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Inhibition of IL-2 Induced IL-10 Production as a Principle of Phase-Specific Immunotherapy

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Leishmania donovani, a protozoan parasite, inflicts a fatal disease, visceral leishmaniasis. The suppression of antileishmanial T cell responses that characterizes the disease was proposed to be due to deficiency of a T cell growth factor, IL-2. We demonstrate that during the first week after L. donovani infection, IL-2 induces IL-10 that suppresses the host-protective functions of T cells 14 days after infection. The observed suppression is concurrent with increased CD4+ glucocorticoid-induced TNF receptor+ T cells and Foxp3 expression in BALB/c mice, implicating IL-2-dependent regulatory T cell control of antileishmanial immune responses. Indeed, IL-2 and IL-10 neutralization at different time points after the infection demonstrates their distinct roles at the priming and effector phases, respectively, and establishes kinetic modulation of ongoing immune responses as a principle of a rational, phase-specific immunotherapy. The Journal of Immunology, 2006, 177: 4636–4643.

Of the basic selection pressures that guided the co-evolution of the parasites and the hosts was the need to evade or eliminate each other, respectively (1). Although Leishmania replicates as amastigotes within the macrophages and evades their antileishmanial functions (2), macrophages present leishmanial Ags to antiparasite T cells (3). Although immune evasion promotes, host-protective T cells ameliorate the infection. Thus, the Leishmania-macrophage interactions are poised to be a dynamic one, regulating immune response parameters differently at different phases of the antileishmanial immune response. Indeed, during the first week of Leishmania major infection, the priming phase when the infection is established, CD4+ T cells release a wide array of cytokines, including IL-2 and IFN-γ (3). These two cytokines were proposed to execute a transient host-protective immunity (3), but during the ensuing effector phase, the host-protective T cell functions are suppressed (3, 4). The observations imply that the immune responses during the priming phase, the first week after infection, may critically influence the onset of the T cell suppression, the characteristic feature of chronic Leishmania donovani infection. However, the mechanism of the T cell suppression remains unknown. Because IL-2 was originally identified as a T cell growth factor, IL-2 infection was proposed to be due to suppressed IL-2 production (6, 7). According to some reports, visceral leishmaniasis patients have decreased IL-2 production (8, 9). By contrast, according to a report on visceral leishmaniasis patients, IL-2 production by nonadherent cells in response to leishmanial Ag was not reduced (10); and in susceptible mice, IL-2 administration during the first week after the infection had no antileishmanial effects (M. Bodas, N. Jain, and B. Saha, unpublished observation). Thus, the role of IL-2 in the induction of host-protective T cells in the first week and in the later period after L. donovani infection remains unknown. Therefore, the effects of anti-IL-2 plus anti-IL-2R Ab treatment of BALB/c mice, a susceptible host, on the course of L. donovani infection were used to probe the dynamicity and evolution of antileishmanial T cell responses during the progressive infection.

Materials and Methods

Leishmania parasite and animals

BALB/c mice were from The Jackson Laboratory and were maintained in the National Centre for Cell Science’s Experimental Animal Facility. Mice were infected (i.v.) with 2 × 107 L. donovani stationary-phase promastigotes (strain LV9). At the end of infection, as indicated, the mice were euthanized and the spleens were collected and weighed. The cut surface of a transverse section of the spleens was stamped onto grease-free microscope glass slides. The stamps were dried, methanol fixed, and Giemsa stained. The slides were examined under a light microscope (E600; Nikon) for enumerating the number of amastigotes per 1000 host cell nuclei. The parasite load was expressed as Leishman-Donovan unit, which is calculated by multiplying the spleen weight (in grams) by the number of amastigotes per 1000 host cell nuclei (11). The experiments accorded with the Committee for the Purpose of Control and Supervision on Experiments on Animals-approved protocols.

Reagents

The Abs and the cytokines were procured from BD Pharmingen and Santa Cruz Biotechnology. Nylon wool was from Robbins Scientific, and the anti-CD3 (clone HB11674; DNAX), anti-IL-10 (clone HB 10739; DNAX), and anti-CD25 hybridomas were from American Type Culture Collection. The Abs were purified using protein G columns. The isotype-specific Abs were procured from BD Pharmingen.

Kinetics of CD4+ T cell proliferation during progressive L. donovani infection

CD4+ T cells were purified from the naive and L. donovani-infected mice on various days after infection. CD4+ T cells were purified (98%) using the murine CD4+ T cell enrichment mixture, as per the manufacturer’s protocol. CD4+ T cells were incubated in 96-well plate at 2 × 105 cells/well with anti-CD3 (0.5 μg/ml) plus anti-CD28 (2 μg/ml) for 60 h.
were pulsed with 1 μCi of [3H]thymidine (BRIT) for 12 h, and proliferation was assessed using liquid scintillation counter (TopCount; Packard Life Sciences).

T cell suppression assay
A total of 5 × 10⁴ CD4⁺ T cells was cocultured with 2.5 × 10⁴ CD4⁺ T cells isolated from the spleen of mice after different days of infection, as indicated (Fig. 1C), and anti-CD3 (0.5 μg/ml) plus anti-CD28 (2 μg/ml) Abs. In other experiments, the indicated numbers of splenic CD4⁺ T cells were added from the naive or L. donovani-infected mice 3 wk after infection (Figs. 2E and 3D). The suppression was expressed as the percentage of decrease of the [3H]Thid incorporation observed in control cultures.

Cytokine ELISA
IL-2, IL-4, IFN-γ, and IL-10 in culture supernatants were assayed by ELISA using paired mAbs (BD Pharmingen).

RT-PCR IL-10 and FoxP3
RT-PCR was performed, as described (12). The primers used are as follows: IL-10 forward, 5’-TCA CTC TTC ACC TGC TCC AC-3’ and reverse, 5’-TCA CTC TTC ACC TGC TCC AC-3’; FoxP3 forward, 5’-CAC CCT ACG ATG CCT TCA G-3’ and reverse, 5’-CAT TTG CCA GCA GTG GGT AG-3’; for equal loading control, β-actin primers forward, 5’-CAG /H11032 TCA CTC TTC ACC TGC TCC AC-3’ and reverse, 5’-TTT CTG CAT CCT GTC GGC AAT-3’ were used.

Flow cytometry
Splenocytes from the uninfected and infected BALB/c mice were stained with FITC- or PE-conjugated mAb to glucocorticoid-induced TNF receptor (GITR), CD25, and CD4, as indicated, and were analyzed by a FACs Vantage (BD Biosciences). The Abs were procured from BD Pharmingen.

IL-2 is required for the generation of the T cells that suppress naive T cell proliferation
Naive BALB/c mice-derived CD4⁺ T cells were stimulated with anti-CD3 plus anti-CD28 in presence or absence of IL-2- or anti-IL-10-neutralizing Ab, followed by a rest for 3 days and restimulations with anti-CD3 plus anti-CD28 for [3H]thymidine incorporation assay.

Macrophage-T cell coculture for parasite-killing assay
Thioglycolate-elicited peritoneal macrophages (5 × 10⁴/well) were cultured with FITC- or PE-conjugated mAb to glucocorticoid-induced TNF receptor (GITR), CD25, and CD4, as indicated, and were analyzed by a FACs Vantage (BD Biosciences). The Abs were procured from BD Pharmingen.

Preparation of crude soluble Ag (CSA) and delayed-type hypersensitivity (DTH) response
The stationary-phase promastigotes were washed in PBS and were subjected to freeze-thaw cycles for six times, followed by sonication, as described earlier (13). The suspension was clarified by microfuging (Brinkman Instruments) at 40°C for 10 min. The supernatants were filtered through a 0.22-μm filter, and the proteins were estimated. The supernatant was used as the leishmanial CSA.

The DTH response was measured 24 h after the s.c. injection of CSA (40 μg) in one of the hind footpads of naive or L. donovani-infected mice. The difference in the thickness between the hind footpad that received the Ag and the other one that received saline was the net swelling providing the measure of DTH response.

For adoptive transfer of DTH by CD4⁺ T cells, the cells from the naive or infected BALB/c mice on different days of infection, as indicated, were transferred s.c. into the left hind footpad of naive BALB/c mice with 40 μg of CSA (2 × 10⁵ cells/mouse). Swelling was measured 24 h after the Ag injection. In some experiments, to test the suppressive effects of the day 21 postinfection CD4⁺ T cells, as described, the indicated number of the T cells was transferred (Fig. 4B).

Statistical analyses
The in vitro experiments were performed at least in triplicates. A minimum of five mice per group was used for any in vivo experiment. The results are described as mean ± SD. The significance of difference between the means was determined by Student’s t test.

Results
L. donovani infection progresses in two phases
We infected BALB/c mice with L. donovani (11) and assessed the weekly progression of infection and T cell functions. We observed that the infection progressed in two phases: during the first 2 wk after the infection, the increase in splenic parasite burden was slow, but during the late phase starting from 14 days after infection, the parasite burden increased rapidly (Fig. 1A). The DTH response to leishmanial Ags was high during the first 2 wk, but died down from 14 days after the infection (Fig. 1A). Similarly, the CD4⁺ T cells proliferated well in response to TCR stimulation during the first 2 wk after infection, but were suppressed in the effector phase (Fig. 1B). In fact, the CD4⁺ T cells isolated from the infected mice were cocultured with the naive CD4⁺ T cells in presence of anti-CD3 and anti-CD28 Ab. It was observed that the CD4⁺ T cells from 14 days postinfection onward suppressed the proliferation of naive CD4⁺ T cells (Fig. 1C). The observed suppression of CD4⁺ T cell proliferation was not due to decreased expressions of CD3 and CD28, as the expressions of CD3 and CD28 on CD4⁺ T cells from both naive and L. donovani-infected mice were comparable (Fig. 1D). These observations suggest that during the first week of L. donovani infection, when the Ag-specific T cells are primed, the host-protective antileishmanial response was intact, resulting in slow parasite growth. However, 2 wk after infection, the host-protective effector functions of these T cells were suppressed by the CD4⁺ T cells with suppressor activities, resulting in the rapid rise in splenic parasite load.

The suppressor activities are contributed by regulatory T (t-reg) cells during L. donovani infection
Because the t-reg cells are shown to have suppressor activity in a variety of settings, including L. major infection (12, 14–16), we examined whether t-reg cells are phenotypically identifiable in spleen 21 days after the infection. We observed that the numbers of CD4⁺ T cells expressing CD25 (IL-2R-α that binds IL-2), a marker of t-reg cells (12), were comparable between the uninfected and L. donovani-infected mice (Fig. 2A). A previous report also suggested that in L. donovani infection, IL-2 binding by T cells is not impaired (17). However, the number of GITR⁺CD4⁺ T cells increased (Fig. 2B), but the Foxp3 expression remained unchanged.
IL-2 IS A SUSCEPTIBILITY FACTOR IN Leishmania INFECTION

(20). The observed rapid parasite growth could be due to less IFN-γ production in that phase of antileishmanial T cell response. Finally, there could be active suppression of the host-protective antileishmanial T cell response due to exaggerated production of counteractive disease-promoting cytokines such as IL-4 and IL-10 (21, 22). Therefore, we examined the kinetics of the production of these cytokines by anti-CD3 plus anti-CD28-stimulated CD4+ T cells during the course of L. donovani infection. First, the production of IL-2 was increased till day 14 after infection, after which its level was maintained (Fig. 3A), suggesting that the observed suppression was not due to deficiency in IL-2 production. In fact, IL-2 supplementation failed to restore CD4+ T cell proliferation in response to anti-CD3 plus anti-CD28 (Fig. 3B). In contrast, the CD4+ T cells from 7 days postinfection produced a higher IFN-γ as compared with that observed during the late phase of infection (Fig. 3A). In contrast, IL-4 production increased till day 14 postinfection and remained steady during the later course of infection (Fig. 3A), whereas the production of IL-10 continued to increase during the course of infection (Fig. 3A). These observations suggested that the observed suppression of CD4+ T cell proliferation was due to exaggerated IL-4 and IL-10 production. Therefore, we assessed the relative importance of these two cytokines in the T cell proliferation assays. It was observed that IL-10, but not IL-4, neutralization restored the CD4+ T cell proliferation significantly (Fig. 3C). In fact, IL-4 appeared to exert antiproliferative effect on naive CD4+ T cells (Fig. 3C). Similarly, the suppression of naive CD4+ T cell proliferation by the infected CD4+ T cells was prevented by IL-10 neutralization, but not by IL-2 supplementation (Fig. 3D). These observations suggested that IL-10 had an antiproliferative effect and that the suppression of CD4+ T cell proliferation was affected by IL-10, but...
Indeed, depletion of CD4⁺CD25⁺ T cells restored the T cell proliferation significantly in response to anti-CD3 plus anti-CD28 stimulation, and addition of CD4⁺CD25⁺ T cells decreased the proliferation; the suppression was preventable with the anti-IL-10 Ab (Fig. 4A). Similar IL-10-dependent suppressor effects of CD4⁺CD25⁺ T cells were observed in the adoptive DTH transfer experiments as well (Fig. 4B). The depletion of CD25⁺ T cells restored the antileishmanial effect of CD4⁺ T cells isolated from the 21-day infected mice, and addition of the titrated numbers of CD4⁺CD25⁺ T cells back to the macrophage-T cell coculture reinstated the suppression of the antiparasitic effect of those CD4⁺CD25⁺ T cells (Fig. 4C). In addition, these CD25⁺ T cells failed to reinstate the suppression in presence of the anti-IL-10 Ab, suggesting that the CD25⁺ T cell-secreted IL-10 was responsible for the suppression of the host-protective effect of the CD4⁺ T cells.

IL-10 abrogates host-protective function of T cells

To test the antiproliferative effect of IL-10, CD4⁺ T cells were incubated with the indicated amount of IL-10 for 12 h, followed by stimulation with anti-CD3 plus anti-CD28. It was observed that the IL-10-pretreated CD4⁺ T cells had a significantly less proliferation than the untreated cells (Fig. 5A), indicating an antiproliferative effect of the cytokine. Thus, it is possible that IL-10 prevents the expansion of the host-protective T cells inhibiting the host-protective functions.

To test the role of IL-10 in the prevention of host-protective T cell functions, we used the adoptive transfer of DTH response against leishmanial Ags (13) and parasite-killing assay using macrophage-T cell coculture system. It was observed that the CD4⁺ T cells from the 21 days postinfection mice inhibited the antileishmanial DTH transferred by CD4⁺ T cells from 10 days postinfection mice and that anti-IL-10 Ab administration rescued the antileishmanial response significantly (Fig. 5B). Similarly, the 24-h infected macrophages were cocultured for additional 48 h with the 10 days postinfection or 21 days postinfection CD4⁺ T cells or in combination, as indicated, and in presence or absence of anti-IL-10

FIGURE 3. CD4⁺ T cell-expressed IL-10 mediates the suppressor function. A, Cytokine profile of splenic CD4⁺ T cells from naive and infected BALB/c mice. The cells (10⁷/well in a 96-well plate) were stimulated with anti-CD3 (0.5 μg/ml) plus anti-CD28 (2 μg/ml) for 36 h. The cell culture supernatants from anti-CD3 plus anti-CD28-stimulated CD4⁺ T cell cultures were assayed for the cytokines by ELISA. B, IL-10 abrogates the suppressive effect of the cytokine. Thus, it is possible that IL-10 prevents the expansion of the host-protective T cells in the adoptive DTH transfer experiment.

FIGURE 4. CD25⁺ T cells in the day 21 infected spleen exert the suppressive effect. A, As described in Materials and Methods, 5 × 10⁶ naive CD4⁺ T cells were cocultured with 2.5 × 10⁵ CD4⁺ or CD4⁺CD25₂ or CD4⁺CD25⁺ cells isolated from the spleen of mice 21 days after infection (D21), and were stimulated with anti-CD3 (0.5 μg/ml) plus anti-CD28 (2 μg/ml) Abs in presence or absence of anti-IL-10 Abs, as indicated. The proliferation assay was performed by a standard [³H]Tdr incorporation assay (*, p < 0.001). B, CD4⁺CD25⁺ T cells from 21-day infected BALB/c mice suppress the adoptively transferable DTH response. The CD4⁺ T cells, unfragmented or fractionated into CD25⁺ or CD25⁻ cells, as indicated, from the 21-day infected BALB/c mice were transferred s.c. into the left hind footpad of naive BALB/c mice with 40 μg of CSA (2 × 10⁶ cells/mouse) in presence or absence of anti-IL-10 Ab (100 μg/mouse). Swelling was measured 24 h after the Ag injection (*, p < 0.001). C, Twenty-one-day infected mice suppress the parasite control. As described in Materials and Methods, the BALB/c-derived peritoneal macrophages were cocultured with the CD4⁺ T cells, either as such or depleted of CD25⁺ T cells or with the addition of the indicated numbers of CD4⁺CD25⁺ T cells, in presence of anti-IL-10 Ab (*, p < 0.002; 10 μg/ml). The control Ab that matched the isotype of the anti-IL-10 Ab did not have any effect on the CD4⁺CD25⁺ T cell-regulated parasite growth in macrophages. The error bars represent mean ± SD. The experiments were performed at least three times, and representative data are shown.
IL-2 deficiency as proposed (6, 7). On the contrary, because the IL-2 production is maintained throughout the infection, it is possible that IL-2 may play a role in the generation of the T cells with suppressor activity (13, 23, 24).

IL-2 is required for IL-10-dependent suppressor activity

We tested whether the presence of IL-2 during primary stimulation resulted in decreased proliferative response upon restimulation. It was observed that the presence of IL-2 during primary stimulation decreased the proliferation upon second stimulation (Fig. 6A). The inhibition was prevented by IL-10 neutralization during primary stimulation (Fig. 6A), suggesting that IL-10 induced during primary stimulation might affect the T cells, as shown earlier (Fig. 5A). Indeed, IL-2 induced IL-10 in T cells in a dose-dependent manner (Fig. 6B).

Because IL-2 induced IL-10 that suppressed the host-protective T cell responses, we examined the therapeutic potential of anti-IL-2 plus anti-IL-2R Ab or anti-IL-10 Ab administration in L. donovani infection in BALB/c mice. We observed that administration of the anti-IL-2 plus anti-IL-2R Ab or anti-IL-10 Ab had significantly ameliorating effects (Fig. 6C). However, the protective effect of anti-IL-2 plus anti-IL-2R Ab was significantly lost at the late phase of infection. In contrast, anti-IL-10 Ab or anti-IL-10 and anti-IL-2 + anti-IL-2R together had a lasting protective effect (Fig. 6C). To find out the mechanism of such protective effect, we assayed IFN-γ and IL-10 production by CD4+ T cells in these mice. It was observed that compared with those from the control mice, the Ab-treated mice had higher IFN-γ, but less IL-10 (Fig. 6D). Like the parasite load, the effect of IL-10 neutralization had a longer lasting effect on the cytokine production by T cells. Therefore, taken together, these data suggested that IL-2 was crucial at the initial priming phase for the induction of IL-10-producing T cells. Because IL-10 works primarily at the effector phase establishing the observed immunosuppression 14 days after infection, it is possible that such treatments with anti-IL-2 plus anti-IL-2R or with anti-IL-10 may have phase-specific effects.

IL-2 works at the priming phase, whereas IL-10 works at the effector phase

To decipher whether IL-2 and IL-10 had any phase-specific effects, we administered the Abs at different time points after L. donovani infection, as indicated. It was observed that the early treatment with anti-IL-2 plus anti-IL-2R had a significant disease-ameliorating effect, but the delay till the second week of infection significantly diminished the protective effect (Fig. 7A). In contrast, even a delayed administration of anti-IL-10 Ab alone or in combination with anti-IL-2 plus anti-IL-2R Ab offered a significant host protection (Fig. 7A). Therefore, the result suggests that IL-2 works primarily at the priming phase, while IL-10 works in the effector phase. However, the protection in both the cases was associated with low IL-10, but high IFN-γ production by the CD4+ T cells (Fig. 7B), suggesting that the suppressive functions were significantly deterred and the host-protective functions of these T cells might have recovered. Next, we isolated CD4+ T cells from the infected mice (control) or from the infected mice that were treated with the indicated Abs immediately after infection or delayed till 14 days after infection and assessed their ability to transfer DTH to syngeneic naive recipients, control amastigote number in macrophages, and suppress the naive CD4+ T cell proliferation. It was observed that the delay in beginning the treatment with anti-IL-2 plus anti-IL-2R Ab significantly reduced the ability of these CD4+ T cells to adoptively transfer the antileishmanial DTH (Fig. 7C) and to control amastigote number in macrophages (Fig. 7D). These
FIGURE 6. IL-2 is required for IL-10-dependent suppressor activity. A, CD4\(^+\) T cells were cultured with the indicated stimuli for 3 days. The cells were given 3 days of rest, followed by anti-CD3 (0.5 \(\mu\)g/ml) plus anti-CD28 (5 \(\mu\)g/ml) stimulation for 48 h. Primary culture of these T cells in absence of IL-2 induced higher proliferation during restimulation. B, IL-2-induced IL-10 production from anti-CD3 plus anti-CD28-stimulated CD4\(^+\) T cells from BALB/c mice, as assessed by RT-PCR (left panel) and ELISA (right panel). The splenic CD4\(^+\) T cells from naive BALB/c mice were stimulated with anti-CD3 plus anti-CD28 and different doses of IL-2, as indicated, for 8 h (for RT-PCR) or 36 h (for ELISA). After 8-h stimulation, the cells were processed for RNA isolation, and the RT-PCR for IL-10 was performed using IL-10-specific primers. The supernatants were collected from the 36-h-old culture and assessed for IL-10 content by ELISA. C, Anti-IL-2 plus anti-IL-2R Ab or anti-IL-10 Ab administration from the day of infection imparted resistance to the BALB/c mice (\(\ast\), \(p<0.001\)). BALB/c mice were infected with the stationary-phase promastigotes (2 \(\times\) 10\(^7\)/mouse) and were treated with anti-IL-2 plus anti-IL-2R and/or anti-IL-10 Ab (each Ab 50 \(\mu\)g/mouse, i.p.) for 5 days. The control mice received only infection and isotype-matched control Abs (50 or 100 or 150 \(\mu\)g/mouse, depending on how much the experimental group received). D, Thirty-five days after infection, the mice were sacrificed and splenic CD4\(^+\) T cells were isolated and stimulated with anti-CD3 plus anti-CD28, as described above. After 36-h culture, the supernatants were estimated by ELISA. The treatment resulted in less IL-10, but higher IFN-\(\gamma\) production by the CD4\(^+\) T cells from the treated mice, as compared with the infected, untreated controls.

FIGURE 7. IL-2 works at the priming phase, whereas IL-10 works at the effector phase. A, A delay in anti-IL-2 plus anti-IL-2R treatment lost its efficiency to reduce splenic parasite burden, but cotreatment with anti-IL-10 Ab restored the efficacy (\(\ast\), \(p<0.001\)). The mice were infected on day 0. Some mice were treated i.p. with the indicated Abs (at doses as described above) from days 0 to 7; the others received the indicated Abs from days 8 to 14, and days 15 to 21. The mice were sacrificed 28 days after infection. The splenic parasite load was determined by the stamp-smear method, as described in Materials and Methods. B, A delayed administration of anti-IL-2 plus anti-IL-2R Ab resulted in the loss of IFN-\(\gamma\)-inducing ability. The splenic CD4\(^+\) T cells from the mice described in A were isolated and were stimulated with anti-CD3 plus anti-CD28 for 36 h, as described in Materials and Methods. The cell culture supernatants were assayed for IL-10 and IFN-\(\gamma\) contents by ELISA. C, A delayed administration of anti-IL-2 plus anti-IL-2R Ab resulted in the loss of adoptively transferable antileishmanial DTH function. The mice from the experiment described in A were sacrificed 28 days after infection. The splenic CD4\(^+\) T cells were isolated and adoptively transferred to a hind footpad of naive syngenic recipients with CSA (40 \(\mu\)g/mouse) in a total volume of 50 \(\mu\)l. The swelling of the injected footpad was compared with that of uninjected footpad. The net swelling was taken as the DTH response. Five mice were used per group of recipients. D, A delayed administration of anti-IL-2 plus anti-IL-2R Ab resulted in the loss of parasite-killing functions. Anti-IL-10 rescues the function. The CD4\(^+\) T cells from the experiment described in A were cocultured with the L. donovani-infected macrophages for 24 h, as described in Materials and Methods. The macrophages were then stained with Giemsa and enumerated under a light microscope for amastigote numbers per 100 macrophages. E, A delayed administration of anti-IL-2 plus anti-IL-2R Ab resulted in the reinstatement of the suppression. The CD4\(^+\) T cells were isolated from the mice groups, which were treated during the first or third week after infection, as shown, with the indicated Abs, as described above. The CD4\(^+\) T cells (2.5 \(\times\) 10\(^3\)) were cocultured with the CD4\(^+\) T cells (5 \(\times\) 10\(^3\)) from naive mice, as described in Materials and Methods. The data, mean \(\pm\) SD, presented here are representatives of three individual experiments.
T cells, however, suppressed the proliferation of naive CD4+ T cells (Fig. 7E).

Thus, the results altogether suggest that during the *L. donovani* infection, IL-2 significantly controls antileishmanial activity of CD4+ T cells. During the first week of infection, when the priming of these cells takes place, IFN-γ is produced that executes the host-protective functions. In contrast, IL-2 induces the T cells with the regulatory functions that are mediated by IL-10. In corroborations with these findings, treatment with anti-IL-2 plus anti-IL-2R works early after infection, whereas the treatment with anti-IL-10 Ab works even late after infection. The ameliorating effects of these phase-specific treatments were associated with the recovery of the host-protective antileishmanial T cell functions. Therefore, our observations suggest kinetic modulation of an ongoing immune response as a principle of the phase-specific immunotherapy.

**Discussion**

The host’s immune response to *Leishmania*, a protozoan parasite, is poised to be a dynamic one just as their history of coevolution appears to be. Although immediately after infection into the host the parasite endeavors to establish the infection, the host mobilizes the T cells against the parasite, resulting in the initial control of the parasitic growth. Later, the parasite uses the host’s immune control mechanism to its own benefit, resulting in its rapid growth. One such immune control mechanism is IL-2-mediated regulation of the CD4+ T cell response. Contrary to the proposition that IL-2 might play a host-protective role in *Leishmania* infection (7), we demonstrate the disease-promoting role of IL-2 in *L. donovani* infection. IL-2 may play a similar role in *L. major* infection as well (25). One possible mechanism of such discrepancy could arise from the form of the parasite used for infection; although Murray et al. (7), used amastigotes, we and Heinzel et al. (25) used promastigotes. However, in our studies, when we compared the effects of promastigotes and amastigotes, we did not find any significant differences in terms of the T cell subset modulation and DTH response (M. Bodas and B. Saha, data not shown).

As far as the role of IL-2 in experimental visceral leishmaniasis is concerned, during the early phase of infection, IL-2 induces both IFN-γ- and IL-10-secreting T cells, perhaps as a function of the available IL-2 concentration, but IL-10 suppresses the IL-12 production by the APCs by differential regulation of MAPKs (26), reduces IL-12R expression on T cells (B. Saha, unpublished observation), and impairs IFN-γ responsiveness of macrophages, resulting in the suppression of IFN-γ-mediated amastigote elimination. Being residents of the IL-10-rich splenic microenvironment, these T cells do not proliferate and suppress the activation of the infiltrating naive T cells, a phenomenon reminiscent of infectious tolerance (27). Thus, *Leishmania* exaggerates and exploits the host’s IL-10-dependent autoregulatory or a feedback servo-mechanism that prevents excessive inflammation-mediated host-tissue pathology, but supports unhindered parasite growth (25, 26). Such a mechanism may operate in those viral infections in which the viral IL-10 (28, 29) may skew the immune response in a similar way. It is possible that our proposed phase-specific immunotherapeutic interferences with the IL-2-dependent expansion of the IL-10-secreting cells early after infection and with their effector functions at a later phase. The previously reported experiments with IL-10 blockade, albeit without any kinetic analyses of the evolution and function of such CD4+ T cells, might also work the same way, resulting in the observed host-protective effect against the parasite (30, 31).

Because parasites coevolved with their hosts to evade the host’s immune responses, the immune system coevolved to adapt to the pressure exerted by these persistent pathogens. Therefore, during an ongoing immune response in an infection, particularly where the constantly changing parasite load modulates the immune response, the host-parasite interaction is rather dynamic than being static and steady state. Based on the kinetics of evolution of the T cells with suppressor functions and their mechanism of action, we demonstrate a novel stage-specific immunotherapy of leishmaniasis that may be applicable to other diseases, such as allograft rejections and autoimmune diseases, particularly where the Ags persist.

**Disclosures**
The authors have no financial conflict of interest.

**References**