Vaccine 27 (2009) 5644–5653

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Strain-specific protective immunity following vaccination against experimental *Trypanosoma cruzi* infection

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ARTICLE INFO

Article history:
Received 3 June 2009
Received in revised form 28 June 2009
Accepted 8 July 2009
Available online 25 July 2009

Keywords:
Trypanosoma cruzi
DNA vaccine
Adenovirus vaccine
CD8

ABSTRACT

Immunisation with Amastigote Surface Protein 2 (*asp-2*) and *trans*-sialidase (*ts*) genes induces protective immunity in highly susceptible A/Sn mice, against infection with parasites of the Y strain of *Trypanosoma cruzi*. Based on immunological and biological strain variations in *T. cruzi* parasites, our goal was to validate our vaccination results using different parasite strains. Due to the importance of the CD8$^+$ T cells in protective immunity, we initially determined which strains expressed the immunodominant H-2K^k-restricted epitope TEWETGQI. We tested eight strains, four of which elicited immune responses to this epitope (Y, G, Colombian and Colombia). We selected the Colombian and Colombia strains for our studies. A/Sn mice were immunised with different regimens using both *T. cruzi* genes (*asp-2* and *ts*) simultaneously and subsequently challenged with blood trypomastigotes. Immune responses before the challenge were confirmed by the presence of specific antibodies and peptide-specific T cells. Genetic vaccination did not confer protective immunity against acute infection with a lethal dose of the Colombian strain. In contrast, we observed a drastic reduction in parasitemia and a significant increase in survival, following challenge with an otherwise lethal dose of the Colombia strain. In many surviving animals with late-stage chronic infection, we observed alterations in the heart's electrical conductivity, compared to naive mice. In summary, we concluded that immunity against *T. cruzi* antigens, similar to viruses and bacteria, may be strain-specific and have a negative impact on vaccine development.

1. Introduction

Genetic vaccination is being pursued as an efficient, long-term, protective form of immunisation against *Trypanosoma cruzi* infection (reviewed in Refs. [1,2]). This strategy is particularly interesting, considering the importance of the CD4$^+$ Th1 and CD8$^+$ cytotoxic T cells during naturally acquired or vaccine-induced immunity to this human intra-cellular, protozoan parasite (reviewed in Refs. [3–5]).

Recently, we studied the immunisation of highly susceptible A/Sn mice with a heterologous prime-boost immunisation regimen, using plasmid DNA followed by a recombinant adenovirus that expressed the Amastigote Surface Protein-2 (ASP-2) of *T. cruzi*. This immunisation protocol generated protective immunity against a lethal challenge with parasites of the Y strain [6]. Vaccinated animals depleted of CD4$^+$ or CD8$^+$ T cells prior to parasitic challenge were highly susceptible to infection, indicating a critical role of these T cells during protective immunity. Protective CD8$^+$ T cells were specific to the immunodominant epitope TEWETGQI, located

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doi:10.1016/j.vaccine.2009.07.013

at amino acids (AA) 320 and 327 of ASP-2. In this mouse model, vaccination with *T. cruzi* genes/antigens not only diminished acute phase parasitemia and mortality, but also reduced chronic phase pathology.

The results obtained with ASP-2 vaccination suggested that this protein could be part of a subunit prophylactic or therapeutic vaccine against Chagas disease. Nevertheless, the fact that *T. cruzi*, like most microorganisms, displays strain polymorphism necessitates critical interpretation of our results. The hypothesis of strain-specific immunity is very plausible and could be applied to most pathogenic microorganisms (reviewed Ref. [7]). Therefore, a similar scenario for *T. cruzi* infection could be possible.

Although plausible, to the best of our knowledge, there has been no proof suggesting that acquired or vaccine-induced, protective immunity against *T. cruzi* is strain-specific. Recent studies describing the epitopes recognised by protective $CD8^+$ T cells suggest that the immunodominant epitopes are, in fact, strain-specific [8–10]. Our studies on genetic vaccination with *asp-2* genes cloned from different parasite strains also confirmed this hypothesis [11]. Due to the possible immunological variations found in parasites strains isolated from different regions of the Americas, the first goal of this study was to determine which strains presented the immunodominant, H-$2K^k$-restricted $CD8^+$ T cell epitope of ASP-2 (TEWETGQI). We then selected two strains that expressed this epitope. These strains corresponded to divergent *T. cruzi* lineage I or II. Using these strains, we evaluated whether our earlier results were valid in susceptible A/Sn mice. These mice were vaccinated with two genes [*asp-2* and *trans*-sialidase (*ts*)] that were previously described as being protective against a challenge with the Y strain of *T. cruzi*. Essentially, we confirmed our hypothesis that both naturally acquired and vaccine-induced protective immunity during experimental *T. cruzi* infection can be strain-specific.

2. Materials and methods

2.1. Mice and parasites

Female 5–8-week-old A/Sn (H-2^a), B10.A (H-2^a) C57BL/6 (H-2^b) were purchased from the Federal University of São Paulo or the University of São Paulo. Parasites of the Sylvio X10/4, Dm28c, CL-Brener, Tulahuen, or G strains of *T. cruzi* trypomastigotes were derived from infected LLC-MK_2, as described [11]. Parasites from the Colombia (COL, Ref. [12]) and Colombian [13] strains had their DNA extracted and rRNA genes sequenced, as described earlier [14]. Bloodstream trypomastigotes of the Y, COL and Colombian strains were obtained from experimentally infected A/Sn mice. The challenge was performed intraperitoneally (i.p.) with the indicated number of trypomastigotes and parasite development was monitored by counting the number of bloodstream trypomastigotes in 5 μl of fresh blood collected from the tail vein [15]. The use of animals and the experimental procedures have been approved by the Ethics Committee for Animal Care of the Federal University of São Paulo. The values of peak parasitemia of each individual mouse were log compared by One-Way Anova followed by Tukey HSD tests available at the website http://faculty.vassar.edu/lowry/VassarStats.html. LogRank test was used to compare mouse survival rate after challenge with *T. cruzi*. The differences were considered significant when the *P* value was <0.05.

2.2. Peptides

Peptides TEWETGQI and TsKb-18 (ANYDFTLV) were purchased from Genscript (Piscataway, NJ). Peptide purity was in a range of 80–90% purity. Their identities were confirmed by Q-TOF Micro™ equipped with an electrospray ionization source (Micromass, UK).

2.3. Recombinant plasmids and adenoviruses used for immunisation

Plasmids pIgSPclone9 and p154/13 expressing ASP-2 and TS of *T. cruzi*, respectively, were generated as described earlier and both lead to expression of the recombinant antigen [15–17]. pAdCMV-*asp*-2 and pAdCMV-*ts* are adenoviral transfer plasmids that contain an eukaryotic expression cassette formed by the cytomegalovirus immediate-early promoter and the SV40 RNA polyadenylation sequences. Inside this cassette we cloned the DNA sequences encoding *T. cruzi* ASP-2 or TS (AdASP-2 and Ad-TS, Ref. [17]). Viruses and plasmids were purified as described earlier [13–15]. Mice were inoculated intra-muscularly (i.m.) in each *tibialis anterioris* muscle with 100 μg of plasmid DNA (pIgSPclone9 and p154/13). Twenty-one days later, these mice received in these same spots a second dose of 100 μg of plasmid DNA or 50 μl of viral suspension containing a total of 4×10^8 plaque forming units of adenovirus (AdASP-2 and Ad-TS). Immunological assays and challenge were performed 14 days after viral inoculation. Some groups of mice received two i.m. doses of 4×10^8 plaque forming units of adenovirus (AdASP-2 and Ad-TS). In this case the doses were administered 8 weeks apart.

2.4. Immunological assays

For the *in vivo* cytotoxicity assays, splenocytes collected from naive B10.A, C57BL/6 or A/Sn mice were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE, Molecular Probes, Eugene, Oregon, USA) at a final concentration of 5 μM ($CFSE_{high}$) or 0.5 μM ($CFSE_{low}$). $CFSE_{high}$ cells were coated for 40 min at 37 °C with 2.5 μM of peptide TEWETGQI or 1.0 μM of peptide TsKb-18. $CFSE_{low}$ cells remained uncoated. Subsequently, $CFSE_{high}$ cells were washed and mixed with equal numbers of $CFSE_{low}$ cells before injecting intravenously (i.v.) $(30–40) \times 10^6$ total cells per mouse. Recipient animals were mice previously immunised with recombinant plasmids or adenoviruses or both. Spleen cells of recipient mice were collected 20 h after transfer as indicated on the legend of the figures, fixed with 1.0% paraformaldehyde and analyzed by fluorescence-activated cell sorting (FACS), using a FacsCanto flow cytometer (BD, Mountain View, CA). Percentage of specific lysis was determined using the formula:

$$1 - \frac{\%CFSE_{high}\ \text{immunised}/\%CFSE_{low}\ \text{immunised}}{\%CFSE_{high}\ \text{naive}/\%CFSE_{low}\ \text{naive}} \times 100\%$$

ELISPOT assay for enumeration of peptide-specific IFN-γ producing cells was performed essentially as described earlier [15]. Antibodies to recombinant ASP-2 (His-65 kDa) or TS were detected by ELISA. To this aim, ELISA plates were coated with 250 ng/well of each recombinant protein and the assay was performed as previously described [15,16].

2.5. Electrocardiogram (ECG) and histology

Mice were i.p. tranquilized with diazepan (20 mg/kg) and transducers were carefully placed under the skin in accordance with chosen preferential derivation (DII). Traces were recorded using a digital system (Power Lab 2/20) connected to a bio-amplifier in 2 mV for 1 s (PanLab Instruments). Filters were standardized between 0.1 and 100 Hz and traces were analyzed using the Scope software for Windows V3.6.10 (PanLab Instruments). We measured heart rate (beats per minute—bpm), duration of the PR, QRS, QT intervals and P wave in ms (millisecond) on 222 dpi. The rela-

tionship between the QT interval and RR interval was individually assessed. To obtain physiologically relevant values for the heart rate-corrected QT interval (QTc) throught Bazzet's formula. Chronically infected vaccinated A/Sn mice and age-matched controls were killed under anesthesia at 120 days post-infection. Hearts were removed and fixed in 4% buffered formalin. Sections were analyzed by light microscopy after paraffin-embedding and standard hematoxylin and eosin staining or Picrosirus-Hematoxilin (*Picrrosirius Red Staining*—EasyPath® Erviegas). The percentage of the colored areas (red) was determined with the aid of the software imageJ. Selected tissue sections were photographed with the aid of Nikon FE2 microscope couple to a Zeiss camera.

2.6. Statistical analysis

The values of peak parasitemia of each individual mouse were log transformed before being compared by One-Way Anova followed by Tukey HSD tests available at the site http://faculty.vassar.edu/lowry/VassarStats.html. Values of ELISPOT assay were also compared by One Way Anova. The LogRank test was used to compare mouse survival rate after challenge with *T. cruzi*. The differences were considered significant when the *P* value was <0.05.

3. Results

3.1. Specificity of the CD8$^+$ T cell immune response during naturally acquired immunity in mice infected with different parasite strains

Our initial studies on the specificity of the CD8$^+$ T cell immune response were performed in infected B10.A mice. We selected this mouse strain because the A/Sn mice that we initially planned to use in our vaccination studies were highly susceptible to infection and died before developing a strong immune response [6]. Both mouse strains have the H-2^a haplotype, a recombinant H-2 haplotype that is homozygous to the H-2K^k and H-2D^d alleles. Therefore, the MHC1a-restricted CD8$^+$ T cells of H-2^a mice recognise epitopes bound to either H-2K^k or H-2D^d molecules.

To uncover the specificity of the naturally acquired CD8$^+$ T cell immune responses, we infected B10.A mice with parasites of eight different strains. The strains used were isolated from different endemic regions of South America (Brazil, Argentina, Chile, and Colombia) and belonged to both of the divergent phylogenetic lineages of *T. cruzi*. The G, Colombian, DM28c, Tulahuén, and Sylvio X/10-4 strains belong to *T. cruzi* lineage I; Strains Y and COL belong to *T. cruzi* lineage II, and CL-Brener is a hybrid of both lineages [14]. Twenty days later, both infected mice and naive controls were used for *in vivo* cytotoxic or *ex vivo* ELISPOT assays to detect specific immune responses to the H-2K^k-restricted epitope TEWETGQI. This epitope is immunodominant, and expressed by several proteins including the ASP-2 of *T. cruzi* [8,10,18,19].

The timing for these experiments was based on earlier studies showing that the CD8$^+$ T cell immune response to *T. cruzi* epitopes has delayed kinetics; being detected at ~14 days after the challenge [7,8,10]. Mice infected with parasites of the Y, G, Colombian, and COL strains displayed strong responses to the peptide TEWETGQI, as measured by both assays (Fig. 1A and B, respectively). The magnitude of the immune responses of mice infected with each of these two parasite strains was comparable. In contrast, mice infected with parasites of the other four strains failed to develop immune responses to this epitope ($P > 0.05$ in all cases).

To confirm that the differences observed during the immune response to TEWETGQI were not due to poor infectivity/immunogenicity by certain *T. cruzi* strains, we infected C57BL/6 mice. The genetic background of this mouse strain is identical to B10.A mice, but they have a different H-2 haplotype (H-2^b). Infection with the parasites of the CL-Brener, Sylvio X/10-4 or DM28-c strains, which failed to induce immune responses to the TEWETGQI epitope in B10.A mice, elicited strong responses to the H-2K^b-restricted peptide TsKb-18, as estimated by the *in vivo* cytotoxic assay or the ELISPOT assay (Fig. 1C and D). Their immune responses were also comparable in magnitude. C57BL/6 mice challenged with other parasite strains also displayed statistically higher immune responses than naive mice (Tulahuén, Y or G), though they were significantly lower than the ones described above.

A single strain, Tulahuén, elicited poor immune responses to the two peptides in both mouse strains. The inability of these mice to generate an immune response to this strain was not due to a lack of infectivity/immunogenicity. BALB/c mice (H-2^d) infected with Tulahuén trypomastigotes responded strongly to a third epitope, IYNVGQVSI, as previously described. This epitope is immunodominant and expressed on the TS antigen of *T. cruzi* (data not shown [20,21]).

Together, these results support our hypothesis and previous observations that the specificity of the acquired immune responses to *T. cruzi* that are mediated by mouse CD8$^+$ T cells vary according to the parasite strain used for infection.

3.2. Protective immunity in highly susceptible A/Sn mice following genetic vaccination with asp-2 and ts

Based on the results above, we restricted our study to three of the eight parasite strains that were distinct from the Y strain and responded to the TEWETGQI epitope. The G strain, isolated from an opossum, was described as poorly infective, leading to low levels of parasitemia [22]. Therefore, we selected the Colombian and COL strains. To confirm the phylogenetic origins of these strains, we sequenced the rRNA genes obtained from the blood of infected mice. As shown in Fig. 2, the results confirmed that Colombian and COL parasites belong to divergent *T. cruzi* phylogenetic lineages I and II, respectively.

To determine whether protective immunity against these two strains could be elicited by genetic immunisation, we compared two different vaccination protocols, using either homologous or heterologous prime-boosting regimens. The homologous protocol consisted of two immunising doses of plasmid DNA (pIgSPCl.9 and p154/13) or recombinant replication-defective adenovirus type 5 (AdASP-2 and Ad-TS). The heterologous immunisation regimen consisted of a priming immunisation with plasmids pIgSPCl.9 and p154/13, followed by a booster injection of the AdASP-2 and Ad-TS. In parallel, control mice were injected with the homologous protocol consisting of two immunising doses of plasmid DNA (pcDNA3) or recombinant adenovirus (Adβ-gal). The control for the heterologous immunisation regimen consisted of a priming immunisation with pcDNA3, followed by the administration the Adβ-gal.

These vaccination protocols were selected based on our previous finding that they could elicit protective immunity after a lethal challenge with parasites of the Y strain of *T. cruzi* [6]. We simultaneously vaccinated mice with *asp-2* and *ts*. This decision was based on our earlier observation that using both genes improved protective immunity in A/Sn mice [24].

To monitor the immunisation efficiency, we determined the antibody titres to recombinant ASP-2 or TS (Fig. 3). Groups of mice that were vaccinated with *T. cruzi* genes presented high levels of specific antibodies, as detected by ELISA. The antibody levels were significantly higher in groups of mice immunised with recombinant adenovirus (2 doses) or heterologous prime-boost (DNA–adenovirus), in comparison to animals that received immunisations with plasmids only. To determine whether CD8 immune responses were elicited, we selected only the group of mice that

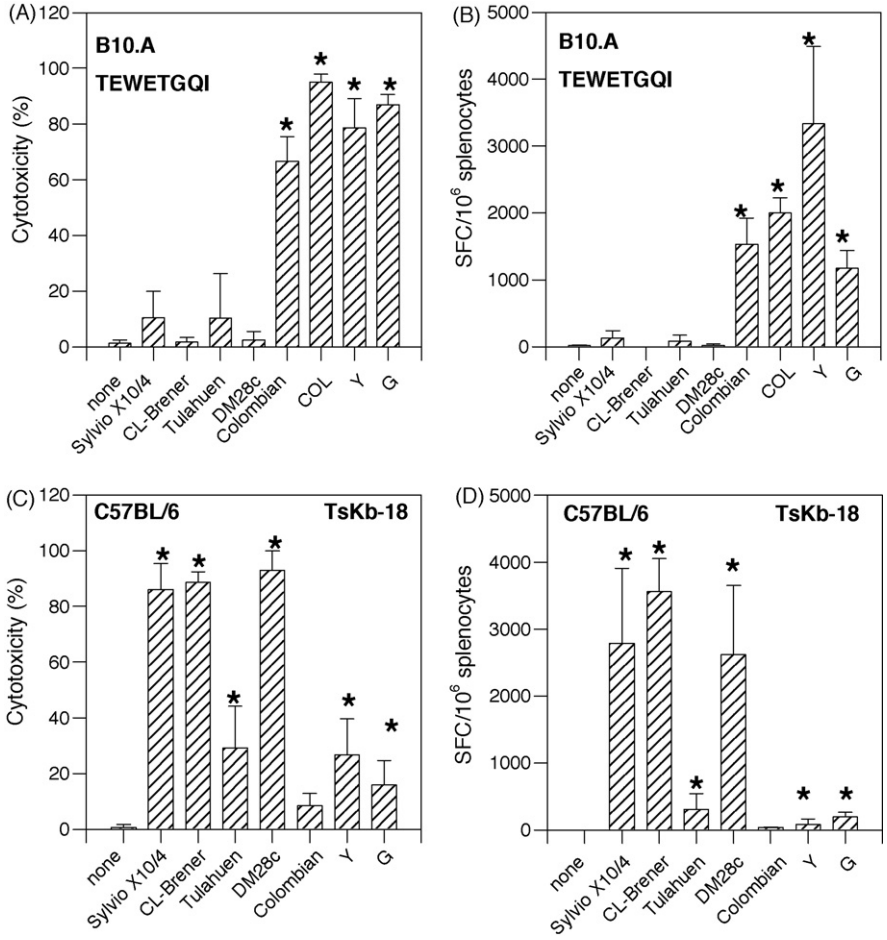

Fig. 1. Specific CD8⁺ T cell mediated immune responses in B10.A or C57BL/6 mice challenged with different strains of *T. cruzi*.
B10.A (Panels A and B) or C57BL/6 (Panels C and D) mice were challenged or not i.p. with bloodstream trypomastigotes (10^4 per mouse) of the Y, Colombian or COL strains or trypomastigote culture forms (10^5 per mouse) of Sylvio X/10 Cl.4, Tulahuén, CL-Brener, DM28c or G strains.
After 20 days, the *in vivo* cytotoxic activity against target cells coated with peptides TEWETGQI (Panel A) or TsKb-18 (Panel B) were determined as described in Section 2. Results represent the mean of four mice ± SD per group. Asterisks denote that *in vivo* cytotoxicity of infected mice against target cells coated with peptide TEWETGQI or TsKb-18 was significantly higher than control naive mice ($P < 0.01$).
After 20 days, IFN-γ producing spleen cells specific for peptides TEWETGQI (Panel B) or TsKb-18 (Panel D) were estimated *ex vivo* by ELISPOT assay. Results represent the mean number of spot forming cells (SFC) per 10^6 splenocytes ± SD ($n = 4$) following *in vitro* stimulation with the indicated peptide or medium only. The asterisks denote that the numbers of SFC specific for peptide TEWETGQI or TsKb-18 of spleen cells from infected mice were significantly higher than the number of SFC of control naive animals ($P < 0.01$).
Results are representative of two or more independent experiments.

received the DNA–adenovirus regimen. As shown in Fig. 4, these mice presented high levels of *in vivo*-specific cytotoxicity (panel A) and peptide-specific IFN-γ producing cells, as detected by ELISPOT (Panel B).

Immunised mice in this experiment were injected with the recombinant adenovirus (2 doses) or heterologous prime-boost protocol. *T. cruzi* gene-injected and control mice were challenged with a lethal dose of *T. cruzi* bloodstream trypomastigotes of the

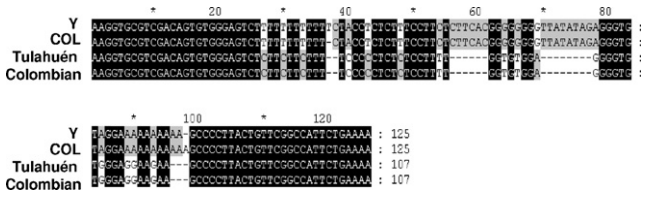

Fig. 2. Sequence of the divergent domain D7 of the large subunit of *T. cruzi* rRNA 24S obtained from distinct parasite strains.
Parasites from the COL and Colombian strains had their DNA extracted and rRNA genes sequenced as described earlier [14]. Their sequences were compared to rRNA genes sequences from Genbank AF301912 and AF239981.

Colombian strain. As shown in Fig. 5A, parasitemia in all four groups progressed steadily. No statistically significant difference was observed among the groups. When mouse survival was followed after the challenge (as shown in Fig. 5B), the group of mice immunised with the heterologous prime-boost regimen survived significantly longer than the other three. Nevertheless, all mice eventually died, and we concluded that no protective immunity was elicited following genetic vaccination against parasites of the Colombian strain.

In subsequent experiments, immunised mice were injected with the homologous protocol consisting of two immunising doses of plasmid DNA (pIgSPCl.9 and p154/13) or recombinant replication-defective type 5 adenovirus (AdASP-2 and Ad-TS). A third group of animals received the heterologous immunisation regimen, consisting of a priming immunisation with plasmids pIgSPCl.9 and p154/13, followed by a booster injection with AdASP-2 and Ad-TS.

Vaccinated and control mice were challenged with a lethal dose of bloodstream trypomastigotes of the COL strain (Fig. 6). All three groups of mice immunised with *T. cruzi* genes (groups 4, 5 and 6) displayed peak parasitemias significantly lower than the controls (groups 1, 2 and 3; Fig. 6, Panel A; $P < 0.01$ in all cases). When mouse

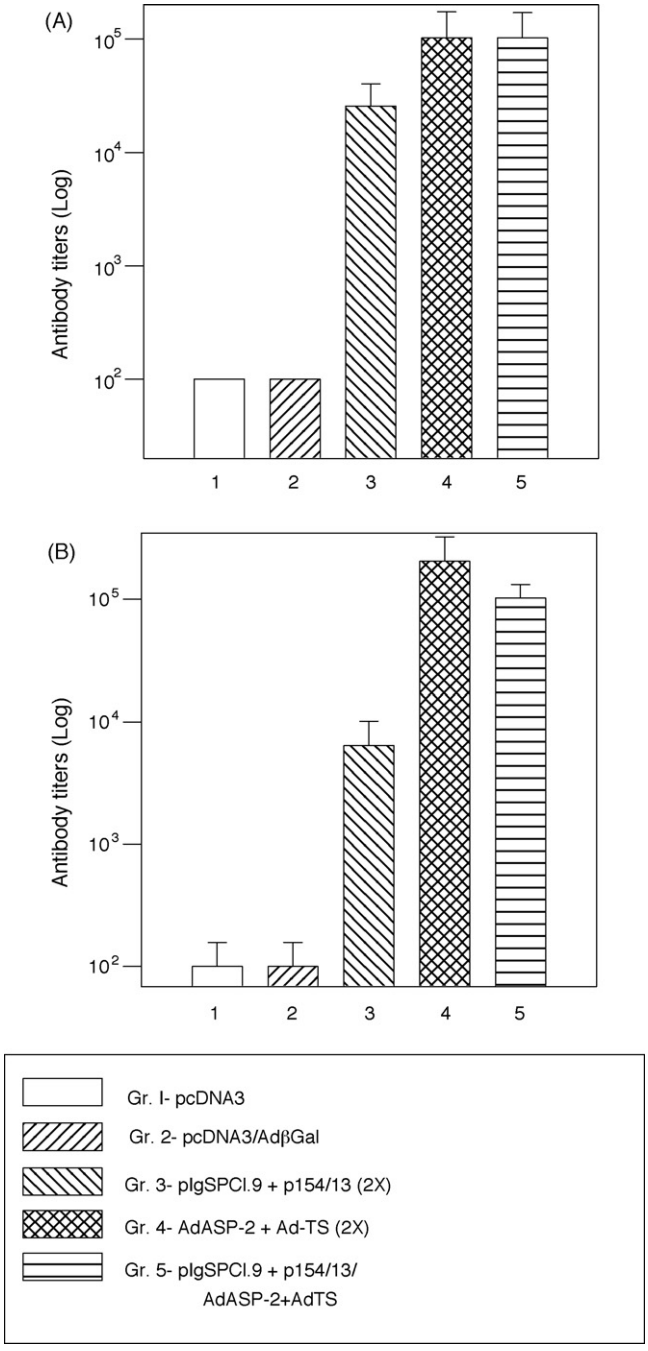

Fig. 3. Specific antibody response in A/Sn mice genetically vaccinated with ASP-2 and TS.
Antibody titers to recombinant TS (Panel A) or ASP-2 (Panel B) were determined in groups of mice ($n = 6$) administered with *T. cruzi* genes, as described in Section 2. Antibody titers as detected by ELISA were significantly higher in groups of mice immunised with recombinant adenovirus (2 doses) or heterologous prime-boost (DNA–adenovirus) when compared to animals that received two immunisations with plasmids ($P < 0.01$ in both cases).

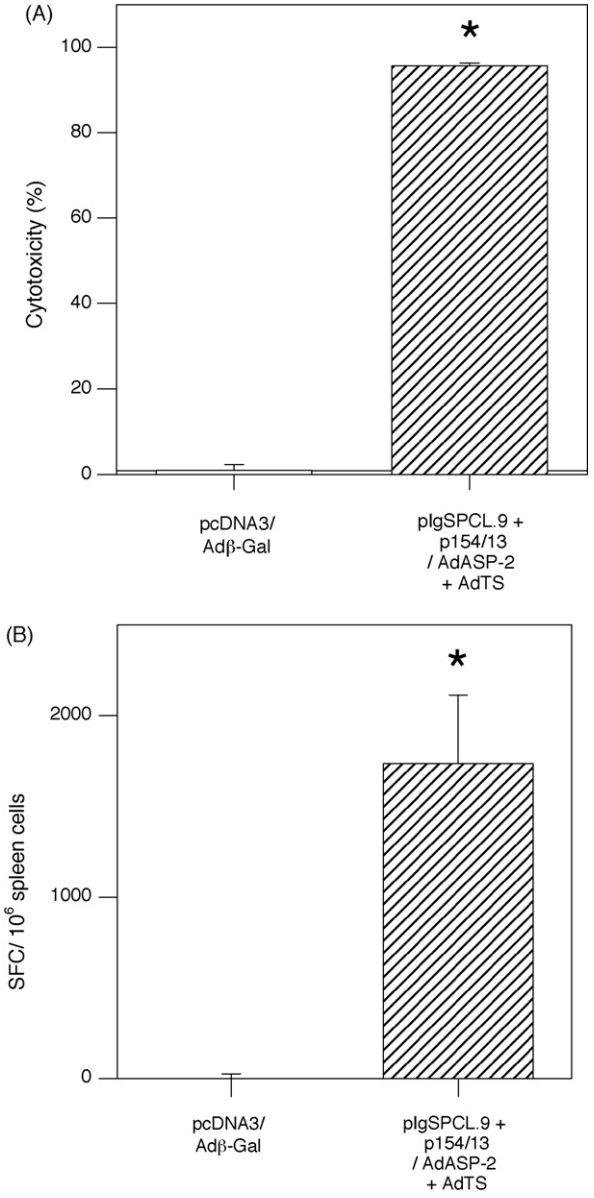

Fig. 4. Specific $CD8^+$ T cell mediated immune responses in A/Sn mice genetically vaccinated with ASP-2 and TS.
A/Sn mice were immunised with the heterologous prime-boost (DNA–adenovirus) protocol, as described in Section 2. After 14 days, the *in vivo* cytotoxic activity against target cells coated with peptides TEWETGQI (Panel A) were determined as also described in Section 2. Results represent the mean of 4 mice ± SD per group. Asterisks denote that *in vivo* cytotoxicity of ASP2/TS immunised mice against target cells coated with peptide TEWETGQI were significantly higher than in control mice injected with pcDNA and Adβ-gal ($P < 0.01$).
After 14 days, IFN-γ producing spleen cells specific for peptide TEWETGQI (Panel B) were estimated *ex vivo* by ELISPOT assay. Results represent the mean number of spot forming cells (SFC) per 10^6 splenocytes ± SD ($n = 4$) following *in vitro* stimulation with the indicated peptide or medium only. The asterisks denote that the numbers of SFC specific for peptide TEWETGQI of spleen cells from ASP-2/TS immunised mice were significantly higher than the number specific SFC of control mice injected with pcDNA and Adβ-gal ($P < 0.01$).
Results are representative of two independent experiments.

survival after the challenge was followed (Fig. 6, Panel B), the results from the parasitemia were largely confirmed. Only 1 of the 18 mice immunised with *T. cruzi* genes succumbed to the acute lethal infection. In contrast, all 18 control mice died by the 30th day after the challenge.

Immunised mice that survived the acute infection were evaluated for cardiac disturbances at 120 days post-infection. Although no differences were found when the statistical analyses were performed (Table 1), qualitative analysis based on cardiac conduction disturbances were detected on the individual electrocardiograms (ECGs; Fig. 7A and B and Table 2). ECG traces observed from the plasmid DNA-vaccinated animals suggested the presence of atrial electrical conduction disturbances due to overload (branch blockage), first or second degree atrioventricular blockage (AVB1 or 2), and sinusal bradycardia. Three of the six mice vaccinated twice with recombinant AdASP-2/Ad-TS (group 5) did not suffer significant car-

Table 1
ECG parameters measured.

Mouse group	Immunol. status	*n*	Heart rate (*n* of beats/min)	P wave duration (ms)	PR segment (ms)	QRS segment (ms)	QTc
	Naive	8	521.6 ± 73.3	16.5 ± 1.9	38.5 ± 7.2	14.2 ± 2.6	31.4 ± 10.4
4	pIgSPCl.9 (2x)	4	463.7 ± 84.4	24.8 ± 11.5	43.9 ± 15.0	15.0 ± 12.5	37.1 ± 6.5
5	AdASP-2 (2x)	6	454.8 ± 41.7	41.7 ± 20.2	52.5 ± 5.0	12.5 ± 2.6	52.5 ± 17.1
6	pIgSPCl.9/AdASP-2	5	413.3 ± 122.2	18.5 ± 3.3	42.9 ± 5.8	14.5 ± 3.4	58.3 ± 29.7

Values represent the means ± SD.

diac alterations at this stage of the disease (Fig. 7C and Table 2). None of the mice vaccinated twice with the heterologous protocol (group 6) developed AVB2 (Table 2).

Histological analyses of the heart tissue following hematoxilin/eosin staining did provide evidence of a link between the observed electrical disturbances and cellular infiltrates. To determine whether the accumulation of fibrosis or tissue repair correlated with the electrical disturbances detected, we stained the interstitial collagen with picrosirius red. The stained area was estimated and the results are presented in Fig. 8A. There was a significant increase in the stained areas in all groups (4, 5 and 6), compared to naive age-matched controls. In contrast, mice immunised with two doses of recombinant adenovirus displayed slightly fewer stained areas ($P < 0.01$).

4. Discussion

In this study, we tested whether immunity against *T. cruzi* infection in mice could be specific to the parasite strain. We found that a single inbred mouse strain (B10.A) responded to the H-2K^k-restricted CD8$^+$ T cell epitope TEWETGQI, after experimental infection with four of the eight parasite strains tested. Mice infected with the other four parasite strains failed to develop significant responses to this epitope. The heterogeneity among the immune responses of CD8$^+$ T cells from B10.A mice to the TEWETGQI epitope was also reproduced with other immunodominant epitopes. As shown in Fig. 1C and D, C57BL/6 mice infected with only three of the seven parasite strains responded strongly to the TsKb-18 epitope. Previous studies have shown that the immunodominant epitope VNHRFTLV is strongly recognised during infection of C57BL/6 mice with the Y or G strains of *T. cruzi*, but not with the CL-Brenner strain [10]. In accordance with these observations, we found that the immunodominant H-2K^d-restricted epitope YNVGQVSI was recognised by T cells of BALB/c mice, after infection with three of the seven strains tested (Y, Colombian and Tulahuen; CC, FT and MR unpublished results). The reason for the heterogeneity in the specificity CD8$^+$ T cell immune responses following infection with different parasite strains is still unknown. It may be related to AA sequence polymorphism, levels of expression of the different epitopes, immunodominance, or immune evasion mechanisms.

We then selected two of the *T. cruzi* strains that expressed the TEWETGQI epitope (Colombian and COL) to test whether protective immunity, elicited by the genetic vaccination with the antigens ASP-2 and TS, could confer protective immunity in highly susceptible A/Sn mice against a lethal challenge with these parasites. Our present results demonstrated that the resulting protective immunity elicited by vaccination with these antigens/genes was strain-specific. Vaccinated mice succumbed to a challenge with parasites of the Colombian strain. In contrast, the vast majority of vaccinated animals survived a challenge with parasites of the COL strain. Like earlier studies, these results demonstrated that immunisation with ASP-2 via distinct delivery systems can elicit protective immunity in C57BL/6, BALB/c and A/Sn mice, after a challenge with the Y or Brazil strain of *T. cruzi* [6,11,15,17–19,23,24]. In the case of TS, successful vaccination studies have been performed in BALB/c or C57BL/6, but not in A/Sn mice, using the Y or Tulahuén strains [16,17,19,21,24–26].

Two possible, non-excluding explanations can account for the variation observed. First, it is possible that these discrepancies could be related to the antigenic polymorphism in the parasites of the Colombian strain, e.g., the absence of epitope(s) critical for the protective immunity. Comparison of the predicted AA sequences for the ASP-2 of Colombian and Y strains revealed high similarity (96.8%, Ref. [11]). In the case of the TS of *T. cruzi*, the transcripts of parasites of the Colombian strain have not yet been isolated and sequenced. However, similar to the ASP-2, the genes encoding the catalytic region of TS used in our study (AA 1–678) did not show high sequence variability among the five other strains. The identities among the predicted AA sequences of TS (Y strain) and other strains were 96, 84, 92, and 97%, in the CL (XP_807431), Sylvio X-10/4 (P23253), FALLS (CAA40511) and Tulahuén "/isolate=" Tul2 (AAA99443) strains, respectively. Nevertheless, these small polymorphic regions may still be critical for inducing protective immunity.

Based on the fact that both strains express the important target epitope TEWETGQI, we should still be able to detect some degree of protective immunity mediated by CD8$^+$ T cells, following a challenge with the Colombian strain. The results obtained after inducing an immune response to infection with both strains also eliminate the possibility that they differ in their capacity to generate similar antigen available for processing and presentation.

Table 2
Summary of the ECG records.

Cardiac alterations	Naive (*n* = 8)	Group 4 (*n* = 4)[a]	Group 5 (*n* = 6)[a]	Group 6 (*n* = 5)[a]
SRA[b]	0	0	0	0
VRA[b]	0	0	0	0
EXT[b]	0	0	0	0
BRAD[b]	0	0	0	2
AVB-1[b]	0	0	0	2
AVB-2[b]	0	3	3	0
DEFLEX[b]	0	0	0	0
INFRA[b]	0	0	0	0
STSD[b]	0	0	0	0

[a] ECG record of A/Sn mouse genetically vaccinated as described in the legend of Fig. 6 and challenged with 250 blood forms of *T. cruzi* 120 days earlier.

[b] Abbreviations: (i) SAR: sinus arrhythmia; (ii) VAR: ventricular arrhythmia; (iii) EXT: ventricular extrasystolis; (iv) BRAD: sinus bradicardya; (v) AVB1: 1° degree atrioventricular block; (vi) AVB2: 2° degree atrioventricular block; (vii) DEFLEX: Q wave deflection (viii) STSD: ST segment depression.

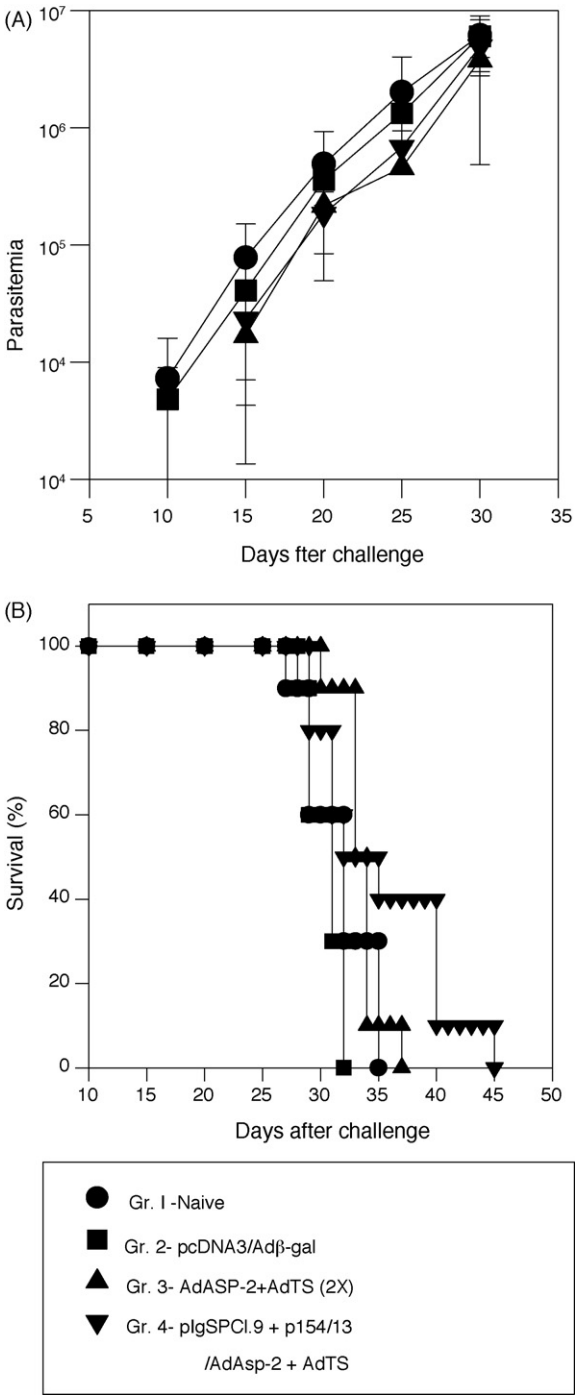

Fig. 5. Parasitemia and mortality in A/Sn (H-2^a) mice immunised with different combination of plasmid DNA and/or rec. adenovirus expressing the ASP-2 and TS of *T. cruzi* and challenged with trypomastigotes of the Colombian strain.
A/Sn mice were immunised as described in Section 2.
Two weeks after the final immunising dose, mice were challenged i.p. with 250 bloodstream trypomastigotes of the Colombian strain.
Panel A: the parasitemia for each mouse group is represented as a mean ± SD (n = 6 or 10). The parasitemias of the different mouse groups were not statistically different.
Panel B: the graph shows the Kaplan–Meier curves for the survival of the mice groups immunised and challenged as described above (n = 6 or 10). Mice from group 4 (▼) survived significantly longer than animals from group 1 (●), 2 (■,) or 3 (▲) (P < 0.01 in all cases, LogRank test).
Pooled results from two experiments are shown.

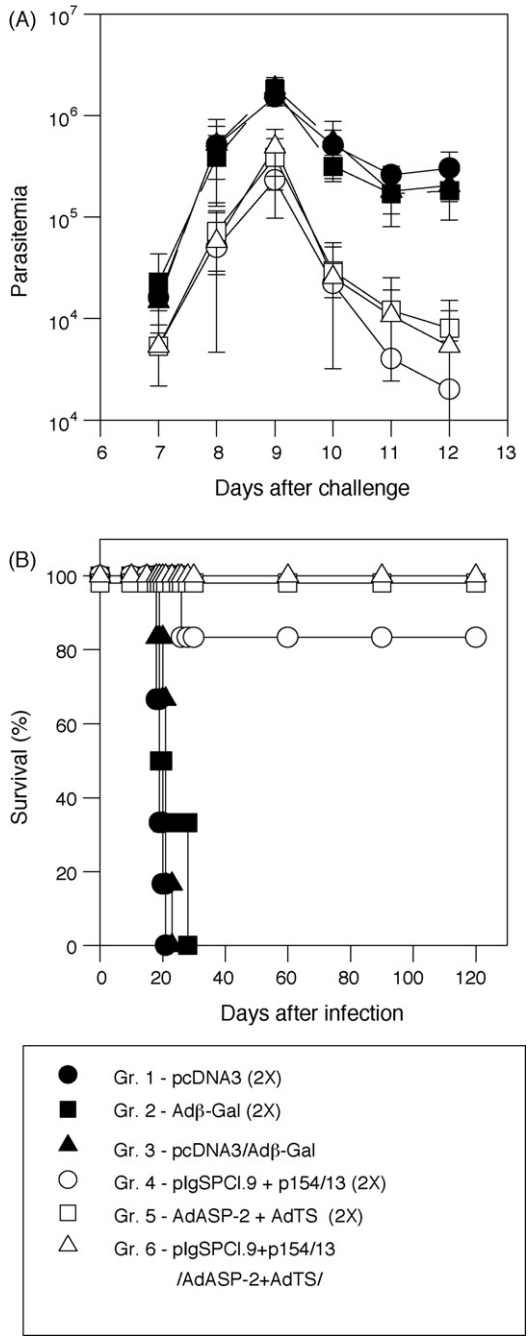

Fig. 6. Parasitemia and mortality in A/Sn (H-2^a) mice immunised with different combinations of plasmid DNA and/or rec. adenovirus expressing the ASP-2 and TS of *T. cruzi* and challenged with trypomastigotes of the COL strain.
A/Sn mice were immunised as described in Section 2.
Two weeks after the final immunising dose, mice were challenged i.p. with 250 bloodstream trypomastigotes of the COL strain.
Panel A: the parasitemia for each mouse group is represented as mean ± SD (n = 6). The parasitemias of mice groups 4, 5 and 6 were statistically lower than mice from groups 1, 2 and 3 (P < 0.01 in all cases).
Panel B: the graph shows the Kaplan–Meier curves for the survival of the mice groups immunised and challenged as described above (n = 6). Mice from groups 4, 5 and 6 survived significantly longer than animals from groups 1, 2 and 3 (P < 0.01 in all cases, LogRank test). Representative results of two independent experiments.

Antigenic polymorphism, as the cause of strain-specific immunity, has been described for many viral and bacterial pathogens, leading to selection of antigenic variants following the use of vaccines [27,28]. In the case of protozoan parasites, it is not clear whether strain-specific immunity plays a major role dur-

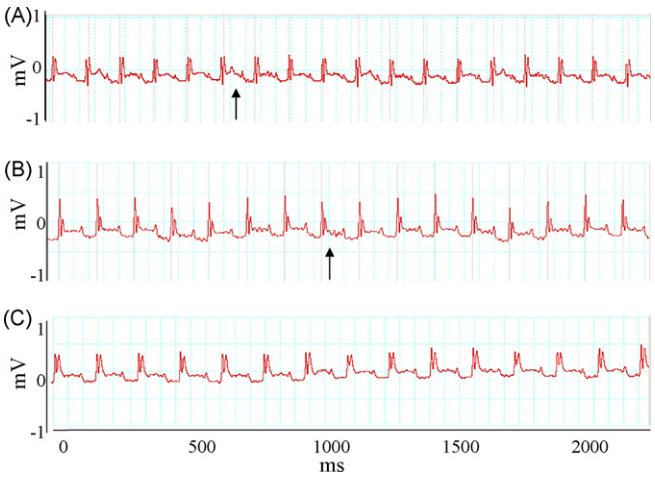

Fig. 7. Examples of ECG from mice which survived the lethal challenge with trypomastigotes of the COL strain of *T. cruzi*.
Vaccinated and challenged A/Sn mice are from groups 4, 5 and 6 of Fig. 6.
Panels A and B: ECG record of a vaccinated A/Sn mouse of group 4 (A) or group 6 (B) and challenged with 250 blood forms of *T. cruzi* 120 days earlier. The electric disturbances indicated by the arrows are AVB 2.
Panel C: ECG record of a vaccinated A/Sn mouse of group 5. No alterations were recorded.
Panel C: ECG record of a vaccinated A/Sn mouse from group 5 and challenged with 250 blood forms of *T. cruzi* 120 days earlier. Fifty percent of these mice did not present any detectable electric disturbance.

ing infection or vaccination, contributing to parasitic evasion of the immune response. Nevertheless, there are examples suggesting that this may be the case during natural infections [29,30]. In addition, during experimental vaccination, strain-specific immunity, attributable to the primary sequence of the vaccine antigens, has been observed [31–33].

The second possible explanation for the observed strain-specificity is that beyond antigenic polymorphism, other forms of biological variability can account for the observed differences. Distinct parasite strains may have different tissue tropisms during the acute phase, multiplying in different target organs. Although many studies have attempted to describe the fate of the *T. cruzi* parasites after experimental infection using histological analyses, only one recent study has tracked the parasites of the Brazil strain, via transfection with a reporter gene. This study found that after i.p. infection of A/J mice, luciferase-expressing *T. cruzi* multiply predominantly in the large intestine [34]. Parasite growth was uncontrolled in those mice, and they all died. No information of this nature is available on other *T. cruzi* strains. Therefore, it is possible that parasites of the distinct strains used in this study (Y, COL and Colombian) vary, with regard to tissue tropism. This fact could explain the differences in the protective immunity observed. In a finding that supports this hypothesis, Leavey and Tarleton (2003, Ref. [35]) described how $CD8^+$ T cells could not be properly activated after penetration into the mouse muscle. In these animals, this unresponsive phenotype contrasted with the spleen $CD8^+$ T, which could still be readily activated after muscle penetration. Therefore, in our experiment, although effector-specific $CD8^+$ T cells were present in the spleen of mice infected with parasites of each strain, it is possible that in the case of the animals infected with the Colombian strain, these cells were not functional at the sites of parasite multiplication, such as within the myocardial muscle. In fact, experimental mouse infection with parasites of the Colombian strain is well known to cause intense myocardial infection [36–42].

In the chronic disease phase (120 dpi), the cardiac electrical conduction system of surviving A/Sn mice infected with the COL strain was evaluated. We observed ECG alterations in most animals. This fact contrasted with our previous experiments in vaccinated A/Sn

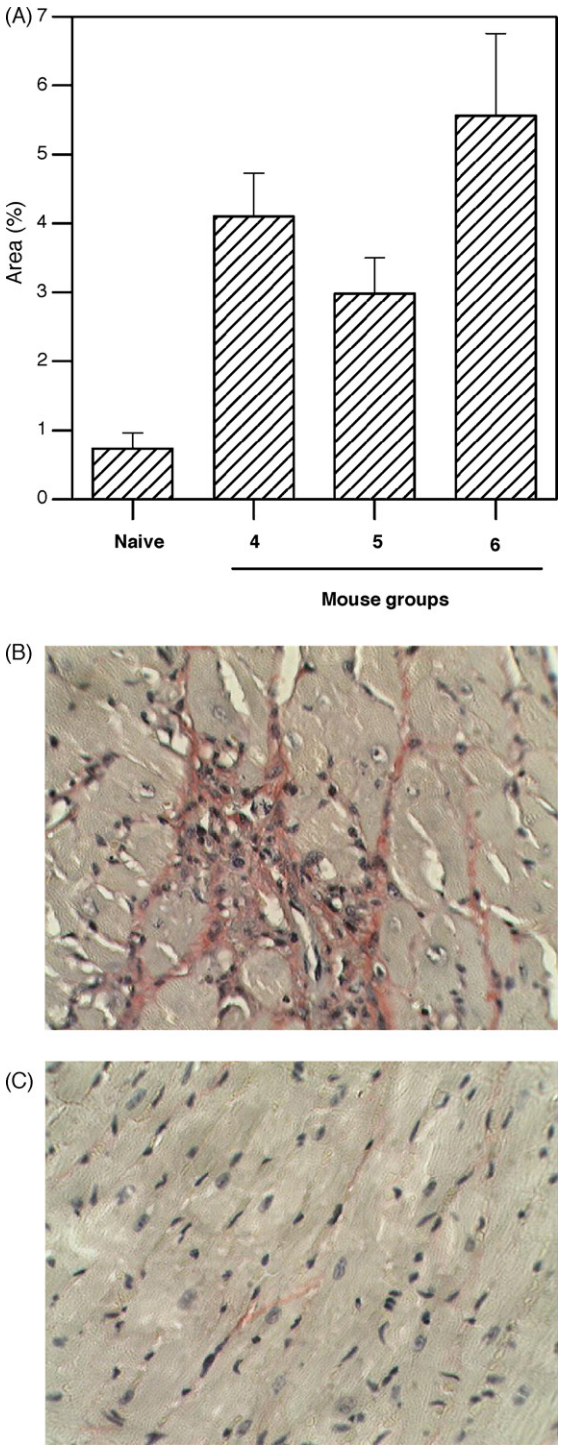

Fig. 8. Red picrosirius staining of heart sections from vaccinated A/Sn mice challenged 120 days earlier.
Vaccinated and challenged A/Sn mice are from groups 4, 5 and 6 of Fig. 6.
Heart tissues sections were stained with red picrosirius/hematoxillin. Sections were observed using microscopy (400×) and the fibrosis areas were estimated using the NIH *ImageJ* software.
Panel A: the fibrosis area of tissues obtained from mice of groups 4, 5 and 6 were higher than the naive mice ($P < 0.01$). However, the fibrosis area of heart sections obtained from mice of 5 were slightly lower than for groups 4 and 6 ($P < 0.01$).
Panel B: example of heart section stained with red picrosirius from vaccinated and challenged mice.
Panel C: example of heart section stained with red picrosirius from naive mice.

mice infected with parasites of the Y strain [6]. Although these mice survived the lethal infection with the COL strain of *T. cruzi*, the vaccinated A/Sn mice exhibited an increased PR interval (ms), suggestive of atrioventricular or branch blockage, somewhat similar to chronic Chagasic cardiomyopathy. In accordance with Punukollu et al. [43], the most common symptoms in the chronic phase of infections are cardiac alterations such as ventricular increase, cardiac insufficiency and myocarditis. We verified electrical disturbances in the PR and QTc intervals by ECG, possibly caused by the infection. This process may have resulted in destruction of the conducting nervous tissues or in local inflammation that led to electrolytic disequilibrium and altered muscle fibre contraction, induced by pro-inflammatory cytokines such as IFN-γ, TNF-α and nitric oxide [44–46].

Few studies have quantified the cardiac area affected with markers that stain the collagen and fibrin produced during the inflammation and tissue repair processes [47–51]. Using picrosirius red staining, we found that all groups showed greater deposition of collagen and fibrin, as well as destroyed cardiomyocytes, corroborating our ECG results. Most of the vaccinated A/Sn mice still developed late-stage symptoms, which is compatible with the chronic Chagasic cardiomyopathy. Thus, our work suggested that, for highly susceptible A/Sn animals, an immune response of higher magnitude or breadth would be required to protect against the chronic stage symptoms.

The fact that immunity can be strain-specific will impose a number of limitations that should be considered during the development of prophylactic or therapeutic vaccines against Chagas disease. Members of the TS super-family of antigens, like the one described here, may not be the best candidates for a universal, simple recombinant vaccine such as, for example, hepatitis B. Nevertheless, they still may be part of a more complex formulation that can be immunogenic against most strains of *T. cruzi*. Recent recombinant vaccines, such as the human papillomavirus vaccine, are a mixture of antigens from four distinct virus types [52]. Nevertheless, they are not expected to have universal coverage against all different types of the human papillomavirus.

Our results also do not exclude that other conserved antigens may show better performance, individually or in combination with ours. To date, there are a number of antigens that are being studied for their potential use in vaccines against *T. cruzi* infections, as was recently reviewed [53]. Some of these antigens are quite conserved; others may have a limited number of alleles that could be used simultaneously. Nevertheless, their relative abilities to elicit protective immunity against *T. cruzi* infection will have to be assessed against multiple parasite strains, in different mouse models.

Finally, we should mention that A/Sn mice are highly susceptible to infection. Most mouse strains, as well as the human population, have a higher innate and/or acquired resistance to infection. It is possible that they may develop better or broader immune responses to recombinant vaccines than was described here.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (2006/1983-4), CNPq, The Millennium Institute for Vaccine Development and Technology (CNPq - 420067/2005-1), The Millennium Institute for Gene Therapy, FAPEMIG (EDT 24.000) (Brazil) and The National Institute for Vaccine Development and Technology (INCTV-CNPq). AVM, RTG, OBR, JLV, RRS, MBPS and MMR were recipients of fellowships from CNPq. CC, BCGA, FT and JRC are recipients of fellowships from FAPESP.

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