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Paraflagellar rod proteins administered with alum and IL-12 or recombinant adenovirus expressing IL-12 generates antigen-specific responses and protective immunity in mice against *Trypanosoma cruzi*

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Abstract

Successful vaccination of mice against an otherwise lethal challenge with the Peru strain of *Trypanosoma cruzi* necessitates the induction of a strong cell mediated immune response. Previously, immunization of mice with the paraflagellar rod proteins from *Trypanosoma cruzi* administered subcutaneously with Freund's adjuvant has been shown to elicit an Ag-specific cellular immune response that reduces parasitemia and provides 100% survival against an otherwise lethal challenge with the parasite. Since this vaccine regimen is unacceptable for use in humans, we have tested immunization regimens using PFR Ag in combination with alum and either recombinant murine IL-12 or a replication deficient adenovirus vector expressing murine IL-12. Here, it is shown that immunization of C57BL/6 mice with PFR Ag coadsorbed to alum with either recombinant IL-12 or adenovirus-expressing IL-12 induces a strong cellular immune response that results in 100% survival and >90% reduction in parasitemia in immunized mice challenged with the bloodstream stage of *Trypanosoma cruzi*.

Keywords: *Trypanosoma cruzi*; Paraflagellar rod proteins; IL-12/Alum

1. Introduction

Trypanosoma cruzi, a hemoflagellate parasite, is the causative agent of American trypanosomiasis or Chagas' disease [1]. In Central and South America, this disease is an important public health problem, and adequate control is hampered by the lack of effective prophylactic or therapeutic agents. In an effort to develop a vaccine to prevent infection, we have found that immunization of mice with a highly purified preparation of paraflagellar rod (PFR) proteins present in the flagellum of *T. cruzi* induces an immune response that results in reduction in the level of circulating parasites and 100% survival against an otherwise lethal inoculum of *T. cruzi* trypomastigotes [2]. The PFR preparation is composed of four distinct proteins, as determined by direct amino acid sequence analysis, immunological analysis with PFR-specific monoclonal antibodies, and analyses of the genes that encode these four proteins [3]. Additional studies have shown that PFR-induced protective immunity requires T cell, but not B cell function, and that survival is strongly associated with a Th-1 type cytokine response [4,5]. Further evidence that INF-γ is critical to the protective response is provided by the observation that PFR-immunized INF-γ knockout mice develop an extremely high parasitemia and do not survive challenge infection.

To date, PFR-mediated protection resulting in 100% survival following challenge infection has only been achieved by subcutaneous immunization of mice with PFR proteins emulsified in Freund's adjuvant [4].

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Immunization of mice with PFR proteins in combination with several other adjuvants (i.e. Ribi-700, QS-21, and murine recombinant IL-12) provided no reduction in parasitemia and no survival against challenge with *T. cruzi* beyond that seen in control animals. However, these studies did reveal that immunization of mice with PFR proteins absorbed to alum provided some survival and a modest decrease in parasitemia compared to *T. cruzi* challenged control animals, thus encouraging additional testing with alum using different immunization regimens, possibly in combination with other immunomodulators.

The cytokine IL-12, a 40 (p40) and 35 (p35) kD dimeric molecule, acts to rapidly and strongly orient the immune response towards a type 1 cytokine pattern [6,7] with a pronounced enhancement of IFN-γ [8–11]. In several disease models, *Leishmania* [12], *Toxoplasma* [13], *Listeria* [14] and *Schistosoma* [15], protective immune responses and Th1 signature cytokines have been enhanced by administration of IL-12 + antigen. Thus, it was surprising that s.c. administration of PFR Ag in combination with rIL-12 failed to protect mice against a subsequent challenge with *T. cruzi* trypomastigotes [4]. Furthermore, T cells from these mice produced low levels of IFN-γ and IL-2 following stimulation in vitro with PFR and substantially higher levels of the Th 2 signature cytokines IL-4 and IL-5 (Wrightsman and Manning, unpublished). Interestingly, the observation that immunization with an Ag in combination with rIL-12 can result in T cells that produce a predominantly Th-2 type cytokine response was also recently observed in studies with the HIV-1 gp 120 protein [16]. Spleen cells from mice immunized s.c. with gp 120 and rIL-12 in solution produced high levels of the Th-2 signature cytokines IL-4, IL-5 and IL-10 and only low levels of IFN-γ. However, the cytokine profile shifted to a strong type 1 response when mice were immunized s.c. with gp 120 protein adsorbed to alum. The striking change in the nature of the T cell response, along with our previous observation that immunization of mice with PFR Ag adsorbed to alum provides some protection against *T. cruzi*, led us to investigate the efficacy of the immune response produced following immunization of mice with either PFR Ag + murine recombinant IL-12 adsorbed to alum or PFR protein + recombinant adenovirus expressing murine IL-12 adsorbed to alum.

2. Materials and methods

2.1. Abbreviations used in this paper

C-DMEM, 25 mMHEPES buffer (pH 7.2), 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, 5×10^{-5} M 2-ME, 50 U/ml penicillin, 50 μg ml streptomycin sulfate, and 10% FBS; NO, nitric oxide; rIL-12, murine recombinant IL-12; PFR, paraflagellar rod; PBS, phosphate buffered saline, pH 7.4; PBST, phosphate buffered saline and 0.05% Tween 20; s.c., subcutaneous.

2.2. Parasites

The Peru clone 3 and Y strains of *Trypanosoma cruzi* was used. Epimastigotes were grown in modified HM [17]. Bloodstream trypomastigotes used for challenge inoculations of mice were obtained by cardiac puncture of female BALB/cByJ mice on day 14 postinfection. Tissue culture-derived trypomastigotes used for macrophage infection were obtained from infected monolayers as described elsewhere [18].

2.3. Purification of PFR proteins

PFR proteins were purified as previously described [18]. 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% PFR proteins and 50% tubulin. The PFR proteins were separated by preparative SDS-PAGE on a Bio-Rad Prep Cell (model 491, Bio-Rad Laboratories, Richmond, CA). Fractions containing the PFR proteins were extensively dialyzed against PBS, concentrated in a Centricon (Amicon, Beverly, MA) and sterilized by 0.45-μm pore filtration. Protein concentrations in the PFR samples were determined using the Pierce protein assay (Pierce Chemical Corp, Rockford, IL) and purity was assessed by SDS PAGE.

2.4. Immunization

Six- to 8-week-old female C57BL/6J mice were immunized by subcutaneous (s.c.) injection with 40 μg PFR protein adsorbed to alum or emulsified with Freund's complete adjuvant. The mice were boosted twice at two-week intervals with 20 μg PFR protein adsorbed to alum or emulsified with Freund's incomplete adjuvant. Alum-PFR Ag mixtures were prepared by combining equal volumes of Rehsorpter aluminum hydroxide adsorptive gel (Intergen Co., Purchase, NY) and PFR Ag at 400 μg/ml in 0.9% saline with gentle mixing for 1 h at room temperature. In some experiments murine recombinant IL-12 (rIL-12; a generous gift from Genetics Institute, Cambridge, MA) was mixed with the PFR protein during the adsorption

procedure. The amount of rIL-12 added to the mixture gave a final concentration of either 0.5 μg rIL-12/40 μg PFR (initial immunization) or 0.5 μg rIL-12/20 μg PFR (boosts). In other experiments recombinant adenovirus AdmIL-12.1 (a generous gift of F.L. Graham, McMaster University, Hamilton, Ont., Canada; 19) or AdmCMVJL.2-βgal (a replication deficient adenovirus expressing bacterial β-galactosidase; a generous gift of Luis Villarreal, University of California, Irvine, CA) were added to the PFR/Alum mixture during the adsorption procedure. The pfu added during adsorption were as described in Results. Recombinant adenovirus was included only in initial immunizations and not subsequent boosts. Two weeks after the last injection, mice were challenged with s.c. injection of 10^2 bloodstream trypomastigotes. Following challenge, mice were checked daily, and survival was recorded at days postinfection. Parasitemia levels were monitored as previously described [20] by removing a blood sample from the tail vein, diluting the sample 1:10 in 0.9% ammonium chloride to lyse the red blood cells, and counting the number of parasites in a Neubauer hemocytometer (American Optical Corp., Buffalo, NY). Parasitemias were monitored every other day from Day 14–26 and weekly from Week 4–6. As previously described [20], peak parasitemia in unimmunized C57BL/6 mice is 5×10^6 trypomastigotes/ml and occurs at days 17–19 postchallenge with a mean survival time of 21 days. Control immunizations included mice injected with regimens of PFR protein adsorbed to alum, rIL-12 adsorbed to alum, AdmIL-12.1 adsorbed to alum, PFR protein plus AdmCMVJL.2-βgal adsorbed to alum. Adsorption of protein to alum was tested by protein assay to ensure that no detectable protein was present in the supernatant.

2.5. Macrophage and T cell cultures

Mice were immunized as described above. Seven to ten days after the last injection, spleens 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, 5×10^{-5} M 2-ME, 50 U/ml penicillin, 50: g streptomycin sulfate, and 10% FBS (C-DMEM). Spleen cell suspensions were enriched for T cells by passage over nylon wool columns [21]. IC-21 macrophages were plated in C-DMEM at 1×10^5 macrophage/well in 96 well plates. Macrophage were allowed to adhere overnight (37°C at 8% CO_2). Cells were either infected overnight with trypomastigotes at a 10:1 parasite to macrophage ratio or incubated with PFR protein (3 μg/ml). Control wells were incubated overnight with C-DMEM containing no parasites. Infected monolayers were washed 3× with DMEM to remove extracellular parasites. Cells were cultured for 7 days at 37°C in an atmosphere of 8% CO_2, supernatants were harvested and assayed for NO_2. All experimental groups were in triplicate. $CD4^+$ and $CD8^+$ T cells were positively selected from nylon wool-purified T cells using MACS MultiSort MicroBeads following procedures recommended by the manufacturer (Miltenyi Biotec, Germany). The purity of the selected cell populations was >98% as determined by FACS analysis.

2.6. Measurement of cytokines

Culture supernatants were taken from triplicate cultures of T cells containing 3 μg/ml of PFR protein and assayed for the presence of IL-4 and IFN-γ by capture ELISA (Pharmingen, San Diego, CA). Briefly, 1–2 μg/ml of cytokine specific capture antibody was bound to 96 well microtiter plates in 0.1 M $NaHCO_3$, pH 8.2 at 4°C overnight, washed with PBST and then blocked with 10% FCS in PBS for 2 h at room temperature. Wells were washed with PBST, 100 μl 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 anticytokine detecting antibody added in a volume of 100 μl and incubated for 45 min at room temperature. The wells were thoroughly washed, 100 μl of strepavidin–peroxidase (2.5 :l/ml) was added, and the reaction was incubated for 30 min at room temperature. After extensive washes in PBST, 100 μl of 2,2′-azino-di-3ethylbenzthiazoline 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 pg/ml.

2.7. Nitrite assays

Nitrite levels in 4- and 7-day culture supernatants were measured using the Greiss reagent as previously described [4]. Briefly, 50 :l culture supernatents were combined in a 96-well plate with a 1/1 mixture of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% naphthylethlenediamide in 2.5% H_3PO_4. Plates were incubated 10 min at room temperature and absorbance was determined at 550 nm using an automated microplate reader. Nitrite concentrations were determined in triplicate using a standard curve of sodium nitrite from 125 to 1 μM prepared in culture media.

2.8. Inhibition of T. cruzi growth in vitro

Parasite titers in the infected IC-21 macrophage/T

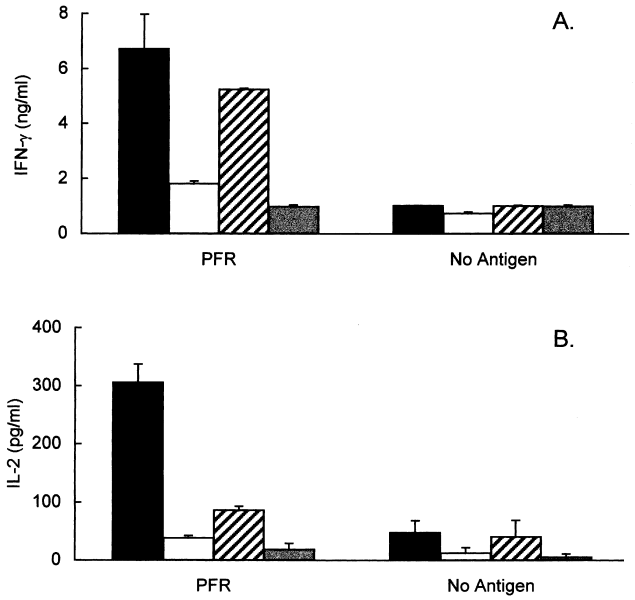

Fig. 1. IFN-γ and IL-2 production by T cells from PFR-immunized mice. Nylon wool-purified T cells from PFR-immunized mice were cultured with PFR antigen and irradiated syngeneic feeder cells. On days 2, 3, 5 and 7 of culture, supernatants were removed and tested in a capture ELISA assay for IFN-γ (A) and IL-2 (B) production. Data shown represents the peak days of IL-2 (day 3) and IFN-γ (day 5) production. Bars represent T cells from mice immunized with PFR proteins emulsified in Freund's adjuvant (black bars), PFR proteins coadsorbed to alum (clear bars), PFR proteins and recombinant IL-12 coadsorbed to alum (diagonal hatched bars), and naïve mice (gray bars). Each value shown represents the mean value from triplicate cultures ± the standard deviation.

cell culture supernatants were determined by pipeting media up and down vigorously several times to resuspend trypomastigotes. Parasite number was determined by counting with a Neubauer hemocytometer.

3. Results

3.1. Effects of coadministration of rIL-12 on PFR Ag-induced cytokine responses

Previous studies have shown that protection of mice against a *T. cruzi* challenge by immunization with PFR Ag is associated with a Th-1 type cell mediated immune response and that IFN-γ plays a particularly critical role in survival and parasite reduction [4,5]. Since significant protection has been observed only with Freund's adjuvant, and this adjuvant cannot be used in humans, it is desirable to explore the efficacy of other adjuvant system that may be acceptable for use in humans and/or other animal models. Because IL-12 has been shown to be an important cytokine in directing Th1 immune responses in mice [6,7] and is in clinical trials for use in humans [22], we first performed experiments to test whether immunization of mice with IL-12 and PFR Ag coadsorbed to alum will promote a type 1 cytokine response similar to that seen with PFR Ag administered s.c. in Freund's adjuvant.

Mice were immunized s.c. with recombinant murine

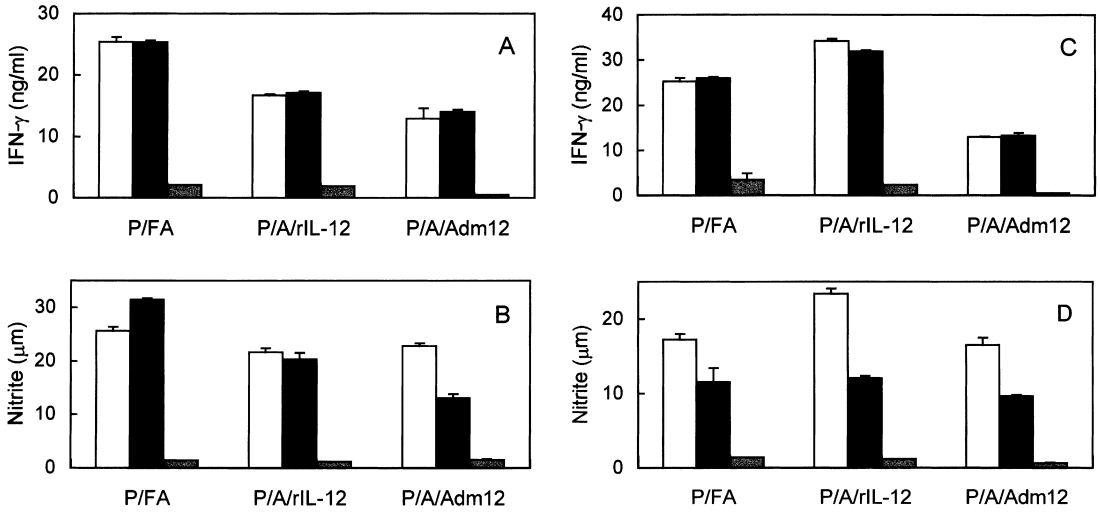

Fig. 2. In vitro induction of NO and IFN-γ by T cells from PFR-immunized mice exposed to either *T. cruzi*-infected macrophages or PFR Ag. IC-21 macrophages were cultured with PFR protein (panels A and B) or trypomastigotes (panels C and D) and T cells from PFR-immunized mice. Nylon wool-purified T cells (clear bars), $CD4^+$ T cells (black bars) or $CD8^+$ T cells (gray bars) from mice immunized with PFR proteins emulsified in Freund's adjuvant (P/FA), PFR proteins and recombinant IL-12 coadsorbed to alum (P/A/rIL-12.1) or PFR proteins and 2×10^8 pfu of AdmIl-12.1 coadsorbed to alum (P/A/AdmIL12) were added to wells with IC-21 macrophages. On day 4 of culture, supernatants were removed and analyzed for nitrite and IFN-γ. Each value shown represents the mean value from triplicate cultures ± the standard deviation.

IL-12 and PFR Ag coadsorbed to alum and were boosted twice at an interval of 14 days. Ten days after the last boost spleen cells were collected and nylon wool-purified T cells were assayed for PFR Ag-induced cytokine production. For controls, T cells from naïve mice and from mice immunized with PFR Ag adsorbed to alum or emulsified in Freund's adjuvant were assayed for cytokine production following stimulation with PFR Ag. As shown in Fig. 1A, T cells from PFR Ag/Freund's immunized mice produced significant levels of IFN-γ, while the PFR Ag/alum immunized mice produce only slightly more IFN-γ than that observed with naïve T cells, consistent with previous observations [4]. Most encouraging was the finding that T cells from PFR Ag/alum/rIL-12 immunized mice produced about the same amount of IFN-γ as T cells from PFR Ag/Freund's immunized mice. No detectable IL-4 or IL-5 were observed in any of the four groups (data not shown), and a significant level of IL-2 was observed only with the Freund's group (Fig.1B), an observation consistent with findings of others which show that IL-12 does not enhance IL-2 synthesis [16,23,24].

3.2. $CD4^+$ T cells from mice immunized by coadministration of PFR and rIL-12 can activate T. cruzi-infected macrophages

Activated macrophages are known to play an important role in resistance to *T. cruzi* infection [25–28], presumably by the production of NO which leads to the killing of the intracellular stages of the parasite [29,30]. Our previous studies have shown that T cells from PFR Ag/Freund's immunized mice recognize *T. cruzi* infected macrophages and release cytokines, particularly IFN-γ, that result in macrophage activation [5]. To determine whether immunization with PFR Ag/alum/IL-12 might also produce T cells with a similar specificity, we examined the levels of INF-γ and NO_2 in the supernatants of nylon wool-purified T cells and MACS-selected $CD4^+$ and $CD8^+$ T cells from PFR Ag/alum/rIL-12 immunized mice. T cells were incubated with IC-21 macrophages that were either infected with *T. cruzi* (Fig. 2C and D) or cultured in the presence of purified PFR Ag (Fig. 2A and B). For controls, INF-γ and NO_2 levels were determined from culture supernatants identical to the above except that T cells were purified from naïve mice (data not shown), or mice immunized with either PFR Ag/Freund's or PFR Ag/alum.

As shown in Fig. 2A and C, T cells and $CD4^+$ T cells from mice immunized with either PFR Ag/Freund's or PFR Ag/alum/IL12 secreted high levels of IFN-γ in the presence of macrophages incubated with PFR Ag or macrophages infected with *T. cruzi*. Only negligible amounts of IFN-γ were observed with the

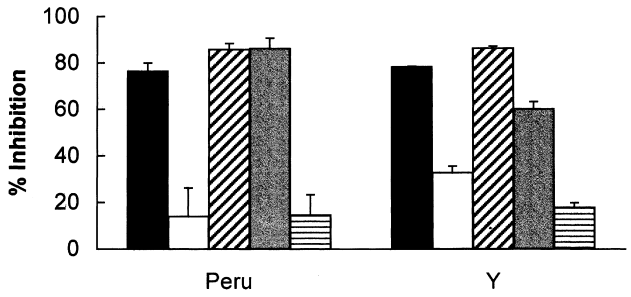

Fig. 3. Inhibition of parasite replication in macrophages cocultured with T cells from mice immunized with PFR Ag. IC-21 macrophages were infected with Peru or Y strain tissue culture-derived trypomastigotes. After overnight incubation, macrophage monolayers were washed 2× with PBS and were refed with media containing T cells from PFR-immunized or naïve mice. On day 4 of culture, supernatants were removed and parasites were counted. The % inhibition was calculated as follows: 100× (1−#parasites/#parasites in wells with no T cells). T cells were tested from naïve mice (horizontal hatched bars), and mice immunized with PFR Ag in Freund's adjuvant (PFR/FA, black bars), PFR Ag adsorbed to alum (PFR/Alum, clear bars), PFR Ag and rIL-12 coadsorbed to alum (PFR/Alum/rIL-12, diagonal hatched bars), and PFR Ag and 2×10^8 pfu AdmIL-12.1 coadsorbed to alum (PFR/Alum/AdmIL-12, gray bars). Each value shown represents the mean value from triplicate cultures ± the standard deviation.

$CD8^+$ T cell population, and no measurable IFN-γ could be detected when any of these T cells were incubated with untreated macrophages (data not shown). IFN-γ levels in the supernatants from cultures containing T cells from PFR Ag/alum immunized mice were also very low (< 2 ng/ml; data not shown).

Consistent with the fact that IFN-γ is important for macrophage activation, high levels of NO_2 were only observed in the culture supernatants of macrophages incubated with T cells or $CD4^+$ T cells from mice immunized with PFR Ag/Freund's or PFR Ag /alum/rIL-12 (Fig.2B and D). No NO_2 above background could be found in cultures with naïve T cells (data not shown), T cells from PFR Ag/alum (data not shown) immunized mice or any of the $CD8^+$ T cell populations (Fig. 2B and D). Consistent with the fact that the parasiticidal activity of activated macrophages involves NO-mediated mechanism(s), T cell/macrophage cultures that produce high levels of IFN-γ and NO_2 exhibited significant antimicrobial activity. As shown in Fig. 3, supernatants of *T. cruzi* infected IC-21 macrophages cultured with T cells purified from mice immunized with either PFR Ag/Freund's or PFR Ag/alum/rIL-12 showed a 75–85% reduction in parasite numbers when compared to control cultures that contained T cells from naïve mice. Although the T cells used in these assays were obtained from mice immunized with PFR Ag isolated from the Peru strain, parasite replication also was inhibited in macrophages infected with the Y strain of *T. cruzi* (Fig. 3), suggesting that T cell recognition of PFR Ag epitopes

Table 1
Effect of IL-12 on protective immunity by vaccination with PFR Ag

Vaccination	PFU (10^8) injected	No. of mice immunized	Mean parasitemia/ml (10^5) ± S.D. at day				No. of survivors	Mean survival time (days)
			14	17	19	22		
Alum	NA	6	20.8 ± 11	43.0 ± 20	44 ± 23	ND	0	21
Freund's	NA	5	8.35 ± 4	15.3 ± 9	28.5 ± 9	25.6 ± 18	0	22.2
PFR Ag + Freund's	NA	6	3.2 ± 0.2	3.8 ± 1.0	5.2 ± 1.6	4.1 ± 2.3	6	> 60
PFR Ag + Alum	NA	6	16.8 ± 1	14.8 ± 19	19.0 ± 10	11 ± 11	3	19[a]; > 60
PFR Ag + Alum + rIL12	NA	6	5.1 ± 0.9	2.5 ± 2.9	1.9 ± 0.7	0.9 ± 0.3	6	> 60
Alum + rIL-12	NA	6	3.5 ± 2.2	9.0 ± 1.9	6.4 ± 4.0	9.0 ± 3.6	1	22[a]; > 60
PFR Ag + Alum + AdmIL-12.1	0.6	6	1.4 ± 0.6	1.7 ± 1.1	2.6 ± 1.1	< 1.0	6	> 60
	2.0	6	2.2 ± 2.1	3.4 ± 1.1	2.1 ± 1.0	< 1.0	6	> 60
	6.0	6	2.2 ± 1.1	2.2 ± 2.1	2.4 ± 1.1	ND	6	> 60
	20	6	1.4 ± 0.9	2.6 ± 0.6	1.6 ± 0.7	< 1.0	6	> 60
Alum + AdmIL-12.1	2.0	6	12.5 ± 7.4	12.9 ± 5.7	16.3 ± 13	17 ± 4.2	1	24[a]; > 60
PAR Ag + Alum + AdCMV.2-βgal	2.0	4	ND	6.5 ± 5	10.0 ± 5.6	17.2 ± 3.6	2	25[a]; > 60

[a] Mean time of death of mice that did not survive > 60 days.

on infected macrophages may not be highly strain specific. Additional control studies also revealed that parasite numbers in supernatants of cultures containing T cells from mice immunized with alum/rIL-12 (data not shown) were not significantly different from those found in cultures containing T cells from naïve mice.

3.3. $CD4^+$ T cells from mice immunized by coadministration of PFR/alum and AdmIL-12.1 can activate T. cruzi-infected macrophages

The observations that significant levels of biologically active IL-12 can be produced in mice following injection of the recombinant replication deficient adenovirus AdmIL-12.1 [19] and that intramuscular injection of an adenovirus-expressing IL-12 (e.g. Ad5IL-12; 30) can induce an immune response that is associated with protection of BALB/c mice against *L. major* infection, lead us to investigate the possibility that the rIL-12 used in the above immunization regimen might be efficaciously replaced by AdmIL-12.1. To explore this possibility, mice were immunized with PFR Ag and 2×10^8 pfu of AdmIL-12.1 coadsorbed to alum. Because immune responses generated against adenovirus vectors have been reported [32–35], administration of AdmIL-12.1 was limited to the initial immunization, and the two boosts that were administered at two-week intervals following the initial injection contained only PFR Ag/alum. For controls, mice were immunized with AdCMV.2-βgal, a replication deficient adenovirus expressing β-galactosidase, coadsorbed to alum with PFR Ag, PFR Ag/Freund's, or PFR Ag/alum/rIL-12. Nylon wool-enriched T cells isolated from experimental and control mice were incubated with IC-21 macrophages that were either infected with *T. cruzi* or cultured in the presence of purified PFR Ag. Culture supernatants were assayed for INF-γ and NO_2 levels. The IFN-γ and NO_2 levels observed for the AdmIL-12.1 immunized mice samples were within 50–80% of those found in identical experiments in which the T cells were isolated from mice immunized with PFR Ag/alum/rIL-12 or PFR Ag/Freund's (Fig. 2). In contrast, the IFN-γ level in cultures containing T cells from mice immunized with PFR Ag/alum/AdCMV.2-βgal were only slightly above those measured in cultures containing T cell from naïve mice, while NO_2 levels in these cultures were below detection. Predictably, parasite numbers in supernatants of *T. cruzi* infected macrophages incubated with T cells obtained from mice immunized with PFR Ag/alum/AdmIL-12.1 were similar to those found in cultures containing T cells from mice immunized with PFR Ag/alum/rIL-12 or PFR Ag/Freund's (Fig. 3) and were < 90% of those found in identical studies containing T cells from mice immunized with the PFR Ag/alum/AdCMV.2-βgal controls (data not shown).

3.4. Immunization of mice with PFR protein coadministered with recombinant IL-12 or recombinant adenovirus producing IL-12 provides protection against T. cruzi infection

We tested the efficacy of the PFR Ag as a vaccine candidate when coadministered with rIL-12/alum or AdmIL-12.1/alum by monitoring the survival and parasitemia burden of immunized mice after challenge with a lethal inoculum of the highly virulent Peru strain of *T. cruzi* (50% lethal dose, ≤5). As shown in

Table 1, 100% (i.e. six of six) of the mice immunized with PFR Ag/alum/rIL-12 survived the *T. cruzi* challenge. In comparison, only 1 of 6 mice in the alum/rIL-12 control group survived challenge, demonstrating that the PFR Ag/alum/rIL-12 vaccine regimen provides significant protection against an otherwise lethal challenge ($p < 0.02$, by Fisher's exact test). Consistent with this result is the finding that the level of circulating parasites in the PFR Ag/alum/rIL-12 immunized mice is significantly lower ($p = 0.05$, by Mann–Whitney U test) than that found in both the alum/rIL-12 and in the PFR Ag/alum immunized mice. The importance of both alum and IL-12 in combination is evidence by the findings that immunization of mice with PFR Ag plus alum provides only partial protection against a *T. cruzi* challenge ([3]; Table 1) and immunization of mice with PFR Ag plus rIL-12 provides no protection against a *T. cruzi* challenge [4].

We examined the efficacy of PFR Ag/alum/AdmIL-12.1 as a vaccine candidate for protection against *T. cruzi* by immunization of mice with four different titers of recombinant virus ranging in dose from 6×10^7 to 2×10^9 pfu coadsorbed with PFR Ag to alum prior to immunization. Two boosts containing only PFR Ag/alum were administered at 14-day intervals following the initial immunization. As shown in Table 1, this immunization regimen provided protection against a *T. cruzi* challenge that was equivalent to that observed with the PFR Ag/alum/rIL-12 regimen (i.e. six of six mice survived with each viral dose). In all four trials the parasitemia levels in the PFR Ag/alum/AdmIL-12.1 immunized mice were significantly less than those observed in the PFR Ag/alum/AdCMV.2-βgal control mice ($p = 0.05$, by Mann–Whitney U test), and were almost identical to those observed when the immunization regimen included rIL-12 rather than AdmIL-12.1. Furthermore, control mice immunized with either PFR Ag/alum/AdCMV.2-βgal or alum/AdmIL-12.1 showed no increase in survival and no significant difference in parasitemia from that observed in mice immunized with Freund's adjuvant alone.

4. Discussion

It is now well established that IL-12 plays a key role in potentiating cellular immune responses by directing the differentiation of $CD4^+$ T lymphocytes towards the Th1 phenotype while concomitantly suppressing differentiation of $CD4^+$ T cells into a subset exhibiting a Th2 phenotype [7,10]. This ability of IL-12 to polarize the direction of the immune response towards cellular immune mechanisms has led to the use of recombinant IL-12 as an adjuvant in vaccines directed against infectious organisms whose control within the host requires Th1 cellular immune responses. Studies with a number of different pathogens have shown that immunization with rIL-12 and Ag(s) in aqueous solution provided significant protection against subsequent challenge; however, similar protection studies using PFR Ag were unsuccessful [4]. In these studies PFR Ag and IL-12 administered either i.p. or s.c. in phosphate buffered saline was shown to provide no protection in mice against a subsequent challenge with *T. cruzi* trypomastigotes. Furthermore, T cells isolated from mice immunized by this regimen produced predominantly Th2 type cytokines upon stimulation in vitro with PFR Ag (Wrightsman and Manning, unpublished).

In contrast to these earlier findings, we now show that s.c. immunization with PFR Ag and either rIL-12 or adenovirus expressing-IL-12 coadsorbed onto alum produces a vaccine regimen that provides 100% survival in mice against an otherwise lethal challenge with *T. cruzi* (Table 1). In comparing the nature of the CMI response generated by this vaccine regimen with that produced by immunization of mice with PFR Ag in Freund's adjuvant, it is apparent that both regimens generate antigen-specific $CD4^+$ T lymphocytes of the Th1 subtype (Fig. 1). $CD4^+$ T cells from mice immunized by these regimens produce substantial levels of INF-γ upon stimulation in vitro with PFR Ag or upon incubation with macrophages infected with *T. cruzi*. Not surprisingly, this leads to macrophage activation, production of NO and a reduction in the ability of the parasite to replicate in the activated macrophage (Figs. 2 and 3).

The contrasting nature of the immune responses generated by immunization of mice s.c. with PFR Ag and IL-12 in solution vs. immunization s.c. with PFR Ag and IL-12 coadsorbed to alum are strikingly similar to the results of previous studies using IL-12 and recombinant gp 120 envelope protein from HIV-1 [16]. In both studies stimulation of T cells in vitro from mice immunized s.c. with Ag and IL-12 resulted in a Th2 type cytokine profile. In contrast, immunization of mice with Ag and IL-12 coadsorbed to alum generated T cells that produced a highly polarized Th1 type cytokine response upon stimulation in vitro. Taken together, these results suggest that antigens that have previously been found to direct differentiation of T lymphocytes towards the Th2 subset might be successfully used to direct differentiation of T lymphocytes towards the Th1 phenotype when coadsorbed to alum with IL-12 prior to immunization.

The use of the adenovirus-producing IL-12 vector in the vaccine regimen with PFR Ag may offer certain advantages. As noted earlier [18,31], the ability of the vector to transduce both quiescent and replicating cells allows for short term, high level synthesis of IL-12, a feature that is particularly attractive since IL-12's influence on the course of the immune response follow-

ing immunization or pathogen invasion occurs within a few days of the initial event [7]. Also, expression of the cytokine is short-term [19] and, therefore, it is unlikely that it will result in pathology that might accompany long term expression of the cytokine. Finally, in a more practical sense, the development of a viral vector suitable for use in humans might represent cost saving over the use of rIL-12, a consideration of some importance when developing a vaccine for a third world disease.

In summary, we have shown that immunization of mice with PFR Ag coadsorbed to alum with either rIL-12 or adenovirus-expressing IL-12 provides 100% survival against an otherwise lethal challenge with *T. cruzi* and results in a reduction in peak parasitemia of $>90\%$ of unimmunized controls. These findings are important for several reasons. First, alum/rIL-12 is potentially useful as an adjuvant formulation in humans, thus, this work represents an important step towards the development of a *T. cruzi* vaccine suitable for testing in humans. Second, with the recent identification of the protein constituents that comprise the PFR antigens, all components of the vaccine regimen tested in this study are chemically defined. Finally, the genes encoding the PFR proteins have been cloned, characterized, and expressed in recombinant vector systems [4], thus allowing the production of this antigen in large amounts, a further step needed prior to vaccine testing in humans. Furthermore, the observation that an adenovirus vector can express a recombinant gene in biologically significant amounts when adsorbed to alum suggests that alum may be an excellent medium for localized presentation of a multicomponent vaccine containing immunomodulatory agents and Ag systems that elicit strong $CD4^+$ and $CD8^+$ T cell responses.

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