo parasite burden. PLoS One 6(8), e23120. doi: 10.1371/journal.pone.0023120.

Winterbourn, C.C., Kettle, A.J., and Hampton, M.B. (2016). Reactive Oxygen Species and Neutrophil Function. Annu Rev Biochem 85, 765-792. doi: 10.1146/annurev-biochem-060815-014442.

Yang, H., Biermann, M.H., Brauner, J.M., Liu, Y., Zhao, Y., and Herrmann, M. (2016). New Insights into Neutrophil Extracellular Traps: Mechanisms of Formation and Role in Inflammation. Front Immunol 7, 302. doi: 10.3389/fimmu.2016.00302.

8. Perspectives

8.1 *Leishmania Major* Inoculation Doses Used in *in Vivo* Mouse Models

In nature, *Phlebotomus* inoculate low numbers of parasites into the dermis and epidermis. In 75% of cases, the *L. major* inocculum has been reported to be lower then 10^3 parasites. However, the number can reach up to 10^5 parasites in rare cases[116]. Upon *Lutzomyia* bites, factors derived from the sand fly have been shown to contribute to neutrophil recruitment[117,118,193]. Upon *Phlebotomus* bites in mice ears, a marked neutrophil recruitment into the dermis was observed (Peters et al., 2008). During our studies of *L. major* infection in mice we tested different inoculation doses. To compensate for the lack of sand fly derived factors, we infected higher parasite numbers than normally transmitted by sandflies. We made the observation that in mice intradermally infected with of 10^4 *L. major* the disease onset was delayed compared to mice injected with 10^5 parasites while the disease outcome did not change. It has been reported for *L. major* Friedlin and *L. major* Seidman that a needle inoculation of as few as 1000 parasites is sufficient to induce a lesion[194]. However, also in these cases the disease onset was delayed in comparison to the inoculation of 10^5 *L. major.* For practical reasons, in the experimental settings where a robust neutrophil recruitment at 24 hours was needed, we injected mice with 10^6 *L. major* parasites which corresponds to the inoculum used in other studies[195,196]. Differences in parasite numbers led to differences in disease dynamics but not in disease outcome. We showed that neutrophils are important determinants of diseases progression. Therefore, it can be assumed that neutrophils are also recruited in relevant numbers upon injection of lower parasite numbers. However, the exact dynamics of neutrophil recruitment and *L. major* needle inoculation has yet to be determined. Finally, to even better determine the neutrophil-parasite dynamics in human disease, it would be desirable to measure neutrophil recruitment and neutrophil infection and neutrophil effector functions in a mouse model where the parasite is transmitted through sand flies.

8.2 Potential Interest in the Development of a Mouse Model of *Leishmania (Viannia) Panamensis* Infection

L. (V.) p. is known to have a poor infectivity in mice. However, the many immunological and genetical tools that are available in mice would make it a worthwhile *in vivo* model to study. Some strains of *L. (V.) p.* injected subcutaneously at a high inoculum (10^7) are able to establish an infection in BALB/c mice[197]. By using monoclonal antibodies that recognize antigens which are upregulated in amastigotes, Castilho et al. developed a BALB/c model of a chronic *L. (V.) p.* infection which can be induced by a low parasite inoculum (10^4)[198]. The laboratory-derived *L. (V.) p.* lines that were used in this thesis were not able to establish an infection in BALB/c mice, neither using a low nor a high parasite inoculum (data not shown). However, we were able to establish infection using clinically isolated drug resistant *L. (V). p.* strains. In preliminary experiments, we could establish that miltefosine resistant and antimony resistant parasites induced bigger lesions than drug susceptible parasites. In order to further elucidate the effect of *L. (V.) p.* drug susceptibility on neutrophils it would be important to assess whether our *in vitro* findings obtained in laboratory-derived drug resistant strains can be reproduced in the *in vivo* mouse model. It would be of interest to determine whether differences in neutrophil activation could be used as surrogate markers for predicting the expected treatment outcome. Furthermore, it could be assessed if a given strain that induces treatment failure in patients also induces treatment failure in mice. Such a mouse model could be used for the adjustment and improvement of treatment modalities. Finally, the mouse model could also be used to further investigate the basic immunological mechanisms and the effects of parasite drug susceptibility phenotype on the immune response against *L. (V.) p.*

8.3 Potential Additional Roles of Toll-Like Receptor 7 in Immunity against *Leishmania major*

pDCs are specialized cells that are able to produce high amounts of Type-1 interferon upon TLR7 and TLR9 signaling[199]. pDCs have been found in the skin and in the draining lymph-nodes of *L. major* infected mice[200]. Furthermore, it has been shown that pDCs pulsed with *L major* lysate and transferred into BALB/c mice induced protection against *L. major* infection[201]. Moreover, pDCs are able to produce IFN-β upon TLR7 activation through bacterial nucleic acids[62]. Indeed, low doses of IFN-β have been shown to have a protective effect against *L. major* infection in BALB/c mice and to lead to an increase of IFN-γ production in T-cells and an increase of IL-12 and iNOS expression in the draining lymph-node of infected mice[202]. Furthermore, it has been reported that iNOS expression early after infection is type-1 IFN dependent[203]. However, other studies show that type-1 signaling is irrelevant for *L. major* infection disease development[204]. In neutrophils, type-1 interferon can activate the expression of the cytotoxic molecule tumor necrosis factor related apoptosis inducing ligand (TRAIL). In tumor-associated neutrophils, IFN-β induces a more antitumoral phenotype [205]. Here, we demonstrated that the absence of TLR7 leads to an increase in disease severity following *L. major* infection. Therefore, it could be hypothesized that in absence of TLR7 a dysfunctional DC response and an alteration in type-1 IFN secretion may contribute to the development of nonhealing lesion and parasite replication[202-204]. Of note, there is evidence indicating that type-1 IFN are capable to modulate neutrophil effector function, which in turn would inflect the leishmaniasis disease progression.

TLR7 activation in a subset of monocytes characterized by low CD14 expression is thought to be involved in the tissue innate immune surveillance. Upon activation these cells secrete IL-1β and TNFα. IL-1β and TNF-α have the ability to prime neutrophils for increased ROS formation which presumably would positively influence disease progression in *L. major* infection[206,207].

Furthermore, CD14^{high} monocytes secrete high levels of IL-8, IL-6 and IL-12 upon TLR7 signaling[64,65]. IL-12 is an important mediator in the shaping of a protective Th1 response and is also important in the NK-cell mediated IFN-γ secretion[65,169]. Moreover, inflammatory monocytes are thought to play an important role in the immune response against *L. mexicana*[155]. It is thus conceivable that following *L. major* infection, TLR7-mediated inflammatory monocyte activation could be beneficial in leishmaniasis disease outcome.

NK-cells secrete IFN-γ upon TLR7 signaling and are involved in the immunity against *L. major* early after subcutaneous infection[65,166]. The absence of TLR7 is possibly a cause of an inadequate NK-cell response to *L. major* infection with an inappropriate IFN-γ secretion and could contribute to the exacerbation of the disease phenotype. However, the role of NK-cells in intradermal *L. major* infection has yet to be established.

Even though we showed that TLR7 signaling in neutrophils early after infection is of utmost importance in the immune response against *L. major,* it has to be considered that TLR7 signaling might also contribute to other pathways involved in a successful immune response against *L. major*.

8.4 Species Specific Differences in Endosomal Single-Stranded RNA Recognition

Here, we used a mouse model on C57BL/6 background to show the importance of TLR7 signaling in neutrophils after *L. major* infection. To transfer these findings to a clinical setting it has to be considered that human neutrophils do not express TLR7[69]. In humans TLR7 and TLR8 both recognize ssRNA[59] but the exact scope of the structures recognized by these receptors is species-specific. It is thought that murine TLR8 is either less functional or does recognize different structures than human TLR8 since *Tlr7*$^{-/-}$ mice do not respond to the TLR7 and 8 agonist resiquimod or human TLR8 RNA ligands[208,209]. However, the human TLR8

agonist 3M-002 and polyT nucleotides were also recognized by murine TLR8[210]. It is thought that in contrast to human TLR8, murine TLR8 plays a negligible role in the recognition of ssRNA[70]. For instance, it is known that guanosine and uridine rich ssRNA oligonucleotides derived from human immunodeficiency virus–1 are recognized by murine TLR7 but human TLR8[191]. Interestingly, it has been reported that mice that lack TLR8 overexpress TLR7 and are hyperresponsive to TLR7 agonists. *Tlr8*$^{-/-}$ mice develop autoimmunity with splenomegaly, increased antibody titers and developed glomerulonephritis while *Tlr7*$^{-/-}$ *Tlr8*$^{-/-}$ double knock-out mice did not show that phenotype[211]. Considering these species specific differences in TLR7 and TLR8 function, we hypothesize that TLR8 compensates for the absence of TLR7 in human neutrophils and that TLR8 signaling in neutrophils early after infection would be important in the human immune response against *L. major* and that our results are also relevant for human disease. However, to confirm this hypothesis, further studies would be needed.

8.5 The Role of the Skin Commensals in Leishmaniasis

It is known that LCL patients infected with *L. braziliensis* have a microbiome that is characterized by an abundance of *Staphylococci* in and around the lesion, which is distinct from the microbiome encountered on healthy skin. In *L. major* infected C57BL/6 mice a similar microbiome was detected[212]. Interestingly, the microbiome changed with disease severity and the biggest abundance of *Staphylococci* was found when the lesion was the largest. This dysbiotic microbiome could be transferred to naïve mice and was shown to have a deleterious effect on disease progression of subsequent *L. major* infection[212]. Furthermore, it has been reported that cutaneous commensals, notably *Staphlyococcus epidermidis* (*S. epidermidis*) promote lesion development in *L. major* infection in a MyD88 and therefore in a TLR signaling dependent manner[213]. *S. epidermidis* by itself does not induce skin inflammation and can even block TLR3 signaling in keratinocytes[214]. Thus, it is thought that the synergistic effect of *S.*

epidermidis and *L. major* leads to the disease phenotype observed in C57BL/6 mice. It would be possible that the absence of TLR7 would lead to an alteration of these mechanisms. Even though, TLR signaling does not have a big impact on physiological skin microbiome composition[215], upon *L. major* infection the absence of TLR7 could lead to a different microbiome-dependent regulation of the disease phenotype observed. It would furthermore be of interest do determine whether there are differences in the microbiota composition in *L. major* infected C57BL/6 and *Tlr7*$^{-/-}$ mice, also because it is known that sand-fly gut microbes are transferred to the host during the sand fly bite [216]. If so, it would be of interest to assess what the dynamics of these changes in microbiota compositions are and how they relate to the lesion development.

Moreover, it would be interesting to assess whether there are changes in the microbiota composition between people that do or do not respond to antileishmanial drug treatment and whether the manipulation of the microbiota would have an effect on the therapeutic outcome.

8.6 The Potential Role of Autophagy in Leishmaniasis

Autophagy is a mechanism of eukaryotic cells that provides a mean for the elimination of unnecessary or dysfunctional intracellular structures or intracellular pathogens[217].

L. major infected macrophages undergo autophagy, which has been shown to restrict parasite replication in a endosomal TLR signaling dependent manner[218]. Chromatin decondensation in NET formation is autophagy and ROS dependent, at least when induced by PMA. In turn it is known that ROS is able to induce autophagy[219-222]. Also, neutrophil degranulation is dependent on autophagy[223]. In addition, autophagy has been shown to be involved in NET formation in sterile inflammation[224].

Here we have demonstrated that *L. major* and *L. (V.) p.* induce ROS production and NET formation in neutrophils. Moreover, we could show that *L. (V.) p.* induces neutrophil degranulation. In *L. (V.) p.* infection, the degree of the induction was dependent on the drug

susceptibility of the parasite. It can therefore be hypothesized that drug resistant *L. (V.) p.* lines would induce the autophagy machinery to a greater extent than drug susceptible *L. (V.) p.* lines. However, the exact role that autophagy plays in the interaction of *l. (V.) p.* and neutrophils would have to be elucidated in future studies.

In *L. major* infection, the induction of neutrophil effector functions was dependent on TLR7 signaling. It is possible, that in addition to the already established differences in neutrophil functions, the induction of autophagy would play an important role in neutrophil mediated parasite control since we have shown that *Tlr7*$^{-/-}$ mice have neutrophils that are more heavily infected and contain more parasites than their wild type counterparts. Previous studies have reported that *Myd88*$^{-/-}$ and *Tlr3*$^{-/-}$ *Tlr7*$^{-/-}$ *Tlr9*$^{-/-}$ triple knock-out macrophages are less capable to induce autophagy and are highly permissive for *L. major* replication[218]. It would be of great interest to determine if the same is the case in neutrophils, since autophagy has been shown to be essential for the main neutrophil effector functions [225].

8.7 The Potential of Toll-like receptor 7 Agonists in Leishmaniasis Treatment

Imiquimod is a synthetic imidazoquinolone that acts as TLR7 and TLR8 agonist. It is commercially available as 3.75% and 5% topical cream. It is approved by Swissmedic for the use against condylomas, actinic keratosis and basal cell carcinoma[226,227]. However, there are several reports on a successful use of Imiquimod against cutaneous leishmaniasis. Imiquimod was shown to increase iNOS production in macrophages *in vitro* and to reduce lesion size in *L. major* infected BALB/c mice [228]. In Peru, an endemic area of New World *Leishmania* spp., there is evidence that the combination therapy of meglumine antimoniate and topical 5% imiquimod against LCL leads to a faster lesion resolution than the therapy with meglumine antimony alone[229-231]. In Iran, an endemic area of Old World *Leishmania* spp., no benefit could

be shown in treatment success by combining antimony with imiquimod[232]. In Syria, topical imiquimod treatment led to a transient effect on lesion size within the first 2-4 weeks of treatment but had no effect on the final disease outcome[233]. Finally, in Kuwait it was reported that topical imiquimod treatment had no effect on disease outcome [234]

*L. majo*r is an Old World species, for which the efficacy of imiquimod in the clinical setting has not yet been sufficiently demonstrated. Considering that early TLR7 signaling in neutrophils is important for disease control, it is no surprise that the earlier studies did not show a beneficial effect of TLR7 signaling as we do here. Unfortunately, it is clinically not feasible to treat the patients during the first hours and days after a sand-fly bite because the disease will not have manifested itself yet. It would be, however, of great interest to determine whether it would be possible to select patients who have a high number of neutrophil present in the lesions and to see whether this specific patient population would respond to treatment with topical Imiquimod. Even though there is already a certain success in the treatment of leishmaniasis induced by New World species, such an approach could also improve patient care in the Americas as well.

8.8 Concluding Remarks

Leishmaniasis is a complex group of diseases against which there is an urgent need for the development of novel effective drugs. Until today the standard of care is often a therapy based on antimony, a treatment modality first described 107 years ago[235]. *Leishmania* infection in immunology has been studied more intensively in the past decades, for instance when the Th1/Th2 paradigm in *L. major* infection was established. However, the critical role of neutrophils in immunity against *Leishmania* infection is just recently becoming apparent. This is not least due to a tendency to think of neutrophils as terminally differentiated killer cells with

a short life-span. In reality, the role of neutrophils in the shaping of immunity is much more complex.

In this thesis, two specific roles of neutrophils in leishmaniasis were elucidated. First, the essential role of neutrophils in leishmaniasis pathogenesis is stressed as well as the importance to Toll-like receptor 7 signaling, notably in neutrophils, is demonstrated. Second, it is established that different lines of *L. (V.) p*, drug-resistant and drug-susceptible ones, interact with neutrophils differently and lead to differences in neutrophil activation. After the establishment of these findings the question of the clinical applicability naturally arises. Given the basic research character of this thesis, this question cannot be precisely answered yet. Nonetheless, given that there is already a Swissmedic, EMA and FDA approved topical TLR7 agonist available, the determination of a relevant role of TLR7 in leishmaniasis potentially opens up the possibility of the development of new adapted treatment modalities. The importance of neutrophils in leishmaniasis generally suggests that these cells have to be more considered during the establishment of treatment modalities.

In the end, the results of this thesis have to be integrated into the totality of the previous findings in the field of immunoparasitology, to strive for the advancement of patient care in neglected tropical disease.

9. Acknowledgements

First and foremost, I want to thank **Prof. Fabienne Tacchini-Cottier**, for taking me under her wing and giving me the opportunity to grow as a scientist and as a person. Thank you for the support during this last years, for the help and understaning in difficult times and the energy, passion and joy in the good times. It was a pleasure to work in your lab and I am grateful to have had such an stimulating working environement and to work with somebody with such a dedication to science.

I would also thank my committee members**. Prof. Daniel Speiser** who already gave me great advice about the MD-PhD program in Lausanne when I was in medical school in Zürich, **Prof. Tamas Laskay** who was ready to take the long journey from Lübeck to provide his expertise about neutrophils and leishmaniasis, **Prof. Blaise Genton** for his clinical point of view on my work, **Prof. Hans Acha-Orbea** for the many stimulating discussion over the past years.

Next, I would like to thank **Dr. Maria Berenice Martínez-Salazar**. Thank you for being there in the good and in the hard times, for your companionship, for your help, for your support, your patience. I am very grateful for all the great moments we could share together.

I want to express my gratitude to all the present and past members of the laboratory. Thank you to **Dr. Benjamin P. Hurrell** for your frienship and all your help especially in the beginning of my MD-PhD. Thanks for introducing me to arts of playing golf, pétanque and all the other games you made up which lightened up the mood in the lab. Thanks to **Chris Neal** who shares my passion and love for skiing, mountaineering and mountainbiking. Sorry, I made you climb 1800 meters on my old bike from Lavey to the Cabane de la Tourche on our first tour. Thank you to **Jordi Mollà Mas,** you were the only student I suppervised in the lab and I really enjoyed working together, sharing a "long-term beer" after long working days and skiing together. I want to thank **Katiuska Passelli** you started your PhD just after me and I very much appreciated to work with you. Thank you to **Yazmin Huayon-La Torre** for your help and support during

these past few years. Thanks to **Dr. Vanessa Carregaro Pereira** and **Anita Bodac** who recently joined the lab and with whom I enjoyed working these past months. I would also like to thank the former lab membres **Dr. Manuel Coutaz, Dr. Marc Descatoire, Jess Coca** and **Manon Beaumann**. I very much appreciated working with you and you made being in the lab a pleasant experience.

I would like to thank **Dr. Nancy Gore Saravia**, **Dr. Olga Lucía Fernández Marulanda, Dr. Maria Adelaida Gómez, Dr. med. María del Mar Castro Noriega, Lady Giovanna Ramírez** and **Alexandra Cossio** from Calí, Colombia with whom we collaborated during my entire MD-PhD and who welcomed me in their team during my stay in Calí.

Thank you to **Prof. Andreas Müller, Prof. Pascale Kropf** and **Dr. Jeremy Di Domizio** for the fruitful collaborations and all the advice. Many thanks to the Flow Cytometry Facility, specialy **Dr. Francisco Sala de Oyanguren, Dr. Stefanie Siegert, Dr. Romain Bedel** and **Dr. Anne Wilson** for all the help during these past years. Thank you to the Cellular Imaging Facility specially to **Florence Morgenthaler** for all the support for my projects.

Thanks to everybody who made my time in the laboratories of Epalinges a good expericence, including **Dmitry Kopelyanskiy, Dr. Alessia Baldo, Dr. Remzi Onur Eren**, **Dr. Matteo Rossi, Dr. Luca Bonsignore, Dr. Borja Prat Luri, Dr. Matthias Stevanin,** all present and former members of the **Prof. Nicolas Fasel Lab,** the **Prof. Hans Acha-Orbea Lab,** the **Prof. Sanjiv Luther Lab,** the **Prof. Greta Guarda Lab** and the **Prof. Joana Joyce Lab.**

Also many thanks for the support of **my friends from** growing up in the Canton of **Zug** even though I was doing my MD-PhD far away in the Suisse romand**.**

Thanks to **Prof. Maries van den Broek, Dr. med Lotta von Boehmer** and **Dr. Urs Wuthier** giving me the inspiration to dedicate myself to research.

Finally, I would like to thank my mother, **Anamaria Regli-Quevedo** for all her love and support and without whom I would not be the person I am today.

10. Other Contributions to the Field of Leishmaniasis

10.1. Published Article

Hurrell, B.P., Beaumann, M., Heyde, S., **Regli, I.B.**, Müller, A.J., and Tacchini-Cottier, F. (2017). Frontline Science: *Leishmania mexicana* amastigotes can replicate within neutrophils. Journal of leukocyte biology. 102(5), 1187-1198.

10.2. Articles in Preparation

Takele, Y., Cruz-Cervera E., **Regli I.B.,** Guleed, S., Tacchini-Cottier, F., Getti,G., Muller, I., Kropf, P. Establishment of *in vitro* models of *Leishmania aethiopica* infections.

Luri, B.P. Neal, C., **Regli, I.B.**, Bovay, E., Hurrell, B.P., Müller, A.J., Petrova, T.V., Tacchini-Cottier, F. Neutrophil lymphatic migration to lymphnodes is regulated by *Leishmania mexicana.*

11. References

1. Doherty M, Robertson MJ. Some early Trends in Immunology. *Trends in immunology.* Dec 2004;25(12):623-631.
2. Modanlou HD. A tribute to Zakariya Razi (865 - 925 AD), an Iranian pioneer scholar. *Archives of Iranian medicine.* Nov 2008;11(6):673-677.
3. Stewart AJ, Devlin PM. The history of the smallpox vaccine. *The Journal of infection.* May 2006;52(5):329-334.
4. Strassburg MA. The global eradication of smallpox. *American journal of infection control.* May 1982;10(2):53-59.
5. Andral MM, Gavarret. Researches on the Quantity of Carbonic Acid Exhaled from the Lungs of the Human Species. *The British and foreign medical review.* Jul 1843;16(31):285-287.
6. Addison W. Experimental and Practical Researches on the Blood: Second Series. *Provincial medical journal and retrospect of the medical sciences.* Aug 26 1843;6(152):444-445.
7. Teti G, Biondo C, Beninati C. The Phagocyte, Metchnikoff, and the Foundation of Immunology. *Microbiology spectrum.* Apr 2016;4(2).
8. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell.* Aug 2002;10(2):417-426.
9. Tacchini-Cottier F, Zweifel C, Belkaid Y, et al. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major. *Journal of immunology.* Sep 1 2000;165(5):2628-2636.
10. Allenbach C, Zufferey C, Perez C, Launois P, Mueller C, Tacchini-Cottier F. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by Leishmania major. *Journal of immunology.* Jun 1 2006;176(11):6656-6664.
11. Charmoy M, Megnekou R, Allenbach C, et al. Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. *Journal of leukocyte biology.* Aug 2007;82(2):288-299.
12. McFarlane E, Perez C, Charmoy M, et al. Neutrophils contribute to development of a protective immune response during onset of infection with Leishmania donovani. *Infection and immunity.* Feb 2008;76(2):532-541.
13. Schuster S, Hurrell B, Tacchini-Cottier F. Crosstalk between neutrophils and dendritic cells: a context-dependent process. *Journal of leukocyte biology.* Oct 2013;94(4):671-675.
14. Hurrell BP, Schuster S, Grun E, et al. Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. *PLoS pathogens.* May 2015;11(5):e1004929.
15. Hurrell BP, Beaumann M, Heyde S, Regli IB, Muller AJ, Tacchini-Cottier F. Frontline Science: Leishmania mexicana amastigotes can replicate within neutrophils. *Journal of leukocyte biology.* Nov 2017;102(5):1187-1198.
16. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Current opinion in immunology.* Feb 2001;13(1):114-119.
17. Moser M, Leo O. Key concepts in immunology. *Vaccine.* Aug 31 2010;28 Suppl 3:C2-13.
18. Kanazawa N, Tchernev G, Wollina U. Autoimmunity versus autoinflammation--friend or foe? *Wiener medizinische Wochenschrift.* Jul 2014;164(13-14):274-277.
19. Hato T, Dagher PC. How the Innate Immune System Senses Trouble and Causes Trouble. *Clinical journal of the American Society of Nephrology : CJASN.* Aug 7 2015;10(8):1459-1469.
20. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. *Science.* Jan 7 2011;331(6013):44-49.
21. Manz MG, Traver D, Miyamoto T, Weissman IL, Akashi K. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood.* Jun 1 2001;97(11):3333-3341.

22. Iwasaki H, Akashi K. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity.* Jun 2007;26(6):726-740.
23. Puhr S, Lee J, Zvezdova E, Zhou YJ, Liu K. Dendritic cell development-History, advances, and open questions. *Seminars in immunology.* Dec 2015;27(6):388-396.
24. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. *Annual review of immunology.* 2015;33:643-675.
25. Yona S, Jung S. Monocytes: subsets, origins, fates and functions. *Current opinion in hematology.* Jan 2010;17(1):53-59.
26. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nature reviews. Immunology.* Aug 2015;15(8):471-485.
27. Segura E, Amigorena S. Inflammatory dendritic cells in mice and humans. *Trends in immunology.* Sep 2013;34(9):440-445.
28. Theoharides TC, Valent P, Akin C. Mast Cells, Mastocytosis, and Related Disorders. *The New England journal of medicine.* Jul 9 2015;373(2):163-172.
29. Romano A, Torres MJ, Castells M, Sanz ML, Blanca M. Diagnosis and management of drug hypersensitivity reactions. *The Journal of allergy and clinical immunology.* Mar 2011;127(3 Suppl):S67-73.
30. Karasuyama H, Yamanishi Y. Basophils have emerged as a key player in immunity. *Current opinion in immunology.* Dec 2014;31:1-7.
31. Ravin KA, Loy M. The Eosinophil in Infection. *Clinical reviews in allergy & immunology.* Apr 2016;50(2):214-227.
32. Segal AW. How neutrophils kill microbes. *Annual review of immunology.* 2005;23:197-223.
33. Downey GP, Doherty DE, Schwab B, 3rd, Elson EL, Henson PM, Worthen GS. Retention of leukocytes in capillaries: role of cell size and deformability. *Journal of applied physiology.* Nov 1990;69(5):1767-1778.
34. McCracken JM, Allen LA. Regulation of human neutrophil apoptosis and lifespan in health and disease. *Journal of cell death.* 2014;7:15-23.
35. Pillay J, Ramakers BP, Kamp VM, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *Journal of leukocyte biology.* Jul 2010;88(1):211-220.
36. Zeidler C, Germeshausen M, Klein C, Welte K. Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. *British journal of haematology.* Feb 2009;144(4):459-467.
37. Boxer LA. How to approach neutropenia. *Hematology. American Society of Hematology. Education Program.* 2012;2012:174-182.
38. Borregaard N. Neutrophils, from marrow to microbes. *Immunity.* Nov 24 2010;33(5):657-670.
39. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunological reviews.* Sep 2016;273(1):11-28.
40. Yin C, Heit B. Armed for destruction: formation, function and trafficking of neutrophil granules. *Cell and tissue research.* Mar 2018;371(3):455-471.
41. Granick JL, Simon SI, Borjesson DL. Hematopoietic stem and progenitor cells as effectors in innate immunity. *Bone marrow research.* 2012;2012:165107.
42. Yanez A, Murciano C, O'Connor JE, Gozalbo D, Gil ML. Candida albicans triggers proliferation and differentiation of hematopoietic stem and progenitor cells by a MyD88-dependent signaling. *Microbes and infection.* Apr 2009;11(4):531-535.
43. Yanez A, Flores A, Murciano C, O'Connor JE, Gozalbo D, Gil ML. Signalling through TLR2/MyD88 induces differentiation of murine bone marrow stem and progenitor cells to functional phagocytes in response to Candida albicans. *Cellular microbiology.* Jan 2010;12(1):114-128.
44. Kim MH, Granick JL, Kwok C, et al. Neutrophil survival and c-kit(+)-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. *Blood.* Mar 24 2011;117(12):3343-3352.

45. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *Journal of leukocyte biology.* Nov 1994;56(5):559-564.
46. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nature reviews. Immunology.* Mar 2013;13(3):159-175.
47. Kruger P, Saffarzadeh M, Weber AN, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS pathogens.* Mar 2015;11(3):e1004651.
48. Roos D, van Bruggen R, Meischl C. Oxidative killing of microbes by neutrophils. *Microbes and infection.* Nov 2003;5(14):1307-1315.
49. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nature reviews. Immunology.* Feb 2018;18(2):134-147.
50. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annual review of pathology.* 2014;9:181-218.
51. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature reviews. Immunology.* Jul 25 2011;11(8):519-531.
52. Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, et al. Cytokine production by human neutrophils: Revisiting the "dark side of the moon". *European journal of clinical investigation.* May 17 2018:e12952.
53. Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *Journal of innate immunity.* 2010;2(3):216-227.
54. Hoving JC, Wilson GJ, Brown GD. Signalling C-type lectin receptors, microbial recognition and immunity. *Cellular microbiology.* Feb 2014;16(2):185-194.
55. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* Mar 19 2010;140(6):805-820.
56. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature.* Oct 18 2007;449(7164):819-826.
57. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell.* Sep 20 1996;86(6):973-983.
58. Vasselon T, Detmers PA. Toll receptors: a central element in innate immune responses. *Infection and immunity.* Mar 2002;70(3):1033-1041.
59. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. *Nature reviews. Immunology.* Jun 2013;13(6):453-460.
60. Satoh T, Akira S. Toll-Like Receptor Signaling and Its Inducible Proteins. *Microbiology spectrum.* Dec 2016;4(6).
61. De Nardo D. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine.* Aug 2015;74(2):181-189.
62. Mancuso G, Gambuzza M, Midiri A, et al. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. *Nature immunology.* Jun 2009;10(6):587-594.
63. Zhang Z, Ohto U, Shibata T, et al. Structural Analysis Reveals that Toll-like Receptor 7 Is a Dual Receptor for Guanosine and Single-Stranded RNA. *Immunity.* Oct 18 2016;45(4):737-748.
64. Cros J, Cagnard N, Woollard K, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity.* Sep 24 2010;33(3):375-386.
65. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *Journal of immunology.* Aug 1 2005;175(3):1636-1642.
66. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *Journal of immunology.* Jan 15 2002;168(2):554-561.
67. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood.* Oct 1 2003;102(7):2660-2669.

68. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nature immunology.* Oct 2004;5(10):987-995.
69. Janke M, Poth J, Wimmenauer V, et al. Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *The Journal of allergy and clinical immunology.* May 2009;123(5):1026-1033.
70. Gantier MP, Tong S, Behlke MA, et al. TLR7 is involved in sequence-specific sensing of single-stranded RNAs in human macrophages. *Journal of immunology.* Feb 15 2008;180(4):2117-2124.
71. Applequist SE, Wallin RP, Ljunggren HG. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *International immunology.* Sep 2002;14(9):1065-1074.
72. Drake MG, Bivins-Smith ER, Proskocil BJ, et al. Human and Mouse Eosinophils Have Antiviral Activity against Parainfluenza Virus. *American journal of respiratory cell and molecular biology.* Sep 2016;55(3):387-394.
73. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. *Nature immunology.* Apr 2015;16(4):343-353.
74. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Frontiers in immunology.* 2014;5:520.
75. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. *The Journal of allergy and clinical immunology.* Apr 2013;131(4):959-971.
76. Harwood NE, Batista FD. Early events in B cell activation. *Annual review of immunology.* 2010;28:185-210.
77. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. *Nature reviews. Immunology.* Mar 2015;15(3):160-171.
78. Elgueta R, de Vries VC, Noelle RJ. The immortality of humoral immunity. *Immunological reviews.* Jul 2010;236:139-150.
79. Singer AL, Koretzky GA. Control of T cell function by positive and negative regulators. *Science.* May 31 2002;296(5573):1639-1640.
80. Gerondakis S, Fulford TS, Messina NL, Grumont RJ. NF-kappaB control of T cell development. *Nature immunology.* Jan 2014;15(1):15-25.
81. Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. *The Journal of investigative dermatology.* Jan 2006;126(1):32-41.
82. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood.* Sep 1 2008;112(5):1557-1569.
83. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* May 30 2008;133(5):775-787.
84. Licona-Limon P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. *Nature immunology.* Jun 2013;14(6):536-542.
85. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity.* Oct 16 2014;41(4):529-542.
86. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmunity reviews.* Jun 2014;13(6):668-677.
87. Cosmi L, Maggi L, Santarlasci V, Liotta F, Annunziato F. T helper cells plasticity in inflammation. *Cytometry. Part A : the journal of the International Society for Analytical Cytology.* Jan 2014;85(1):36-42.
88. Romagnani S. Regulation of the T cell response. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* Nov 2006;36(11):1357-1366.
89. Poinar G, Jr., Poinar R. Paleoleishmania proterus n. gen., n. sp., (Trypanosomatidae: Kinetoplastida) from Cretaceous Burmese amber. *Protist.* Sep 2004;155(3):305-310.
90. Poinar G, Jr. Lutzomyia adiketis sp. n. (Diptera: Phlebotomidae), a vector of Paleoleishmania neotropicum sp. n. (Kinetoplastida: Trypanosomatidae) in Dominican amber. *Parasites & vectors.* Jul 15 2008;1(1):22.
91. Zink AR, Spigelman M, Schraut B, Greenblatt CL, Nerlich AG, Donoghue HD. Leishmaniasis in ancient Egypt and Upper nubia. *Emerging infectious diseases.* Oct 2006;12(10):1616-1617.

92. Frias L, Leles D, Araujo A. Studies on protozoa in ancient remains--a review. *Memorias do Instituto Oswaldo Cruz.* Feb 2013;108(1):1-12.
93. Edrissian G, Rokni MB, Mohebali M, Nateghpour M, Mowlavi G, Bahadori M. History of Medical Parasitology and Parasitic Infections in Iran. *Archives of Iranian medicine.* Aug 2016;19(8):601-607.
94. Tuon FF, Neto VA, Amato VS. Leishmania: origin, evolution and future since the Precambrian. *FEMS immunology and medical microbiology.* Nov 2008;54(2):158-166.
95. Steverding D. The history of leishmaniasis. *Parasites & vectors.* Feb 15 2017;10(1):82.
96. Leishman WB. On the possibility of the occurrence of trypanosomiasis in India. 1903. *The National medical journal of India.* Jul-Aug 1994;7(4):196-200.
97. Donovan C. On the possibility of the occurrence of trypanosomiasis in India. 1903. *The National medical journal of India.* Jul-Aug 1994;7(4):196, 201-192.
98. Ross R. Note on the Bodies Recently Described by Leishman and Donovan. *British medical journal.* Nov 14 1903;2(2237):1261-1262.
99. Ross R. Further Notes on Leishman's Bodies. *British medical journal.* Nov 28 1903;2(2239):1401.
100. Wright JH. Protozoa in a Case of Tropical Ulcer ("Delhi Sore"). *The Journal of medical research.* Dec 1903;10(3):472-482 477.
101. Bray RS, Ashford RW, Bray MA. The parasite causing cutaneous leishmaniasis in Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 1973;67(3):345-348.
102. Espinosa OA, Serrano MG, Camargo EP, Teixeira MMG, Shaw JJ. An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as Leishmania and Endotrypanum. *Parasitology.* Apr 2018;145(4):430-442.
103. Mathers DA, Wan X, Puil E. Barbiturate activation and modulation of GABA(A) receptors in neocortex. *Neuropharmacology.* Mar 2007;52(4):1160-1168.
104. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. *Trends in parasitology.* Dec 2006;22(12):552-557.
105. Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PloS one.* 2012;7(5):e35671.
106. Oryan A, Akbari M. Worldwide risk factors in leishmaniasis. *Asian Pacific journal of tropical medicine.* Oct 2016;9(10):925-932.
107. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. *Lancet.* Oct 29-Nov 4 2005;366(9496):1561-1577.
108. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *The Lancet. Infectious diseases.* Sep 2007;7(9):581-596.
109. Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert review of anti-infective therapy.* Apr 2010;8(4):419-433.
110. Ameen M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. *Clinical and experimental dermatology.* Oct 2010;35(7):699-705.
111. Ardic N, Yesilova Y, Gunel IE, Ardic IN. Leishmaniasis Recidivans in Pediatric Patients. *The Pediatric infectious disease journal.* May 2017;36(5):534.
112. Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature reviews. Microbiology.* Nov 2007;5(11):873-882.
113. Pothirat T, Tantiworawit A, Chaiwarith R, et al. First isolation of Leishmania from Northern Thailand: case report, identification as Leishmania martiniquensis and phylogenetic position within the Leishmania enriettii complex. *PLoS neglected tropical diseases.* Dec 2014;8(12):e3339.
114. Jariyapan N, Daroontum T, Jaiwong K, et al. Leishmania (Mundinia) orientalis n. sp. (Trypanosomatidae), a parasite from Thailand responsible for localised cutaneous leishmaniasis. *Parasites & vectors.* Jun 18 2018;11(1):351.
115. Schonian G, Mauricio I, Cupolillo E. Is it time to revise the nomenclature of Leishmania? *Trends in parasitology.* Oct 2010;26(10):466-469.

116. Kimblin N, Peters N, Debrabant A, et al. Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. *Proceedings of the National Academy of Sciences of the United States of America.* Jul 22 2008;105(29):10125-10130.
117. Teixeira CR, Teixeira MJ, Gomes RB, et al. Saliva from Lutzomyia longipalpis induces CC chemokine ligand 2/monocyte chemoattractant protein-1 expression and macrophage recruitment. *Journal of immunology.* Dec 15 2005;175(12):8346-8353.
118. de Moura TR, Oliveira F, Rodrigues GC, et al. Immunity to Lutzomyia intermedia saliva modulates the inflammatory environment induced by Leishmania braziliensis. *PLoS neglected tropical diseases.* Jun 15 2010;4(6):e712.
119. Rogers M, Kropf P, Choi BS, et al. Proteophosophoglycans regurgitated by Leishmania-infected sand flies target the L-arginine metabolism of host macrophages to promote parasite survival. *PLoS pathogens.* Aug 2009;5(8):e1000555.
120. Peters NC, Egen JG, Secundino N, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science.* Aug 15 2008;321(5891):970-974.
121. Ribeiro-Gomes FL, Sacks D. The influence of early neutrophil-Leishmania interactions on the host immune response to infection. *Frontiers in cellular and infection microbiology.* 2012;2:59.
122. Salei N, Hellberg L, Kohl J, Laskay T. Enhanced survival of Leishmania major in neutrophil granulocytes in the presence of apoptotic cells. *PloS one.* 2017;12(2):e0171850.
123. Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology.* 2008;213(3-4):183-191.
124. Chow C, Cloutier S, Dumas C, Chou MN, Papadopoulou B. Promastigote to amastigote differentiation of Leishmania is markedly delayed in the absence of PERK eIF2alpha kinase-dependent eIF2alpha phosphorylation. *Cellular microbiology.* Jul 2011;13(7):1059-1077.
125. Kohl K, Zangger H, Rossi M, et al. Importance of polyphosphate in the Leishmania life cycle. *Microbial cell.* Jun 22 2018;5(8):371-384.
126. Claborn DM. The biology and control of leishmaniasis vectors. *Journal of global infectious diseases.* May 2010;2(2):127-134.
127. Santos DO, Coutinho CE, Madeira MF, et al. Leishmaniasis treatment--a challenge that remains: a review. *Parasitology research.* Jun 2008;103(1):1-10.
128. Ouellette M, Drummelsmith J, Papadopoulou B. Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy.* Aug-Oct 2004;7(4-5):257-266.
129. Silva LA, Vinaud MC, Castro AM, Cravo PV, Bezerra JC. In silico search of energy metabolism inhibitors for alternative leishmaniasis treatments. *BioMed research international.* 2015;2015:965725.
130. Sereno D, Guilvard E, Maquaire S, et al. Experimental studies on the evolution of antimony-resistant phenotype during the in vitro life cycle of Leishmania infantum: implications for the spread of chemoresistance in endemic areas. *Acta tropica.* Dec 21 2001;80(3):195-205.
131. Lee N, Bertholet S, Debrabant A, Muller J, Duncan R, Nakhasi HL. Programmed cell death in the unicellular protozoan parasite Leishmania. *Cell death and differentiation.* Jan 2002;9(1):53-64.
132. Ponte-Sucre A, Gamarro F, Dujardin JC, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS neglected tropical diseases.* Dec 2017;11(12):e0006052.
133. Vickers TJ, Wyllie S, Fairlamb AH. Leishmania major elongation factor 1B complex has trypanothione S-transferase and peroxidase activity. *The Journal of biological chemistry.* Nov 19 2004;279(47):49003-49009.

134. Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen Leishmania donovani. *The Journal of biological chemistry.* Sep 17 2004;279(38):39925-39932.
135. Wyllie S, Fairlamb AH. Refinement of techniques for the propagation of Leishmania donovani in hamsters. *Acta tropica.* Mar 2006;97(3):364-369.
136. Mohapatra S. Drug resistance in leishmaniasis: Newer developments. *Tropical parasitology.* Jan 2014;4(1):4-9.
137. Sundar S, Jha TK, Thakur CP, et al. Oral miltefosine for Indian visceral leishmaniasis. *The New England journal of medicine.* Nov 28 2002;347(22):1739-1746.
138. Luque-Ortega JR, Rivas L. Miltefosine (hexadecylphosphocholine) inhibits cytochrome c oxidase in Leishmania donovani promastigotes. *Antimicrobial agents and chemotherapy.* Apr 2007;51(4):1327-1332.
139. Barratt G, Saint-Pierre-Chazalet M, Loiseau PM. Cellular transport and lipid interactions of miltefosine. *Current drug metabolism.* Mar 2009;10(3):247-255.
140. Hendrickx S, Mondelaers A, Eberhardt E, et al. Intracellular amastigote replication may not be required for successful in vitro selection of miltefosine resistance in Leishmania infantum. *Parasitology research.* Jul 2015;114(7):2561-2565.
141. Moen MD, Lyseng-Williamson KA, Scott LJ. Liposomal amphotericin B: a review of its use as empirical therapy in febrile neutropenia and in the treatment of invasive fungal infections. *Drugs.* 2009;69(3):361-392.
142. Purkait B, Kumar A, Nandi N, et al. Mechanism of amphotericin B resistance in clinical isolates of Leishmania donovani. *Antimicrobial agents and chemotherapy.* Feb 2012;56(2):1031-1041.
143. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. *Expert opinion on investigational drugs.* May 2008;17(5):787-794.
144. Lai AFEJ, Vrede MA, Soetosenojo RM, Lai AFRF. Pentamidine, the drug of choice for the treatment of cutaneous leishmaniasis in Surinam. *International journal of dermatology.* Nov 2002;41(11):796-800.
145. Basselin M, Denise H, Coombs GH, Barrett MP. Resistance to pentamidine in Leishmania mexicana involves exclusion of the drug from the mitochondrion. *Antimicrobial agents and chemotherapy.* Dec 2002;46(12):3731-3738.
146. Moll H, Fuchs H, Blank C, Rollinghoff M. Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells. *European journal of immunology.* Jul 1993;23(7):1595-1601.
147. Ritter U, Mattner J, Rocha JS, Bogdan C, Korner H. The control of Leishmania (Leishmania) major by TNF in vivo is dependent on the parasite strain. *Microbes and infection.* May 2004;6(6):559-565.
148. Ng LG, Hsu A, Mandell MA, et al. Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS pathogens.* Nov 2008;4(11):e1000222.
149. van Zandbergen G, Klinger M, Mueller A, et al. Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. *Journal of immunology.* Dec 1 2004;173(11):6521-6525.
150. Silva MT. Neutrophils and macrophages work in concert as inducers and effectors of adaptive immunity against extracellular and intracellular microbial pathogens. *Journal of leukocyte biology.* May 2010;87(5):805-813.
151. Charmoy M, Brunner-Agten S, Aebischer D, et al. Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice. *PLoS pathogens.* Feb 5 2010;6(2):e1000755.
152. Goncalves R, Zhang X, Cohen H, Debrabant A, Mosser DM. Platelet activation attracts a subpopulation of effector monocytes to sites of Leishmania major infection. *The Journal of experimental medicine.* Jun 6 2011;208(6):1253-1265.

153. Liu D, Uzonna JE. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. *Frontiers in cellular and infection microbiology.* 2012;2:83.
154. Leon B, Lopez-Bravo M, Ardavin C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity.* Apr 2007;26(4):519-531.
155. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. *Nature reviews. Immunology.* Sep 2016;16(9):581-592.
156. Muraille E, De Trez C, Brait M, De Baetselier P, Leo O, Carlier Y. Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response. *Journal of immunology.* Apr 15 2003;170(8):4237-4241.
157. de Veer MJ, Curtis JM, Baldwin TM, et al. MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *European journal of immunology.* Oct 2003;33(10):2822-2831.
158. Halliday A, Bates PA, Chance ML, Taylor MJ. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis in vivo, but does not require activation by parasite lipophosphoglycan. *Parasites & vectors.* Oct 6 2016;9(1):532.
159. Kavoosi G, Ardestani SK, Kariminia A, Alimohammadian MH. Leishmania major lipophosphoglycan: discrepancy in Toll-like receptor signaling. *Experimental parasitology.* Feb 2010;124(2):214-218.
160. Becker I, Salaiza N, Aguirre M, et al. Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Molecular and biochemical parasitology.* Aug 31 2003;130(2):65-74.
161. Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B. Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. *Clinical and experimental immunology.* Jun 2013;172(3):403-409.
162. Murray HW, Zhang Y, Zhang Y, Raman VS, Reed SG, Ma X. Regulatory actions of Toll-like receptor 2 (TLR2) and TLR4 in Leishmania donovani infection in the liver. *Infection and immunity.* Jul 2013;81(7):2318-2326.
163. Kropf P, Freudenberg N, Kalis C, et al. Infection of C57BL/10ScCr and C57BL/10ScNCr mice with Leishmania major reveals a role for Toll-like receptor 4 in the control of parasite replication. *Journal of leukocyte biology.* Jul 2004;76(1):48-57.
164. Ribeiro-Gomes FL, Moniz-de-Souza MC, Alexandre-Moreira MS, et al. Neutrophils activate macrophages for intracellular killing of Leishmania major through recruitment of TLR4 by neutrophil elastase. *Journal of immunology.* Sep 15 2007;179(6):3988-3994.
165. Zhang WW, Matlashewski G. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against Leishmania major in BALB/c mice. *Infection and immunity.* Aug 2008;76(8):3777-3783.
166. Liese J, Schleicher U, Bogdan C. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *European journal of immunology.* Dec 2007;37(12):3424-3434.
167. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nature reviews. Immunology.* Nov 2002;2(11):845-858.
168. Kropf P, Fuentes JM, Fahnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* Jun 2005;19(8):1000-1002.
169. Reiner SL, Locksley RM. The regulation of immunity to Leishmania major. *Annual review of immunology.* 1995;13:151-177.
170. Barral A, Teixeira M, Reis P, et al. Transforming growth factor-beta in human cutaneous leishmaniasis. *The American journal of pathology.* Oct 1995;147(4):947-954.

171. Bourreau E, Prevot G, Gardon J, Pradinaud R, Launois P. High intralesional interleukin-10 messenger RNA expression in localized cutaneous leishmaniasis is associated with unresponsiveness to treatment. *The Journal of infectious diseases.* Dec 15 2001;184(12):1628-1630.
172. Salhi A, Rodrigues V, Jr., Santoro F, et al. Immunological and genetic evidence for a crucial role of IL-10 in cutaneous lesions in humans infected with Leishmania braziliensis. *Journal of immunology.* May 1 2008;180(9):6139-6148.
173. Pirmez C, Yamamura M, Uyemura K, Paes-Oliveira M, Conceicao-Silva F, Modlin RL. Cytokine patterns in the pathogenesis of human leishmaniasis. *The Journal of clinical investigation.* Apr 1993;91(4):1390-1395.
174. Bosque F, Saravia NG, Valderrama L, Milon G. Distinct innate and acquired immune responses to Leishmania in putative susceptible and resistant human populations endemically exposed to L. (Viannia) panamensis infection. *Scandinavian journal of immunology.* May 2000;51(5):533-541.
175. Bacellar O, Lessa H, Schriefer A, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infection and immunity.* Dec 2002;70(12):6734-6740.
176. Carvalho LP, Passos S, Bacellar O, et al. Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model for pathogenesis. *Parasite immunology.* May 2007;29(5):251-258.
177. Schriefer A, Wilson ME, Carvalho EM. Recent developments leading toward a paradigm switch in the diagnostic and therapeutic approach to human leishmaniasis. *Current opinion in infectious diseases.* Oct 2008;21(5):483-488.
178. Castellano LR, Filho DC, Argiro L, et al. Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon-gamma production. *Human immunology.* Jun 2009;70(6):383-390.
179. Oliveira WN, Ribeiro LE, Schrieffer A, Machado P, Carvalho EM, Bacellar O. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary leishmaniasis. *Cytokine.* Apr 2014;66(2):127-132.
180. Scorza BM, Carvalho EM, Wilson ME. Cutaneous Manifestations of Human and Murine Leishmaniasis. *International journal of molecular sciences.* Jun 18 2017;18(6).
181. Antonelli LR, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, Gollob KJ. Activated inflammatory T cells correlate with lesion size in human cutaneous leishmaniasis. *Immunology letters.* Nov 15 2005;101(2):226-230.
182. Oliveira F, Bafica A, Rosato AB, et al. Lesion size correlates with Leishmania antigen-stimulated TNF-levels in human cutaneous leishmaniasis. *The American journal of tropical medicine and hygiene.* Jul 2011;85(1):70-73.
183. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, Palomo-Cetina A. Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. *Infection and immunity.* Mar 1994;62(3):837-842.
184. Hejazi S, Hoseini S, Javanmard S, Zarkesh S, Khamesipour A. Interleukin-10 and Transforming Growth Factor-beta in Early and Late Lesions of Patients with Leishmania major Induced Cutaneous Leishmaniasis. *Iranian journal of parasitology.* 2012;7(3):16-23.
185. Ehrlich A, Castilho TM, Goldsmith-Pestana K, et al. The immunotherapeutic role of regulatory T cells in Leishmania (Viannia) panamensis infection. *Journal of immunology.* Sep 15 2014;193(6):2961-2970.
186. Bourreau E, Ronet C, Darcissac E, et al. Intralesional regulatory T-cell suppressive function during human acute and chronic cutaneous leishmaniasis due to Leishmania guyanensis. *Infection and immunity.* Apr 2009;77(4):1465-1474.
187. Bacellar O, Faria D, Nascimento M, et al. Interleukin 17 production among patients with American cutaneous leishmaniasis. *The Journal of infectious diseases.* Jul 1 2009;200(1):75-78.

188. Boaventura VS, Santos CS, Cardoso CR, et al. Human mucosal leishmaniasis: neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. *European journal of immunology.* Oct 2010;40(10):2830-2836.
189. Machado PR, Rosa ME, Costa D, et al. Reappraisal of the immunopathogenesis of disseminated leishmaniasis: in situ and systemic immune response. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* Aug 2011;105(8):438-444.
190. Diaz NL, Arvelaez FA, Zerpa O, Tapia FJ. Inducible nitric oxide synthase and cytokine pattern in lesions of patients with American cutaneous leishmaniasis. *Clinical and experimental dermatology.* Jan 2006;31(1):114-117.
191. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.* Mar 5 2004;303(5663):1526-1529.
192. Hurrell BP, Regli IB, Tacchini-Cottier F. Different Leishmania Species Drive Distinct Neutrophil Functions. *Trends in parasitology.* May 2016;32(5):392-401.
193. Silva F, Gomes R, Prates D, et al. Inflammatory cell infiltration and high antibody production in BALB/c mice caused by natural exposure to Lutzomyia longipalpis bites. *The American journal of tropical medicine and hygiene.* Jan 2005;72(1):94-98.
194. Charmoy M, Hurrell BP, Romano A, et al. The Nlrp3 inflammasome, IL-1beta, and neutrophil recruitment are required for susceptibility to a nonhealing strain of Leishmania major in C57BL/6 mice. *European journal of immunology.* Apr 2016;46(4):897-911.
195. Ribeiro-Gomes FL, Roma EH, Carneiro MB, Doria NA, Sacks DL, Peters NC. Site-dependent recruitment of inflammatory cells determines the effective dose of Leishmania major. *Infection and immunity.* Jul 2014;82(7):2713-2727.
196. Charmoy M, Auderset F, Allenbach C, Tacchini-Cottier F. The prominent role of neutrophils during the initial phase of infection by Leishmania parasites. *Journal of biomedicine & biotechnology.* 2010;2010:719361.
197. Neal RA, Hale C. A comparative study of susceptibility of inbred and outbred mouse strains compared with hamsters to infection with New World cutaneous leishmaniases. *Parasitology.* Aug 1983;87 (Pt 1):7-13.
198. Castilho TM, Goldsmith-Pestana K, Lozano C, Valderrama L, Saravia NG, McMahon-Pratt D. Murine model of chronic L. (Viannia) panamensis infection: role of IL-13 in disease. *European journal of immunology.* Oct 2010;40(10):2816-2829.
199. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature reviews. Immunology.* Aug 2008;8(8):594-606.
200. Baldwin T, Henri S, Curtis J, et al. Dendritic cell populations in Leishmania major-infected skin and draining lymph nodes. *Infection and immunity.* Apr 2004;72(4):1991-2001.
201. Remer KA, Apetrei C, Schwarz T, Linden C, Moll H. Vaccination with plasmacytoid dendritic cells induces protection against infection with Leishmania major in mice. *European journal of immunology.* Sep 2007;37(9):2463-2473.
202. Mattner J, Wandersee-Steinhauser A, Pahl A, et al. Protection against progressive leishmaniasis by IFN-beta. *Journal of immunology.* Jun 15 2004;172(12):7574-7582.
203. Diefenbach A, Schindler H, Donhauser N, et al. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity.* Jan 1998;8(1):77-87.
204. Schleicher U, Liese J, Justies N, et al. Type I Interferon Signaling Is Required for CpG-Oligodesoxynucleotide-Induced Control of Leishmania major, but Not for Spontaneous Cure of Subcutaneous Primary or Secondary L. major Infection. *Frontiers in immunology.* 2018;9:79.
205. Andzinski L, Kasnitz N, Stahnke S, et al. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. *International journal of cancer.* Apr 15 2016;138(8):1982-1993.
206. Yagisawa M, Yuo A, Kitagawa S, Yazaki Y, Togawa A, Takaku F. Stimulation and priming of human neutrophils by IL-1 alpha and IL-1 beta: complete inhibition by IL-1 receptor

antagonist and no interaction with other cytokines. *Experimental hematology.* Jul 1995;23(7):603-608.
207. Lewkowicz P, Tchorzewski H, Dytnerska K, Banasik M, Lewkowicz N. Epidermal growth factor enhances TNF-alpha-induced priming of human neutrophils. *Immunology letters.* Jan 31 2005;96(2):203-210.
208. Jurk M, Heil F, Vollmer J, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nature immunology.* Jun 2002;3(6):499.
209. Forsbach A, Nemorin JG, Montino C, et al. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. *Journal of immunology.* Mar 15 2008;180(6):3729-3738.
210. Gorden KK, Qiu XX, Binsfeld CC, Vasilakos JP, Alkan SS. Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides. *Journal of immunology.* Nov 15 2006;177(10):6584-6587.
211. Demaria O, Pagni PP, Traub S, et al. TLR8 deficiency leads to autoimmunity in mice. *The Journal of clinical investigation.* Oct 2010;120(10):3651-3662.
212. Gimblet C, Meisel JS, Loesche MA, et al. Cutaneous Leishmaniasis Induces a Transmissible Dysbiotic Skin Microbiota that Promotes Skin Inflammation. *Cell host & microbe.* Jul 12 2017;22(1):13-24 e14.
213. Naik S, Bouladoux N, Wilhelm C, et al. Compartmentalized control of skin immunity by resident commensals. *Science.* Aug 31 2012;337(6098):1115-1119.
214. Lai Y, Di Nardo A, Nakatsuji T, et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nature medicine.* Dec 2009;15(12):1377-1382.
215. Pasparakis M, Haase I, Nestle FO. Mechanisms regulating skin immunity and inflammation. *Nature reviews. Immunology.* May 2014;14(5):289-301.
216. Dey R, Joshi AB, Oliveira F, et al. Gut Microbes Egested during Bites of Infected Sand Flies Augment Severity of Leishmaniasis via Inflammasome-Derived IL-1beta. *Cell host & microbe.* Jan 10 2018;23(1):134-143 e136.
217. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nature reviews. Immunology.* Oct 2013;13(10):722-737.
218. Franco LH, Fleuri AKA, Pellison NC, et al. Autophagy downstream of endosomal Toll-like receptor signaling in macrophages is a key mechanism for resistance to Leishmania major infection. *The Journal of biological chemistry.* Aug 11 2017;292(32):13087-13096.
219. Remijsen Q, Vanden Berghe T, Wirawan E, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell research.* Feb 2011;21(2):290-304.
220. Mitroulis I, Kourtzelis I, Kambas K, et al. Regulation of the autophagic machinery in human neutrophils. *European journal of immunology.* May 2010;40(5):1461-1472.
221. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. *Cell death and differentiation.* Jul 2009;16(7):1040-1052.
222. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends in biochemical sciences.* Jan 2011;36(1):30-38.
223. Bhattacharya A, Wei Q, Shin JN, et al. Autophagy Is Required for Neutrophil-Mediated Inflammation. *Cell reports.* Sep 22 2015;12(11):1731-1739.
224. Mitroulis I, Kambas K, Chrysanthopoulou A, et al. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. *PloS one.* 2011;6(12):e29318.
225. Skendros P, Mitroulis I, Ritis K. Autophagy in Neutrophils: From Granulopoiesis to Neutrophil Extracellular Traps. *Frontiers in cell and developmental biology.* 2018;6:109.
226. Hanna E, Abadi R, Abbas O. Imiquimod in dermatology: an overview. *International journal of dermatology.* Aug 2016;55(8):831-844.
227. Arzneimittelkompedium der Schweiz. Aldara™ 5% Creme. 2016.

228. Buates S, Matlashewski G. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. *The Journal of infectious diseases.* Jun 1999;179(6):1485-1494.
229. Miranda-Verastegui C, Tulliano G, Gyorkos TW, et al. First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiquimod in combination with pentavalent antimony. *PLoS neglected tropical diseases.* Jul 28 2009;3(7):e491.
230. Arevalo I, Tulliano G, Quispe A, et al. Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* Jun 15 2007;44(12):1549-1554.
231. Miranda-Verastegui C, Llanos-Cuentas A, Arevalo I, Ward BJ, Matlashewski G. Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antimoniate in the treatment of cutaneous leishmaniasis in Peru. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* May 15 2005;40(10):1395-1403.
232. Firooz A, Khamesipour A, Ghoorchi MH, et al. Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: a randomized assessor-blind controlled trial. *Archives of dermatology.* Dec 2006;142(12):1575-1579.
233. Seeberger J, Daoud S, Pammer J. Transient effect of topical treatment of cutaneous leishmaniasis with imiquimod. *International journal of dermatology.* Jul 2003;42(7):576-579.
234. Al-Mutairi N, Alshiltawy M, El Khalawany M, et al. Tropical medicine rounds: Treatment of Old World cutaneous leishmaniasis with dapsone, itraconazole, cryotherapy, and imiquimod, alone and in combination. *International journal of dermatology.* Aug 2009;48(8):862-869.
235. Vianna G. Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. *Anais do 7º Congresso Brasileiro de Medicina e Cirurgia.* 1912;4:426-428.

12. List of Abbreviations

30S ribosomal subunit	30 small ribosomal unit
ACK buffer	Ammonium-Chloride-Potassium Buffer
Acox2	*Acyl-coenzyme a oxidase 2*
Acoxl	*Acyl-coenzyme a oxidase like*
A.D.	Anno domini
et al.	et alii/aliae
Akr1c18	*Aldo-keto reductase family 1 member c18*
Aldh3b2	*Aldehyde dehydrogenase family 3 member b2*
Alox12e	*Arachidonate 12-lipoxygenase, epidermal-type*
ATL	American tegumentery leishmaniasis
AUC	Area under the curve
B.C.	Before Christ
BH	Benjamin Hurrell
BM	Bone marrow
BMS	Berenice Martínez-Salazar
°C	°Celsius
CCL2	C-C chemokine ligand 2
CCL3	C-C chemokine ligand 3
CCR2	C-C chemokine receptor 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11b	Cluster of differentiation 11b (Integrin α-M)
CD11c	Cluster of differentiation 11c (Integrin α-X)
CD14	Cluster of differentiation 14
CD15	Cluster of differentiation 15

CD45	Cluster of differentiation 45 (Protein tyrosine phosphatase, receptor type, C)
CD62L	Cluster of differentiation 62 ligand (L-selectin)
CD66b	Cluster of differentiation 66b (CEACAM8)
cDCs	Classical dendritic cells
CIDEIM	Centro Internacional de Entrenamiento e Investigaciones Médicas
CL	Cutaneous leishmaniasis
CpG	5'— Cytosine—phosphate—Guanine—3'
CTL	Cytotoxic T-cell
CXCL1	C-X-C chemokine ligand 1
CXCL2	C-X-C chemokine ligand 2
CXCL6 / GCP-2	C-X-C chemokine ligand 6 / Granulocyte chemotactic protein 2
Cyp2b19	*Cytochrome p450 2b19*
Cyp2g1	*Cytochrome p450 2g1*
Cyp2j12	*Cytochrome p450 2j12*
Cyp2j8	*Cytochrome p450 2j8*
Cyp3a57	*Cytochrome p450 3a57*
Cyp4j39	*Cytochrome p450 4j39*
DAPI	4′,6-Diamidin-2-phenylindol
DCs	Dendritic cells
DCL	Diffuse cutaneous leishamniasis
e.g.	Exempli gratia
DHR123	Dihydrorhodamine 123
DL	Disseminated leishmaniasis
dLN	Draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

DNase	Desoxyribonuclease
dsDNA	Double stranded deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
EMA	European Medicines Agency
ERK1/2	Extracellular-signal Regulated Kinase 1/2
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
Fa2h	*Fatty acid 2-hydroxylase*
FACS	Fluorescence-activated cell sorting
Far2	*Fatty acyl- coenzyme a reductase 2*
FCS	Fetal calf serum
FDA	Food and Drug Administration (United States)
FRAC	Fluorescence recovery after conversion
FTC / F.T.C	Fabienne Tacchini-Cottier
G-418	Geneticin
GCP-2	Granulocyte chemotactic protein 2
G-CSF	Granulocyte-colony stimulating factor
Gfi1	Growth Factor Independent 1
GFP	Green fluorescent protein
GO-analysis	Gene ontology analysis
h	hours
H-2	Histocompatibility-2
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hephl1	*Hephaestin-like 1*
HIV-1	Human immunodeficiency virus 1
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen – DR isotype

HSC	Hematopoietic stem cell
Hsd17b2	*Hydroxysteroid (17-beta) dehydrogenase 2*
Hsd3b6	*Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6*
HSPC	Hematopoietic stem and progenitor cell
i.d.	Intradermal
i.e.	Id est
IFN	Interferon
IFN-β	Interferon-β
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Intralesional
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
MIP-1β /CCL4	Macrophage inflammatory protein-1β / C-C motif chemokine ligand 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-21	Interleukin 21

IL-22	Interleukin 22
IM	Intramuscular
iNOS	Inducible nitric oxide synthase
IR	Ivo Regli
IV	Intravenous
kg	Kilogram
KP	Katiuska Passelli
L. amazonensis	*Leishmania amazonensis*
L. brazilensis	*Leishmania brazilensis*
L. chagasi	*Leishmania chagasi*
LCF	*Leihsmania chemotactic factor*
LCL	Localized cutaneous leishmaniasis
LCMV	Lymphocytic choriomeningitis virus
LDA	Limiting dilution assay
L. donovani	*Leishmania donovani*
L. evansi	*Lutzomyia evansi*
L. infantum	*Leishmania infantum*
L. major	*Leishmania major*
L. major Sd	*Leishmania major* Seidman
L. martiniquiensis	*Leishmania matiniquiensis*
L. mexicana	*Leishmania mexicana*
L. mex^{SWITCH}	*Leishmania mexicana* photoswitchable
L. longipalpis	*Lutzomyia longipalpis*
L. olmeca	*Lutzomyia olmeca*
L. panamensis	*Lutzomyia panamensis*
LPG	Lipophosphoglycan
LR	Leishmaniasis recidivans

L. tropica	*Leishmania tropica*
L. vallesi	*Lutzomyia vallesi*
L. (V.) p.	*Leishmania (Viannia) panamensis*
L. whitmani / intermedia	*Lutzomyia whitmani / intermedia*
Ly6C	lymphocyte antigen 6 complex, locus C1
Ly6G	Ly6g lymphocyte antigen 6 complex, locus G
LysM	Lysin motif
M199	Medium 199
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MA	Meglumine antimoniate
MAG	Maria Adelaida Gómes
MAPK	Mitogen-Activated Protein Kinase Pathways
MA-R	Meglumine antimoniate resistance
MCL	Mucocutaneous leishmaniasis
mg	Milligram
MHC	Major Histocompatibility Complex
MHC-II	Major Histocompatibility Complex, class II
MIL	Miltefosine
MIL-R	Miltefosine resistance
mL	Milliliter
µL	Microliter
mm	Millimeter
µM	Micromolar
moDCs	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MPO	Myeloperoxidase

mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
n	Sample size
NADPH synthase / oxidases	Dihydronicotinamide-adenine dinucleotide phosphate synthase / oxidases
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Nancy Gore Saravia
NIH/NIAID	National Institutes of Health / National Institute of Allergy and Infectious Disease (United States)
NIK	NF-KappaB Inducing Kinase
NK-cell	Natural killer cell
NKT-cell	Natural killer T cell
NLRP3	Nucleotide-binding and oligomerization domain-like receptor pyrin domain-containing 3
Nm	Nanometer
NNN medium	Novy-MacNeal-Nicolle medium
NO	Nitric oxide
NOD-like receptors	Nucleotide-binding oligomerization domain-like receptors
NOX	NADPH oxidases
NOX2	NADPH oxidase 2
NW	New World species
OF	Olga Fernandez
OW	Old World species
p28	Protein 28
PAMP	Pathogen-associated molecular patterns
P. alexandri	*Phlebotomus alexandri*
P. argentipes	*Phlebotomus argentipes*
P. ariasi	*Phlebotomus ariasi*

PBS	Phosphate-buffered saline
PBMC	Peripheral Blood Mononuclear Cell
P. chinensis	*Phlebotomus chinensis*
pDCs	Plasmacytoid dendritic cells
PDL1	Programmed death-ligand 1
P. dubosqi	Phlebotomus dubosqi
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
P. langeroni	*Phlebotomus langeroni*
P. langeroni orientalis	*Phlebotomus langeroni orientalis*
P. longiductus	*Phlebotomus longiductus*
PMA	Phorbol 12-myristate 13-acetate
P. martini	*Phlebotomus martini*
PMN	Polymorphonuclears (Neutrophils)
P. neglectus	*Phlebotomus neglectus*
P. papatasi	*Phlebotomus papatasi*
P. perniciosus	*Phlebotomus perniciosus*
PRR	Pattern recognition receptors
PSG	Promastigote secretory gel
P. seregentii	*Phlebotomus seregentii*
PSN	Penicillin-Streptomycin-Neomycin
P. syriacus	*Phlebotomus syriacus*
PV	Parasitophorous vacuole
Rdh1	*Retinol dehydrogenase 1*
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI medium	Roswell Park Memorial Institute medium

SbV	Pentavalent antimony
s.c.	subcutaneous
Scd1	*Stearoyl-Coenzyme A desaturase 1*
Scd3	*Stearoyl-Coenzyme A desaturase 3*
Sdr16c5	*Short chain dehydrogenase/reductase family 16C, member 5*
Sdr16c6	*Short chain dehydrogenase/reductase family 16C, member 6*
Sdr42e1	*Short chain dehydrogenase/reductase family 42E, member 1*
SNARF	Seminaphtharhodafluor
spp.	Species
SSG	Sodium Stibogluconate
ssRNA	single stranded ribonucleic acid
STAT-1	Signal transducer and activator of transcription 1
STAT-4	Signal transducer and activator of transcription 4
STAT-6	Signal transducer and activator of transcription 6
TGF-β	Transforming growth factor β
Th	T-helper
Th1	T-helper1
Th2	T-helper2
Th9	T-helper9
Th17	T-helper17
Th22	T-helper22
Tfh	T follicular helper cell
TIR-domain	Toll/interleukin-1 receptor domain
TLR	Toll-like receptor
TLR1	Toll-like receptor 1
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3

TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TLR10	Toll-like receptor10
TLR11	Toll-like receptor 11
TLR12	Toll-like receptor 12
TLR13	Toll-like receptor 13
TMRC	Tropical Medicine Research Centers
TNF-α	Tumor necrosis factor-α
TRAIL	Tumor-necrosis-factor-related-apoptosis-inducing-ligand
Tregs	Regulatory T-cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
UK	United Kingdom
U.S.	United States
USA	United States of America
UV	Ultraviolet
VL	Visceral leishmaniasis
WHO	World Health Organization
WHO-IRTC	World Health Organization – Immunology Research and Trainings Center
WT	Wild type

13. Annex

JLB

Highlighted Article

Frontline Science: *Leishmania mexicana* amastigotes can replicate within neutrophils

Benjamin P. Hurrell, Manon Beaumann,* Sandrina Heyde,† Ivo B. Regli,* Andreas J. Müller,† and Fabienne Tacchini-Cottier*,1*

*Department of Biochemistry, World Health Organization-Immunology Research and Training Collaborative Center, University of Lausanne, Epalinges, Switzerland; and †Otto-von-Guericke-University Magdeburg and Helmholtz Centre for Infection Research Braunschweig, Magdeburg, Germany

RECEIVED APRIL 24, 2017; REVISED JUNE 15, 2017; ACCEPTED JUNE 21, 2017. DOI: 10.1189/jlb.4HI0417-158R

ABSTRACT

Cutaneous leishmaniasis is a neglected tropical disease, causing a spectrum of clinical manifestations varying from self-healing to unhealing lesions that may be very difficult to treat. Emerging evidence points to a detrimental role for neutrophils during the first hours following infection with many distinct *Leishmania* species (spp.) at a time when the parasite is in its nonreplicative promastigote form. Neutrophils have also been detected at later stages of infection in unhealing chronic cutaneous lesions. However, the interactions between these cells and the replicative intracellular amastigote form of the parasite have been poorly studied. Here, we show that *Leishmania mexicana* amastigotes are efficiently internalized by neutrophils and that this process has only a low impact on neutrophil activation and apoptosis. In neutrophils, the amastigotes were found in acidified vesicles. Furthermore, within cutaneous unhealing lesions, heavily infected neutrophils were found with up to 6 parasites per cell. To investigate if the amastigotes could replicate within neutrophils, we generated photoconvertible fluorescent parasites. With the use of flow cytometry imaging and time-lapse microscopy, we could demonstrate that a subset of parasites replicated within neutrophils. Overall, our data reveal a novel role for neutrophils that can act as a niche for parasite replication during the chronic phase of infection, thereby contributing to disease pathology. *J. Leukoc. Biol.* 102: 1187–1198; 2017.

Introduction

Leishmania spp. are protozoan intracellular parasites causing leishmaniasis, a spectrum of neglected tropical diseases ranging from localized cutaneous to deadly visceral forms. Flagellated infectious metacyclic promastigotes are deposited in the host skin by the bite of an infected female sand fly. Parasites rapidly localize within recruited neutrophils and eventually reside in macrophages. Within macrophages, promastigotes transform into amastigotes, the nonflagellated, replicative form of the parasites, where they proliferate. Protection against the disease is linked to the development of $CD4^+$ T cells secreting IFN-γ that induce macrophage microbicidal activity [1]. Some amastigotes can, however, escape macrophage killing and proliferate within PV, contributing to parasite persistence within lesions [2]. There is currently no efficient vaccine preventing leishmaniasis.

Neutrophils are rapidly recruited following infection with various *Leishmania* spp. promastigotes [3–7]. They are the first cells to get infected as they phagocytose promastigotes, accounting at the site of infection for >80% of total infected cells, 24 h after *Leishmania major* or *L. mexicana* intradermal injection [4, 7, 8]. Macrophages can either phagocytose apoptotic neutrophils harboring live promastigotes [9, 10] and/or free parasites released from neutrophils [4]. The neutrophil responses upon promastigote encounter are broad and vary depending on the *Leishmania* spp. They may include induction of the oxidative burst, degranulation, and NET formation, as recently reviewed in Hurrell et al. [11]. Furthermore, neutrophils can release cytokines and chemokines [12] that can affect the local microenvironment, as shown following infection with different *Leishmania* spp. in both humans and mice [9, 13, 14]. The importance of neutrophils in early disease events was highlighted in multiple studies using either neutrophil-depleting antibodies or neutropenic mice. The results were shown to vary depending on the *Leishmania* spp. and the genetic background of the host, reviewed in Hurrell et al. [11] and Carlsen et al. [15]. With the use of *L. mexicana* parasites, a New World *Leishmania* spp., we have previously shown that the early neutrophil recruitment following infection has a negative impact on disease outcome, contributing to the development of chronic lesions; associated absence of an efficient, protective immune response; and failure to control parasite load [7].

Abbreviations: AnV = Annexin V, APC = allophycocyanin, AxAma = axenic amastigotes, BM = bone marrow, CCCP = chlorophenylhydrazone, CD62L = cluster of differentiation 62 ligand, DHR123 = dihydrorhodamine 123, FRAC = fluorescence recovery after conversion, iDMEM = incomplete DMEM, LDA = limiting dilution assay, mKikumeGR = monomeric Kikume Green-Red, MOI = multiplicity of infection, MEG = N-methyl-D_glucamine, NET = neutrophil extracellular trap, PV = parasitophorous vacuole, ROS = reactive oxygen species, spp. = species, WT = wild-type

The online version of this paper, found at www.jleukbio.org, contains supplemental information.

1. Correspondence: Dept. of Biochemistry, WHO Immunology Research and Training Collaborative Center, University of Lausanne, chemin des Boveresses 155, 1066 Epalinges, Vaud, Switzerland. E-mail: Fabienne.Tacchini-Cottier@unil.ch

Whereas early interactions between neutrophils and the infective *Leishmania* promastigotes have been studied extensively, very little data are available on the interactions between neutrophils and the intracellular amastigote form of the parasite within lesions. Neutrophils were found in both human and murine cutaneous lesions. In humans, they were detected in lesion smears of *Leishmania braziliensis* patients [14] and detected by microscopy in tegumentary leishmaniasis patient lesion biopsies [16, 17]. In addition, up-regulation of a pattern of neutrophil attracting chemokines was observed by transcriptional profiling of biopsy specimens with chronic dermal lesions as a result of infection with *Leishmania panamensis* and *L. braziliensis*, strongly suggesting the presence of neutrophils in these lesions [18, 19]. In mice, the presence of neutrophils was reported in lesions of unhealing *L. major* LV39-infected BALB/c mice [3], in lesions of infected *L. major* Seidman C57BL/6 mice [20], and in unhealing lesions of C57BL/6 mice coinfected with *L. major* Friedlin and lymphocytic choriomeningitis virus [21]. Neutrophils were also observed in lesions of *Leishmania chagasi*-infected dogs [22]. Altogether, these findings suggest that neutrophils play a role in the pathogenesis of cutaneous *Leishmania* lesions. The biologic relevance for the presence of neutrophils and subsequent interactions with amastigotes within chronic cutaneous lesions in leishmaniasis, however, still remain largely unknown.

Here, we aimed at investigating the interactions between neutrophils and amastigotes in vitro and in chronic *L. mexicana* lesions of C57BL/6 mice. We first showed that *L. mexicana* amastigotes were efficiently internalized by neutrophils in vitro and that amastigote internalization only had a minor impact on neutrophil viability and activation. Neutrophils present in chronic ear lesions were heavily infected, and 70% harbored >2 intact amastigotes per cell. With the use of newly generated photoconvertible *L. mexicana* parasites, we could demonstrate that amastigotes can replicate ex vivo within neutrophils.

MATERIALS AND METHODS

Ethics statement

All procedures involving human blood were conducted according to the principles specified in the Declaration of Helsinki. All healthy donors provided informed consent, and the study was approved by the Swiss Ethical Committee of the State of Vaud (CER-VD 2017-00182). The data were analyzed anonymously. All animal experimental protocols were approved by the Veterinary Office Regulations of the State of Vaud, Switzerland, (Authorization 1266.6-8, to F.T-C.) and performed in compliance with Swiss ethic laws for animal protection.

Mice

WT C57BL/6 mice were purchased from Envigo (Cambridgeshire, United Kingdom) and bred under specified pathogen-free conditions at the Epalinges Center. Five- to 10-wk-old females (to prevent potential variations as a result of sex hormones) were used for the experiments.

Parasites

L. mexicana (MYNC/BZ/62/M379) WT; *L. mexicana* DsRed, generated as previously described [23] (gift of Prof. Tony Aebischer, Robert Koch Institute, Berlin, Germany); and *Lmex*^{SWITCH} parasites were cultured at 26°C in complete M199. Transgenic parasites were cultured in medium supplemented with Hygromycin B (Sigma-Aldrich, St. Louis, MO, USA) at 50 μg/ml. Infectivity of parasites was maintained by regular passage through C57BL/6 mice.

Generation of mKikumeGR-expressing *L. mexicana*^{SWITCH} parasites

The *Lmex*^{SWITCH} parasite strain was generated by targeted integration of the *mKikumeGR* gene into the recombinant RNA locus of *L. mexicana* WT parasites. For this, the *mKikumeGR* gene sequence [24] was synthesized (Eurofins Scientific, Fresno, CA, USA) and cloned via *BamHI/BgIII* and *HindIII* (Roche, Basel, Switzerland) into a pLEXSY-Hyg2 vector (Jena Bioscience, Jena, Germany). The resulting plasmid was linearized using *SwaI* and electroporated into *L. mexicana* WT parasites. Stable transfectants were selected with 50 μg/ml Hygromycin B (Sigma-Aldrich) in complete M199. Single clones were obtained by LDA and validated by flow cytometry.

Promastigote and amastigote preparations and infections

Infectious metacyclic parasites were prepared from confluent stationary-phase parasites by Ficoll gradient density centrifugation (Sigma-Aldrich), as previously described [7]. For in vivo infections, 1×10^6 metacyclic parasites were needle inoculated in iDMEM into the ear dermis (10 μl), and lesion development was measured weekly using an electronic caliper. Lesion score was established as previously described [25]. In brief, naïve ears have a score of 0; the first signs of inflammation (redness) give the score 0.5. As soon as a lesion is detectable, the length, width, and thickness of the lesions are measured, and the highest value is used to assign a score for each increment of 0.5 mm. A score of 8 signifies tissue destruction. AxAma were obtained after incubation of stationary-phase parasites at 34°C for 3 d in complete M199 medium, supplemented with 50 μg/ml Hygromycin B (Sigma-Aldrich), 10% sodium phosphate buffer 100 mM, pH 5.5, and 10% heat-inactivated FCS. Lesion-derived amastigotes were isolated from lesions of mice infected subcutaneously for >8 wk after mechanical tissue disruption in 1× MEG buffer. In brief, tissue debris was washed away, and parasites were filtered through 40, 8, and 5 μm filters (all BD Falcon; BD Biosciences, San Jose, CA, USA); washed 3 times in the working medium; and used for the experiments. Macrophage-derived amastigotes were used in designated experiments. In brief, BM-derived macrophages were incubated with either *L. mexicana* or *L. major* stationary parasites at an MOI of 10 at 37°C for 2 h. Free parasites were washed away and adherent macrophages further incubated for 72 h at 37°C. Macrophages were then lysed with 0.1% SDS, and released amastigotes were washed before use. The purity of each parasite preparation (>99%) was assessed by microscopy before use.

Immune serum and parasite opsonization

Female C57BL/6 mice were inoculated in iDMEM with 1×10^6 metacyclic parasites in the hind footpad in a final volume of 50 μl. Blood was collected >8 wk postinfection and serum isolated by centrifugation, heat inactivated (30 min at 56°C), and stored at −20°C. In experiments involving opsonization of amastigotes, parasites were incubated for 30 min at 26°C with 1, 5, or 10% of immune serum before being used in the experiments.

Photoconversion

Violet light at 405–430 nm wavelength for photoconversion was obtained by assembling 4 light-emitting diodes (Strato AG, Berlin, Germany) together on an electronic plate. Free parasites or infected neutrophils were photoconverted in 50 μl in a 96-well plate, mounted 2 cm on top of the diodes. Before the experiments, illumination time was calibrated to increase the Red/Green fluorescence ratio in photoconverted parasites and set at 120 s/well (used in all experiments). Photoconverted parasites were analyzed at the indicated times postconversion, by flow cytometry, imaging flow cytometry, or fluorescent videomicroscopy, all without UV laser. In some experiments, parasites were incubated with 0.01% NaN_3 to inhibit parasite proliferation. All incubated photoconverted samples were analyzed side by side with a nonconverted and just-converted sample.

Neutrophil isolation

Mouse neutrophils were isolated from the BM of naive female C57BL/6 mice. In brief, BM neutrophils were isolated by negative or positive MACS selection (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. Neutrophil purity (>95%) was assessed for each experiment by performing a cytospin assay. Human neutrophils were isolated from peripheral venous blood of healthy volunteers. Density gradient centrifugation using PolymorphPrep (Progen Biotechnik, Heidelberg, Germany) was performed, and remaining RBCs were lysed using ACK buffer. Neutrophil purity (>95%) was assessed for each experiment by performing a cytospin assay.

Neutrophil coincubation with parasites

For coincubation experiments, neutrophils were plated in complete RPMI 1640 supplemented with 10% heat-inactivated FCS and let sediment 30 min at 37°C before adding the parasite preparations. Neutrophils were then cultured with transgenic fluorescent promastigotes or amastigotes at a MOI of 2 for 2 or 18 h at 37°C, unless stated differently. When indicated, free parasites were washed away 3 times in 1× PBS before further incubation. Following incubation, neutrophils were collected for the selected readout. In experiments analyzing parasite loads, LDAs were performed as described previously [3].

Flow cytometry

Neutrophils were collected after incubation times and processed for flow cytometry analysis. Parasite-infected neutrophils were identified based on the DsRed, Kikume Green, or Kikume Red positivity, depending on the used transgenic parasites. To measure neutrophil activation, neutrophils were further stained with CD62L. To measure oxidative burst, neutrophils and parasites were coincubated with 1 μM of the free radical sensor DHR123 molecular probe (Thermo Fisher Scientific, Waltham, MA, USA), emitting Green fluorescence when oxidized, used according to the manufacturer's conditions. As a positive control, neutrophils were coincubated with 1 μM PMA, (Roche). To analyze neutrophil apoptosis, neutrophils were further stained with AnV, according to the manufacturer's conditions and DAPI just before analysis. Infected ears were isolated and processed to single-cell suspensions. In brief, ears were recovered, homogenized in iDMEM containing 0.2 mg/ml Liberase TL (Roche) for 2 h at 37°C, and then filtered using 40 μm filters (BD Falcon; BD Biosciences). Cells were then further stained with CD45, Gr1, and Ly6C, and the neutrophil infection rate was identified based on the DsRed positivity of neutrophils. All stained cells were analyzed using a BD LSRFortessa system (BD Biosciences). Analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). The following antibodies and reagents were used: Ly6G-FITC, Ly6C-FITC, and CD45-PerCP-Cy5.5 (BD Biosciences); CD62L-PE-Cy7 (BioLegend, San Diego, CA, USA); AnV-PE-Cy7 and Gr1-APC (eBioscience, San Diego, CA, USA); and DAPI (Sigma-Aldrich).

Intracellular localization of amastigotes

At the indicated times after neutrophil and parasite coincubations, LysoSensor Green DND-189 (Thermo Fisher Scientific) was added to a final concentration of 1 μM to the culture, and cells were further incubated for 15 min at 37°C and 5% CO_2. Neutrophils were then transferred into a poly-L-lysine-coated microslide (μ-Slide VI$^{0.4}$; Ibidi, Munich, Germany) and analyzed by confocal laser-scanning microscopy (TCS SP8; Leica Microsystems, Buffalo Grove, IL, USA). With excitation (458 nm) and 491–526 nm emission, mKikume was photoconverted, as described before acquisition, and detected at 561 nm excitation and 571–620 nm emission. Image analysis was performed with ImageJ software (NIH, Bethesda, MD, USA). To determine the LysoSensor staining around the parasites, the distance between the half-maximal *L. mexicana* mKikume signal and the quarter-maximal LysoSensor signal was determined from fluorescence profile plots. As a negative control, the protonophore carbonyl cyanide 3-CCCP (Sigma-Aldrich) was additionally added to a final concentration of 1 mM to the cell culture before incubation.

Imaging flow cytometry

All samples were analyzed on an ImageStream cytometer (Amnis; Millipore Sigma, Billerica, MA, USA) at low speed and highest magnification (60×). Fifteen thousand events were acquired, based on area and aspect ratio of the brightfield, leaving out debris and free parasites from the acquisition. Data were analyzed in the IDEA software. Internalized vs. noninternalized parasites were defined using the internalization of the bright parasite spots within the membrane marker mask. The number of parasites per neutrophil was defined using the spot count within the parasite channel feature. Further analysis using ImageJ software (NIH) was used for Red/Green ratio calculation and analysis. The following reagents were used: Ly6G-FITC, Ly6C-FITC, and CD45-PerCP-Cy5.5 (BD Biosciences) and Gr1-APC-Cy7 (eBioscience).

Time-lapse videomicroscopy

Poly-L-lysine-coated μ-Slide 8-well ibiTreat chambers (Ibidi) and Phenol Red-free complete RPMI 1640 medium were used. Time-lapse microscopy was performed on a Zeiss inverted microscope (Axio Observer Z1 motorized), equipped with an environmental chamber for temperature, humidity, and 5% CO_2 with a 63× oil objective (EC "Plan-Neofluar" 63×/1.5 Oil M27) and a Photometrics camera (CoolSNAP HQ^2). Neutrophils were identified based on the brightfield, Kikume Red in the Rhodamine channel, and Kikume Green in the GFP channel. A picture was taken every 30 min. Quantitation of fluorescence intensities was performed using ImageJ software.

Statistical analysis

All *P* values were determined using Prism software (GraphPad Software, La Jolla, CA, USA) using the Student's *t* test for unpaired data. A linear regression was performed to analyze Red/Green ratios. The degree of significance is indicated (see figure legends).

RESULTS

L. mexicana amastigotes are internalized by neutrophils in vitro

We first investigated if *L. mexicana* amastigotes were internalized by neutrophils compared with promastigote uptake in vitro. We exposed C57BL/6 BM-sorted neutrophils to DsRed-expressing *L. mexicana* AxAma for 18 h at an MOI of 2 and performed flow cytometry analysis. Following exposure to metacyclic promastigotes, 14% of neutrophils internalized parasites. Amastigotes also infected neutrophils but less efficiently (7%; **Fig. 1A**). By the time chronic lesions develop, the parasite-specific adaptive immune response and associated B cell-derived antibodies are in place [26]. In line with this, prior opsonization of amastigotes with increasing concentrations (1–10%) of immune serum isolated from a chronically infected mouse increased the neutrophil infection rates. Amastigote internalization increased to 12.5 and 14% when opsonized, respectively, with 5 and 10% immune serum, values that are comparable with the ones observed following exposure to promastigotes (Fig. 1A). Thereafter, we used amastigotes opsonized with 10% immune serum. With the use of imaging flow cytometry, amastigote internalization was analyzed both quantitatively and qualitatively. A small frequency of neutrophils harbored parasites external to the cell surface, and these were excluded from the analysis (Fig. 1Ba and Ca). Analysis of infected neutrophils revealed that both promastigotes and opsonized amastigotes were efficiently internalized. Over 70% of neutrophils internalized either 1 promastigote (Fig. 1Bb) or 1 amastigote (Fig. 1Cb), with a minority harboring 2 or more parasites (Fig. 1Bc and d and Cc and d), as

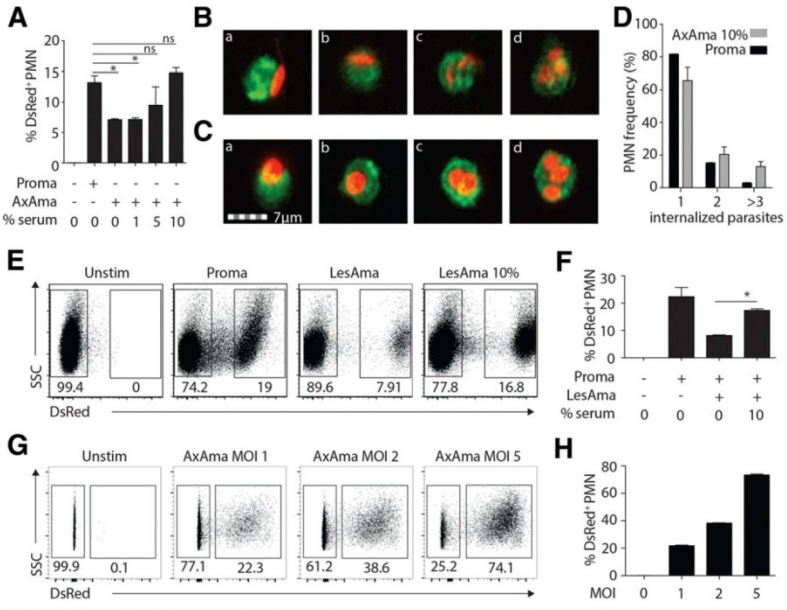

Figure 1. *L. mexicana* amastigotes are internalized by neutrophils in vitro. (A–E) BM neutrophils were cocultured in vitro for 18 h at 37°C at an MOI of 2 with DsRed-expressing *L. mexicana* metacyclic promastigotes (Proma) or AxAma. AxAma were pretreated or not with the indicated concentrations of immune serum from a chronically infected mouse. $Ly6G^+$ $DsRed^+$ neutrophils were analyzed by flow cytometry and by imaging flow cytometry using the Amnis ImageStream. (A) Quantitation of DsRed-expressing neutrophils presented as the mean frequency of infected neutrophils ± SEM. Data shown are representative of >3 experiments with $n = 3$, $^*P < 0.05$; ns, not significant. PMN, Polymorphonuclear leukocyte. Representative ImageStream images of neutrophils infected with (B) metacyclic promastigotes or (C) 10% immune serum-opsonized amastigotes with (a) a parasite outside of neutrophils and (b–d) neutrophils harboring, respectively, 1, 2, or >3 parasites. Green, Ly6G; red, DsRed. (D) Corresponding quantitation presented as the mean frequency ± SEM of neutrophils with 1, 2, or >3 internalized parasites. Data shown are representative of >3 experiments with $n = 3$. (E and F) BM neutrophils were cocultured in vitro for 18 h at 37°C at an MOI of 2 with DsRed-expressing *L. mexicana* metacyclic promastigotes or lesion-derived amastigotes (LesAma), pretreated or not with 10% immune serum. (E) Representative flow cytometry plots of DsRed-expressing neutrophils and (F) corresponding quantitation presented as the mean frequency of infected neutrophils ± SEM. Data shown are representative of 2 experiments with $n = 3$, $^*P < 0.05$. SSC, Side-scatter. (G and H) Human peripheral blood neutrophils were cocultured in vitro for 18 h at 37°C with DsRed-expressing *L. mexicana* AxAma at the indicated MOI, and DsRed-expressing neutrophils were analyzed by flow cytometry. (G) Representative flow cytometry plots of DsRed-expressing neutrophils and (H) corresponding quantitation presented as the mean frequency of infected neutrophils ± SEM. Data shown are representative of 2 experiments with $n = 3$, $^*P < 0.05$.

shown quantitatively in Fig. 1D. As AxAma may differ from lesion-derived amastigotes [27], experiments were repeated using freshly isolated lesion-derived amastigotes. Neutrophil internalization of lesion-derived amastigotes was also less efficient than that of metacyclic promastigotes (Fig. 1E), but opsonization of the amastigotes led to a similar internalization rate than that of promastigotes (Fig. 1F). Similar data were also obtained using intracellular amastigotes isolated from macrophages infected in vitro (data not shown). Collectively, our results reveal that when opsonized with immune serum, neutrophils internalize *L. mexicana* amastigotes as efficiently as metacyclic promastigotes in vitro. Furthermore, *L. mexicana* amastigotes were even more efficiently internalized by blood-derived human neutrophils in the absence of serum with increasing uptake linked to the MOI (Fig. 1G and H). Thus, our findings indicate that both mouse and human neutrophils can efficiently uptake *L. mexicana* amastigotes in vitro.

L. mexicana amastigote uptake has a minimal impact on neutrophil activation

Infection of neutrophils with *Leishmania* spp. promastigotes may affect neutrophil apoptosis in a positive or negative way depending on the strain of parasites or source of neutrophils used [7, 8, 28, 29]. Infection with opsonized amastigotes triggered a small increase in early apoptosis (AnV^+DAPI^-) and late apoptosis/necrosis (AnV^+DAPI^+) in neutrophils, 18 h post-infection (**Fig. 2A**). Nevertheless, ~90% of infected neutrophils were alive (AnV^-DAPI^-; Fig. 2B), showing that *L. mexicana* amastigotes are only weak inducers of neutrophil apoptosis. CD62L is a cell-adhesion molecule down-regulated upon activation. Analysis of CD62L expression on infected neutrophils and bystander (uninfected but parasite exposed) neutrophils revealed that <30% of infected neutrophils were activated (Fig. 2C), with 70% of infected neutrophils not down-regulating CD62L surface expression (Fig. 2D). Oxidative burst is another hallmark of neutrophil activation. ROS production was analyzed by flow cytometry, measuring oxidation of the DHR123 probe in bystander vs. amastigote-infected neutrophils. Amastigote internalization did not trigger ROS production either in bystander or in infected neutrophils, whereas as expected, exposure of neutrophils to PMA induced efficient ROS production (Fig. 2E and F). Collectively, these data reveal that internalization of *L. mexicana* amastigotes in vitro only has a mild effect on neutrophil apoptosis and activation.

Lesion-derived neutrophils harbor multiple parasites

To investigate neutrophil–amastigote interactions within localized cutaneous unhealing lesions, female C57BL/6 mice were needle inoculated in the ear dermis with 1×10^6 metacyclic DsRed expressing *L. mexicana* parasites, and lesion development was assessed weekly (**Fig. 3A**). The ear was isolated 10 wk postinfection at a time when a defined unhealing, cutaneous lesion is present (Fig. 3B). Flow cytometry revealed that 6–10% of $CD45^+$ hematopoietic lesion-derived cells were ($Gr1^+Ly6C^{int}$) neutrophils (Fig. 3C). Furthermore, an elevated frequency (70%) of lesion-derived neutrophils were $DsRed^+$, thus containing *L. mexicana* amastigotes (Fig. 3C and D). We further analyzed

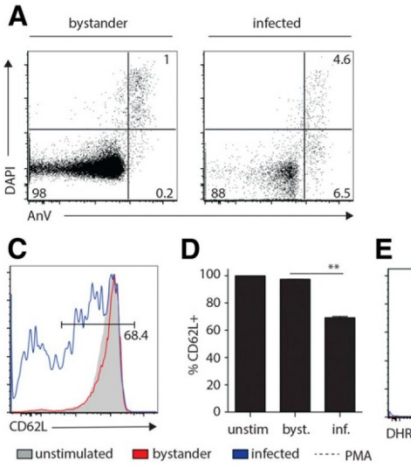

Figure 2. Infection of neutrophils with *L. mexicana* amastigotes has little impact on neutrophil apoptosis and activation. (A–D) BM neutrophils were cocultured in vitro for 18 h at 37°C at an MOI of 2 with DsRed-expressing *L. mexicana* AxAma pretreated with 10% immune serum. (A) Representative flow cytometry plots of AnV and DAPI expression within DsRed$^-$ (bystander) or DsRed$^+$ (infected) Ly6G$^+$ neutrophils and (B) corresponding quantitation presented as the mean frequency ± SEM of AnV$^-$DAPI$^-$ viable, AnV$^+$DAPI$^-$ early-apoptotic, or AnV$^+$DAPI$^+$ late-apoptotic neutrophils. Data shown are representative of 3 experiments with $n = 3$. (C) Representative flow cytometry histograms of CD62L surface expression within unstimulated (unstim; gray), bystander (byst.; red), or infected (inf.; blue) neutrophils and (D) corresponding quantitation presented as the mean expression of CD62L$^+$ neutrophils ± SEM. Data shown are representative of 3 experiments with $n = 3$, $^{**}P < 0.01$. (E and F) BM neutrophils were cocultured in vitro for 2 h at 37°C at an MOI of 2 with DsRed-expressing *L. mexicana* AxAma pretreated with 10% immune serum. (E) Histograms of ROS-sensing DHR123 molecular probe within unstimulated (gray), bystander (red), infected (blue), or PMA-treated neutrophils and (F) corresponding quantitation presented as the mean DHR123 mean fluorescence intensity (MFI) ± SEM. Data shown are representative of 2 experiments with $n = 6$. ns, Nonsignificant.

the number of parasites within infected neutrophils by imaging flow cytometry (Fig. 3E and F). In sharp contrast to our in vitro data, the majority of neutrophils harbored 2 or over 3 intact internalized amastigotes (Fig. 3E), reaching, in rare cases, 6 intact amastigotes within neutrophils (data not shown).

Furthermore, the viability of lesion-derived neutrophils was assessed by flow cytometry, revealing that similar to our in vitro data, ~80% of neutrophils were alive (AnV$^-$DAPI$^-$; Fig. 3G and H). These findings show that within lesions, neutrophils are present, alive, and harbor an elevated number of amastigotes.

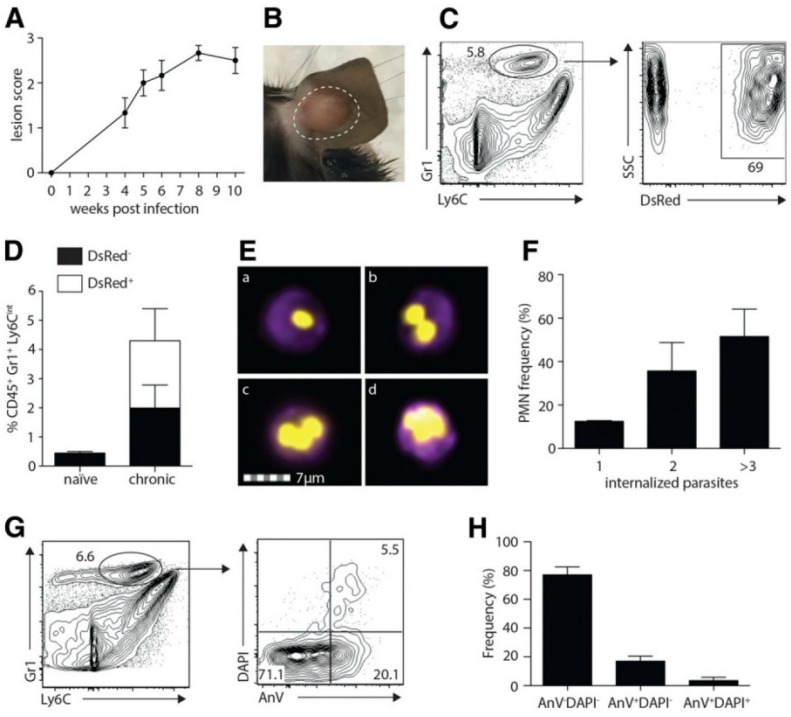

Figure 3. Neutrophils are recruited and infected in chronic *L. mexicana* lesions. (A) Female C57BL/6 mice were inoculated intradermally with 10^6 DsRed-expressing metacyclic *L. mexicana* promastigotes, and lesion score was measured weekly using an electronic caliper. Score of 0: naïve ear to score 3 (3–3.5 mm lesion). (B) Representative 10-wk-old chronic lesion (white dotted line). (C) Ten week postinfection ears were collected, digested, and treated to single-cell suspensions for flow cytometry and imaging flow cytometry analysis. Representative flow cytometry plots of CD45$^+$Gr1$^+$Ly6C^{int} neutrophils and CD45$^+$Gr1$^+$Ly6C^{int}DsRed$^+$ infected neutrophils are shown and (D) corresponding quantitation presented as the mean frequency ± SEM of DsRed$^-$ uninfected (black) or DsRed$^+$ infected (white) CD45$^+$Gr1$^+$Ly6C^{int} neutrophils in naive or chronic ear lesions. Data shown are representative of >3 experiments with $n = 5$. (E) Representative ImageStream images of neutrophils infected with (a) 1, (b) 2, or (-d) >3 amastigotes. Violet, Ly6G; yellow, DsRed. (F) Corresponding quantitation presented as the mean frequency ± SEM of neutrophils with 1, 2, or >3 internalized parasites. Data shown are pooled of 3 experiments with $n = 3$. (G) Representative flow cytometry plots of AnV and DAPI expression of CD45$^+$Gr1$^+$Ly6C^{int} neutrophils within a 10-wk-old chronic lesion and (H) corresponding quantitation presented as the mean frequency ± SEM of AnV$^-$DAPI$^-$ viable, AnV$^+$DAPI$^-$ early-apoptotic, or AnV$^+$DAPI$^+$ late-apoptotic neutrophils. Data shown are representative of 2 experiments with $n = 4$.

L. mexicana amastigotes survive and reside within neutrophil acidic vesicles

We observed multiple amastigotes within neutrophils in chronic *L. mexicana* lesions, and amastigotes were often detected close to each other in a linear manner within neutrophils. In addition, neutrophils were poorly activated by *L. mexicana* amastigotes, with ~90% of infected neutrophils as nonapoptotic in vitro. The mean *L. mexicana* amastigote-doubling time within axenic cultures is estimated to be ~16 h, whereas that observed in murine macrophages was previously reported to be ~33 h [30]. To investigate if amastigotes could replicate within neutrophils ex vivo, we infected neutrophils for 2 h with *L. mexicana* amastigotes, washed away extensively free parasites, and further incubated the infected neutrophils for 3, 24, or 48 h (**Fig. 4A**). During the first 24 h, a proportion of *L. mexicana* amastigotes was killed over time, but between 24 and 48 h, the number of live amastigotes did not decrease further, as analyzed following LDA (Fig. 4B). Similar experiments performed with *L. major* amastigotes also revealed the presence of live parasites, 24 and 48 h postinfection. However, the amastigote number in neutrophils was 10× lower for *L. major* than *L. mexicana*, 48 h postinfection (Supplemental Fig. 1). These data suggest that there exist differences in survival and/or replication between amastigotes of these 2 *Leishmania* spp. We subsequently analyzed the frequency of *L. mexicana* amastigotes per neutrophil after 48 h of incubation. In line with our in vivo data, the majority of neutrophils (80%) harbored 2 or over 3 intact amastigotes (Fig. 4Cb and c, respectively), with only a minority (20%) of neutrophils harboring 1 amastigote (Fig. 4Ca), as shown quantitatively in Fig. 4D. These results suggest that similar to what we observed in chronic lesions of *L. mexicana*-infected mice, neutrophils harbor multiple amastigotes when left 48 h in culture, suggesting that some intracellular parasites may replicate within these cells.

L. mexicana amastigotes are known to replicate in large communal, acidified parasitophorous vesicles, with an acidic pH that favors amastigote intracellular replication within macrophages. To investigate if amastigotes also reside in large acidic compartments within neutrophils, BM-derived, MACS-purified neutrophils were infected with *L. mexicana* at an MOI of 2, washed, and left in culture for 24 or 48 h before analysis. Acidic compartments were then stained with the pH-sensitive LysoSensor Green probe. Confocal laser-scanning microscopic analysis revealed that the parasite (red) resides in an acidic vesicle within neutrophils. Twenty-four hours after initial incubation, the distance between acidic boundaries and the parasite membranes was relatively tight (**Fig. 5A and B**, upper), as quantified in Fig. 5C. To validate the LysoSensor specificity, treatment with the CCCP protonophore was used to abolish LysoSensor staining (Fig. 5A, lower). Forty-eight hours after neutrophil infection, enlargement of the acidic compartment containing the amastigote was observed (Fig. 5D) and further quantified (Fig. 5E, upper, and F). Taken together, these data show that *L. mexicana* amastigotes reside within neutrophil acidic compartments that enlarge over time, somehow resembling macrophage large communal PV that develops following infection with parasites of the *L. mexicana* complex. Collectively, these findings suggest that

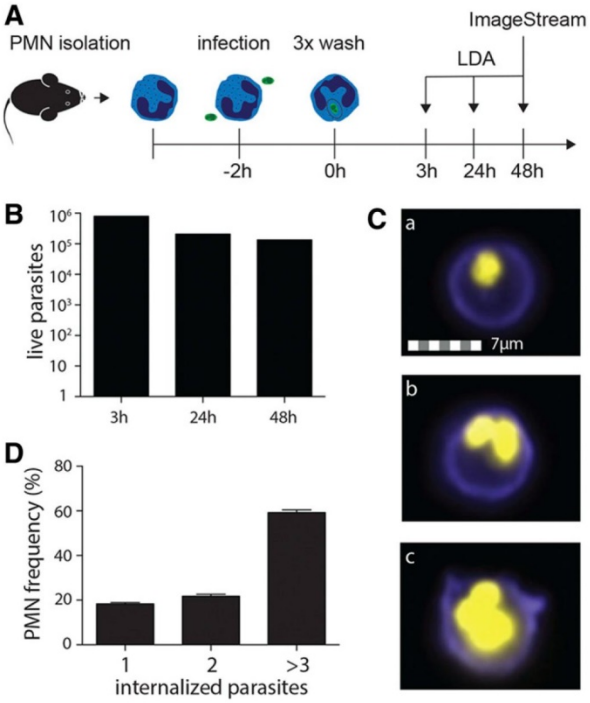

Figure 4. Neutrophils harbor multiple amastigotes that survive 48 h after infection. BM neutrophils were cocultured in vitro for 2 h at 37°C at an MOI of 2 with DsRed-expressing *L. mexicana* AxAma. Free parasites were washed away 3× with PBS 1 ×, and infected neutrophils were further incubated for the indicated times at 37°C. (A) Experimental design. (B) LDA performed 3, 24, and 48 h postinfection. (C) Representative ImageStream images of 48 h incubated-infected neutrophils harboring (a) 1, (b) 2, or (c) >3 amastigotes. Violet, Ly6G; yellow, DsRed. (D) Corresponding quantitation presented as the mean frequency ± SEM of neutrophils with 1, 2, or >3 internalized parasites. Data shown are pooled of 3 experiments with $n = 3$.

L. mexicana amastigotes reside within neutrophil acidic compartments.

To determine firmly if amastigotes replicated within neutrophils, we generated a strain of *L. mexicana* (*Lmex*^{SWITCH}) parasites that constitutively express the photoconvertible GFP mKikumeGR under a ribosomal promoter [24]. The use of parasites expressing this photoconvertible protein is a very efficient approach for tracking pathogen proliferation [31]. *Lmex*^{SWITCH} parasites constitutively express Green fluorescence (520 nm emission), which is converted to almost-complete Red fluorescence (580 nm emission) by a pulse of violet light (405 nm). FRAC in photoconverted *Lmex*^{SWITCH} parasites is associated with parasite proliferation. The principle of FRAC is that upon parasite division, photoconverted Red proteins are diluted, as nonconverted Green mKikumeGR proteins are synthesized de novo. This change in fluorescence reflects the overall increase in protein synthesis required for parasite division (**Fig. 6A**). We first analyzed FRAC by incubating photoconverted *Lmex*^{SWITCH} parasites at 26°C and measuring Kikume Red and Kikume Green fluorescence by flow cytometry, 0, 24, 48, and 60 h after

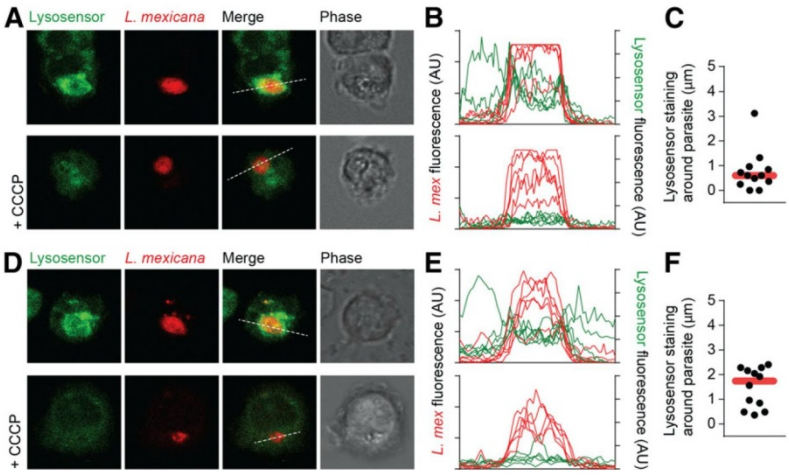

Figure 5. *L. mexicana* amastigotes localize within neutrophil acidic compartments that enlarge over time. BM neutrophils were infected with *Lmex*^{SWITCH} AxAma at an MOI of 2. Twenty-four (A–C) or 48 (D–F) h after infection, parasites were photoconverted, and neutrophil acidic compartments were stained with 1 µM of the pH-sensitive probe LysoSensor Green. Samples were analyzed by confocal laser-scanning microscopy. (A and D, upper) Photoconverted *Lmex*^{SWITCH} amastigotes (red) within acidic compartments (green) of neutrophils, 24 and 48 h, respectively, after infection. (A and D lower) Photoconverted *Lmex*^{SWITCH} amastigotes within neutrophils treated with 1 mM of the protonophore CCCP, 24 and 48 h, respectively, after infection (negative control). (B and E) Fluorescence intensity line plots (normalized to the 3-fold parasite diameter spanned by the plot) of intracellular parasites (red) and the green fluorescent LysoSensor staining (green) surrounding the pathogens, 24 and 48 h, respectively, after infection. (C and F) Quantitative analysis of the distance between the boundaries of the parasite and of the surrounding LysoSensor staining, 24 and 48 h, respectively, after infection. Data shown are representative of 2 experiments with $n > 3$ independent stainings per condition.

photoconversion. Photoconverted parasites recover back from Kikume Red to 77% of Kikume Green, 48 h postincubation (Fig. 6B). Inhibition of parasite mitochondrial respiration by coincubating parasites with sodium azide (NaN_3) inhibited FRAC, confirming that FRAC analysis correlates with parasite proliferation (Fig. 6B, lower right).

We next examined whether FRAC was detectable for intracellular amastigotes within neutrophils. After 2 h of infection with *Lmex*^{SWITCH} amastigotes (MOI of 2) in vitro, free parasites were washed away, and amastigotes within neutrophils were photoconverted and incubated further for 12, 24, and 48 h for flow cytometry analysis of FRAC (Fig. 6C). Following incubation, 20% of the neutrophils were infected. FRAC was not observed following 12 and 24 h in neutrophils (data not shown). After 48 h, however, ~8% of neutrophils were infected with amastigotes that had recovered Kikume Green fluorescence, providing evidence that amastigotes divided within neutrophils. (Fig. 6D, right, and E). As controls, nonconverted and just-converted samples expressed, respectively, all Kikume Green or all Kikume Red fluorescence (Fig. 6D, left).

Visualization of *L. mexicana* amastigote replication within neutrophils

To exclude the possibility that extracellular *L. mexicana* proliferation and subsequent uptake contributed to FRAC detected within neutrophils, we further analyzed and visualized amastigote

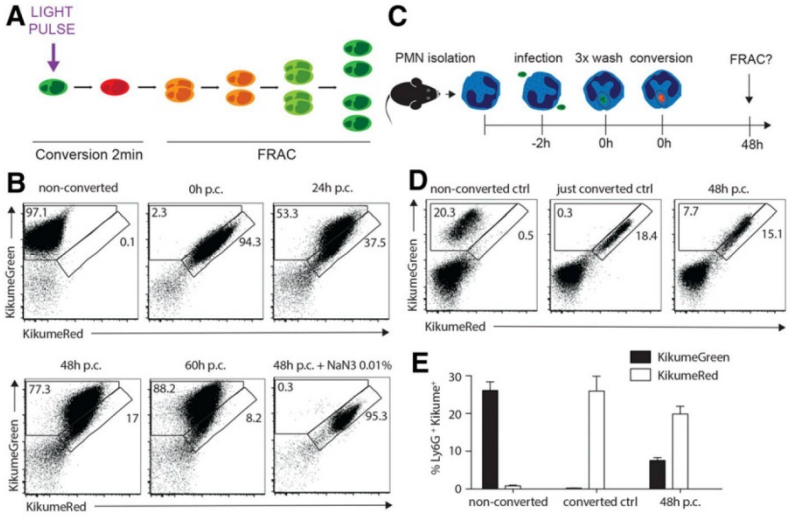

Figure 6. Fluorescence recovery after photoconversion reveals *L. mexicana* amastigote replication within neutrophils in vitro. (A) FRAC using *Lmex*^{SWITCH} parasites. (B) Photoswitchable *Lmex*^{SWITCH} parasites were nonconverted or photoconverted (p.c.) for 2 min and further incubated for the indicated times at 26°C before flow cytometry analysis of FRAC by measuring green (Kikume Green) and red (Kikume Red) fluorescences. (C–E) BM neutrophils were cocultured in vitro for 2 h at 37°C at an MOI of 2 with *Lmex*^{SWITCH} AxAma. Free parasites were washed away 3×, and internalized parasites were photoconverted for 2 min and further incubated for 48 h at 37°C for flow cytometry analysis. (C) Experimental design. (D) Representative flow cytometry plots of Kikume Green and Kikume Red expressions within neutrophils, nonconverted (left), just converted (middle), or 48 h postconversion (right) and (E) corresponding quantitation presented as the means ± SEM expression of Kikume Green and Kikume Red expression within neutrophils. Data shown are representative of 3 experiments with $n = 3$.

FRAC distribution within neutrophils, 48 h after photoconversion and incubation by imaging flow cytometry. With the focus on 100 randomly chosen neutrophils, harboring 2 or more amastigotes, we separately calculated the ratio of photoconverted-to-nonconverted proteins for each amastigote within each neutrophil (Supplemental Fig. 2A and B). If the parasites would have proliferated extracellularly and then entered the neutrophils, then we would have expected a broad distribution of FRAC rates within 1 neutrophil. However, the average distribution of amastigote Red/Green ratios was very narrow within a given cell (Supplemental Fig. 2C). In line with this, we observed a very close correlation between the minimal and maximal amastigote Red/Green ratios within a given cell, looking at all of the infected neutrophils (**Fig. 7A**). These findings suggest that there is a cell-intrinsic mechanism that dictates whether amastigotes recover Kikume Green fluorescence—a process that can be explained only by intracellular replication. We next classified neutrophils according to whether they contained high-proliferating (below-average Red/Green ratio) or low-proliferating (above-average Red/Green ratio) internalized amastigotes and found that there was a clear indication that a given neutrophil mostly contains either high- or low-proliferating amastigotes, as shown by the negative correlation observed between the number of parasites below and above the average Red/Green ratio (Fig. 7B). Furthermore, the population of highly infected neutrophils (>4 amastigotes below-average Red/Green ratio) had the highest replication (Fig. 7B and Ca). Whereas the homogeneity of FRAC rates within many neutrophils suggested that the parasites originated from 1 single invasion event, some neutrophils harbored 2 amastigotes with dissimilar Red/Green ratios (Fig. 7Cb) or even 2 different groups of amastigotes with homogenous Red/Green ratios only within their respective group (Fig. 7Cc). This was possibly a result of 2 amastigotes having invaded the neutrophils independently and thus, exhibiting dissimilar Red/Green ratios within the same cell.

When we scrambled the data, assigning each parasite to a random neutrophil (Supplemental Fig. 2D and E), the correlation between the maximal Red/Green ratio (Fig. 7D) and the negative correlation observed between the number of parasites below and above the average Red/Green ratio was no longer observed (Fig. 7E), further supporting the notion that the characteristic distribution of FRAC rates was a result of parasite replication within neutrophils.

To observe live parasite replication within neutrophils, we went on and imaged FRAC by time-lapse imaging in vitro. Neutrophils infected with *Lmex*^{SWITCH} parasites were imaged 40 h after photoconversion and incubation in vitro. A representative field containing neutrophils imaged during 150 min is shown. Within the neutrophil shown, a photoconverted Red fluorescent amastigote changes to 2 Green fluorescent parasites over the

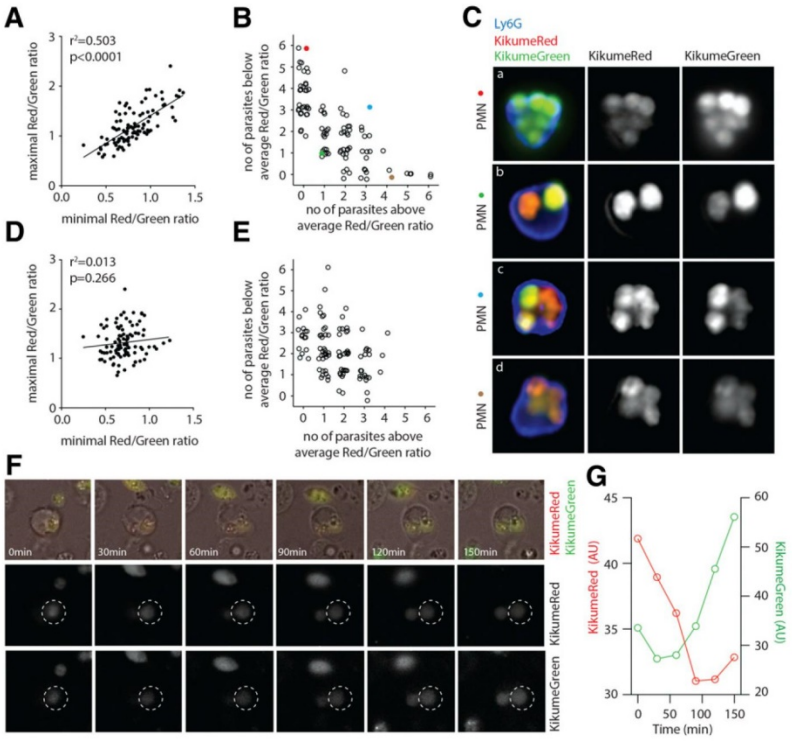

Figure 7. Visualization of *L. mexicana* amastigote proliferation within neutrophils. BM neutrophils were cocultured in vitro for 2 h at 37°C at an MOI of 2 with *Lmex*^{SWITCH} AxAma. Free parasites were washed away 3 times, and internalized parasites were photoconverted for 2 min and further incubated for 48 h at 37°C for imaging flow cytometry analysis. One hundred randomly chosen neutrophils infected with >2 amastigotes were further analyzed. (A) Correlation between amastigote minimal and maximal Red/Green ratios within each neutrophil in a standard analysis (all parasites assigned to their host cell) or (D) in a scrambled analysis (all parasites assigned arbitrarily to a host cell). (B) Classification of neutrophils according to the internalized amastigote fluorescence, either above or below total average Red/Green ratio in a standard analysis or (E) in a scrambled analysis. (C) Representative images are shown of neutrophils harboring (a) 6 amastigotes below-average Red/Green ratio, (b) 1 above and 1 below-average Red/Green ratio, (c) 3 above- and 3 below-average Red/Green ratio, and (d) 4 above-average Red/Green ratio. (F) BM neutrophils were cocultured in vitro for 2 h at 37°C at an MOI of 2 with *Lmex*^{SWITCH} AxAma. Free parasites were washed away 3 times, and infected neutrophils were photoconverted for 2 min and further incubated for 40 h at 37°C. Neutrophils were then adhered on poly-L-lysine-coated microscopy chambers and pictured every 30 min for 150 min on a time-lapse fluorescent microscope. Shown is a neutrophil harboring 1 Kikume Red amastigote dividing into 2 Kikume Green amastigotes (top) over the course of the imaging. Kikume Red (middle) and Kikume Green (bottom) fluorescences are also shown, and the dividing amastigote is circled. (G) Quantitation of the Kikume Red and Kikume Green variations within the dividing (circled) amastigote.

course of imaging, characteristic of FRAC (Fig. 7F, top). Single fluorescence (Kikume Red or Kikume Green) is also shown (Fig. 7F, middle and bottom), and the dividing amastigote is circled. We next quantified fluorescence variations within the dividing amastigote, which is to be expected as a result of dilution of the fluorescence protein between the 2 daughter cells [32]. Concomitantly, an increase of Kikume Green fluorescence occurred, which has previously been shown to be characteristic for parasite division events [31] (Fig. 7F and G). Overall, these results demonstrate that *L. mexicana* amastigotes are able to replicate within neutrophils.

DISCUSSION

Neutrophils are rapidly recruited to sites of infection, where their primary function is to engulf and clear pathogens. To this end, they use a variety of very efficient weapons, ranging from the release of microbial compounds, induction of oxidative burst, and formation of NETs [33, 34]. However, emerging data reveal that several pathogens are able to escape killing by neutrophils and use these cells to establish infection [35]. Following *Leishmania* inoculation, neutrophils are massively and rapidly recruited to the site of parasite deposition [11, 15], and several *Leishmania* spp. were shown to survive within these cells [7, 36–39]. In addition, neutrophils are found later in infection within both human and mouse chronic cutaneous *Leishmania* inflammatory lesions [3, 7, 16, 17, 20, 21, 40]. Neutrophils, through the release of inflammatory mediators and the formation of NETs, can drive inflammation [41]. *L. mexicana* was reported to elicit locally the formation of NETs and to favor the differentiation of Th17 cells recruiting more neutrophils to the lesion [7, 42]. These 2 processes may participate in the development and persistence of *L. mexicana* lesions.

Here, we first showed that *L. mexicana* amastigotes were efficiently internalized by neutrophils—a process that occurred in opsonized or nonopsonized amastigotes, even though phagocytosis of opsonized amastigotes was more efficient in murine neutrophils. The frequency of internalization of amastigotes by human blood-derived neutrophils appeared greater than that of mouse BM-derived neutrophils, with a very high frequency of amastigote internalization (40% at an MOI of 2), in absence of opsonization. These differences likely result from the origin of neutrophils (BM-derived vs. peripheral-extravasated blood neutrophils), as dermal mouse neutrophils found within the lesion were also heavily infected, as well as the differences between human and mouse neutrophils.

Parasite uptake was relatively silent, as revealed by the absence of intracellular ROS induction by the parasite, low apoptosis, and high expression of CD62L on infected neutrophils. Some amastigotes were killed very rapidly by neutrophils after in vitro infection, despite the absence of ROS induction in infected neutrophils. However, a significant subpopulation of parasites survived within murine neutrophils. Inhibition of ROS induction is one of the strategies used by several microorganisms to survive within neutrophils [43], but survival in the presence of ROS has been reported. For instance, amastigotes of the closely related *Leishmania amazonensis* spp. that also cause unhealing lesions in mice were shown to be internalized and survive within murine neutrophils in vitro. However, in contrast to *L. mexicana*, *L. amazonensis* induced neutrophil activation and ROS production, suggesting that the parasites were resistant to neutrophil killing mechanisms [38]. *L. braziliensis* amastigotes were also internalized by murine neutrophils in vitro; however, in sharp contrast to *L. mexicana* and *L. amazonensis*, neutrophils efficiently killed the parasites, at least in vitro [44]. These studies suggest that distinct parasite factors may differently impact neutrophil effector functions. Taken together, these and the present data show that amastigotes are internalized by murine neutrophils and that at least in vitro, a significant proportion of amastigotes from several *Leishmania* spp. are able to survive within neutrophils.

Neutrophil exposure to *Leishmania* amastigotes in vitro does not include either the cell–cell interactions or the inflammatory factors present at the site of infection. Furthermore, during chronic inflammation, the neutrophil lifespan has been reported to be significantly extended [45, 46]. Therefore, we sought to corroborate our in vitro observations in vivo. Consequently, we found that >70% of the neutrophils present in chronic lesions were infected. In contrast to our in vitro findings, the majority of neutrophils remarkably harbored 2 or more parasites. Furthermore, alignment of amastigotes within a given neutrophil, as well as the low levels of apoptotic markers found on lesion-derived neutrophils, suggested possible replication within these cells. In line with this, *L. mexicana* amastigotes were found in neutrophil acidic compartments, a milieu known to be best for amastigote replication. In addition, the size of the intracellular compartment increased with the time of infection, suggesting that *L. mexicana* parasites reside in large vesicular compartments within neutrophils, reminiscent of the giant PVs observed within *L. mexicana* parasitized macrophages [47, 48]. These communal PVs are thought to dilute potential host-derived toxic compounds, thus favoring parasite replication. The replication of *L. mexicana* amastigotes in neutrophils may be linked to the formation of these communal PV.

There likely exist differences between distinct *Leishmania* spp., as far as their potential for replication within neutrophils. For instance, amastigotes from *L. braziliensis* are rapidly killed by neutrophils; thus, parasites will not use these cells to replicate [44]. Here, we show that 48 h after infection in vitro, 10× less *L. major* (LV39) than *L. mexicana* amastigotes were present in C57BL/6 neutrophils. Collectively, these data reveal potential differences in amastigote survival/replication among several *Leishmania* spp.

In addition, other *Leishmania* spp. have developed distinct strategies to survive within neutrophils and may also replicate in these cells. For instance, *Leishmania donovani* parasites establish in neutrophils in a compartment resembling endoplasmic reticulum-like structures, where they are protected from degradation. This process is linked to their surface expression of lipophosphoglycan [37]. In addition, following *L. donovani* and *L. major* engulfment, phagosomes do not fuse with tertiary and specific granules and thus, do not have acidified phagosomes [49], in contrast to what is observed here following engulfment of *L. mexicana* by neutrophils. The generation of photoconvertible *Leishmania*^{SWITCH} for different *Leishmania* spp. will help determine if other *Leishmania* spp. also use neutrophils to replicate.

Neutrophils are also playing important roles in experimental visceral leishmaniasis [6, 50, 51], and a recent study reported the

presence of immature neutrophils with impaired oxidative burst in the blood of visceral leishmaniasis patients [52]. Replication of *Leishmania* amastigotes within these neutrophils would also contribute to parasite burden in this setting.

Although macrophages are the main niche for parasite replication, we provide in vivo evidence that neutrophils are present and parasitized in the chronic phase of infection. Within the *L. mexicana* lesion, 10–20% of all infected cells are neutrophils; other major infected cells are monocytes and macrophages. The interaction between neutrophils and macrophages depends on the apoptotic status of neutrophils, the *Leishmania* spp., as well as host genetic factors [53–55]. Neutrophils were reported to enhance the ability of macrophages to kill *L. amazonensis* [56] and *L. braziliensis* [5]. Within *L. mexicana* lesions, neutrophils were found to express low levels of apoptotic markers; however, these cells eventually should become apoptotic and phagocytosed by macrophages, thus impacting on macrophage function. Therefore, the transient replication of amastigotes within neutrophils may be beneficial for the parasites, allowing them to escape enhanced killing properties by macrophages, resulting from phagocytosis of apoptotic neutrophils, or neutrophil-released factors. With the use of heavy-water labeling, it was recently reported that the replication rate of lesion amastigotes was very slow, with an approximate doubling time of 12 d. However, in that study, the possibility that amastigotes exhibit a wide range of growth rates within lesions was suggested by the detection of a small number of hyperinfected macrophages corresponding to ~20% of the total macrophages [57]. Furthermore, that approach does not provide evidence of single parasite-resolved proliferation rates nor does it allow analysis of host cell-specific amastigote replication. Here, with the use of photoconvertible parasites, we observed heterogeneity of proliferation within neutrophils, suggesting that within the cutaneous *L. mexicana*-infected lesion, some neutrophils contain highly dividing amastigotes, whereas others contain amastigotes with lower proliferation rates.

Survival within neutrophils seems to be a strategy for several bacteria, fungi, and viruses to invade and disseminate into the host [35]. In addition, replication within neutrophils was reported for intracellular bacteria, including *Francisella tularensis* [58]; *Neisseira gonorrhoae* [59]; Gram-negative bacteria, such as *Chlamydia pneumonia* [60]; and more recently, *Yersina* spp. [61]. West Nile virus, an ssRNA flavivirus, was also shown to replicate within mouse and human neutrophils in vitro, suggesting that neutrophils may also be a reservoir for these viruses early in infection, allowing viral spread [62]. However, in some of these studies, it is difficult to exclude that some of the microorganisms released by dead neutrophils in vitro replicated outside of the cell and reinfected neutrophils in vitro, altogether contributing to the elevated pathogen loads within neutrophils.

Several approaches have been established to determine pathogen proliferation in vivo. For *Leishmania* spp., heavy water labeling [57] and in vivo BrdU labeling [63] have been used to identify proliferating parasites by dilution of deuterium isotopes or by DNA synthesis, respectively. However, these approaches had only limited compatibility with single-cell analysis and antibody staining and did not permit in vivo validation of the readout through side-by-side comparison with the actual cell division events. Here, with the use of newly engineered photoconvertible parasites, we could circumvent these problems by combining the approach of cell division-associated dilution of a preformed fluorophore [32] with the measurement of de novo production of fluorescent protein under the control of a ribosomal promoter. As we had shown previously, the high stability of the photoconverted protein results in a strict correlation between the protein dilution rate and the number of cell divisions in individual pathogens, allowing the tracking of several sequential cell-division events [31]. With the use of this approach, we demonstrate that *L. mexicana* amastigotes can replicate within neutrophils ex vivo. Whether replication within a neutrophil is a result of intrinsic properties of a subset of amastigotes remains to be defined. Collectively, our findings strongly suggest that neutrophils may provide a transient reservoir for some *Leishmania* spp. within cutaneous lesions, altogether contributing to parasite persistence rather than clearance of the infection. The targeting of these cells in inflammatory lesions should be considered in treatment of this neglected disease.

AUTHORSHIP

F.T-C designed the study and wrote the manuscript. A.J.M. and B.P.H. contributed to the writing of the manuscript. M.B., I.B.R., and B.P.H. performed the in vitro and in vivo experiments with *L. mexicana* promastigotes and amastigotes. A.J.M. constructed the photoconvertible *L. mexicana* parasites and contributed to the data analysis and to the corresponding figure. S.H. performed the LysoSensor experiments and the related figure.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swiss National Science Foundation (310030 166651 to FTC) and from grants by the Deutsche Forschungsgemeinschaft (SFB854-Z01/02 and MU 3744/2-1 to A.J.M). The authors thank Yazmin Hauyon-La Torre for technical assistance and Daniel Roy for building the *Lmex*^{SWITCH} photoconversion device. The authors also thank Francisco Sala and Dr. Stefanie Siegert from the Flow Cytometry Facility (FCF), as well as Dr. Florence Morgenthaler from the Cellular Imaging Facility (CIF) for technical assistance. All are members of the University of Lausanne.

DISCLOSURES

The authors declare no conflicts of interest. The content is solely the responsibility of the authors.

REFERENCES

1. Scott, P., Novais, F. O. (2016) Cutaneous leishmaniasis: immune responses in protection and pathogenesis. *Nat. Rev. Immunol.* **16,** 581–592.
2. Liévin-Le Moal, V., Loiseau, P. M. (2016) *Leishmania* hijacking of the macrophage intracellular compartments. *FEBS J.* **283,** 598–607.
3. Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., Mukankundiye, C., Vasei, M., Launois, P., Milon, G., Louis, J. A. (2000) An immunomodulatory

function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J. Immunol.* **165,** 2628–2636.
4. Peters, N. C., Egen, J. G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M. P., Germain, R. N., Sacks, D. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* **321,** 970–974.
5. Novais, F. O., Santiago, R. C., Báfica, A., Khouri, R., Afonso, L., Borges, V. M., Brodskyn, C., Barral-Netto, M., Barral, A., de Oliveira, C. I. (2009) Neutrophils and macrophages cooperate in host resistance against *Leishmania braziliensis* infection. *J. Immunol.* **183,** 8088–8098.
6. Thalhofer, C. J., Chen, Y., Sudan, B., Love-Homan, L., Wilson, M. E. (2011) Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan *Leishmania infantum chagasi*. *Infect. Immun.* **79,** 108–117.
7. Hurrell, B. P., Schuster, S., Grün, E., Coutaz, M., Williams, R. A., Held, W., Malissen, B., Malissen, M., Yousefi, S., Simon, H. U., Müller, A. J., Tacchini-Cottier, F. (2015) Rapid sequestration of *Leishmania mexicana* by neutrophils contributes to the development of chronic lesion. *PLoS Pathog.* **11,** e1004929.
8. Ribeiro-Gomes, F. L., Peters, N. C., Debrabant, A., Sacks, D. L. (2012) Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-leishmania response. *PLoS Pathog.* **8,** e1002536.
9. Van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., Laskay, T. (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J. Immunol.* **173,** 6521–6525.
10. Laskay, T., van Zandbergen, G., Solbach, W. (2008) Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology* **213,** 183–191.
11. Hurrell, B. P., Regli, I. B., Tacchini-Cottier, F. (2016) Different *Leishmania* species drive distinct nutrophil functions. *Trends Parasitol.* **32,** 392–401.
12. Cassatella, M. A. (1999) Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* **73,** 369–509.
13. Charmoy, M., Brunner-Agten, S., Aebischer, D., Auderset, F., Launois, P., Milon, G., Proudfoot, A. E., Tacchini-Cottier, F. (2010) Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of *Leishmania major* inoculation in resistant mice. *PLoS Pathog.* **6,** e1000755.
14. Conceição, J., Davis, R., Carneiro, P. P., Giudice, A., Muniz, A. C., Wilson, M. E., Carvalho, E. M., Bacellar, O. (2016) Characterization of neutrophil function in human cutaneous leishmaniasis caused by *Leishmania braziliensis*. *PLoS Negl. Trop. Dis.* **10,** e0004715.
15. Carlsen, E. D., Liang, Y., Shelite, T. R., Walker, D. H., Melby, P. C., Soong, L. (2015) Permissive and protective roles for neutrophils in leishmaniasis. *Clin. Exp. Immunol.* **182,** 109–118.
16. Boaventura, V. S., Santos, C. S., Cardoso, C. R., de Andrade, J., Dos Santos, W. L., Clarêncio, J., Silva, J. S., Borges, V. M., Barral-Netto, M., Brodskyn, C. I., Barral, A. (2010) Human mucosal leishmaniasis: neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. *Eur. J. Immunol.* **40,** 2830–2836.
17. Morgado, F. N., Nascimento, M. T., Saraiva, E. M., de Oliveira-Ribeiro, C., Madeira, Mde. F., da Costa-Santos, M., Vasconcellos, E. C., Pimentel, M. I., Rosandiski Lyra, M., Schubach, Ade. O., Conceição-Silva, F. (2015) Are neutrophil extracellular traps playing a role in the parasite control in active American tegumentary leishmaniasis sesions? *PLoS One* **10,** e0133063.
18. Novais, F. O., Carvalho, L. P., Passos, S., Roos, D. S., Carvalho, E. M., Scott, P., Beiting, D. P. (2015) Genomic profiling of human *Leishmania braziliensis* lesions identifies transcriptional modules associated with cutaneous immunopathology. *J. Invest. Dermatol.* **135,** 94–101.
19. Navas, A., Vargas, D. A., Freudzon, M., McMahon-Pratt, D., Saravia, N. G., Gómez, M. A. (2014) Chronicity of dermal leishmaniasis caused by *Leishmania panamensis* is associated with parasite-mediated induction of chemokine gene expression. *Infect. Immun.* **82,** 2872–2880.
20. Charmoy, M., Hurrell, B. P., Romano, A., Lee, S. H., Ribeiro-Gomes, F., Riteau, N., Mayer-Barber, K., Tacchini-Cottier, F., Sacks, D. L. (2016) The Nlrp3 inflammasome, IL-1β, and neutrophil recruitment are required for susceptibility to a nonhealing strain of Leishmania major in C57BL/6 mice. *Eur. J. Immunol.* **46,** 897–911.
21. Crosby, E. J., Clark, M., Novais, F. O., Wherry, E. J., Scott, P. (2015) Lymphocytic choriomeningitis virus expands a population of NKG2D+CD8+ T cells that exacerbates disease in mice coinfected with *Leishmania major*. *J. Immunol.* **195,** 3301–3310.
22. Verçosa, B. L., Melo, M. N., Puerto, H. L., Mendonça, I. L., Vasconcelos, A. C. (2012) Apoptosis, inflammatory response and parasite load in skin of *Leishmania (Leishmania) chagasi* naturally infected dogs: a histomorphometric analysis. *Vet. Parasitol.* **189,** 162–170.
23. Sörensen, M., Lippuner, C., Kaiser, T., Misslitz, A., Aebischer, T., Bumann, D. (2003) Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria. *FEBS Lett.* **552,** 110–114.
24. Habuchi, S., Tsutsui, H., Kochaniak, A. B., Miyawaki, A., van Oijen, A. M. (2008) mKikGR, a monomeric photoswitchable fluorescent protein. *PLoS One* **3,** e3944.
25. Schuster, S., Hartley, M. A., Tacchini-Cottier, F., Ronet, C. (2014) A scoring method to standardize lesion monitoring following intra-dermal infection of *Leishmania* parasites in the murine ear. *Front. Cell. Infect. Microbiol.* **4,** 67.
26. Buxbaum, L. U. (2013) *Leishmania mexicana* infection induces IgG to parasite surface glycoinositol phospholipids that can induce IL-10 in mice and humans. *PLoS Negl. Trop. Dis.* **7,** e2224.
27. Rochette, A., Raymond, F., Corbeil, J., Ouellette, M., Papadopoulou, B. (2009) Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of *Leishmania infantum*. *Mol. Biochem. Parasitol.* **165,** 32–47.
28. Charmoy, M., Megnekou, R., Allenbach, C., Zweifel, C., Perez, C., Monnat, K., Breton, M., Ronet, C., Launois, P., Tacchini-Cottier, F. (2007) *Leishmania major* induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. *J. Leukoc. Biol.* **82,** 288–299.
29. Falcão, S. A., Weinkopff, T., Hurrell, B. P., Celes, F. S., Curvelo, R. P., Prates, D. B., Barral, A., Borges, V. M., Tacchini-Cottier, F., de Oliveira, C. I. (2015) Exposure to *Leishmania braziliensis* triggers neutrophil activation and apoptosis. *PLoS Negl. Trop. Dis.* **9,** e0003601.
30. Doyle, P. S., Engel, J. C., Gam, A. A., Dvorak, J. A. (1989) *Leishmania mexicana mexicana*: quantitative analysis of the intracellular cycle. *Parasitology* **99,** 311–316.
31. Müller, A. J., Aeschlimann, S., Olekhnovitch, R., Dacher, M., Späth, G. F., Bousso, P. (2013) Photoconvertible pathogen labeling reveals nitric oxide control of *Leishmania major* infection in vivo via dampening of parasite metabolism. *Cell Host Microbe* **14,** 460–467.
32. Helaine, S., Thompson, J. A., Watson, K. G., Liu, M., Boyle, C., Holden, D. W. (2010) Dynamics of intracellular bacterial replication at the single cell level. *Proc. Natl. Acad. Sci. USA* **107,** 3746–3751.
33. Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., Zychlinsky, A. (2012) Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* **30,** 459–489.
34. Kolaczkowska, E., Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13,** 159–175.
35. Urban, C. F., Lourido, S., Zychlinsky, A. (2006) How do microbes evade neutrophil killing? *Cell. Microbiol.* **8,** 1687–1696.
36. Laufs, H., Müller, K., Fleischer, J., Reiling, N., Jahnke, N., Jensenius, J. C., Solbach, W., Laskay, T. (2002) Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect. Immun.* **70,** 826–835.
37. Gueirard, P., Laplante, A., Rondeau, C., Milon, G., Desjardins, M. (2008) Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cell. Microbiol.* **10,** 100–111.
38. Carlsen, E. D., Hay, C., Henard, C. A., Popov, V., Garg, N. J., Soong, L. (2013) *Leishmania amazonensis* amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms. *Infect. Immun.* **81,** 3966–3974.
39. Salei, N., Hellberg, L., Köhl, J., Laskay, T. (2017) Enhanced survival of *Leishmania major* in neutrophil granulocytes in the presence of apoptotic cells. *PLoS One* **12,** e0171850.
40. DeSouza-Vieira, T., Guimarães-Costa, A., Rochael, N. C., Lira, M. N., Nascimento, M. T., Lima-Gomez, P. S., Mariante, R. M., Persechini, P. M., Saraiva, E. M. (2016) Neutrophil extracellular traps release induced by *Leishmania*: role of PI3Kγ, ERK, PI3Kσ, PKC, and [Ca2+]. *J. Leukoc. Biol.* **100,** 801–810.
41. Warnatsch, A., Ioannou, M., Wang, Q., Papayannopoulos, V. (2015) Inflammation. neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* **349,** 316–320.
42. Pedraza-Zamora, C. P., Delgado-Domínguez, J., Zamora-Chimal, J., Becker, I. (2017) Th17 cells and neutrophils: close collaborators in chronic *Leishmania mexicana* infections leading to disease severity. *Parasite Immunol.* **39,** e12420.
43. Dupré-Crochet, S., Erard, M., Nüβe, O. (2013) ROS production in phagocytes: why, when, and where? *J. Leukoc. Biol.* **94,** 657–670.
44. Carlsen, E. D., Jie, Z., Liang, Y., Henard, C. A., Hay, C., Sun, J., de Matos Guedes, H., Soong, L. (2015) Interactions between neutrophils and *Leishmania braziliensis* amastigotes facilitate cell activation and parasite clearance. *J. Innate Immun.* **7,** 354–363.
45. Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Tesselaar, K., Koenderman, L. (2010) In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116,** 625–627.
46. Stasulli, N. M., Eichelberger, K. R., Price, P. A., Pechous, R. D., Montgomery, S. A., Parker, J. S., Goldman, W. E. (2015) Spatially distinct neutrophil responses within the inflammatory lesions of pneumonic plague. *MBio* **6,** e01530–e15.
47. Wilson, J., Huynh, C., Kennedy, K. A., Ward, D. M., Kaplan, J., Aderem, A., Andrews, N. W. (2008) Control of parasitophorous vacuole expansion

jkl

155

by LYST/Beige restricts the intracellular growth of *Leishmania amazonensis*. *PLoS Pathog.* **4,** e1000179.
48. Real, F., Mortara, R. A. (2012) The diverse and dynamic nature of *Leishmania* parasitophorous vacuoles studied by multidimensional imaging. *PLoS Negl. Trop. Dis.* **6,** e1518.
49. Mollinedo, F., Janssen, H., de la Iglesia-Vicente, J., Villa-Pulgarin, J. A., Calafat, J. (2010) Selective fusion of azurophilic granules with *Leishmania*-containing phagosomes in human neutrophils. *J. Biol. Chem.* **285,** 34528–34536.
50. McFarlane, E., Perez, C., Charmoy, M., Allenbach, C., Carter, K. C., Alexander, J., Tacchini-Cottier, F. (2008) Neutrophils contribute to development of a protective immune response during onset of infection with *Leishmania donovani*. *Infect. Immun.* **76,** 532–541.
51. Rousseau, D., Demartino, S., Anjuère, F., Ferrua, B., Fragaki, K., Le Fichoux, Y., Kubar, J. (2001) Sustained parasite burden in the spleen of *Leishmania infantum*-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge. *Eur. Cytokine Netw.* **12,** 340–347.
52. Yizengaw, E., Getahun, M., Tajebe, F., Cruz Cervera, E., Adem, E., Mesfin, G., Hailu, A., Van der Auwera, G., Yardley, V., Lemma, M., Skhedy, Z., Diro, E., Yeshanew, A., Melkamu, R., Mengesha, B., Modolell, M., Munder, M., Müller, I., Takele, Y., Kropf, P. (2016) Visceral leishmaniasis patients display altered composition and maturity of neutrophils as well as impaired neutrophil effector functions. *Front. Immunol.* **7,** 517.
53. Ribeiro-Gomes, F. L., Otero, A. C., Gomes, N. A., Moniz-De-Souza, M. C., Cysne-Finkelstein, L., Arnholdt, A. C., Calich, V. L., Coutinho, S. G., Lopes, M. F., DosReis, G. A. (2004) Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J. Immunol.* **172,** 4454–4462.
54. Allenbach, C., Zufferey, C., Perez, C., Launois, P., Mueller, C., Tacchini-Cottier, F. (2006) Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by *Leishmania major*. *J. Immunol.* **176,** 6656–6664.
55. Afonso, L., Borges, V. M., Cruz, H., Ribeiro-Gomes, F. L., DosReis, G. A., Dutra, A. N., Clarêncio, J., de Oliveira, C. I., Barral, A., Barral-Netto, M., Brodskyn, C. I. (2008) Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*. *J. Leukoc. Biol.* **84,** 389–396.
56. De Souza Carmo, E. V., Katz, S., Barbiéri, C. L. (2010) Neutrophils reduce the parasite burden in *Leishmania (Leishmania) amazonensis*-infected macrophages. *PLoS One* **5,** e13815.
57. Kloehn, J., Saunders, E. C., O'Callaghan, S., Dagley, M. J., McConville, M. J. (2015) Characterization of metabolically quiescent *Leishmania* parasites in murine lesions using heavy water labeling. *PLoS Pathog.* **11,** e1004683.
58. McCaffrey, R. L., Allen, L. A. (2006) *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J. Leukoc. Biol.* **80,** 1224–1230.
59. Simons, M. P., Nauseef, W. M., Apicella, M. A. (2005) Interactions of *Neisseria gonorrhoeae* with adherent polymorphonuclear leukocytes. *Infect. Immun.* **73,** 1971–1977.
60. Van Zandbergen, G., Gieffers, J., Kothe, H., Rupp, J., Bollinger, A., Aga, E., Klinger, M., Brade, H., Dalhoff, K., Maass, M., Solbach, W., Laskay, T. (2004) *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J. Immunol.* **172,** 1768–1776.
61. Spinner, J. L., Winfree, S., Starr, T., Shannon, J. G., Nair, V., Steele-Mortimer, O., Hinnebusch, B. J. (2014) *Yersinia pestis* survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. *J. Leukoc. Biol.* **95,** 389–398.
62. Bai, F., Kong, K. F., Dai, J., Qian, F., Zhang, L., Brown, C. R., Fikrig, E., Montgomery, R. R. (2010) A paradoxical role for neutrophils in the pathogenesis of West Nile virus. *J. Infect. Dis.* **202,** 1804–1812.
63. Mandell, M. A., Beverley, S. M. (2017) Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts. *Proc. Natl. Acad. Sci. USA* **114,** E801–E810.

KEY WORDS:
replication · photoconvertible parasite · promastigotes · granulocytes