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Transgenic *Leishmania* and the immune response to infection

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SUMMARY

Genetic manipulation of single-celled organisms such as the *Leishmania* parasite enables in depth analysis of the consequences of genotypic change on biological function. In probing the immune responses to infection, use of transgenic *Leishmania* has the potential to unravel both the contribution of the parasite to the infection process and the cellular interactions and mechanisms that characterize the innate and adaptive immune responses of the host. Here, we briefly review recent technical advances in parasite genetics and explore how these methods are being used to investigate parasite virulence factors, elucidate immune regulatory mechanisms and contribute to the development of novel therapeutics for the leishmaniases. Recent developments in imaging technology, such as bioluminescence and intravital imaging, combined with parasite transfection with fluorescent or enzyme-encoding marker genes, provides a rich opportunity for novel assessment of intimate, real-time host–parasite interactions at a previously unexplored level. Further advances in transgenic technology, such as the introduction of robust inducible gene cassettes for expression in intracellular parasite stages or the development of RNA interference methods for down-regulation of parasite gene expression in the host, will further advance our ability to probe host–parasite interactions and unravel disease-promoting mechanisms in the leishmaniases.

Keywords

imaging ; immune responses ; *Leishmania*; transgenesis

INTRODUCTION

The leishmaniases, a spectrum of infectious diseases caused by species of the kinetoplastid parasite *Leishmania*, affect man and other mammals in tropical and subtropical regions of the world (1). Transmitted by blood-feeding female sandflies, flagellated *Leishmania* metacyclic promastigotes are phagocytosed by host cells (usually macrophages) at cutaneous sites and differentiate into replicative amastigotes within intracellular phagolysosomal compartments. Maintenance of parasites at dermal sites or subsequent dispersal to internal tissues contributes to disease progression, resulting in the distinct pathologies associated with cutaneous, mucocutaneous, diffuse cutaneous and visceral leishmaniases (CL, MCL, DCL and VL, respectively) (2). While these diseases are often associated with particular parasite species, the immune response to infection in the host, influenced by genetic factors, is the dominant factor determining clinical outcome (3).

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Our understanding of the innate and acquired response to infection with *Leishmania* has been recently reviewed (4,5). While many of these immunological studies have used strains of *Leishmania major* (causative agent of CL) as the infecting parasites, alternative Old World (e.g. *L. donovani*, *L. infantum*) and New World (e.g. *L. mexicana*, *L. braziliensis*) species have been used to unravel immune interactions associated with the different types of disease. Development of transgenic methods for the manipulation of parasite genotypes, together with the recent availability of *Leishmania* genome sequences, have both facilitated in depth analysis of the role of parasite genes in infection and provided new tools for probing immune responses in the host. In this review, we focus on how transgenic *Leishmania* have been used to date to elucidate immunological mechanisms underlying host responses and consider future prospects in this developing research area.

MANIPULATING THE PARASITE

Since the pioneering demonstration of gene targeting by homologous recombination in *L. major* (6), there has been steady progress in construction of a ‘genetic toolkit’ to facilitate research involving transgenic *Leishmania* [reviewed in (7)]. Thus, parasites can be transfected both transiently and stably, using a range of episomal and integrating expression constructs together with suitable markers for both positive and negative selection; genes can be targeted for disruption or replacement by homologous recombination; genetic screens can be facilitated by transposon mutagenesis or functional gene rescue. The development of robust *in vitro* culture systems for growth and differentiation of parasite life cycle stages and well-defined *in vivo* disease models have enhanced the opportunities to address fundamental questions relating to host–parasite interactions. To date, transgenic *Leishmania* have been used to explore three broad overlapping research areas relevant to this review: investigation of parasite gene function, including those sequences implicated in host interactions and virulence; generation of attenuated parasite lines suitable for vaccination studies; parasite ‘tagging’ (using fluorescent and biochemical reporter genes) for analysis post-infection, both *in vitro* and *in vivo*. Examples of each are included in Table 1 and discussed in more detail below.

PARASITE VIRULENCE FACTORS

The main interface between the invading *Leishmania* promastigote and its phagocytic host cell is the parasite glycocalyx, a highly specialized surface coat composed of lipid-anchored glycoconjugates together with some key proteins [reviewed in (8)]. This coat provides a suitably charged surface for opsonization with complement components to facilitate uptake, utilizing a wide range of phagocytic receptors [reviewed in (9)]. The major component of the promastigote glycocalyx is lipophosphoglycan (LPG), a multifunctional molecule required for parasite survival during vector transmission and initial establishment of intracellular infection [reviewed in (10–14)]. Given these critical roles, LPG was one of the first targets for transgenic experimentation in *Leishmania*, an approach that initially required mutagenesis and biochemical screening to identify genes encoding the LPG biosynthetic enzymes (15). Once identified, mutants defective in specific components of the LPG synthetic pathway could be generated and used to probe immune responses to infection both *in vitro* and *in vivo*. For example, infection with *L. major* deficient in *LPG1* (that codes for the galactofuranosyl transferase essential for synthesis of the LPG lipid core) results in poor parasite survival *in vivo* and attenuated virulence in macrophages *in vitro* (16). Using these robust genetic approaches, LPG has been classified as a promastigote virulence factor, although there appear to be some surprising functional differences between the LPGs of different *Leishmania* species. For example, in contrast to the situation in *L. major*, loss of *LPG1* function does not affect disease outcome in *L. mexicana*, with lesion formation comparable to wild-type parasites (17). Interpretation of these and other data is complicated

by the large diversity of *Leishmania* phosphoglycan (PG)-containing macromolecules that share structural domains with LPG, some of which have been characterized only relatively recently. This difficulty is exemplified in a recent paper analysing the virulence profiles of new *L. major* PG mutants null for UDP-galactose transporter genes that facilitate uptake of galactose precursors required for PG synthesis (18). In this study, loss of UDP-galactose transporter gene function did not compromise amastigote virulence, a phenotype similar to that observed with the *L. major LPG1* mutants. In contrast, both *L. major* and *L. mexicana LPG2* transgenic parasites (that are null for the Golgi nucleotide-sugar transporter required for uptake of GDP-mannose, another essential component of the LPG PG repeat units) are avirulent as amastigotes. Further work will be required to determine what other metabolites are compromised in their transport in these mutants and how these might affect parasite macromolecules that are critical for amastigote maintenance in the host. This type of analysis underlines the complexities in resolving the role of specific glycoconjugate species in parasite virulence, especially in intracellular amastigotes that down-regulate LPG but not the more abundant surface glycoinositol phospholipids [although these too are not essential for amastigote virulence (19,20)].

Another class of parasite virulence factors, the cysteine proteases, have been fully characterized using transgenic techniques in *L. mexicana*. Targeted deletion of CPB genes led to attenuated virulence *in vivo* (21), while a series of genetic complementation experiments demonstrated a requirement for multiple cysteine proteases for the full restoration of parasite virulence (22). Similarly, targeted deletion of the *L. major* GP63 gene locus (coding for the major lipid-anchored surface zinc metalloprotease of promastigote stages) resulted in increased parasite sensitivity to complement-mediated lysis and attenuated cutaneous lesion formation in BALB/c mice (23). Both effects could be complemented by episomal expression of a single GP63 gene, confirming GP63 as a virulence factor in *Leishmania* pathogenesis.

Do the factors affecting *Leishmania* virulence differ between parasite species causing different types of disease? Recent sequencing of three *Leishmania* species, *L. major*, *L. infantum* and *L. braziliensis*, has facilitated the comparison of three genomes which result in distinct clinical phenotypes: CL, VL and MCL, respectively (24). This analysis has revealed a striking level of genetic conservation between these three species, with an unexpectedly short list of species-specific genes. The application of targeted gene knockout and re-expression techniques may be useful in determining if any of these sequences play a role in host–parasite interactions and in particular, parasite migration and establishment at visceral sites. To date, only one gene target (A2) has been validated as contributing to viscero-tropism, although the function of the A2 protein is still poorly understood. The A2 gene family members were identified as virulence factors in *L. donovani* (25), with targeted deletion of the A2-A2rel gene cluster resulting in reduced infection of visceral organs (26). Interestingly, in *L. major* the A2 genes are only present in truncated form and are not expressed. Ectopic expression of a single A2 copy in *L. major* results in reduced cutaneous lesion formation, with enhanced splenomegaly and increased parasitization of the viscera (27).

PARASITE ATTENUATION: TARGETS FOR VACCINATION

Despite extensive international research efforts, leishmanization, a practice involving inoculation with viable *Leishmania* parasites, is the only vaccine with proven efficacy in humans (28). Whilst leishmanization has been largely abandoned due to safety concerns, the success of this approach suggests that the use of live attenuated parasite strains may be the way forward for the development of an effective vaccine in man. Parasite transgenesis has been used successfully to further this research goal, with the aim of producing immunogenic

but attenuated organisms that stimulate protective immune responses and confer long-term protection against disease [reviewed in (29)]. Two general approaches have been taken: first, by deletion of genes encoding virulence factors (or their synthetic enzymes) or metabolic pathway components to produce attenuated infective parasites that are incapable of sustaining infection and causing pathology in the host; second, by creating parasites that secrete host immune mediators to boost antiparasite responses and facilitate parasite clearance.

A variety of parasites with attenuated virulence genes have been produced. In the case of *L. major*, these include the LPG2 null mutants described above that are able to infect, but not support lesion development in susceptible mice, and persist in low numbers at the site of infection (30). These same mutants are able to confer resistance to heterologous challenge in susceptible mouse strains (31) but can only induce a potent Th1 response when coadministered with CPG oligodeoxynucleotides (32). In terms of offering wider spectrum protection, inoculation with *L. major* dhfr-ts (dihydrofolate reductase-thymidylate synthase) null mutants has been shown to provide protection against re-challenge with *L. major* (33) and *L. infantum* (34). In contrast, mice infected with a *L. major* mutant defective in *Leishmania* homologue of receptors for activated C kinase (LACK) showed no increased protection against subsequent infection with wild-type parasites. This finding was not perhaps surprising given that mice infected with the mutant showed similar Th1/Th2 cytokine levels as mice that were infected with wild-type parasites (35,36), suggesting that the dominant LACK epitope does not play a role in the aberrant BALB/c response to *L. major* infection.

The CPB cysteine protease mutants of *L. mexicana* described earlier are attenuated in mice and also show decreased pathogenicity in hamsters, together with lower infectivity and growth in human mononuclear phagocytic host cells (37). In the hamster model, CPB-deficient transgenics give rise to significantly lower levels of the Th2-associated cytokines, IL-10 and TGF-β, when compared to wild-type parasites but give comparable protection against homologous challenge at low dose. These data support the feasibility of using these *L. mexicana* mutants to achieve protective immunity *in vivo*.

Only a small number of studies to date have focused on generating attenuated forms of *L. donovani* as a route to the production of an attenuated vaccine against VL. These include mutation of the essential biopterin transporter (BT1) gene in *L. donovani*, producing transgenic parasites with reduced infectivity, when compared to wild-type, that could confer protection to challenge with wild-type *L. donovani* in mice (38). In similar studies, deletion of centrin, a calcium-binding cytoskeletal protein, resulted in reduced viability of *L. donovani* promastigotes *in vitro* and an arrest of axenic amastigotes at the G_2/M stage of the cell cycle, with a resultant inhibition in amastigote growth in macrophages (39). Given the importance of VL as a life-threatening infection in man, further studies in this area are clearly warranted.

INTERACTIONS WITH CELLS OF THE INNATE IMMUNE SYSTEM

Macrophages have long been regarded as the principle host cell for *Leishmania* and a range of studies has employed luminescent or fluorescent parasites to examine aspects of the host–parasite interaction *in vitro* (Table 1). In addition to macrophages, recent attention has focused on two other main cellular hosts, neutrophils and dendritic cells (DC).

In vitro, *L. major* (40) and *L. donovani* (41) promastigotes have both been shown to infect neutrophils. In the case of *L. donovani*, luciferase transgenic parasites have been used to demonstrate parasite retention within an ER-derived compartment, distinct from the classical

phagolysosome (42), which retains metabolic activity (41). In addition to providing a potential means of entry into macrophages, under the guise of an apoptotic neutrophil, the extended life span of neutrophils infected with *L. donovani* promastigotes suggests an alternative means for dissemination from the site of initial infection. Whether this process occur *in vivo* remains to be explored, as does the extent to which *Leishmania* amastigotes interact with neutrophils. Immunohistochemical examination of mice harbouring long-term *L. donovani* infections failed to identify infected neutrophils *in situ*, although single cell *ex vivo* cultures revealed that granulocytes did indeed contain parasites (43).

Using transgenic *L. major* expressing enhanced green fluorescent protein (GFP), Misslitz *et al*. have demonstrated temporal differences in the appearance of parasite antigen in the draining LN (44) as well as the presence of viable intact parasites. DC isolated 16-h post-infection and carrying *L. major* antigen are an important source of IL-12/23p40 and functionally activate $CD4^+$ Th2 cells. While the functional capacity of GFP^+-infected DC was not explored, this study nevertheless highlights an important caveat when using fluorochrome transgenic parasites, namely the potential dissociation between immunologically relevant antigen and parasite localization. In addition, these authors also described a small number of GFP^+ parasites apparently free within the subcapsular sinus, at 4-h post-infection. This is a somewhat surprising finding, given the presence of avidly phagocytic macrophages at this site (45), but one suggesting that direct lymphatic dissemination of *L. major* may occur, at least with high dose infection.

In related studies, the importance of LPG in the induction of DC pro-inflammatory responses has been investigated by transcriptional profiling of *L. mexicana LPG1−/−* mutants. The most striking observation from this work was the almost complete attenuation of interferon-induced and pro-inflammatory cytokine gene expression in the absence of LPG, a result that correlates with a similar lack of responsiveness to amastigote infection. Complementation of this phenotype could be achieved by episomal expression of *LPG1* in *LPG1−/−* promastigotes (46).

ANTIGEN PROCESSING AND THE INFECTED ANTIGEN-PRESENTING CELL

As an intracellular parasite, *Leishmania* provides an interesting model to study the mechanisms that result in the cross-presentation of exogenous antigens to the Class I pathway for the stimulation of $CD8^+$ T cells. Transgenic episomal expression of a reporter antigen, *Escherichia coli* β-galactosidase (β-gal), in *L. mexicana* was first described by Lopez *et al*. (47). Following infection of BALB/c mice, β-gal-specific cytotoxic T cells could be recovered from the spleen with potent killing activity directed towards β-gal-expressing tumour targets. However, specific *in vitro* lysis of macrophages infected with *L. mexicana* was not observed. Although this study was the first to demonstrate *in vivo* that cross presentation of *Leishmania*-derived antigen may occur, the underlying mechanism was not appreciated at the time. Although uptake of infected macrophages (and/or neutrophils) by DC might be involved, more recent identification of specific cross presentation pathways in DC (48) suggests that direct uptake of parasites by these cells may also play a role. The cellular decision controlling entry of ‘exogenous’ antigens into the cross-priming pathway (even in macrophages) may be made early during pathogen invasion (49).

In a similar series of experiments, Garcia *et al*. (50) also failed to demonstrate direct recognition of two reporter antigens, OVA and β-gal, this time in resting and interferon γ/LPS-activated bone marrow-derived macrophages infected with promastigotes of *L. major* and *L. donovani*. Although failure to enter an appropriate cross-priming pathway in these cells may also explain these results, the authors highlighted another potential mechanism, namely the cleavage of the reporter epitope by parasite-derive proteases (in this case, the

major surface protease GP63). In more recent studies, OVA has again been used as a reporter system, with the OVA gene being integrated into the ribosomal DNA locus of *L. donovani* (51), allowing subsequent isolation of OVA-expressing amastigotes for infection in mice. *In vivo* recognition of OVA was confirmed by the capacity of adoptively transferred OVA-specific $CD8^+$ OT-I T cells to cause a reduction in splenic and hepatic parasite burden in mice infected with OVA transgenic but not wild-type *L. donovani* amastigotes (51). Although these studies revealed kinetic differences in the function of naïve, central and effector memory and cytotoxic effector cells, it did not directly address the issue of which cells actually present OVA under these conditions and where these cells are located.

$CD4^+$ T cells are known to be crucial in the clearance of *Leishmania* and antigen processing for $CD4^+$ T cell recognition has also been examined using similar approaches to those described above. Kaye *et al*. (52) generated *L. major* transfectants expressing OVA and β-gal and as neither antigen was detectable as a secreted product, these parasites were used to test the efficacy of phagosomal-targeted antigen compared to that delivered as soluble antigen via endocytosis. Using *in vivo*-activated peritoneal macrophages, phagosomal delivery of OVA using *L. major* promastigotes was as efficient as soluble antigen in driving $CD4^+$ T cell activation (once the response was adjusted for total antigen uptake). However, unlike macrophages pulsed with soluble OVA, which have a short-lived capacity for antigen presentation, presentation by OVA-*L. major* infected macrophages persisted through 24 h. The most likely explanation for these results was that slowly degraded parasites led to a slow-release of OVA into the MHCII processing pathway (52).

ANTIGEN LOCALIZATION AS A DETERMINANT OF PROCESSING EFFICIENCY

Early studies using *E. coli* and *Salmonella* down-played the importance of the site of antigen localization in the infecting micro-organism on the induction of $CD4^+$ T cell responses, stressing antigen abundance as a more important response determinant (53). However, as both *E. coli* and *S. tymphimurium* are rapidly degraded in macrophages, with processing kinetics for $CD4^+$ T cell recognition far faster than with *Leishmania*, the importance of antigen localization and hence accessibility to processing enzymes and/or antigen transport pathways for recognition of *Leishmania* remained unresolved.

In the first of two studies, Wolfram *et al*. (54) generated a T cell line against one of the *L. mexicana* cysteine proteases and used this to show that infected bone marrow-derived macrophages would only present the cysteine protease after intracellular amastigote killing (using either drugs or macrophage-activating cytokine cocktails). As with the studies of Kaye *et al*. (52), these data suggested that internal sequestration of antigen might limit availability to the MHCII processing pathway. To determine whether this was indeed the case, transgenic *L. mexicana* parasites in which the membrane acid phosphatase (MAP) was expressed either as a secreted or membrane antigen or retained in the parasite cytosol were generated. As predicted from their earlier work (54), cytosolic MAP was only recognized after parasites had died within the phagosome, but processing efficiency was strikingly enhanced when MAP was expressed in the membrane or better still, when secreted (55). These studies supported the importance of antigen localization for the recognition of intact amastigotes and suggested that antigen dose following degradation would be critical for effective recognition. This concept has recently been confirmed *in vivo*. Prickett *et al*. (56) utilized the N-terminal targeting sequence of *L. major* hydrophilic acylated surface protein B (HASPB) to generate parasites expressing OVA at equivalent levels in either the plasma membrane or in the cytosol. Surprisingly, given the complexity of potential processing options *in vivo*, only plasma membrane expression of OVA led to significant activation of antigen-specific $CD4^+$ T cell responses.

In contrast to MHCII recognition, an extensive literature suggests that antigen secretion from intracellular pathogens facilitates MHCI loading, most notably in the presence of membrane lytic virulence factors such as listeriolysin (57). To examine this question in *Leishmania*, in which no known lysins have been described, extended peptides of OVA were fused to either the *L. donovani* 3′ nucleotidase/nuclease or the nucleotidase lacking its signal peptide and then expressed in *L. major* (58). Expression of OVA in *L. major* resulted in cross-presentation and priming of OVA-specific T cells by DC but not macrophages *in vitro*. *In vivo*, the transgenic *L. major* NT-OVA primed both CD4 and CD8 responses, demonstrating that this secreted product was able to be processed efficiently into both the Class I and Class II pathways. Secretion of the protein was demonstrated as a requirement of Class I processing, as priming of OT-I cells was significantly reduced *in vitro* when parasites could no longer secrete OVA. Secretion of antigens into the phagolysosome of infected cells therefore enhances the processing of antigens into the Class I pathway (58). Further studies using the NT-OVA *L. major* have examined which pathway of cross-presentation results in *Leishmania* proteins being presented in complex with Class I molecules. If the classical pathway is used, then trafficking of *Leishmania* antigens to the cytosol and the endoplasmic reticulum would occur, in a TAP dependant manner. Infection of DC with NT-OVA *L. major*, however, showed that TAP knockout DC were equally as efficient as wild-type DC in cross-presenting OVA to OT-I T cells *in vitro*. Processing of *Leishmania* antigens into the Class I pathway therefore occurs independently of the TAP pathway with peptides loaded directly into class I molecules within the phagolysosome of the infected cell. Infection of TAP knockout mice also demonstrated that TAP was dispensable for the efficient priming of CD8 responses *in vivo* (59).

IMMUNE MODULATION

Three different studies have focused on the use of transgenic *L. major* which express immunomodulatory proteins predicted to skew the host cytokine production towards a protective Th1 immune response and away from a pathogenic Th2 response. Transgenic *L. major* secreting biologically active murine interferon-γ failed to protect susceptible BALB/c mice from infection, despite a detectable increase in the amount of this cytokine in the infected tissues (60). In a similar study, transgenic *L. major* secreting biologically active GM-CSF showed more promise in promoting a protective host response (101). These GM-CSF expressing parasites survived poorly in macrophages *in vitro*, as a result of increased macrophage pro-inflammatory cytokine production (including IL-1β, IL-18 and IL-6) and infection *in vivo* showed delayed lesion development in susceptible mice.

In a third study, *L. major* engineered to express the extracellular domain of murine CD40-L caused smaller lesions *in vivo* with fewer parasites than controls in susceptible mice and reduced dissemination of transgenic parasites to lymph nodes (61). This protection was associated with reduced levels of IL-4 but similar levels of interferon-γ. However, the mechanism that facilitates access to and activation of host CD40 by parasite-derived CD40-L was not addressed.

Transgenic parasites have also been employed in boosting the innate immune response to infection. Transgenic *L. major* engineered to secrete monocyte chemoattractant protein-1 (MCP-1) have been used effectively to study the role of this chemokine in the early phase of the immune response to infection (62). In susceptible BALB/c mice, MCP-1-*L. major* showed reduced pathogenicity *in vivo* with reduced lesion sizes and smaller parasite burdens than those observed after infection with wild-type parasites. MCP-1 produced by the parasite was sufficient to mediate this effect, as MCP-1 knockout mice showed no difference in disease progression compared to wild-type infected mice. Parasite-produced MCP-1 led to recruitment of CCR2 positive macrophages but this innate immune-mediated effect was

insufficient to protect the host against a subsequent challenge with wild-type parasites. It was therefore not surprising that little cell-mediated immune response was seen in lymphocytes from the MCP-1-*L. major* infected mice upon re-stimulation.

Very few studies to date have used transgenic *Leishmania* to investigate the immunomodulatory effects of parasite proteins on host immune responses. One such study examined cysteine protease B (CPB) derived from *L. mexicana* (63). *Leishmania mexicana* CPB deletion mutants showed similar kinetics of lesion development as wild-type parasites early in infection, but transgenic parasite-infected mice were able to clear the infection following the development of a strong STAT4 and IL-12-dependent Th1 response. CPB was confirmed to have a strong effect on the immune response in further studies where addition of the CPB gene array to *L. major* resulted in suppression of interferon-γ production as a result of degradation of components of the intracellular NF-κB signalling pathway resulting in lower IL-12 levels (63).

VISUALIZING THE IMMUNE RESPONSE

The development of transgenic *Leishmania* species expressing biochemical and fluorescent markers, combined with recent advances in imaging technology, is providing valuable opportunities to directly visualize *Leishmania* infection both *in vitro* and *in vivo*. A combination of approaches to study host–parasite interactions at a cellular, organ and whole body level can provide comprehensive and specific information regarding mechanisms of pathogenesis (Figure 1).

Bioluminescent imaging technology has enabled the study of infectious diseases *in vivo* in real time, utilizing transgenic pathogens expressing the firefly luciferase (LUC) gene [reviewed in Hutchens and Luker (64)]. This marker has been stably transfected into a number of *Leishmania* species, including *L. major* and *L. donovani*, and validated as a sensitive method for measuring parasite numbers in infected macrophages and tissue, as well as in drug screening (65,66).

To date, however, few studies using bioluminescent whole body imaging following *Leishmania* infection have been published. This non-invasive and nondestructive technique can provide real-time information about the kinetics of parasite growth and dissemination as well as quantitatively assessing parasite burdens *in vivo*. During infection with LUC-expressing *L. amazonensis*, Lang *et al*. (66) showed intensity of bioluminescent signal from the lesion correlated with parasite burden. Similarly, dissemination of *L. major* LUC parasites injected into the ear or footpad to the draining lymph node can be observed using the same approach (Figure 2b,c). In addition to the specificity and sensitivity of these experimental methods, they also require lower animal numbers to generate statistically significant data and allow non-invasive monitoring of infection progression, thereby supporting the reduction, refinement and replacement of animals in biomedical research.

While bioluminescent imaging allows examination and quantification of parasite densities on a whole body level, it is limited in terms of resolution. Techniques such as stereomicroscopy allow visualization of both parasites and host cells at the level of individual organs. For example, GFP expressing *L. amazonensis* parasites can be visualized at the site of infection in the footpad (see Figure 2d) and are clearly detectable in the intact excised draining lymph node (see Figure 2e) and, at a higher resolution, within the paracortical area of the node itself (see Figure 2f).

Moving into methods that offer higher resolution of parasite interactions, intravital laser scanning microscopy, principally with 2-photon excitation, is a relatively new technique which has been applied very successfully to extend our understanding of how immune cells

function [recently reviewed in (67)]. The availability of transgenic parasites expressing fluorophores and exogenous antigens for detection, coupled with the use of fluorochrome-labelled antigen-specific host cells, will provide increased power in the study of *Leishmania* immune responses, facilitating studies of parasite behaviour at the site of infection, interactions of immune cells with parasites (both at the initial sites of infection, as well as in visceral organs), interactions between antigen presenting cells and effector cell populations, and mechanisms that result in antiparasite effector responses (for example, see Figure 2g). This *in situ* information further extends the spatiotemporal data that can be obtained by *ex vivo* analysis of T cell responses to reporter antigens (such as analysis of replication by carboxyfluoroscein succinimidyl ester) dilution (56,68); and Figure 2(h).

At a cellular level, transgenic parasites expressing fluorophores and other exogenous proteins have contributed to our understanding of both parasite biology and primary cell biological events relevant to parasite persistence and/or elimination in the host (51,56,59,69–71). Tagging with GFP (or other fluorophores) allows protein detection within the parasite during live cell imaging analysis (see Figure 2i) and has the potential for real-time visualization of antigen shedding and host receptor interactions.

FUTURE DIRECTIONS

As described above, the development of transgenic techniques for manipulation of *Leishmania* has accelerated progress in our understanding of parasite biology and the host's response to infection. Much has been achieved to date and further technical refinements can only increase the power and sensitivity of these approaches. Looking to the future, perhaps the most immediate need is for a robust and highly regulated system for inducible expression of parasite genes in the host. The ability to control the level and timing of transgenic gene expression experimentally would be invaluable in the dynamic study of host–parasite interactions during the time course of infection.

Tetracycline-inducible gene expression in the kinetoplastid parasites was originally pioneered in *Trypanosoma brucei* (72) and is now in widespread use, coupled with RNA interference to knock-down gene expression, to study parasite gene function both in culture and, most importantly, in susceptible hosts *in vivo* (73). Use of similar methods in *Leishmania* species has not been straightforward, not only due to the post-transcriptional regulatory mechanisms common to all kinetoplastids but also, to the lack of identifiable *Leishmania* promoters. The tetracycline-inducible systems developed so far are problematic with respect to background expression levels and low induction efficiencies (74,75), while components of an RNAi machinery are not encoded in the genomes of *L. major* and *L. infantum*. The recent sequencing of the *L. braziliensis* genome has revealed the potential for an active RNAi system however (24,76) and if active, this may be manipulable in the longer term to provide robust experimental methods for the manipulation of gene expression *in vivo*.

In terms of advancing imaging technologies, the ability to boost expression of reporter genes would increase both detection sensitivity and signal-to-noise ratios when examining host–parasite interactions. As an example, the activity of the LUC reporter gene when expressed in the 18 s ribosomal locus is greater than 10-fold down-regulated in *L. amazonensis* amastigotes as compared to metacyclic promastigotes (66), a degree of down-regulation which we have also observed in LUC-transfected *L. major* (K.J. Evans, unpublished data). The reasons for this down-regulation are currently unclear and could relate to metabolic differences between parasite stages, among other factors. Whatever the mechanism, the development of high level regulatable expression systems to boost transgenic protein levels in *Leishmania* amastigotes will be essential to further advance our understanding of host–

parasite interactions during short and long-term infection. Exploiting the synergy between molecular and immunological approaches to the study of infection will lead to fully integrated studies that can significantly enhanced our knowledge and understanding of the leishmaniases and accelerate development of the next generation of therapeutics for management of these deadly diseases.

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Abbreviations

CL	cutaneous leishmaniasis
MCL	mucocutaneous leishmaniasis
DCL	diffuse cutaneous leishmaniasis
VL	visceral leishmaniasis
LPG	lipophosphoglycan
PG	phosphoglycan
OVA	ovalbumin
LUC	luciferase
MAP	membrane acid phosphatase
DC	dendritic cells
TAP	transporter associated with antigen processing
CFSE	carboxyfluoroscein succinimidyl ester

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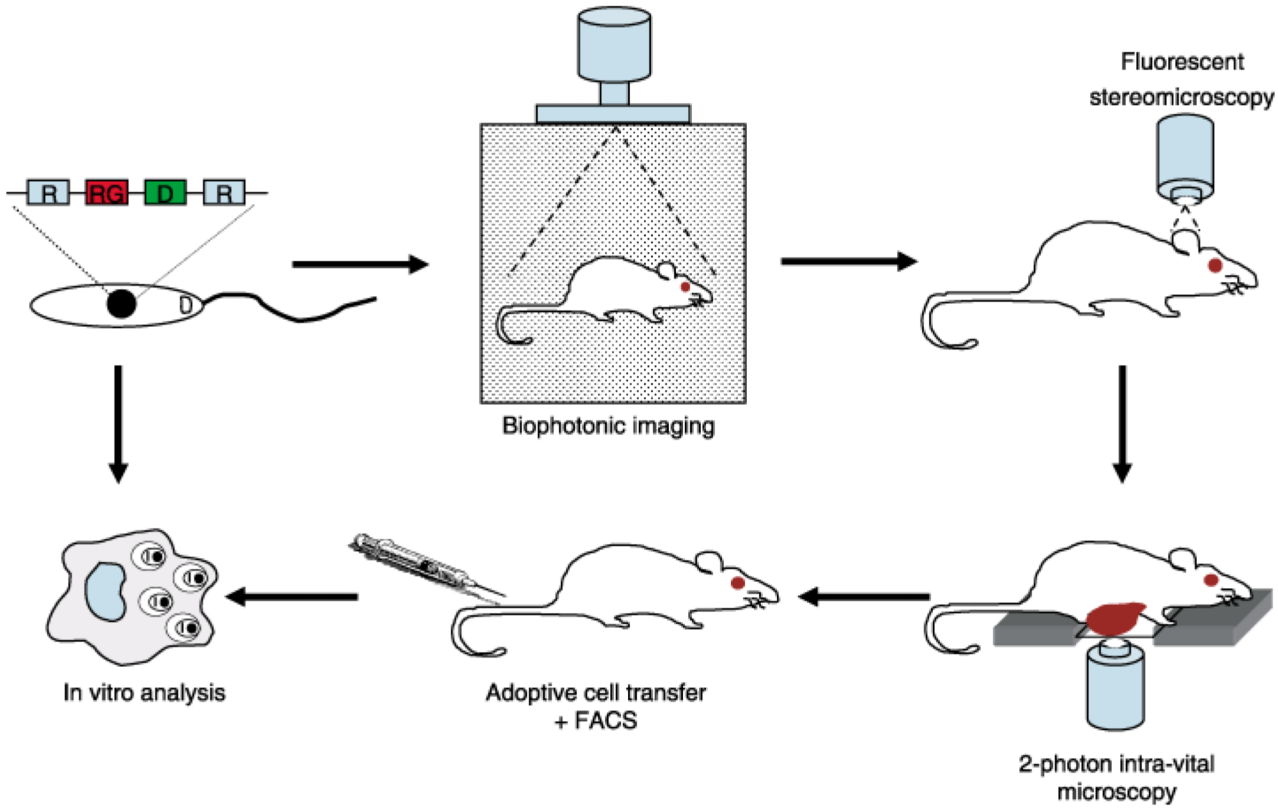

Figure 1. Approaches to analysing the immune response to infection with transgenic *Leishmania.*
Infection with transgenic *Leishmania* carrying one or more 'reporter' genes (RG; here coupled with a drug selection marker (D) and integrated into the ribosomal DNA locus (R) of the parasite nuclear genome) allows progressive in-depth analysis of the immune response to infection, from whole animal to specific tissue to cell-type to individual cell. For examples of the generation of transgenic parasites, see Figure 2 (a); Biophotonic imaging, see (b), (c); Fluorescent stereomicroscopy, see (d)–(f); Photon intravital microscopy, see (g); Adoptive cell transfer and FACS, see (h); Cell biology, see (i).

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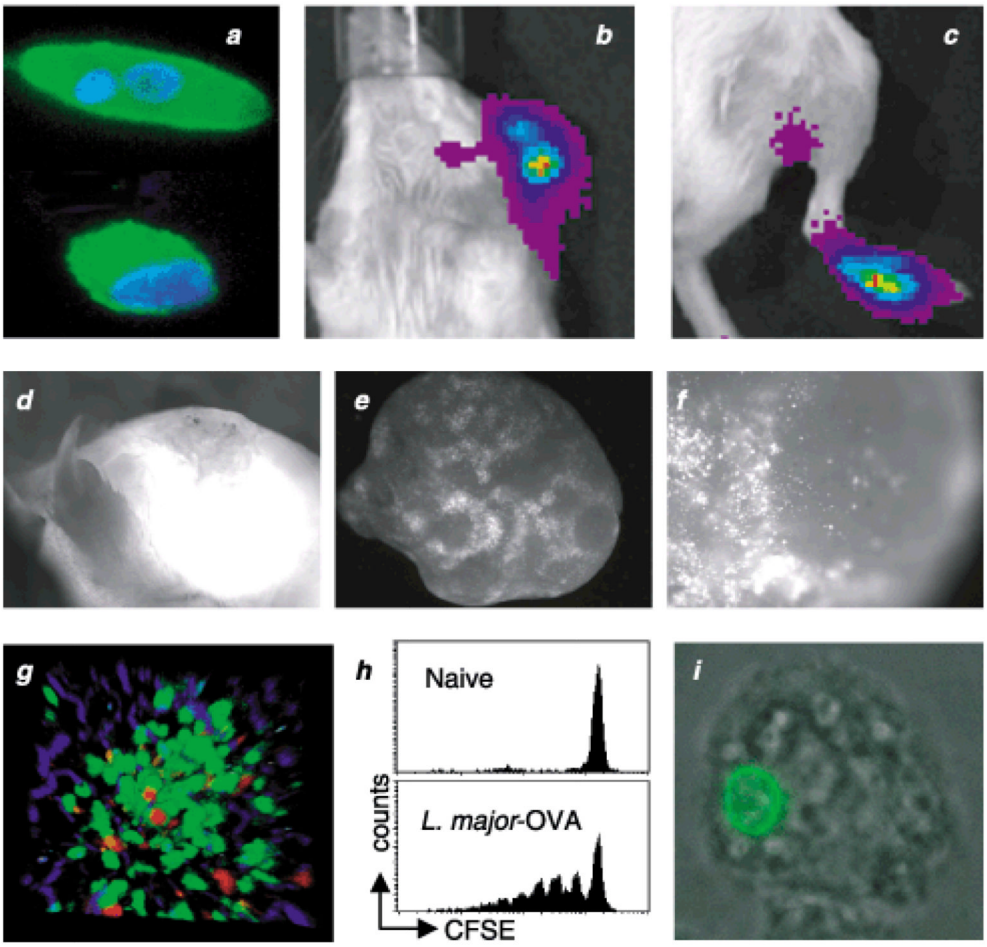

Figure 2. Analysing the immune response to infection with transgenic *Leishmania*.

(a) Transgenic *L. major* promastigote (top)/amastigote (bottom) expressing HASPB18:OVA, labelled with goat anti-OVA followed by biotinylated rabbit antigoat F (ab′)2 and alexa-488-conjugated streptavidin (green). DAPI staining shows the nucleus and kinetoplast (blue). (b–c) BALB/c mice were infected intradermally in the ear, or subcutaneously in the footpad with 1×10^6 *L major* LUC promastigotes. Using a biophotonic imaging system, parasite luciferase signal was detected during the chronic stage of infection from both the lesion at the site of infection and from the draining lymph node, the auricular and popliteal lymph node, respectively. (d–f) Visualization of parasites in the primary lesion and the draining lymph node, by stereomicroscopy, during chronic infection with transgenic *L. amazonensis* expressing GFP. (g) Granuloma in the liver following infection with OVA transgenic *L. donovani*, in a transgenic mouse expressing GFP in T cells (green) with adoptively transferred *in vitro* activated OVA-specific OT-I effector memory cells labelled with CMTMR (red). Images were captured 12-h post-transfer by two-photon laser scanning microscopy. (h) CFSE-labelled OVA-specific $CD4^+$ T cells were adoptively transferred into *L. major-OVA* infected mice and proliferation of the T cells monitored by CFSE dilution (unpublished data provided by P. Scott and P. Gray). (i) Transgenic *L. major* expressing a GFP:am1 gene fusion within a J774 macrophage. The am1 protein contains multiple transmembrane spanning domains that facilitate localization of the fusion protein at the parasite plasma membrane (103).

Table 1
Key studies using transgenic *Leishmania* to study immune responses to infection

Category	Species	Gene/function	References
Parasite manipulation	*L. major*	Gene knockout methods	(6,77)
	L. spp. (various)	Application to drug screening	(78)
	L. spp. (various)	Cosmid vector transfection	(79)
	L. chagasi	rRNA promoter regions	(80)
	L. donovani	LPG	(81,82)
	L. major	HSV-1 thymidine kinase expression	(83)
	L. spp. (various)	Luciferase	(65,66,84,86)
	L. donovani	Luciferase – inducible expression	(74)
	L. donovani	GFP for *in vitro* drug testing	(85)
	L. mexicana and major	Integrated GFP and β-gal	(100)
	L. infantum	GFP	(87)
Parasite virulence factors	*L. mexicana*	Cysteine protease mutants	(21,22,88,89)
	L. major	Leishmanolysin (Gp63) deletion	(23,90)
	L. donovani	A2 mutants	(25–27)
	L. tarentolae	Human tissue-type plasminogen activator expression	(91)
	L. major	MAP-kinase overexpression	(92)
	L. mexicana	Lpg1 knockout	(17)
	L. major	UDP-gal transporter null	(18)
	L. major	*T. cruzi* trans-sialic acid expression	(93)
	L. donovani	LPG	(94,95)
	L. mexicana	GDP-mannose biosynthesis	(96,97)
Parasite attenuation: Targets for vaccination	*L. major*	LPG	(16,30–32,98)
	L. major	*dhfr-ts*	(33)
	L. chagasi and donovani	*dhfr-ts*	(34)
	L. major	LACK antigen mutants	(35,36)
	L. donovani	biopterin transporter (BT1)	(38)
	L. donovani	Centrin mutants	(39,99)
Interactions with cells of the innate immune system	*L. donovani*	Luciferase	(42)
	L. major	eGFP	(44)
	L. mexicana	*Lpg* knockout	(46)
	L. donovani	LPG1 and LPG2	(41)
Antigen processing and presentation	*L. mexicana*	β-galactosidase	(47)
	L. major and donovani	OVA and β-galactosidase	(50,52)
	L. major and donovani	HASPB targeted expression of OVA	(51,56,68)
	L. major	Truncated OVA	(58,59)
	L. mexicana	Membrane bound acid-phosphatase (MAP)	(55)

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Category	Species	Gene/function	References
Immune modulation	*L. major*	Transgenic IFN-γ	(60)
	L. major	Transgenic for human and mouse GM-CSF	(101)
	L. major	murine CD40 ligand (extracellular domain) expression	(61)
	L. major and amazonensis	Murine chemokine monocyte chemoattractant protein 1 (MCP-1)	(62)
	L. mexicana	Cysteine peptidase B (CPB) deletion mutant	(63,102)
In vivo imaging	*L. amazonensis*	Luciferase	(66)