Heterologous Plasmid DNA Prime-Recombinant Human Adenovirus 5 Boost Vaccination Generates a Stable Pool of Protective Long-Lived CD8+ T Effector Memory Cells Specific for a Human Parasite, *Trypanosoma cruzi*[▽][†]

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Recently, we described a heterologous prime-boost strategy using plasmid DNA followed by replication-defective human recombinant adenovirus type 5 as a powerful strategy to elicit long-lived CD8+ T-cell-mediated protective immunity against experimental systemic infection of mice with a human intracellular protozoan parasite, *Trypanosoma cruzi*. In the present study, we further characterized the protective long-lived CD8+ T cells. We compared several functional and phenotypic aspects of specific CD8+ T cells present 14 or 98 days after the last immunizing dose and found the following: (i) the numbers of specific cells were similar, as determined by multimer staining or by determining the number of gamma interferon (IFN-γ)-secreting cells by enzyme-linked immunospot (ELISPOT) assay; (ii) these cells were equally cytotoxic *in vivo*; (iii) following *in vitro* stimulation, a slight decline in the frequency of multifunctional cells (CD107a+ IFN-γ+ or CD107a+ IFN-γ+ tumor necrosis factor alpha positive [TNF-α+]) was paralleled by a significant increase of CD107a singly positive cells after 98 days; (iv) the expression of several surface markers was identical, except for the reexpression of CD127 after 98 days; (v) the use of genetically deficient mice revealed a role for interleukin-12 (IL-12)/IL-23, but not IFN-γ, in the maintenance of these memory cells; and (vi) subsequent immunizations with an unrelated virus or a plasmid vaccine or the depletion of CD4+ T cells did not significantly erode the number or function of these CD8+ T cells during the 15-week period. From these results, we concluded that heterologous plasmid DNA prime-adenovirus boost vaccination generated a stable pool of functional protective long-lived CD8+ T cells with an effector memory phenotype.

Genetic vaccination using the strategy known as the heterologous prime-boost regimen is being pursued as an alternative type of vaccine. This strategy consists of the use of two different vectors, both carrying a gene that encodes the same antigenic protein, for priming and boosting immunizations. This strategy can be particularly important in the case of intracellular pathogens and neoplastic cells, where the effectiveness of the vaccine relies heavily on its capacity to elicit specific immune responses mediated by cytotoxic CD8+ T cells (reviewed in references 22, 31, 38, 39, and 57).

Among possible genetic vector combinations, heterologous prime-boost vaccination, which uses a naked plasmid DNA for priming followed by a booster injection of recombinant replication-deficient human adenovirus type 5 (HuAd5), has succeeded in providing protective immunity in some relevant preclinical experimental models, such as simian immunodeficiency virus (SIV), malaria, and Ebola and Marburg virus models (1, 8, 9, 18, 19, 20, 29, 53). Based on these relative successes obtained with preclinical experimental models, human trials have been initiated (17, 26, 28, 43).

Recently, we reported that this strategy could successfully vaccinate highly susceptible A/Sn mice against systemic infection with the human intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease (American trypanosomiasis). In these experiments, we used a plasmid DNA and a human replication-defective adenovirus type 5, both expressing the amastigote surface protein 2 (ASP-2) of *T. cruzi*. Protective immunity generated by vaccination was medi-

ated by CD4 Th1 and cytotoxic CD8$^+$ TC1 cells. Both T-cell types cooperated to provide the long-lived protective immunity induced by vaccination, as depletion of either CD4$^+$ or CD8$^+$ T cells abolished most of the protective immunity. In addition, perforin and gamma interferon (IFN-γ) were described as critical for the acquired immunity of vaccinated animals against experimental infection with *T. cruzi* (13).

Vaccinated perforin-deficient or wild-type mice were susceptible or resistant to *T. cruzi* infection, respectively. By comparing the CD8$^+$ T-cell immune responses of these two mouse strains following heterologous prime-boost vaccination, we observed that both mice had similar numbers of splenic specific CD8$^+$ T cells (13). Nevertheless, the CD8$^+$ T cells of the susceptible perforin-deficient mice had functional defects detected by immunological assays performed *in vivo* and *in vitro* (13).

Considerable information has been published in recent years about the subsets of memory CD8$^+$ T cells elicited following infections with viruses or bacteria. The current paradigm divides the memory T cells into two major subsets: T effector memory (TEM) and T central memory (TCM) cells. These subpopulations can be identified based on their expression of certain surface markers, such as CD62L and CCR7, and they may differ greatly in terms of functional and migratory properties (reviewed in references 4, 25, 36, and 41). In spite of the fact that rapid knowledge is accumulating on the importance of each of these populations for long-term CD8$^+$ T-cell-mediated immunity against reinfection with viruses and bacteria, limited information is available on the roles of these different memory subsets following vaccination protocols.

Because the functional and phenotypic aspects of long-lived protective CD8$^+$ T cells elicited by heterologous DNA prime-adenovirus boost vaccination are poorly studied, we considered that our model could be of interest to clarify this topic. We accomplished this aim by comparing the functional and phenotypic aspects of the transgene-specific protective CD8$^+$ T cells 14 or 98 days following the last immunizing dose with recombinant HuAd5. We also gained further insights into the requirement of certain signaling pathways for the maintenance of these T cells by using genetically deficient mice that do not express IFN-γ or interleukin-12 (IL-12)/IL-23 (p40). Finally, to test whether these cells established a pool of stable functional CD8$^+$ T cells, we subjected these mice to treatments that could potentially erode either the number or function of specific CD8$^+$ T cells. Overall, we found that these transgene-specific long-lived CD8$^+$ T cells displayed a TEM-like phenotype. They were dependent on the IL-12/IL-23 (p40) signaling pathway and relatively resistant to immunological erosion with vaccination protocols using either a recombinant virus (modified vaccinia Ankara virus [MVA]) or plasmid DNA. These results indicate that long-lived CD8$^+$ TEM-like cells elicited by the heterologous plasmid DNA prime-adenovirus boost vaccination regimen are a stable pool of functional protective T cells that can be pursued for the development of vaccines against *T. cruzi*, and perhaps other protozoan parasites.

MATERIALS AND METHODS

Mice and parasites. Female 8-week-old C57BL/6 wild-type (WT) or A/Sn mice were purchased from CEDEME (Federal University of São Paulo). IFN-γ (12) or IL-12/IL-23 (p40) (33) knockout (KO) mice were raised at the Instituto de Ciências Biomédicas, Universidade de São Paulo. Parasites of the Y strain of *T. cruzi* were used in this study (5). Bloodstream trypomastigotes were obtained from mice infected 7 days earlier. The concentration of parasites was estimated and adjusted to 1,500 parasites/ml. Each A/Sn mouse was inoculated subcutaneously (s.c.) with 0.1 ml. The use of animals and the experimental procedures, including the number of mice per experimental group, have been approved by the Ethics Committee for Animal Care of the Federal University of São Paulo (CEP 0426/09).

Peptide synthesis. The VNHRFTLV peptide and the H-2K^b/VNHRFTLV pentamer were purchased from Genscript (Piscataway, NJ) and ProImmune Inc. (Oxford, United Kingdom), respectively.

Recombinant plasmids and adenoviruses used for immunizations. Plasmid pIgSPclone9 was obtained by inserting the sequence encoding the signal peptide of the mouse immunoglobulin kappa chain in frame with the gene encoding *T. cruzi* amastigote surface protein 2 into the commercial vector pcDNA3 (Invitrogen) (5). pAdCMV-ASP2 is an adenoviral transfer plasmid that contains a eukaryotic expression cassette formed by the cytomegalovirus immediate-early promoter and simian virus 40 (SV40) RNA polyadenylation sequences. We cloned the DNA sequences encoding *T. cruzi* amastigote surface protein 2 obtained by restriction digestion of the plasmid pIgSPclone9 (AdASP-2) (32) within this cassette. Viruses and plasmids were generated and purified as described earlier; both led to the expression of the antigen, as evaluated by *in vitro* transfections (5, 32). Mice were inoculated intramuscularly (i.m.) in each tibialis anterioris muscle with 50 μg of plasmid DNA. Twenty-one days later, these mice received 50 μl of a viral suspension containing 2×10^8 PFU of adenovirus in the same locations. Immunological assays were performed 14 or >98 days after viral inoculation.

Groups of mice vaccinated with the heterologous prime-boost vaccination regimen were subsequently immunized with a recombinant modified vaccinia Ankara virus expressing green fluorescent protein (MVA-GFP) (11). This virus was kindly provided by Flavio da Fonseca of the Federal University of Minas Gerais, Brazil. Mice were injected intravenously (i.v.) with 10^7 PFU on the indicated days (see Fig. 5 and 6). Alternatively, vaccinated mice were immunized with recombinant plasmid DNA containing the *gag* gene of HIV (34). This plasmid was kindly provided by E. T. Marques, Jr., of the Johns Hopkins School of Medicine, Baltimore, MD.

In vivo depletion of CD4$^+$ T cells was achieved by treating vaccinated mice with the GK1.5 monoclonal antibody (MAb) on the indicated days. Mice were injected intraperitoneally (i.p.) with three daily doses of 0.5 mg of anti-CD4 or saline. As determined by fluorescence-activated cell sorter (FACS) analyses, the depletion of CD4$^+$ spleen cells was routinely >90% efficient in anti-CD4-treated mice compared to controls.

Immunological assays. For *in vivo* cytotoxicity assays, splenocytes collected from naïve C57BL/6 mice were treated with ACK buffer (NH$_4$Cl, 0.15 M; KHCO$_3$, 10 mM; sodium EDTA, 0.1 mM; pH 7.4) to lyse the red blood cells. These cells were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Eugene, OR) at a final concentration of 5 μM (CFSE$_{high}$) or 0.5 μM (CFSE$_{low}$). The CFSE$_{high}$ cells were coated for 40 min at 37°C with 1 μM H-2K^b-restricted peptide VNHRFTLV. The CFSE$_{low}$ cells remained uncoated. Subsequently, the CFSE$_{high}$ cells were washed and mixed with equal numbers of CFSE$_{low}$ cells before 40×10^6 total cells were i.v. injected into each mouse. Recipient animals were mice previously immunized with recombinant plasmids and adenoviruses. Spleen cells of recipient mice were collected 20 h after transfer, fixed with 1.0% paraformaldehyde, and analyzed by FACS, using a FacsCanto flow cytometer (BD, Mountain View, CA). The percentage of specific lysis was determined using the following formula: $1 - [(\%CFSE_{high} \text{ immunized}/\%CFSE_{low} \text{ immunized})/(\%CFSE_{high} \text{ naïve}/\%CFSE_{low} \text{ naïve})] \times 100\%$.

Determination of IFN-γ secretion by cultured spleen cells and enzyme-linked immunospot (ELISPOT) assay for enumeration of IFN-γ-producing cells were performed essentially as described previously (5, 48).

For flow cytometry analyses, we used mouse splenocytes treated with ACK buffer. Single-cell suspensions were washed in phosphate-buffered saline (PBS), stained for 10 min at room temperature (RT) with biotinylated major histocompatibility complex class I (MHC I) multimer H-K^b/VNHRFTLV, and finally stained for 20 min at 4°C with avidin-allophycocyanin (avidin-APC) and peridinin chlorophyll protein (PerCP)-labeled anti-CD8 antibodies (both from BD Pharmingen). For analyses of other cell surface markers, single-cell suspensions from spleens of mice were stained at RT with biotinylated MHC I multimer H-K^b/VNHRFTLV and then stained for 20 min at RT with avidin-APC and PerCP-labeled anti-CD8 antibodies or fluorescein isothiocyanate (FITC)-labeled anti-CD11a (clone 2D7), anti-CD11c (clone HL3), anti-CD25 (clone 7D4), anti-CD31 (clone MEC13.3), anti-CD43 (clone S7), anti-CD44 (clone IM7), anti-

CD49d (clone R-12), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.2F3), anti-CD95 (clone Jo2), anti-CD122 (clone TM-β1), anti-CD127 (clone SB/199), or anti-CCR5 (clone C34-3448) (all from BD Pharmingen). Anti-CD27 (clone LG.7F9) and anti-KLRG-1 (clone MAFA) were purchased from eBioscience. At least 100,000 cells were acquired on a BD FacsCanto flow cytometer and then analyzed with FlowJo (Tree Star).

The surface mobilization (expression) of CD107a and the intracellular expression of cytokines (IFN-γ and tumor necrosis factor alpha [TNF-α]) were evaluated after *in vitro* culture of splenocytes in the presence or absence of an antigenic stimulus. Cells were washed three times in plain RPMI and resuspended in cell culture medium consisting of RPMI 1640 medium, pH 7.4, supplemented with 10 mM HEPES, 0.2% sodium bicarbonate, 59 mg/liter of penicillin, 133 mg/liter of streptomycin, and 10% HyClone fetal bovine serum (HyClone, Logan, UT). The viability of the cells was evaluated using 0.2% trypan blue exclusion dye to discriminate between live and dead cells. The cell concentration was adjusted to 5×10^6 cells/ml in cell culture medium containing anti-CD28 (2 μg/ml), brefeldin A (10 μg/ml), monensin (5 μg/ml), and 2 μg/ml FITC-labeled anti-CD107a (clone 1D4B; BD Pharmingen). To half of the cultures, 10 μM (final concentration) VNHRFTLV peptide was added. The cells were cultivated in flat-bottom 96-well plates (Corning) in a final volume of 200 μl in duplicate at 37°C in a humid environment. After a 20-h incubation, cells were stained for surface markers with PerCP- or phycoerythrin (PE)-labeled anti-CD8 on ice for 20 min. To detect IFN-γ or TNF-α by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% bovine serum albumin (BSA), and 2 mM EDTA, fixed in a 4% PBS-paraformaldehyde solution for 10 min, and permeabilized for 15 min in a PBS–0.1% BSA–0.1% saponin solution. After being washed twice, cells were stained for intracellular markers by using APC- or PE-labeled anti-IFN-γ (clone XMG1.2) and PE-labeled anti-TNF-α (clone MP6-XT22) for 20 min on ice. Finally, cells were washed twice and fixed in 1% PBS-paraformaldehyde. At least 300,000 cells were acquired on a BD FacsCanto flow cytometer and then analyzed with FlowJo.

Statistical analysis. When the variances were similar, values were expressed as averages ± standard deviations (SD). These values were compared by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) tests (http://faculty.vassar.edu/lowry/VassarStats.html). When the variances varied substantially, values were expressed as medians (bars) and sample values (dots). Values were compared by the Mann-Whitney or Kruskal-Wallis nonparametric test. The log rank test was used to compare mouse survival rates after challenge with *T. cruzi*. The differences were considered significant when the *P* value was <0.05.

RESULTS

Comparison of functional and phenotypic aspects of transgene-specific CD8+ T cells elicited by heterologous plasmid DNA priming-recombinant adenovirus boosting vaccination 14 or 98 days after the last immunizing dose. In earlier studies, we described that protective immunity against *T. cruzi* infection, as measured by reductions in peak parasitemia and delayed mouse mortality, could be achieved by heterologous plasmid DNA priming-recombinant adenovirus boosting vaccination (13). This protective immunity was long-lived. Mice challenged 14 or 98 days after the last immunizing dose displayed similar degrees of protective immunity. Protective immunity mediated by these cells correlated with the presence of effector CD8+ T cells capable of mediating *in vivo* cytotoxic activity and of expressing, upon short *in vitro* exposure to the antigen, surface CD107a and IFN-γ or IFN-γ and TNF-α (13). Based on this knowledge, we considered that it would be of interest to further characterize important functional and phenotypic aspects of these protective CD8+ T cells elicited by heterologous plasmid DNA priming-recombinant adenovirus boosting vaccination.

To that end, we compared several functional and phenotypic aspects of the specific CD8+ T cells present in the spleens of vaccinated mice 14 or 98 days after the last immunizing dose. We used C57BL/6 mice in our immunological analyses because we were able to obtain the H-2K^b/VNHRFTLV pentamer.
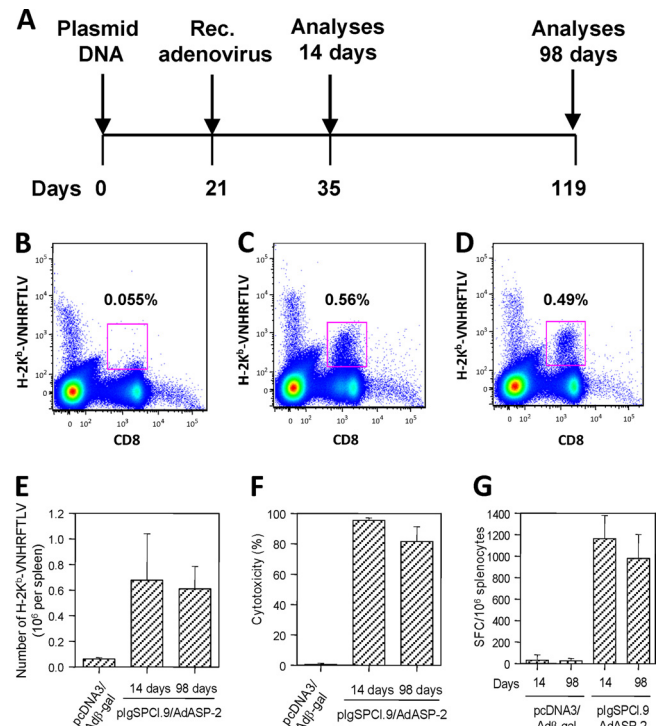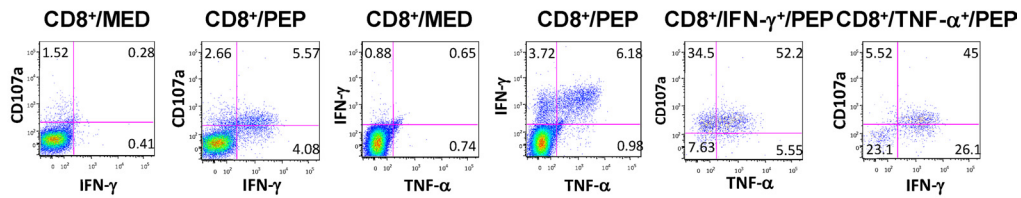

FIG. 1. Comparison of functional aspects of transgene-specific CD8+ T cells elicited by heterologous plasmid DNA priming-recombinant HuAd5 boosting vaccination regimen 14 or 98 days after the last immunizing dose. (A) C57BL/6 mice were immunized as shown. Priming and boosting immunizations were performed i.m. on the indicated days, using 100 μg of plasmid DNA or 2×10^8 PFU of HuAd5. Fourteen or 98 days after the HuAd5 dose, transgene-specific immune responses were analyzed. (B to D) Splenic cells were removed, and the frequencies of CD8+ H-2K^b/VNHRFLTV+ cells were determined. Results are shown as fluorescence intensities following staining with anti-CD8–PerCP, biotinylated H-2K^b/VNHRFLTV, and avidin-APC. The frequencies of double-positive cells inside the gates are depicted as percentages of total splenic cells from the following groups: (i) control mice injected with pcDNA3/Adβ-gal (B), (ii) mice immunized with pIgSPCl.9/AdASP-2 14 days earlier (C), and (iii) mice immunized with pIgSPCl.9/AdASP-2 98 days earlier (D). Results are from one representative of four mice. (E) The total numbers of CD8+ H-2K^b/VNHRFLTV+ cells in the spleens of immunized mice (n = 4) were estimated. No statistically significant difference was found between groups of mice that were immunized with pIgSPCl.9/AdASP-2 14 and 98 days earlier. (F) Fourteen or 98 days after the last immunizing dose, mice were injected with splenic syngeneic cells labeled with CFSE and coated with VNHRFTLV peptide. The specific *in vivo* cytotoxic activity was estimated after 20 h, as described in Materials and Methods. (G) Fourteen or 98 days after the last immunizing dose, spleen cells were collected and the number of splenic IFN-γ spot-forming cells (SFC) was estimated using an *ex vivo* ELISPOT assay. Results from panels E to G are shown as averages ± SD for 4 mice per group and are representative of two or more independent experiments. No statistically significant difference was found between groups of mice that were immunized with pIgSPCl.9/AdASP-2 14 and 98 days earlier.

This reagent is critical for proper visualization of specific CD8+ T cells. The protocol of immunization and the timing of analyses are depicted in Fig. 1A. Initially, we estimated the frequency of specific CD8+ T cells by staining them with anti-CD8 and the H-2K^b/VNHRFLTV pentamer (Fig. 1B to E). Using this methodology, we did not find a significant difference
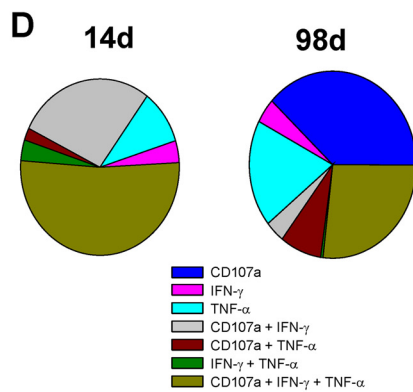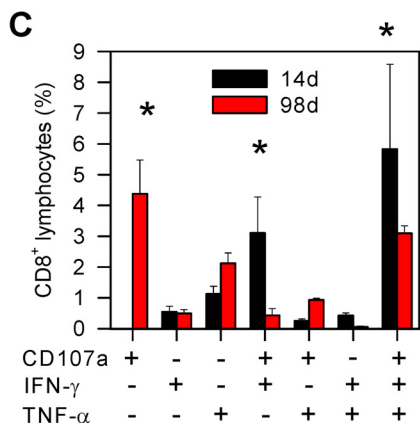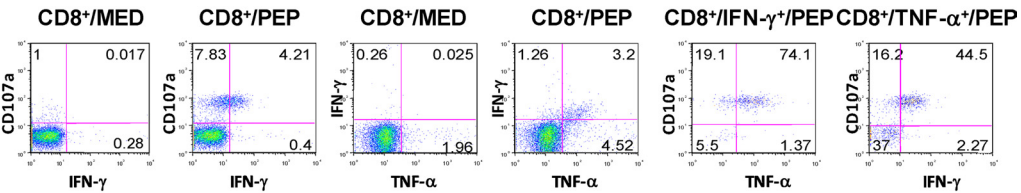
FIG. 2. Comparison of expression of surface CD107a and intracellular cytokines by transgene-specific CD8⁺ T cells induced by heterologous plasmid DNA priming-recombinant HuAd5 boosting vaccination regimen 14 or 98 days after the last immunizing dose. C57BL/6 mice were immunized according to the schedule depicted in Fig. 1A. Fourteen or 98 days after the HuAd5 immunization, splenic cells were collected and cultured in the presence of anti-CD28 and anti-CD107a, with or without the VNHRFTLV peptide. After 12 h, cells were stained with anti-CD8 and then fixed and stained with anti-IFN-γ and anti-TNF-α. Results are shown as fluorescence intensities for the indicated molecules on the *x* and *y* axes. The type of cells and the *in vitro* stimulus are shown at the top of each graphic. Med, medium; Pep, VNHRFTLV. (A and B) FACS analyses of CD8⁺ cells from mice immunized with pIgSPCl.9/AdASP-2 14 (A) or 98 (B) days earlier. (C and D) Frequencies of cell populations in groups of mice immunized with pIgSPCl.9/AdASP-2 14 or 98 days earlier. Asterisks denote statistically significant differences between groups ($P < 0.05$).

in the frequency or total number of splenic antigen-specific cells between the groups of vaccinated mice.

The function of these cells was evaluated using an *in vivo* cytotoxic assay and an ELISPOT assay for *ex vivo* determination of the number of peptide-specific IFN-γ-secreting cells. As shown in Fig. 1F and G, we could not detect significant differences between groups of mice that were boosted with AdASP-2 14 and 98 days prior. These immune responses were elicited by the recombinant vaccine, as control mice injected with pcDNA3 followed by adenovirus beta-galactosidase (Adβ-Gal) displayed minimal antigen-specific responses. Therefore, we concluded that the number and function of the antigen-specific T cells were preserved during the period analyzed.

Subsequently, we evaluated the expression of surface CD107a and intracellular cytokines following a short *in vitro* restimulation with the cognate peptide VNHRFTLV. FACS

analyses of peptide-stimulated splenic CD8 T cells demonstrated that cells from mice primed with pIgSPCl.9 and subsequently boosted with AdASP-2 14 and 98 days prior expressed surface CD107a, IFN-γ, and TNF-α (Fig. 2A and B, respectively). No statistically significant difference was found in the total frequencies of cells stained for any of these molecules when we compared vaccinated mice boosted 14 and 98 days prior. The frequencies of cells positive for membrane CD107a or any of the cytokines varied from 7.49% to 17.63% for the group boosted 14 days earlier and from 11.34% to 13.26% of CD8⁺ T cells for mice boosted 98 days earlier (but the differences were nonsignificant [NS]).

The expression of surface CD107a and intracellular cytokines following *in vitro* restimulation with the cognate peptide VNHRFTLV was dependent on immunization with pIgSPCl.9/AdASP-2, as cells from control mice injected with pcDNA3/Adβ-gal displayed a minimal number of peptide-specific cyto-
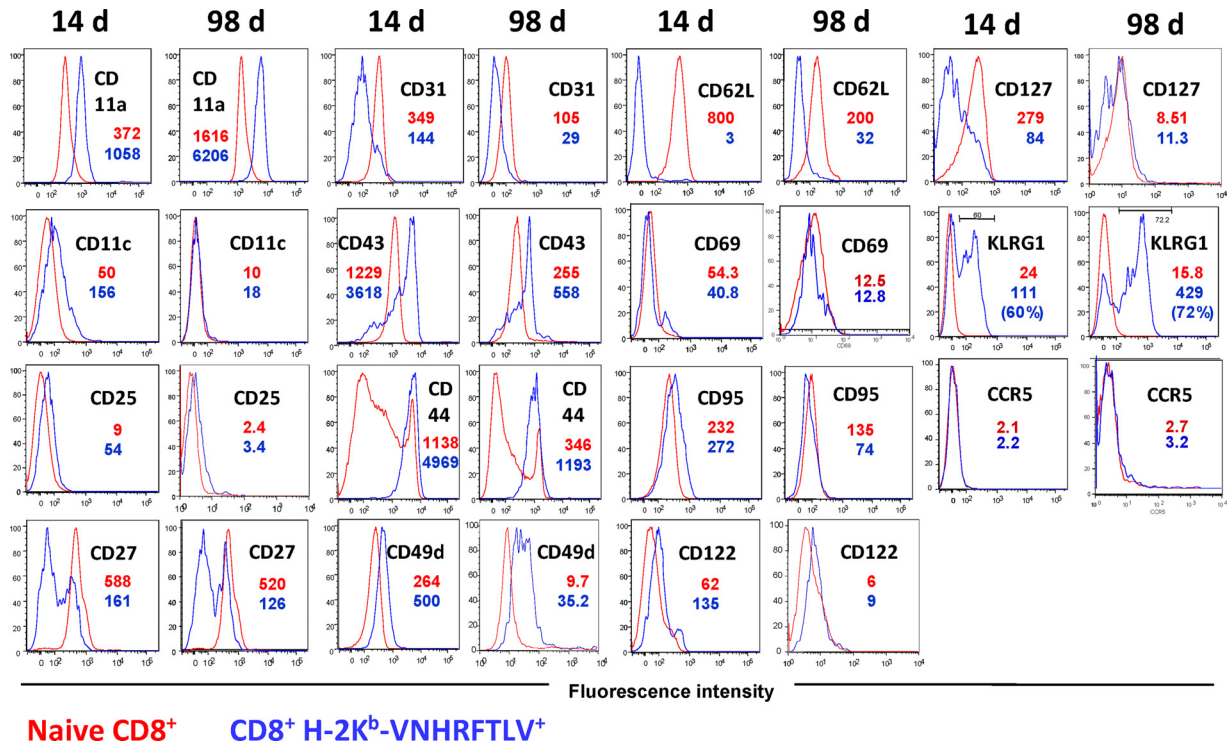
FIG. 3. Phenotypic characterization of splenic transgene-specific CD8$^+$ T cells elicited by heterologous plasmid DNA priming-recombinant HuAd5 boosting vaccination regimen 14 or 98 days after the last immunizing dose. C57BL/6 mice were immunized according to the schedule presented in Fig. 1A. Fourteen or 98 days after the recombinant HuAd5 dose, splenic cells were stained with anti-CD8, H-2K^b/VNHRFTLV, and the indicated marker-specific antibody prior to analysis by FACS. The histograms show the expression of the markers on CD8$^+$ H2K^b/VNHRFTLV$^+$ T cells (blue lines) or control naive CD8$^+$ spleen cells (red lines). Analyses were performed using pools of cells from three mice and are representative of two or more experiments performed independently. Numbers indicate the mean fluorescence intensities. In the case of KLRG-1, percentages of positive cells are indicated.

kine-expressing cells, varying from 0% to 0.16% and from 0.06% to 0.53% of CD8$^+$ T cells from mice boosted 14 and 98 days prior, respectively.

In comparing the subpopulations of cells expressing different combinations of molecules, we found statistically significant differences between cells from vaccinated mice boosted 14 days prior and those from mice boosted 98 days prior. As shown in Fig. 2C and D, the predominant CD8$^+$ T-cell subpopulation in the spleens of mice boosted 14 days earlier comprised CD107a$^+$ IFN-γ^+ TNF-α^+ cells, which accounted for 52.12% of the cells. In mice boosted 98 days earlier with AdASP-2, this subpopulation was reduced to 26.92% of specific CD8$^+$ T cells ($P < 0.05$). Similarly, the frequency of CD107a$^+$ IFN-γ^+ CD8$^+$ T cells was reduced from 27.76% to only 3.75% during the same time span ($P < 0.05$). In contrast, the frequency of CD107$^+$ or TNF-α^+ singly positive or CD107$^+$ TNF-α^+ double-positive CD8$^+$ T cells increased significantly during that period (Fig. 2C and D). In the case of CD107a$^+$ cells, the increase was rather dramatic, from 0% to 38.0% ($P < 0.05$). These results clearly demonstrate that the frequency of total specific T cells changed little in this time span. However, the quality of the effector T cells was not as stable and may have changed significantly. In spite of these changes, it is important to mention that their biologic meaning is not clear because protective immunity was not significantly different (13).

We then compared the surface markers expressed on pro-

tective specific splenic CD8$^+$ T lymphocytes elicited by the heterologous prime-boost regimen. For this purpose, CD8$^+$ T cells obtained from immune mice were stained with anti-CD8, multimer H-2K^b/VNHRFTLV, and one of several different activation, homing, or memory markers. Fluorescence intensity was compared to the expression of the same activation markers on naïve CD8$^+$ T cells. As shown in Fig. 3, compared to naïve CD8$^+$ T cells, the phenotype of double-stained CD8$^+$ H-2K^b/VNHRFTLV$^+$ cells from immune mice was CD11a^{high} CD25$^+$ CD27$^{high/low}$ CD31^{low} CD43^{high} CD44^{high} CD49d$^+$ CD62L^{low} CD69$^-$ CD95$^-$ CD122$^+$ KLRG-1$^{high/low}$ CCR5$^-$. The expression of these markers did not vary according to whether the cells were collected 14 or 98 days after the booster injection with AdASP-2. In fact, only two markers (CD11c and CD127) showed discordant behavior between the two samples. Although specific CD8$^+$ T cells collected from mice boosted 14 days earlier displayed upregulated expression of CD11c and downregulated expression of CD127, their pattern of expression returned to the steady state in cells collected at day 98. In addition to these markers, we also evaluated PD-1, CTLA-4, and BTLA expression on specific CD8$^+$ T cells after 98 days of boosting immunization. We found that PD-1 and CTLA-4 were expressed similarly on naïve and specific CD8$^+$ T cells, whereas BTLA was significantly downregulated (data not shown). Based on these analyses, we concluded that after 14 days, antigen-specific cells displayed a T effector (TE)-like
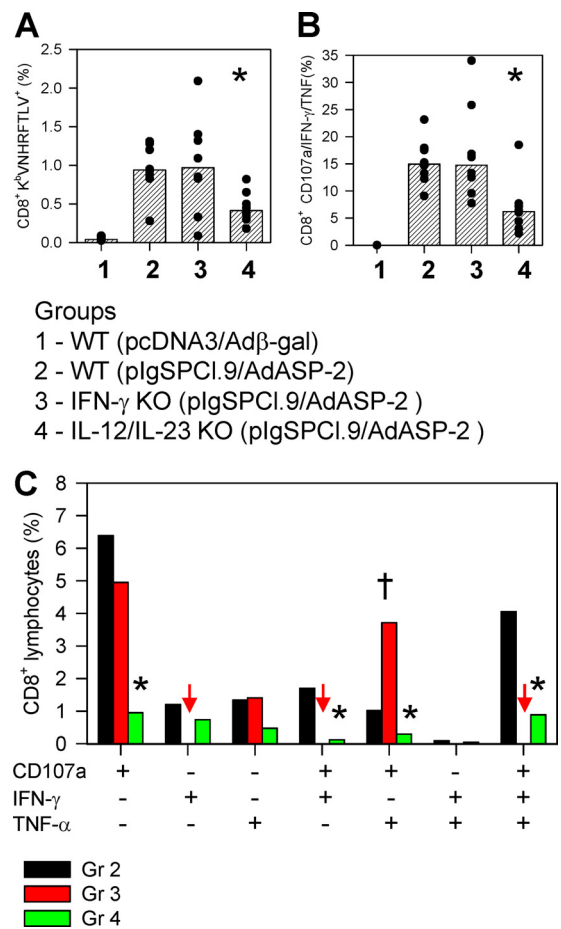
phenotype that subsequently changed to a TEM-like phenotype.

Absence of IL-12/IL-23 (p40), but not IFN-γ, signaling alters the formation of the stable pool of antigen-specific T effector memory cells. Because IL-12/IL-23 (p40) and IFN-γ have previously been implicated in the maintenance of antigen-specific memory T cells (10, 37, 52), we evaluated the fate of these cells in genetically deficient mice 98 days after the last immunizing dose with AdASP-2. We found that compared to that for WT vaccinated controls, the frequency of specific CD8⁺ T cells labeled by the multimer H-2K^b/VNHRFTLV was significantly lower for vaccinated mice deficient for the expression of IL-12/IL-23 (p40) but not for IFN-γ (Fig. 4A). Similarly, these KO mice also had a significant impairment with regard to the number of cells expressing surface CD107a, IFN-γ, or TNF-α after *in vitro* stimulation with the cognate peptide (Fig. 4B and C). In contrast, IFN-γ-deficient mice did not present any impairment in the generation of cells expressing surface CD107a or TNF-α after *in vitro* stimulation (Fig. 4B and C).

Heterologous prime-boost vaccination establishes a pool of antigen-specific CD8⁺ T cells that are relatively resistant to immunological erosion following vaccination with recombinant virus or plasmid DNA. Although memory CD8⁺ T cells traditionally have been considered stable over long periods, recent evidence challenged this concept by showing that subsequent infections or vaccinations may cause attrition and erosion of the memory T-cell pool (14, 24, 44, 45). We tested whether the homogeneous population of transgene-specific TEM-like cells induced by the heterologous prime-boost vaccination regimen could also be the target for this attrition after the exposure of vaccinated mice to other vaccines. We also determined whether CD4⁺ T-cell deficiency induced after immunization could impact the number or function of these cells. To that end, immune mice were subjected to three different treatments. A group of vaccinated mice (group 3) was immunized monthly with recombinant MVA-GFP (12). A second group (group 4) was vaccinated monthly with a plasmid DNA vaccine containing the *gag* gene of HIV (34). A third group of animals (group 5) was treated with depleting anti-CD4 MAb every month for three consecutive months. A schematic diagram of the experimental protocol can be found in Fig. 5A.

One hundred five days after the last immunizing dose with recombinant AdASP-2, splenic cells were analyzed for the frequency of pentamer-stained cells, *in vivo* peptide-specific cytotoxicity, the frequency of peptide-specific IFN-γ-secreting cells, and the frequency of CD8⁺ T cells expressing surface CD107a, IFN-γ, or TNF-α. As depicted in Fig. 5B, despite intragroup variability, no statistically significant difference was observed in the frequency of H-2K^b/VNHRFTLV⁺ CD8⁺ cells when we compared the vaccinated mice of groups 2, 3, 4, and 5. Also, the peptide-specific *in vivo* cytotoxicities of these mice were remarkably high and similar (Fig. 5C).

As shown in Fig. 5D, in the case of the frequency of peptide-specific IFN-γ-secreting cells measured by ELISPOT assay, we also failed to detect statistically significant reductions in the numbers of cells from vaccinated mice subjected to the different treatments. The enumerations of the total frequency of CD8⁺ T cells expressing surface CD107a, IFN-γ, or TNF-α did not differ significantly among groups 2, 3, 4, and 5 (Fig. 5E).

Groups
1 - WT (pcDNA3/Adβ-gal)
2 - WT (pIgSPCl.9/AdASP-2)
3 - IFN-γ KO (pIgSPCl.9/AdASP-2)
4 - IL-12/IL-23 KO (pIgSPCl.9/AdASP-2)

FIG. 4. Importance of IFN-γ or IL-12/IL-23 signaling pathways in the maintenance of transgene-specific CD8⁺ memory T cells elicited by a heterologous plasmid DNA priming-recombinant HuAd5 boosting vaccination regimen. C5BL/6 WT, IFN-γ KO, and IL-12/IL-23 (p40) KO mice were immunized according to the schedule presented in Fig. 1A. Ninety-eight days after the final immunizing dose, splenic cells were analyzed for the frequencies of CD8⁺ H-2K^b/VNHRFTLV⁺ cells (A) or cells expressing surface CD107a, IFN-γ, or TNF-α upon *in vitro* restimulation (B and C). In panels A and B, results are shown as medians (bars), and each dot represents a mouse. Asterisks denote significantly lower frequencies of specific CD8⁺ T cells in the IL-12/IL-23 (p40) KO mice immunized with pIgSPCl.9/AdASP-2 than in the immunized WT mice ($P < 0.05$). The cross in panel C denotes a significantly higher frequency of specific CD8⁺ T cells in the group of IFN-γ KO mice immunized with pIgSPCl.9/AdASP-2 than in the immunized WT mice ($P < 0.05$). The results in panel C are shown as medians for 4 or 5 mice per group and are representative of two independent experiments. Individual mouse results were omitted for clarity but were used for the statistical calculations.

Also, when we estimated the numbers of cells expressing different combinations of these molecules, we found no significant differences among cells collected from vaccinated mice of groups 2, 3, 4, and 5. As shown in Fig. 5F, the predominant CD8⁺ T-cell subpopulations in the spleens of all mice were CD107a singly positive cells and/or CD107a⁺ IFN-γ⁺ TNF-α⁺ triple-positive cells. Other subpopulations also maintained similar frequencies. These results demonstrated that the frequency of each subpopulation of specific T cells was relatively stable despite immunological treatments.
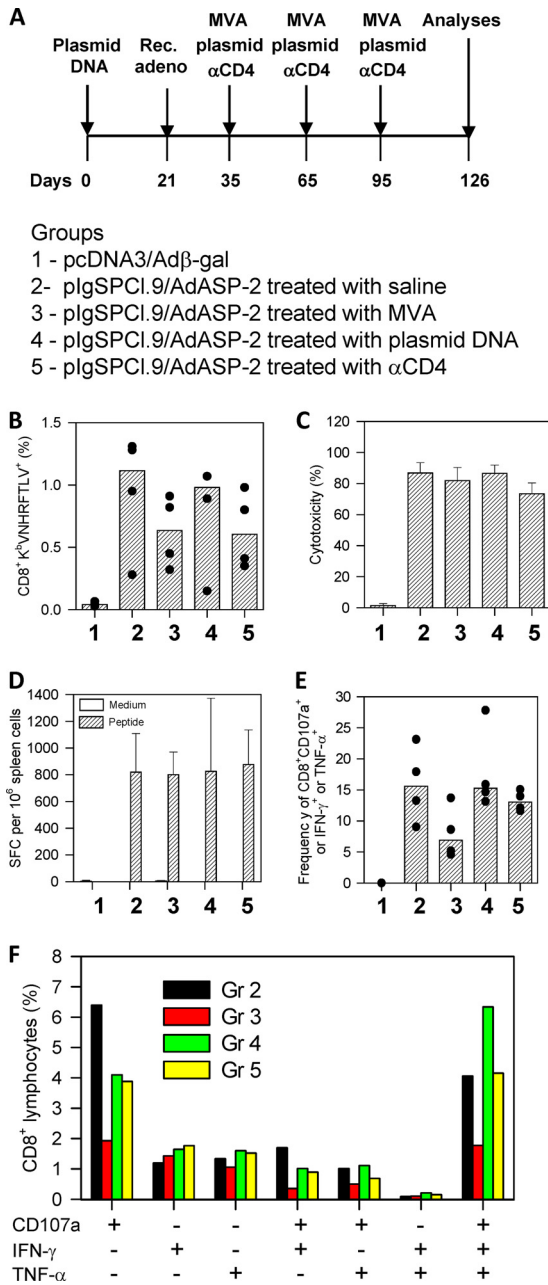
FIG. 5. Immune responses of transgene-specific CD8^{+} T cells elicited by a heterologous plasmid DNA priming-recombinant HuAd5 boosting regimen in mice subjected to immune erosion protocols. (A) Schematic representation of the protocol used for immune erosion in mice immunized with a heterologous prime-boost regimen. Mice were immunized according to the schedule presented in Fig. 1A. On the indicated days, mice were injected with recombinant MVA-GFP (10[7] PFU i.v.) or plasmid DNA (50 μg i.m. per mouse per dose) or treated daily with doses of 0.5 mg of anti-CD4 MAb for three consecutive days. (B) Splenic cells were collected, and the frequencies of CD8^{+} H-2K[b]/VNHRFLTV^{+} cells were determined. Results are expressed as medians (bars), and each dot represents a mouse. (C) Mice were injected with splenic syngeneic cells labeled with CFSE and coated with VNHRFTLV peptide. The specific *in vivo* cytotoxic activity was estimated after 20 h, as described in Materials and Methods. Results are expressed as means plus SD ($n = 4$). (D) Spleen cells were collected, and we estimated the number of splenic IFN-γ spot-forming cells (SFC) by using an *ex vivo* ELISPOT assay. Results are expressed as means plus SD ($n = 4$). (E and F) Splenic cells were collected and
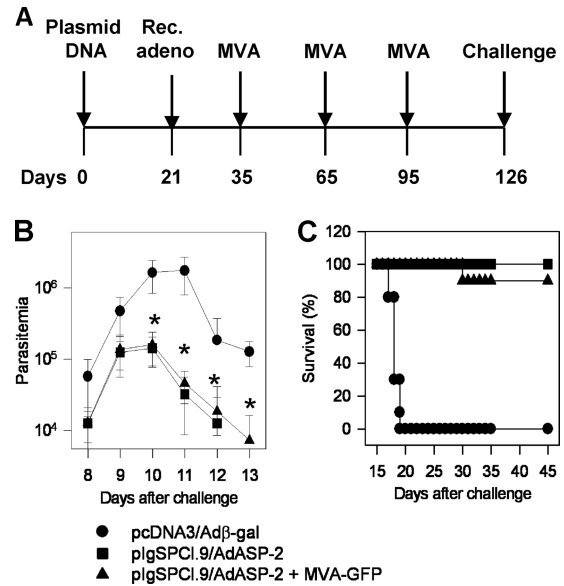

FIG. 6. Protective immune response in mice vaccinated with the heterologous plasmid DNA priming-recombinant adenovirus boosting regimen and subjected to an immune erosion protocol. (A) A/Sn mice were immunized and subsequently treated with recombinant MVA-GFP (10[7] PFU i.v.) on the indicated days. These mice were then challenged s.c. with 150 *T. cruzi* blood trypomastigotes. (B) The number of parasites in the blood was followed from day 8 to day 13. The parasitemia for each mouse group is presented as the mean ± SD ($n = 10$). Asterisks denote groups vaccinated with pIgSPCl.9/AdASP-2 that had significantly lower parasitemia ($P < 0.05$) than control animals injected with pcDNA3/Adβ-gal. No statistically significant difference was observed between each group and mice that had also been treated with recombinant MVA. (C) Kaplan-Meier curves for survival of mouse groups immunized and challenged as described above ($n = 10$). Mice from groups vaccinated with pIgSPCl.9/AdASP-2 survived significantly longer than control animals injected with pcDNA3/Adβ-gal ($P < 0.05$). No statistically significant difference was observed between mice that were treated or not with recombinant MVA. Results were pooled from two experiments.

Analyses of CD44 and CD62L on the surfaces of the H-2K[b]/VNHRFTLV^{+} CD8^{+} cells of each mouse group revealed that their levels were similar, with all cells being CD44^{high} CD62L^{low} (data not shown).

Finally, to test whether these cells were still indeed functional, we evaluated protective immunity against a challenge with a lethal dose of *T. cruzi*. For this purpose, we vaccinated highly susceptible A/Sn mice and subsequently injected these animals with MVA-GFP, as depicted in Fig. 6A. We selected the MVA immunization protocol because it was conceivable that a stronger immune response would be generated by this viral vaccine than by the plasmid. Also, the depletion of CD4

cultured in the presence of anti-CD28 and FITC-labeled anti-CD107a, with or without the VNHRFTLV peptide. After 12 h, cells were stained with PerCP-labeled anti-CD8, fixed, and stained with APC-labeled anti-IFN-γ and PE-labeled anti-TNF-α. Results are expressed as medians (bars), and each dot represents a mouse ($n = 4$). In panel F, individual mouse results were omitted for clarity but were used for the statistical calculations. Results are representative of two independent experiments.

T cells would not provide any information, as we already know that these cells are important for protective immunity after challenge with *T. cruzi* (13).

After an infectious challenge, we followed the parasitemia and survival of the mice that had been vaccinated with pIgSP-Cl.9/AdASP-2 and injected or not with MVA-GFP. In these highly susceptible mice, high parasitemia correlates with intense tissue infection (B. C. de Alencar, J. R. de Vasconcelos, and M. M. Rodrigues, unpublished data). As shown in Fig. 6B, the parasitemia of both groups of mice immunized with ASP-2 was significantly lower than that in control mice injected with pcDNA3 followed by Adβ-Gal ($P < 0.05$). However, no difference was observed between mice vaccinated with pIgSP-Cl.9/AdASP-2 and injected with MVA-GFP and their counterparts that were not injected ($P = $ NS). Similarly, no difference between these two groups of mice was found in terms of survival rates ($P = $ NS). Both groups survived longer than those injected with pcDNA3 followed by Adβ-Gal ($P < 0.05$ in both cases). Histological analyses of the heart tissues of these mice failed to identify an inflammatory reaction or infected cells, as we described previously (13, 49).

DISCUSSION

A key question in the development of new or improved vaccines is how to generate protective long-lived memory T cells by use of nonliving formulations. We previously described that a heterologous prime-boost vaccination regimen using recombinant plasmid DNA followed by a recombinant HuAd5 vector was an effective strategy for the induction of long-lived CD8+ T-cell-dependent protective immunity against systemic infection with a parasitic protozoan (13). In spite of a number of studies characterizing the phenotype and function of immunoprotective memory T cells following infections with viruses and bacteria, the characteristics of memory T cells present in mice subjected to a heterologous prime-boost vaccination regimen using recombinant plasmid DNA followed by recombinant HuAd5 remain to be elucidated.

The lack of information on this subject led us to perform a detailed comparison of the short- and long-term populations of available CD8+ T cells following heterologous prime-boost immunization. We found that in spite of the timing, both populations overlapped greatly with regard to functional and phenotypic characteristics. We observed that the frequency and total number of splenic specific CD8+ T cells were maintained during this time span. Not only was the number of splenic cells preserved, but these cells also exhibited strong cytotoxic activity and secreted IFN-γ when target cells coated with the cognate peptide were encountered *in vivo* and *ex vivo* (ELISPOT assay), respectively. The functional properties retained after extended periods are likely linked to the protective capacity of these cells because, as we observed previously, both perforin and IFN-γ are important for the effector mechanisms mediated by CD8+ T cells against *T. cruzi* infection (13). It is important to mention that we studied only H-2K^b-restricted CD8+ T cells specific for the immunodominant epitope VNHRFTLV. Attempts to find other subdominant epitopes in ASP-2 recognized by H-2^b-restricted CD8+ T cells have failed so far (M. R. Dominguez and M. M. Rodrigues, unpublished data). Nevertheless, in other mouse strains where there might

be dominant and subdominant CD8 epitopes, similar analyses of the phenotype may provide differences between populations of distinct specificities.

Although the number and frequency of specific splenic T cells did not change during the time span studied, the expression of surface CD107a, IFN-γ, or TNF-α upon *in vitro* stimulation with the cognate peptide did. Two weeks after the last immunizing dose, the frequency of triple-positive multifunctional cells was ~50% of specific CD8+ T cells. This frequency was reduced to approximately half that in the spleens of mice immunized 14 weeks earlier. The reason for this change is not clear. Whether these cells failed to survive or differentiated to another phenotype will be interesting to study further. A similar phenomenon was also observed among the population of CD8+ T cells simultaneously expressing surface CD107a and TNF-α. In contrast, the frequency of singly positive cells expressing surface CD107a increased during the same period. Whether this increase was due to accelerated proliferation or due to differentiation to another T-cell subpopulation remains unknown. In spite of these changes, we do not consider that they had a significant biological meaning, as mice vaccinated 14 or 98 days previously were equally well protected against an infectious challenge (13) (Fig. 6).

Not only did specific CD8+ T cells retain their functional characteristics, but the surface phenotypes of the specific CD8+ T cells were also quite similar 14 and 98 days after the last immunizing dose with AdASP-2. The phenotype of the first group was similar to that previously described for short-lived effector cells (CD44^high CD11a^high CD62L^low CD127^− KLRG-1^high) (reviewed in references 4, 25, 36, and 41). The latter subpopulation resembled TEM cells (CD44^high CD11a^high CD62L^low CD127^+ KLRG-1^low) (reviewed in references 4, 25, 36, and 41). However, in our case, after staining for the KLRG-1 marker, we found that cells in both groups expressed various levels of this molecule. A similar population of specific CD8+ T cells has been described following heterologous prime-boost immunizations with vesicular stomatitis virus (or plasmid DNA) followed by recombinant vaccinia virus (35). In contrast, we did not observe the emergence of typical TCM cells (CD44^high CD11a^high CD62L^high CD127^+ KLRG-1^low), even after an extended period of 6 months (data not shown). This observation also corroborates with previously published observations from Masopust et al. (35). Together, these data suggest that the heterologous prime-boost regimen may change the CD8+ T-cell differentiation program, significantly delaying or perhaps even blocking the development of TCM-like cells. We also evaluated the surface phenotype of these specific CD8+ T cells after a challenge. Essentially, we found that these cells maintained the TEM-like phenotype for up to 6 months after the challenge with *T. cruzi* (data not shown).

The idea that fully active TEM-like cells can survive for relatively long periods may have an important impact on the design of vaccines against pathogens that require TEM cells for resistance to reinfection. In fact, our results support the idea that TEM cells may contribute greatly to the protective immune response, as these cells can still provide immunity against *T. cruzi*-mediated systemic infection (13) (Fig. 6B and C). Previous observations that TEM cells can mediate protective immunity were provided in the cases of reinfections with

vaccinia and Sendai viruses (3, 40). However, the TCM cell subset was shown to be the most relevant for protective immunity against certain viruses, protozoan parasites, and tumors (27, 51, 56). This also seems to be the case during infection with *T. cruzi*. Following successful drug therapy of infected mice, there is the emergence of a TCM-like subpopulation (CD62L^{high}) that is highly effective in protecting against reinfection (6). Similar experiments could not be performed with our mouse model because treatment of mice with drugs did not prevent parasite infection (M. M. Rodrigues, unpublished observation). The parasite strain that we used seems to be resistant to conventional drug therapy.

The reason that these cells retained the TEM-like phenotype and the mechanism by which they did so may reflect persistent expression of the transgene. Earlier studies of mice vaccinated with a simian replication-defective adenovirus demonstrated unequivocally that the transgene product was still available for antigen presentation as long as 16 weeks after a single immunizing dose (46). Antigen persistence was confirmed by experiments demonstrating a causal relationship between the duration of transgene expression following immunization with a HuAd5 vaccine and maintenance of the specific CD8$^+$ T cells (16). The same group previously reported that maintenance of the CD8$^+$ T-cell memory population following HuAd5 immunization does not require active thymopoiesis, arguing against continual recruitment of naïve T cells (55). The authors of that study proposed that the early memory population produced by HuAd5 is sustained by continual restimulation; this is consistent with the high levels of KLRG-1 among the members of the memory population, because KLRG-1 is considered to be an indicator of recent and repetitive antigenic stimulation (47). The precise site where antigen is produced continually or whether pulsed APC retains the ability to stimulate T cells over the long term is unknown at present.

One of the limitations of our analyses was the fact that we presented results solely for splenic T cells. Compatible with the absence of CD62L on these cells, we could not find significant amounts of specific CD8$^+$ T cells in the draining lymph nodes 98 days after the last immunizing dose (data not shown). The absence of specific CD8$^+$ T cells in the lymph nodes was also noted by Masopust et al. following heterologous prime-boost immunizations (35). Nevertheless, following heterologous prime-boost immunizations, specific CD8$^+$ T cells were found residing in the gut, lungs, peripheral blood, and liver (35). It is possible that these cells may also reach the skin (54). Also, up to now, we have limited information with regard to where the CD8$^+$ T cells encounter and eliminate parasite-infected cells, a subject that is critical for understanding the immune response.

Because our model provided an interesting system to evaluate the requirements for the maintenance of long-lived protective CD8$^+$ TEM-like cells, in the second part of our study we addressed the question of whether IL-12/IL-23 (p40) or IFN-γ activation pathways are important, using genetically deficient mice. We found that expression of IL-12/IL-23 (p40), but not IFN-γ, was critical for the long-term survival of these mice. It is important that the absence of IL-12/IL-23 (p40) resulted in smaller amounts of specific CD8$^+$ T cells, as measured by staining with the H-2k^b/VNHRFTLV pentamer 14 days after the booster vaccination. Nevertheless, at that time, the frequencies of IFN-γ-producing cells were not statistically different between WT and IL-12/IL-23 (p40) KO mice (data not shown). Therefore, it seems that IL-12/IL-23 (p40) reduces the total number of specific cells at early time points, but it also diminishes the number of transgene-specific cytokine-producing cells after extended periods (Fig. 4). Consistent with the small number of specific CD8$^+$ T cells, immunized IL-12/IL-23 (p40) KO mice succumbed to infection after challenge (data not shown). This result should be interpreted with caution, however, because it would be impossible to attribute the high susceptibility of the IL-12/IL-23 (p40) KO mice only to a defect of specific CD8$^+$ T cells. The absence of this cytokine impairs a number of other immunological functions, including the expansion of Th1 CD4$^+$ T cells and NKT and NK cells, all of which actively participate during the immune response to *T. cruzi* infection (7, 15, 23, 30, 42).

A critical role of the IL-12 signaling pathway in the induction and maintenance of memory CD8$^+$ T cells was recently studied in several experimental models, and differences were noticed (2, 37, 52). These differences probably reflect a distinct interface of host-pathogen interactions, such as the tissue distribution, multiplication rate, etc.

The idea that memory CD8$^+$ T cells are stable was challenged recently by a number of publications showing that subsequent infections or vaccinations may cause attrition and erosion of the memory T-cell pool (14, 24, 44, 45). The fact that we had a homogeneous population of specific CD8$^+$ TEM-like cells allowed us to test their stability. To this end, we immunized the mice with unrelated recombinant MVA or plasmid DNA. We observed that both treatments had a limited impact on the number or quality of these memory TEM-like cells, as measured by different functional immunological assays. Most importantly, the presence of protective CD8$^+$ cells was not altered in mice vaccinated with recombinant MVA. Our results are in agreement with more recent data showing that T-cell receptor (TCR)-transgenic activated CD8$^+$ T cells are not eliminated by subsequent infections with recombinant viruses or bacteria (50). Therefore, we concluded that the intense immune response obtained by subsequent immunization with viruses or plasmid DNA will probably not compromise the efficiency of the heterologous prime-boost immunization regimen. Whether immunizations with other strong vaccines will interfere with TEM-like cell stability remains to be determined. This question is also valid for other viral, bacterial, protozoan, and helminthic infections.

In previous studies, it was reported that the main subset of CD8$^+$ T cells targeted for erosion are TEM cells (14, 24). Our results argue against this finding, as we observed that not all CD8$^+$ TEM cells are targeted for MVA-induced erosion as described previously (24). It is plausible to speculate that the "quality" of the TEM cells elicited by the heterologous prime-boost regimen involving plasmid DNA and recombinant HuAd5 may differ from that, for example, of TEM cells generated by *Listeria monocytogenes* infection (24). Notably, a certain degree of heterogeneity has already been noted in the TEM CD8$^+$ cell subpopulation: these cells can be subdivided further into CD43$^-$ CXCR3$^-$, CD43$^-$ CXCR3$^+$, and CD43$^+$ CXCR3$^+$ groups (21).

We also evaluated the requirement of CD4$^+$ T cells for the maintenance of TEM-like CD8$^+$ T cells. We previously ob-

served that the expansion of these cells was dependent on the presence of CD4$^+$ T cells during priming. In CD4-deficient mice, we observed a reduction of >10-fold in the number of IFN-γ-secreting CD8$^+$ T cells measured by ELISPOT assay (13). In spite of the dependence of CD4$^+$ T cells on priming, their depletion following vaccination did not significantly modify their number or function (Fig. 5). On one side, we observed that specific CD8$^+$ T-cell expansion in our model system was dependent on CD4$^+$ T cells. On the other hand, the depletion of CD4$^+$ T cells after boosting did not significantly reduce the number of CD8$^+$ T cells. Together, these observations suggest that the specific CD8$^+$ T cells we observed after 98 days were not primed recently. Most likely, these cells were maintained by either homeostatic proliferation, antigenic restimulation, or both.

In summary, our study describes some important characteristics of the memory CD8$^+$ T-cell population elicited by heterologous prime-boost vaccination that might be key for the generation of long-lived protective immunity against intracellular parasitic protozoans. This model also establishes a population of protective TEM-like cells that may be interesting for studies of T-cell transcriptional adaptations for long-term survival and function, which may be distinct from those of TCM CD8$^+$ cells.

REFERENCES

1. **Acierno, P. M., et al.** 2006. Preservation of functional virus-specific memory CD8$^+$ T lymphocytes in vaccinated, simian human immunodeficiency virus-infected rhesus monkeys. J. Immunol. **176:**5338–5345.
2. **Agarwal, P., et al.** 2009. Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory. J. Immunol. **183:**1695–1704.
3. **Bachmann, M. F., P. Wolint, K. Schwarz, and A. Oxenius.** 2005. Recall proliferation potential of memory CD8$^+$ T cells and antiviral protection. J. Immunol. **175:**4677–4685.
4. **Belz, G. T., and A. Kallies.** 2010. Effector and memory CD8+ T cell differentiation: toward a molecular understanding of fate determination. Curr. Opin. Immunol. **22:**279–285.
5. **Boscardin, S. B., S. S. Kinoshita, A. E. Fujimura, and M. M. Rodrigues.** 2003. Immunization with cDNA expressed by amastigotes of *Trypanosoma cruzi* elicits protective immune response against experimental infection. Infect. Immun. **71:**2744–2757.
6. **Bustamante, J. M., L. M. Bixby, and R. L. Tarleton.** 2008. Drug-induced cure drives conversion to a stable and protective CD8$^+$ T central memory response in chronic Chagas disease. Nat. Med. **14:**542–550.
7. **Cardillo, F., J. C. Voltarelli, S. G. Reed, and J. S. Silva.** 1996. Regulation of *Trypanosoma cruzi* infection in mice by gamma interferon and interleukin 10: role of NK cells. Infect. Immun. **64:**128–134.
8. **Casimiro, D. R., et al.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J. Virol. **77:**6305–6313.
9. **Casimiro, D. R., et al.** 2005. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. J. Virol. **79:**15547–15555.
10. **Curtsinger, J. M., and M. F. Mescher.** 2010. Inflammatory cytokines as a third signal for T cell activation. Curr. Opin. Immunol. **22:**333–340.
11. **Da Fonseca, F., and B. Moss.** 2003. Poxvirus DNA topoisomerase knockout mutant exhibits decreased infectivity associated with reduced early transcription. Proc. Natl. Acad. Sci. U. S. A. **100:**11291–11296.
12. **Dalton, D. K., et al.** 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science **259:**1739–1742.
13. **De Alencar, B. C., et al.** 2009. Perforin and gamma interferon expression are required for CD4$^+$ and CD8$^+$ T-cell-dependent protective immunity against a human parasite, Trypanosoma cruzi, elicited by heterologous plasmid DNA prime-recombinant adenovirus 5 boost vaccination. Infect. Immun. **77:**4383–4395.
14. **Dudani, R., K. Murali-Krishna, L. Krishnan, and S. Sad.** 2008. IFN-gamma induces the erosion of preexisting CD8 T cell memory during infection with a heterologous intracellular bacterium. J. Immunol. **181:**1700–1709.
15. **Duthie, M. S., et al.** 2002. During *Trypanosoma cruzi* infection CD1d-restricted NK T cells limit parasitemia and augment the antibody response to a glycophosphoinositol-modified surface protein. Infect. Immun. **70:**36–48.
16. **Finn, J. D., et al.** 2009. Persistence of transgene expression influences CD8$^+$ T-cell expansion and maintenance following immunization with recombinant adenovirus. J. Virol. **83:**12027–12036.
17. **Freel, S. A., et al.** 2010. Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination. J. Virol. **84:**4998–5006.
18. **Geisbert, T. W., et al.** 2010. Vector choice determines immunogenicity and potency of genetic vaccines against Angola Marburg virus in nonhuman primates. J. Virol. **84:**10386–10394.
19. **Gilbert, S. C., et al.** 2002. Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunization regimes. Vaccine **20:**1039–1045.
20. **Hensley, L. E., et al.** 2010. Demonstration of cross-protective vaccine immunity against an emerging pathogenic ebolavirus species. PLoS Pathog. **6:**e1000904.
21. **Hikono, H., et al.** 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. J. Exp. Med. **204:**1625–1636.
22. **Hill, A. V., et al.** 2010. Prime-boost vectored malaria vaccines: progress and prospects. Hum. Vaccin. **6:**78–83.
23. **Hoft, D. F., and C. S. Eickhoff.** 2005. Type 1 immunity provides both optimal mucosal and systemic protection against a mucosally invasive, intracellular pathogen. Infect. Immun. **73:**4934–4940.
24. **Huster, K. M., et al.** 2009. Cutting edge: memory CD8 T cell compartment grows in size with immunological experience but nevertheless can lose function. J. Immunol. **183:**6898–6902.
25. **Jameson, S. C., and D. Masopust.** 2009. Diversity in T cell memory: an embarrassment of riches. Immunity **31:**859–871.
26. **Jaoko, W., et al.** 2010. Safety and immunogenicity study of multiclade HIV-1 adenoviral vector vaccine alone or as boost following a multiclade HIV-1 DNA vaccine in Africa. PLoS One **5:**e12873.
27. **Klebanoff, C. A., et al.** 2005. Central memory self/tumor-reactive CD8$^+$ T cells confer superior antitumor immunity compared with effector memory T cells. Proc. Natl. Acad. Sci. U. S. A. **102:**9571–9576.
28. **Koup, R. A., et al.** 2010. Priming immunization with DNA augments immunogenicity of recombinant adenoviral vectors for both HIV-1 specific antibody and T-cell responses. PLoS One **5:**e9015.
29. **Letvin, N. L., et al.** 2006. Preserved CD4$^+$ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science **312:**1530–1533.
30. **Lieke, T., S. E. Graefe, U. Klauenberg, B. Fleischer, and T. Jacobs.** 2004. NK cells contribute to the control of *Trypanosoma cruzi* infection by killing free parasites by perforin-independent mechanisms. Infect. Immun. **72:**6817–6825.
31. **Lu, S.** 2009. Heterologous prime-boost vaccination. Curr. Opin. Immunol. **21:**346–351.
32. **Machado, A. V., et al.** 2006. Long-term protective immunity induced against *Trypanosoma cruzi* infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. Hum. Gene Ther. **17:**898–908.
33. **Magram, J., et al.** 1996. IL-12-deficient mice are defective in IFN γ production and type 1 cytokine responses. Immunity **4:**471–481.
34. **Marques, E. T., Jr., et al.** 2003. HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. J. Biol. Chem. **278:**37926–37936.
35. **Masopust, D., S. J. Ha, V. Vezys, and R. Ahmed.** 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J. Immunol. **177:**831–839.
36. **Obar, J. J., and L. Lefrançois.** 2010. Early events governing memory CD8$^+$ T-cell differentiation. Int. Immunol. **22:**619–625.
37. **Pearce, E. L., and H. Shen.** 2007. Generation of CD8 T cell memory is regulated by IL-12. J. Immunol. **179:**2074–2081.
38. **Radosević, K., A. Rodriguez, A. Lemckert, and J. Goudsmit.** 2009. Heterologous prime-boost vaccinations for poverty-related diseases: advantages and future prospects. Expert Rev. Vaccines **8:**577–592.
39. **Ranasinghe, C., and I. A. Ramshaw.** 2009. Genetic heterologous prime-boost vaccination strategies for improved systemic and mucosal immunity. Expert Rev. Vaccines **8:**1171–1181.

40. **Roberts, A. D., and D. L. Woodland.** 2004. Cutting edge: effector memory CD8^{+} T cells play a prominent role in recall responses to secondary viral infection in the lung. J. Immunol. **172:**6533–6537.

41. **Sallusto, F., J. Geginat, and A. Lanzavecchia.** 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. **22:**745–763.

42. **Sardinha, L. R., et al.** 2006. Contribution of NK, NK T, gamma delta T, and alpha beta T cells to the gamma interferon response required for liver protection against *Trypanosoma cruzi*. Infect. Immun. **74:**2031–2042.

43. **Schooley, R. T., et al.** 2010. AIDS Clinical Trials group 5197: a placebo-controlled trial of immunization of HIV-1-infected persons with a replication-deficient adenovirus type 5 vaccine expressing the HIV-1 core protein. J. Infect. Dis. **202:**705–716.

44. **Selin, L. K., K. Vergilis, R. M. Welsh, and S. R. Nahill.** 1996. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. J. Exp. Med. **183:**2489–2499.

45. **Selin, L. K., et al.** 1999. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. Immunity **11:**733–742.

46. **Tatsis, N., et al.** 2007. Adenoviral vectors persist in vivo and maintain activated CD8^{+} T cells: implications for their use as vaccines. Blood **110:**1916–1923.

47. **Thimme, R., et al.** 2005. Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation. J. Virol. **79:**12112–12116.

48. **Tzelepis, F., et al.** 2008. Infection with *Trypanosoma cruzi* restricts the repertoire of parasite-specific CD8^{+} T cells leading to immunodominance. J. Immunol. **180:**1737–1748.

49. **Vasconcelos, J. R., et al.** 2004. Protective immunity against *Trypanosoma cruzi* infection in a highly susceptible mouse strain following vaccination with genes encoding the amastigote surface protein-2 and *trans*-sialidase. Hum. Gene Ther. **15:**878–886.

50. **Vezys, V., et al.** 2009. Memory CD8 T-cell compartment grows in size with immunological experience. Nature **457:**196–199.

51. **Wherry, E. J., et al.** 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. **4:**225–234.

52. **Wilson, D. C., et al.** 2010. Differential regulation of effector- and central-memory responses to Toxoplasma gondii infection by IL-12 revealed by tracking of Tgd057-specific CD8+ T cells. PLoS Pathog. **6:**e1000815.

53. **Wilson, N. A., et al.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. **80:**5875–5885.

54. **Woodland, D. L., and J. E. Kohlmeier.** 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. Nat. Rev. Immunol. **9:**153–161.

55. **Yang, T. C., et al.** 2006. The CD8^{+} T cell population elicited by recombinant adenovirus displays a novel partially exhausted phenotype associated with prolonged antigen presentation that nonetheless provides longterm immunity. J. Immunol. **176:**200–210.

56. **Zaph, C., J. Uzonna, S. M. Beverley, and P. Scott.** 2004. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. Nat. Med. **10:**1104–1110.

57. **Zavala, F., et al.** 2001. A striking property of recombinant poxviruses: efficient inducers of *in vivo* expansion of primed CD8^{+} T cells. Virology **280:**155–159.

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