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Epitope mapping and protective immunity elicited by adenovirus expressing the *Leishmania* amastigote specific A2 antigen: Correlation with IFN-γ and cytolytic activity by CD8+ T cells

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ABSTRACT

A2 was identified as an amastigote virulence factor of *Leishmania* (*Leishmania*) *donovani* and as a candidate antigen for vaccine development against visceral leishmaniasis. Here, predicted hydrophilic, class I and II MHC-binding synthetic peptides were used to define epitopes recognized by A2-specific antibodies, CD8+ T and CD4+ T cells, respectively. Immunization of BALB/c mice with adenovirus expressing A2 (AdA2) resulted in low antibody response, contrasting with high levels of IFN-γ producing CD4+ T and CD8+ T cells specific for A2. Further, A2-specific CD8+ T cells from immunized mice were capable of lysing sensitized target cells *in vivo*. Finally, we demonstrated an association of A2-specific T cell responses and reduced parasitism in both liver and spleen from mice immunized with AdA2 and challenged with *L.* (*L.*) *chagasi*.

1. Introduction

Leishmania parasites are distributed worldwide, and in some geographic areas more than one species can be found causing different clinical manifestations of tegumentary and visceral leshmaniasis. Leishmaniasis is endemic in many countries in Asia and South America [1], and its control is difficult due to zoonotic features of transmission and the sylvatic nature of reservoirs and vectors [2]. In this context, an efficient vaccine would be desirable to control the disease.

A2 proteins are predominantly expressed in the amastigote stage of *Leishmania* parasites, first described in *Leishmania* (*Leishmania*) *donovani*. These proteins are composed mostly of a variable number of 10-amino-acid repeats and their molecular weights may range from 45 to 100 kDa [3]. Thus, structurally, A2 has some resemblance to the circumsporozoite protein (CS) from *Plasmodium* spp. [4], a main vaccine candidate for an anti-malaria vaccine [5]. A2 was identified as an important virulence factor of the parasite [6], but its role in infection process remains to be defined [7]. Interestingly, *L.* (*L.*) *major* parasites, which express a truncated form of A2 and are associated with cutaneous leishmaniasis, after transfection to express the *L.* (*L.*) *donovani A2* gene displayed an increased potential to survive in the resident macrophages of the spleen and liver, suggesting that expression of *A2* genes may be important to visceralization of parasites in mice [8].

Antibody responses to A2 are observed in a significant number of humans and dogs infected with old world and new world species of *Leishmania* [9]. Moreover, in a recent study, comparing different proteins for diagnosis of leishmaniasis in dogs, A2 showed the best results to identify asymptomatic dogs, suggesting that it is associated with protective immunity [10]. In addition, A2 was identified as capable of stimulating in vitro CD4+ T and CD8+ T cells from infected C3H/HeJ mice, during a systematic screening of

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L. (*L.*) *chagasi* amastigote antigens [11]. It was also demonstrated that A2, in DNA or protein formulations, can protect mice against *L.* (*L.*) *donovani* [12,13], *L.* (*L.*) *amazonensis* [14,15] and *L.* (*L.*) *chagasi* [15].

Vaccines against leishmaniasis should be capable of eliciting immune responses mediated by IFN-γ producing CD4+ T cells [16,17]. It was also reported that CD8+ cytotoxic T lymphocytes could promote killing of infected cells, therefore contributing to disease control [18–21]. In this context, recombinant DNA plasmids and/or viruses seem attractive vehicles for generation of vaccines against this parasite, since they are efficient inducers of protective immunity mediated by both antigen-specific CD4+ Th1 cells as well as CD8+ T lymphocytes [17,22,23].

In this work, we defined and characterized the structure of the main B cell epitope in A2 and, more importantly, we mapped MHC-I, for the first time in a *Leishmania* antigen, and MHC-II binding peptides derived from A2 sequence. Finally, we vaccinated mice with a recombinant adenovirus encoding the *A2* gene from *L.* (*L.*) *donovani*, and showed that homologous prime/boost protocol is effective in inducing both IFN-γ-secreting CD4+ T and CD8+ T cells as well as cytolytic CD8+ T lymphocytes, which protect mice against *L.* (*L.*) *chagasi* infection.

2. Materials and methods

2.1. Mice

Two pairs of congenic mouse strains were used: (i) BALB/c (H2d) (parental lineage) and C.B10-H2 (H2b) (CB10); and (ii) C57BL/6 (H2b) (parental lineage) and C57BL/KsJ (BKS) (H2d); on the (BALB/c) and (C57BL/6) genetic background, respectively. Female, 6–8 weeks old mice were obtained from the animal facilities from Rene Rachou Institute, Oswaldo Cruz Foundation, Belo Horizonte, MG, Brazil and housed according to institutional standard guidelines.

2.2. Parasites and antigen preparations

L. (*L.*) *chagasi* (MHOM/BR/1975/M2682) was kindly provided by Maria Norma Mello (Department of Parasitology, Federal University of Minas Gerais, MG, Brazil). Parasites were grown at 23 °C in supplemented Grace's Medium (Sigma, St. Louis, MO). Soluble *Leishmania* antigen (SLA) was prepared from late-log-phase promastigotes of *L.* (*L.*) *chagasi* after few passages in liquid culture as previously described [24]. rA2 was purified using a nickel affinity chromatography, as described elsewhere [9].

2.3. Synthetic peptides

We used the BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/) and ProtScale (http://www.expasy.ch/cgi-bin/protscale.pl) softwares to predict, respectively, the MHC class I binding peptide (CD8) and B cell epitope (B-1) from *L.* (*L.*) *donovani* A2 sequence (GenBank S69693) [25]. Two MHC class II binding epitopes (CD4-1 and CD4-2), with 17 residues each, were designed by overlapping of the non-repetitive segment of the protein. Peptides B-1, CD8, CD4-1 and CD4-2 were purchased from Biosynthesis, Inc. (Lewisville, TX). Other peptides derivated from B-1 and CD4-1 (SP0511-14, SP0517-18, SP0528-29 and SP0547) were synthesized according to a standard *Nα*-9-ethyloxycarbonyl (Fmoc) strategy on a PSSM8 multispecific peptide synthesizer (Shimadzu, Kyoto, Japan) by solid-phase synthesis and were purified by high performance liquid chromatography and confirmed with a Micromass Q-Tof Micro (Micromass MS Technologies, Division of Waters, Milford, MA). Peptides obtained by this method were all C-terminal amides and, for ELISA, were conjugated with bovine serum albumine (BSA), in glutaraldehyde, according to a standard protocol previously described [26].

2.4. Solution nuclear magnetic resonance (NMR)

Multidimensional solution nuclear magnetic resonance (NMR) experiments were carried out in order to determine the three-dimensional structures of SP0529 and SP0547. Total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC), and ^{1}H–^{15}N HSQC spectra were recorded. The NMR spectra were analysed using NMRVIEW, version 5.0.3 [27]. Nuclear Overhauser effect (NOE) intensities obtained at 200 ms mixing times were converted into semi-quantitative distance restrains using the calibration by Hyberts et al. [28]. The upper limits of the distances restrains thus obtained were 2.8, 3.4 and 5.0 Å (strong, medium, and weak NOEs, respectively). Structure calculations were performed using the Xplor-NIH software, version 2.17.0 [29]. Starting with the extended structure, 200 structures were generated using a simulated annealing protocol. This was followed by 18,000 steps of simulated annealing at 1000 K and a subsequent decrease in temperature in 9000 steps in the first slow-cool annealing stage. The display, analysis, and manipulation of the three-dimensional structures were performed with the programme MOLMOL [30].

2.5. Generation of recombinant adenovirus expressing A2 (AdA2)

The gene encoding the A2 antigen was cut from construction pcDNA3-A2 (pA2) (kindly provided by Dr. Greg Matlashewski, McGill University, Quebec, Canada), cloned into the expression cassete of an adenovirus shuttle vector (pAd-A2) and tested for protein expression in HEK 293 cells (CRL-1573; American Type Culture Collection [ATCC], Manassas, VA). The recombinant adenovirus was generated by intracellular homologous recombination between pAd-A2 and plasmid pJM17, which carries a non-replicative ΔE1 adenovirus type 5 genome [31], co-transfected into permissive E1-transgenic/HEK 293 cells, using calcium chloride as previously described [32]. The AdA2 was purified by cesium chloride isopycnic banding, and was frozen at −70 °C, 100 μM Tris, pH 8.0. An adenovirus (AdCtrl) expressing the amastigote protein (ASP) from *Trypanosoma cruzi* was used as control [33].

2.6. Western-blot and ELISA

For Western-blot analysis, antigen samples (10 μg protein/sample) were run in 12% polyacrylamide gels under denaturing conditions and transferred onto nitrocellulose membranes, incubated with anti-A2 mAb (kindly provided by Dr. Greg Matlashewski, McGill University, Quebec, Canada) and revealed with peroxidase-conjugated goat anti-mouse IgG and the detection chemiluminescence reagent (ECL Western-blot detection system; Amersham Biosciences, Little Chalfont, UK) by exposure to X-ray film (Hyperfilm; Amersham Bioscience). The ELISA was performed with plates coated overnight at 4 °C with purified recombinant A2 (2.5 μg/ml) or peptides corresponding to B cell epitopes (10 μg/ml). Mice serum samples or anti-A2 mAb were diluted 1:100 in PBS-0.05% Tween 20 and 0.25% skimmed milk (incubation buffer) and reaction developed using a peroxidase-conjugated goat anti-mouse IgG (Sigma) and 3,3′,5,5′-tetramethylbenzidine (TMB) reagent (Sigma) as substrate.

2.7. ELISPOT

Nitrocellulose bottom 96-well plates (Millipore, Bedford, MA) were pre-incubated with an anti-IFN-γ monoclonal antibody (clone R4-6A2; BD Biosciences Pharmingen, San Diego, CA), and blocked for 2 h with Dulbecco's modified Eagle's medium (DMEM, Sigma). Spleen cells were prepared in complete DMEM supplemented with recombinant IL-2 (100 U/ml) and added to plates at 10^6 cells per well, for 20 h stimulation, with A2-derived peptides (5 μM). A biotin conjugated monoclonal anti-IFN-γ antibody (clone XMG1.2; BD Biosciences Pharmingen) was used to detect cytokine spots in combination with streptavidin-peroxidase conjugate (BD Biosciences Pharmingen), and revealed with 1 mg/ml 3,3′-diaminobenzidine (DAB; Sigma) [34].

2.8. In vivo cytotoxicity assays and flow cytometric analyses

BALB/c spleen cells were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE) at a concentration of 20 μM ($CFSE_{high}$) or 1 μM ($CFSE_{low}$). $CFSE_{high}$ cells were pulsed for 30 min at 37 °C with A2-specific peptide, mixed with equal amounts of $CFSE_{low}$, and intravenously injected at 4×10^7 cells per mouse. Recipient mice were BALB/c, 2 weeks after the second immunization with AdA2 or AdCtrl, or immunized mice, 35 days after i.v. challenge with 10^7 promastigotes of *L.* (*L.*) *chagasi*. Spleen cells of recipient mice were collected 20 h after transfer, fixed and analysed by fluorescence-activated cell sorting (FACS), using FacScan cytometer (BD Biosciences Immunocytometry Systems, Mountain View, CA). The percentage of specific lysis was determined with the following formula: $1 - [(\%CFSE_{high}$ infected $- \%CFSE_{low}$ infected$)/(\%CFSE_{high}$ naïve $- \%CFSE_{low}$ naïve$)] \times 100$.

2.9. Depletion of CD4+ or CD8+ T cells in mice

Female BALB/c (four animals per group) were treated with three doses of 500 μg of anti-CD8+ (YTS169) or 200 μg of anti-CD4+ (GK 1.5) mAbs by intraperitoneal route, 3 days apart. Control groups received 500 μg of purified monoclonal antibody anti-β-galactosidase (GL113) or PBS. Depleted and mock-depleted mice were immunized with 10^9 PFU AdA2 or AdCtrl. After immunization, mice received additional doses of monoclonal antibodies once a week. Spleens were collected for ELISPOT 2–3 weeks after immunization.

2.10. Immunizations and challenge

BALB/c mice received two doses of 10^9 PFU of AdA2 or AdCtrl, 6 weeks apart, according to a protocol described elsewhere [35,36]. Serum samples were collected 11 days after booster immunization. Mice were challenged i.v., 14 days after the last vaccine administration, with 10^7 late-log-phase *L.* (*L.*) *chagasi* promastigotes. The number of viable parasites in the liver and spleen was determined by a limiting dilution assay, as described previously [37]. The number of viable parasites per milligram of tissue was determined from the highest dilution at which promastigotes had grown after 14 days of incubation. To obtain anti-rA2 sera for epitope mapping, mice were immunized s.c. in the right footpad with two doses of 50 μg of rA2 and 1 μg of alum, within a 3 weeks interval.

2.11. Statistical analysis

All data comparisons were tested for significance by non-parametric Mann–Whitney test with SigmaStat (version 2.03), and $p < 0.05$ was considered significant.

3. Results

3.1. In silico analysis of T and B cell epitopes encompassed within A2 protein

Analysis of A2 sequence from *L.* (*L.*) *donovani* on BIMAS and ProtScale softwares indicated the presence of, respectively, a high score class I MHC H2-D^d-binding (named CD8), located within the repetitive units, and a B cell (named B-1) epitope (Fig. 1). The epitope B-1 is composed of the entire non-repetitive segment plus the complete first repetitive unit, resulting in 21-amino-acid peptide. Further, two putative class II MHC-binding sequences, 17 amino-acids each, were generated by analysis of the N-terminal fragment of A2. The peptide denominated CD4-1 corresponds to a sequence running from the fifth amino-acid residue downstream the non-repetitive segment until the last amino-acid residue of the first repetition. The epitope named CD4-2 corresponds to the entire

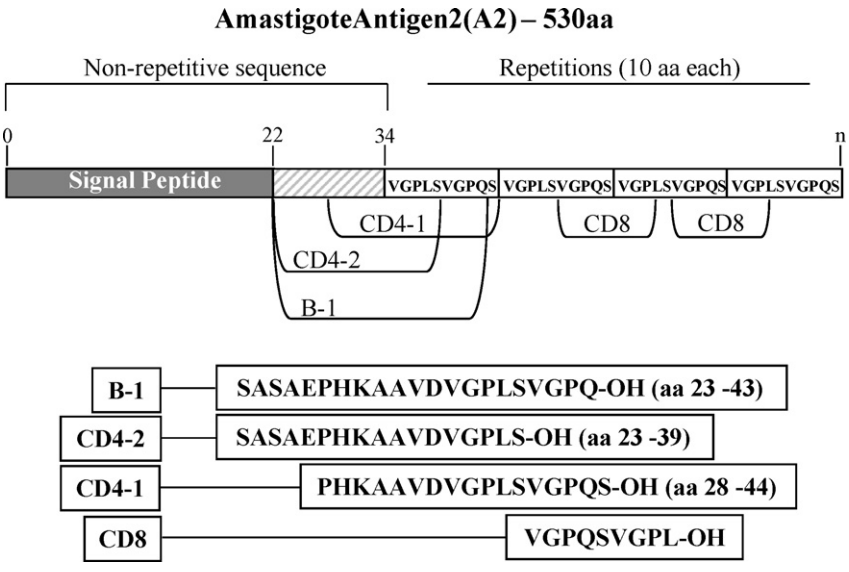

Fig. 1. Virtual B and T cell epitope mapping of *L.* (*L.*) *donovani* A2 protein.

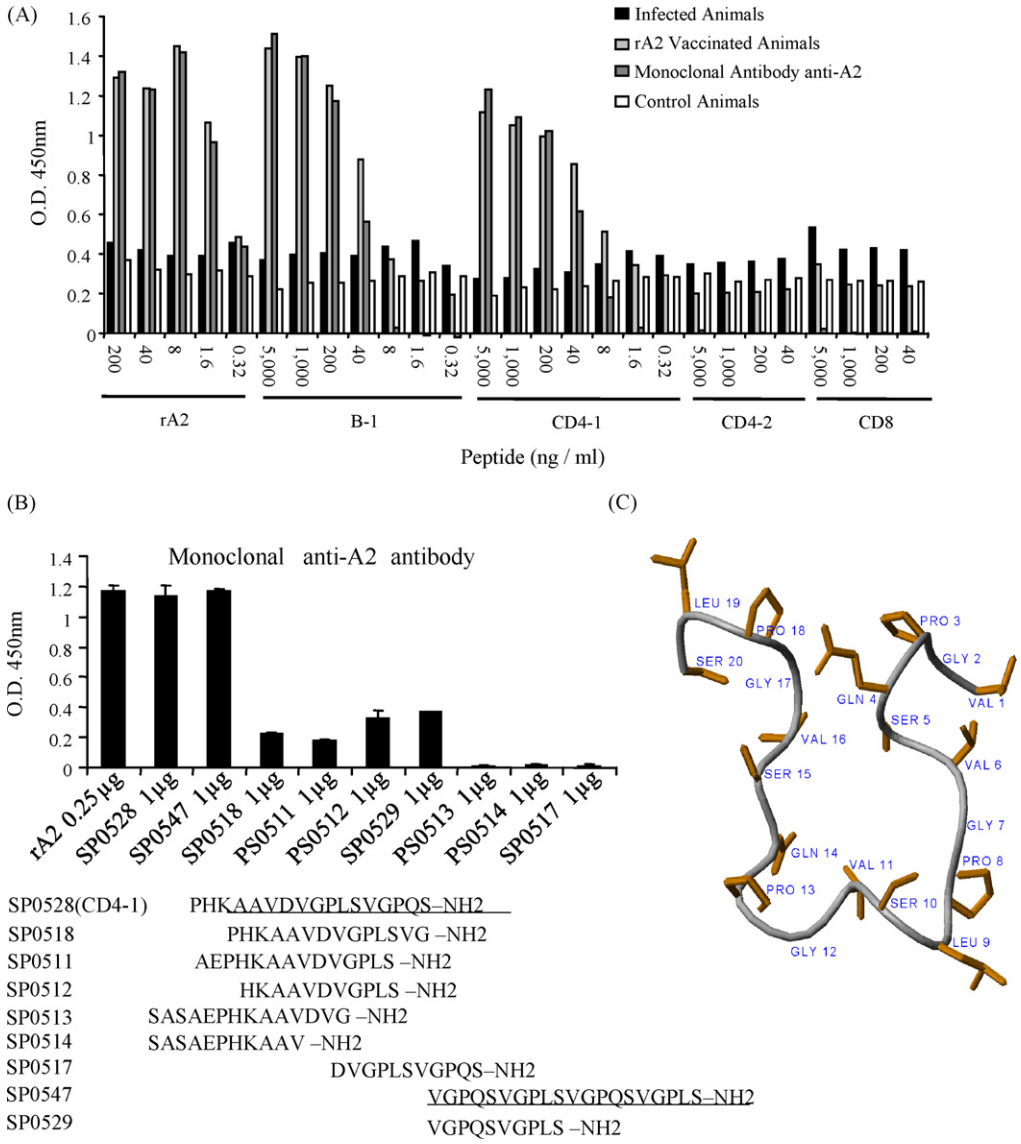

Fig. 2. B cell epitope mapping of A2. (A) The original synthetic peptides derived from A2 (B-1, CD4-1, CD4-2 and CD8) were tested in ELISA employing sera from infected and vaccinated mice (pool of three samples per group) as well as anti-A2-specific monoclonal antibody. (B) Shorter sequences based on reactive peptides CD4-1 and B-1 and peptides composed of A2 repetitions were tested in ELISA against anti-A2 monoclonal antibody in order to determine the minimal B epitope. In (A and B) results are representative of four independent experiments. (C) SP0547 was submitted to NMR, which revealed its lowest energy structure at 4.0 mM in TFE:H_2O (10:90) – pH 7.0 (20 mM phosphate buffer), 20 °C as determined by NOE connectivity diagram.

non-repetitive segment of the protein plus five amino-acid residues from the first repetitive segment (Fig. 1).

3.2. Experimental analysis of B cell epitopes identified in A2 sequence

As shown in Fig. 2A, the B-1 epitope reacts positively with antibodies present in sera from rA2-vaccinated mice and with the A2-specific monoclonal antibody. The level of reactivity observed in reactions with B-1 was similar to that observed when rA2 was used as antigen in control reactions. Sera from mice infected with *L.* (*L.*) *chagasi* (Fig. 2A) did not react with B-1 or rA2. When the predicted T cell epitopes named CD8, CD4-1, and CD4-2 were tested, the only additional peptide that showed a comparable reactivity with sera from immunized mice and anti-A2 mAb (as seen with B-1 or rA2) was the CD4-1 (Fig. 2A), which except for the five last amino-acids in the N-terminal had a major aa sequence overlap with the B-1 epitope.

In an attempt to define the minimal B epitope, we designed smaller peptides based on B-1 sequence, as shown in Fig. 2B. Peptides were conjugated to BSA to enhance binding to ELISA plates. However, BSA reacted to mice serum and generated false-positive reactions in ELISA (not shown). Therefore, epitope mapping was performed with anti-A2 monoclonal antibody, since a comparable reactivity with peptide B1 was observed for sera of vaccinated mice and the anti-A2 monoclonal antibody (Fig. 2A). We observed that, in all peptides (SP0511, SP0512 and SP0518) in which at least part of the repetitive segment VGPLSVGPQS was deleted, the recognition by the anti-A2 monoclonal antibody was largely affected. Peptides SP0513 and SP0514, which had the repetitive unit deleted, showed no reaction with the monoclonal antibody. Surprisingly, the peptides that had only one repetitive unit SP0517 and SP0529 showed low or no reactivity with the anti-A2 mAb. Importantly, the SP0547, which had two repetitive units, reacted as much as rA2 and peptides B-1 and CD4-1 (SP0528).

The peptide SP0547 was submitted to magnetic resonance. Sequence-specific chemical shift assignments have been performed for peptide SP0547 from the correlations observed in TOCSY and NOESY spectra using standard procedures [38]. Some inter-residual NOEs were observed in peptide SP0547 (not shown). Importantly, three correlations of the sort NN(i,i+2) observed at the central portion of the peptide sequence (Leu-9 and Val-11, Ser-10 and Gly-12, Val-12 and Pro-14) may be an indicative of a specific structural arrangement. Two long-range NOEs have been determined – Hα(i)-HN(j) Ser-5 and Gly-17, Hβ(i)-Hβ(j) Ser-5 and Val-16. These correlations indicate the spatial proximity between these sites and therefore characterize a sort of turn in the peptide structure (Fig. 2C). No inter-residual correlations have been determined in the NOESY spectrum of peptide SP0529, what is in agreement with a nonrigid conformation. Since the primary sequence of SP0547 represents the sequence of SP0529 twice, we suggest that the activity observed for SP0547 could be attributed to its structural arrangement, once the unordered SP0529 does not show significant biological activity.

3.3. Experimental analysis of predicted T cell epitopes in A2

We then generated a non-replicative adenovirus encoding A2 protein (AdA2). Expression of A2 was tested in AdA2-infected cells by Western-blot with an anti-A2 monoclonal antibody. Multiple forms of rA2, with molecular masses under 53 kDa, were detected in infected cell extracts (Fig. 3A, lane 3). A similar pattern of bands was observed in rA2 purified from *E. coli* (Fig. 3A, lane 1).

Based on the score indicated by software BIMAS (Fig. 3B, upper left), peptide CD8 had a high probability of binding to D^d molecules. On the other hand, this same epitope had lower probability of showing affinity to molecules D^b and K^b. To test this hypothesis, we

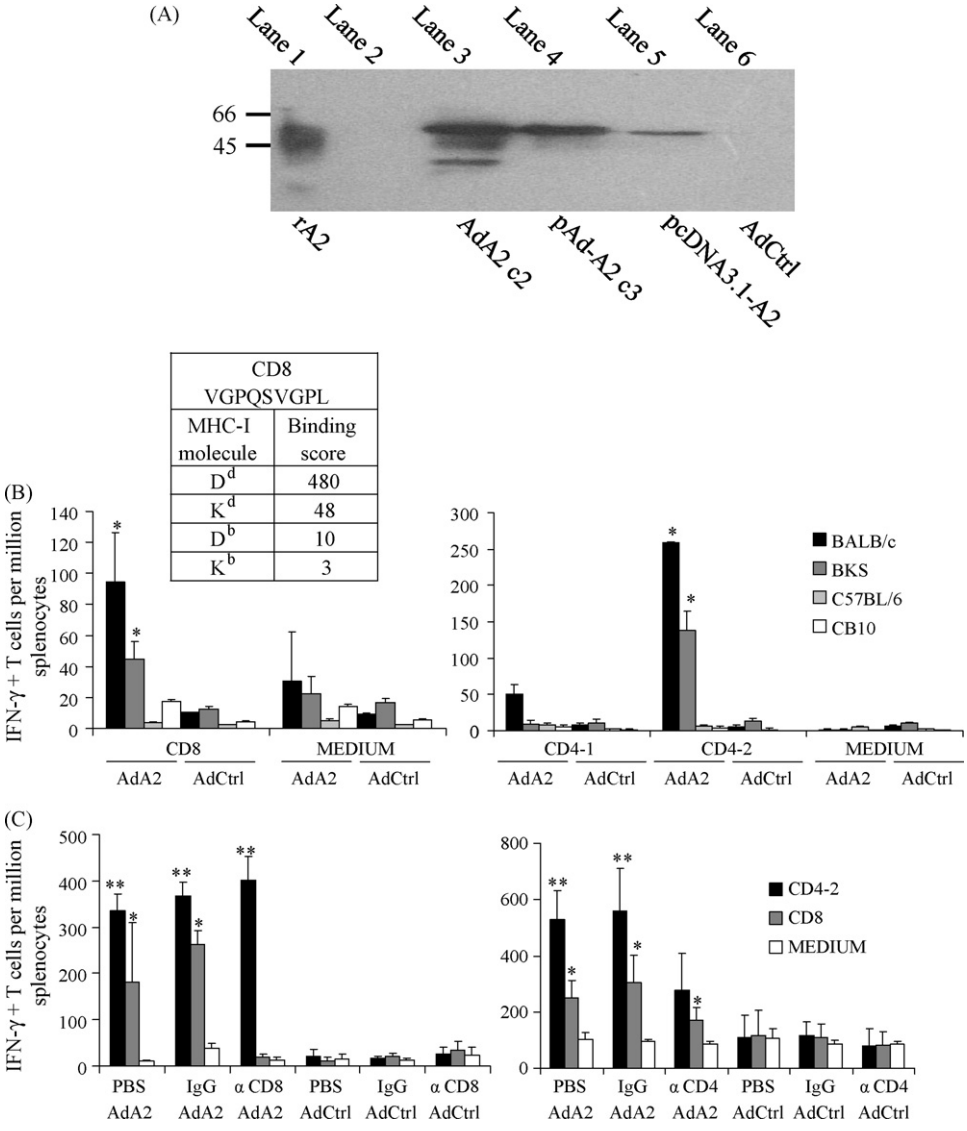

Fig. 3. Construction of A2-recombinant adenovirus and characterization of T cell epitopes of A2 recognized by vaccinated mice. (A) Expression of A2 protein in AdA2-infected or pA2-transfected cell lysates was tested in Western-blot assay against a monoclonal antibody specific for A2 protein. (B) Cellular responses against different epitopes predicted in A2 were tested in ELISPOT assay performed with spleen cells of BALB/c, BKS, C57BL/6 and CB10 mice collected 15 days after prime dose of different adenoviruses. The score values in upper panel indicate the probability of interaction between the CD8 peptide and different MHC-I haplotypes, and were defined by BIMAS software. (C) ELISPOT assay performed with spleen cells of BALB/c mice submitted to depletion of CD8+ or CD4+ T cells followed by immunization with one dose of adenovirus. In (B and C) ELISPOT assays were performed with three samples per group, tested individually, and results are representative of two independent experiments. (*) or (**) indicates statistically significant difference between experimental and control groups. pAd-A2, transfer vector carrying the *A2* gene sequence; AdCtrl, recombinant adenovirus encoding a gene not related to *Leishmania*; rA2, recombinant A2 protein; AdA2, recombinant adenovirus encoding the *A2* gene.

immunized with one dose of AdA2, BALB/c and C57BL/KsJ (BKS) mice, both strains showing haplotype $H2^d$, as well as C57BL/6 and C.B10-H2 (CB10) mice, which show $H2^b$ haplotype. In agreement with predictions, spleen cells from BALB/c and BKS mice reacted specifically (producing IFN-γ) to re-stimulation with peptide CD8 (Fig. 3B, left). We also tested if peptides CD4-1 and CD4-2 were haplotype-restricted. We demonstrated that CD4-2 peptide was presented only in association with BALB/c and BKS mice MHC-II molecules, and that peptide CD4-1 did not react with molecules D^d or D^b (Fig. 3B, right).

Confirmation that peptides CD4-2 and CD8 were recognized, respectively, by CD4+ and CD8+ T cells was achieved in ELISPOT performed with spleen cells from mice submitted to selective depletion of CD4+ or CD8+ T cells, followed by immunization with one dose of AdA2 (Fig. 3C). The depletion of CD8+ T cell subpopulation abrogated only the response to the counterpart MHC-I epitope (Fig. 3C, left). In a similar extent, depletion of CD4+ T cells reduced IFN-γ production after in vitro stimulation with peptide CD4-2, since IFN-γ production detected was not statistically significant when compared to control group (Fig. 3C, right).

3.4. Immune responses and protection against challenge in mice vaccinated with AdA2 in homologous prime/boost protocols

Finally, we evaluated the immune response elicited by a homologous prime/boost protocol employing AdA2. Fig. 4A shows that following vaccination, high levels of A2-specific IFN-γ producing CD4+ T and CD8+ T cells from parental BALB/c lineage ($H2^d$), but not from the congenic CB10 mice ($H2^b$) were induced. Induction of CTLs, employing an *in vivo* cytotoxicity assay, in which target cells were sensitized with peptide CD8 and transferred to immunized mice, was also evaluated. As seen in Fig. 4B, vaccination induced activation of anti-A2-specific CTLs in BALB/c mice, but not in CB10 mice. CTLs leaded to almost 40% specific lysis of target cells. No cytotoxic activity was observed when we used the CD4-2 peptide (Fig. 4C). Even after two doses, AdA2 did not induce significant levels of anti-A2 antibodies in BALB/c or CB10 mice (not shown).

In the next step, we tested whether immunization with AdA2 is able to induce protection against challenge with a virulent strain of *L.* (*L.*) *chagasi*. After i.v. challenge of BALB/c mice with 10^7 promastigotes of *L.* (*L.*) *chagasi*, we observed a sustained immune response, with peptide-specific cytotoxic activity (Fig. 5A, left) and IFN-γ

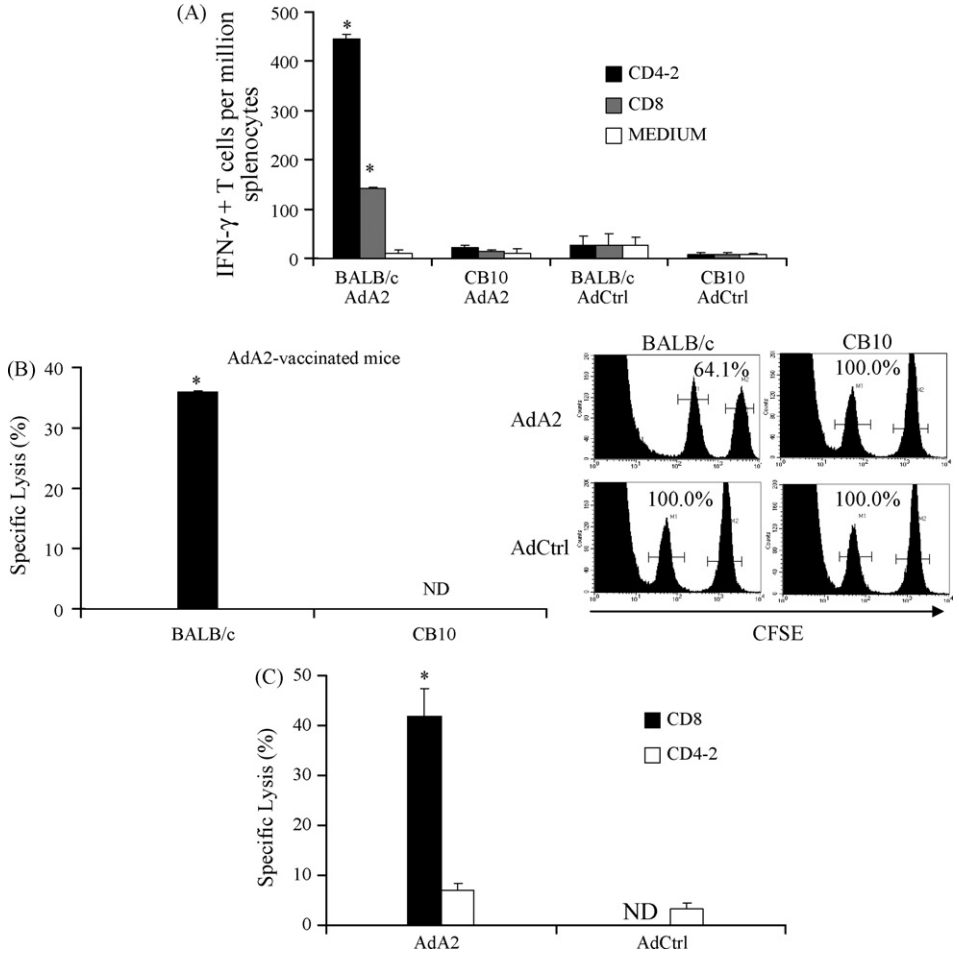

Fig. 4. T cell responses elicited by vaccination with adenovirus expressing A2 protein. (A) ELISPOT assay performed with spleen cells of BALB/c and CB10 mice collected 15 days after second dose (boost) of different adenoviruses. Assays were performed with three samples per group, tested individually. (B) *In vivo* cytotoxicity assay. Splenocytes from naïve BALB/c or CB10 donors were labeled with two concentrations of CFSE ($CFSE_{high}$ and $CFSE_{low}$) and inoculated i.v. into the tail vein of immunized mice. $CFSE_{high}$ cells were pulsed with peptide CD8, and $CFSE_{low}$ cells remained unpulsed (internal controls). Percentage of cell lysis was measured 20 h later by FACS. Percentage numbers on histogram refer to $CFSE_{high}$ cells, pulsed with peptide. (C) *In vivo* cytotoxicity assay with $CFSE_{high}$ cells were pulsed with peptide CD4-2 or CD8 and inoculated in BALB/c mice vaccinated with two doses of AdA2. In (B and C) two receptors were individually tested per group, and the percentage of specific lysis was determined with the following formula: $1 - [(\%CFSE_{high}\ \text{infected} - \%CFSE_{low}\ \text{infected})/(\%CFSE_{high}\ \text{naïve} - \%CFSE_{low}\ \text{naïve})] \times 100$. All experiments were repeated two times, independently. (*) indicates statistically significant difference between experimental and control groups. ND, not detected.

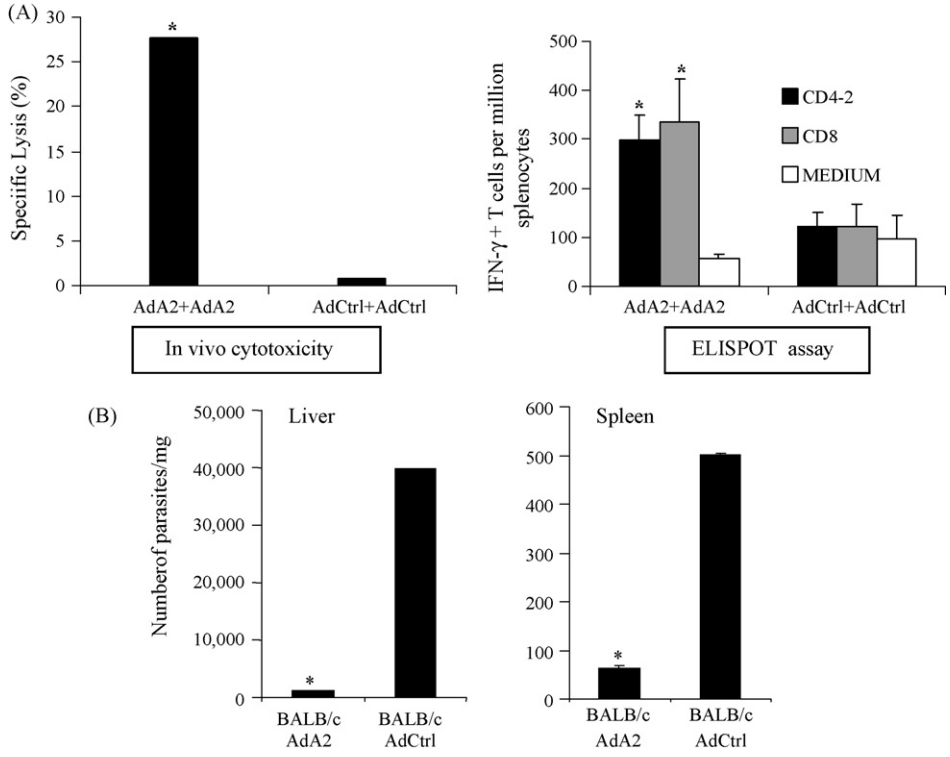

Fig. 5. Immune responses after challenge with *L.* (*L.*) *chagasi* and protection assays. (A) *In vivo* cytotoxicity assay and ELISPOT assay performed 30 days after i.v. challenge of AdA2-immunized mice with 10^7 *L.* (*L.*) *chagasi* parasites. Two animals per group were individually tested, and the experiments were repeated two times, independently. (B) Mice (six animals per group) were challenged i.v. with 10^7 *L.* (*L.*) *chagasi* promastigotes 15 days after boost vaccination. Parasite burdens were determined by limiting dilution, 35 days after infection. (*) indicates statistically significant difference between experimental and control groups. These results are representative of two different experiments.

production (Fig. 5A, right). Immunization with AdA2 in a standard homologous prime/boost vaccination protocol in BALB/c mice resulted in highly significant reduction of parasite burdens in liver and spleen (Fig. 5B), 35 days after infection. At this stage of infection in this model, spleen parasite burdens are low but tend to increase, while parasite numbers in liver are higher and tend to decrease [39]. Thus, demonstration of reduction of parasite burdens upon vaccination with A2 at both sites in this animal model is an important aspect.

4. Discussion

A2 was identified as an important amastigote virulence factor of *L.* (*L.*) *donovani* parasites [6], and has recently emerged as a candidate for vaccine development against visceral leishmaniasis [12–15]. Here, we characterized the dominant B as well as CD4+ T and CD8+ T cell epitopes present in A2, and demonstrated that the adenovirus vector expressing A2 induces a strong CD4+ T and CD8+ T cell response and protective immunity against experimental infection with *L.* (*L.*) *chagasi*.

The B cell epitope in A2 was located within the repetitive units, since Ab recognition was dependent on maintenance of integrity of the repetitive segment. Importantly, nuclear magnetic resonance indicated that peptide SP0547, which has two repetitive units, folds into a beta-sheet like structure. Thus, it is possible that the activity observed for SP0547 is related to this structural feature, since peptide SP0529 that consists of single repetitive unit, does not fold and is immunologically inert. Importantly, in both humans and dogs, the state of "resistance" to *Leishmania* is associated with development of cell-mediated responses, whereas active disease is associated with high antibody levels and suppressed T cell responses [40,41]. Nevertheless, a recent study has demonstrated that humoral response to A2 in dogs, although low, is associated with asymptomatic infection, and presumably, protective immunity against *Leishmania* parasites [42]. Thus, the results of B cell epitope mapping of A2, obtained here, could be eventually applied in the field, to evaluate the development of protective immune responses in humans and dogs, which are the main target populations for a vaccine against visceral leishmaniasis. If reactivity to the main B cell epitope in A2 is consistently traced and associated with protective immunity, for example, it would be possible to identify protected dogs vaccinated with A2 and asymptomatic dogs naturally infected with *Leishmania* from naïve and susceptible animals that are infected, but did not develop protective immunity. Further, the high levels of anti-A2 antibodies in sera from dogs vaccinated with rA2 may help to discriminate these animals from naturally infected dogs, which is valuable information in geographical areas or Countries where elimination of seropositive infected dogs is the current policy.

We also successfully characterized the sequence VGPQSVGPL as a dominant CD8+ T cell epitope in A2. This peptide reacted strongly and specifically with corresponding IFN-γ producing CD8+ T cells in ELISPOT as well as *in vivo* cytotoxic assays of mice immunized with AdA2. Indeed, as far as we know, this is the first study where a CD8+ T cell epitope is mapped in a *Leishmania* derived antigen. We also identified a CD4+ T epitope by testing overlapping epitopes of the A2 sequence. The CD4-2 peptide (SASAEPHKAAVDVGPL) was shown to be the main epitope recognized by IFN-γ producing CD4+ T cells in ELISPOT.

Control of visceral infection is often associated with formation of granulomas composed of T cells and macrophages in liver [41]. The essential effector mechanism appears to involve parasite killing by

macrophages activated by IFN-γ [16]. The main source of IFN-γ is normally attributed to CD4+ T lymphocytes, nevertheless there are evidences that CD8+ T cells are also involved in protective immunity against leishmaniasis [43]. Thus, despite of intracellular location of parasites in the vertebrate host, the role of protective CD8+ cytolytic T cells induced by anti-leishmania vaccines is less established as compared to function of CD4+ T cells [19–21]. Importantly, peak of CTL activity coincided with regression of parasite loads in spleen and liver from mice experimentally infected with *L.* (*L.*) *infantum* and were capable of killing target cells pulsed with total *Leishmania* antigen or macrophages infected with *Leishmania* [19]. Here, we detected for the first time *in vivo* CTL activity displayed by CD8+ T cells against a well-defined *Leishmania* target, the A2-specific VGPQSVGPL repetitive unit.

A2 has at least one epitope recognized by CD4+ T lymphocytes and, normally, has over 40 copies of the VGPQSVGPL repetitive unit that was now identified as a potent CD8+ T cell epitope. A major question here is how to induce a potent A2-specific T cell-mediated immunity in immunization protocol. Considering the well-established properties of adenovirus vector in eliciting both CD4+ T as well as CD8+ T cells in vaccine protocols, we constructed a non-replicative adenovirus vector expressing A2. AdA2 vaccination induced IFN-γ production as well as CTL activity by CD8+ T cells from mice expressing the MHC haplotype $H2^d$ (BALB/c and BKS). In agreement, a homologous prime/boost protocol employing AdA2 was highly efficient in inducing A2-specific IFN-γ producing CD4+ T and CD8+ T cells as well as CD8+ T cells displaying cytotoxic activity, leading to host protection in BALB/c mice challenged with *L.* (*L.*) *chagasi*. The lack of responsiveness of CD8+ T cells from CB10 or C57BL/6 mice is not surprising, since the predicted score of CD8 peptide binding to MHC class I molecule D^b is only 10, as compared to 480 for binding to molecule D^d. Nevertheless, *in silico* analysis showed presence of other peptides in A2 that could bind to MHC class I molecule D^b (not shown). Randomly designed MHC-II binding peptides were also not able to stimulate CD4+ T cells from CB10 or C57BL/6 mice. However, to thoroughly address this question, it is necessary to generate new peptides, with differences in one or two overlapping amino-acids, instead of five amino-acids, as in CD4-1 and CD4-2 sequences. In fact, vaccination of C57BL/6 mice with recombinant A2 showed to induce protection against challenge with *L.* (*L.*) *donovani*, which could be interpreted as indirect demonstration of T cell response against A2 in this mice strain [12].

In conclusion, in the present work we characterized B and T epitopes on A2, which account for its immunogenicity in BALB/c mice. Further, we demonstrated that protective Th1-biased immune response is efficiently induced using AdA2. Further, IFN-γ production and CTL activity mediated by CD8+ T cells, and not antibody production, are associated with induced protective immunity against experimental infection with *L.* (*L.*) *chagasi*. These results further suggest that A2 is an important candidate antigen for development of a vaccine against visceral leishmaniasis. Additional analysis of immune response to the defined B lymphocyte, CD4+ T as well as CD8+ T cell epitopes allowing comparison of symptomatic versus asymptomatic patients/dogs, may help evaluate the role of anti-A2 immune responses to natural *Leishmania* infection.

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