Macrophages as host, effector and immunoregulatory cells in leishmaniasis: Impact of tissue micro-environment and metabolism

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ABSTRACT

Leishmania are protozoan parasites that predominantly reside in myeloid cells within their mammalian hosts. Monocytes and macrophages play a central role in the pathogenesis of all forms of leishmaniasis, including cutaneous and visceral leishmaniasis. The present review will highlight the diverse roles of macrophages in leishmaniasis as initial replicative niche, antimicrobial effectors, immunoregulators and as safe hideaway for parasites persisting after clinical cure. These multiplex activities are either ascribed to defined subpopulations of macrophages (e.g., Ly6C^{high}CCR2$^+$ inflammatory monocytes/monocyte-derived dendritic cells) or result from different activation statuses of tissue macrophages (e.g., macrophages carrying markers of of classical [M1] or alternative activation [M2]). The latter are shaped by immune- and stromal cell-derived cytokines (e.g., IFN-γ, IL-4, IL-10, TGF-β), micro milieu factors (e.g., hypoxia, tonicity, amino acid availability), host cell-derived enzymes, secretory products and metabolites (e.g., heme oxygenase-1, arginase 1, indoleamine 2,3-dioxygenase, NOS2/NO, NOX2/ROS, lipids) as well as by parasite products (e.g., leishmanolysin/gp63, lipophosphoglycan). Exciting avenues of current research address the transcriptional, epigenetic and translational reprogramming of macrophages in a *Leishmania* species- and tissue context-dependent manner.

1. Introduction

Leishmania are protozoan parasites that are transmitted as flagellated, infective (so-called metacyclic) promastigotes to mammalian organisms by the bites of sand flies. Within the mammalian host, the promastigotes are rapidly taken up by different types of myeloid cells, notably neutrophils, macrophages and dendritic cells, in which the parasites transform into a non-flagellated, amastigote form. Depending on the species, strain and inoculated dose of *Leishmania*, the site of infection as well as on the genetic background, immune and health status, age and sex of the host, the transferred parasites will either lead to an asymptomatic or symptomatic infection. The latter can manifest itself as a localized, frequently self-healing skin lesion (localized cutaneous leishmaniasis [LCL]) or as a systemic, non-healing disease (mucocutaneous leishmaniasis, diffuse cutaneous leishmaniasis [DCL], disseminated cutaneous leishmaniasis, visceral leishmaniasis [kala azar, VL], post-kala azar dermal leishmaniasis [PKDL]) [1–3].

Amongst the myeloid host cells for *Leishmania* parasites, macrophages undoubtedly play a paramount role, both from a historical as well as from a clinical, diagnostic and immunological perspective. The description of the close alliance between macrophages and *Leishmania* dates back to the early work of the Russian borne military doctor Peter Borovsky (1863–1932) from Taschkent, who in 1898 provided the first histological account on the pathogenesis of cutaneous leishmaniasis (CL; termed "sart sore" in his

Fig. 1. Schematic overview of macrophage functions in cutaneous leishmaniasis. Most of the functions depicted have been established in the *L. major* mouse infection model of cutaneous leishmaniasis. For details see the respective sections in the text.

country). He not only correctly classified the underlying infectious agent as a protozoan parasite, but also recognized and graphically illustrated its size (on average 1.5 to 2 μm) and localization within host cells, which he described as "lymphoid and epithelioid cells" as he presumably was unaware of Metschnikow's characterization of macrophages [4]. The microscopical detection of oval-shaped *Leishmania* amastigotes (with the typical disc-formed kinetoplast adjacent to the flagellar basal body) within tissue macrophages ("histiocytes") of cutaneous, splenic, hepatic or bone marrow biopsies is still a central pillar of the microbiological diagnosis of both CL and VL.

From an immunological point of view, macrophages proved to be much more than simple target cells for *Leishmania* parasites. In leishmaniasis, macrophages not only function as a replicative niche during the acute phase of infection, but also operate as anti-leishmanial effector cells, as immunoregulators and as permissive host cells for long-term survival of persistent parasites (Fig. 1). These diverse activities are carried out by defined subpopulations of macrophages or result from different activation statuses of tissue macrophages that are shaped by immune and stromal cell-derived cytokines, micro milieu factors, host cell-derived metabolites as well as by parasite products.

In the following, I will review some key findings that illustrate the phenotypic and functional heterogeneity of macrophages in leishmaniasis and also document the impact of microenvironmental and parasite-derived factors. Results on myeloid cells other than macrophages will also be discussed, if they are of principal conceptual relevance. For detailed summaries of the role of neutrophils and dendritic cells in leishmaniasis the reader is referred to previous review articles [5–8].

2. Macrophages as replicative niche to establish *Leishmania* infection

2.1. Experimental cutaneous leishmaniasis

When *Leishmania* promastigotes are inoculated into the dermis of

mammalian hosts by the bite of sand flies or – experimentally – by intradermal needle injection, they immediately have to cope with the humoral and cellular antimicrobial machinery of the host. Especially in the case of natural infection, the promastigotes initially end up in a blood pool due to the laceration of capillaries in the *stratum papillare* of the dermis [9]. In the case of mouse infections with *L. major,* one of the species causing CL, promastigotes were rapidly taken up by **neutrophils that served as vehicles** for subsequent infection of monocyte-derived or tissue-resident macrophages [10,11]. Although *L. major* promastigotes were reported to delay the apoptosis of neutrophils [12], *L. major*-infected neutrophils ultimately died and became a target of macrophages. This process was facilitated by the release of the monocyte-attracting chemokine CCL4 (MIP-1β) by neutrophils. Phagocytosis of apoptotic, infected neutrophils that still carry viable promastigotes, not only shuttled the parasite into macrophages, but at the same time suppressed their anti-leishmanial effector function by the release of transforming-growth factor (TGF)-β [10,13]. TGF-β is a known inhibitor of the production of reactive oxygen species (ROS) by the phagocyte NADPH oxidase (Phox) [14] and of nitric oxide (NO) by type 2 (or inducible) NO synthase (NOS2, iNOS) [15] and secures at least the transient survival of *Leishmania* by these and other mechanisms [16]. It is important to emphasize that the role of neutrophils as "trojan horses" causing susceptibility to infection is neither observed with all *Leishmania* species nor in all mouse models [17] and reflects only part of their function in leishmaniasis. In addition to serving as replicative niche, neutrophils actively impede the recruitment of monocyte-derived dendritic cells, thereby impair the development of a protective T cell response and lead to disease chronicity as seen in *L. mexicana*-infected mice [18]. Furthermore, as reviewed in detail elsewhere, neutrophils also act as leishmanicidal effector cells during the innate phase of infection (see also below) [6,7,17,19].

The inoculated *Leishmania* parasites can also be *directly* endocytosed by host cells located in the epidermis or dermis. It has been longstanding question whether *Leishmania* promastigotes sneak into host

cells that are particularly permissive to *Leishmania* replication and survival. Early *in vitro* studies identified dermal macrophages as targets of *L. major* promastigotes [20], whereas epidermal Langerhans cells could only be infected by *L. major* amastigotes [20,21]. Neither the dermal macrophages nor the epidermal Langerhans cells produced leishmanicidal effector molecules (ROS or NO) in response to *L. major* infection [20,21] and were therefore considered as potential niches for the initiation of the skin infection.

More recent studies characterized the phenotype of **dermal macrophages** that allow for parasite replication. Lee *et al.* observed that parasites of the highly virulent *L. major* Seidman strain that cause non-healing CL despite development of a type 1T-helper (Th1) cell immune response, were preferentially taken up by dermal macrophages expressing high levels of the C-type lectin receptor CD206 (mannose receptor) [22], which is a known receptor for *Leishmania* parasites [23]. The CD206^high dermal macrophages (a) were positive for additional markers (e.g., CD36, CD209, CD301) that signify alternatively activated or M2 macrophages [24,25], but were negative for arginase 1 as well as NOS2 [22]; (b) were not replenished by recruited monocytes or other bone-marrow-derived cells, suggesting an embryonic origin; (c) required IL-10 and eosinophil-derived IL-4 for their CD206 expression and local proliferation (Fig. 1); and (d) accounted for the non-healing course of infection as shown by depletion experiments [22,26]. Importantly, CD206^high dermal macrophages were not a preferential target of a less virulent *L. major* strain (Friedlin) [22], which offers an explanation why the course of cutaneous infection with *L. major* Friedlin was comparable in wild-type and CD206-deficient mice [27].

In CL, the susceptibility of macrophages to *Leishmania* and the development of a non-healing course of infection has also been linked to M2 markers other than CD206, such as **arginase (Arg) 1** (Fig. 1), which is induced by IL-4, IL-13, TGF-β1 or IL-10 [28,29]. Arg1 is a cytosolic enzyme found in both non-hematopoietic (e.g., hepatocytes) and hematopoietic cells (e.g., macrophages). Arg1 converts L-arginine into urea and ornithine, a precursor of polyamines that promote parasite proliferation and the synthesis of trypanothione, a dithiol required for parasite protection against oxidants [30]. At the same time, Arg1 competes with NOS2 for the common substrate L-arginine (see below) and thereby impedes NO production [29,31]. Earlier studies that relied on global arginase inhibitors (blocking Arg1 as well as the Arg2 isoform without cell-type selectivity) provided first evidence for a disease-promoting role of Arg1 in *L. major*-infected BALB/c mice, a mouse strain that develops visceral and lethal disease following cutaneous infection with *L. major* [32,33]. Hölscher *et al.* showed that conditional deletion of the IL-4 receptor alpha chain (IL-4Rα) on neutrophils and macrophages using a lysozyme M promoter-driven Cre-recombinase (LysM^Cre) delayed the skin swelling and reduced the parasite burden in *L. major*-infected susceptible BALB/c mice, but ultimately still allowed for progressive disease as seen in the respective BALB/c wild-type controls. Resident peritoneal macrophages isolated from *L. major*-infected LysM^Cre IL-4Rα^flox/− mice and co-cultured with draining lymph node cells showed a reduced arginase activity in response to *in vitro* stimulation with lipopolysaccharide (LPS) [34]. Direct genetic proof that Arg1 expression accounts for progressive disease in *L. major*-infected BALB/c mice came from a study by Schleicher *et al.*, who observed that Tie2^Cre Arg1^flox/flox BALB/c mice lacking Arg1 in endothelial cells and all hematopoietic cells were protected from a non-healing course of *L. major* infection. Importantly, Arg1 expression in the skin and draining lymph nodes of *L. major*-infected BALB/c wild-type mice (3–4 weeks post infection [p.i.]) was largely dependent on IL-4 and IL-13, restricted to CD45^+ (i.e. hematopoietic) cells and exclusively found within a **myeloid population** that carried surface **markers of both monocytes/macrophages and -dendritic cells** (CD11b^+CD11c^+Ly6C^+CCR2^+MHCII^+Ly6G^+F4/80^+CD64^+). These Arg1^+ cells were also positive for additional M2 markers (CD206, resistin-like molecule alpha [also termed RELM-alpha or Fizz1], programmed death ligand 2 [PDL2]), but deficient for CD207 (langerin) and CCR7. Although approximately 75% of the Arg1^+ cells co-expressed intracellular NOS2 protein, the enzymatic activity of Arg1 largely

suppressed the generation of NO by NOS2 and thereby allowed for parasite survival [29,35]. In accordance with our findings, two subsequent studies identified CD11b^+Ly6C^+CCR2^+CX3CR1^+CD64^+CD24^low cells (which were termed "inflammatory monocytes"; [36]) and CD11c^+Ly6C^+CCR2^+F4/80^+ cells (categorized as "monocytes" or "monocyte-derived dendritic cells"; [37]) as central host cells during the innate (≤ 4 days p.i.) and acute phase of *L. major* infection (3 weeks p.i.). Using an elegant photoconversion-based biosensor for measuring *in vivo* proliferation, Heyde *et al.* demonstrated that the CD11c^+Ly6C^+CCR2^+F4/80^+ cells were highly permissive for *L. major* replication [37]. A recently published third study, applying the *L. amazonensis* mouse model of chronic CL, again came to the conclusion that CD11b^+Ly6C^+CCR2^+CX3CR1^+CD64^+ monocytes, coexpressing MHC class II, CD206, PDL2 and Arg1, were the primary reservoir for *Leishmania* parasites during the first 3 weeks of infection. Paradoxically, the recruitment and expansion of these permissive host cells turned out to be dependent on IFN-γ from day 4 p.i. onwards [38]. Together, all these studies convincingly show that blood monocyte-derived phagocytes in the infected skin form a replicative niche for *Leishmania* parasites as has been proposed by early analyses many years ago (reviewed in Ref. [39]).

Other macrophage molecules or products that support the establishment of a *Leishmania* infection include the **complement receptor type 3 (CR3)** and IL-10. CR3, which was originally identified to be a major receptor for the phagocytosis of *L. major* promastigotes by peritoneal macrophages *in vitro* [40], later turned out to be non-essential for parasite uptake, but critical for delaying phagosome maturation [41]. *In vivo*, however, mice deficient for CD11b and, thus, for the heterodimeric protein CR3 (CD1b/CD18) showed an unaltered or only slightly ameliorated course of infection in C57BL/6 or BALB/c mice, respectively [42]. **IL-10**, a known co-inducer of Arg1 [28] and inhibitor of NO, ROS and TNF production [43], was released by macrophages in response to antibody-opsonized *L. major* promastigotes in an Fcγ-receptor-dependent manner both *in vitro* and *in vivo* [44,45]. In a *L. mexicana* mouse model, in contrast, the LysM^Cre-mediated deletion of IL-10 in macrophages and neutrophils had no impact on the chronic course of infection, whereas a CD4^Cre-mediated deletion of IL-10 in T cells caused healing of the skin lesions [46]. Thus, in CL the cellular source of IL-10 that is critical for macrophage deactivation appears to be dependent on the parasite species.

2.2. Experimental visceral leishmaniasis

In mouse VL, due to *L. donovani* or *L. infantum* infections or resulting from the systemic dissemination of *L. major* in highly susceptible BALB/c mice, immature monocytes and macrophage precursors as well as certain phagocyte subpopulations have been suspected early on as "safe targets" for the parasite in spleen, liver or bone marrow [47]. In the bone marrow, *L. donovani* caused the expansion of cells resembling hematopoietic stem cells as well as of Sca1^+ emergency myeloid progenitor cells that gave rise to **CD11c^+Ly6C^+ monocytes** exhibiting a permissive and immunosuppressive phenotype [48,49]. Kupffer cells in the liver and macrophages of the red pulp and of the marginal zone of the spleen (marginal zone macrophages [MZM] and marginal metallophilic macrophages [MMM]) have been known for a long time to be host cells for *Leishmania* parasites (Refs. [50–54] and references therein). Hepatic Kupffer cells showed a defective ROS production in response to IFN-γ and consequently a reduced anti-leishmanial activity [51]. Splenic red pulp and white pulp macrophages infected with *L. donovani* amastigotes expressed CD68 (macrosialin) and MOMA-2, but also MHC class II, the latter suggesting their possible interaction with CD4^+ T cells [53]. In the bone marrow, stromal macrophages were highly susceptible to infection by *L. donovani*, allowed for parasite replication, and generated TNF and granulocyte macrophage-colony stimulating factor (GM-CSF), which further stimulated myelopoiesis and therefore potential host cells for *Leishmania* [55].

More recently, it was observed that following intravenous infection

of mice with *L. donovani* amastigotes blood monocytes were newly recruited into the liver and spleen, where they became target for the parasites. The surface phenotype of these inflammatory monocytes (CD11b$^+$CD11c$^-$Ly6C^{high}Ly6G$^-$CCR2$^+$MHCII^{int}F4/80^{int}) closely resembled the monocytes mobilized into the infected dermis during CL (see above), except for the lack of CD11c [56]. The recruitment of the cells was not only strictly dependent on C-C chemokine receptor type 2 (CCR2), but also required the signal transducer and activator of transcription 1 (STAT1), a key transcription factor of the IFN-γ signaling cascade. Treatment of *L. donovani*-infected mice with a CCR2-antagonist that blocked the influx of inflammatory monocytes, led to parasite and disease control in both liver and spleen [56]. Another study on *L. donovani*-infected mice came to the conclusion that during the chronic phase of infection (> day 14 p.i.) the percentage and absolute number of CD11b$^+$CD11c$^+$Ly6C^{high} inflammatory monocytes increased in the spleen. The vast majority of these monocytes co-expressed CCR2, F4/80, and MHC II. Total CD11b$^+$ myeloid cells purified from chronically infected spleens strongly suppressed Th1 cell differentiation *in vitro* and therefore functionally resembled myeloid-derived suppressor cells [49].

Similar to chronic CL, alternative macrophage activation also occurs in experimental VL. In the spleen of *L. donovani*-infected mice, arginase enzyme activity and the expression of Arg1 protein, one of the prototypic M2 markers, closely paralleled the steadily increasing parasite burden in the spleen. Treatment of the mice with an arginase inhibitor or with anti-IL-4 (+/- anti-IL-10) interrupted disease progression, restored NOS2 expression and induced parasite control. IL-4 and IL-10 synergized in the induction of Arg1, which was partly due to the upregulation of IL-4Rα by IL-10 [57]. These findings were largely confirmed by Terrazas et al. [56], who reported that the Arg1/NOS2 mRNA ratio was ca. 15-fold higher in the spleen than in the liver of *L. donovani*-infected mice, correlating with the progressive and self-limiting organ manifestation, respectively. Similarly, in *L. donovani*-infected Syrian hamsters, which is considered the best model for human VL, high Arg1 and low NOS2 levels characterized the infected splenic tissue and macrophages [58,59].

An unusual intraperitoneal mouse infection model investigated the role of the macrophage mannose receptor (CD206) in VL. LysM^{Cre}$^+$CD206$^{flox/flox}$ mice, which lack mannose receptor in myeloid cells, had a significantly higher parasite burden in the spleen than wild-type mice at day 14 after intraperitoneal injection of *L. infantum*. *In vitro*, CD206-deficient peritoneal macrophages showed, despite a slightly reduced initial binding and uptake of *L. infantum* or *L. donovani* promastigotes, an enhanced parasite content after 48 h of infection, which correlated with a reduced production of ROS and IL-1β as compared to wild-type macrophages [60]. These data suggest that in VL, unlike to *L. major* Seidman infections described above, crosslinking of CD206 by *Leishmania* parasites cause macrophage activation rather than deactivation.

2.3. Human leishmaniasis

The potential role of **(inflammatory) monocytes** as host cells for *Leishmania* parasites has not only been investigated in mice, but also in humans. While peripheral blood monocytes from healthy volunteers were activated by IFN-γ for antileishmanial activity (see below), a series of studies revealed that prior infection of normal human monocytes with *L. donovani* impaired their ability to produce ROS or cytokines (e.g., IL-1) or to express MHC class II in response to IFN-γ [61–63]. In line with these results, peripheral blood monocytes from Indian patients with VL showed an attenuated generation of ROS and NO, a reduced expression of MHC class II and certain chemokine receptors and co-stimulatory molecules, and a decreased production of IL-1 and IL-6 as compared to healthy endemic controls [64,65]. At the same time, the plasma concentrations of IL-4, IL-10 and IL-13 were elevated [64]. Patients with early, non-ulcerated CL caused by *L. braziliensis* exhibited increased frequencies of intermediate (CD14$^+$CD16$^+$) and non-

classical monocytes (CD14^{dim}CD16$^+$) in the peripheral blood, whereas the percentage of classical monocytes (CD14$^+$CD16$^-$) was similar to healthy controls. Importantly, the intermediate monocytes were positive for the chemokine receptor CCR2, and one of its ligands, the chemokine CCL2, was elevated in the skin lesions as compared to normal skin, suggesting that these monocytes will be attracted to the site of infection and contribute to pathology via their ability to produce TNF [66].

To date, there is no direct proof that **alternatively activated or M2 monocytes or macrophages** represent the preferential host cell reservoir for *Leishmania* parasites in humans. However, several studies provided evidence that Arg1 activity is associated with disease progression in human leishmaniasis. In Ethiopian patients with LCL, Arg1 activity in lesional tissue was significantly higher than in control skin biopsies, but was present in granulocytes and not in monocytes or macrophages. Whether Arg1$^+$ granulocytes harboured *Leishmania*, was not analysed [67]. Similarly, in an Iranian study population, skin lesions of patients with acute LCL (< 1 year duration) showed increased levels of Arg1 compared to biopsies from healthy controls [68], with higher Arg1 activities detected in *L. tropica*-infected than in *L. major*-infected patients. mRNA and immunohistochemical analyses of skin lesions from *L. amazonensis*-infected patients with DCL not only revealed a significant reduction of IFN-γ, TNF and IL-1β, but also higher levels of Arg1 and ornithine decarboxylase and a preponderance of transcripts that are characteristic for regulatory macrophages [69] when compared to lesions of patients with LCL [70,71]. Skin lesions from *L. mexicana*-infected patients with DCL were reported to contain macrophages with more prominent Arg1-staining than lesions of LCL patients [72], whereas the frequency of NOS2-positive macrophages was significantly lower in DCL lesions [73]. The Arg1 activity of peripheral blood mononuclear cells from Ethiopian patients with VL was again restricted to granulocytes, elevated compared to uninfected controls, and declined after treatment, suggesting that Arg1 expression is associated with disease [74]. In Indian patients with VL, blood monocytes showed an enhanced expression of certain "anti-inflammatory" markers (e.g., transglutaminase-2, vitamin D signalling receptor, peroxisome proliferator-activated receptor [PPAR] γ) compared to healthy endemic controls [65].

Finally, in patients with PKDL, there was an increase of the M2 markers Arg1 (associated with CD68$^+$ macrophages), CD206 and PPARγ in the skin lesions and in peripheral blood monocytes, which returned to normal levels upon treatment. Similarly, the reduced generation of NO and ROS by peripheral blood monocytes of PKDL patients was partly restored by successful therapy [75].

3. Macrophages as antileishmanial effector cells in leishmaniasis

The transformation of macrophages from permissive host cells into leishmanicidal effector cells during the course of *Leishmania* infection requires activation by cytokines, notably by IFN-γ, which is produced by a variety of cell types (e.g., natural killer [NK] cells, CD4$^+$ or CD8$^+$ T cells, certain types of NKT cells; Fig. 1) and already released during the early phase of infection (reviewed in [76–79;80]). The **canonical activity of IFN-γ** not only causes transcriptional induction of antimicrobial effector pathways in both uninfected and infected macrophages (reviewed in [81]), but also primes the responsiveness of macrophages by epigenetic remodelling of pre-existing enhancers and latent enhancers to subsequent stimuli (reviewed in [82]), including exposure to *Leishmania* parasites [62].

The current immunological concept for the control of intracellular *Leishmania* amastigotes entails the differentiation of Th1 cells, following the immunosynaptic interaction between infected and antigen-presenting dendritic cells and macrophages and naïve CD4$^+$ T cells. The IFN-γ released by Th1 cells then triggers various effector mechanisms in neighbouring phagocytes (see below) and was proven to be essential for healing of experimental and human leishmaniasis [83]. The dominant

role of Th1 cells, IFN-γ, dendritic cells and macrophages was mainly elucidated in the *L. major* mouse model of CL. A number of elegant studies using adoptive cell transfers, conditional cell ablations, bone marrow chimeric as well as transgenic mice, revealed that (1) IFN-γ release by CD4$^+$ T cells was necessary and sufficient for achieving parasite control and could not be replaced by IFN-γ production by non-T cells [84]; (2) different populations of dendritic cells were required for a protective T cell response [85–87]; (3) IFN-γ responsiveness of CD68$^+$ macrophages was critical for parasite containment *in vivo* [88]; and (4) the effect of IFN-γ was not restricted to macrophages with direct contact to the IFN-γ-producing CD4$^+$ T cells but also reached more distant by-stander macrophage targets [89].

It is important to point out that **IFN-γ** can also exert **paradoxical effects** on macrophages and other myeloid cells, as already discussed for acute CL (see above, Ref. [38]). In mouse macrophages infected with *L amazonensis* amastigotes, stimulation with IFN-γ alone failed to induce NOS2, but increased the uptake of L-arginine as well as the intracellular parasite burden. Both effects were dependent on the host cell cationic amino acid transporter (CAT) 2B, indicating that L-arginine supported the growth of the parasites [90], e.g., via enhanced generation of polyamines [30,91]. Likewise, in cultures of mouse bone marrow-derived immature monocytes infected with *L. donovani* amastigotes, the addition of IFN-γ caused an increase of the percentage of infected cells [49]. Similarly, IFN-γ promoted parasite replication in a STAT3-and Arg1-dependent manner when added to splenic macrophages isolated from *L. donovani*-infected hamsters [59]. Conversely, there is compelling evidence that IL-4 and IL-4Rα, which normally drive M2 gene expression [92], block classical macrophage activation [93] and characterize Th2 responses, can also synergize with IFN-γ and promote *Leishmania* control by macrophages and inflammatory dendritic cells [94–96]. These and other findings (reviewed in Refs. [97,26]) point to the coexistence of classical and alternative macrophage activation during leishmaniasis and question the conventional Th1/Th2 scheme for protection versus susceptibility to disease.

3.1. Reactive oxygen species

The instant generation of ROS by myeloid cells after priming by cytokines and exposure to microbial products (e.g., N-formylmethionyl-leucyl-phenylalanine [FMLP]) or protein kinase C activators (e.g., phorbol myristate acetate) is termed "oxidative burst" and is due to the activation of the multimeric enzyme **phagocyte NADPH oxidase (Phox)** [19,98]. The seminal discovery that recombinant IFN-γ primed human monocytes for ROS production and antileishmanial activity and was superior to 12 other cytokine preparations tested [99–101] was the starting point for a series of studies documenting the dominant role of IFN-γ in monocyte and macrophage activation [102,103]. Later analyses demonstrated that mouse and human blood monocytes as well as inflammatory monocytes newly recruited into infected tissues (Ly6C$^+$CCR2$^+$CX3CR1$^+$F4/80^{int}) functioned as antileishmanial effectors and that the killing of different *Leishmania* species (e.g., *L. major*, *L. donovani*, *L. braziliensis*) *in vitro* was dependent on ROS formation (reviewed in Refs. [39;19,104–106]). The use of mouse strains deficient in the 91 kDa heme-containing β-subunit (NOX2) of the catalytic flavocytochrome b$_{558}$ of Phox (gp91$^{phox−/−}$) revealed an organ-specific contribution of Phox to parasite control *in vivo*, with absent or weak effects in the skin (*L. major, L. amazonensis*), draining lymph node (*L. major, L. amazonensis*) and liver (*L. donovani*), but strong effects in the spleen (*L. major*) [107–110]. Several case reports on VL in patients with NOX2-deficiency indicate a protective role of ROS in humans (Refs. [111,112] and references therein).

3.2. Reactive nitrogen species

IFN-γ is the key inducer of NOS2 in macrophages [113,114]. Several observations in mouse models have provided convincing evidence for a

critical role of **NOS2-derived NO** in controlling *Leishmania* parasites. These include (a) the inverse correlation between tissue expression of NOS2 and disease severity [115]; (b) the prevention of parasite killing by macrophages in the presence of NOS2 inhibitors [116]; (c) the development of progressive disease in mice lacking NOS2 or treated with NOS2 inhibitors [107,117–119]; and (d) the reactivation of disease in clinically cured mice, when NOS2 activity was blocked [120]. More recent *in vivo* analyses, using elegant reporter systems for tracing *Leishmania* replication, suggested that during the acute phase of *L. major* infection NO restricts parasite metabolism and proliferation in the absence of killing and that it acts not only cell-intrinsically in NOS2-positive cells, but also reaches parasites residing in NOS2-negative cells [121,122]. An equivalent concept was previously established for the chronic (latent) phase of *L. major* infection based on detailed *in situ* studies [120,123,124].

The induction of NOS2 in mouse macrophages by IFN-γ was critically dependent on **endogenous TNF** [94,125,126]. TNF supports the induction of NOS2 and the production of NO by activating mitogen-activated protein kinases and the transcription factors NF-κB and AP-1 [127,128]. A new facet of TNF action was recently discovered by Schleicher *et al.*, who found that TNF not only supported the expression of NOS2, but also inhibited the IL-4-induced expression of Arg1 in both uninfected and *L. major*-infected mouse macrophages or dendritic cells as well as in *L. major*-infected mice. Mechanistically, TNF reduced the acetylation of lysine 27 of histone H3 and thereby hindered the accessibility of phosphorylated STAT6 to the Arg1 enhancer and promoter region (Fig. 2). The TNF-mediated suppression of Arg1 increased the availability of L-arginine and thereby promoted the production of NO by NOS2 at the site of infection *in vivo*. TNF also counteracted the IL-4-mediated increase of mitochondrial oxidative phosphorylation (OXPHOS) and shifted the macrophage metabolism towards glycolysis [35]. The implications of this metabolic shift will be discussed in Section 5.4.

In addition to IFN-γ, there is also evidence for a protective role of **type I IFNs (IFN-α/β)** in leishmaniasis. In mouse CL following *L. major* infection, the expression of IFN-α/β by dermal macrophages was associated with early NOS2 expression, NK cell activation and parasite containment [119], but was dispensable for long-term parasite control and clinical cure during primary or secondary *L. major* infection in self-healing C57BL/6 mice [129]. In *L. major*-infected BALB/c mice, deletion of IFN-β or of the type I IFN receptor (IFNAR) did not alter the non-healing course of disease [129], although exogenous IFN-β was able to convey protection in these mice [130], and macrophages exposed simultaneously or sequentially to *L. major* promastigotes and IFN-α/β expressed NOS2 and antileishmanial activity [78,131]. Besides these protective effects of IFN-α/β, a series of studies indicated that an exuberant production of type I IFNs can also cause aggravation of CL or VL (see Section 5.3. below). As discussed elsewhere, the function of type I IFNs is clearly concentration-dependent [130–132].

3.3. Impact of microenvironmental and metabolic factors on oxygen-dependent parasite defense

The activity of both Phox and NOS2 is dependent on the presence of sufficient amounts of **oxygen**. In many tissues, the oxygen concentration is already low under steady-state conditions, e.g., in the skin (pO$_2$ ∼ 5–6%) [133], liver (portal vein pO$_2$ ∼ 7%) [134] and the bone marrow (pO$_2$ ∼ 1.3–4%) [135]. *In situ* analyses of *L. major*-infected mice revealed that the transcutaneously measured oxygen tension (p$_{tc}$O$_2$) in skin lesions dropped to levels (∼2.8%) which in bone marrow-derived macrophages caused upregulation of IL-4/IL-10-driven Arg1 expression and impeded the oxygen-dependent generation of NO and parasite control [133]. Clinical and parasitological resolution of CL was accompanied by recovery of the p$_{tc}$O$_2$ concentrations. When mice were kept under a normobaric, but hypoxic atmosphere (pO$_2$ ∼ 9%), the p$_{tc}$O$_2$ levels were significantly reduced compared to control mice exposed to ambient air (pO$_2$ ∼ 20%), the tissue parasite burdens were

A. Macrophage plus IL-4

B. Macrophage plus IL-4 and TNF
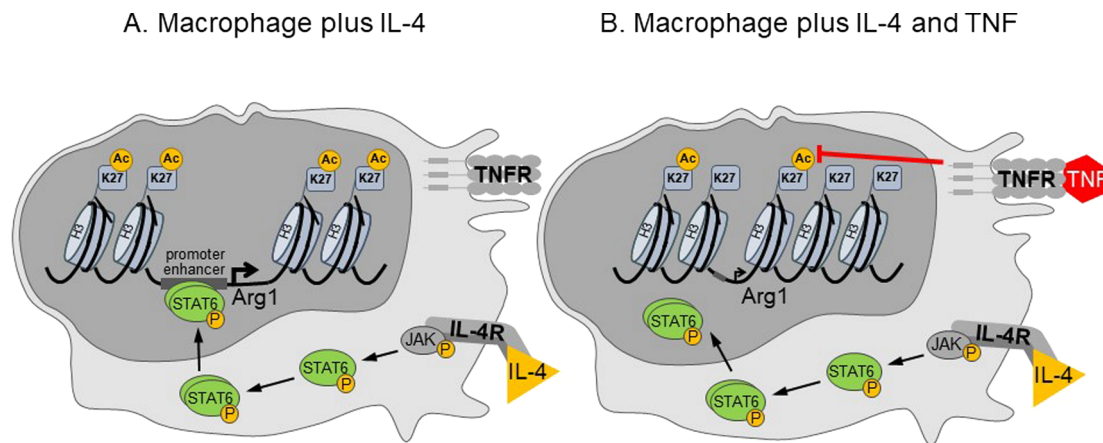
Fig. 2. TNF downregulates Arg1 gene transcription by inhibiting the access of the transcription factor STAT6. IL-4, an alternative macrophage activator, caused tyrosine phosphorylation and nuclear translocation of STAT6, which, together with other transcription factors (not shown), bound to the promoter and enhancer regions regulating the transcription of Arg1 (and other M2 genes; not shown). The binding required prior remodelling and opening of the chromatin, which was associated with IL-4-induced acetylation of lysine 27 of histone 3. Co-stimulation of macrophages with IL-4 plus TNF reduced the histone acetylation and impaired the opening of the gene locus and the binding of phosphorylated STAT6 (Ref. [35] and data not shown).

increased and the resolution of the skin lesions delayed [133]. *In vitro*, hypoxia led to the expression of hypoxia-inducible factor (HIF) 1α which was enhanced in the presence of *L. major* and further increased by the addition of TNF and IFN-γ [136]. HIF-1α is a transcription factor that regulates the expression of genes necessary to cope with reduced oxygen conditions, such as vascular endothelial growth factor (VEGF)-A (that promotes angiogenesis and lymphangiogenesis) [137] or NOS2 (that leads to the production of vasodilatory NO) [138,139]. During *L. major*-induced CL, macrophages were the dominant cell type expressing HIF-1α and VEGF-A [137]. In *L. major*-infected mice lacking HIF-1α in myeloid cells (LysM^{Cre} HIF-1α$^{flox/flox}$), the tissue expression of NOS2 was reduced and the skin lesion sizes and parasite loads were increased [136].

The findings made in the *L. major* model of CL contrast with results obtained in another model of experimental CL. In *L. amazonensis*-infected J774 macrophage-like cells or in *L. amazonensis*-infected peritoneal exudate macrophages from both wild-type and NOS2-deficient mice, hypoxia ($pO_2 \sim 3\%$) led to an NOS2/NO-independent reduction of the number of intracellular parasites as compared to normoxic culture conditions [140,141]. Unexpectedly, hypoxia not only upregulated the HIF-1α-dependent production of the cytokine migration inhibitory factor (MIF) and the protein expression of gp91^{phox} (NOX2), but also enhanced the generation of ROS detected by 2′,7′-dichlorodihydrofluorescein diacetate. Silencing of HIF-1α or addition of diphenylene iodonium chloride, an inhibitor of Phox (as well as of NOS2), prevented the improved parasite control under hypoxia [141].

In experimental VL, the analysis of the role of HIF-1α yielded discrepant results, depending on the conditional knock-out mouse system used. Deletion of HIF-1α in granulocytes, monocytes and mature macrophages (using LysM^{Cre} HIF-1α$^{flox/flox}$ mice) led to increased susceptibility to *L. donovani*, which resulted from a mTOR (mammalian target of rapamycin)- and SREBP-1c (sterol regulatory element-binding protein 1c)-driven intracellular accumulation of lipids following the upregulation of lipogenic enzymes. Lipids may serve as energy source for amastigotes or protect them against microbicidal effector molecules of macrophages [142]. In contrast, deletion of HIF-1α in CD11c$^+$ myeloid cells (CD11c^{Cre}HIF-1α$^{flox/flox}$ mice) increased the expansion, but reduced the immunosuppressive potential of Ly6C^{high} inflammatory monocytes, caused an upregulation of NOS2 and ROS production by CD11b$^+$ splenic cells, promoted the release of IL-12 by dendritic cells, and supported the differentiation of CD4$^+$ Th1 cells and the expansion of CD8$^+$ T cells. Consequently, the splenic parasite burden in CD11c^{Cre}HIF-1α$^{flox/flox}$ mice was significantly lower than in the

respective wild-type controls [49,143,144]. These results argue for a fundamentally different role of HIF-1α in lysozyme-positive versus CD11c-positive myeloid cells.

Tissue osmolarity is a micro milieu factor that has only recently attracted the interest of infectious disease immunologists. Pioneering studies had provided evidence that hypertonicity, as it, for example, prevails in the skin and inflamed tissues [145], has a strong regulatory impact on the secretion of cytokines [146], the function of macrophages [147,148] and the differentiation of different T cell subsets [149–152]. In the context of CL, it was shown that mice lacking the tonicity-responsive enhancer binding protein (TonEBP) (also termed nuclear factor of activated T-cells [NFAT] 5) displayed reduced levels of NOS2 in dermal macrophages and increased parasite numbers in skin lesions, draining lymph nodes and spleen of *L. major*-infected mice. However, whether these findings reflected the impact of tissue tonicity on macrophage responses, was not addressed [153]. Jantsch *et al.* provided direct and firm evidence that increased concentrations of Na$^+$ in the tissue following a high-salt diet improved control of *L. major in vivo* via NFAT5-dependent induction of NOS2 [154]. The Na$^+$/Ca^{2+} exchanger 1 (NCX1, also known as solute carrier family 8 member A1 [SLC8A1]) accounted for the Na$^+$ influx during exposure of macrophages to high salt conditions [155]. Recent data indicate that the effect of tissue osmolarity on phagocyte functions might not be restricted to classically activated (M1) macrophages, but could also pertain to alternatively activated (M2) macrophages [156].

3.4. Other antileishmanial effector mechanisms

In human monocytes and monocyte-derived macrophages, degradation of the amino acid L-tryptophan by the IFN-γ-inducible enzyme **indoleamine 2,3-dioxygenase (IDO)** was found to partially contribute to the killing of intracellular *L. donovani*. As the effect was reversed by the addition of L-tryptophan, depletion of the amino acid rather than accumulation of potentially toxic tryptophan metabolites (kynurenines) were thought to account for the anti-leishmanial activity of IDO. No IDO activity was detectable in mouse macrophages [157], presumably due to the inactivation of IDO by high levels of NOS2-derived NO [158]. Later studies established a role for IDO during the interaction of dendritic cells with T cells in mouse and human leishmaniasis. IDO-mediated tryptophan depletion suppressed T cell proliferation and stimulated the generation of regulatory T cells, thereby promoting the chronicity of disease [159–162] (see also Table 1). This immunomodulatory function of IDO is dependent on the generation of

superoxide anion by Phox [158,163].

Neutrophils are known to generate extracellular traps (so-called **NETs**) that consist of DNA decorated with histones, neutrophil elastase, myeloperoxidase and other antimicrobial molecules and serve to contain infections with bacteria and fungi [164]. Human neutrophils also formed NETs in response to *Leishmania* infection in both ROS-dependent and ROS-independent processes [165–167]. Whereas *L. amazonensis* turned out to be susceptible to NETs [165,167], other *Leishmania* species (e.g., *L. mexicana*, *L. major*, *L. infantum*, *L. donovani*) resisted killing by NETs via their expression of surface lipophosphoglycan (LPG) [166], the secretion of 3′-nucleotidase/nuclease [168] or by unknown mechanisms [18]. Although macrophages have been reported to produce **extracellular DNA traps** [169], there is no published evidence to date that they play a role in the defense against *Leishmania* parasites.

In the past, various **antimicrobial peptides** (AMP) from natural sources showed cytotoxic activity against cultured extracellular *Leishmania* promastigotes [170–172]. While these findings are potentially relevant for novel therapeutic approaches, only few AMPs are endogenously produced by macrophages [173] and therefore could contribute to the control of intracellular amastigotes. Kulkarni *et al.* found that cathelicidin-type antimicrobial peptides (CAMP) exerted cytolytic effects on *L. amazonensis* promastigotes and that CAMP-deficient mice developed significantly more severe skin lesions with higher parasite burdens [171,174]. Crauwels *et al.* recently found an increased CAMP gene expression in skin lesions of *L. aethiopica*-infected patients as compared to skin biopsies from healthy controls. LL37, the bioactive cleavage product of human cationic antimicrobial protein (hCAP18) that is encoded by CAMP, caused apoptosis of *L. aethiopica* and *L. major* promastigotes *in vitro*. Treatment of human macrophages (derived from rhGM-CSF-treated monocytes) with 1α,25-dihydroxyvitamin D3 enhanced CAMP mRNA expression and the production of hCAP18, which was paralleled by reduced survival of intracellular *Leishmania* [175].

4. Macrophages as immunoregulators in leishmaniasis

During the innate and acute phase of *Leishmania* infections, macrophages not only serve as host cells and antileishmanial effector cells, but also fulfil immunoregulatory functions due to their expression of MHC class II and costimulatory molecules, presentation of antigens, secretion of cytokines and release of RNS and ROS (Fig. 1). These processes can be positively or negatively affected by the infection with *Leishmania*, depending on the parasite species, its developmental stage and the experimental set-up, as illustrated by the following examples (see also Table 1).

4.1. Macrophage cytokine production

The effects of *Leishmania* parasites on macrophage cytokine production are far from uniform. *L. major* or *L. donovani* promastigotes impaired the **IL-12 production** of macrophages *in vitro* [176], even in the presence of IFN-γ [177]. Similarly, *L. major* or *L. mexicana* promastigotes attenuated the production of IL-1β by bone marrow-derived macrophages after stimulation with synthetic hemozoin, an agonist of the inflammasome NLRP3; the effect resulted from the proteolytic activity of leishmanolysin, a 63 kDa surface metalloprotease of *Leishmania* parasites [178]. On the other hand, *L. major*, *L. braziliensis* or *L. amazonensis* promastigotes were reported to trigger the activation of NLRP3, the **production of IL-1**β and the generation of NO by bone marrow-derived macrophages primed with lipopolysaccharide (LPS), Pam3CSK4 or IFN-γ [179,180,280]. When, however, the macrophages were infected with *L. amazonensis* amastigotes in the presence of the NLRP3 agonists LPS and ATP, the activation of NLRP3 and the release of IL-1β and IL-18 were prevented [181]. At the same time, the production of TNF was enhanced [181] as previously also seen with IFN-γ-stimulated macrophages following infection with *L. major* amastigotes

[94,125,126].

The analysis of the functional role of NLRP3 or IL-1β during CL *in vivo*, using C57BL/6 mice deficient for the IL-1 receptor or its adaptor molecule MyD88, for NLRP3 or ASC (an adaptor molecule of NLRP3) or for caspase 1 (processing pro-IL-1β to mature IL-1β), also yielded discrepant results: intradermal infections with (i) *L. amazonensis* or *L. braziliensis*, (ii) *L. major* (LV39 strain) or (iii) *L. major* (Seidman strain) led to aggravated, unaltered or reduced skin lesion pathology, respectively, compared to wild-type controls [179,180]. Subcutaneous infection of NLRP3-, ASC- or caspase 1/11-deficient mice on a non-healing BALB/c background with *L. major* (IR173 strain) ameliorated the course on infection [182], whereas in a *L. guyanensis* footpad infection model the inflammasome activation was without impact on lesion pathology or parasite burden [281]. In a natural mouse model of VL, transmission of *L. donovani* by sand fly bites along with gut bacteria of the vector caused NLRP3 activation, IL-1β production by neutrophils rather than macrophages, and parasite dissemination to the spleen, which was prevented by antibiotic treatment of the sand flies or anti-IL-1β treatment of the host [183]. In contrast, intraperitoneal infections of caspase 1- or ASC-deficient mice with *L. infantum* led to increased parasite loads in spleen and liver [179]. Together, these data illustrate that in CL and VL endogenous IL-1β can be protective, disease-promoting or without clinical effect, depending on the parasite species and strain and the mouse genetic background.

4.2. Immunoregulation by RNS and ROS

One of the functional characteristics of NO is its ability to positively or negatively regulate a broad spectrum of immune responses due to its chemical reactivity with enzymes, signaling molecules and transcriptions factors [114,184–187]. Early studies already reported that **NOS2-derived NO** exerts negative feedback-regulatory effects on the differentiation and proliferation of Th1 cells [188] as well as on the production of numerous cytokines (reviewed in [189]). Consequently, *L. major*-infected mice with a reduced but not absent NOS2 gene expression showed a striking expansion of Th1 cells and upregulation of IFN-γ production [118,190], and in *L. major*-infected wild-type treated with an NOS2-inhibitor the expression of NOS2 protein in the infected tissue was strongly upregulated [120,191]. More recently, it was demonstrated that in *L. major*-infected mice the production of NO by NOS2$^+$ macrophages impeded mitochondrial respiration and ATP generation (a long-known effect of NO [192–194]), thereby limited the production of proinflammatory cytokines (e.g., IL-1β, TNF) and chemokines (e.g., CCL2, CCL3) and inhibited the further recruitment of inflammatory cells [195]. *In vitro* co-culture experiments with NOS2$^{+/+}$ and NOS2$^{-/-}$ macrophages as well as mixed bone marrow chimeric mice revealed that the negative regulatory activity of NO required a certain density of NOS2$^+$ cells and, due to the diffusion capacity of NO, affected both NOS2$^+$ as well as NOS2$^-$ cells in the microenvironment [195].

Similar to NOS2-derived NO, ROS generated by Phox (NOX2) function as stimulatory or inhibitory signaling molecules and secondary messengers in the immune system [196–199]. Recent studies with *L. amazonensis*-infected mice illustrated that in this model of CL **Phox-derived ROS** operate mainly as inhibitors of neutrophil influx and as executers of neutrophil apoptosis rather than as antileishmanial effector molecules [200].

The ability of ROS as well as RNS to restrict ongoing immune responses is critical for the termination and resolution of inflammatory processes and for preventing immunopathologies. However, it is important to emphasize that their effects vary between stimulatory and inhibitory, depending on the *in situ* concentrations. Therefore, it is not surprising that during the innate phase of *L. major* infection the small amounts of NO produced by macrophages mainly serve to initiate IL-12-signaling, NK cell activation and early parasite containment [78,119,201], whereas later during the acute clinical phase of infection

the antiparasitic and T cell-dampening of NO effect prevails [195].

5. Macrophages as niche for long-term parasite persistence

5.1. Immunological consequences of Leishmania persistence

One of the enigmas in both CL and VL is the persistence of parasites even after treatment and clinical cure of the disease. From an immunological point of view, parasite persistence has two major consequences, as revealed by mouse studies and clinical observations. First, in the *L. major* mouse model surviving amastigotes lead to continuous stimulation of Ly6C$^+$T-bet$^+$CD4$^+$ and IFN-γ-producing effector T cells [202] that mediate induction of NOS2 [120] and protect against secondary *disease* [202,203], although secondary *infections* are not prevented [204]. Immunity following primary infection has been the basis for the use of leishmanization (injection of parasites at cosmetically non-critical locations) to avoid ulcerative and scar-forming CL, e.g., in the face (reviewed in Ref. [205]). Second, persisting parasites can be the starting point for the reemergence of clinical disease once their containment by the immune system is impaired (e.g., following iatrogenic immunosuppression due to an autoimmune or malignant disease) (reviewed in Refs. [2,123,124,206]). The underlying mechanisms of parasite persistence and reactivation have been of major interest to *Leishmania* researchers.

5.2. Host cells for persisting Leishmania

In mouse **CL**, parasites persisted in **mature tissue macrophages** (positive for F4/80 [syn. EMR1, EGF-like module-containing mucin-like hormone receptor-like 1], BM-8 and MOMA-2, negative for Gr-1 and Ly6C; [120,123,207,208]), dendritic cells (positive for CD205 [DEC-205, NLDC-145] and CD11c; [120,207,209]) and in reticular fibroblasts of the lymph node draining the former skin lesion (positive for ER-TR7; [120,123,207,208]). In three independent studies [120,207,208], persisting *L. major* parasites were associated with both NOS2$^+$ cells (mostly macrophages and dendritic cells) and NOS2$^-$ cells (macrophages, dendritic cells, reticular fibroblasts). Although the percentages of *L. major* amastigotes residing in NOS2$^+$ and NOS2$^-$ cells ranged from 15% to 60% and from 85% to 40%, respectively, and the quantitative distribution of the parasites across the different host cell types significantly varied between these studies, the data obtained uniformly support the following scenario: (1) The persisting parasites are under continuous control by NOS2. The prompt reappearance of clinical disease upon inhibition of NOS2 and the absence of spontaneous episodes of reactivation strongly argue against the development of genuine NO resistance amongst the persisting parasite population [120,123,124,208]. (2) The persisting parasites are not dormant, but are metabolically active and proliferate [207]. Persistence is a dynamic process and characterized by ongoing parasite replication, killing (by NOS2$^+$ cells) and evasion (via retreat into NOS2$^-$ cells) that occur in parallel [120,123]. (3) NO-mediated control of *Leishmania* is not restricted to parasites located in NOS2$^+$ cells, but also reaches parasites in NOS2$^-$ cells [122], due to the known diffusion capacity of NO [210].

In **VL**, the chronicity of infection is accompanied by remarkable structural changes in the spleen changes, notably the **loss of marginal zone macrophages**, which form an important splenic macrophage population phagocytosing *Leishmania*, and the loss of stromal cells in the periarteriolar lymphoid sheath, which impairs the generation of lymphocyte-attracting chemokines such as CCL19 and CCL21 (reviewed in: [211]). In the bone marrow, *L. donovani* infections cause secondary hemophagocytosis (erythrophagocytosis) by macrophages, which is at least partially due to the downregulation of signal regulatory protein (SIRP) α, a receptor transmitting "don't eat me signals", on macrophages [212]. This process contributes to the anemia observed in VL [2], but also leads to increased susceptibility to secondary infections because heme (released from phagocytosed erythrocytes) inhibits

endocytosis, oxidative burst and migration of mouse and human neutrophils and macrophages [213–215]. Importantly, macrophages that have taken up erythrocytes also showed improved survival of intracellular *L. donovani* amastigotes [212].

5.3. Molecular mechanisms of Leishmania persistence and survival: Role of cytokines

Persistence of *L. major* parasites in experimental CL was dependent on **regulatory CD25$^+$CD4$^+$ T cells and IL-10** [203,216], which led to the hypothesis that IL-10-induced Arg1 might deprive macrophages of L-arginine and thereby impede NO production or even NOS2 protein expression [29,124]. However, the analysis of mice lacking Arg1 expression in all hematopoietic and endothelial cells unequivocally showed that *L. major* parasites persisted in Arg1-deficient mice to the same degree and in the same host cells as in wild-type mice [208]. Arg2 is also unlikely to be decisive for the long-term survival of *L. major* because, similar to Arg1, Arg2 was not detectable in the draining lymph nodes after clinical cure of the infection [208]. With respect to the arginase of the parasite, Mou *et al.* reported that an arginase-deficient mutant of *L. major* showed an unaltered ability to persist in long term-infected mice [217].

As IL-10 does not seem to cause *Leishmania* persistence via induction of arginases, other mechanisms of action need to be considered. IL-10 is known to suppress the production of NO, ROS, TNF and IL-1 by primary macrophages [43,218], the production of IL-1, IL-6 and TNF by macrophage cell lines [219] and the cytokine-mediated activation of Th1 cells by antigen-presenting cells [220]. It is conceivable that all these activities of IL-10 support the survival of *Leishmania* in macrophages and other host cells.

In addition to IL-10, other cytokines might facilitate *Leishmania* persistence in macrophages. Several studies with *L. tropica*-, *L. infantum*- or *L. donovani*-infected mice and with humans from *Leishmania*-endemic areas suggested a pathogenic role for **TGF-β** [16,221–223], a known suppressor of macrophage functions and Th1 differentiation [93,224,225]. For **type I IFNs (IFN-α/β)**, at least five different categories of mechanisms of action have been described that could support parasite survival *in vivo*. (a) Resident or inflammatory peritoneal macrophages exposed to IFN-α/β for as little as 30 min showed a strongly reduced expression of NOS2 mRNA, protein and activity in response to subsequent infection with *L. major* promastigotes. The effect of IFN-α/β was due to an impaired activation and nuclear translocation of STAT1α and NF-κB, prevented parasite killing and could not be reverted by IFN-γ treatment [131]. However, in *L. major*-infected C57BL/6 mice deficient for IFNAR1 or IFN-β, parasite persistence was fully maintained [129]. (b) Bone marrow-derived macrophages infected with *L. guyanensis* promastigotes carrying an endogenous *Leishmania* RNA virus 1 (LRV1) expressed reduced levels of IFN-γ-receptor (IFN-γR) on their surface compared to macrophages infected with LRV1-negative *Leishmania*. As the double-stranded RNA of LRV1 is a ligand of TLR3 and known inducer of IFN-α/β, the downregulation of IFN-γR was also seen with macrophages infected with LRV1-negative parasites *and* treated with IFN-α or IFN-β. In vivo, LRV1$^+$ *L. guyanensis* caused more severe skin lesions than LRV1$^-$ *Leishmania* in wild-type, but not in IFNAR1-deficient mice [226]. An additional disease-promoting effect of LRV1 originated from the TLR3-dependent induction of microRNA155 and the activation of the phosphatidylinositol 3-kinase/Akt signaling pathway that fosters macrophage survival and thereby parasite persistence [227]. Furthermore, it was observed that LRV1-induced type I IFNs triggered autophagy, which led to the degradation of NLRP3 and the inhibition of inflammasome activation, thereby supporting parasite survival [282]. (c) IFN-β promoted the growth of *L. amazonensis* or *L. braziliensis* in human monocyte-derived macrophages, which was due to the induction of superoxide dismutase 1 and an ensuing suppression of ROS production [228]. (d) Stimulation of bone-marrow-derived macrophages with IFN-α raised the uptake of *L. donovani* or *L. infantum*

promastigotes, which was causally linked to an increased surface expression of sialoadhesin (Siglec-1/CD169) [229]. (e) In VL due to *L. donovani* or *L. infantum,* type I IFNs, which were mainly produced by plasmacytoid DCs and not by myeloid DCs or macrophages [78,230], strongly acted on myeloid DCs to suppress the production of IFN-γ and to enhance the expression of IL-10 by parasite-specific CD4$^+$ T cells, leading to increased parasite loads in liver and spleen [231].

5.4. Molecular mechanisms of Leishmania persistence and survival: Role of metabolism

Over the years, numerous further strategies of immune evasion and subversion have been identified by which *Leishmania* parasites transform macrophages into a niche for initial infection, but possibly also for long-term survival. The underlying molecular mechanisms include reprogramming of the transcriptome by interference with signaling cascades and/or by epigenetic remodelling, cleavage of host proteins and inhibition of macrophage translation, and remodelling of the metabolome including the degradation of substrates and cofactors required for anti-leishmanial effector pathways (reviewed in [232–239]). Some pertinent examples are listed in Table 1.

A particularly exciting area of research is the effect of *Leishmania* parasites on host cell metabolism and, conversely, the impact of host cell microenvironment on *Leishmania* metabolism and survival. It has become evident that the **metabolic reprogramming of macrophages by *Leishmania*** parasites differs between the species and developmental stage of the parasite, but also depends on the time-point of infection and the genetic background of the host cell (Table 1). In bone marrow-derived macrophages of BALB/c mice, infection with *L. infantum* promastigotes caused an early peak (6 h p.i.) of glycolysis due to the upregulation of glycolytic enzymes, which was followed by increased mitochondrial respiration (OXPHOS) (18 h p.i.), paralled by an enhanced expression of the PPAR-γ coactivator-1α (PGC-1α) [240]. This metabolic switch was dependent on sirtuin-1 deacetylase, the serin-threonine-kinase 11 (LKB1) and the **AMP-activated kinase** (AMPK). Importantly, macrophages or mice deficient for any of these enzymes showed reduced numbers of *L. infantum,* indicating that OXPHOS, which signifies alternatively activated macrophages (M2) [241], favours parasite survival [240]. Why macrophage skewing towards OXPHOS supports parasite persistence, has not yet been studied in detail. One possibility is that ATP generation by the host cells via mitochondrial respiration reduces the consumption of glucose and thereby increases the **availability of glucose** for intracellular *Leishmania*. The importance of glucose for *Leishmania* virulence *in vivo* has been demonstrated for *L. major* and *L. mexicana* using mutants defective for gluconeogenesis [242] or glucose uptake [243], respectively. Data obtained in the *L. mexicana* model suggest that parasites which maintain glycolysis may become less sensitive to the action of NO by avoiding the NO-sensitive Krebs cycle [244] and mitochondrial respiration [193].

One of the central hubs of macrophage carbon metabolism is **mTOR**, which functions as a sensor of amino acid concentrations, promotes mRNA translation and **lipid synthesis** [245]. Several studies found that *L. donovani* activated the mTOR pathway in macrophages, which was associated with increased parasite survival [142,246], M2 macrophage polarization [246] and lipid synthesis [142]. Recently, it was demonstrated that *Leishmania* parasites express a 2,4-dienoyl-CoA reductase (DECR), an enzyme that is essential for β-oxidation of polyunsaturated fatty acids. A DECR-deletion mutant of *L. major* failed to survive in bone marrow-derived macrophages and did not cause disease in BALB/c mice, which are highly susceptible to wild-type *L. major*. These data suggest that *Leishmania* parasites thrive on lipids accumulating in macrophages. However, the metabolic role of mTOR appears to be far more complex in leishmaniasis, because other studies (using mTOR inhibitors) came to the conclusion that activation of mTOR contributes to the control of *L. donovani* [247] and *L. major* [248] (see Table 1).

Leishmania and macrophages also compete for **amino acids**, best exemplified by L-arginine. As detailed in Sections 2.1 and 3.2, macrophages express Arg1 and/or NOS2 depending on their activation status. Both Arg1 and NOS2 are L-arginine metabolizing enzymes that are capable to deprive *Leishmania* parasites of L-arginine. However, *Leishmania* promastigotes, axenic amastigotes as well as intracellular amastigotes residing in macrophages were shown to respond to arginine deprivation by upregulation of a high-affinity, monospecific L-arginine transporter (amino acid permease, AAP3) [249–251]. Thereby, the parasites secure their L-arginine supply for the parasite-arginase-dependent synthesis of L-ornithine and polyamines even in an L-arginine-deficient environment.

6. Concluding remarks

- A common theme in both CL and VL in mice and humans is the function of Ly6C^{high}CCR2$^+$ inflammatory monocytes as initial reservoir for parasite replication. These and other myeloid cells such as M2 macrophages qualify as replicative niche for *Leishmania* parasites, either due to the expression of specific uptake receptors or an impaired antimicrobial activity. The latter results from the remodelling of the host cell by the parasites or from specific microenvironmental conditions in the infected tissues such as hypoxia or the prevailing cytokine milieu.

- Markers of classical (M1) and alternative (M2) macrophage activation are not mutually exclusive. The co-expression of NOS2 and Arg1 on a single cell level, e.g., during acute *L. major* CL, supports the concept that a continuous and bidirectional transition of macrophage activation statuses rather than the development of macrophage subpopulations with an irreversible function and fate determines the outcome of *Leishmania* infections. "M2 → M1 plasticity" of macrophages has been seen during therapy with conventional anti-leishmanial drugs (e.g. Ref. [75]).

- While the effect of cytokines and tissue milieu factors on macrophage metabolism has been studied quite extensively in recent years (reviewed in [252] and [253]), our understanding of the mutual impact of *Leishmania* and macrophage metabolites on each other *in vitro* and *in vivo* is still limited [254,255]. Recently applied innovative techniques for the detection of host cell and parasite metabolites, such as *in vivo* heavy water labelling [256] or *in vitro* surface-enhanced Raman scattering [257], will certainly help to further unravel the intimate metabolic relationship between macrophages and *Leishmania*.

- In experimental mouse models, host cytokine factors that are required for long-term *Leishmania* persistence *in vivo* have been identified, but the exact molecular mechanisms that enable *Leishmania* to survive still remain to be defined.

- The application of the knowledge of different macrophage phenotypes for therapeutic purposes will be a major challenge. Many of the cytokine, effector and metabolic pathways discussed fulfil diverse functions, depending on the responsible parasite species, the clinical form and disease stage of leishmaniasis. Ideally, macrophage-modulatory as well as anti-parasitic therapeutics should act in a tissue- and host cell-specific, non-toxic manner. Possible concepts and prototypic drugs have been put forward [258,259].

Credit authorship contribution statement

Christian Bogdan: Conceptualization, search and analysis of published literature, writing and editing of the entire manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

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