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Immune Responses against a Single CD8$^+$-T-Cell Epitope Induced by Virus Vector Vaccination Can Successfully Control *Trypanosoma cruzi* Infection

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In order to develop CD8$^+$-T-cell-mediated immunotherapy against intracellular infectious agents, vaccination using recombinant virus vectors has become a promising strategy. In this study, we generated recombinant adenoviral and vaccinia virus vectors expressing a single CD8$^+$-T-cell epitope, ANYNFTLV, which is derived from a *Trypanosoma cruzi* antigen. Immunogenicity of these two recombinant virus vectors was confirmed by the detection of ANYNFTLV-specific CD8$^+$ T cells in the spleens of immunized mice. Priming/boosting immunization using combinations of these two recombinant virus vectors revealed that the adenovirus vector was efficient for priming and the vaccinia virus vector was effective for boosting the CD8$^+$-T-cell responses. Moreover, we also demonstrated that the ANYNFTLV-specific CD8$^+$-T-cell responses were further augmented by coadministration of recombinant vaccinia virus vector expressing the receptor activator of NFκB (RANK) ligand as an adjuvant. By priming with the adenovirus vector expressing ANYNFTLV and boosting with the vaccinia virus vectors expressing ANYNFTLV and RANK ligand, the immunized mice were efficiently protected from subsequent challenge with lethal doses of *T. cruzi*. These results indicated, for the first time, that the induction of immune responses against a single CD8$^+$-T-cell epitope derived from an intrinsic *T. cruzi* antigen was sufficient to control lethal *T. cruzi* infection.

Trypanosoma cruzi is the etiological agent of Chagas' disease in Central and South America (8, 20, 21, 35). As it invades and replicates in essentially all types of cells of mammalian hosts, T-cell-mediated immunity is critical for resolving the infection (3, 9). In accordance with this notion, the depletion of CD8$^+$ or CD4$^+$ T cells results in unrelenting parasitemia and a fatal outcome in mice (38, 42, 43, 44). Considering the paucity of therapeutic drugs against *T. cruzi* (46), the development of a vaccine to induce effective T-cell-mediated immunity has been eagerly expected (50, 51). We previously identified a major epitope of trans-sialidase surface antigen (TSSA) recognized by CD8$^+$ T cells in *T. cruzi*-infected C57BL/6 mice and have demonstrated that vaccination with plasmid DNA encoding TSSA can induce CD8$^+$-T-cell-mediated protective immunity against lethal *T. cruzi* infection (19, 27, 28, 29).

Vaccination using recombinant virus vectors has become a promising strategy to induce T-cell immunity against intracellular infectious agents (37, 40). Adenovirus and vaccinia virus have been shown to be the most efficient vectors for inducing protective immune responses against human immunodeficiency virus (12, 15, 16, 39) and malaria (22, 30, 31, 36, 54). The generation of replication-deficient virus mutants makes this strategy safer and more effective for containing the threat and spread of infections. The recombinant virus vector vaccination was also demonstrated effective for conferring protective immunity against *T. cruzi*, which was artificially engineered to express a well-characterized, immunogenic foreign antigen (26). However, it remains to be determined if the recombinant virus vectors expressing an intrinsic *T. cruzi* antigen are really effective for conferring immunological protection. In addition, it also remains to be determined whether the vaccine-induced CD8$^+$-T-cell responses are sufficient for controlling the *T. cruzi* infection.

In the present study, we demonstrated that vaccination with recombinant adenoviral and vaccinia virus vectors expressing a single CD8$^+$-T-cell epitope, ANYNFTLV, which is derived from a *T. cruzi* TSSA antigen, was effective for protecting mice from lethal *T. cruzi* infection. We also found that recombinant vaccinia virus expressing RANK ligand exhibited an adjuvant effect for enhancing the induction of ANYNFTLV-specific CD8$^+$ T cells. These findings demonstrate that the immune response directed against a single CD8$^+$-T-cell epitope is sufficient for controlling the lethal *T. cruzi* infection, providing a new basis for improving vaccine strategies against Chagas' disease.

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MATERIALS AND METHODS

Animals and parasite. Female C57BL/6 (*H-2^b*) mice, 5 to 8 weeks of age, were purchased from SEAC Yoshitomi (Yoshitomi, Fukuoka, Japan). Blood-form

trypomastigotes of *T. cruzi* Tulahuen strain (24) were maintained in outbred CD1 or inbred BALB/c mice by intramuscular or intraperitoneal inoculation of 5,000 trypomastigotes into naïve mice every 2 week. An institutional review committee at Juntendo University has approved the animal studies described here.

Cells and culture. The C57BL/6-derived thymoma cell line EL-4 was used as antigen-presenting cells for $CD8^+$-T-cell cultures and assays. The cell line has been widely used for $CD8^+$-T-cell assays, since it was well reported that it expresses only class I antigens but not class II (23, 49), which was confirmed by our own hands (data not shown). In that respect, the EL4 cells are suitable antigen-presenting cells for the detection of antigen-specific $CD8^+$ T cells during the gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. BHK-21 cell (American Type Culture Collection, Manassas, VA) was used for growing highly attenuated vaccinia virus strain called modified vaccinia virus Ankara (MVA) (American Type Culture Collection) (41) or MVA-derived recombinant viruses. The transformed human embryonic kidney cell line 293 (American Type Culture Collection) was used for growing replication-deficient adenovirus or recombinant adenoviruses. These cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Life Technologies/BRL, Rockville, MD) supplemented with 10% fetal calf serum, 2 g/liter sodium bicarbonate (Sigma-Aldrich Co., St. Louis, MO), 200 mg/liter L-arginine hydrochloride (Life Technologies/BRL), 36 mg/liter L-asparagine (Life Technologies/BRL), 2.6 g/liter HEPES (Sigma-Aldrich), 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), and antibiotics (complete Dulbecco's modified Eagle's medium). The medium used for ELISPOT assays and the culture of lymphocytes was supplemented with phorbol myristate acetate-stimulated EL-4 cell culture supernatant as a source of 30 U/ml interleukin (IL)-2 (complete Dulbecco's modified Eagle's medium-IL-2) (25).

Peptide. An H-$2K^b$-restricted $CD8^+$-T-cell epitope peptide, ANYNFTLV, derived from TSSA (19, 27, 28, 29) (Fig. 1A) was synthesized and used for immunological assays. The gene encoding for TSSA was first identified to be present among the clusters of genes encoding for enzymes involved in de novo pyrimidine biosynthesis in *T. cruzi* (11). One of the figures in the report (11) showed a schematic representation of the 25-kb segment containing five genes that encode all six enzymes of de novo pyrimidine biosynthesis. In that scheme, however, there was an additional gene, *orf*, which was described only as a surface protein of *T. cruzi* (DNA accession number AB010287). We designated the "surface protein" TSSA, since its amino acid sequence was highly homologous to the *T. cruzi* trans-sialidase superfamily protein. Although we have not determined yet whether the TSSA actually has neuraminidase and trans-sialidase activities, we demonstrated that the DNA encoding TSSA was highly immunogenic, conferring protective immunity in C57BL/6 mice against *T. cruzi* infection (19, 27, 28, 29).

Generation of recombinant adenoviruses. To construct a minigene encoding the peptide MANYNFTLV, synthesized oligonucleotides 5′-ATGGCCAACTA CAACTTCACCCTGGTGTAA-3′ and 5′-GATCTTACACCAGGGTGAAGT TGTAGTTGGCCATTGCA-3′ were annealed and inserted into pIRES2-EGFP (Becton Dickinson and Company, Franklin Lakes, NJ), which was predigested with PstI and BamHI (Fig. 1B). The sequences of oligonucleotides were modified to optimize the expression of peptides in mammalian cells. The resulting plasmid DNA was designated as pIRES-MANY.

To generate recombinant adenovirus vectors, the NotI-XhoI fragments of pIRES-MANY or pIRES2-EGFP were treated with Klenow fragment (Takara Bio Inc., Shiga, Japan) and inserted into SwaI site of the cosmid vector pAxCAwt (Takara). The resulting cosmid vectors were designated pAdex/MANY and pAdex/GFP, respectively. Using the adenovirus expression vector kit (Takara), recombinant adenoviruses, designated Ad-MANY and Ad-GFP, were generated by homologous recombination between the cosmid vector pAdex/MANY or pAdex/GFP and adenovirus genomic DNA, respectively. After picking up green fluorescence-emitting, virus-infected cells and six rounds of purification, recombinant viruses were amplified in 293 cells, and then purified by centrifugation through a cushion of cesium chloride (18). The titer of the virus stocks was determined by detecting green fluorescence-emitting cells in 293 cell cultures.

Generation of recombinant vaccinia viruses. For the generation of recombinant MVAs, the HindIII-Psp1406I fragment of the pIRES-MANY was treated with KOD DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) and inserted into the PmeI site of the vaccinia virus insertion vector, pMCO3 (6), downstream of a strong synthetic early/late virus promoter (Fig. 1B). The resulting plasmid or the unmodified pMCO3 was used to transfect BHK-21 cells, which were coinfected with the MVA. Cells that stained blue upon addition of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (Clontech, Palo Alto, CA) were selected. After six rounds of plaque purification, recombinant viruses were amplified in BHK-21 cells and purified by centrifugation through a cushion of 36% sucrose (10). They were designated MVA-MANY and MVA-p3, respectively. The titer of purified viruses was determined by staining virus-infected BHK-21 cells with X-Gluc.

In order to generate the recombinant MVA expressing RANK ligand (RANKL), murine RANKL cDNA (29) was inserted into the PmeI site of the pMCO3. The isolation and purification of the recombinant MVA expressing RANKL, designated as MVA-RANKL, was performed as described above. The expression of RANKL by the MVA-RANKL was confirmed by staining the virus-infected BHK-21 cells with biotin-conjugated anti-RANKL monoclonal antibody (IK22-5) (29), or rat IgG isotype control (BD PharMingen, San Diego, CA). After washing with PBS twice, the cells were incubated with PE-labeled streptavidin (BD PharMingen), washed with PBS twice, and then analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). The data were processed using the CellQuest program (BD Biosciences).

Quantification of antigen-specific T cells by ELISPOT assay. The frequency of antigen (ANYNFTLV)-specific T cells was determined by ELISPOT assay for IFN-γ-secreting cells essentially as described previously (25). Briefly, serial dilutions of freshly isolated splenocytes or cultured T cells (1×10^4 to 100×10^4) were cocultured with irradiated EL-4 cells that had been pulsed with 1 μM ANYNFTLV peptide in anti-IFN-γ monoclonal antibody-coated plates for 24 to 28 h. The spots formed by IFN-γ-secreting cells were detected with biotinylated anti-IFN-γ monoclonal antibody followed by peroxidase-labeled streptavidin and diaminobenzidine. The developed spots were counted under a microscope and expressed as the number of spots per 10^6 cells.

Vaccination schedule, dosages, and challenge infection. All the vaccination schedules and dosages are described in detail in each figure legend. The number of ANYNFTLV-specific $CD8^+$ T cells was quantified by ELISPOT assay. The immunized mice were challenged with lethal or sublethal dose of Tulahuen strain of *T. cruzi* blood-form trypomastigotes 10 to 14 days after the last immunization. Blood from all infected mice was collected periodically from the tail vein, and the number of parasites in 5 μl blood (parasitemia) was counted microscopically. Survival of host mice was monitored daily.

Statistical analyses. Statistical analyses were performed by the unpaired Student's *t* test or Dunnett's two-tailed *t* test for the ELISPOT assays and for the parasitemia. The unpaired Mann-Whitney *U* test was used to determine significant differences in survival data. *P* values less than 0.05 were considered significant.

RESULTS

Immunogenicity of recombinant adenovirus expressing ANYNFTLV. In order to test the immunogenicity of recombinant adenovirus expressing the H-$2K^b$-restricted $CD8^+$-T-cell epitope of TSSA (ANYNFTLV), we immunized C57BL/6 mice intramuscularly or intraperitoneally with different doses of Ad-MANY or Ad-GFP. The induction of ANYNFTLV-specific $CD8^+$ T cells in the spleen of Ad-MANY-immunized mice was demonstrated by ELISPOT assay using freshly isolated or in vitro expanded splenocytes as effector cells in a dose-dependent manner (Fig. 2A to D). In contrast, Ad-GFP did not induce the ANYNFTLV-specific $CD8^+$ T cells at any doses. When the immunization was done via intraperitoneal route, the numbers of induced antigen-specific $CD8^+$ T cells were consistently lower than those induced by intramuscular immunization (Fig. 2A and C) (354 ± 34 versus 712 ± 224 after inoculating 5×10^8 PFU viral load and 64 ± 15 versus 284 ± 30 after inoculating 5×10^7 PFU viral load, determined by ELISPOT assay using freshly isolated splenocytes as effector cells). It has been demonstrated that the induction of antigen-specific $CD8^+$ T cells was significantly affected by the route of immunization (14, 34). We therefore decided that the immunization with Ad-MANY should be done via the intramuscular route in the following experiments.

Immunogenicity of recombinant MVA expressing ANYNFTLV. We next tested the immunogenicity of recombinant MVA expressing the ANYNFTLV epitope. When C57BL/6 mice were immunized either intramuscularly or intraperitoneally with two

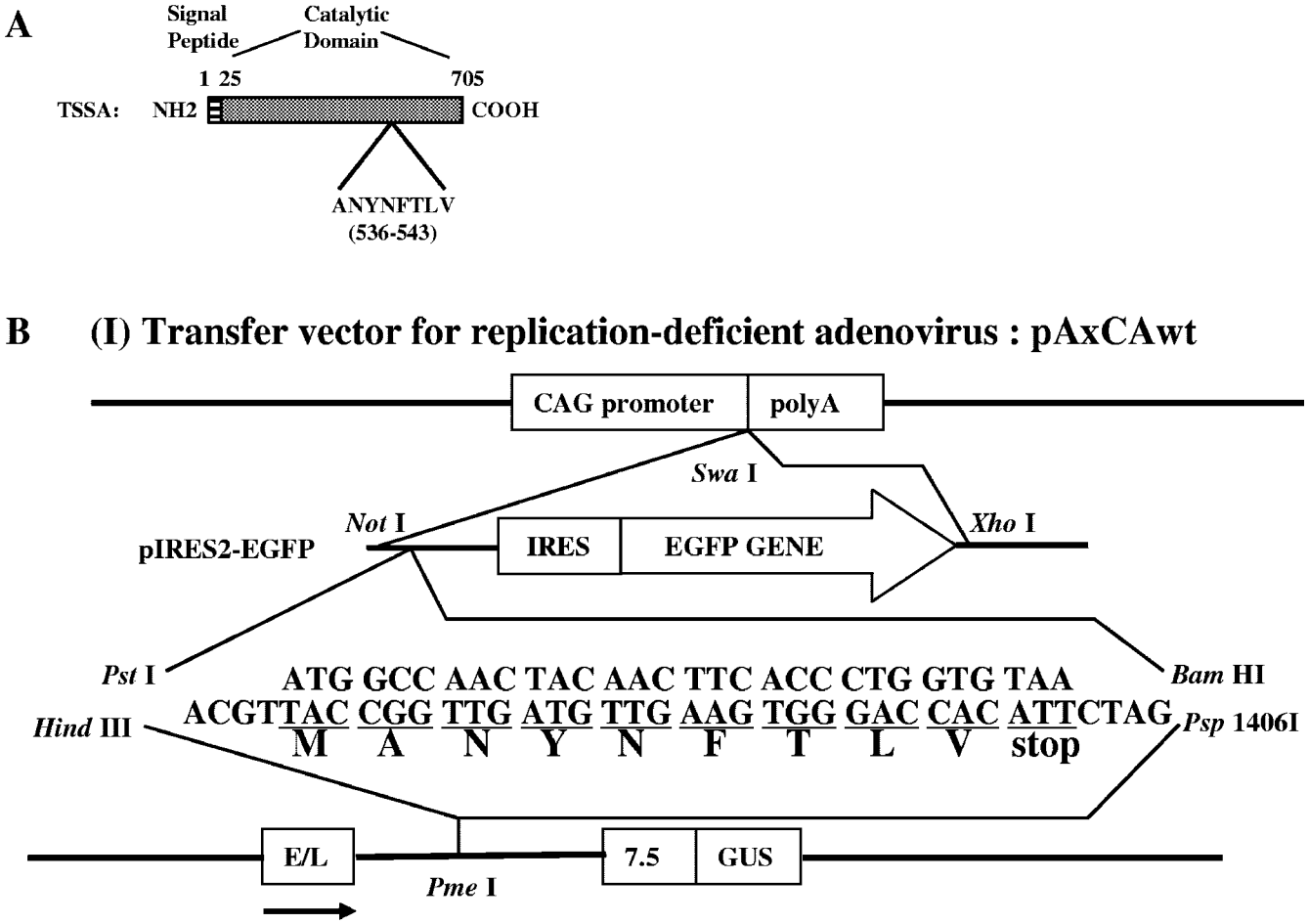

FIG. 1. Generation of recombinant virus vectors. A. Primary structure of *T. cruzi* trans-sialidase surface antigen (TSSA) and an H-2K^b-restricted CD8$^+$-T-cell epitope, ANYNFTLV. The gene encoding for TSSA was first identified to be present among the clusters of genes encoding for enzymes involved in de novo pyrimidine biosynthesis in the genome of *T. cruzi* Tulahuen strain (11). The report (11) showed a schematic representation of the 25 kb segment containing not only five genes that encode all six enzymes of de novo pyrimidine biosynthesis but also an additional gene, *orf*, which was described only as surface protein of *T. cruzi* (DNA accession number: AB010287). We designated the surface protein as TSSA, since its amino acid sequence was highly homologous to the *T. cruzi* trans-sialidase superfamily protein. The *T. cruzi* trans-sialidase usually consists of four parts; i.e., signal peptide, catalytic domain, C-terminal repeats, and hydrophobic region for GPI anchor. TSSA, however, consists of only two parts, signal peptide and catalytic domain. An H-2K^b-restricted CD8$^+$-T-cell epitope, ANYNFTLV (536–543), was identified on TSSA (19). B. A minigene encoding the MANYNFTLV peptide was inserted either into pAxCAwt, a transfer vector for replication-deficient adenovirus, or into pMCO3, a transfer vector for highly attenuated vaccinia virus (MVA). Detailed procedures for generating recombinant viruses are described in the Materials and Methods. CAG, modified chicken β-actin promoter with the cytomegalovirus immediate-early enhancer (32); poly A, poly(A) addition signal; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; E/L, synthetic early/late MVA promoter; 7.5, MVA P7.5 promoter; GUS, gene encoding *Escherichia coli* β-glucuronidase.

different doses of MVA-MANY, few ANYNFTLV-specific CD8$^+$ T cells were detected in freshly isolated splenocytes by ELISPOT assay (Fig. 3A and C). However, a significant expansion of the specific CD8$^+$ T cells was observed when the immune splenocytes were stimulated with the ANYNFTLV peptide in vitro for one week (Fig. 3B and D). In contrast, MVA-p3 did not significantly induce the specific CD8$^+$ T cells. Since a larger number of antigen-specific CD8$^+$ T cells were induced by intraperitoneal immunization compared to intramuscular immunization, the intraperitoneal route was used for MVA-MANY in the following experiments.

Ad-MANY is efficient for priming and MVA-MANY is efficient for boosting the ANYNFTLV-specific CD8$^+$-T-cell responses. The above results indicated that Ad-MANY was more efficient than MVA-MANY for priming the ANYNFTLV-specific CD8$^+$-T-cell response (Fig. 2 and Fig. 3). To further expand the ANYNFTLV-specific CD8$^+$ T cells, we next examined the effect of a boost immunization with Ad-MANY or MVA-MANY after the Ad-MANY priming. As shown in Fig. 4A, the boost immunization with Ad-MANY augmented the expansion of ANYNFTLV-specific CD8$^+$ T cells only modestly compared to that with Ad-GFP. In con-

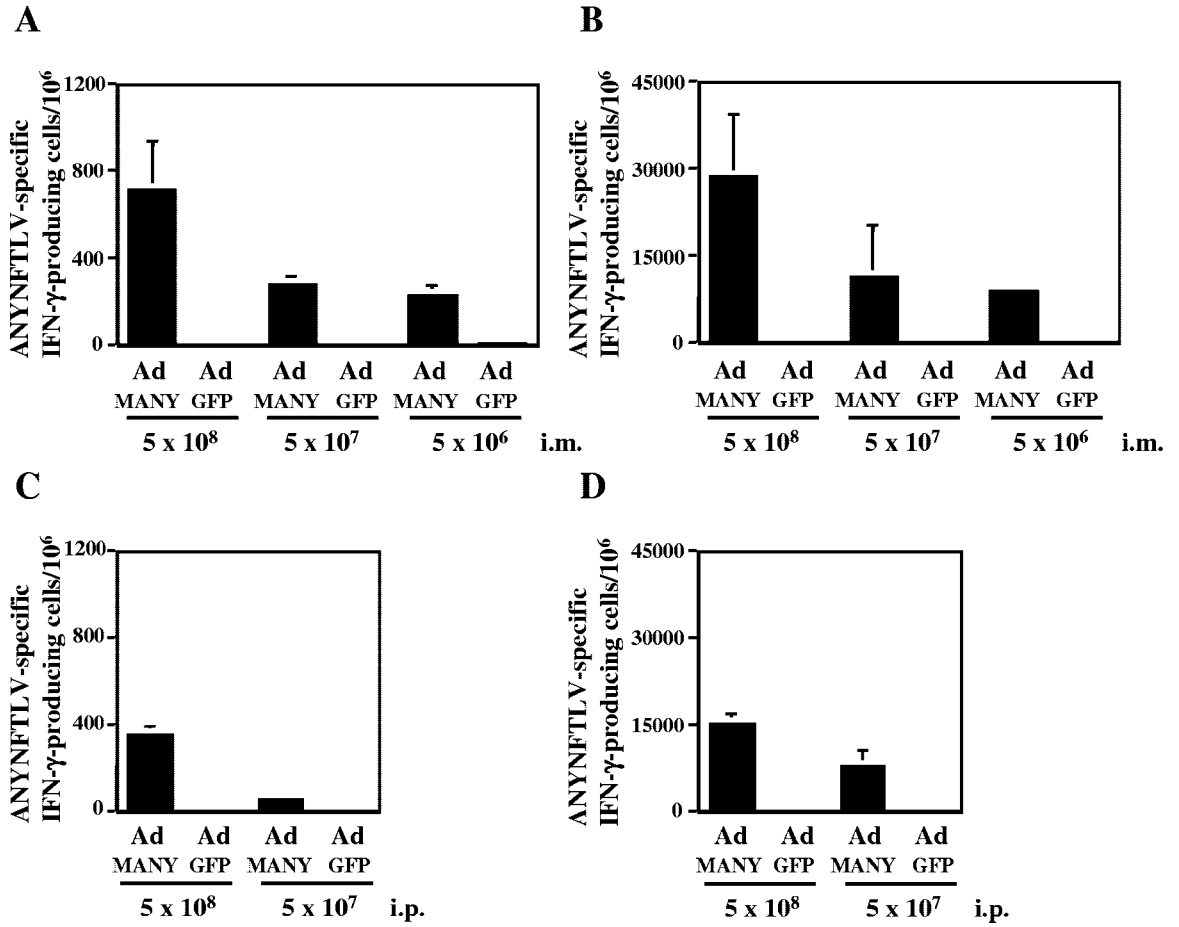

FIG. 2. Immunogenicity of recombinant adenovirus expressing ANYNFTLV. C57BL/6 mice were administered with either Ad-MANY or Ad-GFP intramuscularly at three different doses (A, B) or intraperitoneally at two different doses (C, D). The mice were sacrificed 14 days after the immunization, and their spleens were removed. A half of splenocytes from individual mice were cultured with irradiated EL-4 cells pulsed with ANYNFTLV peptide for one week. The freshly isolated splenocytes (A, C) or the 1-week cultured splenocytes (B, D) were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells/10^6 cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean ± standard deviation of three mice in each group. The data are representative one of three independent experiments.

trast, the boost immunization with MVA-MANY markedly increased the frequency of ANYNFTLV-specific $CD8^+$ T cells, while that with MVA-p3 did not (Fig. 4A). These results indicated that MVA-MANY was much more efficient than Ad-MANY for boosting the ANYNFTLV-specific $CD8^+$-T-cell response after Ad-MANY priming. In addition, the induction of ANYNFTLV-specific $CD8^+$ T cells by the Ad-MANY priming/MVA-MANY boost was superior to the one induced either by the MVA-MANY priming/Ad-MANY boost or by the MVA-MANY priming/MVA-MANY boost described as follows.

When mice were first immunized with one of the recombinant viruses followed by the booster injection of Ad-MANY or Ad-GFP, we didn't detect significant differences in the induced numbers of ANYNFTLV-specific $CD8^+$ T cells between a group of mice immunized with MVA-MANY priming/Ad-MANY boost and the one immunized with MVA-p3 priming/Ad-MANY boost (472 ± 28 versus 512 ± 147 per 10^6 splenocytes). Repeated immunization of Ad-MANY was also not effective for enhancing the induction of antigen-specific $CD8^+$ T cells, and on the contrary, it was even significantly lower than that induced by MVA-p3 priming/Ad-MANY boost immunization (184 ± 41 versus 512 ± 147 per 10^6 splenocytes, $P < 0.05$). When the mice were first immunized with one of the recombinant viruses followed by the booster injection of MVA-MANY or MVA-p3, we detected the enhanced induction of antigen-specific $CD8^+$ T cells by the combined immunization of Ad-MANY priming/MVA-MANY boost (832 ± 79 per 10^6 splenocytes) compared to either Ad-MANY priming/MVA-p3 boost (125 ± 65 per 10^6 splenocytes) or Ad-GFP priming/MVA-MANY boost (11 ± 2 per 10^6 splenocytes). Repeated immunization with MVA-MANY was also effective, against our expectation, for enhancing the induction of antigen-specific $CD8^+$ T cells compared to that induced by MVA-p3 priming/MVA-MANY boost immunization (350 ± 105 versus 5 ± 8 per 10^6 splenocytes, $P < 0.05$), however, its immunogenicity is still inferior to the Ad-MANY priming/MVA-MANY boost immunization.

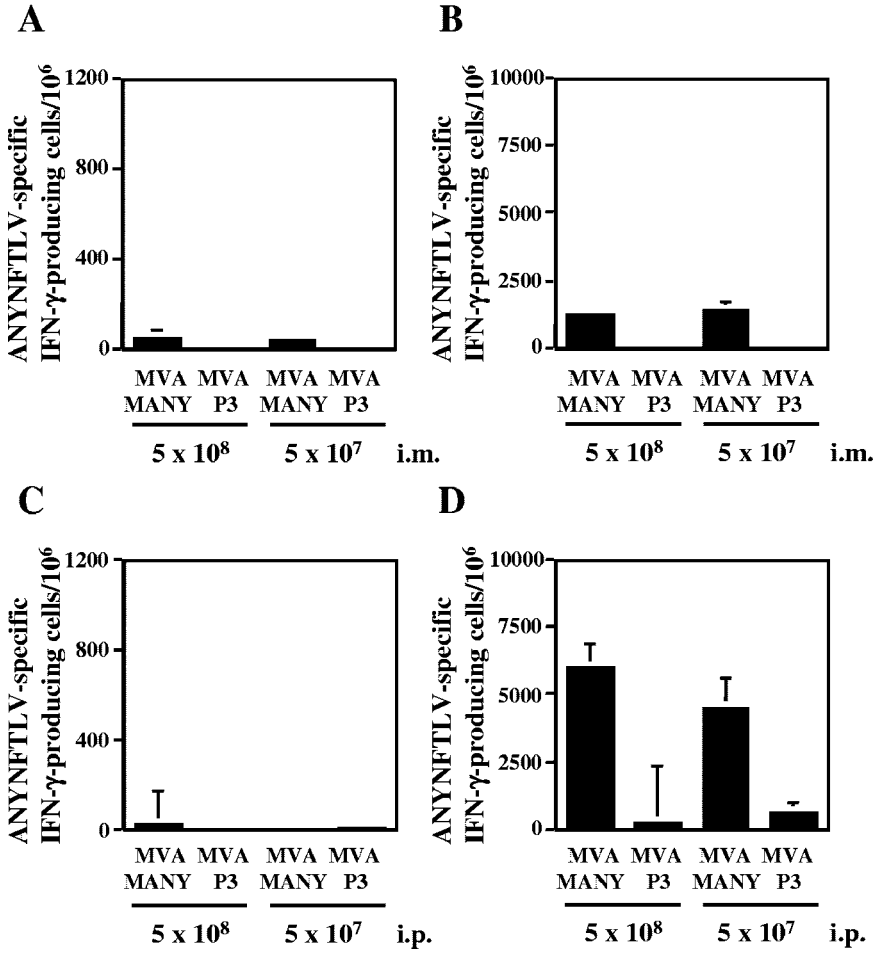

FIG. 3. Immunogenicity of recombinant MVA expressing ANYNFTLV. C57BL/6 mice were administered intramuscularly (A, B) or intraperitoneally (C, D) with two different doses of either MVA-MANY or MVA-p3. The mice were sacrificed 11 days after the immunization, and their spleens were removed. A half of splenocytes from individual mice were cultured with irradiated EL-4 cells pulsed with ANYNFTLV peptide for 1 week. The freshly isolated splenocytes (A, C) or the one-week cultured splenocytes (B, D) were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells/10^6 cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean ± standard deviation of three mice in each group. The data are representative one of three independent experiments.

To determine the induction of protective immunity mediated by the ANYNFTLV-specific $CD8^+$ T cells in vivo, we challenged the mice with a lethal dose of *T. cruzi* blood-form trypomastigotes after the prime/boost immunization with Ad-MANY/MVA-MANY. As shown in Fig. 4B and C, the parasitemia at 11, 14, and 20 days postinfection was significantly suppressed and the survival was significantly prolonged by the prime/boost immunization with Ad-MANY/MVA-MANY. The priming with Ad-MANY and boosting with MVA-p3 also significantly suppressed the parasitemia, but survival was not significantly prolonged.

Prime/boost immunization could be enhanced by increased viral loads. In the above experiments, the parasitemia was significantly suppressed and the survival was significantly prolonged by the prime/boost immunization with 5×10^7 PFU of Ad-MANY and 5×10^7 PFU of MVA-MANY, but all of the immunized mice eventually succumbed to *T. cruzi* infection. We then examined whether a prime/boost immunization with 10-fold higher doses (5×10^8 PFU) of Ad-MANY and MVA-MANY could induce a higher ANYNFTLV-specific $CD8^+$-T-cell response. The priming with 5×10^8 PFU of Ad-MANY and the subsequent boosting with 5×10^8 PFU of MVA-MANY was not toxic, since the immunized mice did not show an apparent pathological symptom such as ruffled fur or hunched posture. As shown in Fig. 5, the prime/boost immunization with 5×10^8 PFU of Ad-MANY/MVA-MANY markedly increased the frequency of ANYNFTLV-specific $CD8^+$ T cells compared to that with 5×10^7 PFU of Ad-MANY/MVA-MANY. In contrast, priming with 5×10^8 PFU of Ad-GFP or boosting with 5×10^8 PFU of MVA-p3 did not significantly induce the ANYNFTLV-specific $CD8^+$ T cells.

Adjuvant effect of recombinant MVA expressing murine RANKL. To further increase the frequency of ANYNFTLV-specific $CD8^+$ T cells, we next tried to use recombinant MVA expressing RANKL as an adjuvant, since we previously demonstrated a potent adjuvant effect of RANKL-expressing plas-

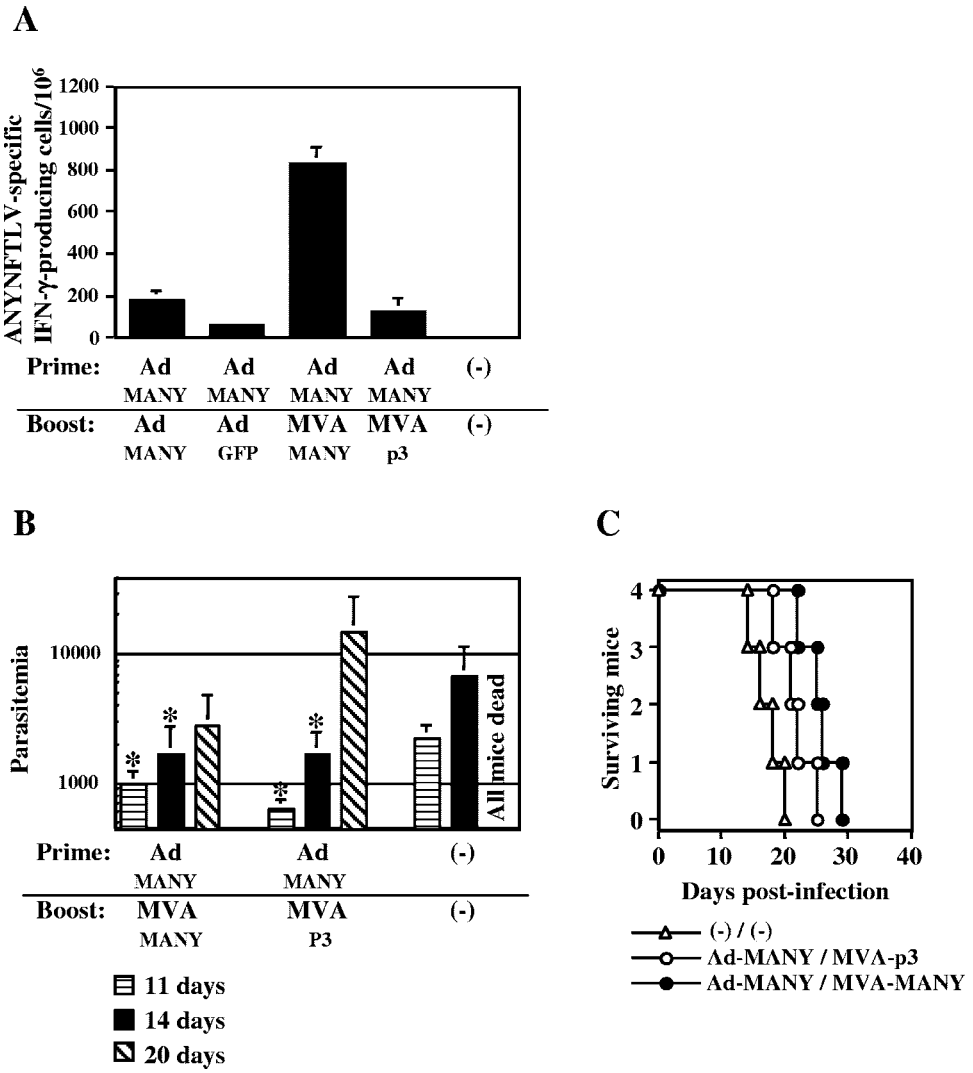

FIG. 4. Immune responses induced by prime/boost immunization using Ad-MANY and MVA-MANY. A. C57BL/6 mice were primed intramuscularly with 5×10^7 PFU of Ad-MANY. Twelve days later, the mice were boosted intramuscularly with 5×10^7 PFU of Ad-MANY or Ad-GFP, or intraperitoneally with 5×10^7 PFU of MVA-MANY or MVA-p3. The mice were sacrificed 14 days after the boost immunization, and their spleens were removed. The freshly isolated splenocytes were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells$\times 10^6$ cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean ± standard deviation of three mice in each group. B. Some groups of the prime/boosted mice ($n = 4$) were infected intramuscular with 10,000 *T. cruzi* blood-form trypomastigotes at 10 days after the boost immunization. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted at 11, 14, and 20 days postinfection. *, $P < 0.05$ compared to unimmunized mice by the unpaired Student's *t* test. Statistical analysis at 20 days postinfection was not achievable due to the death of all unimmunized mice. C. Survival was monitored daily. The survival of Ad-MANY/MVA-MANY-immunized mice was significantly different ($P < 0.05$ by the unpaired Mann-Whitney *U* test) from that of unimmunized mice. The data are representative one of two independent experiments.

mid for the induction of ANYNFTLV-specific CD8+-T-cell response by DNA vaccination (29). As shown in Fig. 6A, recombinant MVA inserted with RANKL cDNA was very efficient for expressing murine RANKL on the surface of BHK-21 cells as well as various mouse cells (data not shown).

We then included 5×10^7 PFU of MVA-RANKL as an adjuvant in the boosting with 5×10^8 PFU of MVA-MANY after the priming with 5×10^8 PFU of Ad-MANY. As shown in Fig. 6B, the inclusion of MVA-RANKL significantly increased the frequency of ANYNFTLV-specific CD8+ T cells induced by prime/boost immunization with Ad-MANY/MVA-MANY compared to that of MVA-p3.

Protection from lethal *T. cruzi* infection. We finally challenged the mice with a lethal (10,000) or sublethal (2,000) dose of *T. cruzi* blood-form trypomastigotes after the prime/boost immunization with high doses (5×10^8 PFU) of Ad-MANY/MVA-MANY or Ad-GFP/MVA-p3 and the inclusion of 5×10^7 PFU of MVA-RANKL or MVA-p3 (control) as an adjuvant. As shown in Fig. 7A and C, the parasitemia was significantly suppressed by the high doses of Ad-MANY/MVA-

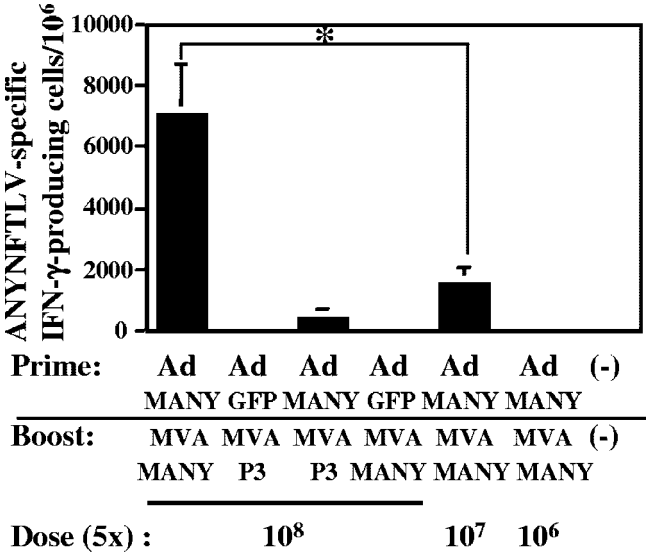

FIG. 5. Induction of ANYNFTLV-specific CD8$^+$ T cells by prime/boost immunization with high doses of Ad-MANY and MVA-MANY. C57BL/6 mice ($n = 3$) were first primed with different doses (5×10^8, 5×10^7, or 5×10^6 PFU) of Ad-MANY or 5×10^8 PFU of Ad-GFP, and boosted 14 days later with different doses (5×10^8, 5×10^7, or 5×10^6 PFU) of MVA-MANY or 5×10^8 PFU of MVA-p3. The mice were sacrificed 10 days after the boost immunization, and their spleens were removed. The freshly isolated splenocytes were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells$\times 10^6$ cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean ± standard deviation of three mice in each group. *, $P < 0.05$ determined by the Dunnett's two-tailed t test. The data are representative one of three independent experiments.

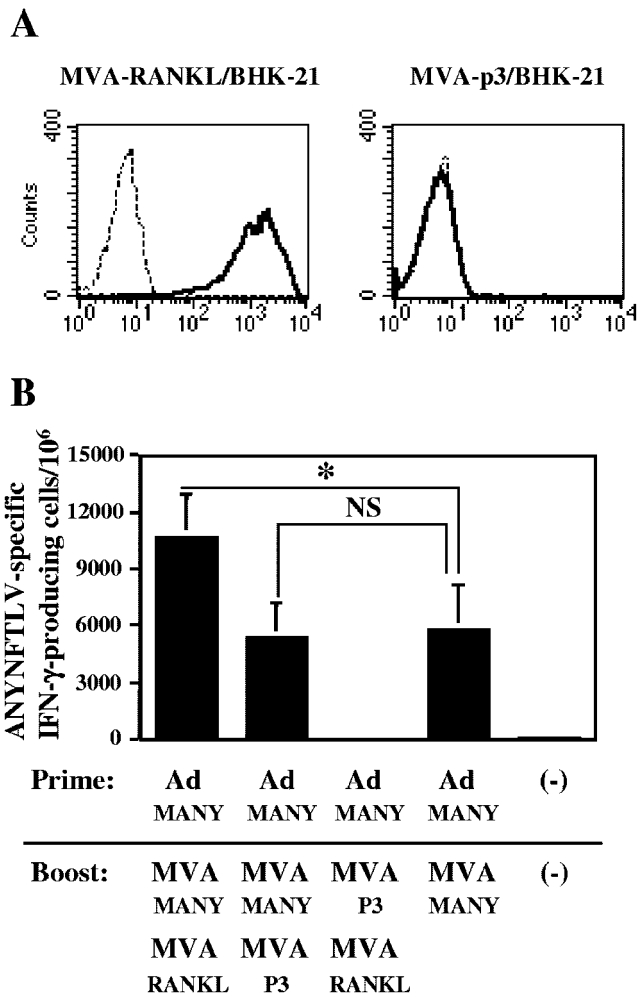

FIG. 6. Adjuvant effect of recombinant MVA expressing murine RANKL. (A) BHK-21 cells were infected with either MVA-RANKL or MVA-p3 and then were stained with biotinylated anti-RANKL monoclonal antibody followed by PE-labeled streptavidin. The bold histograms indicate the staining with anti-RANKL monoclonal antibody and the thin histograms indicate the staining with isotype-matched control rat immunoglobulin G. (B) C57BL/6 mice ($n = 3$) were first primed with 5×10^8 PFU of Ad-MANY and then boosted 11 days later with 5×10^8 PFU of MVA-MANY or MVA-p3 and 5×10^7 PFU of MVA-RANKL or MVA-p3. The mice were sacrificed 10 days after the boost immunization, and their spleens were removed. The freshly isolated splenocytes were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells/10^6 cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean ± standard deviation of three mice in each group. *, $P < 0.05$ by the Dunnett's two-tailed t test. NS, not significantly different. The data are representative one of two independent experiments.

MANY with or without MVA-RANKL, but protection of all mice from lethal *T. cruzi* infection was only achieved by the high doses of Ad-MANY/MVA-MANY with MVA-RANKL. These results indicated that the CD8$^+$-T-cell response to a single epitope (ANYNFTLV) of an intrinsic *T. cruzi* antigen (TSSA) could control lethal *T. cruzi* infection, if it could be efficiently induced by recombinant virus vector vaccination and adjuvant.

DISCUSSION

In the present study, we generated two recombinant virus vectors (Ad-MANY and MVA-MANY) expressing a single CD8$^+$-T-cell epitope (ANYNFTLV) derived from an intrinsic *T. cruzi* antigen (TSSA). In order to explore CD8$^+$-T-cell-mediated immunotherapeutic strategies against *T. cruzi* infection, we evaluated the immunogenicity of these recombinant viruses with regard to the induction of protective immune responses.

The expression of ANYNFTLV peptide by Ad-MANY and MVA-MANY was verified by detecting the ANYNFTLV-specific CD8$^+$ T cells in immunized mice (Fig. 2 and Fig. 3). However, the immunogenicities of Ad-MANY and MVA-MANY were different, especially when we evaluated the induction of ANYNFTLV-specific CD8$^+$ T cells in freshly isolated splenocytes. The induction of ANYNFTLV-specific CD8$^+$ T cells by Ad-MANY was robust, reaching up to approximately 800 cells (intramuscularly) (Fig. 2A) or 400 cells (intraperitoneally) (Fig. 2C) per 10^6 splenocytes. In contrast, few ANYNFTLV-specific CD8$^+$ T cells were detected in freshly isolated splenocytes when mice were immunized with

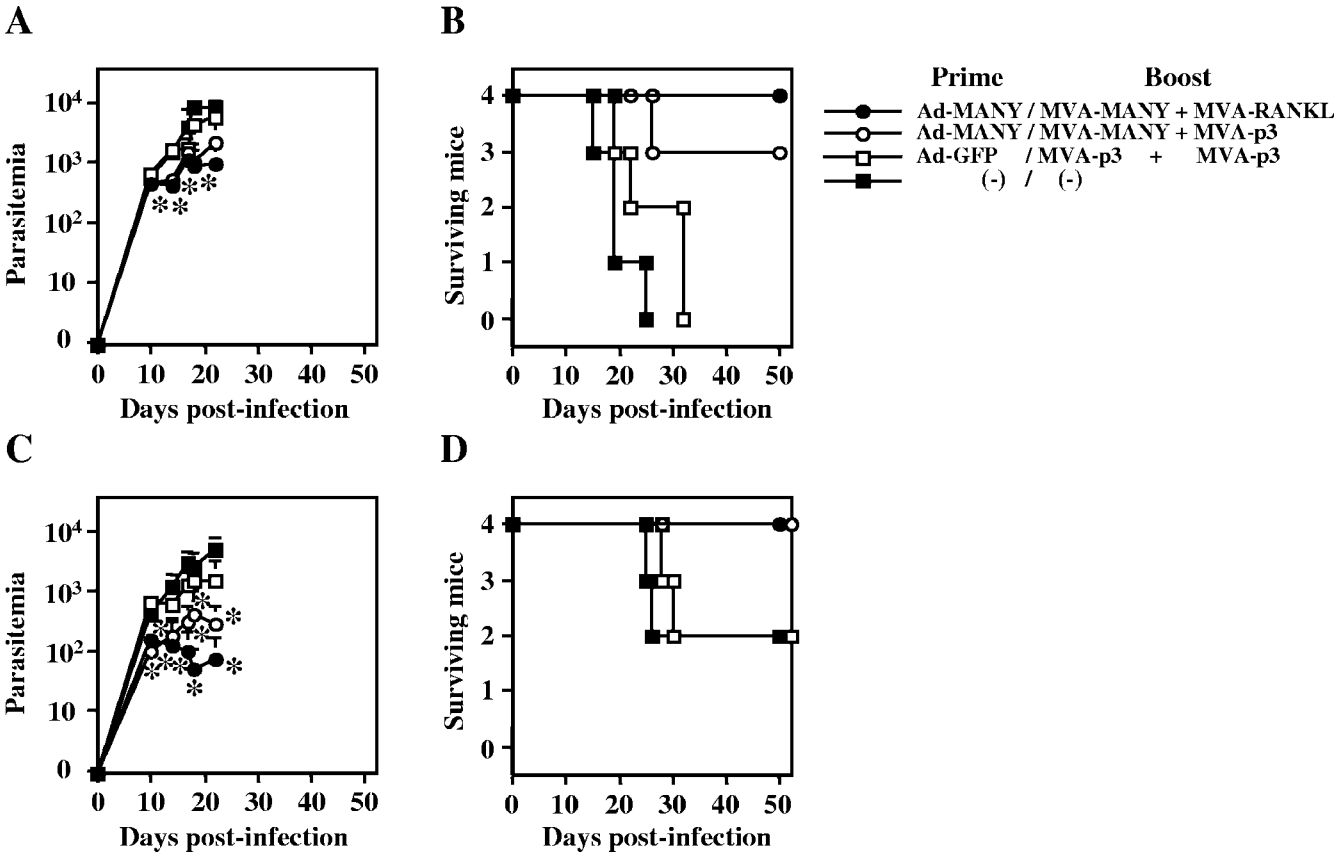

FIG. 7. Prime/boost immunization with Ad-MANY/MVA-MANY + MVA-RANKL can control lethal *T. cruzi* infection. C57BL/6 mice ($n = 4$) were first primed with 5×10^8 PFU of Ad-MANY or Ad-GFP, and then boosted with 5×10^8 PFU of MVA-MANY or MVA-p3 and 5×10^7 PFU of MVA-RANKL or MVA-p3 11 days later. The mice were infected intraperitoneally with 10,000 (A, B) or 2,000 (C, D) Tulahuen strain of *T. cruzi* blood-form trypomastigotes 14 days after the boost immunization. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically, and the data represent the mean ± standard deviation of four mice in each group (A, C). Survival was monitored daily (B, D). *, $P < 0.05$ compared to the unimmunized mice by the Dunnett's two-tailed *t* test (A, C). The longer survival of Ad-MANY/MVA-MANY + MVA-RANKL group was significantly different ($P < 0.05$ by the unpaired Mann-Whitney *U* test) from that of Ad-GFP/MVA-p3 plus MVA-p3 group of mice (B). The survival of other groups was not significantly different from that of the Ad-GFP/MVA-p3 plus MVA-p3 group of mice (B, D).

MVA-MANY (Fig. 3A and C). However, modest but substantial induction of ANYNFTLV-specific CD8+ T cells by MVA-MANY was detectable after the stimulation of immune splenocytes with the ANYNFTLV peptide in vitro for a week. This inferior ability of MVA-MANY to prime ANYNFTLV-specific CD8+-T-cell response in naïve mice seems most likely due to the immunological phenomenon called "immunodominance" which is potentially a serious pitfall in using virus vectors to deliver foreign antigens for the induction of immune responses (53). Thus, in the MVA-MANY-immunized mice, T-cell responses against MVA antigens might predominate over and hinder the ANYNFTLV-specific CD8+-T-cell response.

To augment the induction of ANYNFTLV-specific CD8+ T cells, we next evaluated the effects of boost immunization with Ad-MANY or MVA-MANY after Ad-MANY priming. In a sharp contrast with the priming, MVA-MANY was far more effective than Ad-MANY for the boosting (Fig. 4A). This superior ability of MVA-MANY at boosting might be due to circumvention of the "immunodominance" by the Ad-MANY priming so that the expanded ANYNFTLV-specific CD8+ T cells by the Ad-MANY priming predominated over MVA-specific T cells. In contrast, the boosting effect of Ad-MANY was only modest (Fig. 4A). This seems at least partly due to inhibition of adenoviral reinfection by neutralizing antibodies against adenovirus induced by the Ad-MANY priming. Therefore, the use of two different virus vectors expressing the same target antigen for prime/boost immunization is an efficient strategy to circumvent this problem.

The vaccination strategy using recombinant adenovirus for priming followed by the booster injection of recombinant vaccinia virus was also effective against other infections such as malaria (4, 13) and human immunodeficiency virus (7). To our experiences, the use of two recombinant virus vectors for adenovirus priming/MVA boosting was more effective for the induction of ANYNFTLV-specific CD8+ T cells than the use of TSSA gene-encoding DNA vaccine for priming followed by the booster injection of MVA-MANY. Even though the induction of ANYNFTLV-specific CD8+ T cells of DNA vaccine priming/MVA-MANY boosting was significantly enhanced (145 ± 50 per 10^6 splenocytes) when it was compared to those induced by either DNA vaccine alone (-12 ± 11 per 10^6

splenocytes) or MVA-MANY alone (24 ± 23 per 10^6 splenocytes), the number of ANYNFTLV-specific $CD8^+$ T cells induced by that regimen was constantly and significantly lower than that induced by the combined immunizations of two recombinant virus vectors.

When the immunized mice were challenged with a lethal dose of *T. cruzi* after the prime/boost immunization with 5×10^7 PFU of Ad-MANY/MVA-MANY, the parasitemia was significantly suppressed (Fig. 4B) and the survival was significantly prolonged but all mice eventually died (Fig. 4C). To achieve survival of all immunized mice from lethal *T. cruzi* infection, we further enhanced the induction of ANYNFTLV-specific $CD8^+$ T cells by the prime/boost immunization with 10-fold higher doses (5×10^8 PFU) of Ad-MANY/MVA-MANY (Fig. 5) and the inclusion of MVA-RANKL as an adjuvant (Fig. 6B).

Since the Ad and MVA vectors used here were highly attenuated, no apparent adverse effect manifested by pathological symptoms was observed after the priming with 5×10^8 PFU of Ad-MANY and the boosting with 5×10^8 PFU of MVA-MANY, while the frequency of ANYNFTLV-specific $CD8^+$ T cells was dramatically increased (approximately 7,000 per 10^6 splenocytes). Moreover, the inclusion of MVA-RANKL at the boosting further increased the frequency of ANYNFTLV-specific $CD8^+$ T cells (approximately 10,000 per 10^6 splenocytes). RANKL is a member of the tumor necrosis factor family, which has been implicated in immune regulation and bone homeostasis (2, 45). A major target for RANKL in the immune system is dendritic cells (DCs) that are potent antigen-presenting cells and express a high level of RANK (1, 52). It has been reported that RANKL not only enhances the survival of DCs but also up-regulates the production of cytokines, such as IL-12 (2, 17), and the expression of costimulatory molecules, such as B7, by dendritic cells thereby augments T-cell responses (47, 48). It would be interesting to determine the profiles of cytokine production in vivo with regard to the protective immunity against *T. cruzi* infection, since cytokines could modify the course of infection in combination with cellular components including $CD8^+$ T cells. We previously demonstrated that plasmid expressing RANKL was a potent genetic adjuvant for enhancing the induction of TSSA-specific $CD8^+$ T cells by DNA vaccination (29). We have now shown that MVA expressing RANKL is also useful as an adjuvant for recombinant MVA vaccination.

By the prime/boost immunization with 5×10^8 PFU of Ad-MANY/MVA-MANY and the inclusion of MVA-RANKL as adjuvant, all the immunized mice now survived against lethal *T. cruzi* infection (Fig. 7B). Although we previously demonstrated that recombinant virus vector vaccination against a highly immunogenic murine malaria antigen could protect mice from genetically modified *T. cruzi* expressing the same malaria antigen (26), this is the first indication that the $CD8^+$-T-cell response against a single $CD8^+$-T-cell epitope of intrinsic *T. cruzi* antigen was sufficient for controlling lethal *T. cruzi* infection, if it could be efficiently induced by recombinant virus vector vaccination and adjuvant. It is obvious that we should further define the optimal immunization protocols such as the immunization intervals of both recombinant viruses (4) and should determine how long the induced protective immune responses would last after the booster immunization. It would be also interesting to analyze if the enhanced T-cell-mediated immune responses could have detrimental effects both in the acute and in the chronic phase of *T. cruzi* infections, since there are few reports regarding this issue in the context of developing T-cell-mediated vaccination strategies. We are particularly interested in the immunopathological outcome caused by T cells during the chronic stage of Chagas' disease. This important issue will be analyzed thoroughly in closely combined with the development of effective T-cell-mediated vaccination strategies.

Our vaccination strategy targeting the induction of immune responses against a single $CD8^+$-T-cell epitope may be applicable for the induction of protective immunity against intracellular pathogens such as human immunodeficiency virus, which tends to be resistant to neutralizing antibodies. Moreover, since antigen-specific $CD8^+$-T-cell responses play a pivotal role not only in protective immunity against infections but also in antitumor immunity, a similar strategy using recombinant viral vaccines and adjuvant may be also useful for efficient induction of tumor-specific $CD8^+$-T-cell responses, for which many $CD8^+$-T-cell epitopes have been identified (5, 33). Further studies are now under way to address these possibilities.

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