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Induction of cell-mediated immunity during early stages of infection with intracellular protozoa

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Abstract

Toxoplasma gondii and Trypanosoma cruzi are intracellular parasites which, as part of their life cycle, induce a potent cell-mediated immunity (CMI) *maintained by* Th1 *lymphocytes and* IFN-γ. *In both cases, induction of a strong* CMI *is thought to protect the host against rapid parasite multiplication and consequent pathology and lethality during* the *acute phase of infection*. However, the *parasitic infection is not eliminated by* the *immune system and* the *vertebrate host serves as a parasite reservoir*. *In contrast*, *Leishmania sp*, *which is a slow growing parasite*, *appears to evade induction of* CMI *during early stages of infection as a strategy for surviving in a hostile environment* (*i*.e., *inside* the *macrophages which are their obligatory niche in* the *vertebrate host*). *Recent reports show that* the *initiation of* IL-12 *synthesis by macrophages during these parasitic infections is a* key *event in regulating* CMI *and disease outcome*. *The studies reviewed here indicate that activation/inhibition of distinct signaling pathways and certain macrophage functions by intracellular protozoa are important events in inducing/modulating* the *immune response of their vertebrate hosts*, *allowing parasite and host survival and therefore maintaining parasite life cycles*.

Key words
- Leishmania
- Toxoplasma gondii
- Trypanosoma cruzi
- Macrophages
- Cytokines
- Nitric oxide

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Introduction

*T*he *parasites Trypanosoma cruzi and Toxoplasma gondii belong to distinct families of* the *protozoa phylum*, the *Trypanosomatidae* (*order Kinetoplastida*) *and Coccidia* (*order Apicomplexa*), *respectively*. *Although they are phylogenetically distinct*, these *two species of protozoa have several common features in terms of their biology and their life cycle in* the *vertebrate hosts*. Both *T. cruzi and T. gondii infect a wide range of mammals including humans*. *In addition*, these *intracellular organisms can infect and replicate inside any kind of nucleated cell of their vertebrate hosts* (1,2). *As suggested by their host cell specificity in* the *absence of immunity*, these *parasites are highly virulent*. *Thus*, *during* the *early phase of infection and before development of specific im-*

munity *T. gondii* and *T. cruzi* can be found in different tissues and organs, and are normally accompanied by a mononuclear inflammatory reaction in small necrotic foci. With the rapid formation of parasite-specific cell-mediated immunity (CMI), most parasites are cleared from the host tissues, the necrotic foci are regenerated and parasite distribution tends to be more localized. Whereas latent forms of *T. gondii* will localize mainly in the central nervous system (CNS), during chronic infection *T. cruzi* amastigotes are more easily found in cardiac tissue. In the case of either infection, pathogen-induced CMI maintained by Th1 lymphocytes and IFN-γ is thought to be a major event protecting the host against rapid parasite multiplication and consequently against pathology and lethality during the acute phase of infection (3,4).

IL-12 and IFN-γ are thought to be essential cytokines for the establishment of protective Th1-mediated immunity during infection with *T. gondii* or *T. cruzi* (3-6). In addition, IFN-γ appears to be the key cytokine in activating macrophage effector function, thought to be a major mechanism responsible for controlling *T. gondii* or *T. cruzi* replication and parasite dissemination within the vertebrate host tissues during the acute phase of infection (7,8). After the initial phase of disease, acute infection normally evolves to a chronic and asymptomatic stage, and parasite replication is supposed to be controlled by multiple mechanisms, such as complement fixing antibodies and cytotoxic $CD8^+$ lymphocytes, which are maintained by $CD4^+$ Th1 lymphocytes (3,5,9-11). However, the parasitic infection is not eliminated by the immune system and the vertebrate host will serve as a parasite reservoir, being clearly important for *T. gondii* or *T. cruzi* dissemination and maintenance of their life cycle. The importance of T cell-mediated immunity in controlling parasite replication in the chronic stage of infection is clearly observed during reactivation of chronic infection in patients with the acquired immunodeficiency syndrome (AIDS) (12-14).

The intracellular parasites of the genus Leishmania use a quite distinct strategy to perpetuate their life cycle. In the first place, in their vertebrate hosts Leishmania parasites are slow-growing parasites that reside and proliferate in a very hostile environment. In contrast to *T. cruzi* and *T. gondii* which are facultative macrophage residents, Leishmania sp reside primarily inside macrophages (15). Thus, suppression of macrophage effector functions appears to be a crucial strategy for establishing infection and parasite persistence in the vertebrate host. Several studies indicate that Leishmania may use different strategies to evade induction of macrophage effector functions (16,17). Probably the most powerful strategy is to inhibit the synthesis of IFN-γ, a cytokine which leads to the activation of macrophage effector functions (18,19). A second strategy would be the inhibition of effector functions in macrophages exposed to IFN-γ (20).

In addition to establishing infection, Leishmania parasites must replicate to attain sufficiently high levels and to persist in the vertebrate host tissues in order to favor the encounter with their phlebotomine vector. Therefore, parasites that are able to impair the induction or function of IFN-γ would be favored for transmission. Consistent with this hypothesis, in many vertebrate hosts infection with Leishmania sp is characterized by a period of latency followed by lesion development and parasite proliferation (15). Eventually, after the initial infection, a healing phase follows, which is accompanied by the development of *T* cell-mediated immunity and a decrease in parasite load in vertebrate host tissues. Again both IL-12 and IFN-γ have been shown to play an essential role in the healing phase associated with the establishment of protective immunity against different Leishmania species (21,22).

In this review we analyze the studies

performed in our laboratories and elsewhere, dealing with the initiation of immune responses and effector mechanisms responsible for parasite control during early stages of infection with *T. cruzi*, *T. gondii* and *Leishmania sp* in their vertebrate hosts.

Induction of IL-12 synthesis by macrophages exposed in vitro to live T. gondii or T. cruzi or products derived from these protozoa

It is clear from many studies that, in common with other pathogens, *T. gondii* and *T. cruzi* have the capacity to nonspecifically trigger cytokine synthesis by macrophages (6,23-25). We have shown that infection with either live tachyzoites or live trypomastigotes induces cytokine synthesis by different types of macrophages. Our data show that these live parasite forms trigger the synthesis of a wide range of cytokines (i.e., IL-1ß, IL-10, IL-12 and TNF-α) by inflammatory macrophages in vitro (23,25). It is important to note that the pattern of cytokine expression by macrophages exposed to either *T. cruzi* or *T. gondii* is highly influenced by IFN-γ (24,26) (Figure 1). Production of the same cytokines also occurs in vivo during acute infection with either parasite and these cytokines are important mediators of resistance (26,27).

Interestingly, some particular fractions from these parasites retain the ability to trigger cytokine synthesis by macrophages. A major interest of our research is to identify the molecules of *T. gondii* and *T. cruzi* which activate distinct macrophage functions (i.e, cytokine synthesis, nitric oxide release and parasiticidal activity). We have recently shown that glycoconjugates isolated from *T. cruzi* trypomastigotes are highly enriched for their macrophage-activating potency (23,28,29), as compared to crude parasite extracts. These glycoconjugates are rather complex molecules which possess different chemical structures (i.e., alkylacylglycerol, glycosylphosphatidylinositol (GPI) membrane anchor linked to a mucin-like glycoprotein). Our data also suggest that GPI anchors from these molecules are responsible for triggering the synthesis of different cytokines (including IL-12) by inflammatory macrophages (23,28,29). Studies defining the molecules of *T. gondii* which trigger cytokine synthesis by macrophages are currently in progress. Our preliminary data also indicate the hydrophobic nature of these molecules and that the activity is destroyed by periodic acid oxidation, acid deamination and alkaline hydrolysis (23,25,30). It is important to mention here that in order to induce optimal levels of IL-12 synthesis by macrophages, priming with IFN-γ is always required prior to stimulation with glycoconjugates derived from either *T. gondii* or *T. cruzi*.

Impairment of IL-12 synthesis by macrophages infected in vitro with Leishmania sp

Interestingly, in vitro infection of macrophages with either L. major or L. donovani does not result in induction of IL-12 synthesis by macrophages, even when the cells are primed with IFN-γ (18,19,31) (Figure 1). In fact, infection with Leishmania leads to complete and selective inhibition of IL-12 synthesis by macrophage host cells activated with different microbial stimuli (19), but has no significant inhibitory effect on the expression of other inducible genes such as IL-1α, IL-1ß, TNF-α, inducible nitric oxide synthase (iNOS) or IL-10. A possible mechanism for selective inhibition of IL-12 synthesis by macrophages would involve binding to complement receptors, since triggering of complement receptors has been shown to selectively induce IL-12 synthesis by macrophages (32,33). In agreement with this hypothesis are studies showing that the infective stages of Leishmania parasites use the complement receptors for C3 products in

order to invade macrophages (34). We have also tested the ability of the major L. major surface GPI-anchored molecules from promastigotes as well as amastigotes for their ability to trigger cytokine synthesis by macrophages. Even at concentrations which were two or three logs higher than the optimal doses of *T. cruzi* trypomastigote- or *T. gondii* tachyzoite-derived molecules, we were unable to induce cytokine synthesis by inflammatory macrophages using Leishmania-derived glycoconjugates (i.e., lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs)) (28,29,35).

Induction of T cell-independent IFN-γ synthesis by microbial stimuli

Studies initially performed with Listeria monocytogenes indicated that natural killer (NK) cells can be triggered to synthesize IFN-γ in the absence of T cells (36). This T cell-independent pathway of IFN-γ production has been extensively studied using splenocytes from mice with severe combined immunodeficiency (SCID) (37). In addition to Listeria, a wide variety of intracellular organisms, including T. gondii and T. cruzi, have been shown to stimulate T cell-independent IFN-γ synthesis by NK cells (6,24, 38,39).

The protozoan-induced IFN-γ response was found to be mediated by NK cells and to be dependent on soluble factors released from macrophage accessory cells (6,38). Follow-up studies with T. gondii and T. cruzi have defined IL-12 as the key cytokine produced by pathogen-activated macrophages and responsible for induction of IFN-γ synthesis by NK cells in the absence of T lym-

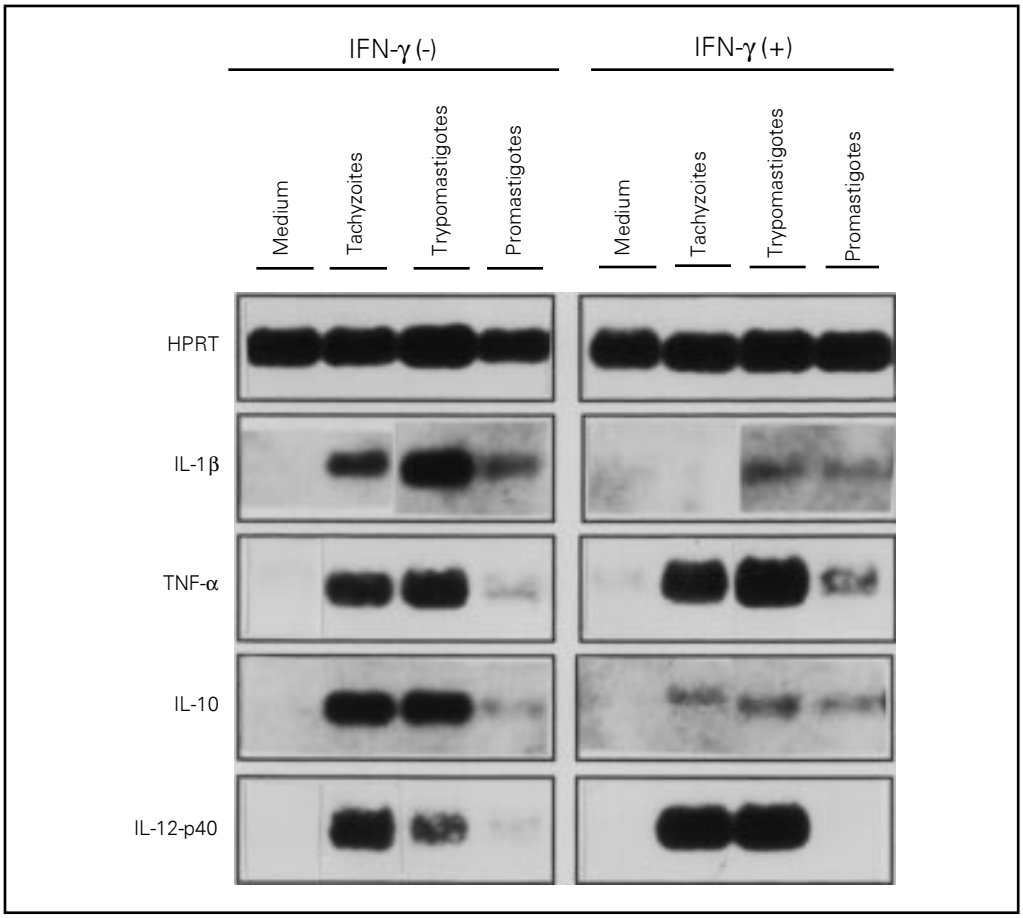

Figure 1 - Induction of monokine mRNA expression by inflammatory macrophages exposed to live forms of *Toxoplasma gondii*, *Trypanosoma cruzi* and *Leishmania major*. Thioglycolate-elicited macrophages were incubated in the presence (right panel) or absence (left panel) of IFN-γ, left uninfected (medium) or infected with tachyzoite, trypomastigote or promastigote forms of *T. gondii, T. cruzi* or *L. major*, respectively. After 6 h of incubation, total RNA was extracted from macrophage cultures and expression of the house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) and monokine (i.e., IL-1ß, TNF-α, IL-10 and IL-12-p40) genes was measured by the reverse transcriptase polymerase chain reaction.

phocytes (Figure 2) (6,24). The importance of this pathway in resistance to infection has also been revealed in studies using T. gondii. Thus, SCID mice treated with anti-IL-12 antibodies have enhanced susceptibility to infection with T. gondii (5).

An important paracrine positive feedback loop is observed between NK cells and macrophages. Thus, the IFN-γ produced by NK cells is a strong enhancer of IL-12 synthesis by macrophages. However, IFN-γ by itself is not sufficient to trigger IL-12 synthesis, and microbial products appear to be essentially required to initiate IL-12 production by macrophages. Recent studies have also shown that the monokines TNF-α, IL-1ß and IL-15 potentiate the effects of IL-12 on inducing IFN-γ synthesis by NK cells (24,38,40,41). In contrast, IL-10 and TGF-ß are potent inhibitors of parasite stimulation of IFN-γ synthesis by the T cell-independent pathway (38,39,42).

In contrast to T. gondii and T. cruzi, both L. major and L. donovani promastigotes fail to induce IFN-γ synthesis by NK cells in the absence of T cells (43,44). Moreover, Scott (45) showed that treatment with anti-CD4 monoclonal antibody (mAb) abolishes in vivo induction of IFN-γ by infection with L. major. It is noteworthy that the ability of Leishmania sp to evade (or to inhibit) IL-12 synthesis by macrophages in vitro is consistent with the incompetence of the parasite in triggering IFN-γ synthesis by NK cells in the absence of T cells.

IL-12 bias T cell differentiation towards the Th1 phenotype

Infection of C57BL/6 or BALB/c mice with L. major has become an important paradigm for the study of in vivo differentiation of Th precursor cells into Th1 or Th2 lymphocytes. Whereas infected C57BL/6 mice develop a Th1-mediated immune response and heal, BALB/c mice develop a Th2 response and eventually succumb to infection with L. major (46). Treatment of BALB/c mice with anti-IL-4 mAbs switches the differentiation of parasite-specific lymphocytes into the Th1 phenotype and makes the animals resistant to infection with L. major. In contrast, treatment of C57BL/6 mice with anti-IFN-γ switches T cell differentiation into the Th2 phenotype leading to enhanced animal susceptibility to infection (47,48). Consistent with these findings is the study showing that adoptive transfer of parasite-specific Th1 cell lines makes BALB/c mice resistant to infection, whereas parasite-specific Th2 cell lines favor susceptibility and uncontrolled lesion development during infection with L. major (49).

Because IFN-γ is known to play a major role favoring the differentiation of Th cells into the Th1 phenotype (45,50) and IL-12 has been reported to directly promote IFN-γ synthesis by Th cells (51,52), the involvement of IL-12 in the generation of Th1 lymphocytes was tested in different systems (5,22,52-55). Most studies indicate that IL-12 but not IFN-γ acts directly on T cells to enhance priming for IFN-γ synthesis, whereas the high levels of IFN-γ produced by NK cells and/or T cells exposed to IL-12 clearly favor T cell differentiation towards the Th1

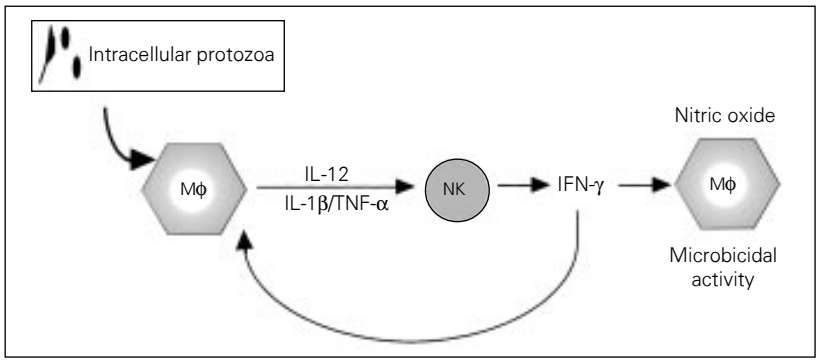

Figure 2 - Induction of the T lymphocyte-independent interferon-γ (IFN-γ) pathway by intracellular protozoa. The first target of IL-12 released from macrophages (Mφ) exposed to intracellular protozoan products are natural killer (NK) cells. After exposure to IL-12, NK cells produce high levels of IFN-γ, even in the absence of T cells. IFN-γ derived from NK cells is responsible for the activation of microbicidal effector functions (i.e., nitric oxide synthesis) exhibited by macrophages during early stages of infection. The monokines interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) potentiate the effects of IL-12 on NK cells and IFN-γ enhances the synthesis of IL-12 by macrophages exposed to microbial products.

phenotype by inhibiting expansion and cytokine synthesis by Th2 lymphocytes (Figure 3). As previously determined (56,57), Th precursor cells will develop into Th2 cells when incubated with IL-4 during primary stimulation with antigen.

Since many microorganisms including the protozoa T. cruzi and T. gondii have been shown to trigger IL-12 synthesis by cells of the monocyte/macrophage lineage it was suggested that this event is responsible for Th1 differentiation during different microbial infections. In fact, experiments performed with naive CD4+ T cells derived from transgenic mice expressing only one αß+ T cell receptor have shown that exposure of antigen-presenting cells (macrophages) to heat-killed L. monocytogenes favored T cell differentiation towards the Th1 phenotype (53). Simultaneous addition of anti-IL-12 blocked the effects of heat-killed Listeria on T cell differentiation.

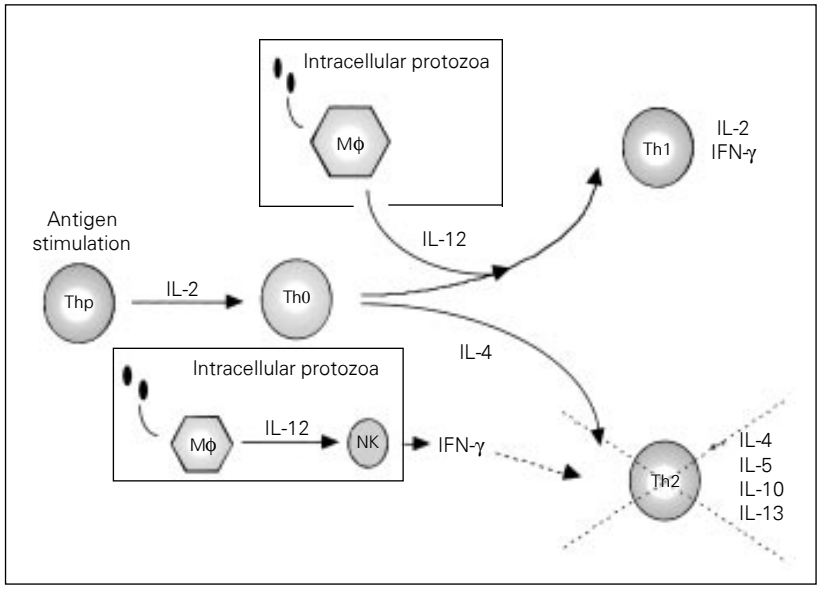

Figure 3 - T helper cell subset selection during infection with intracellular protozoa. Stimulation of Th precursor cells (Thp) by professional antigen-presenting cells such as dendritic cells induces IL-2 synthesis and Thp proliferation and initiates the differentiation of Thp towards the final effector stages, Th1 or Th2, via Th0. Exposure of antigen-stimulated Thp or Th0 stages to IL-12 favors differentiation towards the Th1 phenotype and development of cell-mediated immunity. In contrast, exposure of Thp or Th0 cells to IL-4 favors differentiation towards the Th2 phenotype. IFN-γ produced by natural killer (NK) cells or Th1 cells is a potent inhibitor of the development of Th2 lymphocytes, favoring outgrowth of a predominant Th1 subset. Mφ, Macrophage.

Experiments were also performed to address the involvement of IL-12 in T cell differentiation in vivo. BALB/c mice infected with L. major develop a strong Th2 response towards the parasite antigen. However, if vaccinated with parasite extracts in combination with rIL-12 (55), or treated with rIL-12 during early stages of infection with L. major, BALB/c mice develop an immune response dominated by Th1 cytokines (22,58).

The studies described above indicate the essential requirement of IL-12 for the inducion of T cell differentiation towards the Th1 phenotype. In addition, these studies suggest that to exert its effects on T cell differentiation, IL-12 is needed during the initial encounter of T cells with the antigen. To further analyze the requirement of IL-12 in the maintenance of an already established Th1 response we studied the role of IL-12 in cytokine synthesis as well as resistance in mice either acutely or chronically infected with T. gondii. Our results show that IL-12 synthesis is required for IFN-γ production and resistance to T. gondii during acute but not during chronic toxoplasmosis. In contrast, treatment with anti-IFN-γ mAb enhances susceptibility to this parasite in both stages of infection (5). Altogether these results indicate that IL-12 is required to initiate IFN-γ synthesis by lymphocytes, but after a Th1 response is established, IL-12 is no longer required.

Different species of Leishmania induce IL-12 and IFN-γ production in vivo

In contrast to the results obtained in in vitro experiments, different studies have indicated that in vivo infection with Leishmania sp does, in fact, trigger the synthesis of IL-12 by macrophages and IFN-γ by cells from the lymphocytic lineage and possibly NK cells (31,55,59,60). In addition, it is also known that treatment of resistant mice with neutralizing mAbs against either IL-12 or

IFN-γ enhances animal susceptibility to infection with different Leishmania species (21,22,47,61,62).

As mentioned in the previous section, parasite-specific Th precursor cells differentiate into the Th2 phenotype in BALB/c mice and into the Th1 phenotype in C57BL/6 mice, when these mouse strains are infected with L. major. These findings are consistent with the hypothesis that whereas BALB/c mice have a predisposition to developing immune responses dominated by the Th2 phenotype, C57BL/6 mice normally develop immune responses dominated by Th1 lymphocytes (63). Nevertheless, in either mouse strain, infection with T. cruzi or T. gondii will lead to the development of Th1 responses (4,5,64,65 and our unpublished observations). Taken together with the results described in the sections above, these results indicate that during infection with T. gondii or T. cruzi, parasite-induced IL-12 dictates the development of Th precursor lymphocytes into Th1 cells.

During infection with L. major the situation appears to be quite different. Thus, during pregnancy C57BL/6 mice become more susceptible to L. major infection developing the Th2 response instead of the predicted Th1 response in non-pregnant females (66). These results may be explained in part by studies indicating that during pregnancy there is a modulation of Th1 lymphocytes. In contrast, infection with T. gondii (35) or treatment with rIL-12 (22,58), which induces systemic levels of IFN-γ, renders BALB/c mice resistant to infection with L. major, as determined by the development of uncontrolled lesions. At the same time, these animals switch their parasite-specific Th responses towards the Th1 phenotype. Taken together, these findings indicate that during infection with L. major, the status of the host immune system rather than parasite-induced IL-12 and IFN-γ is critical in determining the differentiation of Th precursor lymphocytes into Th1 or Th2 effector stages.

In order to compare the ability of Leishmania sp, T. cruzi and T. gondii to induce IL-12 and/or IFN-γ, we performed in vivo or ex vivo experiments with BALB/c mice (susceptible to L. major) and C57BL/6 mice (resistant to L. major). When spleen cells were cultured in vitro at several parasite to splenocyte ratios, both T. cruzi and T. gondii triggered the synthesis of IL-12 and IFN-γ. In contrast, neither L. major nor L. amazonensis induced detectable levels of either cytokine. Consistent with these observations, we observed that in vitro stimulation with major surface glycoconjugates extracted from T. cruzi or T. gondii but not Leishmania sp induced high levels of IL-12 synthesis by murine macrophages (35).

Studies performed in vivo gave us a quite different answer. When parasites were injected intraperitoneally, only T. cruzi and T. gondii induced IL-12 and IFN-γ production by splenocytes peaking 5 days postinfection. However, all parasites induced similar levels of IL-12 and IFN-γ production by the draining lymph node cells when mice were injected subcutaneously into the hind footpad. Surprisingly, even BALB/c mice did produce high levels of IL-12 under these conditions. Similar results were obtained when parasites were injected intraperitoneally and IL-12 and IFN-γ synthesis was measured in the resident peritoneal cells. Based on these studies, we suggest that, whereas T. cruzi and T. gondii trigger systemic cytokine synthesis during acute infection, parasites from the Leishmania genus have a more local effect activating immune cells at the site of infection.

In summary, our data indicate that systemic induction of IL-12 and IFN-γ may be required for prompt T cell differentiation into Th1 phenotypes. Alternatively, although infection with L. major might trigger local synthesis of IL-12 and IFN-γ, the levels are not sufficiently high or produced for long enough periods of time to switch Th precursor cells to differentiate into the Th1 instead

of the Th2 phenotype in BALB/c mice, which are predisposed to developing immune responses dominated by Th2 cells.

Induction of chemokine synthesis during acute infection with T. gondii or T. cruzi

Chemokines are a group of cytokines related to IL-8 that can be produced by a wide range of cells (e.g., lymphocytes, mast cells, macrophages, fibroblasts, endothelial cells and smooth muscle cells) and are known to have chemotactic as well as activating functions for different types of inflammatory cells of the immune system (67-70). In contrast to the classical chemotactic agents (e.g., C5a, PAF), chemokines are selective attractants that activate distinct leukocyte populations. The hallmark for this family of pro-inflammatory proteins is the conservation of four cysteine residues that are important for the tertiary structure. The chemokines can be divided into two subfamilies depending on whether the first two cysteines are adjacent (C-C chemokines) or not (C-X-C chemokines). In addition, the C-X-C chemokines can be grouped into those containing the ELR motif (e.g., KC and MIP-2) and those lacking the ELR motif (e.g., MIG and IP-10). The ELR motif is common to all C-X-C chemokines that act on neutrophils. Whereas the major target of the first group of C-X-C chemokines are neutrophils, the chemokines belonging to the latter group of C-X-C chemokines are major attractants and activators of T lymphocytes and NK cells (67-70). Although, C-C chemokines (e.g., MCP-1, MIP-1α, MIP-1ß and RANTES) are thought to act on monocytes, recent studies indicate that this group of chemokines has a much wider range of biological activity since they can also act on lymphocytes, eosinophils and basophils.

In addition to different microbial products (i.e., LPS, virus dsRNA and Staphylococcus enterotoxin A), the monokines IL-1 and TNF-α are potent inducers of chemokine synthesis in different cell types. In fact, many of the original chemotactic effects attributed to IL-1 and TNF-α were shown later to be mediated by different chemokines. As shown above, both *T. gondii* and *T. cruzi* are potent stimulators of IL-1 and TNF-α synthesis by macrophages. In addition, we have also evaluated the ability of these parasites to directly induce chemokine synthesis by different types of cells (i.e., macrophages, fibroblasts and endothelial cells). So far, our data indicate that live tachyzoites or live trypomastigotes as well as glycoconjugates isolated from either parasite are able to trigger the synthesis of C-X-C chemokines (i.e., CRG-2 and KC) as well as C-C chemokines (MIP-1α, MIP-1ß and RANTES) by inflammatory macrophages (71). It is noteworthy that the cytokine IFN-γ has a dual role in the induction of different chemokines. Whereas IFN-γ is essentially required for induction and/or augmentation of two C-X-C chemokines lacking the ELR motif (i.e., MIG and IP-10), it seems to be a potent inhibitor of certain chemokines such as JE (a C-C chemokine) and KC (a C-X-C chemokine). One of the questions raised by these studies is whether the inducibility and/or inhibitory effects of IFN-γ would extend to the other members of the C-C and C-X-C chemokines and whether a profile of chemokines could be determined in part by the early synthesis of IFN-γ by NK cells.

Our in vivo studies show that acute infection with *T. gondii* is a potent inducer of chemokines MIG and CRG-2 (72). The maximal expression of MIG occurs in different organs/tissues at 7 days of infection and independently of local production of IFN-γ. Nevertheless, expression of MIG is strictly dependent on IFN-γ synthesis and no MIG expression is observed in mice lacking the gene for IFN-γ and infected with *T. gondii*. Whereas macrophages appear to be the major source of MIG in spleens, the major source of this chemokine in other organs has not been defined.

We have also studied the expression of different chemokines in the cardiac tissue of mice acutely infected with T. cruzi (73). We observed that among the C-X-C chemokines only those induced or potentiated by IFN-γ (i.e., CRG-2 or MIG) were significantly increased in the cardiac tissue of mice infected with T. cruzi (data not shown). A slight or undetectable augmentation in expression of other C-X-C chemokines was observed in the cardiac tissue of the same animals. In contrast, expression of all C-C chemokines tested was augmented in the cardiac tissue of animals acutely infected with this protozoan. Interestingly, the chemokine pattern expressed in the host cardiac tissues during the acute phase of experimental Chagas' disease was closely similar to that expressed by inflammatory macrophages exposed to live trypomastigotes (or trypomastigote GPI-mucins) plus IFN-γ. As discussed above, it is possible that the pattern of chemokine expression is determined by cytokines such as IL-1 and TNF-α, as well as IFN-γ, which are produced during infection with T. cruzi. We are currently investigating the effect of in vivo treatment with anti-IFN-γ and/or anti-TNF-α mAbs on chemokine profile and leukocyte infiltrate in the tissue of animals infected with T. cruzi.

Although there are studies indicating that in vitro infection with Leishmania may trigger the synthesis of certain chemokines in both human and mouse macrophages (74,75), our studies indicate that in contrast to T. gondii and T. cruzi parasites, L. major is a very poor inducer of different chemokines by inflammatory macrophages primed or not with IFN-γ (our unpublished data).

Role of down-regulatory cytokines (i.e., IL-4, IL-10 and TGF-ß) in modulating induction of cell-mediated immunity and macrophage effector functions

Another important aspect of the strong immune response induced by intracellular protozoa is that under certain conditions, if uncontrolled, it can be the major cause of immunopathology and lethality. Therefore, it seems that the potency of host immune responses triggered by these parasites has to be tightly regulated, displaying the ability to limit the infection but at the same time lacking pathogenicity. Therefore, the elucidation of the molecular basis involved in the induction of the protective immune response and its regulation may yield important clues for a better understanding of successful long-term interaction between parasites and their vertebrate host.

At the same time that macrophages are responsible for induction of CMI, these cells promote regulation of this response through the production of IL-10 and TGF-ß, which regulates the expression or function of IL-12 and other monokines (Figure 4), as well as through the synthesis of nitric oxide (see next section) which has an antiproliferative effect on cells from the lymphocytic lineage. In addition to regulating monokine synthesis by macrophages (76), IL-10 has been shown to be an important modulator of macrophage effector functions against different parasites (4,77). We have also shown that IL-10 inhibits the synthesis of reactive nitrogen intermediates (RNI) by macrophages activated by IFN-γ and this inhibitory activity is enhanced by TGF-ß and IL-4 (78).

In the specific case of infection with T. cruzi, IL-10 has been shown to antagonize IFN-γ activity rather than IL-12 synthesis (4). Thus, resistant and susceptible mouse strains produce similar levels of IFN-γ in response to T. cruzi infection. The levels of IL-10 produced by susceptible animals in response to T. cruzi infection are much higher than those produced in resistant animals. Interestingly, treatment with anti-IL-10 renders susceptible animals more resistant to infection with T. cruzi (79). Similarly, IL-10 KO mice are more resistant to infection with T. cruzi than their wild-type controls (80).

In recent studies we have assessed the role of IL-10 synthesis in regulating IL-12 and Th1-type cytokine in vivo as well as in establishing the parasite-host equilibrium during acute infection with *T. gondii*. Our approach was to infect IL-10 KO mice (81) with an avirulent strain of the parasite and to analyze cytokine responses and host resistance to this pathogen. All of the infected IL-10 KO mice died by day 14 postinfection, in contrast to 100% survival of infected wild-type animals. The IL-10 KO mice infected with *T. gondii* showed a dramatic increase in serum levels of IL-12 and IFN-γ as compared to uninfected IL-10 KO or infected wild-type mice. Our results also show that the parasite burden was similar or decreased in the IL-10 KO mice as compared to wild-type animals. In agreement with the RT-PCR results, no evidence of parasite expansion was observed in tissue sections from the IL-10 KO mice. Nevertheless, the infected IL-10 KO mice showed clear evidence of enhanced tissue pathology as demonstrated by increased frequency and intensity of cellular infiltration and necrosis in the liver and, to a lesser extent, in the lungs. Taken together, these results suggest that the increased mortality of IL-10 KO mice is not due to uncontrolled parasite growth, but to an abnormal pathologic response to infection. The histopathology studies demonstrated that infected IL-10 KO animals presented pathology similar to that of wild-type animals under continuous treatment with high doses of IL-12 (82). These studies indicate that, if uncontrolled, high levels of IL-12 synthesis during microbial infection can be harmful to the host and, under some conditions, may cause a high incidence of lethality.

TGF-ß, on its own, is a potent regulator of macrophage effector functions. Thus, mouse treatment with recombinant TGF-ß makes animals more susceptible to infection with *T. cruzi* (83), different species of Leishmania (84,85) and *T. gondii* (42). In the case of Leishmania infection, the modulatory role of TGF-ß appears to be important for parasite replication and lesion development, since high levels of active TGF-ß are found in the lesions containing Leishmania amastigotes (85).

Finally, IL-4 has been initially shown to block macrophage parasiticidal activity in macrophages activated by IFN-γ (86). However, different studies indicate that the major mechanism of action of IL-4 in regulating induction of CMI during L. major infection is to favor the differentiation of parasite-specific Th precursor cells towards the Th2 phenotype (46). Interestingly, IL-4 KO mice are more susceptible to infection with *T. gondii* than wild-type mice. The results obtained by Roberts et al. (87) show that after infection with *T. gondii* a greater mortality is observed in animals lacking IL-4 functional gene. Paradoxally, this enhanced mortality was accompanied by higher levels of IFN-γ synthesis and lower tissue parasitism. This study suggests that during acute infection with *T. gondii* induction of IL-4 synthesis may play an important role in regulating an overwhelming parasite-induced Th1 response that can cause host tissue damage and lethality.

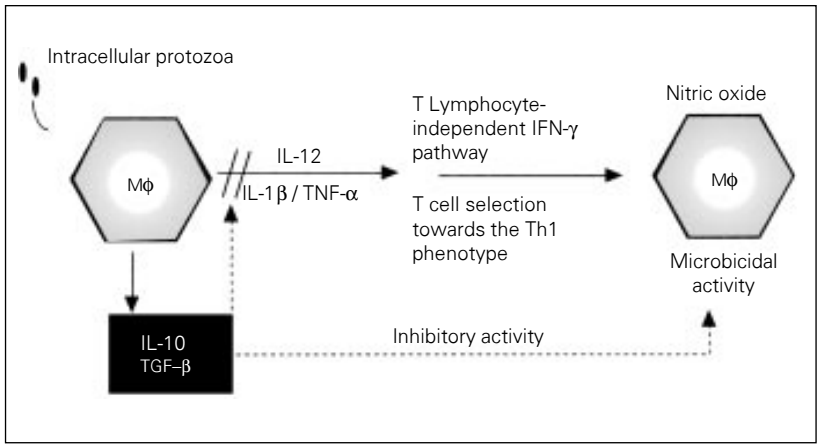

Figure 4 - Down-regulatory role of IL-10 and TGF-ß in IFN-γ synthesis by NK cells and/or T lymphocytes and microbicidal activity by macrophages (Mφ). Macrophages exposed to microbial products will produce IL-10 as well as active TGF-ß that will modulate the synthesis of IL-12, IL-1ß and TNF-α by macrophages, thus inhibiting induction of IFN-γ synthesis by NK cells and Th1 lymphocytes. The microbicidal activity (i.e., synthesis of nitric oxide) displayed by IFN-γ-activated macrophages is also inhibited by IL-10 and TGF-ß.

Role of nitric oxide in the control of parasite replication and regulation of cell-mediated immunity induced by Leishmania sp, T. gondii and T. cruzi

After exposure to IFN-γ, macrophages exposed to microbial products produce high levels of RNI. In addition to contributing to immune regulation during protozoan infections, the RNI are also important effector molecules responsible for controlling parasite replication by murine macrophages (88).

Our recent in vitro studies have shown that GPI-mucins isolated from trypomastigote membranes are potent inducers of nitric oxide synthesis by IFN-γ-primed macrophages, even at concentrations as low as 10 ng/ml. Consistent with this observation, GPI-mucins enhanced the killing of different Leishmania species by macrophages. Glycoconjugates isolated from *T. gondii* tachyzoites have similar activity in inducing nitric oxide synthesis by IFN-γ-primed macrophages. In contrast, our experiments show that glycolipids (*i*.e., LPG and GIPLs) obtained from L. major or L. donovani are unable to potentiate the nitric oxide synthesis and/or microbicidal activity displayed by IFN-γ-primed macrophages, even at concentrations as high as 20 µg/ml (28,29). In studies performed by Proudfoot and colleagues (20), it was demonstrated that GIPLs extracted from L. major amastigotes inhibit the synthesis of nitric oxide by a murine macrophage cell line. Consistent with these findings are our studies showing that infection of IFN-γ-primed macrophages with trypomastigotes (or exposure to parasite surface glycoconjugates) leads to macrophage death due to the synthesis of high levels of nitric oxide (Camargo MM and Gazzinelli RT, unpublished observations). Yet infection with L. major has a quite opposite effect, preventing apoptosis in IFN-γ-activated macrophages (89).

Apoptosis of $CD4^+$ *T* lymphocytes (90) induced by high levels of nitric oxide produced by activated macrophages (91) appears to be an important immunoregulatory mechanism acting during the acute phase of infection with either *T. cruzi* or *T. gondii* (91,92) (Figure 5). The importance of nitric oxide immunoregulatory functions as well as in controlling parasite load is revealed in studies using iNOS KO mice or animals treated with specific inhibitors for nitric oxide synthesis by iNOS (88). Animals infected with *T. cruzi* and treated with iNOS inhibitors are unable to control parasitemia and mortality can reach 100% at 25 days postinfection, whereas 100% of untreated animals survived (7). Similarly, animals infected with *T. gondii* and treated with iNOS inhibitors, or iNOS KO mice infected with *T. gondii*, are more susceptible to infection than control animals treated with PBS or wild-type animals infected with *T. gondii* (93,94). The increase in cyst numbers observed in mice infected with *T. gondii* and treated with iNOS inhibitors also results in an intense inflammatory reaction in the CNS (93) indicating that in vivo inhibition of iNOS may also block the immunoregulatory activity of RNI.

Although we were unable to induce nitric oxide synthesis by IFN-γ-primed macrophages stimulated with LPG or GIPLs, studies performed in our laboratory and elsewhere indicate that infection with Leishmania triggers the synthesis of nitric oxide both in vitro and in vivo (29,95). In agreement with these findings, in vivo treatment with inhibitors of

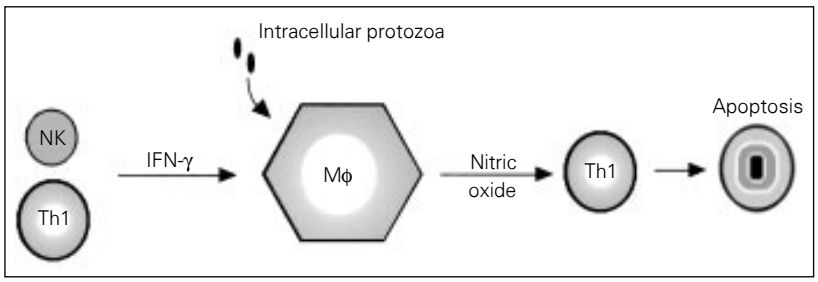

Figure 5 - Antiproliferative effect of nitric oxide on Th1 lymphocytes. Inflammatory macrophages (Mφ) exposed to products derived from intracellular protozoa as well as IFN-γ from NK cells or Th1 lymphocytes produce high levels of nitric oxide. At high concentrations, nitric oxide causes stasis as well as apoptosis of Th1 lymphocytes.

nitric oxide or the lack of a functional iNOS gene results in enhanced susceptibility to infection with L. major (96,97). Nevertheless, our studies indicate that in vitro (29) and in vivo (Gazzinelli RT, unpublished observations) infection with Leishmania is less potent than infection with either T. cruzi or T. gondii in inducing nitric oxide synthesis.

Activation/inhibition of distinct signaling pathways in macrophages infected with or exposed to intracellular protozoa

Major questions raised from the studies described above are related to the nature of macrophage receptor(s) and characterization of signaling pathways stimulated by T. gondii and T. cruzi products that lead to the induction of cytokine synthesis and activation of distinct macrophage functions. Recent studies indicate that glycoconjugates extracted from T. gondii and GPI anchors extracted from Plasmodium falciparum (98-100) or Trypanosoma brucei (101) induce protein kinase C (PKC) and protein tyrosine kinase activity which is important for the synthesis of cytokines such as IL-1 and TNF-α by macrophages. In addition, these studies suggest that exposure of macrophages to protozoan GPI anchors also induces phosphorylation of IκB, resulting in the release of NF-κB, which is essential for the induction of cytokine synthesis and certain macrophage functions.

In contrast, several reports indicate that macrophage infection with Leishmania sp does in fact intercept different signaling pathways such as PKC, leading to the blockage of various macrophage functions (16,17,20, 89,102-105). Some of the inhibitory effects on signaling pathways and macrophage functions are mediated by Leishmania glycolipids (i.e., LPG and GIPLs) and appear to favor parasite survival inside macrophages. Further understanding of interferences of Leishmania parasites in signaling pathways and functions displayed by macrophages may be instructive as to how modulation of immune responses dictated by macrophages can be achieved.

Conclusions

A large variety of pathogens and microbial products have been shown to trigger macrophages to produce a diversity of chemoattractant and pro-inflammatory cytokines. Such events appear to be essential in initiating the inflammatory process and determining the nature of the immune responses against invasive organisms. Thus, the initial interaction of intracellular parasites with macrophages may play a major role in determining parasite-host equilibrium and disease outcome during protozoan infections. In this review, we attempted to analyze the interaction of intracellular protozoa (i.e., T. gondii, T. cruzi and Leishmania) with macrophages and the role of this event in the initiation of inflammation and establishment of protective immune responses during infection with such parasites. The tachyzoite forms of T. gondii or trypomastigote and amastigote forms of T. cruzi are strong inducers of the synthesis of different monokines (including IL-12) and chemokines, as well as effector functions by macrophages. We propose that these two intracellular protozoa, which can invade many types of nucleated vertebrate host cells, and therefore are potentially highly virulent, activate macrophages in order to regulate their own numbers, thereby ensuring both host and parasite survival. In contrast, Leishmania parasites are unable to induce a strong cytokine response by macrophages and are less potent in inducing microbicidal activity by macrophages. These findings are consistent with the need for this parasite to evade cell-mediated immunity early in infection, and to persist in the host in high numbers as long as possible, in order to perpetuate its life cycle.

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