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Differential Induction of TGF-β Regulates Proinflammatory Cytokine Production and Determines the Outcome of Lethal and Nonlethal *Plasmodium yoelii* Infections[1]

Fakhreldin M. Omer,* J. Brian de Souza,*† and Eleanor M. Riley[2]*

Transforming growth factor-β is an essential moderator of malaria-induced inflammation in mice. In this study, we show that the virulence of malaria infections is dependent upon the cellular source of TGF-β and the timing of its production. C57BL/6 mice infected with a nonlethal (Py17X) strain of *Plasmodium yoelii* produce TGF-β from 5 days postinfection; this correlates with resolution of parasitemia, down-regulation of TNF-α, and full recovery. In contrast, infection with the lethal strain Py17XL induces high levels of circulating TGF-β within 24 h; this is associated with delayed and blunted IFN-γ and TNF-α responses, failure to clear parasites, and 100% mortality. Neutralization of early TGF-β in Py17XL infection leads to a compensatory increase in IL-10 production, while simultaneous neutralization of TGF-β and IL-10R signaling leads to up-regulation of TNF-α and IFN-γ, prolonged survival in all, and ultimate resolution of infection in 40% of Py17XL-infected animals. TGF-β production can be induced in an Ag-specific manner from splenocytes of infected mice, and by cross-linking surface CTLA-4. CD25$^+$ and CD8$^+$ cells are the primary source of TGF-β following Py17X stimulation of splenocytes, whereas Py17XL induces significant production of TGF-β from adherent cells. In mice immunized against Py17XL, the early TGF-β response is inhibited and is accompanied by significant up-regulation of IFN-γ and TNF-α and rapid resolution of challenge infections. *The Journal of Immunology*, 2003, 171: 5430–5436.

The pathology and clinical symptoms of malaria are associated with the intraerythrocytic phase of infection. Malaria merozoites invade RBC and undergo repeated cycles of asexual replication, erythrocyte lysis, and reinvasion of new red cells. In the absence of appropriate immune responses, parasitemia rises exponentially, leading to red cell destruction and impairment of blood flow by sequestered parasitized erythrocytes, giving rise to severe anemia, metabolic acidosis, and cerebral malaria, with frequently fatal results (1). Data from murine malaria infections, increasingly supported by data from human studies, indicate that an early inflammatory cytokine response, comprising IL-12, IFN-γ, and TNF-α, is required to control the initial burst of intraerythrocytic parasite multiplication (2–5); resistance is absolutely dependent on IFN-γ (6), and failure to maintain early Th1 responses can lead to a rapid increase in parasite load (7). IFN-γ and TNF-α act synergistically to induce parasite killing inside phagocytic cells. However, in excess, proinflammatory cytokines are the major mediators of severe disease in both humans and mice (8). Plasma levels of bioactive TNF-α correlate with susceptibility to cerebral malaria syndromes in mice infected with *Plasmodium berghei* (9), and mice with genetic defects in the proinflammatory cytokine cascade (IFN-γ receptor, TNF-α receptor 2, IFN-γ regulatory factor) are resistant to cerebral malaria (10–12). However, a recent report that the cerebral pathology of *P. berghei* may be mediated by lymphotoxin-α rather than (immunologically cross-reactive) TNF-α (13) may mean that some of these data needs to be reinterpreted. It has recently been reported (14) that blockade of the negative regulator of T cell activation, CTLA-4, exacerbates the severity of *P. berghei* infections, indicating that failure to regulate T cell activation contributes to pathology. In humans, risk of death from cerebral malaria correlates with circulating TNF-α levels (15, 16); death in adults is associated with high ratios of plasma IL-6 to IL-10 (17); severe anemia in children is linked to high plasma ratios of TNF-α to IL-10 (18, 19); and high ratios of proinflammatory (TNF-α, IFN-γ, IL-12) to anti-inflammatory (TGF-β) cytokine production in vitro are associated with decreased risk of infection, but increased risk of fever (20). Optimizing the host immune response to malaria thus depends on fine tuning the balance of proinflammatory to anti-inflammatory cytokine responses (21).

We have previously shown that, in BALB/c mice, the ability to survive infection with either *P. berghei* K173 or *Plasmodium chabaudi chabaudi* A/J is positively correlated with the ability to secrete TGF-β (22). A causal relationship between TGF-β and survival was demonstrated by administration of a neutralizing Ab to TGF-β to *P. chabaudi*-infected mice (which led to 100% mortality) and administration of rTGF-β to *P. berghei*-infected mice (which now survived the acute infection, although they died later from overwhelming parasitemia). Changes in TGF-β concentration were accompanied by reciprocal changes in TNF-α concentration, indicating that TGF-β may either directly or indirectly down-regulate TNF-α production (22). We have therefore postulated that TGF-β may play a crucial role in establishing the proinflammatory/anti-inflammatory cytokine balance that is required for successful resolution of malaria infections (21).

However, it is apparent that the timing and magnitude of the TGF-β response are crucially important in determining the outcome of infection. Thus, Tsutsui and Kamiyama (23) observed that very high doses of rTGF-β, administered at the start of a malaria

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infection, inhibited parasite clearance, leading to overwhelming parasitemia and death. Thus, tightly regulated production of bioactive TGF-β, in the right amounts and at the correct time, may be required to achieve the appropriate balance of inflammatory and anti-inflammatory responses.

To test this hypothesis, we have compared the kinetics and induction requirements of TGF-β in infections with two closely related strains of the same parasite species, lethal *Plasmodium yoelii* 17XL (Py17XL)[3] and the nonlethal variant *P. yoelii* 17X (Py17X) (24) in C57BL/6 mice. In mice infected with (nonlethal) Py17X, TGF-β production increased steadily, peaking at 10 days postinfection (p.i.), and was associated with down-regulation of IFN-γ and TNF-α, allowing the infection to be resolved without severe pathology. By contrast, mice infected with (lethal) Py17XL produced a very early burst of TGF-β (within 24 h) that appeared to inhibit the normal IFN-γ and TNF-α response, leading to unconstrained growth of the parasite and 100% mortality. We have therefore conducted a series of experiments to determine the relationship between the cellular source and timing of TGF-β production and the outcome of infection with these two strains of parasite. Importantly, we show that the virulence of malaria infections is dependent upon both the cellular source of TGF-β and the timing of its production.

Materials and Methods

Malaria infections

P. yoelii 17XL (Py17XL, lethal) and Py17X (nonlethal) (24) were maintained in vivo by regular passage in naive mice. C57BL/6 mice (4–6 wk old; Harlan, Oxford, U.K.) were injected with 10^5 Py17XL- or 10^5 Py17X-parasitized RBC (pRBC) suspended in 100 μl saline citrate. Control mice received an equivalent number of uninfected erythrocytes (uRBC). Parasitemia was monitored by examination of Giemsa-stained thin smears of tail blood. For in vitro studies, parasitized and nonparasitized erythrocytes and their lysates were prepared from platelet-depleted blood to minimize inadvertent TGF-β contamination. Freshly collected mouse erythrocytes were allowed to settle, platelet-rich plasma was removed, and the sedimented blood was spun at 250 $\times$ *g* for 10 min to remove any remaining platelets before washing extensively (4$\times$) with RPMI.

Py17XL immunization

C57BL/6 mice were inoculated with 10^5 Py17XL pRBC and treated with 15 mg/kg body weight chloroquine phosphate for 3 days starting on day 3 p.i. Parasitemia was monitored by microscopy until no parasites were seen. Mice were reinfected with 10^6 Py17XL pRBC, drug cured, infected with10^7 Py17XL pRBC, and treated again. After three rounds of infection and cure, over 6 wk, mice were completely refractory to reinfection. Mice that spontaneously recovered from Py17X infection were considered immune, as they were resistant to further infection with Py17X.

Neutralization of TGF-β and IL-10R

Groups of C57BL/6 mice infected with 10^5 17XL pRBC received either 20 μg per mouse per day of neutralizing anti-TGF-β IgG1 mAb (clone1835; R&D Systems, Abingdon, U.K.), 20 μg per mouse per day of neutralizing anti-IL-10R Ab (clone 1B1.3a; BD PharMingen, Cowley, U.K.) (25), a combination of anti-TGF-β and anti-IL-10R Abs, or equivalent amounts of isotype-matched irrelevant control Abs (R&D Systems), 1 day before infection, on the day of infection, and for up to 4 days following infection.

Plasma collection

Blood was collected from the tail vein into heparinized tubes and centrifuged initially at 250 $\times$ *g* for 10 min and then at 750 $\times$ *g* for 15 min to obtain platelet-depleted plasma.

Spleen cell cultures

Spleen cells from five mice per group were pooled and purified on Lympho-Sep (Harlan-Seralab, Loughbrough, U.K.), washed, and resuspended in RPMI 1640 supplemented with glutamine, penicillin, and streptomycin (Sigma-Aldrich, Poole, U.K.) and 2% Ultroser (Life Technologies, Paisley, U.K.). Plastic-adherent cells (1 h at 37ºC) were released by incubation for 30 min on ice in Ca^{2+}-free HBSS and resuspended at a concentration of 2×10^6/ml in RPMI + 1% FCS. $CD25^+$ cells were depleted by panning on petri dishes precoated with anti-CD25 mAb (Serotec, Oxford, U.K.) (250 μg/ml; 1 h at 37ºC); cells were checked by flow cytometry and found to contain $<0.6\%$ $CD25^+$ cells. Unbound, $CD25^-$ cells were collected, washed, and resuspended (2×10^6/ml) in RPMI + 1% FCS. $CD4^+$ and $CD8^+$ T cells were positively selected with magnetic beads (Dynal, Bromborough, U.K.), according to manufacturer's instructions. Briefly, for CD4 isolation, 1 ml washed beads were incubated (1 h at 4ºC on a roller) with 7×10^7 spleen cells. Selected cells were magnetically separated and washed, and beads were released by incubation with Detachabead Ab (1 h at 4ºC). Cells were washed and resuspended (2×10^6/ml) in RPMI + 1% FCS. $CD8^+$ cells were purified by incubation with rat anti-mouse CD8 attached to dynabeads by a DNA linker. Bound cells were magnetically recovered, and beads were released by incubation with DNase-releasing buffer and magnetically removed (CeLLection; Dynal). As APCs, peritoneal macrophages from normal C57BL/6 mice were washed, adjusted to 10^6/ml, and γ irradiated (2000 rad). $CD4^+$ and $CD8^+$ populations were checked for purity by flow cytometric analysis and were between 91 and 95% pure.

Cells (2×10^6 cells/well, with or without 10^5 irradiated APCs) were aliquoted into sterile 24-well, microtiter plates (Nalge Europe, Hereford, U.K.) and cultured at 37ºC in 5% CO_2 for up to 24 h together with Con A (5 μg/ml) (Sigma-Aldrich), cross-linking anti-mouse CTLA-4 Ab (anti-CTLA-4, 20 μg/ml) (BD PharMingen), isotype-matched control Ab (Serotec; 20 μg/ml), live pRBC (10^6 cells/ml), parasite lysates (at an equivalent concentration), live uRBC, or control lysates. All assays were conducted in triplicate. Culture supernatants were stored at -80ºC.

Cytokine ELISAs

Indirect sandwich ELISA for TNF-α, IFN-γ, and IL-10 were conducted using pairs of capture and detection Abs (BD PharMingen), according to the manufacturer's instructions, as described previously (22). Recombinant mouse IL-10 and IFN-γ standards were from BD PharMingen, and the rTNF-α standard was from R&D. For TGF-β assays, plasma samples or supernatants were acid activated (as described previously (22)) to release the mature form of TGF-β. ELISA were performed essentially as described (22), except that chicken anti-human TGF-β1 (2 μg/ml) was used as the coating Ab; monoclonal mouse anti-human TGF-β1, -β2 , and -β3 (2 μg/ml) as the detecting Ab; and human rTGF-β1 as the standard (all reagents from R&D Systems). As far as we are aware, the TNF-α reagents do not differentiate between TNF-α and lymphotoxin-α, and we cannot thus differentiate between the potential role of these two closely related cytokines (13).

Results

P. yoelii *infections in C57BL/6 mice*

C57BL/6 mice were infected with 10^5 Py17X or Py17XL pRBC by i.p. injection. Parasitemia was monitored by microscopy, and circulating levels of TGF-β, IFN-γ, and TNF-α were determined by ELISA (Fig. 1).

C57BL/6 mice infected with Py17X developed a moderate parasitemia that increased steadily from approximately day 4 to reach a peak of ~45% at day 15 p.i. (Fig. 1*a*); parasitemia resolved spontaneously and was cleared by day 27 p.i. These mice were completely immune to reinfection (data not shown). As expected, mice infected with spontaneously resolving Py17X showed a marked and immediate rise in plasma IFN-γ concentration that peaked at 5 days p.i. and remained high for the duration of the infection (Fig. 1*b*). In the same mice, TNF-α levels began to rise from 3 days p.i., peaking at day 7 and declining sharply after day 10 (Fig. 1*c*). IL-10 levels rose much more gradually over the course of the infection, reaching a peak concentration of ~10 ng/ml at ~16 days p.i. (Fig. 1*d*). Increasing parasitemia was accompanied by a gradual increase in plasma TGF-β concentration that plateaued from days 10 to 16 postinfection and remained above normal until at least 20 days p.i. (Fig. 1*e*).

By contrast, infection with Py17XL led to a rapidly increasing parasitemia, reaching 80% by 5 days p.i. By day 7, all infected

[3] Abbreviations used in this paper: Py, *P. yoelii*; p.i., postinfection; pRBC, parasitized RBC; uRBC, uninfected RBC.

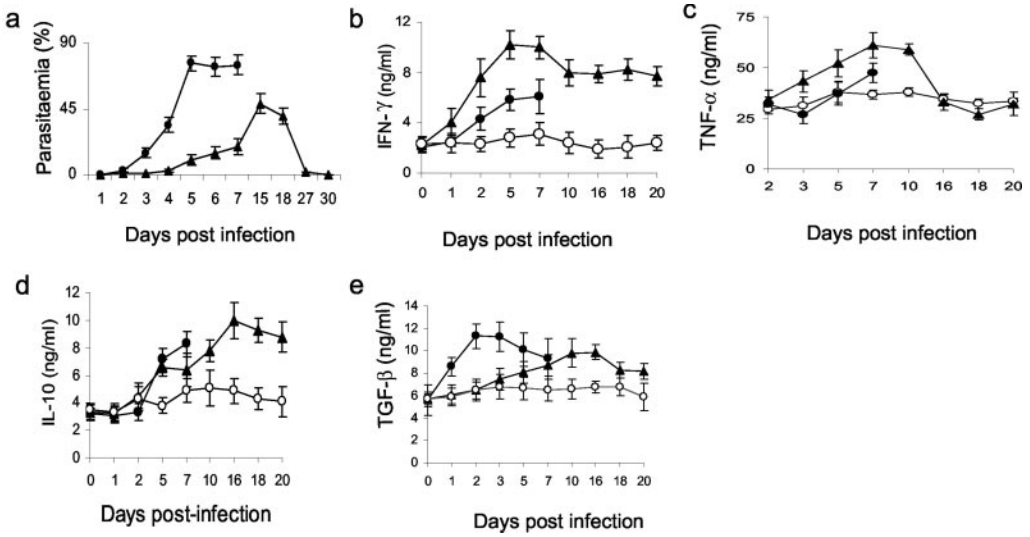

FIGURE 1. Course of infection and cytokine production in C57BL/6 mice infected with either lethal (Py17XL) or nonlethal (Py17X) *P. yoelii* malaria. Mice ($n = 5$) were infected with 5×10^5 Py17XL (●) or PY17X (▲) pRBC or uRBC (○). Blood was collected from the tail vein and used for determination of parasitemia (*a*) or plasma levels of IFN-γ (*b*), TNF-α (*c*), IL-10 (*d*), and TGF-β (*e*) by ELISA. Values represent the mean ± SE of data from five mice per group.

mice were showing severe clinical signs and, as death was considered to be inevitable, mice were humanely killed (as required by United Kingdom legislation). Surprisingly, in these mice, both the IFN-γ and the TNF-α response were noticeably muted and cytokine concentrations were significantly lower than in Py17X-infected mice (e.g., day 7, $t \geq 5.380$, 8 df; $p < 0.001$). IL-10 levels were not significantly different between the two groups of mice (day 5, $t = 2.23$, $p > 0.05$; day 7, $t = 1.49$, $p = 0.17$). However, the most remarkable observation was an extremely early burst of TGF-β production, detectable as early as 24 h p.i., peaking on days 2–3, and remaining above normal until death. This could not be attributed to higher levels of parasitemia in the first 2 days of infection, as parasite counts on these days were not significantly different from mice infected with Py17X.

Thus, these data suggest that the rapid rise in parasitemia, and subsequent death, of Py17XL-infected mice is linked to an early burst of TGF-β production, leading to inhibition of the antiparasitic proinflammatory cytokine response.

Does neutralization of the early burst of TGF-β promote IFN-γ and TNF-α production and reduce early parasite growth in Py17XL-infected mice?

Mice (five per group) received neutralizing Ab to TGF-β on the day before and on days 1, 2, 3, and 4 after infection with 10^5 17XL pRBC (Fig. 2). Anti-TGF-β Ab treatment reduced plasma TGF-β levels to those seen in uninfected mice (Fig. 2*c*), but there was no effect on either parasite density (Fig. 2*a*) or the survival of the mice (Fig. 2*b*). In keeping with this, neither IFN-γ nor TNF-α levels were increased in anti-TGF-β-treated mice. However, on days 2–4, IL-10 levels were significantly higher in mice receiving anti-TGF-β Ab than in isotype control mice (Fig. 2*c*), suggesting that in the absence of TGF-β there is a compensatory rise in IL-10 that is able to down-regulate the proinflammatory response.

To determine whether IL-10 can compensate for loss of TGF-β, the experiment was repeated (using 10 mice per group) with the addition of a group of mice receiving anti-IL-10R Ab and a group

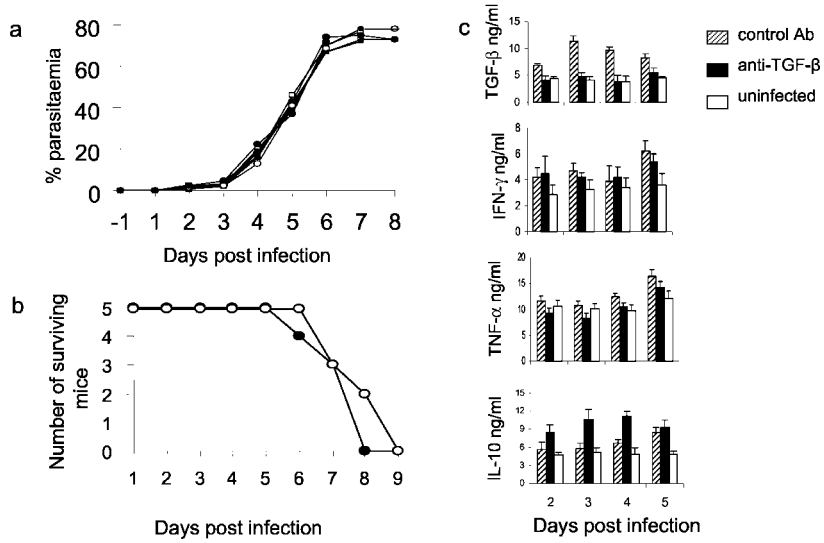

FIGURE 2. Neutralization of TGF-β in Py17XL-infected mice does not affect the course of infection, but does lead to up-regulation of IL-10. Mice (five per group) were injected with neutralizing anti-TGF-β IgG1 mAb (●) or control IgG (○) 1 day before infection and on days 1, 2, 3, and 4 following infection with 5×10^5 Py17XL pRBC. Parasitemia, shown as a single line for each animal (*a*), survival (*b*), and plasma cytokine concentrations (*c*) were monitored daily. Cytokine levels in naive, uninfected mice are also shown; cytokine values represent the mean + SE of data from five mice per group.

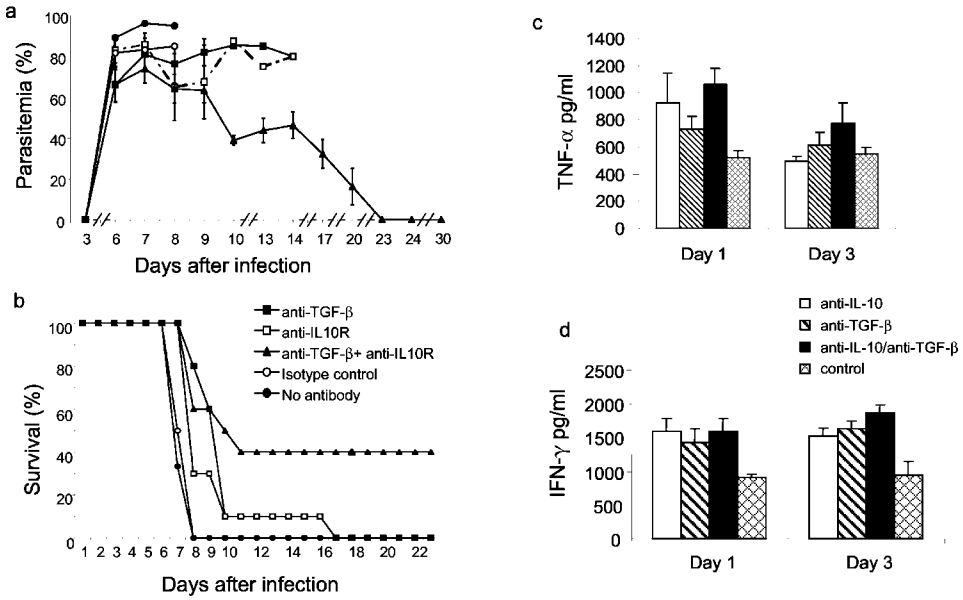

FIGURE 3. Simultaneous neutralization of TGF-β and IL-10R reduces parasitemia and mortality in Py17XL-infected mice. Mice (10 per group) were injected with neutralizing anti-TGF-βIgG1mAb,anti-IL-10RAb,anti-TGF-β plus anti IL-10R, and isotype control IgG (or were not injected) 1 day before infection and on days 0, 2, and 3 following infection with 5×10^5 Py17XL pRBC. Parasitemia (mean $\pm$ SE) (*a*) and survival (*b*) were monitored daily; plasma levels of TNF-α (*c*) and IFN-γ (*d*) were measured on days 1 and 3 p.i.

receiving both anti-TGF-β and anti-IL-10R (Fig. 3). Control mice (no Ab or isotype-matched irrelevant Ab) developed parasitemia of >80% and died or were sacrificed by day 8 p.i. Animals receiving either anti-TGF-β or anti-IL-10R Abs developed somewhat lower peak parasitemias, but, in each group, 9 of the 10 mice died by day 10; all mice in these groups were dead by day 17. By contrast, in mice receiving both anti-TGF-β and anti-IL-10R Abs, parasitemias peaked at below 80% and parasites were gradually cleared; 4 of the 10 mice completely cleared their infections and remained healthy until at least 34 days p.i., when the experiment was terminated. Immediately after infection, serum TNF-α levels were ~40% higher in mice treated with both anti-IL-10 and anti-TGF-β than in mice receiving just anti-TGF-β (1 day p.i.: 1065 $\pm$ 118 pg/ml vs 735 $\pm$ 88 pg/ml), and this difference was statistically significant ($p = 0.03$); TNF-α levels remained higher in the anti-IL-10/anti-TGF-β mice than in controls for at least 3 days p.i. (Fig. 3*c*). IFN-γ levels were 75–98% higher in mice receiving both anti-IL-10 and anti-TGF-β than in isotype control mice, and this difference was statistically significant (1 day p.i., 1590 $\pm$ 178 pg/ml vs 900 $\pm$ 63 pg/ml, $p < 0.001$; 3 days p.i., 1875 $\pm$ 111 pg/ml vs 950 $\pm$ 196 pg/ml, $p = 0.02$), but not significantly higher than in mice receiving anti-IL-10 alone or anti-TGF-β alone (Fig. 3*d*).

In vitro induction of TGF-β from splenocytes

The differing kinetics of TGF-β production in Py17XL- and Py17X-infected C57BL/6 mice suggest that the trigger for TGF-β release, and its cellular source, may differ. To determine whether *P. yoelii* Ags directly induced the production of TGF-β from murine spleen cells, mice were infected with 10^5 Py17X or 10^5 Py17XL pRBC and spleens were harvested at 5 days p.i.; cells from naive, spleens from uninfected mice were used as controls. Cells were restimulated in vitro for 18 h with an extract of Py17X-infected, Py17XL-infected, or uninfected mouse erythrocytes. Cells incubated in culture medium alone were used as a negative control. To determine whether TGF-β production could be induced by stimulation via the cell surface receptor CTLA-4, which might implicate a population of regulatory T cells in the production of TGF-β in this system (26), an activating, cross-linking anti-CTLA-4 Ab was added to some cultures. Cell supernatants were collected and tested for TGF-β by ELISA (Fig. 4*a*).

Spleen cells from infected mice spontaneously produced TGF-β (medium control) with cells from Py17XL-infected mice producing higher levels of TGF-β than cells from Py17X-infected mice ($t = 5.38$, 8 df, $p < 0.001$). Cells restimulated with Py Ags produced significantly more TGF-β than medium controls. Py17XL Ag induced TGF-β production from normal spleen cells (i.e., uninfected mice) and induced significantly more TGF-β from spleen cells of 17XL-infected mice than from spleen cells of 17X-infected mice. In contrast, Py17X Ag did not induce TGF-β from uninfected spleen

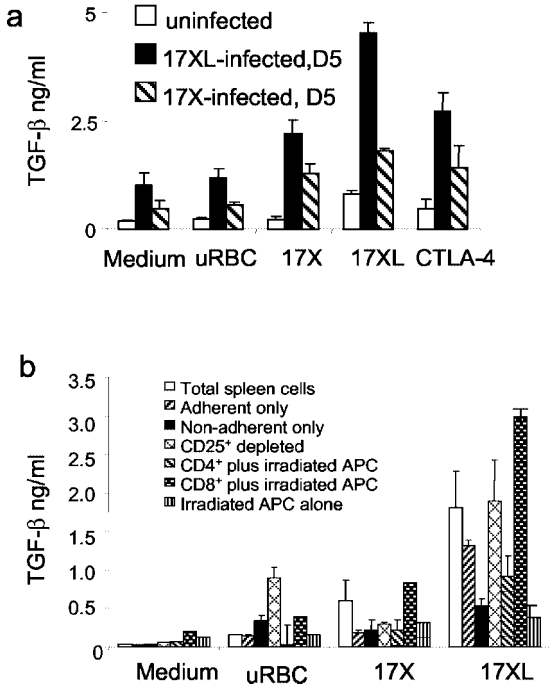

FIGURE 4. TGF-β production in vitro by spleen cells from malaria-infected and uninfected C57BL/6 mice. *a*, Spleen cells were collected 5 days p.i. with 5×10^5 uRBC, Py17XL pRBC, or Py17X pRBC, and restimulated in vitro for 18 h with medium alone, uninfected mouse RBCs (mRBC), Py17X, or Py17XL Ag, or a cross-linking Ab to CTLA-4. *b*, Intact spleen cells or separated spleen cell populations from naive (uninfected) mice were restimulated in vitro for 18 h with medium alone, uninfected mouse RBCs (mRBC), Py17X, or Py17XL Ag. TGF-β in culture supernatants was measured by ELISA. Data represent mean + SE of triplicate assays and are representative of at least four independent experiments.

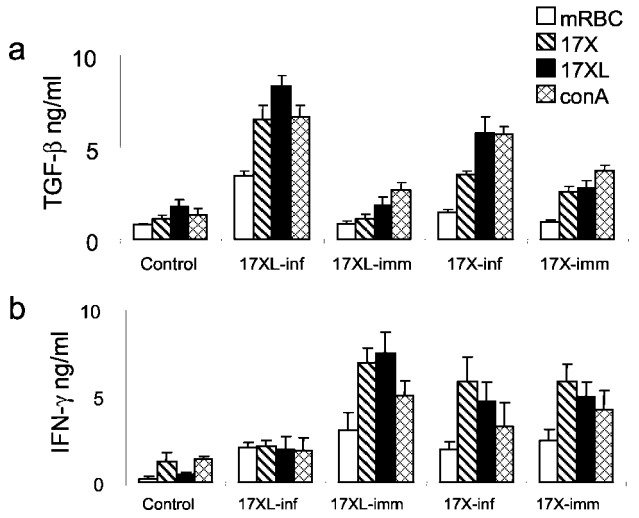

FIGURE 5. TGF-β and IFN-γ production in vitro by spleen cells from malaria-infected and malaria-immune C57BL/6 mice. Spleen cells from mice undergoing a primary infection with Py17XL (Py17XL-inf) or Py17X (Py17X-inf) or from immune mice reinfected with Py17XL (Py17XL-imm) or Py17X (Py17X-imm), or uRBC-infected (control) mice, were collected 5 days p.i. with 5×10^5 pRBC or uRBC and restimulated in vitro for 18 h with uninfected mouse RBCs (mRBC), Py17X, Py17XL, or Con A. TGF-β and IFN-γ in culture supernatants were measured by ELISA. Data represent mean + SE of triplicate assays and are representative of two independent experiments.

cells, and although TGF-β was induced from spleen cells of both Py17X- and Py17XL-infected mice, levels were significantly lower than those induced by Py17XL Ag ($p < 0.001$ in all cases). Thus, Py17XL Ag appears to induce TGF-β in a spontaneous manner from unprimed cells and also induces high levels of TGF-β in an Ag-specific manner. In contrast, Py17X Ag only induces TGF-β in an Ag-specific manner from primed spleen cells.

Restimulation with a cross-linking Ab to CTLA-4 (in the absence of malaria Ag, anti-CD3, or other activators of TCR signaling) induced TGF-β secretion from spleen cells of infected, but not uninfected, mice, and levels were similar to those achieved by restimulation with Py17X Ag. As T cell activation via cross-linking of CTLA-4 is dependent upon signaling via the TCR (27), this suggests first that there is ongoing presentation of malaria Ag to specific T cells in vitro, and second that TGF-β is being produced by malaria Ag-specific, CTLA-4$^+$ T cells.

TGF-β production from adherent cells and from Ag-specific CD25$^+$ T cells

To determine the cellular source of TGF-β, cytokine production was compared for total spleen cells, adherent and nonadherent cell populations, purified CD4$^+$ and CD8$^+$ T cells, and spleen cells depleted of CD25$^+$ cells by panning. Irradiated APCs were added back to purified CD4$^+$ and CD8$^+$ populations. Cells were incubated for 24 h with pRBC lysates or uRBC or medium alone, and supernatants were tested for TGF-β (Fig. 4*b*). As expected, Py17XL pRBC induced much higher levels of TGF-β production from unseparated spleen cells than did Py17X pRBC. For some reason that we cannot explain, depletion of CD25$^+$ cells led to increased TGF-β production by spleen cells incubated with uRBC.

For cells stimulated with nonlethal Py17X pRBC, TGF-β was detected only in supernatants of whole spleen cells or of CD8$^+$ cells (plus irradiated APC); TGF-β production was abolished by CD25$^+$ depletion and was not seen in separated populations of adherent cells, nonadherent cells, or CD4$^+$ T cells. Thus, Py17X appears to induce TGF-β from a CD8$^+$ T cell population, which may also be CD25$^+$. TGF-β production from CD8$^+$ T cells appears to be dependent on Ag presentation, as TGF-β production was not seen in purified nonadherent cell populations. By contrast, Py17XL pRBC induced high levels of TGF-β from adherent cells (but not from purified nonadherent cells) and from purified CD8$^+$ cells in the presence of irradiated APCs. Depletion of CD25$^+$ cells from total splenocytes had no effect on TGF-β induction by Py17XL, presumably because much of the TGF-β in this cell population is coming from adherent cells.

Taken together, these data suggest that both Py17X and Py17XL induce TGF-β production from CD8$^+$ (CD25$^+$) T cells, but that Py17XL also induces TGF-β from adherent cells. This rapid induction of TGF-β from adherent cells may explain the very early burst of TGF-β production in Py17XL-infected mice.

Immunization of C57BL/6 mice against Py17XL reduces the early burst of TGF-β production and increases IFN-γ production

Mice infected with Py17X spontaneously clear their infections and are resistant to reinfection. Mice infected with Py17XL typically die within the first 7 days after infection; however, three rounds of infection at escalating doses of pRBC with drug treatment before peak parasitemia render the mice immune to further infection (F.M. Omer, unpublished). We hypothesized that immunity may be due to the development of a rapid, Ag-specific IFN-γ (and/or TNF-α) response, leading to elimination of parasites before they reached patency; in Py17XL infections, this enhanced IFN-γ response might follow from down-regulation of the early burst of TGF-β. We therefore compared the splenic TGF-β and IFN-γ responses of immune mice with those of mice undergoing a primary infection (5 days p.i. with Py17X or Py17XL) and those of naive, uninfected mice (Fig. 5). Py17X-immune mice had spontaneously recovered from a single infection of 10^5 infected erythrocytes, while Py17XL-immune mice had received three rounds of infection and drug treatment.

As expected, in the primary infections, cells from Py17XL-infected mice produced higher levels of TGF-β than cells from Py17X-infected mice, TGF-β production could be enhanced by restimulation in vitro with malaria Ags or Con A, and Py17XL Ag induced higher levels of TGF-β than Py17X Ag (Fig. 5*a*). Similarly, IFN-γ production was higher in Py17X-infected mice than in Py17XL-infected mice. IFN-γ production could be enhanced by malaria Ag or Con A stimulation of spleen cells from Py17X-infected mice, but not of cells from Py17XL-infected mice (Fig. 5*b*).

By contrast, spleen cells from Py17XL-immune mice produced markedly lower levels of TGF-β ($t = 19.3$, df $= 8$, $p < 0.001$), and markedly higher levels of IFN-γ ($t = 8.9$, df $= 8$, $p < 0.001$) than Py17XL-infected mice in response to all stimuli. In Py17X-immune mice, IFN-γ responses were similar to those in infected mice, although the TGF-β response (especially following Py17XL or Con A stimulation) was moderately reduced. Thus, the ability to control parasitemia in Py17XL-immunized mice is associated with inhibition of the early burst of TGF-β and up-regulation of IFN-γ.

Discussion

Successful resolution of blood stage malaria infections in mice depends, first, on their ability to mount an early proinflammatory cytokine response and, second, on their ability to down-regulate this inflammatory response before the onset of immune pathology. We have previously shown that TGF-β plays a critical role in this process. At very low concentrations, TGF-β is proinflammatory, but as its concentration increases it becomes profoundly immunosuppressive, down-regulating the production of IFN-γ and TNF-α

(28). In murine malaria infections, if TGF-β fails to be produced in sufficient amounts once the primary peak of parasitemia has been controlled, immunopathology occurs and mice die from a syndrome similar to endotoxic shock (22). In contrast, the administration of large doses of TGF-β at the beginning of a malaria infection completely suppresses the early inflammatory response, leading to unconstrained parasite growth and death (23).

Interestingly, as we have shown in this work, infection of C57BL/6 mice with the lethal rodent malaria parasite Py17XL leads to a very early burst of TGF-β production that is associated with suppression of IFN-γ and TNF-α and rapid parasite replication. Mice die from overwhelming parasitemia. The early burst of TGF-β production appears to emanate principally from adherent cells. In contrast, mice infected with the nonlethal strain of *P. yoelii* (Py17X) make a delayed and more moderate TGF-β response, which presumably serves to down-regulate the proinflammatory cytokine response once the acute phase of parasite replication has been controlled. In Py17X infections, TGF-β production is restricted to a $CD8^+$and $CD25^+$ cell population.

We were unable to reverse the mortality of Py17XL-infected mice by neutralization of TGF-β alone; we believed this was due to a compensatory increase in IL-10, another immunoregulatory cytokine, when TGF-β is suppressed. In support of this, simultaneous neutralization of TGF-β and inhibition of IL-10R signaling led to a reduction in parasitemia and mortality in Py17XL-infected mice; this was accompanied by increased serum concentrations of IFN-γ and TNF-α. The complementary roles of TGF-β and IL-10 in moderating the outcome of murine malaria infections are further supported by our recent finding that neutralization of TGF-β in IL-10-deficient (but not wild-type) C57BL/6 mice infected with (normally nonlethal) *P. chabaudi* AS leads to 100% mortality (29).

We were able to confirm the link between TGF-β, regulation of inflammatory cytokines, and survival by immunization of mice with Py17XL; induction of protective immunity was associated with a decrease in the early TGF-β peak and a concomitant increase in IFN-γ production. Although a variety of effector mechanisms (including protective Ab responses) contributes to protective immunity in this model (30), we believe that restoration of the early IFN-γ response contributes to the ability to control parasitemia. This is supported by the observation that administration of rIFN-γ protects mice from lethal *P. yoelii* infection, but has little effect on the course of nonlethal infections (31). We suspect that the early burst of TGF-β activation may be inhibited in Py17XL-immune mice by specific Abs that neutralize the parasite-derived molecules (metalloproteases and thrombospondin-like molecules) that directly activate latent TGF-β (42).

Py17XL Ags induce significant TGF-β production from naive (uninfected) murine spleen cells within 12–18 h, whereas Py17X Ags induce little, if any, TGF-β at this time. The early TGF-β appears to derive from a population of adherent cells that we believe to be monocytes/macrophages, although this does need to be confirmed. Other potential sources of rapid TGF-β production include mast cells and platelets, but these were essentially absent from the adherent cell populations. An alternative explanation is that Py17XL parasites are able to directly activate constitutively produced TGF-β, leading to an immediate immunosuppressive effect (42). In contrast, cells from mice infected with nonlethal *P. yoelii* do produce TGF-β later in infection. The Ag-specific nature of TGF-β production in vitro at 5 days p.i. suggests that a memory T cell population may be a significant source of TGF-β at this stage of the infection; the observation that cells from infected mice could be induced to secrete TGF-β by incubation with a cross-linking Ab to CTLA-4 suggests that these TGF-β-producing T cells may belong to a regulatory T cell population.

Regulatory T cells were initially characterized as $CD3^+$, $CD4^+$, and IL-2Rα chain $(CD25)^+$; these cells proliferate poorly after activation via the TCR, fail to produce characteristic T cell cytokines, and are suppressive to other memory T cell populations (26, 32–34). Regulatory T cells are now known to be a rather heterogeneous population; suppressive activity can be induced by signaling via CTLA-4, a negative regulator of T cell activation, and is mediated either by cytokines, most notably IL-10 and TGF-β (35), or by cell-cell contact (36). Both $CD4^+$ and $CD8^+$ regulatory T cells have been described (37, 38), and several examples of pathogen-specific regulatory cells have now been reported (reviewed in Ref. 39). Our data indicate that Py17X induces TGF-β from a population of cells that are both $CD8^+$ and express $CD25^+$ in the resting state; cells from infected mice can be induced to secrete TGF-β via CTLA-4 signaling or by reactivation with Py Ags in the presence of APCs. Thus, nonlethal Py infections induce TGF-β from cells with the characteristics of $CD8^+$ regulatory T cells.

Interestingly, TGF-β (with or without IL-10) production from malaria-specific $CD8^+$ cells may offer an explanation for previous observations in malaria-immune humans that depletion of $CD8^+$ T cells restores IFN-γ responses to malaria Ags in otherwise nonresponding donors (40, 41), and suggests that there are strong parallels between regulation of antimalarial immunity in murine and human infections.

Taking all this data together, we propose the following model for TGF-β production during murine malaria infection. In Py17X-infected mice, we believe that the TGF-β response derives from an Ag-specific $CD8^+$ regulatory T cell population and serves to down-modulate the inflammatory response once the primary peak of parasitemia has been resolved. In the case of Py17XL-infected mice, an early burst of TGF-β (perhaps due to activation of a constitutive source of latent TGF-β by Py17XL Ags) (42) completely suppresses the proinflammatory response and the parasite cannot be contained. Py17XL immunization either neutralizes the Ags that induce the early burst of TGF-β or primes an additional source of IFN-γ that overrides and suppresses the TGF-β response.

In conclusion, we have demonstrated that the timing of TGF-β production is crucial for the successful resolution of murine malaria infections, and that the virulence of some malaria infections is causally associated with their ability to induce very rapid TGF-β release from constitutive sources. In addition, we have shown that $CD8^+$ $CD25^+$ cells with all the characteristics of a regulatory T cell population can be specifically induced to secrete TGF-β in response to both lethal and nonlethal malaria infections. Importantly, we have shown that the TGF-β response to malaria infection can be modified by immunization. If confirmed, these findings have important implications for the development of novel immunotherapies for severe malaria and for the development of malaria vaccines that are able to induce parasite clearance without precipitating immunopathology.

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