Immunosuppression and in Trypanosome-Elicited γIFN-Cells in the Production of IL-4, IL-10, and IFN-γ and in Trypanosome-Elicited Immunosuppression

Jude E. Uzonna, Radhey S. Kaushik, Ying Zhang, John R. Gordon and Henry Tabel

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Experimental Murine Trypanosoma congolesi Infections. II. Role of Splenic Adherent CD3+ Thy1.2+ TCR-αβ– γδ– CD4+8– and CD3+ Thy1.2+ TCR-αβ– γδ– CD4–8– Cells in the Production of IL-4, IL-10, and IFN-γ and in Trypanosome-Elicited Immunosuppression1

Jude E. Uzonna, Radhey S. Kaushik, Ying Zhang, John R. Gordon, and Henry Tabel2

Trypanosome-induced suppression of T and B cell responses to parasite-related and -unrelated Ags is considered a major mechanism of evasion of the host’s immune defenses by the parasite. Reduced T and B cell responses have been attributed to suppressor T cells, suppressor macrophages, or both. We have previously shown that endogenously produced IL-10 and IFN-γ mediate the suppression of T cell responses in Trypanosoma congolesi-infected mice. Here, we show for the first time that splenic CD3+ Thy1.2+ αβ– γδ– CD4+8– and CD3+ Thy1.2+ αβ– γδ– CD4–8– cells that copurify with plastic-, nylon wool-, or Sephadex G-10-adherent cell populations, in synergy with adherent Thy1.2– cells, are the major producers of IL-4, IL-10, and IFN-γ in T. congolesi-infected mice. We further demonstrate the involvement of these cells in the suppression of T cell proliferative responses to mitogen and in B cell responses to a parasite-unrelated Ag. The Journal of Immunology, 1998, 161: 6189–6197.

African trypanosomes are protozoan parasites that cause disease and death in humans and animals. Trypanosoma congolesi infections result in severe acute or chronic debilitating disease in cattle and other domestic animals. T. congolesi is an extracellular parasite living in the bloodstream of the host, and as such, to survive it must have developed very sophisticated mechanisms to evade the host’s immune defenses. Trypanosome-induced immunosuppression is considered one of the major mechanisms of evasion from the host’s immune defenses by the parasite (reviewed in Refs. 1–4). Immunosuppression has been described in both natural and experimental infections of domestic mammals as well as laboratory rodents and is suggested to be responsible for the poor responsiveness of trypanosome-bearing hosts to vaccinations (5). Lymphocytes from infected hosts give a lower response to T cell mitogens or allogeneic cells in vitro (4). Both in vivo (1, 3) and in vitro (6, 7) T cell-dependent and T cell-independent B cell responses are suppressed. Similarly, suppression of Ag-specific T cell proliferative responses to unrelated Ags has been reported in infected animals (8–10). There is as yet no consensus about the mechanisms underlying trypanosomically-induced suppression of lymphoid cells. Reduced T and B cell responsiveness has been attributed to suppressor T cells (6, 8, 9, 11), suppressor macrophages (2, 4, 12–14), or a combination of these cell types (1, 15).

In response to trypanosomal Ags generated during infection, the host’s immune system produces an array of molecules, including cytokines. In Trypanosoma brucei infections, endogenous IFN-γ produced by CD8+ T cells has been associated with the down-regulation of lymph node cell proliferative responses to mitogens (10) and enhanced susceptibility to the disease (16, 17). Depletion of CD8+ cells has been shown to suppress both the growth of T. brucei and IFN-γ secretion in infected rodents (16). In this system it was reported that T. brucei releases a molecule that binds to the CD8 molecule on T cells and polyclonally activates the CD8+ T cells to secrete copious amounts of IFN-γ, which, in turn, has been reported to stimulate parasite growth (17).

BALB/c mice are highly susceptible to experimental T. congolesi infections, while C57BL/6 mice are relatively resistant, as measured by the degree and pattern of parasitemia and the survival period (18, 19). Genetic analysis indicates that the efficiency of control of the first wave of parasitemia in mice infected with T. congolesi correlates with the survival period (19). The patterns of cytokine responses during some parasite infections determine or are at least strongly correlated to the susceptibility of the host (20). We have recently shown that during experimental T. congolesi infections, the plasma of the highly susceptible BALB/c mice as well as the supernatants fluids of their spleen cell cultures contain significantly higher levels of IL-4, IL-10, and IFN-γ than those from resistant C57BL/6 mice (21, 22). In addition, we found that IL-10 and IFN-γ contributed to the suppression of splenocyte proliferative responses to Con A in these mice (21, 22). Furthermore, in vivo neutralization of these cytokines significantly enhanced the resistance of the highly susceptible BALB/c mice to T. congolesi infections, as measured by reductions in parasitemia and increased mean survival periods. However, in contrast to the report on T. brucei (17), we have been unable to demonstrate any growth stimulatory effect of either IFN-γ or IL-10 on T. congolesi (23), indicating that the beneficial effects of in vivo neutralization of these cytokines were indirect at least in part due to a

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trypanosomiasis infections result in severe acute or chronic debilitating disease in cattle and other domestic animals. T. congolesi is an extracellular parasite living in the bloodstream of the host, and as such, to survive it must have developed very sophisticated mechanisms to evade the host’s immune defenses. Trypanosome-induced immunosuppression is considered one of the major mechanisms of evasion from the host’s immune defenses by the parasite (reviewed in Refs. 1–4). Immunosuppression has been described in both natural and experimental infections of domestic mammals as well as laboratory rodents and is suggested to be responsible for the poor responsiveness of trypanosome-bearing hosts to vaccinations (5). Lymphocytes from infected hosts give a lower response to T cell mitogens or allogeneic cells in vitro (4). Both in vivo (1, 3) and in vitro (6, 7) T cell-dependent and T cell-independent B cell responses are suppressed. Similarly, suppression of Ag-specific T cell proliferative responses to unrelated Ags has been reported in infected animals (8–10). There is as yet no consensus about the mechanisms underlying trypanosomically-induced suppression of lymphoid cells. Reduced T and B cell responsiveness has been attributed to suppressor T cells (6, 8, 9, 11), suppressor macrophages (2, 4, 12–14), or a combination of these cell types (1, 15).

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reversal of immunosuppression. Here, we show that in contrast to T. brucei infections, most of the IL-4, IL-10, and IFN-γ production in T. congolense-infected BALB/c mice is mediated by small numbers of splenic CD3+ Thy-1.2+ αβ− γδ− CD4+ 8+ and CD3+ Thy-1.2+ αβ− γδ− CD4− 8− cells that copurify with plastic-, nylon wool-, or Sephadex G-10-adherent cells. We also show that the secretion of these cytokines requires cooperation between Thy-1.2+ and Thy-1.2− spleen cells of infected mice. We further demonstrate that these cells are involved in the in vitro suppression of B cell responses to a T cell-dependent Ag as well as in the suppression of T cell proliferative responses to Con A.

**Materials and Methods**

**Mice**

Female BALB/c (AnNCiBR) (BALB/c) and outbred CD-1 mice were obtained from the Animal Resource Center of the University of Saskatchewan. BALB/c mice were between 8 and 10 wk old, and CD-1 mice were 5–6 wk old. All mice were maintained according to the recommendations of the Guide to the Care and Use of Experimental Animals of the Canadian Council for Animal Care.

**Parasites**

The origin of the T. congolense, variant antigenic type TC13 used in the present study has been previously described (18). Cloned trypanosome populations were stored as frozen stabilates in liquid nitrogen. Parasites were passaged in CD-1 mice as previously described (24). The parasites for infection of BALB/c mice were isolated from the blood of CD-1 mice 3 days after passage by DEAE-union exchange chromatography (25).

**T. congolense lysate**

Parasites isolated by the above method were washed twice (5000 × g, 5 min) with PBS (pH 7.2) supplemented with 10% heat-inactivated FBS. The parasites were subsequently resuspended in complete medium consisting of RPMI 1640 (Sigma, Oakville, Canada) supplemented with 10% FBS, 2 mM L-glutamine, 50 mM 2-ME, and 100 IU each of penicillin and streptomycin (Life Technologies, Grand Island, NY). The parasites (10⁷/ml) were subjected to three cycles of freezing and thawing at −80°C and were sonicated using a Vibra Cell (Sonics & Materials, Danbury, CT) 10 times (30 s each time) at 50% duty cycle at 30-s intervals. The lysates were stored at −80°C until used.

**Experimental design**

BALB/c mice were infected i.p. with 10⁵ organisms of T. congolense variant antigenic type TC13 in 0.1 ml of Tris-saline-glucose supplemented with 10% FBS. Infected mice had a prepatent period of 4 days and developed a fulminating and uncontrollable parasitemia that peaked on day 7 postinfection. Their mean survival period was 8 ± 0.5 days (21, 22). On day 7 postinfection, groups of six to eight infected and control uninfected mice were killed with CO₂, and their spleens were made into single-cell suspensions by teasing in complete medium, filtered through eight layers of sterile gauze, and washed twice by centrifugation at 600 × g for 5 min.

**Antibodies**

For cellular deletions, the following Abs were used: anti-CD4 (clone GK 1.5, American Type Culture Collection, Manassas, VA), anti-CD8 (clone TIB 211, American Type Culture Collection), anti-Thy-1.2 (clone TIB 99, American Type Culture Collection), anti-TCR-αβ (clone H57-597, Pharmingen, San Diego, CA), anti-TCR-γδ (clone UC7-1D3, Pharmingen), anti-CD3 (clone 17A2, Pharmingen), and anti-NK-1.1 (clone DX5, Pharmingen). For FACS analyses, the following Abs were purchased from Pharmingen: anti-CD4−FITC (clone RM4-5), anti-CD8−PE (clone 53-67), anti-Thy-1.2−PE (clone 30-412), anti-TCR-αβ−FITC (clone H57-597), anti-TCR-γδ−FITC (clone GL3), anti-CD3−FITC (clone 144-2C11), anti-NK-1.1−FITC (clone DX5), anti-CD16/CD32 (FcγRIII/II receptor, Fe Block) mAb and hamster IgG−FITC.

**Depletion of splenocyte subsets**

Splenocyte suspensions (10⁷ cells/ml) in complete medium were incubated on ice for 45 min with previously determined optimal amounts of purified mAb to mouse CD4, CD8, Thy-1.2, CD3, TCR-αβ, TCR-γδ, or NK-1.1. The cells were then washed with RPMI 1640 medium supplemented with 5% FBS (RPMI-5), resuspended in RPMI-5 containing LOW-TOX-M rabbit complement (10/1; Cedarlane, Hornby, Canada) and incubated at 37°C for 45 min. Washed cells were resuspended in complete medium, and aliquots were stained with labeled Abs for FACS analyses. In all cases, the specific cellular deletions were >99% effective, while nontarget cells were not significantly affected by these treatments.

**Separation of cells by adherence or nonadherence to plastic dishes**

Splenocytes from infected mice were separated into plastic-adherent and nonadherent populations as described by Khan et al. (26) with minor modifications. Briefly, the cells (10⁷/ml) in complete medium were seeded into tissue culture-treated plastic petri dishes (Falcon 3001, VWR, Edmonton, Canada) and incubated at 37°C in a 5% CO₂ incubator. After 3 h, the nonadherent cells were removed by pipetting and careful washing, and the adherent cells were dislodged with ice-cold versene (0.02% EDTA in PBS, pH 7.2) and gentle scraping. All cells were washed with complete medium, and their purity was determined by nonspecific esterase (27) and Giemsa staining. On all occasions, the adherent cells were >85% macrophages as determined by their microscopic morphology and <5–8% T cells (CD3+, Thy-1.2+) as assessed by FACS.

**Enrichment of T cells by nonadherence to nylon and Sephadex G-10**

Nylon wool and Sephadex G-10 columns were prepared as previously noted (28, 29). Briefly, packed sterile nylon wool (8 ml) or Sephadex G-10 (30 ml; Pharmacia, Uppsala, Sweden) in 10- or 35-ml syringes, respectively, were equilibrated with complete medium. Then 2 or 5 ml of splenocyte suspension in complete medium (5 × 10⁷ cells/ml) was applied to the nylon wool or Sephadex G-10 columns, respectively. The cells were incubated on the columns for 30 min at 37°C, and the nonadherent (i.e., effluent) cells were obtained by washing the columns with 10 ml (nylon wool) or 20 ml (Sephadex G-10) of complete medium. The nonadherent cells were >80 and 65% (nylon wool and Sephadex G-10, respectively) Thy-1.2+ as assessed by FACS. To obtain the adherent cells, column matrices were dislodged and incubated with warm medium for 10 min at room temperature. The viability of the eluted cells was >95% as assessed by trypan blue dye exclusion. The adherent cells from all columns contained between 8 and 10% Thy-1.2+ cells as assessed by FACS.

**Positive enrichment of CD4+ and Thy-1.2+ cells**

Enrichment of CD4+ and Thy-1.2+ cells was performed by positive selection using MACS technology (Miltenyi Biotec, Auburn, AL). Splenocytes (4 × 10⁷/ml and 3.0 × 10⁷/ml for CD4+ and Thy-1.2+ enrichments, respectively) were washed and resuspended in PBS supplemented with 5% FBS and 2 mM EDTA, pH 7.2, and then incubated for 20 min with either rat anti-mouse Thy-1.2+ or anti-mouse CD4-paramagnetic beads (Milenyi Biotec). Cells were washed in 0.5 ml of buffer, and applied to magnetized MiniMACS Type MS+ columns (Miltenyi). Nonadherent (i.e., negative) cells were collected by washing the magnetized columns with buffer, while the marker-positive cells were eluted by flushing the demagnetized columns with medium. Both effluent and eluate cells were washed twice with complete medium; their viability, as assessed by trypan blue dye exclusion, was always >99%. Positively enriched cells were >97% positive for their respective markers, as assessed by FACS analyses, while the negative (effluent) populations contained between 5 and 8% Thy-1.2+ cells.

**FACS analysis**

Splenocytes (10⁷ cells/ml) in complete medium were blocked for 15 min with rat anti-mouse CD16/CD32 (FcγRIII/II receptor, Fe Block) mAb and incubated for 30 min on ice with the appropriate Abs diluted in FACS wash solution (2% BSA in PBS, pH 7.2). The cells were washed, resuspended in FACS wash, fixed with paraformaldehyde (1% final concentration), and analyzed by flow cytometry using the LYSIS II program (FACScan, Becton Dickinson, Mountain View, CA).

**Splenocyte cultures and assays for cell cooperation and Ag dependency of cytokine secretion**

Splenocytes were cultured at 2.5 × 10⁶ cells/ml in 96-well tissue culture plates (200 µl/well) in a humidified CO₂ incubator. All cultures were harvested after 48 h by centrifugation, and the supernatants were stored at −35°C. In one set of experiments, MACS negatively selected Thy-1.2− cells containing 5–8% residual Thy-1.2+ cells, were depleted of residual Thy-1.2+ cells by treatment with anti-Thy-1.2 Abs and complement, which
removed all FACS-detectable Thy-1.2^+ cells from these populations. MACS positively selected Thy-1.2^+ cells were then cultured either alone or in combination with varying numbers of Thy-1.2^− cells. In another series of experiments, Thy-1.2^+ cells from infected or uninfected mice were cultured with Thy-1.2^− splenocytes from uninfected mice (i.e., T cell-depleted APCs) and pulsed with *T. congolense* lysates (10^6 trypanosome-equivalent/culture).

**Cytokine assays**

The levels of IL-4, IL-10, and IFN-γ in the supernatant fluids of splenocyte cultures were determined by routine sandwich ELISA using commercial recombinant standards and capture-detection Ab pairs (PharMingen) (21, 22). The limits of detection of the ELISAs were 15 pg/ml for IL-4 and 31 pg/ml for IL-10 and IFN-γ. All experiments were repeated at least twice.

**Splenocyte proliferation assay**

Splenocyte proliferation was quantified by the [3H]Tdr uptake assay. Quadruplicate cultures of pooled cells from groups of four uninfected and 7-day-infected mice were cultured at 3 × 10^5 cells/well in 200 μl of complete medium in 96-well flat-bottom plates (Falcon VWR, Edmonton, Canada). To assess the suppressive effects of the Sephadex G-10-adherent cell-depleted APCs, T cell-depleted cultures were cocultured with 10^5 spleen cells from uninfected mice (i.e., T cell-depleted APCs) and pulsed with *T. congolense* lysates (10^6 trypanosome-equivalent/culture).

**Assay for hemolytic plaque-forming cells (PFC)**

B cell responses to SRBC were assayed by a PFC assay using the slide modification of Jerne technique (30). Groups of four mice were primed i.v. with 200 μl of 0.1% SRBC in PBS and were boosted 2 days later with same volume of 10% SRBC. Seven days after the primary immunization, the mice were killed, the spleens were collected, and suspensions of single spleen cells were prepared. The cells (10^7/well) were cultured in 200-μl volumes in 96-well tissue culture plates in the presence of the absence of SRBC (2.5 × 10^5/well). Some cultures received an additional 2.5 × 10^5 undepleted or Thy-1.2-depleted Sephadex G-10-adherent cells from uninfected or infected BALB/c mice. After 4 days of in vitro culture, the cells were harvested, washed, and used for PFC assay against SRBC.

**Statistical analysis**

Data are presented as the mean ± SE. The significance of differences was determined by Student’s *t* test using StatView SE Software (Abacus Concepts, Berkeley, CA).

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1 Abbreviation used in this paper: PFC, plaque-forming cell.
mitogens, and their supernatant fluids were assayed for IL-4, IL-10, and IFN-γ. Treatment with specific Ab and complement. The resulting cell populations as well as whole spleen cell suspensions (splenocytes) were then cultured without IL-4 and 1.5-fold more IL-10 and IFN-γ (Fig. 2). Indeed, the plastic-adherent cells produced 3- to 4-fold more IL-4-, IL-10-, and IFN-γ-producing cells at high levels if any IL-4, IL-10, or IFN-γ was present. Similar results were obtained with either nylon wool- or Sephadex G-10-adherent cells. The set of results presented is one of two similar experiments.

**FIGURE 2.** IL-4-producing (A), IL-10-producing (B), and IFN-γ-producing (C) cells copurify with plastic-, nylon wool-, and Sephadex G-10-adherent cell populations. Splenocytes from BALB/c mice infected with T. congolense for 7 days were separated into adherent and nonadherent populations by incubating on a plastic petri dish for 3 h or passage over nylon wool or Sephadex G-10 columns (see Materials and Methods). Plastic-adherent (Pla-adhr) and -nonadherent (Pla non-adhr) as well as nylon wool- or Sephadex G-10-adherent (nylon adhr or sephad adhr, respectively) and -non-adherent (nylon non-adhr or sephad non-adhr, respectively) cells were cultured for 48 h, and the supernatant fluids were assayed for IL-4, IL-10, and IFN-γ. Cultures of unfractionated spleen cell suspensions (splenocytes) were also included. Plastic-, nylon wool-, or Sephadex G-10-adherent or -nonadherent cells from uninfected mice do not secrete any detectable amount of IL-4, IL-10, or IFN-γ in cultures (data not shown). The set of results presented is one of three similar experiments.

T cell-enriched populations from infected mice secreted almost no IL-4, IL-10, or IFN-γ when cultured without mitogen (Fig. 2, A–C), suggesting that the responsible cytokine-secreting cells were effectively removed by passage through the T cell enrichment columns.

**IL-4-, IL-10-, and IFN-γ-producing cells copurify with cell populations adherent to plastic, nylon wool, or Sephadex G-10.**

Because the column-enriched T cells failed to secrete IL-4, IL-10, or IFN-γ, we hypothesized that the cytokine-producing cells from the first experiments would have copurified with the column-adherent cells. To test this, we cultured both the adherent and nonadherent cells from plastic petri dish, nylon wool, or Sephadex G-10 processes for 48 h and assayed their supernatants for IL-4, IL-10, and IFN-γ. In each case, the nonadherent cells secreted little if any IL-4, IL-10, or IFN-γ (Fig. 2, A–C). In contrast, the adherent cell populations produced each of these cytokines at high levels (Fig. 2). Indeed, the plastic-adherent cells produced 3- to 4-fold more IL-4 and 1.5-fold more IL-10 and IFN-γ than did the unfractionated spleen cells (Fig. 2, A–C), and the nylon wool- and Sephadex G-10-adherent adherent cells produced more IL-10 and as much IFN-γ as did the unfractionated cells (Fig. 2). Neither plastic-, nylon wool-, nor Sephadex G-10-adherent cells from uninfected mice produced detectable levels of IL-4 (<15 pg/ml) or IL-10 or IFN-γ (<31 pg/ml) in culture, indicating that the secretion of these cytokines was dependent on the T. congolense infection.

**FIGURE 3.** Thy-1.2+CD4+8− and Thy-1.2+CD4+CD8− adherent cells produce and/or regulate the production of IL-4, IL-10, and IFN-γ by splenocytes of T. congolense-infected BALB/c mice

Since depletion of CD4+ and Thy-1.2+ cells from splenocytes of T. congolense-infected mice abolished or markedly reduced IL-4, IL-10, and IFN-γ production, it was surprising that cells that copurified with plastic- or matrix-adherent cell populations produced these cytokines at high levels. Such cells are normally considered to comprise mostly macrophages and few other accessory cells (29), none of which secrete IL-4 or IFN-γ. Because further analysis revealed that these adherent cells contained 8–10% Thy-1.2+ cells (data not shown), we repeated the experiments using plastic-adherent cells that we depleted of residual CD4+, CD8+ or Thy-1.2+ cells (Fig. 3, A–C). Depletion of CD4+ cells substantially, but incompletely, reduced IL-4, IL-10, and IFN-γ production by the cells, while depletion of CD8+ cells had no effect on IL-10 production, significantly increased IL-4 secretion (p < 0.01), and minimally decreased IFN-γ secretion (p < 0.05). In contrast, depletion of Thy-1.2+ cells completely abolished the secretion of each of these cytokines by the adherent cells.
Table I. Effects of treatment of matrix-adherent splenocytes from T. congolense-infected mice with various mAbs and complement on IL-10 and IFN-γ secretion

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Concentration of Cytokine (pg/ml)</th>
<th>IL-10</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected splenocytes</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Infected splenocytes</td>
<td>874 ± 17</td>
<td>5,479 ± 111</td>
<td></td>
</tr>
<tr>
<td>Nonadherent cells</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Adherent cells</td>
<td>1,664 ± 51</td>
<td>9,144 ± 230</td>
<td></td>
</tr>
<tr>
<td>Adherent cells, CD3+</td>
<td>174 ± 10</td>
<td>340 ± 46</td>
<td></td>
</tr>
<tr>
<td>Adherent cells, TCR-αβ+</td>
<td>464 ± 36</td>
<td>940 ± 180</td>
<td></td>
</tr>
<tr>
<td>Adherent cells, TCR-αβ+</td>
<td>1,890 ± 51</td>
<td>11,384 ± 411</td>
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</tr>
<tr>
<td>Adherent cells, TCR-γδ+</td>
<td>1,562 ± 51</td>
<td>9,196 ± 122</td>
<td></td>
</tr>
<tr>
<td>Adherent cells, NK1.1+</td>
<td>1,876 ± 89</td>
<td>10,612 ± 312</td>
<td></td>
</tr>
</tbody>
</table>

*Sephadex G-10-adherent cells from T. congolense-infected mice were treated with various mAbs and complement and cultured at 5 x 10^5 cells/well. After 48 h, the supernatant fluids were collected and assayed for IL-10 and IFN-γ by ELISA. The set of results presented is one of three similar experiments. ND, not detected (i.e., below the assay sensitivity."

To determine whether the cytokine-secreting Thy-1.2+ cells that copurified with the adherent cells simply comprised contaminating T cells that could be replaced by conventionally purified T cells, we again depleted the plastic-adherent cells from infected mice of residual Thy-1.2+ cells, but then added back 10% (final concentration) nylon wool-purified T cells (≥80% Thy-1.2+) and assessed the cytokine secretion of these T cell-reconstituted, Thy-1.2-depleted, plastic-adherent spleen cells. This T cell reconstitution restored only about 3% of the IFN-γ synthesis (320 ± 25 vs 10,500 ± 490 pg/ml for Thy-1.2-depleted T cell-reconstituted adherent and undepleted adherent cells, respectively; p < 0.0001) and had no restorative effect on IL-4 or IL-10 synthesis. Thus, the Thy-1.2+ cytokine-secreting cells among the adherent cell populations of the infected mice appeared to be of a unique phenotype. Since Thy-1.2+ cell depletion completely ablated cytokine production, but CD4+ or CD8+ depletion only moderately affected IL-4, IL-10, and IFN-γ secretion, we concluded that there exists among the adherent cells a small, but important, subpopulation of Thy-1.2+ CD4− CD8− cytokine-secreting cells.

The important IL-10- and IFN-γ-secreting Thy-1.2+ splenocytes from T. congolense-infected mice are CD3+, TCR-αβ+, TCR-γδ+, and NK1.1+.

Double-negative (i.e., CD4− CD8−) TCR-αβ+, NK1.1+ (32), and TCR-γδ+ (33, 34) T cells have been reported to play some regulatory roles in many parasitic infections. To further characterize the adherent cytokine-secreting cells, we depleted the matrix-adherent cells of CD3-, αβ-, γδ-, or NK1.1-bearing cells by specific Ab-dependent complement-mediated lysis and again assessed the abilities of the residual cells to secrete IL-10 and IFN-γ. Depletion of CD3+ cells very markedly reduced the ability of the adherent cells to secrete IL-10 and IFN-γ, while depletion of αβ-, γδ-, or NK1.1-bearing cells had no negative effects (Table I). Collectively, these results show that adherent CD3+ Thy-1.2+ αβ− γδ− NK1.1+ CD4− CD8− cells in the spleens of T. congolense-infected BALB/c mice are the major producers of IL-10 and IFN-γ.

IL-4, IL-10, and IFN-γ production requires synergy of Thy-1.2+ and Thy-1.2− cells

An apparent paradox associated with African trypanosomiasis is the observation that despite the impairment of their proliferative capacity, lymphoid cells from infected animals secrete large amounts of cytokines, particularly IFN-γ (4, 10, 17, 21, 22, 35). This has led to speculation that the cytokine-producing cells are induced or activated in an Ag-nonspecific manner, perhaps by a mechanism such as polyclonal T cell activation mediated by a direct interaction of trypanosome-derived molecules with the T cells (36). To address this issue, we positively selected CD4+ and Thy-1.2+ splenocytes from T. congolense-infected BALB/c mice using MACS. While the unfraccionated splenocytes secreted high levels of IL-4, IL-10, and IFN-γ, neither the Thy-1.2-positive (≥97% purity) nor the Thy-1.2-negative populations secreted much of either cytokine relative to the unfraccionated spleen cells (Fig. 4, A–C), suggesting that the production of these cytokines by Thy-1.2+ cells might require cooperation with Thy-1.2− cells. Thus, Thy-1.2+ cells were subsequently cultured either alone or in the presence of varying concentrations of Thy-1.2-negative cells. Supplementation of Thy-1.2− cells from infected mice with Thy-1.2+ splenocytes from infected (Fig. 5), but not uninfected (data not shown), mice resulted in a dose-dependent restoration of cytokine production by Thy-1.2− cells. Thy-1.2− cells from infected mice did not induce Thy-1.2+ spleen cells from uninfected mice to secrete detectable levels of IFN-γ and induced only very low production of IL-10 (Table II). These results demonstrate that the secretion of IL-10 and IFN-γ by splenocytes from T. congolense-infected mice operates via a requisite cooperation of Thy-1.2+ and Thy-1.2− cells.

To test whether the production of IL-10 and IFN-γ by Thy-1.2+ splenocytes from infected mice might depend on classical Ag presentation, we supplemented cultures of Thy-1.2+ cells from infected mice with Thy-1.2− spleen cells from uninfected mice (i.e.,...
As shown in Table III, APC supplementation restored the ability of the Thy-1.2^+ cells from infected mice to secrete IL-10 and IFN-γ in response to T. congolense lysates. Direct pulsing of Thy-1.2^+ cells from either uninfected or T. congolense-infected mice with T. congolense lysates did not induce either IL-10 or IFN-γ secretion. These results suggest that this IL-10 and IFN-γ production might require Ag presentation rather than direct binding of T. congolense molecules to T cells, as has been reported for T. brucei (31, 36).

Adherent Thy-1.2^+ spleen cells from T. congolense-infected mice suppress T and B cell responses to mitogen and parasite-unrelated Ag.

One of the prominent features of African trypanosomiasis is a suppression of T and B cell responses to parasite-related and unrelated Ags (1, 2, 4). Suppressor T cells (6–9, 11), macrophage-like cells (2, 4, 12, 14), or a combination of these (1, 15) have variously been implicated in this effect. Because IL-10 (21) and IFN-γ (3, 10, 22) are involved in the suppression of T cell responses in trypanosome-infected animals, we wished to know whether our adherent IL-10- and IFN-γ-secreting Thy-1.2^+ cells might effect such suppression. We tested the impact of our Thy-1.2^+ adherent cells from either infected or uninfected mice on Con A-driven proliferation of normal mouse splenocytes. Coculture of spleen cells from uninfected mice with Sephadex-adherent cells from infected, but not uninfected, mice resulted in a significant (48 ± 5%; p < 0.01) suppression of the proliferative response to Con A. Depletion of Thy-1.2^+ cells from the Sephadex G-10-adherent cells reversed this suppression (Fig. 6A).

In another set of experiments, spleen cells from BALB/c mice immunized 7 days previously with SRBC were stimulated in vitro with SRBC in the absence or the presence of Sephadex G-10-adherent cells from either uninfected or T. congolense-infected mice. Four days later, the B cell responses to SRBC were measured using a hemolytic PFC assay. As shown in Fig. 6B, adherent cells from the spleens of infected, but not those from uninfected, mice significantly suppressed (80 ± 3%; p < 0.001) the B cell response to SRBC. Again, depletion of Thy-1.2^+ cells from the adherent cells of infected mice effectively reversed this suppression.

Table II. Optimal secretion of IL-10 and IFN-γ requires cooperation of positively enriched Thy-1.2^+ cells and Thy-1.2^− cells derived from the spleens of T. congolense-infected, but not uninfected mice

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Concentration of Cytokine (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-10</td>
</tr>
<tr>
<td>Infected splenocytes</td>
<td>1805 ± 42</td>
</tr>
<tr>
<td>Infected Thy-1.2^+ cells</td>
<td>412 ± 6b</td>
</tr>
<tr>
<td>Infected Thy-1.2^− cells</td>
<td>39</td>
</tr>
<tr>
<td>Infected Thy-1.2^+ cells + infected Thy-1.2^− cells</td>
<td>2342 ± 25</td>
</tr>
<tr>
<td>Infected Thy-1.2^+ cells + normal Thy-1.2^− cells</td>
<td>130 ± 18</td>
</tr>
<tr>
<td>Normal splenocytes</td>
<td>65 ± 12</td>
</tr>
<tr>
<td>Normal Thy-1.2^+ cells</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>Normal Thy-1.2^− cells</td>
<td>103 ± 56c</td>
</tr>
<tr>
<td>Infected Thy-1.2^− cells</td>
<td>56 ± 12</td>
</tr>
</tbody>
</table>

a Single-cell suspension of spleens from uninfected or infected mice were separated into Thy-1.2^+ and Thy-1.2^− populations using anti-Thy-1.2 Abs coated on paramagnetic beads. The Thy-1.2^− populations were further treated with anti-Thy-1.2 Ab and complement to completely deplete them of any residual Thy-1.2^+ cells. The enriched Thy-1.2^+ cells were cultured at 5 × 10^6 cells/well either alone or with 5 × 10^5 Thy-1.2^− for 48 h, and the supernatant fluids were assayed for IL-10 and IFN-γ by ELISA. The set of results presented is one of two similar experiments. ND, not detected (i.e., below the sensitivity of the ELISA).

b p < 0.001 vs whole splenocytes from infected mice.

c p < 0.001 vs infected, Thy-1.2^+ + 5 × 10^6 infected, Thy-1.2^− cells.

Table III. Secretion of IL-10 and IFN-γ by Thy-1.2^+ cells in the spleens of T. congolense-infected BALB/c mice is trypanosome specific and requires splenic APC

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Concentration of Cytokine (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-10</td>
</tr>
<tr>
<td>Infected splenocytes</td>
<td>726 ± 12</td>
</tr>
<tr>
<td>Infected Thy-1.2^+ cells</td>
<td>ND</td>
</tr>
<tr>
<td>Infected Thy-1.2^− cells + Tryps</td>
<td>ND</td>
</tr>
<tr>
<td>Infected Thy-1.2^+ cells + APC + Tryps</td>
<td>1020 ± 41</td>
</tr>
<tr>
<td>Normal splenocytes</td>
<td>ND</td>
</tr>
<tr>
<td>Normal Thy-1.2^+ cells</td>
<td>ND</td>
</tr>
<tr>
<td>Normal Thy-1.2^− cells</td>
<td>ND</td>
</tr>
<tr>
<td>APC + Tryps</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Enriched Thy-1.2^+ cells (5 × 10^6 cells/well) were supplemented with 10^8 spleen cells (APC) from uninfected mice, completely depleted of Thy-1.2^+ cells by treatment with anti-Thy-1.2 Abs and complement, and cultured in 96-well tissue culture plates in the presence or absence of T. congolense lysate (Tryps). After 48 h, the supernatant fluids were collected and assayed for IL-10 and IFN-γ by ELISA. Values presented represent the mean ± SE of four wells. The set of results presented is one of two similar experiments. ND, not detected (i.e., below the sensitivity of the ELISA).
have been shown to be the major producers of IFN-γ (16, 31). In that system, direct binding of a trypanosomal component to the CD8+ T cells was reported to trigger the production of large amounts of IFN-γ (16, 17). IFN-γ was further reported to have a direct growth stimulatory effect on the parasite (16, 37). Depletion of CD8+ cells in vivo was associated with a reduction in parasitemia and enhanced resistance to the disease (16). In addition, CD8+, but not CD4+, knockout mice exhibited an enhanced resistance to T. brucei infections (38), and this effect was reportedly mediated via a suppression of IFN-γ secretion. In T. congolense-infected mice, we found higher levels of IL-10 (21) and IFN-γ (22) mRNA induction and cytokine expression by spleen cells of infected highly susceptible BALB/c than by those of relatively resistant C57BL/6 mice. However, we were unable to show any growth stimulatory effect of IFN-γ on T. congolense (23). Our data, rather, indicate that the production of higher levels of IL-10 and IFN-γ by the susceptible mice contributes to their enhanced susceptibility to the disease, in part through the immunosuppressive effects of these cytokines (21, 22). Our current data also differ from those obtained in the T. brucei-infected mice, in that they show that CD3+ Thy-1.2+ αβγδ CD4+8+ and CD3+ Thy-1.2+ αβ−γδ+ CD4−8− cells are the major producers and/or regulators of IL-4, IL-10, and IFN-γ in BALB/c mice infected with T. congolense. This finding is in agreement with recent observations that depletion of CD8+ cells in T. congolense-infected susceptible zebu cattle has no effect on the outcome of the disease (39) despite the fact that spleen and lymph node cells from infected animals secrete high levels of IFN-γ in vitro (40). Considering their close structural and molecular similarities, it is surprising that such marked differences exist between the effects of T. brucei and T. congolense infections. We suspect that, among other things, the inherently invasive nature of T. brucei as well as the differences in some crucial enzymes could be important distinguishing factors between the two model systems.

Several lines of evidence suggest that the cells producing IL-4, IL-10, and IFN-γ in the spleens of T. congolense-infected BALB/c mice are not conventional T cells. First, nylon wool- and Sephadex G-10-nonadherent cells that are enriched for conventional T cells did not secrete these cytokines (Fig. 2), and the production of these cytokines by cells nonadherent to plastic was dramatically reduced. Second, plastic-adherent cell populations produced two- to fivefold more of each cytokine than did the nonadherent cells despite the fact that they contained only about 5–8% of CD3+ Thy-1.2+ cells. Furthermore, nylon wool- and Sephadex G-10-adherent cells containing <10% CD3+ Thy-1.2+ cells produced as much or even more of each cytokine as unfractionated splenocytes from infected mice. Moreover, depletion of αβ+, γδ+ CD4+ or CD8+ cells from the adherent cell populations had little or no significant effect on the secretion of these cytokines. Only the depletion of CD3+ or Thy-1.2+ cells from the adherent cells could completely abolish cytokine secretion (Table I and Fig. 3).

We tested whether IL-4-, IL-10-, and IFN-γ-secreting cells were NK1.1+ T cells, NK1.1+ T cells, which can either be Thy-1.2+ CD4+8− (60%) or Thy-1.2− CD4+8+ (40%), have been shown to secrete large amounts of IL-4, IL-10, and IFN-γ upon appropriate stimulation and comprise about 0.5–1% of splenocytes from normal mice (reviewed in Refs. 32, 41, and 42). Using a pan NK mAb (DX5) (43), we detected no significant increase in the numbers of DX5+ cells either among unfractionated splenocytes from infected mice or among our enriched matrix-adherent (i.e., cytokine-producing) cells. Furthermore, treatment of these enriched matrix-adherent cells with cytotoxic DX5 Ab and complement had no effect on the secretion of IL-10 and IFN-γ by these

Taken together, these results suggest that adherent CD3+ Thy-1.2+ TCR-αβγδ NK1.1− CD4−8− cells from the spleens of T. congolense-infected mice that secrete large amounts of IL-10 and IFN-γ are involved in the suppression of the T and B cell responses observed in these animals. They demonstrate also that this suppression is not effected in an Ag-specific fashion.

**Discussion**

This work identified a unique set of Thy-1.2+ cells that is involved in the regulation and/or secretion of IL-4, IL-10, and IFN-γ in the spleens of BALB/c mice infected with T. congolense. According to the data presented here, both CD3+ Thy-1.2+ αβ− γδ+ CD4+8− and CD3+ Thy-1.2+ αβ− γδ− CD4−8− cells are involved in this process. In T. brucei-infected rodents, CD8+, but not CD4+, cells

**FIGURE 6.** Suppression of Con A-induced proliferation of spleen cells from uninfected mice (A) and of the B cell response to SRBC (B) by adherent cell populations from T. congolense-infected mice. Sephadex G-10-adherent cell populations were obtained from single-cell suspensions of spleens from uninfected (norm.) or BALB/c mice infected for 7 days with T. congolense (infect.). Depletion of residual Thy-1.2+ cells was conducted by treatment with Ab and complement of adherent cells derived from uninfected (Thy-1.2− norm.) or infected (Thy-1.2− infect.) mice. A, Whole spleen cell suspensions from uninfected or infected mice were cultured either alone (3 × 10^6 cells/well) or with undepleted or Thy-1.2-depleted Sephadex G-10-adherent cells (10^5/well) from uninfected or infected mice in the presence of 5 μg/ml Con A. After 48 h, the cultures were pulsed with 0.5 μCi of [H]TdR overnight, and the uptake of [H]TdR was determined. B, BALB/c mice were primed with 0.1% SRBC and were boosted with 10% SRBC 3 days later. Seven days after primary immunization, the mice were euthanized, and single-cell suspensions were made from their spleens. Splenocytes (10^6/well) were cultured in vitro in the presence of 2.5 × 10^5 SRBC and 2.5 × 10^5 undepleted or Thy-1.2-depleted Sephadex G-10-adherent cells from normal or infected mice. After 4 days of culture, the number of anti-SRBC PFC was determined. Note that depletion of Thy-1.2+ cells from the adherent cell population ablates suppression. *, p < 0.01; **, p < 0.001.
cells (Table I). These results unequivocally indicate that the adherent CD3 " Thy-1.2" cytokine-secreting cells are not NK1.1 " T cells.

We further explored the possibility that these unique cells could be γδ T cells. Double-negative (CD4-8-) TCR-γδ+ T cells have been shown to play an important early role in determining the outcome of many parasitic infections (33, 34). We also failed to detect any significant increase in the percentages of TCR-γδ+ cells in either the whole splenocyte or matrix-adherent cell populations from infected mice. Furthermore, depletion of TCR-γδ-bearing cells by treatment with specific Ab and complement did not affect the secretion of IL-10 and IFN-γ by the matrix-adherent cells. These observations strongly indicate that the IL-10- and IFN-γ-secreting matrix-adherent cells do not bear a TCR-γδ.

We established a potential role for the adherent Thy-1.2+ cells in the pathogenesis of African trypanosomiasis by demonstrating their involvement in the suppression of T and B cell responses to mitogen and SRBC, respectively. Coculture of the adherent cells from infected mice with whole spleen cells from uninfected mice resulted in a suppression of the normal proliferative responses to Con A, and this effect was abolished by depletion of Thy-1.2+ cells (Fig. 6A). Furthermore, the marked suppression of B cell response to SRBC in vitro by the adherent cell populations was also ablated by depletion of these Thy-1.2+ cells (Fig. 6B). Adoptive transfer experiments have shown that both T cells (6, 7, 11) and adherent macrophage-like cells (12, 13, 14) are involved in the suppression of lymphoid cell responses in trypanosome-infected mice. In a review of the mechanisms of trypanosome-induced immunosuppression, Roelants and Pinder (1) suggested that both cell types are important. Our present data demonstrate that adherent Thy-1.2+ cells are involved in the pathogenesis of immunosuppression observed in T. congolense-infected mice.

We have previously shown that IL-10 (21) and IFN-γ (22) produced during T. congolense infections mediate suppression of lymphoid cell proliferative responses. Our finding that the secretion of IL-10 and IFN-γ by spleen cells from infected mice requires cooperation between adherent Thy-1.2+ and Thy-1.2+ cells further supports the reports of Corsini et al. (15) and the suggestions of Roelants and Pinder (1) that both Thy-1.2+ cells and adherent macrophage-like cells are important in trypanosome-induced immunosuppression. We hypothesize that adherent (regulatory) Thy-1.2+ cells could alter (via cytokines and possibly cell to cell contact) macrophage functions and cytokine production patterns, thereby rendering these cells suppressive. In line with this, we have obtained recent evidence that simultaneous exposure of bone marrow-derived macrophages from trypanosome-susceptible, but not trypanosome-resistant, mice to IFN-γ and T. congolense lyse increases their IL-10 secretion by two- to fourfold (R. S. Kaushik, J. E. Uzonna, J. R. Gordon, and H. Tabel, manuscript in preparation). IL-10 is a potent immunosuppressive cytokine (44). Expression of the costimulatory molecule, B7, on APCs is necessary for effective T cell activation (45–47). IL-10 down-regulates the expression of B7 on APCs (48, 49). Thus, our data are consistent with our working model in which the induction of regulatory cells during African trypanosomiasis occurs by a trypanosome Ag-specific mechanism, whereas immunosuppression occurs by a non-specific mechanism mediated by cytokines.

The mechanisms of induction and activation of adherent CD3+ Thy-1.2+ αβ- γδ+ CD4-8- cells during African trypanosomiasis is as yet unknown. However, we provided clues that secretion of cytokines (IL-4, IL-10, and IFN-γ) by these cells might occur by an Ag-specific manner rather than via polyclonal activation mediated by direct binding of T. congolense molecules to T cells (as was reported for T. brucei infections) (16, 36). Several lines of evidence support this. First, cytokine secretion was restricted to a small percentage of Thy-1.2+ cells present in the plastic-, nylon wool-, or Sephadex G-10-adherent cell populations. Second, positively selected Thy-1.2+ cells could not produce IL-4, IL-10, or IFN-γ unless they were supplemented with Thy-1.2+ cells from infected mice. Supplementation with Thy-1.2+ spleen cells (i.e., APC) from uninfected mice did not restore cytokine secretion by Thy-1.2+ cells from infected mice. However, in the presence of T. congolense lysates, these APC restored cytokine secretion by Thy-1.2+ splenocytes from infected, but not uninfected, mice (Table III). Furthermore, the cytokine secretion response could not be restored by pulsing purified Thy-1.2+ T cells with optimal concentrations of T. congolense lysate alone. Third, purified Thy-1.2+ cells from infected mice were unable to induce cytokine secretion by positively enriched Thy-1.2+ cells from uninfected mice. This is probably due to the fact that the few Ag-specific Thy-1.2+ cells that would be expected to exist in the spleens of uninfected mice would require much more than 48 h of primary in vitro culture for activation and/or expansion and cytokine secretion, whereas those from mice infected for 7 days have already been primed and expanded in vivo. However, since the reactive Thy-1.2+ cells do not appear to have either TCR-αβ or TCR-γδ (Table I), we speculate that the activating trypanosomal molecule, presented by the APC, binds to an as yet unidentified molecule present only on the surface of these unique Thy-1.2+ cells.

The nature of the trypanosomal Ag(s) that activates the adherent Thy-1.2+ cells for cytokine synthesis, the mechanisms of Ag processing and presentation and the restricting elements for these processes, if any, remain unknown. However, we have clearly demonstrated that this activation for cytokine synthesis by the Thy-1.2+ cells requires cooperation with CD3- Thy-1.2+ adherent cells (APCs) from the spleens of either infected or uninfected mice (Table III). We speculate that these former cells may be induced following recognition of some conserved trypanosomal antigen, e.g., the glycolipid portion of the variant surface glycoprotein presented by nonclassical restricting elements. The variant surface glycoprotein of African trypanosomes is inserted into the plasma membrane via a lipid, the glycosylphosphatidylinositol (50, 51).

In conclusion, our results indicate that adherent CD3+ Thy-1.2+ αβ- γδ+ CD4-8- cells derived from the spleens of T. congolense-infected mice, in cooperation with Thy-1.2+ cells, produce IL-4, IL-10, and IFN-γ and that these cells mediate the trypanosome-induced immunosuppression observed in infected mice.

Acknowledgments
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References