Influence of Parasite Load on the Ability of Type 1 T Cells To Control *Leishmania major* Infection

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BALB/c mice infected with *Leishmania major* developed a type 2 immune response which failed to control parasite replication. We found that *scid* mice that received splenocytes from BALB/c mice that had been infected for 3 weeks with *L. major* (a type 2 cell population) and that were subsequently infected with *L. major* were protected when they were treated with interleukin 12 (IL-12). In contrast, IL-12 was ineffective at protecting BALB/c mice infected for 3 weeks, suggesting that a high parasite load regulates the development of protective immunity. To determine how this regulation operates, we performed a series of adoptive transfers of naive, type 1 or type 2 splenocytes into *scid* mice. The recipient *scid* mice were infected either for 5 weeks prior to cell transfer (and thus had a high parasite load) or at the time of cell transfer. *scid* mice that were infected for 5 weeks and received a type 1 cell population were able to cure their lesions. However, when 5-week-infected *scid* mice received both type 1 and 2 cell populations, they were unable to control their infections. In contrast, the same type 1 and 2 cells transferred to naive *scid* mice, which were subsequently infected, provided protection. In addition, we found that naive cells mediated protection in *scid* mice with established lesions. These results show that high parasite numbers do not block type 1 protective responses or the development of type 1 responses. Instead, the influence of a high parasite load is dependent on the presence of a type 2 cell population.

The outcome of infection with *Leishmania major* depends on the type of immune response that develops. A type 1 response favors resistance, while a type 2 response promotes susceptibility (32, 35). Most studies have focused on the importance of CD4+ Th1 and Th2 cells, although CD8+ T cells may also contribute to resistance (7, 10, 24). Cytokines, specifically interleukin 4 (IL-4) and IL-12, play a critical role in T-helper-cell differentiation (25, 28), although other factors also influence T-helper-cell subset development. In leishmaniasis, the dose of parasites used to initiate infection is particularly important. Thus, high doses of parasites can convert normally resistant animals to a susceptible phenotype, while low doses of parasites can promote healing and Th1-cell development in normally susceptible mice (3, 18). Parasite numbers also appear to play a role in regulating T-helper cells once they have developed. For example, IL-12 or anti-IL-4 monoclonal antibody treatment was unsuccessful as an immunotherapy in *L. major*-infected BALB/c mice, but healing and switching from a Th2- to a Th1-type response occurred when either treatment was combined with chemotherapy (26, 27).

At present, the mechanism by which a high parasite dose inhibits the development of a protective type 1 response is unknown. Parasites, or products of infected cells, such as IL-10, transforming growth factor β (TGF-β), and prostaglandin E2, might directly act to downregulate Th1 cells. Alternatively, the influence of parasite dose may relate to the amount of antigen presented to the immune system. Several studies have addressed the role of antigen in Th-cell differentiation, although the conclusions reached have been varied. For example, one study with T-cell-receptor-transgenic mice found that high doses of peptide favored a Th1 response but that lower doses favored a Th2 response (4). In contrast, in a different T-cell-receptor-transgenic mouse, high and very low concentrations of peptide favored a Th2 response while intermediate levels of antigen favored a Th1 response (14). The in vivo studies with *L. major* suggest that a high dose of antigen favors a Th2 response. Moreover, a classic study done by administering different doses of flagellin found that high doses favored antibody responses but that lower doses favored a delayed-type hypersensitivity response (29).

In this study, we were interested in further defining how the parasite load influences the nature of the immune response, since in previous experiments we found that parasite load influenced the ability of IL-12 to promote a protective immune response. These results suggested that the large number of parasites present in susceptible BALB/c mice might directly inhibit the protective capacity of type 1 cells. To test this hypothesis, we transferred splenocytes from *L. major*-infected BALB/c mice with a dominant type 1 or type 2 response into *scid* mice with either a low parasite burden (infected at the time of cell transfer) or a high parasite burden (infected for 5 weeks) and monitored the disease. Surprisingly, we found that high numbers of parasites do not directly inhibit a type 1 population from controlling *Leishmania*-induced lesions or the...
development of a protective type 1 response from a naïve cell population. Rather, high numbers of parasites suppress only type 1 responses when type 2 cells are present. These results suggest that elimination of type 2 cells may be as useful in developing a successful treatment for chronic diseases associated with a type 2 immune response as decreasing the pathogen load is.

MATERIALS AND METHODS

Animals. Female BALB/cByJ and BALB/cByJSmm-scid/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The animals were maintained in a specific-pathogen-free environment. The animal colony was screened regularly for the presence of murine pathogens and consistently tested negative. The work done in this study was approved by an institutional animal care and use committee and followed relevant federal guidelines on animal care.

Parasites and antigens. L. major (MHOM/IL/80/Friedlin) was used for all infections. Parasites were grown in Grace’s insect cell culture medium (Life Technologies, Grand Island, N.Y.) with 20% fetal bovine serum (≥0.125 endotoxin units/ml; HyClone, Inc., Logan, Utah) and 2 mM glutamine. Stationary-phase promastigotes were harvested, and metacyclic-stage parasites were negatively selected with Arachis hypogaea agglutinin (Sigma Chemical Co., St. Louis, Mo.) as described previously (33). Mice were injected in the hind footpad with 10⁶ metacyclic-stage L. major parasites. Soluble leishmanial antigen was prepared as described previously (36). The numbers of parasites in the spleens were determined by limiting dilution analysis of single-cell suspensions made from individual spleens. Briefly, single-cell suspensions of the spleens were plated in log-fold serial dilutions in Grace’s insect cell culture medium, starting with a 1:100 dilution. Each sample was plated in triplicate, and the mean of the negative log parasite titers was calculated 5 days after culture initiation.

Cell transfers. In order to obtain a type 1 cell population, BALB/c mice were infected with 10⁶ metacyclic-stage L. major parasites and treated intraleSIONALLY with IL-12 (Genetics Institute, Cambridge, Mass.) six times over the first 2 weeks of infection. To do this, 10⁶ parasites were transferred into a single cell suspension from these mice and then transferred, both to intravenously infected and 5-week infected BALB/c scid mice infected with L. major by intravenous injection (type 2 population) and were simultaneously infected with 10⁷ L. major parasites. Mice were then injected with 0.2 µg of IL-12 intraleSIONALLY six times during the first 2 weeks of infection. Type 2 cell populations were obtained from BALB/c mice that had been infected for 3 weeks with L. major, which exhibited a dominant type 2 response (IFN-γ, 6.5 ng/ml; IL-4, 0.4 U/ml). Type 2 cell populations were obtained from BALB/c mice that had been infected for 3 weeks with L. major that exhibited a dominant type 2 response (IFN-γ, <0.1 ng/ml; IL-4, 3.7 U/ml), and naïve spleocytes were obtained from uninfected BALB/c mice. These mice were transferred approximately one spleen cell equivalent (6 x 10⁸ to 1 x 10⁹ spleocytes) to scid mice.

IgE and antigen-specific IgG1 and IgG2a ELISAs. The immunoglobulin E (IgE) enzyme-linked immunorbent assay (ELISA) used R35-72 (PharMingen, San Diego, Calif.) at 5 µg/ml in phosphate-buffered saline (PBS) and an anti-IgE-hors eradish peroxidase (Southern Biotechnology, Birmingham, Ala.) antibody at a 1:1,000 dilution to detect IgE in mouse serum. A purified mouse IgE (anti-trinitrophenol) was used as the standard. In order to detect antigen-specific IgG1 and IgG2a, ELISA plates were first coated with 1 µg of soluble leishmanial antigen in PBS per ml and then mouse serum, starting at a dilution of 1:150, was added, after which threefold dilutions were made. The goat anti-mouse IgG1 or IgG2a-hors eradish peroxidase-labeled antibodies were purchased from Southern Biotechnology and were used at a 1:1,000 dilution.

Statistical analysis. Student’s t test was used in comparative analysis, and a P value of <0.05 was considered significant.

RESULTS

scid mice reconstituted with a type 2 cell population and treated with IL-12 control L. major infection. Chemotherapy and IL-12 treatment of mice infected with L. major induced a switch from a dominant type 2 to a dominant type 1 immune response and healing of the infection (26). To demonstrate that the efficacy of IL-12 when combined with chemotherapy was due to low numbers of parasites rather than an unknown effect of the drug, we set up an experimental model where a type 2 response would exist in the presence of low numbers of parasites. This was accomplished by reconstituting scid mice with spleen cells from BALB/c mice infected for 3 weeks (a type 2 cell population). The reconstituted scid mice were then infected with L. major. The scid mice that received the type 2 cell population were treated with IL-12 for 2 weeks, and the course of infection was monitored. As a control, BALB/c mice that had been infected for 2 weeks were treated with IL-12. scid mice reconstituted with type 2 cells and treated with IL-12 were able to control the disease, while those that were not treated with IL-12 were completely susceptible (Fig. 1). At 3 weeks, the parasite burden in the spleen was low or undetectable, but there remains the likelihood that either antigen or some parasites might have been transferred with the type 2 spleocytes. However, if this was the case, it did not block the protective effects of IL-12 treatment. As previously reported, IL-12 could not protect 3-week-infected BALB/c mice (26), although the same treatment given to BALB/c mice at the time of infection was protective (data not shown) (12, 38). Interestingly, IL-12-treated and PBS control scid mice had similar courses of infection, indicating that IL-12 does not appear to have any protective effect against L. major in the absence of T cells.

To assess the in vivo immune response in these mice, we took advantage of the fact that both T and B cells were trans-
ferred to scid mice and monitored IgE levels as a measure of a type 2 response. scid mice which received type 2 spleen cells had levels of IgE comparable to those of the 3-week-infected BALB/c mice. However, in the IL-12-treated spleen cells from 5-week-infected BALB/c mice. However, in the IL-12-treated spleen cells that received type 2 spleen cells, IgE levels were significantly lower than in the controls (data not shown). Although scid mice can be protected by T-cell transfers, lymph nodes draining the site of infection were not found and thus cytokine responses could not be directly assessed. Taken together, these data suggest that a type 2 cell population can be inhibited by IL-12, but only when parasite numbers are reduced.

Type 1 cell populations can resolve lesions from scid mice infected for 5 weeks with L. major. We hypothesized that high parasite numbers would directly inhibit a type 1 response. To test this hypothesis, we transferred spleenocytes from healing BALB/c mice, which had been treated with IL-12 for the first 2 weeks of infection (a type 1 cell population), into either 5-week-infected scid mice, or naïve scid mice that were subsequently infected. To confirm that the parasite load was quite different in the scid recipient mice, some animals were sacrificed 3 days after cell transfer and the numbers of parasites in the lesions were quantitated. Lesions from 5-week-infected scid mice had significantly more parasites (>10^7) than lesions from 3-day-infected scid mice (10^5).

scid mice that received type 1 spleenocytes and were subsequently infected were able to heal their lesions (Fig. 2A). These results confirm previous findings that the transfer of Th1 cell lines protects scid mice against L. major (13). Surprisingly, type 1 cells were also able to promote lesion resolution in mice that had been infected for 5 weeks (Fig. 2B). The resolution of infection was quite dramatic since lesions were substantial in size (approximately 3 mm) and contained >10^5 parasites when the transfer took place. These data suggest that parasites do not have a direct inhibitory effect on an established type 1 cell population.

A mixture of type 1 and type 2 cells protects scid mice with low numbers of parasites but is unable to heal chronically infected scid mice. Since type 1 cells control L. major in an environment with a high parasite load in the absence of a type 2 response, we hypothesized that a type 1 cell population might not be able to control L. major lesions in the presence of a type 2 cell population. Therefore, we transferred equal numbers of spleenocytes from IL-12-treated (type 1) and untreated (type 2), infected BALB/c mice into scid mice that had been infected for 5 weeks or were infected at the time of cell transfer and monitored the course of infection. In these experiments we also transferred naïve cells into uninfected or 5-week-infected scid mice. Five-week-infected scid mice that received both cell populations (type 1 and type 2) could not control the disease and exhibited a course of infection that was comparable to that of mice that received only type 2 cells. In contrast, the same mixed cell population of type 1 and type 2 cells was protective in scid mice that were infected at the time of cell transfer (Fig. 3).

Interestingly, naïve spleen cells transferred into 5-week-infected scid mice provided a significant amount of protection (Fig. 3). These results were unexpected, since we thought that the high parasite load would favor a type 2 response, just as the parasite dose can influence whether a type 1 or 2 response develops at the initiation of an infection in BALB/c mice (3). To examine the immune response in mice that received naïve cells, we collected sera and measured the levels of antigen-specific IgG1 and IgG2a as measures of type 2 and type 1 immune responses, respectively. The 5-week-infected scid mice that received naïve spleen cells had titers of IgG1 similar to those of mice that had received type 1 cells (approximately 1/1,000) and had titers of IgG2a (approximately 1/1,000) higher than those of mice that had received type 2 cells (<1/100). These data suggest that the scid mice that received naïve spleen cells developed a type 1 response and were able to control an L. major infection, regardless of the number of parasites present.

As another measure of protection, parasite numbers in the spleens of mice were quantitated in the scid recipients. Parasite counts were not obtained from the footpad, since many of the animals that had progressive disease exhibited severely necrotic lesions. There were no significant differences in the numbers of splenic parasites in 5-week-infected scid mice that received type 1 and type 2 cells, only type 2 cells, or no cells (Fig. 4); all of these mice had >10^7 parasites in their spleens. In contrast, scid mice that were infected at the same time as the transfer of type 1 and type 2 cells exhibited low numbers of parasites in their spleens. In fact, three of four mice had no
detectable parasites in their spleens. All recipient groups of mice were found to have similar numbers of T cells in their spleens, indicating that the cell transfers were equally effective under each experimental condition.

DISCUSSION

How T-cell subsets are regulated during an ongoing immune response is an area of active investigation. In this study, we investigated how a type 1 cell population, as well as the presence of a type 2 cell population, is influenced by parasite load. We have shown that a type 2 cell population which exacerbates infection in scid mice can be switched to a protective phenotype by the administration of IL-12 if the parasite load is low. These results extend our previous findings obtained by combined IL-12 treatment and chemotherapy by directly linking the success of the immunotherapy to low numbers of parasites. Unexpectedly, we found that the protective capacity of type 1 cells is not directly inhibited by a high parasite load and that naive cells are not blocked in their ability to become a protective cell population in the presence of a very high parasite burden. However, type 1 cells in the presence of a type 2 cell population were unable to provide protection in an environment with a high load of parasites, although the same mixed cell populations were protective in an environment with a low load of parasites. These results suggest that parasites have an indirect role in modulating protective T-cell responses, primarily acting to tip the balance towards a type 2 response rather than directly acting on type 1 cells.

There are several ways that a high parasite load might block the development of a protective immune response. Our first hypothesis was that a type 1 response would be suppressed in the presence of a large number of parasites and that this suppression might be due to a high antigen load associated with the infection or the ability of parasites to stimulate the production of inhibitory factors by the infected cells. Several observations are consistent with this hypothesis. Thus, in a model of experimental encephalomyelitis, when repeated high doses of myelin basic protein were administered to diseased mice, there was an increase in T-cell apoptosis and an amelioration of autoimmune disease (5). In addition to regulating high antigen levels, a large parasite load might regulate type 1 cells by stimulating the production of inhibitory cytokines due to the increase in the number of infected macrophages or...
dendritic cells. *L. major*-infected cells produce several factors, such as IL-10, TGF-β, and prostaglandin E₂, that have been shown to inhibit Th1 responses (1, 2, 6, 9, 16, 31, 34, 37). One example where parasite load has been shown to influence the level of these cytokines is a study where *L. major*-infected BALB/c mice that were treated with a leishmanial drug had lower levels of IL-10 and TGF-β mRNA than those that had not received the drug (17). However, our results indicate that a high parasite load has no overriding influence on the ability of a type 1 cell population to resolve an established infection, since a type 1 cell population transferred to *scid* mice with established lesions containing greater than 10⁸ parasites was able to promote healing of the infection. The potency of this type 1 cell population may also be enhanced by the presence of dendritic cells in the donor population that have been exposed to IL-12 and can present leishmanial antigens. Dendritic cells exposed to leishmanial antigens and IL-12 were shown to induce substantial protection when they were transferred to immunocompetent mice (8, 40), and in this case, dendritic cells may ensure that any nonpolarized Leishmania-specific T cells develop into IFN-γ producers.

In contrast, this same type 1 cell population failed to provide protection in 5-week-infected mice when it was given with a type 2 cell population, although the mixed type 1 and type 2 cells were protective when they were transferred to a naïve *scid* mouse that was subsequently infected. It is unclear how a type 2 cell population regulates a type 1 response in the presence of high numbers of parasites but not in the presence of low numbers of parasites. One possibility is that the higher numbers of parasites leads to greater type 2 cell activation and production of IL-4, which might directly suppress type 1 cells. However, since Th1 cells have been shown to be unresponsive to IL-4, this may be unlikely (15). Interestingly, Powrie et al. found that cells from *L. major*-infected BALB/c mice contained both Th2 cells and precursors of Th1 cells (30). It is possible that in a low-level-antigen environment, these Th1 precursors may have an advantage in developing. Another possibility is that IL-4 promotes type 2 cell development from uncommitted and/or naïve T cells within these heterogeneous cell populations, which then shifts the balance of the entire T-cell population toward a type 2 phenotype. However, it is unclear why this would occur only in the context of an established infection. A third possibility is that type 2 cells or their products provide a signal to *L. major*-infected cells to produce different, or greater amounts of, factors that alter the overall phenotype of the T-cell population. One scenario might involve IL-10, which has been shown in recent studies with IL-10 knockout and transgenic mice to be a critical factor in susceptibility to leishmaniasis (9, 16). The production of IL-10 can be triggered by ligation of the Fcγ receptor on macrophages, and amastigotes opsonized with antileishmanial antibody stimulate IL-10 production by macrophages (16). At 5 weeks, when there are a large number of amastigotes that are reinfecting cells in the presence of high antibody levels, there may be more IL-10 produced than at the initiation of the infection, when there are fewer parasites.

In these studies, we also investigated whether a high parasite load would block the differentiation of naïve T cells towards a type 1 phenotype, which might be required to switch from a dominant type 2 to type 1 phenotype. Several previous studies have shown that transfer of splenocytes, CD3⁺ T cells, or CD4⁺ T cells from naïve mice into either nude or *scid* mice, followed by a challenge with *L. major*, can promote a healing infection (19–21, 23, 30, 39). The striking observation from these previous studies is that such adoptive transfer is protective even in genetic backgrounds (e.g., BALB/c) where the donor mice would be susceptible. Only by transferring large numbers of cells can one reconstitute a BALB/c *scid* mouse to exhibit a susceptible phenotype (21). At the doses of cells we used in this study, we anticipated that *scid* recipients would be resistant to *L. major* infection, which turned out to be the case. Data on the effects of parasite dose suggest that naïve cells might develop a Th2 phenotype when they are transferred into *scid* mice with established *L. major* infections (3), in contrast to what occurs after transfers into uninfected mice. However, we found that naïve T cells transferred to *scid* mice were able to provide significant control over the infection. Similarly, naïve T cells were able to provide some protection in nude mice (22). This finding raises the question of why parasite dose influences T-helper-cell development at the initiation of the infection (3) but not in the studies described here. A likely explanation for these differences is that, although we transferred a large number of cells into *scid* mice, the total number of T cells present in the *scid* recipients were still substantially less than that in the BALB/c donors. We argue that the influence of parasite dose is critically dependent on T-cell numbers. Indeed, this idea is consistent with a large body of literature on leishmaniasis where any treatment that decreases the number of T cells present in BALB/c mice leads to resistance (reviewed in reference 34). If one assumes that IL-12 is limiting, then sufficient IL-12 might be present to promote a type 1 response when a low number of cells respond but not when a large number of cells respond.

Our data suggest that either getting rid of an ongoing type 2 response or decreasing parasite load may be important in successfully treating patients with chronic leishmaniasis. Indeed, a previous study found that CD4⁺-T-cell depletion accompanied by IL-12 treatment (or anti-IL-4 monoclonal antibody treatment) can promote resolution of lesions in BALB/c mice infected for 3 weeks with *L. major*, which had lesions of 0.5 to 1 mm in diameter (11). Our findings that a type 1 cell population could heal *L. major*-infected *scid* mice with established lesions that were ≥3 mm suggests that it may be possible to successfully treat leishmaniasis even when the parasite load is quite high.

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