# Protection of Mice Against *Trypanosoma cruzi* by Immunization with Paraflagellar Rod Proteins Requires T Cell, but Not B Cell, Function[1]

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**Previous studies have shown that immunization of mice with the paraflagellar rod proteins (PAR) of *Trypanosoma cruzi* induces an immune response capable of protecting mice against an otherwise lethal challenge with this parasite. Herein, we define immunologic responses that do or do not play a critical role in PAR-mediated protection. Firstly, PAR-immunized Ab-deficient ($\mu$MT) strain mice survived an otherwise lethal *T. cruzi* challenge, indicating that a B cell response is not required for PAR-induced immunity. However, $\beta_2$m −/− mice, which are severely deficient in MHC class I and TCR$\alpha\beta^+$CD8$^+$CD4$^-$ T cells, did not survive challenge infection following PAR immunization, indicating that MHC class I/CD8$^+$ T cell function is necessary for protection induced by PAR immunization. Surprisingly, PAR-immunized mice depleted of CD4$^+$ T cells survived a *T. cruzi* challenge for >84 days postinfection while maintaining a parasitemia that is generally thought to be lethal (i.e., >10$^6$ trypomastigotes/ml), thus associating CD4$^+$ T cell function with the process of parasite clearance. Consistent with this association, CD4$^+$ T cells from PAR-immunized mice released INF-$\gamma$ and stimulated *T. cruzi*-infected macrophages to release nitric oxide. The importance of IFN-$\gamma$ in PAR-induced protective immunity is further indicated by the observation that PAR-immunized INF-$\gamma$ knockout mice developed an extremely high parasitemia and did not survive a challenge infection. Thus, while Ab-mediated immune mechanisms are not required for protection induced by PAR immunization, T cell responses are necessary for both elimination of bloodstream parasites and survival.** *The Journal of Immunology,* **1997, 158: 5330–5337.**

*Trypanosoma cruzi* is a parasitic protozoan that causes Chagas' disease, a major human health problem in Central and South America. Chagas' disease is the major cause of heart disease within endemic areas, affecting approximately 18 million people (1, 2). Chemotherapeutic agents have limited effectiveness against the disease, and no vaccine to prevent infection is available. In an effort to develop such a vaccine, we have found that the paraflagellar rod proteins (PAR)[4] present in the flagellum of *T. cruzi* induce an immune response capable of reducing the level of circulating parasites in the bloodstream and protecting mice against an otherwise lethal inoculum of *T. cruzi* trypomastigotes (3). In these studies, immunization with PAR s.c. resulted in a low titer of parasite-specific serum Abs, but 100% of the mice survived an otherwise lethal challenge of the parasite. In contrast, immunization of mice with PAR i.p. produced a high titer of serum Ab against the parasite; however, no protection was provided against a subsequent *T. cruzi* challenge. These data suggest that PAR-mediated protective immune responses may involve cellular, rather than humoral, immune mechanisms.

In both humans and the mouse experimental systems, immune control of *T. cruzi* is thought to be mediated by several different cell populations: B cells, T cells, and macrophages (4–9). In mice, the passive transfer of *T. cruzi* immune serum to naive animals results in partial protection to challenge infection, indicating the role of circulating Ab in controlling infection (10, 11). Further evidence of the importance of B cell activity is seen in the increased susceptibility of mice treated with anti-IgM antiserum and in the high susceptibility of inbred mouse strains with impaired ability to produce Ab (6, 12). The role of cell-mediated immunity to *T. cruzi* has been demonstrated by both cell depletion and direct genetic studies. Mice receiving either anti-CD4 or anti-CD8 antiserum have increased susceptibility to infection, as do athymic or cyclosporin A-treated mice (13–17). More recently, genetic knockout mice lacking either CD4 or CD8 molecules as well as mice deficient in $\beta_2$m were shown to develop increased parasitemias and have decreased survival following infection (18–20), showing that both T cell populations are involved in resistance against *T. cruzi*.

There are several mechanisms by which T cells may reduce the parasite level in the infected host. T cell lines derived from *T. cruzi*-infected mice can lyse infected target cells in an MHC-restricted manner and can transfer protection to acute stage infected mice (21, 22), suggesting that T cells may directly destroy infected cells. They also may release cytokines (INF-$\gamma$, TNF-$\alpha$, IL-3, and granulocyte-macrophage CSF) (23–30) that activate macrophages, leading to the killing of the intracellular stage of *T. cruzi*, presumably by the production of NO (31, 32). Of these cytokines, INF-$\gamma$ has been found to be the most effective in reducing the level of

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[4] Abbreviations used in this paper: PAR, paraflagellar rod proteins; NO, nitric oxide; $\mu$MT, C57BL/6-IgH-6$^{tm1}$; GKO, BALB/c-Ifg$^{tm1}$; $\beta_2$m, $\beta_2$-microglobulin; $\beta_2$m$^{-/-}$, C57BL/6GphTacBr-[KO]; C-DMEM, 25 mM HEPES buffer (pH 7.5), 1 mM Na pyruvate, nonessential amino acids, L-glutamine, 5 × 10$^{-5}$ M 2-ME, 50 U/ml penicillin, 50 $\mu$g/ml streptomycin sulfate, and 10% FBS in Dulbecco's modified Eagle's medium; N$^G$MMA, N$^G$-monomethyl L-arginine; FWB, 1% fetal bovine serum and 0.1% $NaN_3$ in phosphate-buffered saline; PBST, phosphate-buffered saline and 0.05% Tween-20.



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circulating parasites in the bloodstream and increasing the survival time of mice challenged with the parasite (27). T cells also may enhance the production of Abs against specific parasite Ags, which, in turn, can decrease the level of circulating parasites (8, 22, 33).

In the present work we have studied the role of B, $CD4^+$, and $CD8^+$ cells in the protective immune response mediated by immunization of mice with the PAR proteins. Survival and levels of bloodstream trypomastigotes of mice immunized with PAR and challenged with a lethal inoculum of infective trypomastigotes were monitored in mice administered neutralizing anti-CD4 Abs in vivo or in mice with either genomic deletions of $\beta_2$m or the $\mu$-chain of IgM. Also, changes in the amounts of INF-$\gamma$ released by $CD4^+$ or $CD8^+$ T cells from naive and PAR-immunized mice incubated in vitro with macrophages that were incubated with either *T. cruzi* or PAR Ag were analyzed as was the specific release of NO by macrophages.

## Materials and Methods

### Parasites

The Peru strain of *T. cruzi* was used in all experiments. Epimastigotes were grown in modified Leibovitz's L-15 medium (Life Technologies) with 0.05 mg/ml Hemin and 10% FBS (34). Bloodstream trypomastigotes used for challenge inoculations of mice were obtained by cardiac puncture of female BALB/cByJ mice on day 14 postinfection. An inoculum of $10^3$ trypomastigotes was used for all experiments.

### Mice

Four- to six-week-old female BALB/cByJ, C57BL/6J, C57BL/6-IgH-$6^{tm1Cgn}$ ($\mu$MT) and BALB/c-$Ifg^{tm1}$ (GKO) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). MHC class I-deficient C57BL/6GphTacBr-[KO]$\beta_2$m ($\beta_2$m $-/-$) mice were obtained from Taconic Farms (Germantown, NY). All mice were 8 to 12 wk of age at the time of parasite inoculation.

### Ag preparation

PAR proteins were purified as previously described (35). Briefly, $10^{11}$ Peru strain epimastigotes were harvested by centrifugation, washed in PBS, and lysed in 0.1 M tricine, pH 8.5, containing 1% Nonidet P-40. The pellet was extracted with high salt buffer consisting of 0.1 M tricine, pH 8.5, 1 M NaCl, and 1% Triton X-100, using sonication. This crude flagellar pellet was successively extracted with 2.0 and 6.0 M urea in 10 mM tricine, pH 8.5. The resulting supernatant contains approximately 50% PAR and 50% tubulin. The PAR proteins were separated by preparative SDS-PAGE on a Bio-Rad Prep Cell (model 491, Bio-Rad Laboratories, Richmond, CA). Fractions containing the PAR proteins were extensively dialyzed against PBS, concentrated by centrifugation in a Centricon (Amicon, Beverly, MA), and sterilized by 0.45-$\mu$m pore filtration.

### Immunization

Mice were immunized by s.c. injection of 40 $\mu$g of PAR proteins emulsified with CFA and were boosted twice at 2-wk intervals with 20 $\mu$g of protein with IFA. Control groups were injected with adjuvant plus PBS. Two weeks after the last injection, mice were challenged with an s.c. injection of $10^3$ bloodstream trypomastigotes. Following challenge, mice were checked daily, and survival was recorded daily postinfection. Parasitemias were monitored every other day from days 14 to 28 and weekly thereafter.

### Macrophage and T cell cultures

Mice were immunized as described above. Seven to 10 days after the last injection, spleens and inguinal and axillary lymph nodes were removed, and single-cell suspensions were prepared in DMEM supplemented with 25 mM HEPES buffer (pH 7.2), 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, $5 \times 10^{-5}$ M 2-ME, 50 U/ml penicillin, 50 $\mu$g streptomycin sulfate, and 10% FBS (C-DMEM). Spleen cell suspensions were enriched for T cells by passage over nylon wool columns (36). For enrichment of $CD4^+$ and $CD8^+$ T cells, $2 \times 10^7$ enriched T cells were labeled with either 300 $\mu$l of L3T4-Dynabeads or 60 $\mu$l of Ly-2-Dynabeads (Dynal A.S., Oslo, Norway) and negatively selected using the Dynal MPC-1 magnetic particle concentrator. The purity of $CD4^+$ and $CD8^+$ cells was >98%.

Bone marrow macrophages were harvested from the femurs of BALB/cByJ mice and seeded at approximately $5 \times 10^4$ large nonlymphocyte-like cells/well of a 96-well plate. The cells were allowed to adhere for 2 days (37°C at 8% $CO_2$) in the presence of 30% conditioned medium from 3T3 cells and 70% C-DMEM. Nonadherent cells were removed by gentle washing, and the adherent cells were allowed to differentiate for 3 additional days, again in the presence of 30% conditioned medium from 3T3 cells and 70% C-DMEM. The final concentration of macrophages was approximately $1 \times 10^5$/well, creating about a 90% confluent layer of macrophages per well. Bone marrow macrophages were either infected at a 10:1 parasite to macrophage ratio with *T. cruzi* trypomastigotes overnight, then washed free of extracellular parasites, or cultured in the presence of 5 $\mu$g/ml PAR. Control wells were either untreated or cultured with 5 $\mu$g/ml Con A.

Peritoneal macrophage were harvested from BALB/cByJ mice 3 days following an i.p. injection of 3% thioglycolate medium and plated in C-DMEM at $1 \times 10^5$ macrophage/well in 96-well plates. Macrophage were allowed to adhere overnight (37°C at 8% $CO_2$), nonadherent cells were removed by gentle washing, and the adherent cells were infected overnight with trypomastigotes at a 10:1 parasite to macrophage ratio. Control wells containing adherent macrophage were incubated overnight with C-DMEM containing no parasites. Infected monolayers were washed with DMEM to remove extracellular parasites.

Splenic T cells or lymph node cells ($1 \times 10^6$/well) were added to both the infected and the uninfected monolayers. $N^G$-monomethyl L-arginine ($N^GMMA$), an inhibitor of NO synthase, was added to a concentration of 500 nM to some experimental groups. Cells were cultured for 3 days at 37°C in an atmosphere of 8% $CO_2$, and supernatants were harvested and assayed for IFN-$\gamma$ and $NO_2$. All experimental groups were studied in triplicate.

### Inhibition of T. cruzi growth in vitro

Parasite titers in the infected peritoneal macrophage/T cell culture supernatants were determined by pipetting media up and down vigorously several times to resuspend trypomastigotes. Parasite number was determined by counting with a Neubauer hemocytometer.

### Measurement of parasitemias

Parasitemia levels were determined as previously described (13) by removing a blood sample from the tail vein and counting the number of trypomastigotes with a Neubauer hemocytometer (American Optical Corp., Buffalo, NY).

### FACS analysis

Nylon wool-enriched splenic T cells or lymph node cells ($1 \times 10^6$) were washed with FACS wash buffer, PBS (pH 7.4), 1% FBS, and 0.1% $NaN_3$ (FWB). Cells were stained in 100-$\mu$l volumes with the recommended concentrations of the appropriate mAbs against several cell surface markers (CD4, CD8, or CD3) or with isotyped matched control Abs (PharMingen, San Diego, CA) at 4°C in the dark. Cells were washed twice with FWB, fixed in 500 $\mu$l of FWB containing 1% paraformaldehyde, and analyzed by FACScan using the LYSIS II program (Becton Dickinson, San Jose, CA).

### Measurement of cytokines

Culture supernatants were collected at 3, 5, and 7 days, and IFN-$\gamma$ was measured by capture ELISA (PharMingen). Briefly, 1 to 2 $\mu$g/ml of cytokine-specific capture Ab was bound to 96-well microtiter plates in 0.1 M $NaHCO_3$, pH 8.2, at 4°C overnight, washed with PBST (PBS plus 0.05% Tween-20), and then blocked with 10% FCS in PBS for 2 h at room temperature. Wells were washed with PBST, 100 $\mu$l of either standards or samples were added, and the reaction was incubated at 4°C overnight. Wells were again washed with PBST and the appropriate concentration of biotinylated anti-cytokine-detecting Ab, added in a volume of 100 $\mu$l, and incubated for 45 min at room temperature. The wells were thoroughly washed, 100 $\mu$l of strepavidin-peroxidase (2.5 $\mu$g/ml) was added, and the reaction was incubated for 30 min at room temperature. After extensive washes in PBST, 100 $\mu$l of 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (Boehringer Mannheim Biochemicals, Indianapolis, IN) substrate was added, and plates were read at 405 nm in an automated ELISA plate reader. Concentrations were calculated from linear regions of a titration curve of cytokine standards, values for control wells were subtracted, and final concentrations were expressed as nanograms per milliliter.

### Nitrite assays

Nitrite levels in 3-, 5-, and 7-day culture supernatants were measured using the Griess reagent (37). Briefly, 50-$\mu$l culture samples were combined in a 96-well plate with a 1/1 mixture of 1% sulfanilamide in 2.5% $H_3PO_4$ and

Table I. *Role of immune effector mechanisms on protective immunity by vaccination with PAR proteins*

| Mouse Strain | Vaccination | No. of Mice Immunized | Mean Parasitemia ($10^5$) ± SD at Day 17 | 19 | 21 | 24 | No. of Survivors | Mean Survival Time (days) |
|---|---|---|---|---|---|---|---|---|
| C57BL/6 | Freund's | 6 | 12.0 ± 11 | 12.9 ± 11 | 18.5 ± 12 | 13.5 ± 5.6 | 1 | 22 |
| C57BL/6 | Freund's + PAR | 6 | 3.8 ± 1 | 5.2 ± 1.6 | 5.3 ± 1.6 | 0.8 ± 0.3 | 6 | >120 |
| μMT | Freund's | 6 | 32.3 ± 21 | 34.5 ± 9 | 27.4 ± 10 | 20.0 ± 8 | 0 | 25 |
| μMT | Freund's + PAR | 9 | 4.7 ± 2.7 | 3.5 ± 2.6 | 1.3 ± 1.1 | 0.6 ± 0.3 | 9 | >120 |
| $\beta_2$m −/− | Freund's | 3 | 84.0 ± 30 | 113 | | | 0 | 18 |
| $\beta_2$m −/− | Freund's + PAR | 6 | 14.5 ± 6 | 50.3 ± 14 | | | 0 | 20 |
| GKO | Freund's | 6 | 420 ± 120 | | | | 0 | 18 |
| GKO | Freund's + PAR | 6 | 640 ± 270 | | | | 0 | 18 |

0.1% naphthylethlenediamide in 2.5% $H_3PO_4$. Plates were incubated for 10 min at room temperature, and absorbance was determined at 550 nm. Nitrite concentrations were determined in triplicate using a standard curve of sodium nitrite from 125 to 1 μM prepared in culture medium.

## Results

### *Ab is not required for protection of mice immunized with PAR*

Our previous studies on immunization of BALB/c mice with PAR have suggested that the immune response that provides protection against a subsequent challenge with *T. cruzi* may not require Ab participation (3). To directly test whether Abs are required for the protective immune response induced by PAR immunization, B cell-deficient mice (μMT) in which the membrane exon of the Ig μ-chain gene has been genetically disrupted (38) were immunized with PAR and subsequently challenged with an otherwise lethal dose of *T. cruzi*. To confirm that Abs against the PAR proteins were not being produced by the immunized μMT mice, serum from immunized mice was shown by both Western blot and capture ELISA analysis to lack detectable levels of anti-PAR Abs (data not shown). As shown in Table I, 100% (i.e., nine of nine) of the PAR-immunized μMT mice survived the *T. cruzi* challenge. In contrast, none of the μMT mice that received only Freund's adjuvant survived beyond 25 days postchallenge. Furthermore, the PAR-immunized μMT mice showed an 85 to 95% reduction in parasite burden compared with the Freund's adjuvant-treated control mice.

Since all previous studies with PAR immunization have been conducted on the BALB/c mouse (13), which is a high parasitemia strain (i.e., peak parasitemia is $>5 \times 10^6$) (13), and the μMT mouse has a C57Bl/6 genetic background, which is a low parasitemia strain (i.e., peak parasitemia is $<5 \times 10^6$), a direct comparison of the survival and parasitemia burden in the two groups of PAR immunized mice cannot be made. Therefore, to further analyze the effects of immunization of the μMT mice with PAR, C57Bl/6 mice were immunized with PAR and Freund's adjuvant or with only Freund's adjuvant and subsequently challenged with *T. cruzi*. As shown in Table I, 100% of the PAR-immunized C57Bl/6 mice survived the challenge, while only one in six of the adjuvant control mice survived. Therefore, since the numbers of bloodstream parasites and the level of survival (i.e., 100%) in both the PAR-immunized C57Bl/6 and PAR-immunized μMT mice are essentially the same, it appears that B cells or Abs do not play a significant role in the PAR-mediated mechanism(s) that leads to survival and reduction of parasitemia in *T. cruzi*-infected mice.

### *$CD8^+$ T cells are necessary for survival of* T. cruzi*-infected PAR-immunized mice*

To assess the role of $CD8^+$ cells in resistance of PAR-immunized mice to *T. cruzi*, mice ($\beta_2$m −/−) that have a genomic disruption of the $\beta_2$m gene (39) and are severely deficient in both the cell surface expression of class I MHC molecules and $TCR\alpha\beta^+CD8^+CD4^-$ cells were immunized with PAR and challenged with *T. cruzi*. As shown in Table I, both the PAR-immunized mice and the Freund's control mice developed high parasitemia and died by day 20, showing that $CD8^+$ cell function is essential for PAR-mediated protective immunity. The two groups of $\beta_2$m −/− mice both exhibited significantly higher parasitemia values than their C57Bl/6 counterparts ($p = 0.05$, by Mann-Whitney $U$ test), indicating that $CD8^+$ cells also play a role in reducing the level of circulating parasites in both PAR-immunized and control mice. Interestingly, the PAR-immunized $\beta_2$m −/− mice have a significantly lower level of circulating parasites than the Freund's control mice ($p = 0.05$).

### *$CD4^+$ T cells are required for clearance of circulating parasites in PAR-immunized mice*

To explore the role that $CD4^+$ T cells may play in PAR-mediated protective immunity, BALB/c mice were depleted of their $CD4^+$ cells 1 wk before immunization, throughout the immunization period, and for 12 wk postchallenge by i.p. injection with rat anti-CD4 mAb GK1.5 (40) (Fig. 1). To insure that the $CD4^+$ cell population was depleted, the percentages of $CD4^+$ and $CD8^+$ cells present in lymph nodes and enriched splenic T cell preparations were determined by FACS analysis from mice taken at the following points: the first day of immunization with PAR (day −42), the day the mice were challenged (day 0), and on days 60, 70, and 84 postchallenge. In these preparations, the percentage of $CD4^+$ cells exhibited a range of values from 0.28 to 3.07% of the enriched splenic T cells (Fig. 2). Similar values were observed with lymph node preparations (data not shown). On day 84 postchallenge, administration of the GK1.5 Ab was terminated, and the extent of repopulation of lymph nodes and spleen by $CD4^+$ cells by days 137 and 154 postchallenge was monitored by FACS analysis. Parasitemia and survival were monitored throughout the experiment.

All the $CD4^+$-depleted/PAR-immunized mice (i.e., six of six) survived the infection on day 60 postchallenge, when the first infected mouse was killed for FACS analysis. In contrast, mice in the three control groups (i.e., Freund's adjuvant, $CD4^+$-depleted plus Freund's adjuvant, and Freund's adjuvant plus normal rat IgG) died by day 20 postchallenge. Of the remaining five $CD4^+$ depleted/PAR-immunized mice, none succumbed to the infection, but individual mice were killed for FACS analysis as described above. Particularly surprising was the observation that the level of circulating parasites in the $CD4^+$-depleted/PAR-immunized mice remained $>10^6$/ml throughout the 3-mo period during which the

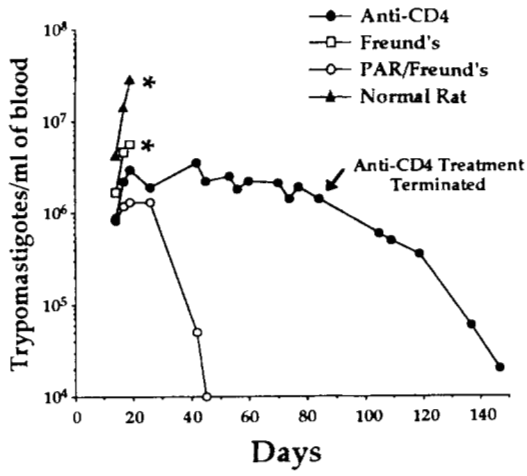


**FIGURE 1.** Effect of depletion of $CD4^+$ T cells on parasitemia in PAR-immunized BALB/c mice infected with *T. cruzi*. BALB/c mice were depleted of $CD4^+$ T cells by i.p. injection of the rat mAb GK1.5. The percentage of $CD4^+$ T cells was monitored by FACS analysis (see Fig. 2). Parasitemia levels were monitored starting on day 14 postinfection. Depletion began 1 wk before PAR immunization and was continued until day 84 postinfection, when administration of GK1.5 mAb was terminated. Control groups were mice immunized only with Freund's adjuvant (s.c.), mice injected with normal rat IgG, and mice immunized with PAR and Freund's (s.c.). The asterisk denotes all mice have died in the Freund's adjuvant-immunized and normal rat IgG control groups.

anti-CD4 mAb was administered. In comparison, normal PAR-immunized BALB/c mice were capable of resolving their parasitemia by day 45. These results clearly indicate that $CD4^+$ cell activity is not essential for the survival of mice for a time of at least several weeks postchallenge; however, $CD4^+$ T cells do play a significant role in reducing the level of circulating parasites in PAR-immunized mice. To determine whether the regrowth of $CD4^+$ T cells would allow the depleted mice to resolve their parasitemia, the GK1.5 treatment was terminated on day 84 of infection, and the repopulation of $CD4^+$ T cells was assessed by FACS analysis on days 137 and 154 post challenge (Fig. 2, *G* and *H*). By day 137, approximately 50% of the normal level of $CD4^+$ T cells had repopulated both spleen and lymph nodes. Associated with the repopulation of $CD4^+$ T cells was a steady decrease in parasitemia with an eventual clearance of the parasite from the blood, further confirming the critical involvement of $CD4^+$ T cells in the clearance of circulating parasites.

### *$CD4^+$ T cells from PAR-immunized mice can activate* T. cruzi*-infected macrophages*

The observation that PAR-immunized $\mu$MT mice can effectively clear circulating *T. cruzi* indicates that T cell participation in the absence of Ab function can efficiently clear the parasite from the bloodstream. One mechanism that may contribute to this eradication is the release of cytokines, which, in turn, activate the parasiticidal mechanisms of phagocytes. Since both in vivo (41, 42) and in vitro (27, 43) experiments have shown that macrophages are important in resistance to *T. cruzi* infection, and T cells from PAR-immunized mice secrete high levels of INF-$\gamma$ in response to PAR in vitro (44), we examined the possibility that $CD4^+$ and/or $CD8^+$ T cells from PAR-immunized mice might specifically recognize and mediate the activation of *T. cruzi*-infected macrophages. Nylon wool-purified T cells or Dynabead-selected $CD4^+$ or $CD8^+$ T

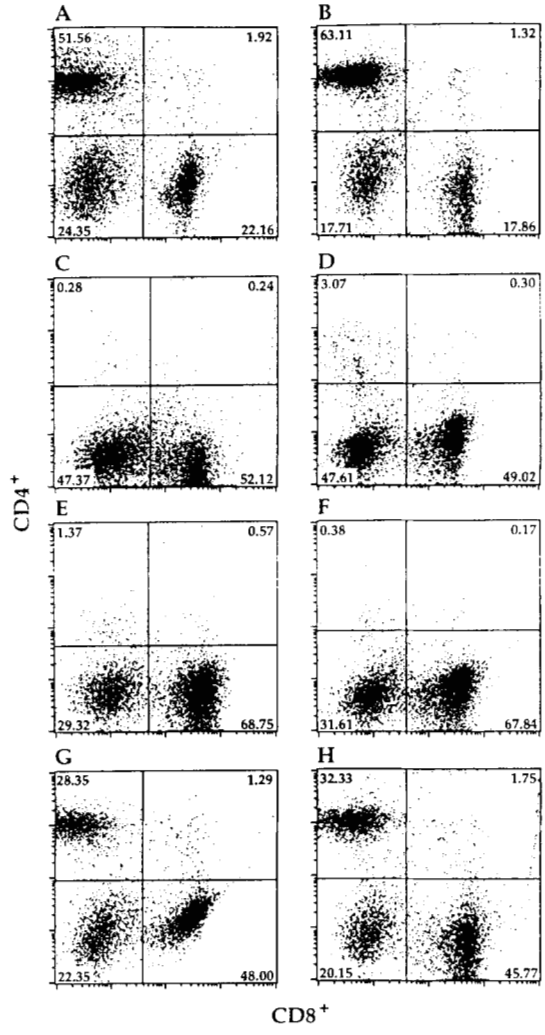


**FIGURE 2.** Flow cytometric analysis of splenic T cells from BALB/c mice following PAR immunization, $CD4^+$ depletion, and *T. cruzi* infection. *A–D*, $CD4^+$ and $CD8^+$ profiles of enriched splenic T cells from normal mice that were unimmunized (*A*), PAR immunized (*B*), unimmunized and GK1.5 treated (*C*), and PAR immunized and GK1.5 treated (*D*). *E–H*, $CD4^+$ and $CD8^+$ profiles of enriched splenic T cells from mice that were PAR immunized, *T. cruzi* infected, and GK1.5 treated both during immunization and for 12 wk of the *T. cruzi* infection. Cells were stained at 10 wk of infection (*E*), 12 wk of infection (*F*), 16 wk of infection (*G*; 4 wk after $CD4^+$ depletion was discontinued), and 20 wk (*H*; 8 wk after $CD4^+$ depletion was discontinued). The percentage of T cells for each quartant is given in the respective corner.

cells from both PAR-immunized and naive BALB/c mice were cultured with syngeneic bone marrow macrophages. The macrophages were infected with *T. cruzi*, untreated, or cultured in the presence of PAR Ag or Con A. T cell/macrophage culture supernatants were collected on days 3, 5, and 7, and INF-$\gamma$ levels and nitrite concentrations were determined. As shown in Figure 3, both purified T cells and $CD4^+$ T cells from PAR-immunized mice secreted INF-$\gamma$ in the presence of *T. cruzi*-infected macrophages, PAR Ag, or Con A, while no measurable INF-$\gamma$ could be detected when these T cells were cultured with untreated macrophages. However, $CD8^+$ T cells or T cells from naive mice produced negligible amounts of INF-$\gamma$ when incubated with infected macrophages or PAR Ag. Consistent with the fact that INF-$\gamma$ is important for macrophage activation, high levels of $NO_2$ were observed

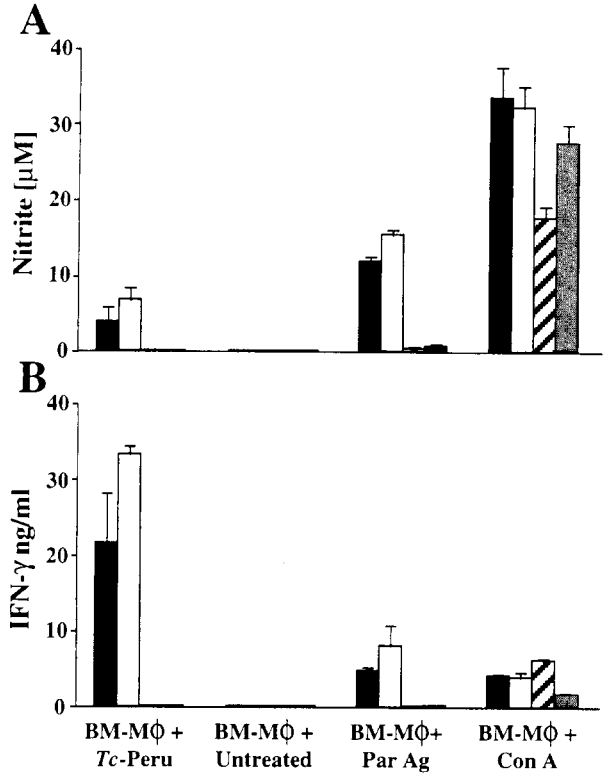


**FIGURE 3.** In vitro induction of NO and IFN-γ by $CD4^+$ T cells from PAR-immunized mice exposed to either *T. cruzi*-infected bone marrow macrophages or PAR Ag. Infected or uninfected bone marrow macrophage from BALB/c mice were cultured for 72 h with nylon wool-purified T cells (■), $CD4^+$ T cells (□), or $CD8^+$ T cells (▨) from PAR-immunized mice or with T cells from naive syngeneic mice (▩). In each panel the experimental groups are the T cells cultured with *T. cruzi*-infected bone marrow macrophage, untreated bone marrow macrophage, bone marrow macrophage in the presence of PAR (5 μg/ml), or bone marrow macrophage in the presence of Con A (5 μg/ml). Culture supernatants also were collected on days 5 and 7, and similar patterns of NO and IFN-γ production were seen (data not shown).

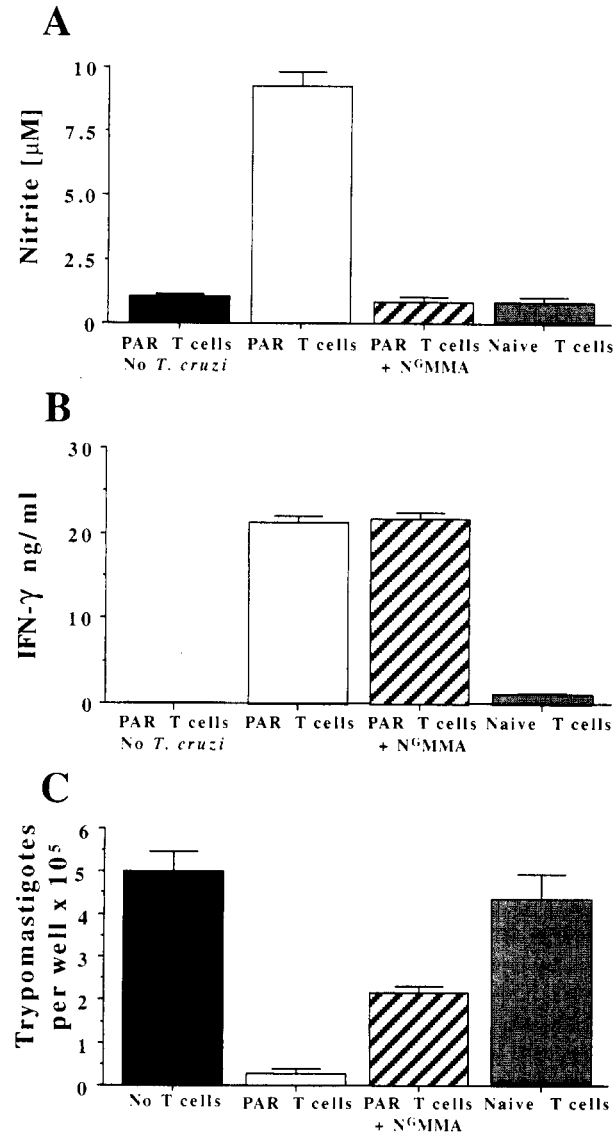


**FIGURE 4.** In vitro induction of NO, IFN-γ, and reduced parasite titers in cultures containing both *T. cruzi*-infected peritoneal macrophage and T cells from PAR-immunized mice. Infected or uninfected peritoneal macrophage from BALB/c mice were cultured for 72 h with T cells from either PAR-immunized or naive syngeneic mice. In each figure the experimental groups are T cells from PAR-immunized mice cultured with uninfected peritoneal macrophage, infected peritoneal macrophage, infected peritoneal macrophage in the presence of $N^G$MMA, or naive T cells cultured with infected peritoneal macrophage. In *A*, micromoles of nitrite in culture supernatants was determined; in *B*, nanograms per milliliter of IFN-γ in culture supernatants was measured; in *C*, the PAR-T cell group was replaced with cultures containing only infected macrophage, and the numbers of free-swimming trypomastigotes in culture supernatants in each of the four groups were calculated.

in the culture supernatant of both *T. cruzi*-infected macrophages and macrophages treated with PAR in the presence of purified T cells or $CD4^+$ T cells from PAR-immunized mice. In contrast, no $NO_2$ above background levels could be detected in cultures with $CD8^+$ T cells or naive T cells.

It has been shown that parasiticidal activity of INF-γ- plus LPS-activated macrophages against *T. cruzi* involves an L-arginine-dependent, NO-mediated mechanism (32). Although the above study did not involve T cells, our observation that T cells from PAR-immunized mice elicit NO production in infected macrophages accompanied by a reduction in parasite replication (Fig. 4) suggests that this antimicrobial activity may result from L-arginine-dependent NO production. To test this possibility, the inhibitor of NO synthase, $N^G$MMA, was added to the culture containing infected macrophages and T cells from PAR-immunized mice. As shown in Figure 4, the addition of $N^G$MMA had no effect on INF-γ production; however, NO synthesis was reduced to background levels, and the number of parasites present in the culture supernatant was approximately 10 times that observed in the absence of inhibitor.

### *INF-γ is essential for survival of* T. cruzi*-infected PAR-immunized mice*

Since the above studies suggest that INF-γ may play an important role in PAR-mediated immunity, we examined the ability of PAR-immunized mice that have a genomic disruption of the gene for INF-γ (GKO) to survive a challenge with *T. cruzi*. As shown in Table I, both the PAR-immunized mice and the Freund's control mice developed high parasitemias and died by day 19, demonstrating that IFN-γ function is essential for PAR-mediated protective immunity. Also, both groups of GKO mice had extremely high levels of circulating parasites compared with the C57Bl/6 control groups ($p = 0.01$), consistent with the inference from the above

studies that INF-$\gamma$ produced by $CD4^+$ T cells in PAR-immunized mice plays a major role in reducing the level of parasitemia following *T. cruzi* challenge.

## Discussion

We have examined the ability of PAR to provide protective immunity against *T. cruzi* in mice that have been severely (i.e., >94%) depleted of their $CD4^+$ cell population, possess a genetic deficiency that abrogates their ability to produce Abs, have a genetic deletion in the gene coding for the cytokine INF-$\gamma$, or carry a genetic deficiency that prevents the presentation of Ag via the MHC class I pathway. Our principle findings are as follows. 1) PAR-immunized mice challenged with an otherwise lethal inoculum of *T. cruzi* do not require Ab function for either survival against infection or clearance of the parasite from the bloodstream. 2) The inability to present Ag by MHC class I abolishes the ability of PAR-immunized mice to survive a challenge by the parasite and severely limits their capacity to control the level of circulating parasites. 3) The absence of the cytokine INF-$\gamma$ also severely limits the capacity of PAR-immunized mice to control the level of circulating parasites and survive a challenge. 4) The depletion of >94% of the $CD4^+$ cells in PAR-immunized mice drastically reduces their capacity to control the level of circulating blood parasites, but, surprisingly, the immunized mice can survive the infection for $\geq$84 days postchallenge while maintaining a blood parasite burden of $>10^6$ parasites/ml. 5) $CD4^+$ T cells from PAR-immunized mice are capable of stimulating *T. cruzi*-infected macrophages to produce NO, which results in strong inhibition of parasite growth.

The importance of Abs in the control of parasitemia in both acute and chronic stages of experimental Chagas' disease is well documented. As early as 1938, it was shown that passive immunization with anti-*T. cruzi* antiserum can protect mice against a lethal challenge (45). Later studies using passive transfer of convalescent mouse serum (46) or specific anti-*T. cruzi* Abs (10), confirmed the earlier findings, and additional investigations looking at the susceptibility of high and low Ab responder mice (6) and rats depleted of IgM by administration of anti-$\mu$ antiserum (12) also demonstrated the importance of Ab for survival and parasite clearance. These studies and the proposal by Brenner and coworkers that trypanolytic Abs elicited by an active infection are the major and possibly sole immune effector mechanism controlling murine and human *T. cruzi* infections (47) have led to the general belief that anti-*T. cruzi* Abs are essential for expression of protective immunity against the parasite, and concomitantly, that an efficacious vaccine against *T. cruzi* must contain a protective B cell epitope(s). While Abs undoubtedly play an important role in providing protection during a natural infection, we believe that the observation that $\mu$MT mice immunized with PAR both survive a lethal challenge and reduce the level of circulating parasites below detectable levels demonstrates that when mice are vaccinated with an efficacious immunogen, B cell responses are not essential for survival and/or parasite clearance. We do not, however, exclude the possibility that B cell responses are important in reducing the pathology that is associated with the chronic stage of the infection, and this issue is currently being investigated.

The fact that some inbred strains of mice when infected with *T. cruzi* exhibit significantly higher parasitemia levels and shorter survival times than other inbred strains of mice (13) has lead to the generally accepted belief that parasitemia is predictive of mortality. We believe, however, that this is not necessarily the case. Our conclusion is based on the observation that PAR-immunized mice depleted of >94% of their $CD4^+$ T cells survive an otherwise lethal challenge by the parasite, but do not substantially reduce the level of circulating parasites for up to day 84 of infection, at which time depletion of the $CD4^+$ T cells was terminated. Furthermore, there was no indication that the mice could not have survived well beyond the 84-day experimental period, since there was no evidence of physical distress, such as lethargy, loss of appetite, or scruffy coat. Thus, this result in addition to demonstrating that $CD4^+$ T cells play an important role in parasite clearance, also reveals that mice can tolerate high levels of parasitemia for at least several months without succumbing to the infection. A plausible explanation for this latter observation is that high parasitemia burden may not be the primary factor that results in the tissue damage that eventually leads to death during the acute stage, but, rather, pathologic events that occur in the early stages of infection may generate ongoing tissue damage that eventually results in death independent of the level of parasitemia that might occur. Consistent with the speculation that high parasitemia levels may not necessarily lead to death are the results of Rottenberg et al. (48), which show that when genetically altered mice that lack CD4 were infected with the low virulent CA-I strain of *T. cruzi*, a blood parasitemia of $>10^7$ parasites/ml (i.e., 1000 times that observed in $CD4^+$ control mice) was present by day 30 of infection. Furthermore, the parasitemia remained at this high level until at least day 50 of infection, yet 70% of the mice survived. In contrast, infection of $CD4^-$ mice with the virulent RA strain resulted in 100% lethality by about day 18 of infection, yet blood parasitemia attained a level of only $2 \times 10^6$ parasites/ml.

$CD4^+$ T cells may contribute to the reduction of circulating parasites by several mechanisms. They may directly interact with specific B cells to enhance B cell proliferation and Ab production, which then reduces circulating parasites by either complement-mediated lysis (10) or other Ab-mediated mechanisms (49, 50). They may also function as effector cells that recognize infected macrophages and release lymphokines, which, in turn, activate the phagocyte to either kill or inhibit the replication of the intracellular form of *T. cruzi*. In the case of PAR-immunized mice, while we cannot exclude the possibility that parasite reduction may be occurring due to enhanced B cell activity following repopulation of $CD4^+$ T cells, we suspect that much of the reduction in parasite burden is probably a result of $CD4^+$ T cells functioning as effector cells. We base this belief on the observations that PAR-immunized mice do not require Ab function to clear the parasite from the bloodstream, and that $CD4^+$ cells from PAR-immunized mice can specifically activate *T. cruzi*-infected macrophages, leading to NO production and a concomitant reduction of parasite numbers (Fig. 4). Consistent with this finding are the observations that 1) $CD4^+$ T cells from PAR-immunized mice produce high levels of IFN-$\gamma$, a potent macrophage-activating cytokine, in vitro when stimulated with purified PAR Ag (Figs. 3 and 4) (44); and 2) PAR-immunized mice whose $CD4^+$ T cell population has been depleted lack the ability to control parasitemia following a *T. cruzi* challenge (Fig. 1).

Since nonactivated macrophages infected with *T. cruzi* are not capable of damaging the intracellular form of the parasite (32) and thus causing the release of intracellular proteins such as PAR, which may then function as immunogens, the question arises of why T cells from PAR-immunized mice should recognize these macrophages in an Ag-specific fashion. One plausible explanation lies in the differences in the various stages of the life cycle of the mammalian forms of the parasite. The bloodstream form of the parasite, the trypomastigote, is in $G_0$ and must invade the host cell before propagation. Upon invasion and before cell division, there is an obligatory transformation of the trypomastigote into the amastigote, which is the intracellular dividing form of the parasite.

During this transformation, the flagellum of the trypomastigote undergoes a reduction in length of >90%, presumably as a result of catabolic mechanisms. Since the flagellum is the intracellular location of PAR, and loss of the flagellum occurs at least in part while the parasite is in the phagolysosome, it seems likely that PAR might be proteolyzed and available for association with MHC class II molecules. Following degradation of the phagolysosome, it is equally possible that PAR fragments might be released into the cytoplasm of the macrophage and be available for entry into the MHC class I pathway. If this should be the case, the obligatory transformation of the intracellular trypomastigote before propagation may result in PAR being displayed in association with MHC molecules on the surface of infected cells as well as possibly other flagellar proteins.

The absolute requirement of MHC class I and presumably $CD8^+$ T cells for the survival of PAR-immunized mice is revealed by the striking susceptibility of the $\beta_2$m-deficient mice to *T. cruzi* infection. Both the PAR-immunized and Freund's control mice succumbed to infection by day 20. The PAR-immunized mice showed a somewhat lower parasitemia than the Freund's control animals, consistent with the view that PAR-specific $CD4^+$ T cells may play a role in lowering the parasite burden in the immunized $\beta_2$m-deficient mice. The parasitemia of the $\beta_2$m $-/-$ Freund's control group was also significantly ($p = 0.05$) higher than that of the Freund's C57Bl/6 control group, indicating, as have previous studies (16, 18), that both $CD8^+$ and $CD4^+$ T cells provide important functions for the control of parasitemia.

It is interesting that an immunization regimen typically considered to induce an MHC class II-restricted response would induce protection that apparently requires $CD8^+$ T cells. It seems unlikely that the requirement for $CD8^+$ T cells is for the production of IFN-$\gamma$, since spleenocytes highly enriched for $CD8^+$ T cells and lacking $CD4^+$ T cells do not produce IFN-$\gamma$ in the presence of either PAR Ag or *T. cruzi*-infected macrophages (Fig. 3). One possible role of the $CD8^+$ T cells could be that they possess cytolytic function and protect through killing of *T. cruzi*-infected cells. The induction of CTL by immunization with soluble Ag has been demonstrated using several different viral Ags (51–53), the protozoan *T. gondii* (54), and highly purified OVA (55). The studies with OVA are particularly relevant, since it was found that while detergent-denatured OVA was capable of inducing CTLs in immunized mice, neither native, heat-denatured, nor urea-denatured OVA was able to induce measurable CTL responses following immunization, thus indicating that detergent denaturation was necessary for the soluble protein to induce CTLs. The purification process of PAR preparations involves separation by SDS-PAGE techniques; therefore, all PAR Ag given in the immmunization regimen is denatured by SDS. The possibility that PAR, like OVA, may be capable of inducing CTLs in vivo is being examined.

In summary, the results of these experiments lead to several unique findings. Firstly, the observation that parasitemia in PAR-immunized mice can be controlled in the absence of B cell function, but not in the absence of $CD4^+$ T cell function, was unexpected. Secondly, the finding that $CD4^+$ T cells from PAR-immunized mice are capable of activating parasitized macrophages and induce killing through what appears to be NO-mediated mechanism is the first indication that immunization with a defined Ag can generate effective trypanocidal activity. Thirdly, the observation that MHC class I-restricted T lymphocytes are critical for the protection of mice immunized with PAR introduces the possibility that immunization with PAR can induce an Ag-specific CTL response. All of the above findings have important implications for the development of a vaccine against *T. cruzi*.

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