THE IMMUNOLOGY OF SUSCEPTIBILITY AND RESISTANCE TO *LEISHMANIA MAJOR* IN MICE

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Established models of T-helper-2-cell dominance in BALB/c mice infected with *Leishmania major* — involving the early production of interleukin-4 by a small subset of *Leishmania*-specific CD4+ T cells — have been refined by accumulating evidence that this response is not sufficient and, under some circumstances, not required to promote susceptibility. In addition, more recent studies in *L. major*-resistant mice have revealed complexities in the mechanisms responsible for acquired immunity, which necessitate the redesign of vaccines against *Leishmania* and other pathogens that require sustained cell-mediated immune responses.

T HELPER 1/T HELPER 2 (T_H1/T_H2). A classification of $CD4^+$ T cells on the basis of the patterns of cytokines that they secrete. T_H1 cells secrete large amounts of IFN-γ and associated pro-inflammatory cytokines. T_H2 cells secrete large amounts of IL-4 and associated cytokines that promote antibody production by B cells. T_H1/T_H2 cytokines can cross-regulate each other's responses. An imbalance of T_H1/T_H2 responses is thought to contribute to the pathogenesis of various infections, allergic responses and autoimmune diseases.

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doi:10.1038/nri933

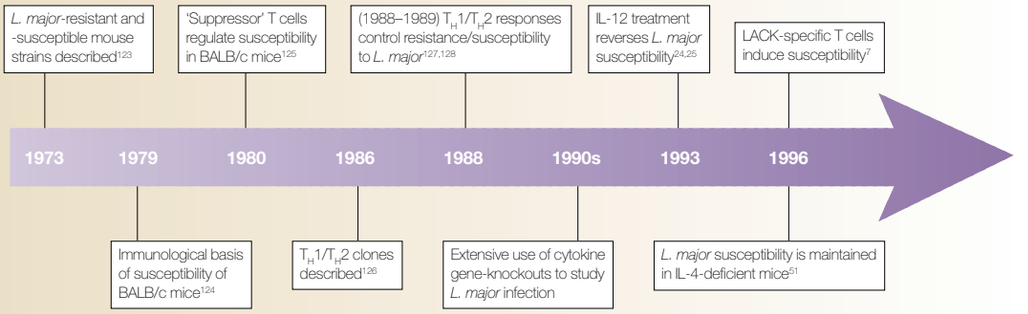
The first direct demonstration of the relevance of the T HELPER 1/T HELPER 2 (T_H1/T_H2) balance to the regulation of disease outcome *in vivo* arose from studies in the *Leishmania major* mouse model (see TIMELINE). Natural transmission of cutaneous leishmaniasis caused by *L. major* infection is by the sandfly vector *Phlebotomus papatasi*, which inoculates a small number (100–1,000) of infectious-stage metacyclic promastigotes into the skin (FIG. 1). *L. major* is distributed widely through northern Africa, the Middle East and central Asia. Infection of natural rodent reservoirs and of human hosts leads invariably to the development of localized cutaneous lesions that eventually heal, and to the generation of life-long immunity to re-infection. In the laboratory, most mouse genotypes control *L. major* infection also, which is initiated typically by needle inoculation of a large number (10^4–10^7) of parasites into subcutaneous sites, such as the footpad or base of the tail (BOX 1). However, certain strains — such as BALB/c mice — fail to control infection and develop progressive lesions and systemic disease. These mice are thought to be a model of non-healing forms of the human disease — such as kala-azar or diffuse cutaneous leishmaniasis — that are associated with infection by other species of *Leishmania* (for the genetics of *L. major* infection, see FIG. 2).

The genetic predisposition for susceptibility or resistance to *L. major* infection in mice correlates with the dominance of an interleukin-4 (IL-4)-driven T_H2 response that causes disease or an IL-12-driven, interferon-γ (IFN-γ)-dominated T_H1 response that promotes healing and parasite clearance, respectively (for a model of T_H1/T_H2 development, see FIG. 3). Recent data, however, have challenged the simplicity of this model and have revealed further complexities in cytokine regulation and the mechanisms of acquired resistance and immune escape. In this review, we consider recent findings in the *L. major* mouse model in the context of earlier studies, and attempt to reconcile apparent differences and emphasize those aspects of the T_H1/T_H2 model that have been altered or refined.

T_H2-cell development and susceptibility

T_H2-cell development in BALB/c mice. The apparent resolution of infection with *L. major* in BALB/c mice treated at the time of infection with an anti-IL-4 monoclonal antibody[1,2] or in IL-4-deficient BALB/c mice[3,4] helped to establish the view that early production of IL-4 drives the polarized T_H2 response that is responsible for suppressing T_H1-cell development and inhibiting the high-level secretion of IFN-γ that is required to activate infected macrophages for parasite killing. There is also convincing evidence that the early IL-4 response is confined largely to an oligoclonal population of $CD4^+$ T cells with a Vβ4Vα8 T-cell receptor (TCR) that recognize the

Timeline | **The *Leishmania major* mouse model**



IL, interleukin; LACK, *Leishmania* homologue of receptors for activated C kinase; T_H1, T helper 1.

Leishmania antigen LACK (*Leishmania* homologue of receptors for activated C kinase)[5]. This conclusion is based on the observation that infected Vβ4-deficient BALB/c mice mount stronger T_H1 responses than wild-type BALB/c mice and control their lesions[6], as do BALB/c mice that are tolerant of LACK as a result of the transgenic expression of the protein[7]. It has been proposed that LACK-specific Vβ4Vα8 $CD4^+$ T cells form a unique lineage in BALB/c mice that is biased to produce IL-4, because their TCRs have relatively low affinity for peptide–MHC[8]. So, to the extent that inherent differences in the T-cell compartment control resistance or susceptibility to *L. major*[9], a model has emerged in which the susceptibility of BALB/c mice is determined by a relatively high frequency of LACK-reactive cells that are biased to produce IL-4 after early exposure to *L. major*.

Importance of the early T_H2 response. The importance of early IL-4 production by LACK-reactive $CD4^+$ T cells as the determining variable in susceptibility to *L. major* infection is not, however, consistent with several findings. In earlier studies, Vβ4Vα8 TCR usage was found to be similar in *L. major*-infected BALB/c and C57BL/6 mice[10], and LACK-specific T cells were found to produce a burst of IL-4 in resistant B10.D2 mice[11]. Most recently, in studies in IL-4 REPORTER MICE — which contain a knock-in gene encoding IL-4 linked to enhanced green fluorescent protein (GFP) — the frequency and kinetics of IL-4-producing cells induced by *L. major* that bind a LACK–MHC CLASS II TETRAMER were found to be similar in resistant and susceptible strains[12]. Indeed, early, albeit transient, IL-4 responses after *L. major* infection in resistant mouse strains have been a fairly consistent finding[13–17] (FIG. 4). Furthermore, although injections of IL-4 (REF. 2) or anti-IL-12 antibodies[18] at the time of parasite challenge promote a strong T_H2 response in C3H mice, the response is transient and does not reverse the normal resistant phenotype of these mice in the long term. Taken together, these data indicate that the ability to redirect an early T_H2 response is the more probable determinant of resistance in the mouse model.

The necessity to ablate T_H2 cytokine production to effect a cure has been shown using transgenic resistant-background mice with constitutive expression of either IL-4 (REFS 19,20) or IL-10 (REF. 21). In each case, the mice failed to control *L. major* infection despite generating a relatively strong T_H1 response. Even from the strong T_H2-polarizing environment of *L. major*-infected BALB/c mice, an effector $CD4^+CD45RB^{hi}$ T-cell subset could be recovered that could transfer immunity to SEVERE COMBINED IMMUNODEFICIENT (SCID) mice[22]. So, the sustained production of T_H2 cytokines, particularly in resistant-background mice, does not necessarily prevent the development of a T_H1 response. Nevertheless, the T_H2 response dominates the clinical outcome, presumably as a result of powerful deactivating effects on infected cells.

IL-4 REPORTER MICE
Genetically engineered knock-in mice in which the gene encoding IL-4 has been replaced by sequences that encode a reporter molecule, such as green fluorescent protein (GFP). When the IL-4 promoter region is activated, GFP is expressed and living cells can be visualized by flow cytometry.

MHC CLASS II TETRAMERS
A method of visualizing antigen-specific $CD4^+$ T cells by flow cytometry. Typically, four MHC class II molecules with their associated peptides are held together by streptavidin, which has four binding sites for biotin, which is attached to the tail of the MHC molecule. These four peptide–MHC complexes (tetramers) can bind peptide-specific T-cell receptors. The streptavidin molecules are often labelled with a fluorochrome so that binding can be assessed by flow cytometry. Similarly, MHC class I tetramers can be engineered to track $CD8^+$ T-cell receptors.

SEVERE COMBINED IMMUNODEFICIENCY (SCID). Mice of this phenotype lack functional T and B cells owing to a spontaneous mutation in the *Prkdc* gene (protein kinase, DNA activated, catalytic polypeptide) located on chromosome 16. These mice are often used for the reconstitution of T-cell subsets to study their functions *in vivo*.

Box 1 | **Variables of the *Leishmania major* infection model**

The conventional *Leishmania major* mouse model uses a high dose of parasites, usually stationary-phase promastigotes, injected into a subcutaneous site (shaved rump or hind footpad). Several variables have been introduced to the model, which, in some cases, have altered the outcome. These include the developmental stage of the parasites used for injection (purified metacyclic promastigotes compared with heterogeneous populations of stationary-phase promastigotes[122]), the injection of a small number of parasites[44], alternative routes of inoculation (including intravenous, intradermal and intranasal routes[14,35,36]) and a natural form of infection using the sandfly vector[119]. The *L. major* substrains that are used might influence the outcome of infection also[49]. Substrains of *L. major* used in mouse models can be derived from human or rodent isolates, and they are classified generally in terms of their geographic origin. Also, *L. major* substrains might undergo spontaneous variations owing to routine passages or length of time in culture. Some of the common *L. major* substrains that are used in mouse models are Friedlin (Jordan Valley, WHOM/IL/80/FN), IR173 (Iran, WHOM/IR/-173), LV39/Neal (southern Russia, MRHO/SU/59/P), NIH/Seidman (West Africa, MHOM/SN/74/S), World Health Organization reference strain 5-ASKH (Turkmenskaya, MHOM/SU/73/5-ASKH) and CC-1 (Iran, MHOM/IR/83/LT252).

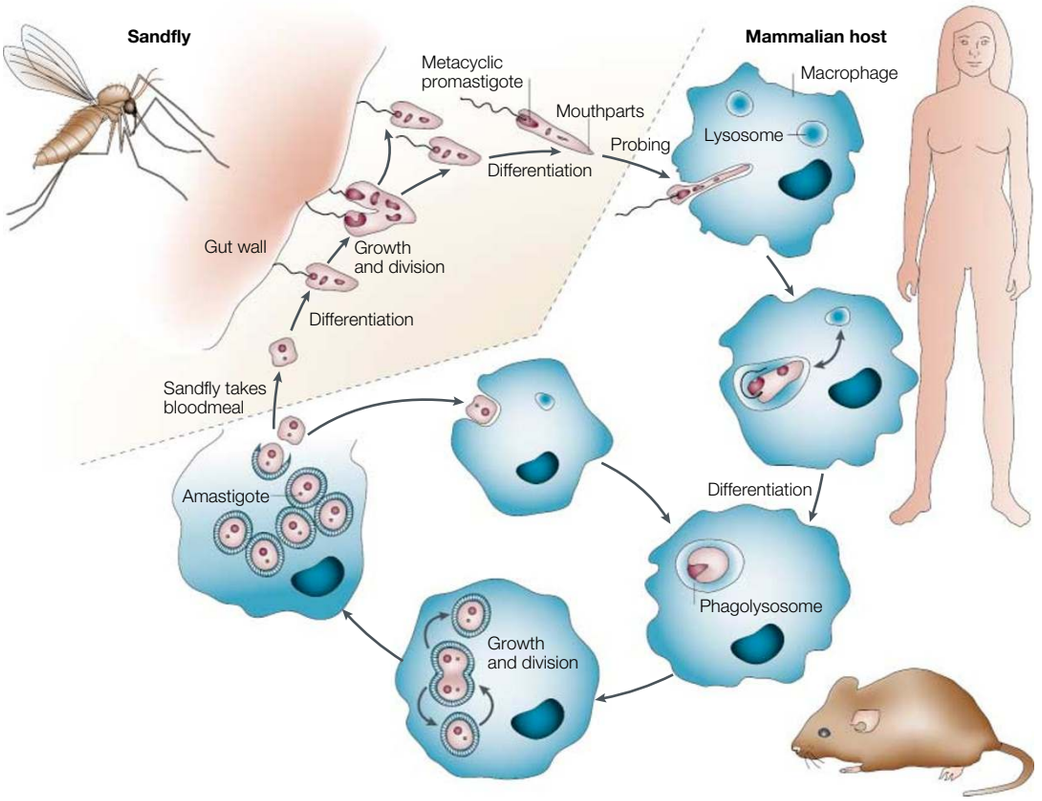


Figure 1 | **Life cycle of *Leishmania major* infection.** *Leishmania* parasites are transmitted by the bites of infected female sandflies, which inject a small number of infectious-stage, metacyclic promastigotes into the skin. These forms are opsonized efficiently by serum components and taken up by macrophages, where they reside in phagolysosomes and transform into replicating amastigotes. Infected macrophages are taken up by sandflies during blood feeding; they are lysed in the fly midgut, releasing parasites that transform into rapidly dividing, non-infectious-stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration that is accompanied by their differentiation to non-dividing, metacyclic promastigotes that can be transmitted when the sandfly takes another blood meal.

For example, the production of REACTIVE NITROGEN INTERMEDIATES (RNIs) by IFN-γ-activated macrophages is known to be inhibited by IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β)[23], and the downregulation of these cytokines might be crucial to the development of acquired resistance (see below).

IL-12: redirecting the early T_H2 response. The ability of exogenous IL-12 to redirect the early T_H2 response to *L. major* in BALB/c mice and to promote resistance is well supported[24,25], as is the effect of genetic disruption of IL-12 on upregulating the expression of IL-4 and establishing progressive disease in normally resistant mice[14,26]. A stronger, more sustained T_H2 response and disease exacerbation have been observed also in resistant mice treated with anti-IL-12 antibodies[17,24], although unless the treatment is maintained, the IL-12 response recovers and the infection is controlled. The fact that anti-IL-12 antibody treatment has the greatest effect when delayed until seven days after infection[17] reinforces the findings from IL-12-deficient C57BL/6 mice after a low-dose challenge, in which parasite growth was identical to that in wild-type mice over the first 4–5 weeks[14]. This indicates that the production of IL-12 is delayed normally even in resistant mice. The inability of *L. major* to drive early IL-12 production in either resistant or susceptible mouse strains might explain why their initial response to *L. major* defaults to a T_H2 pathway.

Sustained T_H2-cell dominance in BALB/c mice. If IL-12 production is necessary to redirect the early T_H2 response, then some failure of this response pathway in BALB/c mice seems likely to underlie their susceptibility to *L. major* (FIG. 5). Selective loss of IL-12 signalling owing to downregulated expression of the IL-12 receptor β2-chain (IL-12Rβ2) has been proposed to explain the defective IL-12 response in BALB/c mice[27]. The IL-12R comprises two components, IL-12Rβ1 and IL-12Rβ2, which are both expressed by T cells after TCR engagement. The instability of IL-12Rβ2 expression in BALB/c mice is thought to occur through an IL-4-dependent process[28] and an as-yet-undefined, genetically controlled IL-4-independent mechanism[29]. The relevance of this process has been questioned, however, by the finding that BALB/c mice that express an IL-12Rβ2 transgene maintain a non-healing phenotype, despite stable IL-12 signalling and the activation of signal transducer and activator of transcription 4 (STAT4)[30].

REACTIVE NITROGEN INTERMEDIATES (RNIs). Primarily nitric oxide, these are generated by nitrogen oxidation of L-arginine, and can have potent activity to destroy intracellular pathogens such as *Leishmania*.

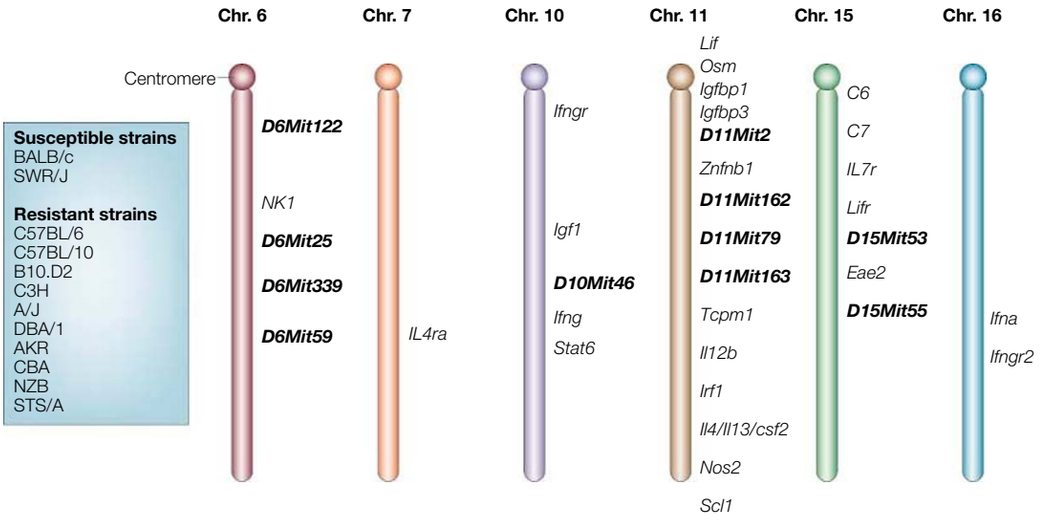


Figure 2 | **The genetics of resistance to *Leishmania major*.** Several mouse loci have been found to be associated with resistance to *L. major* infection. The linkage data shown were compiled from studies of infection of recombinant inbred strains[49,129] or serial backcross mapping[48], and they are based on measurements of lesion size. The loci in bold are the markers that were found to be linked highly to resistance. The plausible candidate genes that are located on these chromosomes or have been mapped in the marker regions are shown also. However, it should be noted that no polymorphisms of these candidate genes have been identified that would indicate resistance to *L. major* infection. The placement of loci in relation to the centromeres represents the relative order of the loci and not the actual map distances. For information on the actual maps and gene symbols see the Mouse Genome Informatics website. Chr., chromosome.

The inflammatory or tissue environment in which the early T_H2 response is induced might also preclude its extinction in BALB/c mice. For example, neutrophils — which are maintained for several weeks as a high proportion of the inflammatory infiltrate in the inoculated footpad of BALB/c mice, but only transiently in C57BL/6 mice[31] — might contribute to the sustained induction of a T_H2 response, because depletion of neutrophils at the time of *L. major* challenge in BALB/c mice inhibited the IL-4 response and promoted partial resistance[32]. Additional host-strain differences have been found in the manner in which parasites disseminate from the site of inoculation to the draining lymph nodes and visceral organs; dissemination occurs rapidly in BALB/c mice, whereas early parasite containment in the footpad and draining lymph nodes is observed in resistant mice[33]. As a result of this dissemination, $CD4^+$ T cells that produce IL-4 spontaneously *in vitro* can be found in the liver and spleen of BALB/c mice two weeks after infection, whereas these cells are not found in the viscera of C57BL/6 mice[34]. The same distinctive patterns of early parasite trafficking have been observed recently in a comparison of BALB/c SCID and C57BL/6 SCID mice, which indicates that the differences are not secondary to an adaptive immune response (T. Kamala and P. Matzinger, personal communication).

That the site of antigen delivery can influence T-cell priming has been shown clearly in the *L. major* model; parasites that are delivered intravenously or intranasally can elicit sustained T_H2 responses and produce non-healing infections in normally resistant mice[35,36]. This indicates that owing to differences in parasite dissemination between resistant and susceptible mice, distinct populations of dendritic cells (DCs) with the capacity to induce preferential priming of either T_H1 or T_H2 cells might become activated. The existence of tolerogenic or T_H2-inducing DCs in peripheral tissues has been indicated strongly by studies of DCs derived from the liver, lung and Peyer's patches[37]. Such populations might not be distinct lineages of antigen-presenting cell (APC), but might, instead, be owing to the modulation of APC function by specific tissue environments (such as cytokines and chemokines). It is, for example, possible that the chemokine monocyte chemotactic protein 1 (MCP1; also known as CCL2) — the deletion of which prevents T_H2-cell polarization and confers partial resistance to *L. major* infection in BALB/c mice[38] — might be overexpressed in certain tissues of susceptible mouse strains. Finally, the observations that interactions between co-stimulatory molecules and their receptors (such as cytotoxic T-lymphocyte antigen 4 (CTLA4)/CD28–B7 (REFS 39–41) and OX40–OX40L[42]) can, under certain conditions, have crucial roles in the development of a T_H2 response to *L. major in vivo* raise the possibility that the differential expression of these co-stimulatory molecules might define functionally distinct subsets of APC in different tissues.

As skin-derived DCs that can produce IL-12 p40 in response to *L. major in vitro* and prime for protective T-cell responses *in vivo* are readily obtainable from BALB/c mice[43], it does not seem that the IL-12 defect in BALB/c mice is intrinsic to these cells. This is reinforced by the important observation that BALB/c mice develop stable resistance to small parasite inocula in a subcutaneous site[44]. A small number of parasites administered to BALB/c mice might mimic the events

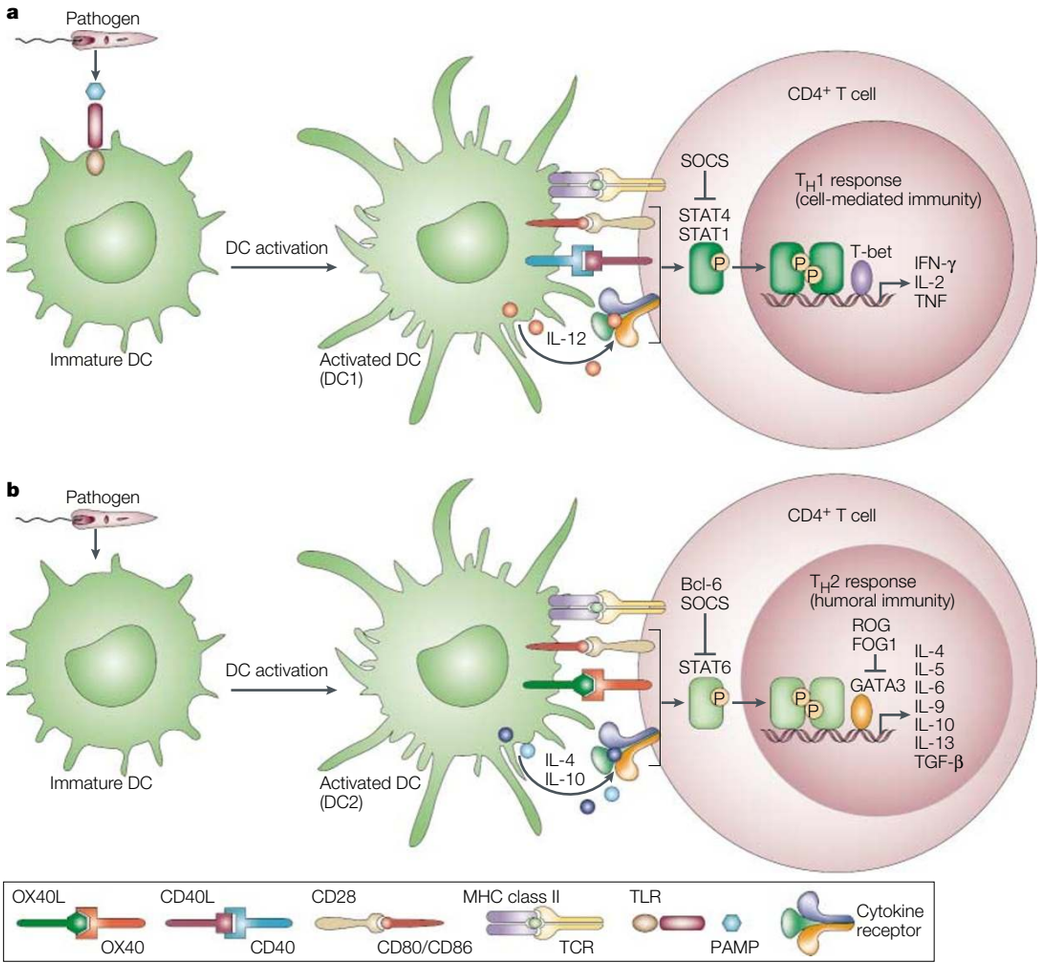


Figure 3 | **Model of T_H1-/T_H2-cell development.** For both T helper 1 (T_H1)- and T_H2-cell differentiation, antigens are presented to naive $CD4^+$ T cells by dendritic cells (DCs). The interaction of co-stimulatory molecules with their respective ligands (CD40–CD40L, OX40–OX40L and/or CD80–CTLA4/CD28), together with the local cytokine environment, promotes the differentiation of naive T cells into interferon-γ (IFN-γ)-secreting T_H1 cells or interleukin-4 (IL-4)-secreting T_H2 cells. It has been proposed also that distinct subsets of dendritic cells, known as DC1 and DC2, might exist, which, in turn, direct T_H1 and T_H2 differentiation pathways, respectively. **a** | In T_H1-cell development, certain pathogens or pathogen-associated molecular patterns (PAMPs) trigger antigen-presenting cells, through Toll-like receptors (TLRs), to secrete IL-12, which promotes the differentiation of naive T cells into IFN-γ-secreting T_H1 cells. Signal transducer and activator of transcription 4 (STAT4) and STAT1 are activated by IL-12 and IFN-γ, respectively. T-bet, a T-box transcription factor is T_H1-lineage specific. **b** | In T_H2-cell development, the inability of antigen to activate DCs to produce IL-12 results in a default pathway of naive T-cell differentiation into IL-4-secreting T_H2 cells. In addition, antigen and/or specific tissue environments might activate DCs to produce IL-4 or IL-10, which will instruct T_H2-cell development. STAT6 is activated specifically by IL-4-receptor binding. The T_H2-lineage-specific transcription factor GATA3 binds to consensus GATA-binding sites (AGATAG). The factors c-Maf and NFATc (nuclear factor of activated T cells) have been associated with T_H2 differentiation also. Bcl-6, ROG (repressor of GATA) and FOG1 (friend of GATA1) negatively regulate T_H2 differentiation by repressing the activity of STAT6 and GATA3. Suppressor of cytokine signalling (SOCS)-family members inhibit T_H1 and T_H2 responses by blocking STAT activity. CTLA4, cytotoxic T-lymphocyte antigen 4; TCR, T-cell receptor; TGF-β, transforming growth factor-β; TNF, tumour-necrosis factor.

that occur normally in resistant mice, in which there is no dissemination of parasites beyond the local draining lymph nodes. It is interesting that the successful immunization of BALB/c mice using irradiated *L. major* promastigotes depends crucially on a high dose and intravenous route of injection[45]. There is evidence to indicate that the protection induced in this model is due not to immunization *per se*, but to tolerization of the *L. major*-specific $CD4^+$ T cells that would normally be activated along a T_H2 developmental pathway in the viscera[46]. Such inherent host-strain differences in parasite dissemination and inflammation might be relevant to the observation that cells in the non-T-cell compartment[9] and at least six genetic loci contribute to resistance to *L. major*[47,48] (FIG. 1).

IL-4-independent T_H2 pathways. Although an early T_H2 response might not be the distinguishing event in the development of non-healing disease in BALB/c mice, there is little doubt that a sustained T_H2 response

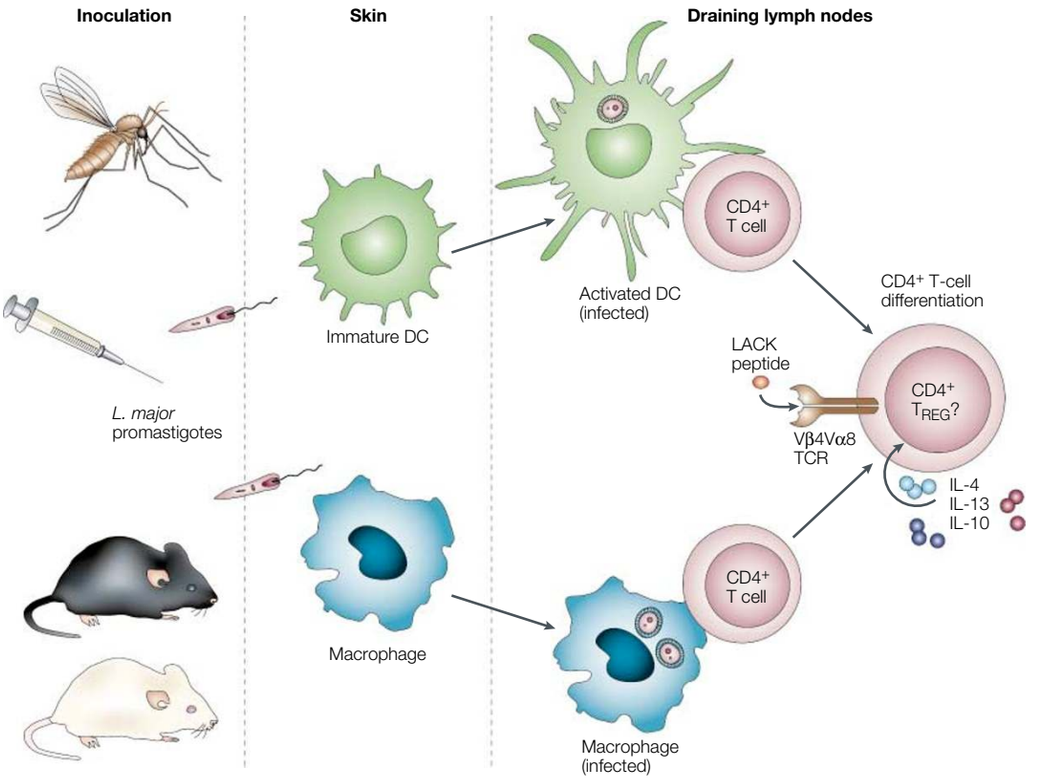


Figure 4 | **Early events in susceptible and resistant mice.** In both susceptible and resistant mouse strains, inoculation of *Leishmania major* metacyclic promastigotes by needle or by infected sandflies results in the early production of interleukin-4 (IL-4) and other type-2 cytokines by $CD4^+$ T cells in lymph nodes draining the site of infection. The early T helper 2 (T_H2) response is due, in part, to the activation of an oligoclonal population of T cells expressing a Vβ4Vα8 T-cell receptor (TCR) that recognizes the LACK (*Leishmania* homologue of receptors for activated C kinase) peptide. These cells might be pre-committed to produce IL-4 and IL-10 — possibly, they are regulatory T cells (T_{REG}) — or they might default to the T_H2 pathway owing to the absence of *L. major*-driven IL-12 production by macrophages or dendritic cells.

is responsible ultimately for this outcome. There are, however, accumulating data to indicate that the IL-4 component of this response is not sufficient and, in some cases, not necessary for susceptibility (FIG. 5). Using genetically pure BALB/c IL-4- or IL-4Rα-deficient mice, it was shown that infection with the *L. major* substrain IR173 was controlled only partially in the IL-4-deficient mice, but highly controlled in IL-4Rα-deficient mice[49]. As the IL-4R α-chain is shared between the receptors for IL-4 and IL-13, these results indicate a potential role for IL-13 in mediating susceptibility to *L. major*. Studies in an IL-13-deficient mouse strain, as well as an IL-13-transgenic mouse strain, confirmed that IL-13 is a susceptibility factor in *L. major* infection and that there is an additive effect of deleting both IL-4 and IL-13 (REF. 50). Remarkably, both IL-4-deficient mice[51] and IL-4Rα-deficient mice are as susceptible as wild-type mice when infected with another *L. major* substrain, LV39. A similar lack of resistance to another substrain, NIH/Seidman, was observed in IL-4-deficient BALB/c mice treated with a soluble IL-13Rα2–Fc fusion protein to block the biological activity of IL-13 (REF. 52). So, under some circumstances, IL-4Rα signalling is not required for T_H2 priming to occur, which indicates that other pathways exist to promote parasite survival.

Under some conditions, TGF-β has been shown to suppress T_H1-cell development and to inhibit macrophage activation. Its role in *L. major* infection is indicated by the exacerbation of infection in C57BL/6 × BALB/c F1 mice treated during the chronic phase with TGF-β and, more convincingly, by the enhanced resistance of these mice to infection after treatment with anti-TGF-β antibody[53]. Although antibody treatment did not alter the pattern of IL-4 or IFN-γ production, it did increase the production of nitric oxide by macrophages in parasitized lesions. An effect of TGF-β on the differentiation of T_H1 cells is indicated by a recent report involving BALB/c-background mice that express a dominant-negative form of TGF-β receptor type II in T cells[54]. Formation of *L. major*-induced lesions was delayed in these mice and the lesions progressed more slowly. Although the mice had an enhanced T_H1 response in draining lymph-node cells, they also maintained strong T_H2-type cytokine production, which is consistent with the fact that they failed to heal the lesions ultimately.

An additional cytokine that can suppress T_H1 responses and the activation of macrophages is IL-10. IL-10 was thought initially not to be important in *L. major* infection, because treatment of BALB/c mice

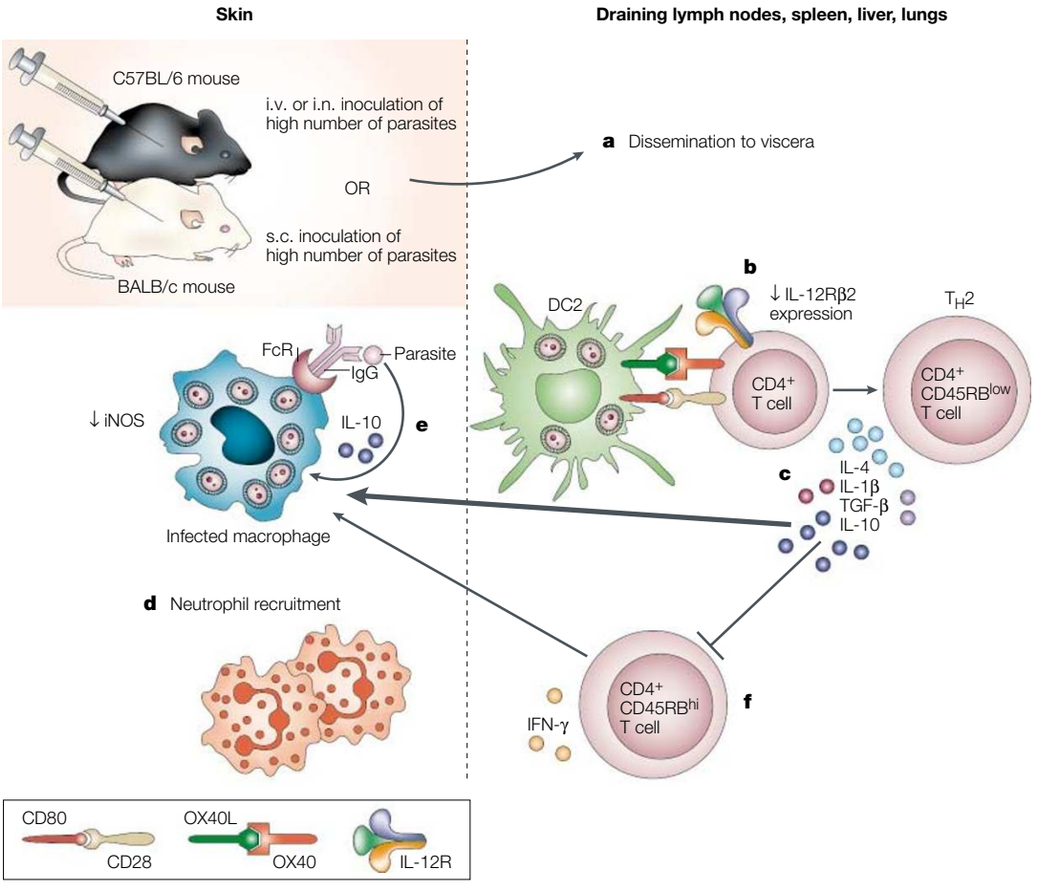


Figure 5 | **Late events in susceptible mice.** The evolution of susceptibility to *Leishmania major*, as occurs after sub-cutaneous (s.c.) inoculation of a relatively high number of parasites into BALB/c mice, or intravenous (i.v.) or intranasal (i.n.) inoculation of a high number of parasites into C57BL/6 mice, is due to the failure of an interleukin-12 (IL-12)-dependent redirection of the early T helper 2 (T_H2) response, resulting in the clonal expansion and dominance of $CD4^+CD45RB^{low}$ T_H2 cells. The T_H1-response defect might be due to several mechanisms, including: **a** | dissemination of parasites to the viscera, where T_H2-priming conditions are maintained. The class of immune response in these tissues might be biased by the presence of lineage-specific type-2 dendritic cells (DC2s) that express the appropriate co-stimulatory molecules and cytokines required to drive T_H2-cell development, or by the cytokine milieu that conditions DCs to promote T_H2-cell development; and/or **b** | unstable expression of IL-12 receptor β2-chain (IL-12Rβ2) on activated $CD4^+$ T cells. Type-2 cytokines (**c**) contribute to susceptibility by downregulating T_H1-cell differentiation and by conditioning infected macrophages to become unresponsive to activation signals required for nitric oxide (NO)-dependent killing. Sustained neutrophil recruitment to the inflammatory site (**d**) might be inhibitory to T_H1-cell development, owing, for example, to the local secretion of transforming growth factor-β (TGF-β) or IL-10. IL-10 produced by macrophages as a consequence of Fc-receptor (FcR) ligation by parasite-specific antibodies (**e**) might contribute to the pool of deactivating cytokines in infected tissues. A minor population of interferon-γ (IFN-γ)-producing $CD4^+CD45RB^{hi}$ effector cells (**f**) remain active even during the progressive stages of disease. IgG, immunoglobulin G; iNOS, inducible nitric oxide synthase.

with an anti-IL-10 monoclonal antibody had little effect on reversing disease progression[22,55]. However, the role of IL-10 in *L. major* susceptibility needs to be reconsidered in the light of several recent studies: resistant mice expressing an IL-10-encoding transgene under the control of the MHC class II Ea promoter, which directs the expression of IL-10 mainly to APCs that display MHC class II molecules, were more susceptible to infection with *L. major*[21]; and the stable T_H1 phenotype that is established in BALB/c mice infected with a low dose of *L. major* could be overcome by transfection of a plasmid encoding IL-4 at the time of challenge, but only if co-administered with a plasmid encoding IL-10 (REF. 56). Conversely, IL-10-deficient mice crossed for several generations to a BALB/c background were markedly more resistant to *L. major* infection than wild-type BALB/c mice[57]. Most recently, IL-10-receptor blockade has been shown to confer partial resistance to *L. major* in wild-type BALB/c mice, regardless of the *L. major* substrain (N.N.-T., unpublished observations).

Macrophages have been proposed as an important source of IL-10 in the BALB/c model, on the basis of *in vitro* findings that *Leishmania* amastigotes coated with immunoglobulin G mediate ligation of FcγRs on macrophages, which, in conjunction with lipopolysaccharide, triggers them to produce large amounts of IL-10 and suppresses the production of IL-12 (REF. 57) (FIG. 5). However, as anti-CD4 antibody treatment abolished the

expression of IL-10 in lymph-node cells four days after infection[15] and as depletion of CD4 allowed IL-4-deficient mice to control infections with LV39 (REF. 58), it is probable that the crucial source of IL-10 *in vivo* is $CD4^+$ T cells. Indeed, $CD4^+$ T cells from *L. major*-infected BALB/c mice express high levels of both IL-4 and IL-10 messenger RNA[15], and the $CD4^+$ T cells that suppress *L. major* immunity in BALB/c mice were found to belong to an IL-4- and IL-10-producing population of cells that were $CD45RB^{low}$ and also inhibited colitis[22]. In light of the well-described phenotype and function of naturally occurring $CD4^+CD25^+CD45RB^{LOW}$ REGULATORY T CELLS[59], it seems possible that the T_H2 cells that are responsible for progression of *L. major* infection might be activated from a distinct lineage of IL-4- and IL-10-secreting immunoregulatory T cells (FIG. 5).

The relative contributions of T_H2 cytokines. The results from IL-4- and IL-4Rα-deficient BALB/c mice seem to undermine a basic tenet of the T_H1/T_H2 model regarding the instructional role of IL-4 in T_H2-cell development. It is now clear, however, that IL-4Rα–STAT6 signalling is not essential for priming $CD4^+$ T cells to produce T_H2 cytokines *in vivo*, because in STAT6- or IL-4Rα-deficient mice, T_H2 responses are decreased, but significant amounts of IL-4 and other T_H2-related cytokines are still present[60]. Given the redundancy of T_H2 cytokines able to inhibit T_H1 responses and/or macrophage activation, and given the inherent T_H1-cell developmental defects that have been ascribed to BALB/c mice, it is, perhaps, not surprising that IL-4 production and IL-4R signalling are not required for susceptibility to *L. major* in these mice. These outcomes should not, however, be interpreted to indicate that the IL-4–IL-4R pathway does not contribute to susceptibility in every case that it remains intact. It might be possible to reconcile the data by using a more rigorous definition of 'resistance.' Often, the term has been applied to mice that can control progressive lesion development, whether or not they actually heal or have a marked reduction in parasite load over time. A careful quantitative analysis of parasite load at the inoculation site has shown that, regardless of the *L. major* strain used for challenge, impairment of IL-4, IL-10 or IL-13 responses individually does not achieve the same level of resistance to infection as seen in IL-4Rα-deficient mice. In turn, IL-4Rα-deficient mice are not as resistant as IL-4Rα and IL-10 double-deficient mice or IL-4Rα-deficient mice treated with anti-IL-10R antibody, which have a resistant phenotype comparable to that of C57BL/6 mice (N.N.-T., unpublished observations). It is not known why certain *L. major* substrains (for example, LV39) require a more global reduction in T_H2 cytokines, as is achieved by anti-CD4 antibody treatment, to be controlled. As these substrains do not seem to induce higher levels of these cytokines[49], they might have a preference for replicating in cell types that are more sensitive to the effects of deactivating cytokines. Alternatively, they might have intrinsically greater resistance to immune-mediated killing mechanisms, and therefore require a greater increase in the T_H1:T_H2 cytokine ratio for killing to occur.

Finally, to what extent do the factors that control susceptibility to *L. major* infection in BALB/c mice also operate in non-healing and/or systemic forms of human disease? In this context, the overproduction of IL-10 — as has been detected in chronic cutaneous lesions[61], in lesion tissue from kala-azar patients[62] and in plasma from patients with post-kala-azar dermal leishmaniasis[63] — seems to provide a much better correlate of susceptibility than production of IL-4. It has not been determined whether $CD4^+$ T cells produce the IL-10 in these clinical settings.

T_H1-cell development and resistance

Regulation of T_H1 responses. Advances in gene-knockout and transgenic technologies have been particularly helpful in advancing our understanding of the pathways that are involved in the induction of acquired immunity to *L. major* in resistant mice; they have not, however, fundamentally altered the view that type-1 cytokines, particularly IFN-γ, are essential and will be the key to vaccine development and immunotherapy for leishmaniasis (FIG. 6). Consistent with the concept of inflammatory type-1 cytokines as mediators of protection, the genetic ablation of cytokines (IL-12, IFN-γ and tumour-necrosis factor, TNF), receptors (IFN-γR), transcription factors (T-bet and STAT4) or co-stimulatory molecules (CD40–CD40L) that are involved in the development or function of T_H1 cells will lead to susceptibility to *L. major*. For mice that are deficient in IL-12 (REF. 26), IFN-γ[64], T-bet[65] or CD40–CD40L interactions[66,67], the immune response to *L. major* defaults to the T_H2 pathway, which is associated with defective IL-12 production. In the case of mice that are deficient in IFN-γR[68] or STAT4 (REF. 69), no T_H2 default response was observed, which indicates that the impaired production or activity of effector cytokines, rather than the upregulation of expression of deactivating cytokines, is responsible for their inability to heal. Alternatively, in the absence of IL-12 signalling, other T_H1-inducing cytokines, such as IL-18, might inhibit the T_H2 default response. Indeed, the IL-4 response to *L. major* was enhanced in IL-18-deficient mice[70], as was the early growth of parasites in these mice. It is clear, however, that in the absence of IL-12, IL-18 is not sufficient to drive immunity to *L. major*, and it is not required in IL-12-competent mice, because IL-18-deficient C57BL/6 mice were able to heal ultimately and to extinguish the IL-4 response that is induced early after infection in these mice[70]. It seems probable that the susceptibility to *L. major* of IL-18-deficient mice reported in the study by Wei *et al.*[71] was a result of the early termination of the experiment before the mice had had a chance to heal. An additional T_H1-response pathway involving the class-1 cytokine receptor WSX1 has been found to influence resistance to *L. major* in a manner similar to IL-18 (REF. 72). Taken together, these data indicate that although alternative factors can cooperate with IL-12 to redirect the early T_H2 response and can affect the early stages of *L. major* infection in resistant mice, IL-12–IL-12R signalling is essential to establish and maintain a curative T_H1 response.

$CD4^+CD25^+CD45RB^{LOW}$ REGULATORY T CELLS
A specialized subset of $CD4^+$ T cells that can suppress other T-cell responses. These cells are characterized by expression of the IL-2 receptor β-chain (CD25). In some instances, suppression has been associated with the secretion of IL-10, TGF-β or both.

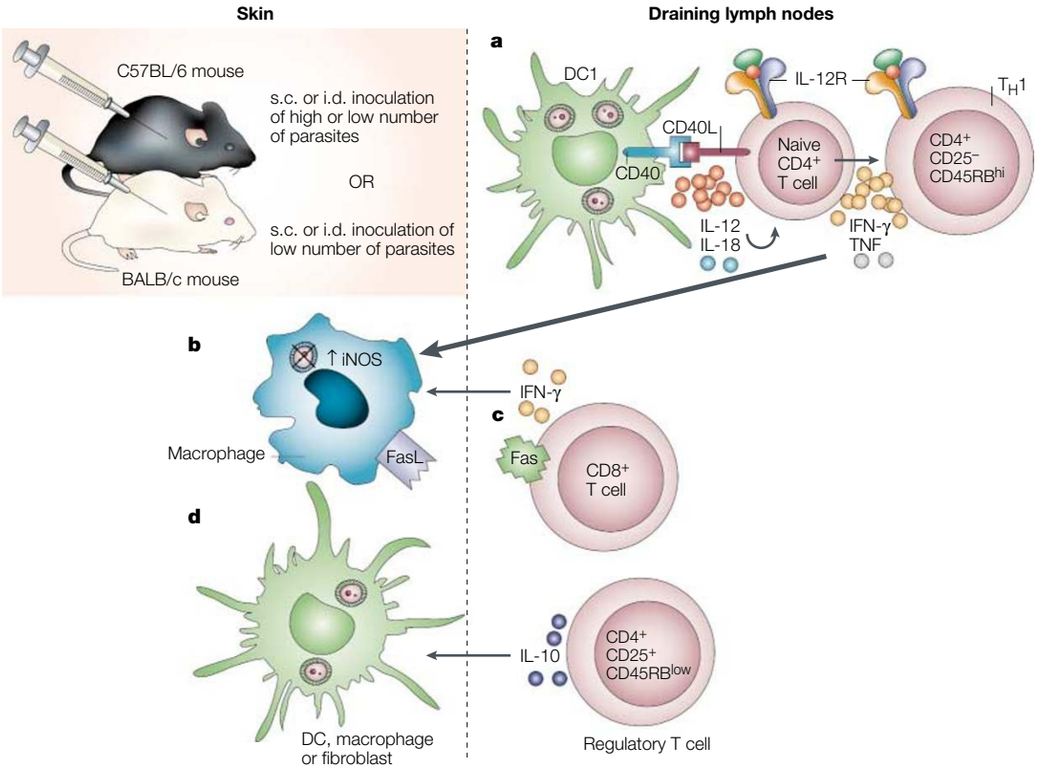


Figure 6 | **Late events in resistant mice.** The evolution of resistance to *Leishmania major*, as occurs after sub-cutaneous (s.c.) or intradermal (i.d.) inoculation of a high or low number of parasites in C57BL/6 mice, or a low number of parasites in BALB/c mice, also involves the early production of interleukin-4 (IL-4) and other type-2 cytokines by $CD4^+$ T cells. However, the parasites remain restricted to the site of infection and to the local draining lymph nodes, where, in response to accumulating amastigotes and endogenous agonists such as CD40L and interferon-γ (IFN-γ), dendritic cells (DCs) are activated to upregulate expression of CD40 and other co-stimulatory molecules and to produce IL-12 (**a**). These local priming conditions generate a predominant T helper 1 (T_H1) response characterized by $CD4^+CD25^-CD45RB^{hi}$ effector cells that produce high levels of IFN-γ and tumour-necrosis factor (TNF) to upregulate the expression of inducible nitric oxide synthase (iNOS) and activate infected macrophages for intracellular killing (**b**). $CD8^+$ T cells cooperate to control infection by their production of IFN-γ and by their lytic activities, which might be mediated by a Fas–Fas ligand (FasL)-dependent pathway (**c**). After healing, low numbers of amastigotes persist at the site of infection in macrophages, DCs and fibroblasts, owing to the production of IL-10 by $CD4^+CD25^+$ regulatory T cells (**d**).

The *L. major* model has been particularly useful to clarify the role of IL-12 in not only initiating the development of a T_H1 response, but maintaining a T_H1 response once it has been established. In particular, IL-12-deficient mice treated transiently with IL-12 develop progressive lesions eventually[73], and the lesions of healed C57BL/6 mice harbouring persistent parasites reactivate after treatment with anti-IL-12 antibodies[74]. Most convincingly, primed T_H1 cells from healed mice cannot transfer immunity to IL-12-deficient mice[73]. It seems probable that IL-12 is required to prevent the differentiation of newly emerging uncommitted precursors to T_H2 cells, because the conditions that promote the early T_H2 response at the time of infection, before the onset of IL-12 production, will be re-established at later times whenever IL-12 is depleted[75].

Given its central role in reconditioning the adaptive immune response, the source of IL-12 is an important issue to address. Although macrophages ingest *Leishmania* efficiently, they are not activated by ingestion and their ability to produce IL-12 in response to strong pro-inflammatory stimuli is selectively impaired[15,76,77]. This might explain the delayed onset of immunity in resistant mice, particularly after low-dose challenge[14], because macrophages are the primary targets of infection. In contrast to macrophages, mouse DCs, including epidermal Langerhans cells (LCs), take up *L. major* parasites, acquire a mature phenotype and release IL-12 p40 *in vitro*[78–80]. Their role in producing IL-12 *in vivo* and promoting the development of *L. major*-specific T_H1 immunity has been shown also[14,43,81]. The ability of LCs to transport *L. major* from infected skin to the draining lymph nodes[82] is thought to rely, at least in part, on the expression of CC-chemokine receptor 2 (CCR2), because resistant-background mice deficient in CCR2 are highly susceptible to *L. major* infection, and LC migration is impaired markedly in these mice[83].

Natural killer (NK) cells are an additional cellular component of the innate immune response that have been implicated in the development of a T_H1 response, primarily through their ability to produce IFN-γ, which can optimize the production of IL-12 by DCs and the expression of IL-12R by activated T cells. Rapid development of a T_H1 response and the early control of

L. major infection after high-dose footpad injection, as occurs in C3H mice, is associated with an early NK-cell response, whereas C57BL/6 and BALB/c mice that lack an early NK-cell response have delayed or absent development of a T_H1 response, respectively[84]. It is clear, however, that although early NK-cell activity might influence the kinetics of the T_H1 response, these cells are not required ultimately for resistance, because immune-deficient, T-cell-reconstituted mice that lack NK cells selectively have efficient IL-12-dependent IFN-γ production by $CD4^+$ T cells and heal their lesions[85,86].

Effector molecules in acquired resistance. In addition to deficiencies in T_H1 cytokines that are essential to redirect the T_H2 response along a T_H1 developmental pathway, animals with defects in effector molecules that are involved in macrophage activation and microbicidal response pathways are susceptible to *L. major*. The upregulation of expression of inducible nitric oxide synthase (iNOS) and the subsequent production of RNIs in response to activation by IFN-γ has, in many systems (including *L. major* infection), been thought to use TNF as a co-factor[87], and animals that are deficient in either iNOS[88] or TNF[89] cannot control infection. Interestingly, TNFR1- and TNFR2-deficient mice can control parasite replication, although they fail to resolve their lesions[90], which indicates that TNF signalling can be compensated for through a third receptor, but that the ligand is needed to confer protection. The induction of iNOS and production of nitric oxide seem not to be sufficient for microbicidal function, as mice deficient in Fas (CD95) or Fas ligand (FasL) cannot eliminate *L. major* despite enhanced production of nitric oxide[91,92]. On the basis of these findings, it was concluded that the apoptosis of infected macrophages through the Fas–FasL pathway might contribute to host resistance.

Role of $CD8^+$ T cells. With regard to the T-cell subsets that are involved in acquired resistance to *L. major*, the crucial role of $CD4^+$ T cells has been a consistent finding[14,93,94]. The requirement for $CD8^+$ T cells, however, is a recent observation that does not agree with earlier studies. Although $CD8^+$ T cells were shown to be important for immunity to re-infection in mice that had healed their primary lesions[95], C57BL/6 mice deficient in β2-microglobulin or CD8 maintained their ability to heal[96], as did anti-CD8 antibody-treated mice[97], which indicates that $CD8^+$ T cells are not required for the control of primary infection involving a high-dose, subcutaneous challenge. By contrast, using a challenge system that reproduces two key features of natural transmission — low dose (100 metacyclic promastigotes) and intradermal inoculation (in the mouse ear dermis) — the outcome of *L. major* infection in anti-CD8 antibody-treated and CD8-deficient mice showed that, in addition to $CD4^+$ T cells, $CD8^+$ T cells are required for the control of primary *L. major* infection in the skin[98] (FIG. 6). These data are consistent with clinical studies that report high numbers of antigen-specific $CD8^+$ T cells in lesions and peripheral blood during the acute stage of lesion formation and during the healing process[99,100]. Although it is assumed that the main protective function of antigen-specific $CD8^+$ T cells is to contribute to the release of IFN-γ in the *L. major*-loaded dermis, the cytolysis of infected host cells that are themselves defective in intracellular killing might release parasites and make them available for uptake by cells that are more responsive to activation signals (for example, macrophages). The observations regarding the importance of the Fas–FasL pathway might be relevant to the possible role of $CD8^+$ T-cell cytolytic activity in immunity to *L. major*.

L. major persistence in healed mice. The fact that *L. major* is sequestered in fibroblasts[101] and DCs[102] has been offered as an explanation for the important observation that latent infections are established in resistant mice after clinical cure[103]. Despite their inability to achieve sterile immunity, healed mice maintain life-long immunity to re-infection. Immune pressure during the chronic phase is maintained by $CD4^+$ and $CD8^+$ T cells, IL-12, IFN-γ and iNOS, because impairment of these responses during latency has been shown, in each case, to promote parasite growth and the reappearance of lesions[74,104,105]. IL-10 was shown to have a crucial role in chronicity by the inability of the parasite to establish a persistent infection after healing in IL-10-deficient C57BL/10 mice and by the sterile immunity that was achieved in wild-type mice treated during the chronic phase with anti-IL-10R antibody[105]. So, the persistence of *L. major* does not seem to be explained adequately by the SAFE-TARGET MODEL[101], because regardless of the nature of the cells harbouring the parasites, the absence of IL-10 allowed these cells to be activated for effective killing or, perhaps, to become sensitive to apoptotic pathways, thereby releasing parasites for uptake and killing by other cells. The clinical findings that are most relevant to these data are those indicating that even in healed cases of visceral or localized cutaneous disease, IL-10 continues to be produced together with IFN-γ[106,107,108], which might explain the failure of these individuals to achieve sterile cure. Recently, the source of the IL-10 in C57BL/6 mice was found to be a population of $CD4^+CD25^+CD45RB^{low}$ immunoregulatory T cells[109] (FIG. 6), which links the $CD4^+CD45RB^{low}$ T-cell subset that suppresses *L. major* immunity in BALB/c mice[22] with the naturally occurring suppressor cells that prevent sterile cure in resistant mice. Importantly, the IL-10-deficient and anti-IL-10R antibody-treated mice that achieved sterile cure were no longer immune to re-infection[109], which indicates that the maintenance of effector memory T cells requires antigen persistence. Similar findings have been published recently[110], in which the transfer of immune cells from sub-clinical mice could protect naive BALB/c mice against a pathogenic challenge and could completely clear the parasite, leaving the mice susceptible to a re-challenge infection.

SAFE-TARGET MODEL
A model to explain the role of cell types such as fibroblasts, which *L. major* parasites might infect and replicate in without activating immune responses.

Implications for vaccine design

The observations regarding the need for both $CD4^+$ and $CD8^+$ T cells for acquired resistance, as well as the requirements for sustained IL-12 production and parasite persistence to maintain immunity, have important

Table 1 | **Defined subunit vaccines against *Leishmania major****

Antigen	Vaccine form	Challenge model	Result	References
gp63	rgp63 expressed in *Salmonella*	BALB/c × C57BL/6	Partial protection	130
	rgp63 expressed in BCG	BALB/c, CBA	Partial protection	131
	gp63 synthetic peptide + poloxamer adjuvant	BALB/c	Protection	132
	gp63 DNA	BALB/c	Partial protection	133
PSA2	rPSA2 with *C. parvum*	C3H, BALB/c	Protection	134
	PSA2 DNA	C3H	Complete protection	135
LACK	rLACK + rIL-12	BALB/c	Protection	74,136
	LACK DNA	BALB/c	Long-lived protection	114
TSA	rTSA + rIL-12	BALB/c	Protection	137
	TSA DNA	BALB/c	Protection	138
LmSTI1	LmSTI1 DNA	BALB/c	Protection	138
LmSTI1 + TSA + LACK	LmSTI1 DNA + TSA DNA + LACK DNA	C57BL/6 (low-dose i.d.)	Complete, long-lived protection	115
CP type I and II	CPI DNA + CPII DNA	BALB/c	Long-lived protection	139
Histone H1	rH1	BALB/c	Partial protection	140
SP15‡	SP15 DNA	BALB/c, C57BL/6 (low-dose i.d. + SGH)	Protection	120
Maxadilan§	rMaxadilan	CBA (high-dose s.c. + SGH)	Protection	121

*All vaccinated mice were evaluated using high-dose, subcutaneous (s.c.) challenge 2–4 weeks after vaccination, unless indicated otherwise. Partial protection refers to outcomes in which only a fraction of the vaccinated animals had reduced lesion scores or in which BALB/c mice had a delayed, but still progressive, lesion development. Protection refers to moderated lesion scores in all vaccinated mice. Complete protection refers to the absence of lesions in all mice. Long-lived protection refers to protection achieved in mice challenged at least three months after vaccination. ‡From *Phlebotomus papatasi* saliva. §From *Lutzomyia longipalpis* saliva. BCG, *Mycobacterium bovis* bacillus Calmette-Guerin; CP, cysteine proteinase; *C. parvum*, *Cryptosporidium parvum*; gp, glycoprotein; i.d., intradermal; IL-12, interleukin-12; LACK, *Leishmania* homologue of receptors for activated C kinase; PSA2, promastigote surface antigen 2; r, recombinant; SGH, salivary-gland homogenate; TSA, thiol-specific antioxidant.

implications with regard to vaccination strategies. The concern that non-living, protein-based vaccines will elicit poor $CD8^+$ T-cell responses and be less potent and durable than live vaccines has, to some extent, been substantiated in human trials. Live vaccination, or leishmanization as it is known — which involves the inoculation of virulent organisms in the arm to protect against the development of severe or multiple lesions, particularly on the face — provides virtually complete and life-long protection[111]. By contrast, a safe, non-living vaccine made up of whole-cell killed *Leishmania* inoculated with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) as an adjuvant failed to confer substantial protection to humans against cutaneous disease[112,113]. The underlying problems with this vaccine are indicated by recent results from the *L. major* mouse model; mice immunized with killed promastigotes or recombinant proteins plus IL-12 as an adjuvant had a high level of protection when challenged 2–4 weeks after vaccination, but they had already lost a substantial degree of protection when challenged after 12 weeks[114,115]. Immunity could be maintained by repeated administration of antigen or IL-12 (REF. 74), or by antigen and/or IL-12 delivered by plasmid DNA[114,115]. Immunization using plasmid DNA encoding single or multiple *Leishmania* antigens is a particularly effective approach to generate strong and long-lasting protection against *L. major*, owing to its ability to induce $CD4^+$ and $CD8^+$ T-cell responses, its sustained delivery of antigen and its provision of a strong T_H1-promoting adjuvant in the form of unmethylated CpG dinucleotide motifs[116]. TABLE 1 provides a summary of many of the defined, subunit vaccines — either recombinant protein- or DNA-based — that have shown some degree of efficacy in the *L. major* mouse model.

As more natural challenge models have revealed differences in the immune mechanisms that are required to control infection, it will be important to evaluate candidate vaccines using sandfly challenge. In addition to the inoculum size and site of challenge, sandfly-transmitted infections differ from those initiated by a needle in that infected sandflies also inoculate small amounts of saliva. Many studies have reported that the co-injection of parasites with salivary-gland homogenates of vector sandflies produces a substantial increase in lesion size and/or parasite burden that is due, in large part, to an upregulation of type-2 responses by components in the salivary-gland lysate[117]. Furthermore, pre-exposure of mice to sandfly saliva was found to neutralize the enhancing effects of saliva[118] and to confer powerful protection against *L. major* infection transmitted by sandfly bite[119]. Protection was associated with a strong delayed-type hypersensitivity response, including the production of IFN-γ and IL-12 at the site of the bite, which indicates that in this inflammatory setting, infected macrophages might be activated for early killing of the parasites. These results have prompted the use of defined salivary antigens, delivered as either recombinant proteins or as plasmid DNA, to vaccinate mice against a challenge inoculation containing *L. major* plus sandfly saliva[120,121].

Concluding comments

The mouse *L. major* infection model remains a popular tool for immunologists to investigate the contribution of various factors — for example, cytokines, receptors or signalling molecules — to the development of a T_H1 or T_H2 response *in vivo*. Although some surprises have emerged from these studies that challenge some basic tenets of the T_H1/T_H2 model, important unifying themes can be discerned. *L. major* seems to initiate an early T_H2 response that is redirected effectively in resistant mice by IL-12-dependent mechanisms. By contrast, in susceptible mice — either because of inherent instabilities in IL-12R expression and/or because the parasites disseminate to tissues that preferentially drive T_H2 development — the T_H2 response is maintained and dominates the clinical outcome. The ability of redundant deactivating cytokines, including IL-4, IL-13, IL-10 and TGF-β, to prevent effective killing of the parasite, even when T_H1 responses are induced also, might explain why ablation of IL-4 or IL-4R signalling in BALB/c mice is, in some cases, insufficient to reverse susceptibility, particularly as IL-4 is not necessary to initiate T_H2-cell development *in vivo*. Whereas defects in T_H1-response development might produce an imbalance in the number and activity of parasite-driven T_H2 cells (possibly, regulatory T cells) in BALB/c mice, these same cells might operate in more dynamic equilibrium with T_H1 effector cells in resistant mice, preventing the ability of the mice to achieve sterile cure, rather than the development or expression of resistance. Although there is an obvious advantage to the parasite to express epitopes (for example, LACK) that might exploit the activation of these T_H2 cells, it seems equally probable that their function is of benefit to the host by moderating the tissue damage that is associated with powerful immune responses in the skin and by favouring parasite persistence so as to maintain life-long immunity to re-infection. The need for persistent antigen and sustained IL-12 production to maintain the $CD4^+$ and $CD8^+$ effector T cells that are responsible for acquired immunity will pose serious challenges to the development of a safe and effective non-living vaccine.

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Acknowledgements
We thank A. Sher and D. Jankovic for critical review of the manuscript.

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β2-microglobulin | B7 | Bcl-6 | CCR2 | CD8 | CD28 | CD40 | CD40L | c-MAF | CTLA4 | Fas | FasL | FOG1 | GATA3 | IFN-γ | IFN-γR | IL-4 | IL-4Rα | IL-10 | IL-10R | IL-12 | IL-12 p40 | IL-12Rβ1 | IL-12Rβ2 | IL-13 | IL-13Rα2 | IL-18 | iNOS | MCP1 | NFAT-c | OX40 | OX40L | ROG | STAT1 | STAT4 | STAT6 | T-bet | TGF-β | TGF-β receptor type II | TNF | TNFR1 | TNFR2 | WSX1
Swiss-Prot: http://ca.expasy.org/sprot/sprot-top.html
GFP

FURTHER INFORMATION
Mouse Genome Informatics: http://www.informatics.jax.org/
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