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Review

CD4+ T Cell-Mediated Immunity against the Phagosomal Pathogen *Leishmania*: Implications for Vaccination

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The generation of an efficacious vaccine that elicits protective CD4+ T cell-mediated immunity has been elusive. The lack of a vaccine against the *Leishmania* parasite is particularly perplexing as infected individuals acquire lifelong immunity to reinfection. Experimental observations suggest that the relationship between immunological memory and protection against *Leishmania* is not straightforward and that a new paradigm is required to inform vaccine design. These observations include: (i) induction of Th1 memory is a component of protective immunity, but is not sufficient; (ii) memory T cells may be protective only if they generate circulating effector cells prior to, not after, challenge; and (iii) the low-dose/high-inflammation conditions of physiological vector transmission compromises vaccine efficacy. Understanding the implications of these observations is likely key to efficacious vaccination.

Vaccination and Immune Memory

Conventional vaccination strategies currently in use are based on the concept of immunological memory, whereby exposure of the immune system to a vaccine generates immunity against an infectious pathogen without the need to undergo the complete infectious process. Upon subsequent exposure to the pathogen, the memory state elicited by the vaccine results in a more efficient immune response, protecting the vaccinee from disease. In the conventional scenario, memory cells of the adaptive immune system mediate the expression of immunological memory. These memory cells are long-lived, and once generated via short-term exposure to a vaccine they provide long-lasting protection. The concept of immunological memory has provided the foundation for the generation of those vaccines that have seen widespread use in people, including the polio, smallpox, and measles vaccines, among others [1]. While our understanding of immunological memory is evolving [2], these vaccines are generally thought to work by generating memory B cells that, upon exposure to infectious challenge, rapidly produce antibodies (Abs) that mediate pathogen clearance by phagocyte- or complement-mediated pathways.

Many intracellular pathogens cannot be controlled by extracellular circulating Abs and instead require cell-mediated immunity. However, the efficacy and successes of Ab-mediated vaccination strategies has yet to translate to T cell-mediated vaccination. The bacillus Calmette–Guérin (BCG) vaccine against tuberculosis is the only licensed vaccine believed to work primarily through cell-mediated immunity, and both its efficacy and mode of action are a matter of debate [3,4]. Attempts to improve the BCG vaccine, evaluated in part based on their ability to generate enhanced CD4+ T cell memory [5], have fallen short of expectations in clinical trials [3,4]. The failure to translate the successes of antibody-mediated vaccination to T cell-mediated vaccination suggests that the relationship between protection and memory in the

Highlights

Th1 memory is a component of protective immunity against phagosomal infections, but it is not sufficient.

Protection via CD4-mediated concomitant immunity is the gold standard of protective immunity against phagosomal infections.

Chronic primary infection is required for protection against secondary infection due to the requirement for persistent antigen exposure to maintain protective T_{EFF} cells.

Memory T cells may mediate protection against infection only if they generate circulating effector cells prior to, not after, challenge.

Modeling the low-dose/high-inflammation conditions of physiological vector transmission of *Leishmania* has implications for the interpretation of vaccine efficacy.

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latter case may be considerably different, and that a new paradigm is required to inform cell-mediated vaccine design [3,4,6].

The Immunobiology of *Leishmania* Infection

The *Leishmania* parasite is a vector-transmitted obligate intracellular pathogen and the causative agent of the disease leishmaniasis (Box 1). Despite the spectrum of clinical manifestations caused by *Leishmania* spp., in each case infection is initiated when an infected sand fly vector deposits the **metacyclic promastigote** (see Glossary) form of the parasite into the skin during a blood meal [7,8]. Parasites are deposited in very low numbers, often ≤600 [9], and are rapidly engulfed − predominantly by neutrophils, recruited in large numbers in response to the sand fly bite – but remain viable [7,8,10–13]. Similar to *Mycobacterium, Cryptococcus*, *Salmonella enterica,* and *Ehrlichia, Leishmania* specifically targets phagocytic cells for infection and expansion. Upon internalization, the parasite resides in a phagosome that typically undergoes a maturation process into a microbicidal phagolysosome via phagosome–lysosome fusion [14]. While the phagolysosome has a well-established role in pathogen killing [15], **phagosomal pathogens** such as *Leishmania* are well adapted to prevent or withstand this innate response [16–19]. Sequential intravital imaging of sand fly bite sites has revealed that *Leishmania major* does not undergo proliferation during this early neutrophil phase and that a single parasite can initiate the entire course of infection [13,20]. The majority of parasites then transition from neutrophils into inflammatory monocytes, between 10 and 48 h, where they again resist killing by innate defense mechanisms such as reactive oxygen species (ROS) and undergo robust proliferation as intracellular amastigotes [13]. Infection is propagated at later stages when intracellular amastigotes infect new phagocytic cells, resulting in ongoing chronic infection [13,21–24]. In addition to the parasite itself, several additional parasite, host and vector-associated factors have been implicated in infection outcome and disease severity (Box 2) [25].

While *Leishmania* interference with phagosome–lysosome fusion events can also result in altered antigen processing in the phagolysosome of antigen-presenting cells [26,27], disease is ultimately controlled by interferon (IFN)-γ-producing T helper 1 (Th1) CD4$^+$ T cells of the adaptive immune system. This is largely due to the recognition of phagosome-derived antigenic peptides in the context of MHC II molecules by the αb T cell receptor (TCRs) on CD4$^+$ T cells [26–29]. While both antibody- and CD8$^+$ T cell-mediated immunity have roles during

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Box 1. Clinical Manifestations of Leishmaniasis

Localized cutaneous leishmaniasis (CL) results in disfiguring, but typically self-resolving, lesions and extensive scarring, often on the hands and face. Localized CL is the most common form of leishmaniasis. Diffuse CL occurs during a defective cell-mediated immune response and does not spontaneously resolve. WHO estimates 0.7–1 million new cases of CL annually. In 2017, >95% of CL cases occurred in Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq, and Syria.

Mucocutaneous leishmaniasis (MCL) causes inflammatory destruction of the mucus membranes, specifically the nasal and oropharyngeal mucosa and cartilage. This form of leishmaniasis is more commonly seen in the Americas, and is associated with New World strains of *Leishmania* such as *L. braziliensis*, *L. amazonensis*, and *L. guyanensis*. WHO estimates that >90% of MCL occurs in Bolivia, Brazil, Ethiopia, and Peru.

Visceral leishmaniasis (VL) features parasite dissemination to, and expansion in, the internal organs such as the liver, spleen, and bone marrow. VL is fatal if left untreated. Hallmark symptoms include splenomegaly, persistent fluctuating fever, weight loss, and anemia. Even after treatment, patients are at risk of developing post-Kala-azar dermal leishmaniasis (PKDL). In recent years, VL cases have sharply declined, from an estimated 200 000–390 000 new cases in 2012 [97] to 50 000–90 000 new cases in 2018 (https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis), though VL exhibits cyclical epidemiological patterns [98], and under-reporting is common.

Box 2. Factors Contributing to Inflammation and Leishmaniasis Severity

  (i) Vector saliva, which contains antihemostatic and immunomodulatory molecules, has been shown to enhance cutaneous leishmaniasis when coinjected with *Leishmania major* [99,100].
 (ii) Parasite-secreted proteophosphoglycans (PPGs) form a plug within the sand fly gut called the promastigote secretory gel (PSG) which promotes parasite regurgitation at the feeding site. Regurgitated PPG/PSG enhances *Leishmania* infection and worsens disease parameters at the site of infection [101,102].
(iii) Parasite-derived viruses, such as the cytoplasmic virus LRV1, have been shown to exacerbate disease in the *L. guyanensis* model [103].
(iv) Host skin microbiota. Germ-free (GF) mice have reduced lesion size and *Leishmania*-specific IFN-γ production following *L. major* infection [104]. *L. braziliensis* infection has been shown to promote a dysbiotic skin microbiota, and a pre-existing dysbiotic skin microbiota results in an enhanced inflammatory response to *L. major* without altering parasite load [105].
 (v) Parasite-derived exosomes, which are regurgitated with *Leishmania* during feeding and contain parasite-derived immune-modulatory molecules, have been found to increase footpad swelling through enhanced induction of inflammatory cytokines when coinjected with *L. major* [106].
(vi) Vector-derived microbiota, components of which enhance neutrophil recruitment and induce neutrophil IL-1β production, are associated with more efficient parasite dissemination to the spleen [8].
(vii) Tissue damage is significantly different when comparing needle and sand fly infections; sand fly-mediated damage drives the formation of a neutrophil plug and is associated with prolonged detection of neutrophils at a bite site [45]. Neutrophils enhance the ability of *L. major* to establish infection [7].

infection. The maintenance of T_{EFF} cells is antigen-dependent.

T effector memory (T_{EM}) cells: CD4$^+$ T cells which circulate through the periphery, blood, and secondary lymphoid organs. T_{EM} cells express CD44, but not CD62L or Ly6C. These cells are longer lived than T_{EFF} cells in the absence of antigen, but shorter lived than T_{CM} cells, and may produce effector cytokines (such as IFN-γ and TNF-α).

Tissue resident memory (T_{RM}) cells: T cells which are resident in the tissue and do not recirculate. T_{RM} cells may act independently of the circulating T cell pool to provide rapid immunity and may produce effector molecules (such as IFN-γ and TNF-α). CD8$^+$ T_{RM} cells express CD69 and CD103; however, T_{RM}-defining markers on CD4$^+$ T cells appear to vary between tissue types.

phagosomal infections [30–32], they cannot control infection in the absence of the CD4$^+$ T cell response, as these effector mechanisms struggle to 'see' phagosomal antigen [33]. Subsequent exposure of infected phagocytes to CD4$^+$ **T effector cell (T_{EFF})**-derived IFN-γ leads to increased oxygen free radical production within lysosomes and increased phagosome–lysosome fusion. In mouse models, this activation process has primarily been associated with the induction of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide (NO) production from L-arginine [34], but it is important to note evidence that both NO and ROS are required for parasite clearance *in vivo* [35–37]. During primary infection, simultaneous exposure of phagocytes to tumor necrosis factor (TNF)-α has also been found to favor parasite clearance by counteracting the proparasitic effects of arginase, which includes competing with iNOS for L-arginine [38]. In humans, parasite elimination has largely been attributed to ROS, but iNOS production in cutaneous leishmaniasis (CL) patient lesions has been shown to correlate with disease control [39]. While cutaneous lesions caused by *L. major* are typically self-healing, sterile immunity is not achieved, and affected individuals carry the parasite for life. This is partly due to the ability of the parasite to take advantage of interleukin-10 (IL-10) and/or T regulatory-cell-mediated modulation of Th1 immunity [40–43], and in some cases, targeting specific phagocyte populations that are refractory to cell-mediated immune activation [44].

Leishmania Vaccination
The Rationale for *Leishmania* Vaccination
Remarkably, both mice and humans who recover from cutaneous disease following infection with *L. major* but maintain chronic subclinical infection in the skin are highly resistant to sand-fly-transmitted infection at a cutaneous site of secondary challenge [45–50]. In animal models, chronic *L. major* infection also protects against visceral infection caused by *Leishmania infantum* [51], and antidotal epidemiological observations in people suggest that a similar cross-protective phenomenon exists [52,53]. This is a classic example of **concomitant immunity**, in which the immune response cannot clear the primary infection but facilitates powerful immunity at a secondary site [33,54,55]. In the case of *Leishmania* infection, concomitant immunity is mediated primarily by IFN-γ-producing CD4$^+$ Th1 cells [33,56,57], and emulating the protective response mediated by concomitant immunity has been a key objective of prophylactic vaccines against all forms of leishmaniasis.

The *Leishmania* Vaccine Effort

Protection conferred by concomitant immunity in the context of leishmaniasis is so effective that the practice of '**leishmanization**', whereby virulent parasites obtained from an infected individual are deliberately inoculated into a recipient without the disease-exacerbating factors associated with sand fly mediated transmission, was employed in the past as a live vaccine [58–60]. For example, during the 1960s, military recruits in the former Soviet Union deployed to endemic areas were routinely leishmanized with a 'high' virulence strain of *L. major*, resulting in virtually complete prevention of subsequent disease versus non-leishmanized endemic controls [59,60]. Despite the large number of preclinical vaccine candidates, and approaches designed to emulate this protective response [61], the successful transition of *Leishmania* vaccines into human trials has remained elusive, though considerable efforts are underway [62–67]. At this time, first-generation vaccines using inactivated whole-parasite antigen, with or without BCG as an adjuvant, are the only *Leishmania* vaccine candidates to have completed Phase III clinical trials. These formulations proved to be safe and, in some cases, elicited detectable cell-mediated immunity, but failed to provide protection against natural exposure [68]. More recent use of immunogenic recombinant *Leishmania* proteins or polyproteins and/or powerful Th1 adjuvants approved for use in people have resulted in more defined vaccines [62–65,69,70]. Multiple formulations of these 'second-generation' vaccines, as well as other preclinical vaccination strategies, have been shown to successfully generate *Leishmania*-specific cells that exhibit characteristics of memory Th1 cells, namely a **T central memory** (T_{CM}) phenotype (CD44$^+$ CD62L$^+$), cytokine production upon re-exposure to parasite antigen weeks to months after vaccination, and enhanced Th1 responses upon challenge when compared with nonvaccinated controls [46,63,70–72]. Therefore, evaluation of these formulations employing multiple parameters of memory cell generation suggests that these vaccines would be successful. However, observations in animal models of CL, in which more advanced vaccine formulations were also unable to provide protection against sand fly transmitted infections, have raised the possibility that the failure of *Leishmania* vaccines, and the failure of CD4$^+$ T cell-mediated vaccination in general, is not due to a lack of appropriate Th1 memory-inducing antigens and adjuvants, although these components are essential; rather, it is due to a more fundamental lack of knowledge regarding the relationship between immune memory and protection against phagosomal pathogens.

Immunity at Secondary Sites of *Leishmania* Challenge: A Blueprint for Vaccination

Comparison of Challenge Models Reveals a Deficiency in Vaccine-Mediated Immunity

A series of studies comparing *Leishmania* vaccine efficacy with concomitant immunity in mice following needle versus infected sand fly challenge revealed that several protein + adjuvant-based vaccines provided intermediate protection against needle challenge and failed to provide protection against experimental infected sand fly challenge [45,46,69,73]. In contrast, concomitant immunity in mice with a fully resolved but persistent primary infection provided robust and significantly better protection against both needle and sand fly challenge when compared with vaccinated mice. This protective concomitant response is maintained for at least 23 weeks postinfection in mouse models, approximately 12–13 weeks after resolution of the primary lesion [46], and is believed to be life-long [58,74]. Vaccination fails to protect against infected sand fly challenge despite generating antigen-specific Th1 immune responses 'prior' to and/or following challenge [45,46,75,76]. These observations suggest that there exist fundamental differences in the nature of vaccine-induced/memory-mediated immunity versus concomitant immunity, and while vaccination is likely emulating some aspects of the protective response, some essential properties are missing.

Employing Concomitant Immunity to Define Biomarkers of Protective Immunity

Immunity at a secondary site of challenge in individuals with an ongoing primary infection is often referred to as a memory response. However, this is a misrepresentation of what happens in chronic infections such as leishmaniasis due to the long-term persistence of replicating parasites and antigen availability. Dependent upon their localization in the body, *Leishmania*-specific T cells will have periodic exposure to antigen and be comprised of cells with different memory and effector phenotypes and different ontogenies. In light of this, we and others have interrogated the expression of concomitant immunity at sites of secondary challenge in mice with a healed primary infection in an attempt to better identify the phenotype and functionality of those CD4$^+$ T cells that mediate the optimal protective response [33,45,51,56,57,77,78]. These studies have revealed several biomarkers that should inform successful vaccine design, including those described below.

Rapid Immunity at the Site of Secondary Challenge

The most striking feature of protective concomitant immunity versus nonprotective vaccine-mediated immunity in the *Leishmania* model is the speed of the CD4$^+$-mediated immune response in the former [33,45]. Increases in the absolute number of IFN-γ-producing cells in dermal sites of challenge distal to the primary chronic site of infection can be detected as early as 10 h postchallenge (the earliest time point tested) versus naïve and nonchallenged chronic controls and increases exponentially over the first week of infection [13,33,45]. In contrast, vaccine-mediated responses in the skin are not detectable until 7 days postchallenge and are still peaking when the concomitant response is beginning to wane, likely as a result of robust parasite clearance [46].

Rapidly Recruited IFN-γ$^+$ Cells Are Ly6C$^+$T-bet$^+$Ki-67$^-$CD44$^+$CD62L$^-$Cells

Phenotypic analysis of IFN-γ$^+$ cells directly *ex vivo* or employing parasite-specific CD4$^+$ T cell tetramers reveals that rapidly (≤4 days) recruited cells associated with protection have a Ly6C$^+$T-bet$^+$Ki-67$^-$CD44$^+$CD62L$^-$phenotype. The lack of Ki-67 expression in these cells indicates that they have not undergone proliferation in the time since challenge and are therefore unlikely to be derived from T$_{CM}$ memory cells that have proliferated in response to challenge antigen. Ly6C expression defines CD4$^+$ T$_{EFF}$ in the mouse [33,79–81] and following adoptive transfer (A.T.) and needle challenge of naïve recipient wild-type mice, only CD44$^+$CD62L$^-$Ly6C$^+$ cells derived from chronic mice emulate the protective response observed following infected sand fly challenge of chronically infected intact mice. These cells are also highly enriched for the Th1 transcription factor T-bet and the Th1-associated cell surface marker CXCR3 [33]. These observations suggest that the protective population possesses an effector phenotype prior to challenge.

Rapidly Recruited IFN-γ$^+$ Cells Are Predominantly Single-Cytokine Producers

The protective capacity of memory Th1 cells can be directly correlated to their ability to produce multiple cytokines upon antigen or pharmacological restimulation prior to challenge [71]. Consequently, production of multiple cytokines has been employed as a correlate of efficient CD4$^+$ vaccination and memory generation. Interestingly, direct intracellular staining (dICS), which measures *in situ* cytokine production directly *ex vivo* without antigen or pharmacological restimulation, of CD4$^+$ T cells derived from a dermal site of secondary challenge, are predominantly IFN-γ$^+$ single producers [33]. Analysis of cytokine gene expression in the skin also revealed a rapid upregulation of *ifng*, but not *tnfa* concurrent with the arrival of recruited CD4$^+$ T cells [13]. These results suggest that multicytokine production predicts the protective capacity of memory cells following needle challenge, but may not be a requirement for the delivery of optimal effector function by rapidly recruited T$_{EFF}$ cells.

Chronically Infected Mice Contain Tissue Resident Memory (T$_{RM}$) Cells

Parasites in mice with a chronic *L. major* infection are highly localized to the primary site of dermal infection. Despite this, CD4$^+$ cells with the capacity to produce IFN-γ upon exposure to parasite antigen [33,78] or stain with a parasite-specific peptide:MHC II tetramer [33], can be found at peripheral skin sites distal to the primary site. These cells are noncirculating CD4$^+$ **T$_{RM}$** cells that can enhance recruitment of circulating CD4$^+$ T$_{EFF}$ cells following high-dose needle challenge [78]. While the ability of *Leishmania* vaccines to generate T$_{RM}$ cells has yet to be reported, they represent an intriguing target of vaccination due to their potential to enhance protective cell recruitment [35,78].

Rapid Recruitment of Leishmania-Specific Cells to the Skin Can Be Antigen-Independent

In mice with a healed but chronic *L. major* infection, cells with the capacity to produce IFN-γ upon exposure to antigen are efficiently recruited to a secondary dermal site of challenge with phosphate-buffered saline (PBS) or exposure to uninfected sand fly bites [33]. In addition, adoptively transferred Ly6C$^+$ T cells [33] or bulk CD44$^+$CD62L$^-$ 'effector' cells [33,57], obtained from chronically infected mice efficiently migrate into sites of challenge in naïve recipient mice, indicating that while T$_{RM}$ cells can enhance their recruitment, circulating cells can migrate to sites of challenge independently of antigen or T$_{RM}$ function. This recruitment is likely mediated by tissue damage.

Circulating Ly6C$^+$CD4$^+$ T$_{EFF}$ Cells Pre-exist Secondary Challenge and Mediate Optimal Immunity

Employing A.T. and needle challenge models, sort purified polyclonal CD44$^+$CD62L$^-$Ly6C$^+$ T$_{EFF}$ cells from mice with a chronic primary infection were found to mediate highly significant levels of parasite control. In contrast, CD44$^+$CD62L$^+$ T$_{CM}$ cells alone mediate late (6 weeks postchallenge) protection against high-dose challenge but no protection following high- or low-dose challenge at early (3 weeks postchallenge) time points [33,57]. Total CD4$^+$ populations from chronic mice depleted of the Ly6C$^+$ T$_{EFF}$ population [33], or T$_{RM}$ cells by themselves [36], can mediate minor but significant levels of protection at 3 or 4 weeks postchallenge, but these suboptimal levels of immunity do not emulate the optimal protective response. The levels of protection mediated by 'non'-Ly6C$^+$ T$_{EFF}$ populations following needle challenge is reminiscent of the levels mediated by vaccines that have ultimately failed to protect against infected sand fly challenge [45,46].

Protective Ly6C$^+$Ki-67$^-$ Cells Are Short-Lived in the Absence of Antigen

A fundamental property of memory cells in the context of conventional vaccine-induced immune memory is that, once generated, memory cells are long-lived. In contrast, 'parking' sorted Ly6C$^+$ T cells derived from chronically infected mice in naïve uninfected recipients revealed that these cells dramatically declined over a 2-week period while CD44$^+$CD62L$^+$ T$_{CM}$ cell numbers were maintained [33]. This observation corroborated, at the cellular level, older observations in which manipulation of experimental animals to achieve sterile cure of the primary infection results in the loss of concomitant immunity upon challenge [57,82,83]. Presumably, this loss of protection is due to both the loss of existing T$_{EFF}$ cells in the absence of antigen and a cessation of the generation of new T$_{EFF}$ cells.

Tissue-Infiltrating (Intravenous$^-$) Ly6C$^+$ T$_{EFF}$ Cells Are Not 'Site-Specific'

Employing an experimental model of dermal *L. infantum* challenge, which leads to infection and expansion of parasites in the spleen and liver, revealed that mice with a healed primary *L. major* infection in the skin exhibited robust protection in the viscera [51]. Analysis of the IFN-γ-producing population in the liver and spleen employing dICS revealed that these cells were

once again Ly6C$^+$, and employing an intravenous (i.v.) labeling technique, were also tissue-infiltrating cells rather than cells trapped in the vasculature. Therefore, the ability of Ly6C$^+$ cells to home to multiple sites of infection correlates with their highly protective capacity.

Rapid Activation of Tissue-Infiltrating Monocytes Mediates Protective Immunity

IFN-γ induces both *cxcl9* and *cxcl10* gene expression, resulting in monocyte recruitment to the skin and production of NO [13] and ROS, both of which have been shown to be important for parasite killing in recruited monocytes [36]. CCR2$^+$Ly6C$^+$CX3CR1$^+$ inflammatory monocytes represent approximately 80% of all iNOS$^+$ cells at secondary sites of challenge and are essential for parasite control [13,36]. At sites of secondary challenge, infected Ly6C$^+$CX3CR1$^+$CCR2$^+$ monocytes had increased IFN-γ-dependent MHC II expression [13] whereas these same cells at sites of primary infection had lower MHC II expression versus uninfected Leishmania-red fluorescent protein (RFP)$^-$ monocytes derived from the same infection site. Therefore, the correlation between the rapidity of CD4$^+$ T cell-mediated immunity and protection is likely related to the ability of early IFN-γ to prevent the parasite from successfully establishing a permissive intracellular niche in monocytes [13] (Figure 1).

IL-10 Can Be a Component of the Concomitant Immune Response

IL-10 production can slow, but does not abrogate, concomitant immunity at a secondary site of *Leishmania* challenge [77,84], and the magnitude of this IL-10 response is significantly influenced by the anatomical site of primary infection, that is, leishmanization [77]. Despite the role of IL-10 in the inhibition of Th1 immunity [42], whether IL-10 would be considered a fully 'negative-biomarker' is debatable as some IL-10 production may limit IFN-γ-mediated immunopathology, as evidenced by acute dermal swelling during highly protective concomitant immune responses [13,33,46].

Taken together, these observations suggest that while infection induces the generation of antigen-specific cells with multiple phenotypes, these cells have different relative capacities to mediate immunity against needle versus infected sand fly challenge (Figure 2). These observations have also revealed three key concepts with implications for CD4$^+$ T cell-targeted vaccination against leishmaniasis and other phagosomal pathogens.

Three Key Concepts That Define a New Th1 Vaccine Efficacy Paradigm

Th1 Memory Is a Component of Protective Immunity, but It Is Not Sufficient

The inability of vaccines or T$_{CM}$ cells to match the level of protection observed in mice with a healed but chronic primary infection combined with their inability to mediate protection against infected sand fly challenge suggests that, while generation of Th1 memory is a component of protective immunity, it is not sufficient. Rather, additional parameters beyond the detection of cytokine production by parasite-specific memory cells must be incorporated into vaccine evaluation, with the most obvious being: (i) how quickly vaccine-mediated T cell immunity appears at the challenge site, (ii) the ability of a vaccine to maintain a population of cells with the functional characteristics of the Ly6C$^+$ T$_{EFF}$ cell population observed in mice, and (iii) the rapid activation of inflammatory monocytes. Attention to these immune parameters is likely to result in the generation of vaccines that ultimately protect.

Memory T Cells May Mediate Protection Only If They Generate Circulating Effector Cells Prior to, Not after, Challenge

The data reviewed here employing the *Leishmania* model strongly suggest that, in the context of those infections that are most dependent upon CD4$^+$ T cell-mediated immunity, traditional immune memory is not sufficient for protection. Jameson and Masopust [85] have suggested
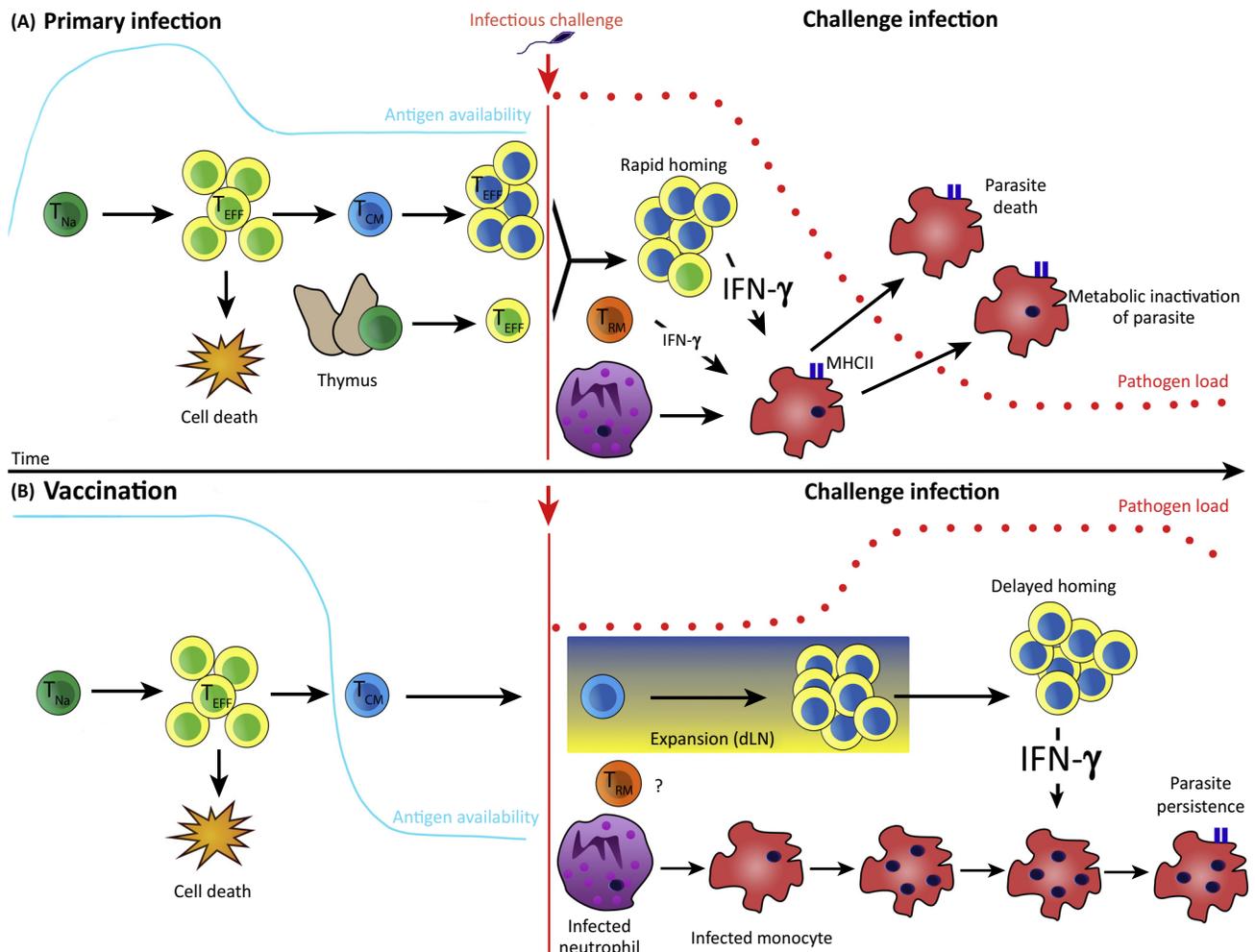
Trends in Parasitology

Figure 1. Immunity at Secondary Sites of Leishmania Challenge following Persistent Primary Infection (A) or Vaccination (B). After both primary infection (A) and vaccination (B), CD4$^+$ *Leishmania*-specific T_{Na} cells (green) are activated to become effector CD4$^+$ T cells (T_{EFF}, yellow). In (A), primary infection, antigen persists and continued exposure of T_{CM} and T_{Na} populations to antigen maintains a population of circulating T_{EFF} cells (yellow). In (B), Vaccination, T_{EFF} cells undergo a period of attrition that coincides with antigen clearance, wherein the majority of T_{EFF} cells die, leaving a pool of *Leishmania*-specific T_{CMs} (blue). Upon infectious challenge, in (A), persistent primary infection, circulating T_{EFF} cells are rapidly recruited to the site of challenge in response to tissue damage, a process that can be enhanced by antigen recognition by T_{RM} cells (orange). In (B), vaccination, T_{CMs} in the dLN must await antigen delivery and undergo proliferation and differentiation into T_{EFF} cells before homing to the site of challenge and exerting effector functions. In the presence of rapid T_{EFF}-mediated IFN-γ production in (A), persistent primary infection, *Leishmania* transitions into inflammatory monocytes exposed to IFN-γ and are activated to express MHC II and become iNOS$^+$, preventing the establishment of a parasite niche and mediating parasite killing or metabolic inactivation and infection control. In the absence of rapid T_{EFF}-mediated IFN-γ production in (B), vaccination, *Leishmania* transitions from neutrophils into inflammatory monocytes and establishes a permissive niche, as evidenced by a lack of MHC II expression on infected monocytes undergoing maturation, resulting in poorly controlled infection. Adapted and updated from [107].

that the most important aspect of a 'memory' T cell is the ability to provide an enhanced immune response upon secondary infection. Thus, from a functional perspective, it could be argued that these Ly6C$^+$ T_{EFF} cells mediate immune 'memory' at the level of the individual, yet the short-lived nature of the T_{EFF} cells themselves is contrary to the classical requirement at the cellular level for a memory cell to be long-lived in the absence of antigen. This view begs the question as to why the immune system would maintain large numbers of CD4$^+$ memory T cells, that is, CD62L$^+$ T_{CM} cells, if they do not mediate sufficiently protective immunity upon
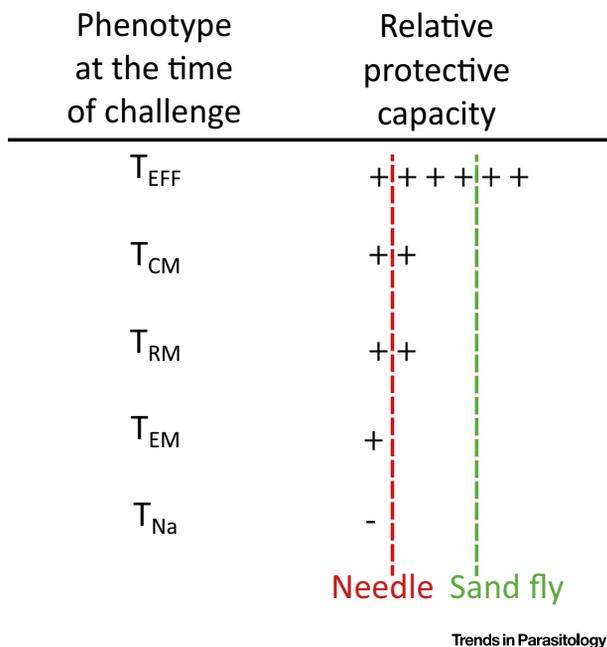
Phenotype at the time of challenge	Relative protective capacity
T_{EFF}	+ + + + + +
T_{CM}	+ +
T_{RM}	+ +
T_{EM}	+
T_{Na}	-

Needle   Sand fly

Trends in Parasitology

Figure 2. Relative Protective Capacities of T_{Na}, T_{EFF}, T_{CM}, T_{RM}, and T_{EM} Populations against *Leishmania* Challenge. T cell subsets are described in the glossary. $CD4^+$ T_{EM} cells are $CD44^+CD62L^-Ly6C^-$ in mice but are not further defined [33,79]. The dashed needle (red) and sand fly (green) lines represent the threshold above which the relative protective capacity of a given T cell population must exist in order to prevent disease. (−) represents the baseline protective capacity of naïve T cells upon challenge (i.e., no protection). (+) represents the relative protective capacity of the indicated antigen-experienced populations based on the integration of parasite load data from the following publications [13,33,36,45,46,57,78,82,83], with a greater number of +s indicating a greater protective capacity. Abbreviations: T_{CM}, T central memory cells; T_{EFF}, T effector cells; T_{EM}, T effector memory cells; T_{RM}, tissue resident memory cells; T_{Na}, naïve T cells.

challenge. The answer to this question likely lies in the ontogeny of circulating T_{EFF} cells themselves. In experiments designed to determine the source of $Ly6C^+T-bet^+$ T_{EFF} cells, the $Ly6C^+$ T_{EFF} cells themselves, T_{CM}, and $CD44^-CD62L^+Ly6C^-$ **T effector memory (T_{EM})** cells were independently adoptively transferred into infection-matched recipients along with $CD44^-CD62L^+$ naïve cells as an internal control. On day 14 post-transfer, the T_{CM} population had proliferated and generated a robust population of daughter cells with the $CD44^+CD62L^-Ly6C^+T-bet^+$ T_{EFF} phenotype [33]. Adoptively transferred $CD44^-CD62L^+$ **naïve T cells (T_{Na})** did not proliferate or generate $Ly6C^+T-bet^+$ T_{EFF} cells over the 2 weeks of the experiment, and in ongoing *L. donovani* infection, $CD4^+$ **recent thymic emigrants (RTEs)** exhibit stunted functional differentiation [86]. These data suggest that, in the presence of a chronic infection, T_{CM} cells serve as a pool for the maintenance of memory-derived $Ly6C^+$ T_{EFF} cells, thereby explaining the role of $CD4^+$ T_{CM} cells maintained during chronic infection (Figure 1). While T_{RTE} cells have the potential to contribute to the T_{EFF} pool, T_{CM} cells provide a preselected, antigen-specific, and Th1-differentiated reservoir of cells which are more efficiently stimulated by chronic *Leishmania* infection to generate T_{EFF} prior to challenge [33]. While it has only currently been examined in the $CD8^+$ T cell context [87], an examination of the chromatin availability of $T_{RTE}-$ versus T_{CM}-derived T_{EFF} cells would be extremely informative to determine the source of pre-existing T_{EFF} cells in chronically infected individuals.

Modeling Vector Transmission of *Leishmania* Has Implications for Interpreting Vaccine Efficacy

Vector transmission of *Leishmania* delivers low numbers of parasites into the skin while inducing a robust and prolonged inflammatory response that includes neutrophils and monocytes [7–9,13]. In the context of vaccination, the impact of this inflammatory response, and in particular neutrophil recruitment, is critically important as neutrophils have been shown to abrogate IFN-γ production by $CD4^+$ T cells and vaccine efficacy [45,88]. The apparent requirement for the early expression of T cell immunity following vector transmission is likely related to the need to overcome: (i) sand fly-mediated recruitment of permissive phagocytes;

(ii) modulation of infected phagocytes concurrently engaged in antigen presentation and efferocytosis, whereby phagocytes clear apoptotic neutrophils or neutrophil blebs resulting in suppressed T cell activation [45,88,89]; and (iii) the paucity of antigen available to drive immunity, including activation of T_{CM} cells in the draining lymph node. Therefore, replicating the low-dose/high-inflammation setting of sand fly transmission would appear to be essential for interpreting the efficacy of protective immunity, and by extension, vaccination. However, sand fly colonies are rare, and replicating vector transmission in a reductionist experimental setting without sand flies remains exceedingly difficult. This is due in large part to the numerous parasite, host, and vector-associated factors that have been shown to contribute to inflammation [7,8,45] (Box 2) and a fledgling understanding of the relative importance of each of these factors following vector transmission (Figure 3, Key Figure). Even experimental sand fly transmission is hampered by the complexity of generating flies that consistently transmit infection and the potential implications of using too many flies to overcome this. High-dose needle inoculation elicits inflammation akin to sand fly bites, but is also likely to overemphasize antigen-driven phenomena and bypasses the ability of the parasite to sequester antigen, an

Key Figure

Potential Outcomes of Experimental Models of *Leishmania* Infection and the Implications of Each Model on Data Interpretation
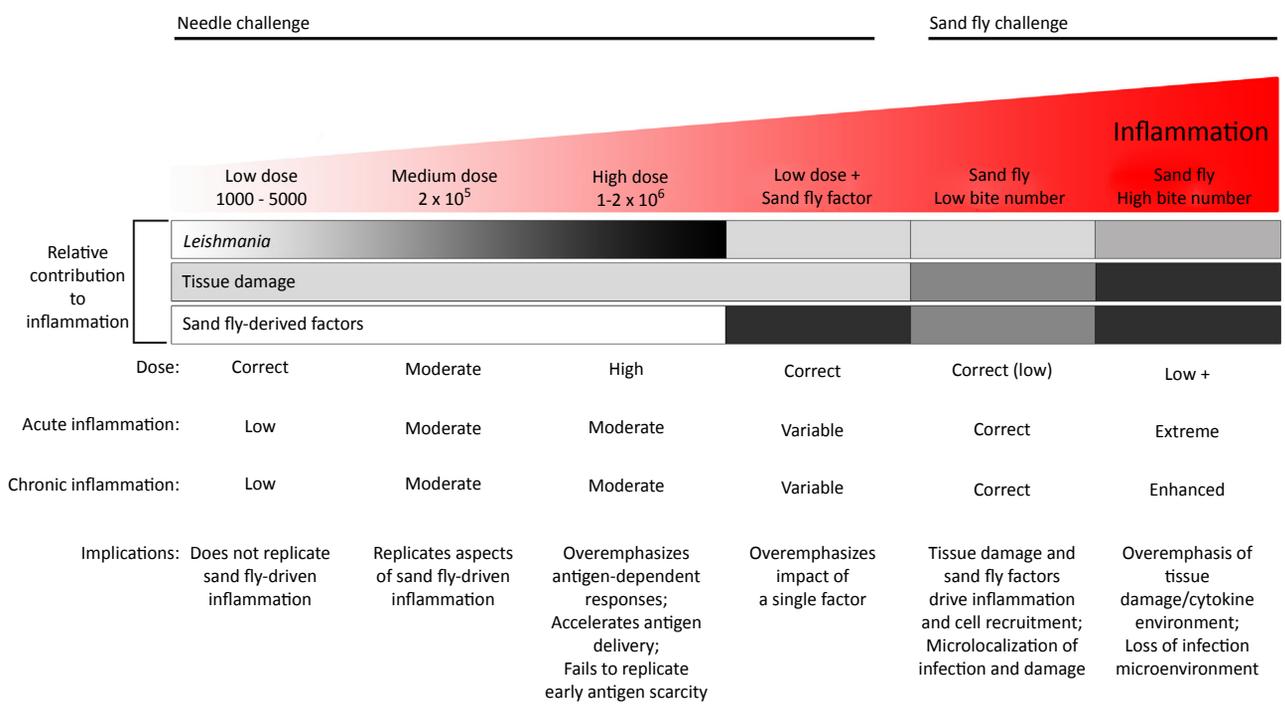
Figure 3. The relative contributions of *Leishmania* dose, tissue damage, and sand fly-derived factors (Box 2) ranging from low contribution, shown in white, to high contribution, shown in black, on total relative inflammation, shown in red. Implications of the experimental models are explained within the context of the gold standard of infection (low bite number sand fly challenge), and are evaluated based on their ability to replicate tissue damage and sand fly factor-driven inflammation.

evasion mechanism central to infection [90,91]. 'Add-back' experiments, in which a parasite or vector-associated factor is inoculated into the skin with *Leishmania* can result in exacerbated disease or overemphasize the role of a single factor in isolation. One solution to these caveats would be to incorporate an analysis of the biomarkers that define the concomitant immune response as outlined above. While experimental sand fly challenge should remain a gold standard of preclinical research, analysis of these parameters does not require the use of an infected sand fly challenge model per se. Rather, they emphasize the importance of including mice with a healed primary infection as a positive control and analyzing protection early, thereby accounting for the ability of *Leishmania* to establish an intracellular niche in phagocytes that, once established, is very difficult to overcome [92–96] (Figure 1). This difficulty is emphasized by the basic observation that these pathogens are able to maintain chronic primary infections in the first place, despite the generation of a robust Th1 immune response.

Concluding Remarks

Characterization and application of the biomarkers associated with protective concomitant immunity is likely to result in a more efficient preclinical pipeline for the generation of a *Leishmania* vaccine (see Outstanding Questions). The *Leishmania* sand fly vector-transmission model has been instrumental in identifying the impact of inflammatory cell recruitment on vaccine efficacy and the importance of immune-mediated host cell activation at very acute time points, thereby preventing the formation of a permissive phagosomal pathogen niche. While *Leishmania* may have specifically adapted to rely upon the sand fly bite to recruit a permissive phagocyte niche, other phagosomal pathogens are likely to have evolved strategies to achieve the same goal. Therefore, the rapidity of protective CD4$^+$-mediated immunity against phagosomal pathogens may be essential, and we suggest that the vaccination approach most likely to succeed will be one that either maintains rapidly recruited T_{EFF} cells or replicates this critical characteristic. A persisting but attenuated parasite vaccine may be the approach most likely to maintain the T_{EFF} population [108]. We hope that the novel considerations raised in this review will facilitate a more efficient preclinical pipeline for CD4$^+$ T cell-based vaccination strategies.

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