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Quantitative and qualitative features of heterologous virus-vector-induced antigen-specific CD8+ T cells against *Trypanosoma cruzi* infection

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Abstract

We studied some aspects of the quantitative and qualitative features of heterologous recombinant (re) virus-vector-induced, antigen-specific $CD8^+$ T cells against *Trypanosoma cruzi*. We used three different, highly-attenuated re-viruses, i.e., influenza virus, adenovirus and vaccinia virus, which all expressed a single, *T. cruzi* antigen-derived $CD8^+$ T cell epitope. The use of two out of three vectors or the triple virus vector vaccination regimen not only confirmed that the re-vaccinia virus, which was placed last in order for sequential immunization, was an effective booster for the $CD8^+$ T cell immunity in terms of the number of antigen-specific $CD8^+$ T cells, but also demonstrated that i) the majority of cells exhibit the effector memory (T_{EM}) phenotype, ii) robustly secrete IFN-γ, iii) express higher intensity of the CD122 molecule and iv) present protective activity against *T. cruzi* infection. In contrast, placing the re-influenza virus last in sequential immunization had a detrimental effect on the quantitative and qualitative features of $CD8^+$ T cells. The triple virus vector vaccination was more effective at inducing a stronger $CD8^+$ T cell immunity than using two re-viruses. The different quantitative and qualitative features of $CD8^+$ T cells induced by different immunization regimens

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support the notion that the refinement of the best choice of multiple virus vector combinations is indispensable for the induction of a maximum number of $CD8^+$ T cells of high quality.

Keywords

$CD8^+$ T cells; *Trypanosoma cruzi*; Recombinant adenovirus; Recombinant influenza virus; Recombinant vaccinia virus

1. Introduction

Development of a $CD8^+$ T cell vaccine strategy has been intensely investigated in recent years (Masopust et al., 2007; Masopust, 2009) not only due to the emergence of neutralizing antibody (Ab)-resistant pathogens (Appay et al., 2008; Overstreet et al., 2008) but due to the obvious recognition of imminent pandemic infection and bioterrorism (McMurry et al., 2007; Doherty and Kelso, 2008; Stambas et al., 2008). The rationality of this approach is supported by observations which demonstrated that $CD8^+$ T cells could suppress the proliferation of several infectious pathogens (Goulder and Watkins, 2004; Masopust et al., 2007; Appay et al., 2008).

Trypanosoma cruzi is the etiological agent of Chagas' disease in Central and South America (Chagas, 1909). Since T cell-mediated immunity is critical for resolving the infection (Tarleton et al., 1992; Miyahira, 2008), a *T. cruzi* infection model is useful for defining a $CD8^+$ T cell-dependent vaccine strategy. We previously identified a major epitope of trans-sialidase surface antigen (TSSA) recognized by $CD8^+$ T cells in *T. cruzi*-infected C57BL/6 (B6) mice and have demonstrated that vaccination, either with plasmid DNA encoding TSSA (Katae et al., 2002; Miyahira et al., 2003a, b, c) or with recombinant (re) viruses expressing a single $CD8^+$ T cell-inducing epitope (Miyahira et al., 2005), could confer $CD8^+$ T cell-mediated protective immunity against lethal *T. cruzi* infection.

Vaccination using heterologous re-virus vectors (Stephenson, 2001) has been effective for inducing protective immunity against several infectious pathogens such as HIV (Shiver and Emini, 2004), malaria (Oliveira-Ferreira et al., 2000; Bruna-Romero et al., 2001; Zavala et al., 2001) and *T. cruzi* (Miyahira et al., 1999, 2005). A heterologous primary/booster vaccine strategy (Miyahira et al., 1998, 1999, 2005; Oliveira-Ferreira et al., 2000) demonstrated that $CD8^+$ T cell immunity can be boosted, offering potential for future clinical applications. Despite its dramatic success demonstrated in animal studies, its efficacy in humans was rather discouraging (Bejon et al., 2007; Sekaly, 2008), and needs to be improved for human application. By considering the limited physiological and spatial capacity for hosts' immunity (Vezys et al., 2009), the improvement of not only the quantitative attribute but the qualitative features of $CD8^+$ T cell immunity must be achieved (Masopust et al., 2006, 2007, 2009; Araki et al., 2009).

Since there is still limited information regarding the qualitative features of $CD8^+$ T cells against infectious pathogens (Masopust et al., 2006; Araki et al., 2009; Masopust, 2009), we elucidated, in the present study, some aspects of the phenotypic and immunological features of antigen-specific $CD8^+$ T cells which were induced by the use of three different, highly-attenuated re-viruses, all expressing a single *T. cruzi* antigen-derived $CD8^+$ T cell epitope, ANYNFTLV. During the course of our investigation, we verified the remarkable boosting capacity of vaccinia virus for the induction of higher numbers of $CD8^+$ T cells, which exhibit $CD122^{dull}$, $CD44^+$, $CD62L^-$, IFN-γ^+ phenotype, when it was placed last for sequential vaccination. We also verified the superior efficacy of a triple virus vector vaccination regimen for the induction of $CD8^+$ T cell immunity compared with the combination of two viruses. Our study supports the

importance and need to refine the choice of multiple virus vector combinations to achieve the induction of a maximum number of $CD8^+$ T cells of high quality.

2. Materials and methods

2.1. Animals and parasites

Female B6 (H-2^b) mice, 5-8 weeks of age, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Blood-form trypomastigotes of *T. cruzi* Tulahuen strain (Miyahira and Dvorak, 1994) were maintained in BALB/c mice by i.m. inoculation of 5,000 trypomastigotes into naïve mice every 2 weeks. An institutional review committee at the National Defense Medical College, Saitama, Japan, approved the animal studies described here.

2.2. Cells and culture

The B6 mouse-derived thymoma cell line EL-4 was used as antigen-presenting cells for $CD8^+$ T cell cultures and assays. The BHK-21 cell line (ATCC, Manassas, VA, USA) was used for growing a highly-attenuated vaccinia virus strain called modified vaccinia virus Ankara (MVA) (ATCC) (Sutter and Moss, 1992) -derived re-virus. The transformed human embryonic kidney cell line 293 (ATCC) was used for growing replication-deficient re-adenoviruses. These cells were cultured in medium as described previously (Miyahira et al., 2005). The medium used for immunological 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 IL-2 (Miyahira et al., 1995).

2.3. Peptide

An H-$2K^b$-restricted $CD8^+$ T cell epitope peptide, ANYNFTLV, derived from TSSA (Katae et al., 2002; Miyahira et al., 2003a, b, c, 2005) was synthesized and used for immunological assays.

2.4. Generation of re-viruses

Re-influenza viruses were generated using an established eight-plasmid influenza reverse genetics system (Hoffmann et al., 2000; Quinlivan et al., 2005). The plasmids containing the segments of A/Puerto Rico/8/34 influenza strain (PR8) were co-transfected in 293T cells and 24 h later cells were harvested and inoculated into 7-8 days-old embryonated eggs. The allantoic fluids containing the virus preparations were collected 48 h post-inoculation. The $CD8^+$-T-cell epitope, ANYNFTLV, was inserted into the neuraminidase (NA) of A/PR/8/34 (H1N1) at residue 42 by PCR mutagenesis using the following primers: 5′-CAATTCAAACTGGAAGT*GCC*AAC*TAC*A*ACTTC*A*CCCTGGTG*CAAAACATCATTAC-3′ and 5′-GTAATGATGTTTTG*CACCAGGG*T*GAAGT*T*GTA*GTT*GGC*ACTTCCAGTTTGAATTG-3′. A corresponding number of coding amino acids were eliminated from the NA segment in order to maintain the length equal to the wild-type NA. To generate NS1 of the PR8 strain containing only the first 126 aminoacids (NS1-D126), we used a strategy described previously (Solorzano et al., 2005). NS1-D126 was constructed by ligation into pDZ vector (Quinlivan et al., 2005) of two PCR fragments obtained using two sets of primers. The 5′end portion of the segment was obtained with the following primers: 5′-GCGCTTAATTAAGAGGGAGCAATTGTTGGCG-3′ (NS1-153) and 5′-CATCGCTCTTCTATTAGTAGAAACAAGGGTGTTTTTTATTATTAAATAAG-3′. The 3′ end portion containing the first 126 amino acids of NS1 was obtained using the following primers: 5′-GCGCTTAATTAATCAAGATCTACTTATCCATGATCGCCTGG-3′ (NS1-126) together with 5′-GATCGCTCTTCTGGGAGCAAAAGCAGGGTGACAAAGAC-3′. NS1 truncations did not

affect the sequence of NEP/NS2. The highly-attenuated re-influenza virus expressing an ANYNFTLV epitope is designated as re-FLU.

The re-adenovirus and re-vaccinia viruses expressing an ANYNFTLV epitope (Miyahira et al., 2005) are designated as re-Ad or re-MVA, respectively. The genetically-modified viruses which do not express an ANYNFTLV epitope were used as control re-virus vectors, which were described as FLU, Ad (Miyahira et al., 2005) or MVA (Miyahira et al., 2005).

2.5. Quantification of antigen-specific T cells by ELISPOT assay

The frequency of antigen-specific T cells was determined by the IFN-γ enzyme-linked immunospot (ELISPOT) assay for IFN-γ-secreting cells essentially as described previously (Miyahira et al., 1995).

2.6. Monoclonal antibodies (mAbs)

The following mAbs were used: FITC-conjugated anti-mouse CD62L / L-selectin /LECAM-1 / Ly-22 mAb (MEL-14), peridinin chlorophyll-a protein (PerCP)-Cy5.5-conjugated anti-mouse CD8α mAb (53-6.7), allophycocyanin (APC)-conjugated anti-mouse CD44 /Pgp-1 /Ly-24 mAb (IM7), phycoerythrin (PE)-conjugated anti-mouse IFN-γ mAb (XMG1.2), PerCP-Cy5.5-conjugated anti-CD4 mAb (RM4-5), PE-conjugated anti-CD122 mAb (TM-β1), biotin-conjugated anti-CD25 mAb (7D4), APC-labeled streptavidin, PE-conjugated anti-NK1.1 mAb (PK136), FITC-conjugated anti-T cell receptor (TCR)β chain mAb (H57-597) (BD Biosciences Mountain View, CA, USA), APC-conjugated anti-mouse CD8α mAb (53-6.7) (eBioscience, San Diego, CA, USA), PE-labeled H-2K^b/ANYNFTLV pentamer (Proimmune, Oxford, United Kingdom).

2.7. Flowcytometric analyses

Splenocytes (2×10^6) were stained with each fluorescent dye-conjugated mAb. After washing with 2% FCS-supplemented PBS containing with 1 mM Na_2EDTA and 0.1 % sodium azide (washing buffer), the specimens were analyzed on a FACSCalibur™ (BD Biosciences). To quantify the ANYNFTLV-specific, IFN-γ-producing CD8$^+$ T cells, splenocytes (2×10^6) were co-cultured with 1×10^5 of irradiated, 5 mM ANYNFTLV-pre-pulsed EL-4 cells in medium containing 10 mg/ml Brefeldin A for 16 h. They were then stained with APC-conjugated anti-mouse CD8α mAb, fixed and permeabilized with BD FACS Lysing Solution together with FACS Permeabilizing Solution 2 (BD Biosciences), further stained with the PE-conjugated anti-mouse IFN-γ mAb, and were finally analyzed on FACSCalibur. The data were processed using CellQuest software (BD Biosciences).

2.8. Vaccination schedule, dosages, and challenge infection

All of the vaccination schedules, dosages and the protocols for *T. cruzi* challenge infection are described in each figure legend.

2.9. Statistical analyses

Statistical analyses were performed using the StatView software (SAS Institute, Cary, NC, USA). An unpaired Student's *t* test was applied to determine the significant differences between two groups for the ELISPOT assays, FACS analyses and for the parasitemia. Significant differences in survival data were calculated by the Kaplan-Meier method with Mantel-Cox logrank. *P* values less than 0.05 were considered significant.

3. Results

3.1. Phenotypic features of immune responses induced by the use of re-Ad and re-MVA

The ELISPOT assay (Fig. 1A) and the K^b/ANYNFTLV pentamer technology (Fig. 1B) confirmed the superior induction of antigen-specific $CD8^+$ T cells by the heterologous re-Ad/re-MVA to the homologous re-Ad immunization. In addition, we found that those induced by the heterologous immunization consisted of higher portions of ANYNFTLV-specific $CD44^+CD62L^-CD8^+$ T cells (T_{EM} phenotype) (98.2%) than those induced by the homologous one (83.6%) (Fig. 1C). A similar trend in cell type composition was observed in the ANYNFTLV-non-specific $CD8^+$ T cells (13.8% vs. 5.0%) (Fig. 1C). The composition of "regulatory" cell populations such as the $CD4^+CD25^+$ T cells, $NK1.1^+\alpha\beta TCR^+$ T (NKT) cells and $CD8^+CD122^+$ T cells did not show dramatic differences between experimental groups (Fig. 1D) except that we noticed the increase of $CD8^+CD122^{dull}$ cell populations in the heterologous re-Ad/re-MVA-immunized mice (arrow in Fig. 1D). Since the expression of a CD122 molecule on $CD8^+$ T cells is viewed as one of the indications for the maturation of these cells (Khanolkar et al., 2004;Mbitikon-Kobo et al., 2009), we compared its expression between the $CD8^+$ T cells derived from different immunization protocols (Fig. 1E). The CD122 expression on ANYNFTLV-specific $CD8^+$ T cells induced by either of these double re-virus vector immunization regimens exhibit similar levels of flow cytometric mean fluorescence intensity (MFI) (62.5 in heterologous immunization versus 61.5 in the homologous one) (Fig. 1E).

3.2. Efficient induction of IFN-γ-secreting, ANYNFTLV-specific $CD8^+$ T cells by the heterologous re-Ad/re-MVA immunization

We next elucidated the capacity for IFN-γ secretion by the ANYNFTLV-specific $CD8^+$ T cells which were induced by either the heterologous or homologous immunizations. The re-Ad/re-MVA immunization could induce a significantly higher portion of IFN-γ-secreting, ANYNFTLV-specific $CD8^+$ T cells than the homologous re-Ad immunization (4.8% ± 0.4 versus 2.7% ± 0.8) (Fig. 2A, B). In addition, the $CD8^+$ T cells induced by the re-Ad/re-MVA immunization exhibited significantly higher MFI of IFN-γ content than those induced by the homologous immunization (217 versus 128) (Fig. 2C), indicating that the IFN-γ content per $CD8^+$ T cell is significantly different depending on the vaccination regimen used for its induction.

3.3. Characterization of re-FLU for the induction of antigen-specific $CD8^+$ T cells

The immunogenicity of the highly-attenuated re-FLU for the induction of ANYNFTLV-specific $CD8^+$ T cells was confirmed by the ELISPOT assay after immunizing B6 mice with different viral doses (Fig. 3A). When we combined the re-FLU with one of the other re-viruses for immunization, both the ELISPOT assay (Fig. 3B) and the pentamer technology (data not shown) presented similar results, showing that the heterologous re-Ad/re-MVA double immunization was better for the induction of $CD8^+$ T cell numbers than any other re-FLU-combined heterologous immunization regimens. The re-FLU-combined heterologous immunization induced a lower percentage of $CD8^+$ T cells of T_{EM} phenotype than those induced by the heterologous re-Ad/re-MVA immunization (Fig. 3C, D). This feature was particularly conspicuous when the re-FLU was used as a booster immunogen, exhibiting T_{EM} phenotype only in 45.9% in cells induced by the re-Ad/re-FLU vaccination (Fig. 3C) and at 39.7% induced by the re-MVA/re-FLU vaccination (Fig. 3D).

3.4. Phenotypic and immunological features of ANYNFTLV-specific CD8+ T cells induced by heterologous triple virus vector immunization

We then tested whether there are any advantages to using three different re-viruses for the induction of $CD8^+$ T cells. Since the best order for sequential double immunization to induce the maximum number of antigen-specific $CD8^+$ T cells was the heterologous re-Ad/re-MVA (Miyahira et al., 2005), we combined the newly-generated re-FLU with two other re-viruses in different heterologous vaccination orders as shown in Fig. 4A and B. We found that vaccination in the order of re-FLU/re-Ad/re-MVA was the best for maximum induction of ANYNFTLV-specific $CD8^+$ T cell numbers as determined by both the ELISPOT assay (Fig. 4A) and the pentamer technology (Fig. 4B). In contrast, the immunogenicity of re-Ad/re-MVA/re-FLU was significantly impaired compared not only with those demonstrated in two other triple vaccination regimens but with that observed in the re-Ad/re-MVA double vaccination (Fig. 4A, B). Fig. 4C shows the representative data of pentamer analyses demonstrating that the percentage of $CD8^+$ T cells of T_{EM} phenotype was higher when the re-MVA was placed last in the triple sequential vaccination regimen (95.9% and 93.9%). In contrast, the percentage of the T_{EM} phenotypic cell population was the lowest when mice were immunized with re-Ad/re-MVA/re-FLU (81.1%). The percentage of T_{EM} phenotype of ANYNFTLV-non-specific $CD8^+$ T cells showed a similar trend of differences among different regimens (Fig. 4C). Since we found that the expression of CD122 (Fig. 1D) and the capacity for IFN-γ secretion (Fig. 2) by the induced $CD8^+$ T cells were different depending on the vaccination regimens, we therefore characterized these phenotypes among $CD8^+$ T cells induced by each regimen (Fig. 4D). As expected, the quantitative features of the ANYNFTLV-specific $CD8^+$ T cells induced by the re-FLU/re-Ad/re-MVA vaccination (8.18%) and the re-Ad/re-FLU/re-MVA vaccination (6.81%) coincided with the higher expression of CD122 (MFI = 56.9 and 54.0, respectively) and the elevated IFN-γ content per $CD8^+$ T cell (MFI = 55.3 and 53.3, respectively) (Fig. 4D). The inferior induction of ANYNFTLV-specific $CD8^+$ T cells by the re-Ad/re-MVA/re-FLU vaccination (4.86%), on the contrary, coincided with the lower expression of CD122 on the $CD8^+$ T cells (MFI = 42.5) and the lower content of IFN-γ per $CD8^+$ T cell (MFI = 43.5) (Fig. 4D).

3.5. Increased immunization doses enhanced the induction of ANYNFTLV-specific CD8+ T cell quantity without changing their memory phenotype

By the 10-fold-increase of the immunizing doses of re-Ad and re-MVA, we found that the induction of ANYNFTLV-specific $CD8^+$ T cells was significantly enhanced as evidenced by both the ELISPOT assay (Fig. 5A) and the pentamer technology (Fig. 5B). This quantitative enhancement of $CD8^+$ T cell immune responses, however, did not coincide with the T_{EM} phenotypic change of the ANYNFTLV-specific $CD8^+$ T cells (90.3% versus 95.9%) (Fig. 5C), implying that the antigenic dose might not influence the qualitative features of induced $CD8^+$ T cells.

3.6. The heterologous triple virus vector vaccination regimen using three different re-viruses could induce CD8+ T cell-mediated protective immunity

Since the clinical course of *T. cruzi* infection differs depending on the infection doses, we lastly chose three different infection dose; i.e., 5,000 per mouse (Fig. 6A), 1,000 per mouse (Fig. 6B) and 100 per mouse (Fig. 6C), to assess the immunological efficacy of heterologous triple virus vector vaccination against lethal *T. cruzi* infection. We found that the protective efficacy of the triple re-FLU/re-Ad/re-MVA vaccination (●) was better, although slightly, than that of the double re-Ad/re-MVA (○) as assessed by both the parasitemia and the survival of infected mice. These protection assays strongly suggest that the heterologous triple virus vector vaccination regimen using three different re-viruses could hold immunological advantages for improving the outcome of lethal *T. cruzi* infection (Fig. 6).

4. Discussion

Development of an effective $CD8^+$ T cell vaccine has not yet been clinically achieved despite the epoch-making discovery of a primary/booster vaccination strategy (Vuola et al., 2005; Reyes-Sandoval et al., 2007; Radosević et al., 2009). One of explainations for this difficulty is that the quantitative enhancement of $CD8^+$ T cell immunity might not be sufficient to achieve $CD8^+$ T cell-mediated immunological protection against infectious pathogens. $CD8^+$ T cells consist of an enormously heterogeneous population including cells which might not be capable of controlling infectious diseases (Harari et al., 2006; Kaech and Wherry, 2007; Kedzierska et al., 2008; Hansen et al., 2009). Although hosts' immune cells must countermeasure against, literally, limitless numbers of infectious pathogens in their environment, the capacity for immunity is, on the contrary, not limitless. In this regard, the qualitative considerations for immunity are necessary under the condition that the quantitative enhancement for $CD8^+$ T cells is not achievable in an unrestricted manner due to the physiological and spatial restrictions (Vezys et al., 2009). In the present study, by taking advantage of the *T. cruzi* infection model, we have aimed to refine a new $CD8^+$ T cell vaccine protocol which should be optimized both in a quantitative and in a qualitative manner for controlling infectious diseases. Although it is worth elucidating the homologous re-virus vector vaccination-induced immune responses, in the current study we have focused on the analyses of heterologous re-virus vector vaccination-induced responses.

The assays employed to assess the $CD8^+$ T cell immune responses in the current study were the ELISPOT assay and pentamer technology. The two assays have fundamental differences which require careful interpretations of the obtained data. The ELISPOT assay detects the functional aspect of activated $CD8^+$ T cells, which is the secretion of IFN-γ. However on the contrary, the pentamer technology detects the $CD8^+$ T cells which could physically bind to the pentamer via TCR. The capacity for physical binding to the TCR does not necessarily mean that the peptide could trigger the activation of $CD8^+$ T cells. It is therefore possible that some of the $CD8^+$ T cell epitopes could physically bind to the TCR on the $CD8^+$ T cells, but could not properly activate those, resulting in the absence of fully-functional, IFN-γ-secreting $CD8^+$ T cells. Although we have no concrete data to explain why there is a discrepancy in the number of cells induced by either the re-Ad/re-Ad homologous vaccination or the single re-Ad one depending on the assays employed for the detection of ANYNFTLV-specific $CD8^+$ T cells (Fig. 1A, B), we speculate that some vaccination regimens tend to induce more antigen-specific $CD8^+$ T cells which exhibit poorer immunological function and secrete lower amounts of IFN-γ.

When analyzing the phenotypic features of $CD8^+$ T cells induced by the re-virus vaccination regimens, we found a strong association between the numerical enhancement of ANYNFTLV-specific $CD8^+$ T cell immunity and the $CD8^+$ T cells of T_{EM} phenotype (Gattinoni et al., 2009; Vezys et al., 2009) upon the heterologous re-Ad/re-MVA sequential immunization (Fig. 1C), re-FLU-combined double vaccinations (Fig. 3C, D) and the heterologous triple virus vector immunizations (Fig. 4C). While examining additional phenotypic markers on cells, we found that the induction of ANYNFTLV-specific $CD8^+$ T cells was associated with the emergence of a $CD8^+CD122^{dull}$ T cell population (Fig. 1D). Since the CD122 is an IL-2/IL-15 receptor β-subunit (Létourneau et al., 2009), its critical role for the induction of immunologically functional $CD8^+$ T cell immunity is conceivable. The $CD8^+$ T cells induced by the heterologous re-Ad/re-MVA/re-FLU vaccination unexpectedly exhibited lower MFI of CD122 than those induced by two other triple vaccinations (Fig. 4D), indicating that the CD122 expression would be one of the phenotypic markers of qualitative features of induced $CD8^+$ T cells. CD122 is thought to be correlated with the maturation of $CD8^+$ T cells (Khanolkar et al., 2004; Mbitikon-Kobo et al., 2009), suggesting that the phenotypic changes might possibly result from interference with the maturation of $CD8^+$ T cells. However, since we did not find

any differences in CD122 expression between the heterologous re-Ad/re-MVA-induced and the homologous re-Ad/re-Ad-induced $CD8^+$ T cells (Fig. 1E) despite the numerical expansion of $CD8^+$ T cells of both being significantly different (Fig. 1A, B), the expression of CD122 on $CD8^+$ T cells might not be directly linked to their capability for cell proliferation.

Interestingly, the capacity for IFN-γ secretion, which is obviously crucial to combat infectious pathogens (Tsuji et al., 1995), per induced ANYNFTLV-specific $CD8^+$ T cell as predicted by the MFI of IFN-γ staining was higher when the re-MVA was used last in the sequential order both in the double (Fig. 2) and in the triple virus vector vaccination (Fig. 4D). In contrast, the $CD8^+$ T cells induced by the heterologous re-Ad/re-MVA/re-FLU vaccination, again unexpectedly, exhibited lower MFI of IFN-γ staining than those induced by two other triple vaccinations (Fig. 4D). These results indicate that the order of virus vector vaccination affects not only the number of induced antigen-specific $CD8^+$ T cells but their qualitative features. The down-regulation of induced numbers of ANYNFTLV-specific $CD8^+$ T cells, the decreased percentages of T_{EM} phenotype, the down-regulation of CD122 expression and the decreased IFN-γ content in $CD8^+$ T cells induced by the heterologous re-Ad/re-MVA/re-FLU triple vaccination (Fig. 4D) were surprising and unexpected. The precise mechanisms for the induction of these phenotypic changes are not clear. One possible explanation for this qualitative change might be that the re-FLU immunization, which was placed last in the sequence, could induce more $CD8^+$ T cells of T_{EM} phenotype which could accumulate in tissues other than spleen, such as lung and liver (Wherry et al., 2003). The qualitative features of antigen-specific $CD8^+$ T cells in several different tissues must be characterized to clarify this question. Another possible explanation for this is “immunodominance”, an immunological phenomenon by which all of the $CD8^+$ T cell-inducing epitopes compete with one another, leading to the selection of one or a few that actually could induce $CD8^+$ T cell immune responses (Yewdell, 2006). If the re-FLU contains intrinsic antigens which encode stronger $CD8^+$ T cell-inducing epitope(s), it could dominate the $CD8^+$ T cell immunity which would outnumber the induction of ANYNFTLV-specific $CD8^+$ T cells and could suppress the maturation of primed but subdominant $CD8^+$ T cells. In contrast, MVA has been well known both for its outstanding boosting capacity for $CD8^+$ T cell immunity and its safety for use in vaccination (Sutter and Moss, 1992; Hanke et al., 2007), although there has been no concrete evidence to explain its uniqueness in $CD8^+$ T cell immunity (Zavala et al., 2001; Miyahira et al., 2005). The hosts' immune responses against vaccinia virus were recently analyzed in detail (Laouar et al., 2008; Zhao et al., 2009), particularly elucidating phenotypic features of vaccinia virus-specific $CD8^+$ T cells. The coincidence of the enhanced induction of ANYNFTLV-specific $CD8^+$ T cell numbers and their phenotypic changes indicated that the MVA might have an immunological capability which could accelerate the differentiation and maturation of “primed” antigen-specific $CD8^+$ T cells, resulting in the improved protective immunity.

Our results contradicted a previous report (Vuola et al., 2005) which demonstrated that immunization with three vectors showed no improvement over an optimal two vector regimen. We assume that the immunogenicity and immunological efficacy could be different depending on vectors used for sequential heterologous vaccinations. Besides this contradiction, even if the immunological protection against *T. cruzi* infection induced by the heterologous triple virus vector vaccination was significantly improved, the parasitemia and the survival of these mice exhibited only minimal differences compared with the clinical *T. cruzi* infection course of double virus vector-vaccinated ones (Fig. 6). This observation might not be surprising, since the infection with *T. cruzi* itself, which expresses the ANYNFTLV epitope as an intrinsic antigen, could rigorously boost the ANYNFTLV-specific $CD8^+$ T cell immune responses. If the double virus vector vaccination already induced high enough numbers of antigen-specific $CD8^+$ T cells, the *T. cruzi* infection itself could be sufficient to enhance the antigen-specific $CD8^+$ T cells, enabling the improvement of pathogenic outcome.

Our current study has focused on the elucidation of only some aspects of the quantitative and qualitative features of heterologous re-virus-vector-induced, antigen-specific $CD8^+$ T cells against *T. cruzi*. There are several other qualitative features such as the expression of multiple cytokines, perforin, granzymes and other T cell phenotypic surface markers (Muller et al., 2003; Bengsch et al., 2007; Precopio et al., 2007). There is an emerging concept that vaccinia virus could induce "polyfunctional" $CD8^+$ T cells (Precopio et al., 2007; Reyes-Sandoval et al., 2010). The addition of a $CD4^+$ T cell epitope for immunizations might change the qualitative features of $CD8^+$ T cells and might improve vaccine efficacy (Machado et al., 2006; Duan et al., 2009; Haolla et al., 2009). In addition, the change of vaccination protocol such as the extension of 2-week vaccination intervals to 8-weeks might also affect both the quantitative and qualitative features of $CD8^+$ T cells (Bruna-Romero et al., 2001). Despite there being many other possible factors which could affect the outcome of heterologous re-virus vaccination, however, our study clearly suggests that the order of multiple virus vector vaccination must be carefully defined to maximize the vaccine efficacy and to avoid an unexpected detrimental outcome.

We are also fully aware that our experimental approach has limitations in achieving the final goal, which is the development of a new vaccine strategy against infectious pathogens. The immune system consists of a variety of immune effector mechanisms. They are closely linked to one another and act as an organized entity to combat invading pathogens. In this regard, ideally vaccines should induce all of the immune effector mechanisms as a whole. We should not lose sight of this, however we believe it is necessary to perform full and thorough analyses on each of the immune effector mechanisms one by one to accumulate incremental knowledge. We believe that this approach will also contribute to the development of innovative future vaccine strategies. Again, we do not consider it effective to use a single peptide for the practical application of virus vector vaccines. To overcome the major histocompatibility complex (MHC) restriction, the inclusion of multiple $CD8^+$ T cell peptides and their combination with $CD4^+$ T cell and B cell peptides will be indispensable to induce maximal and optimal vaccine-induced immune responses. The refinement of optimal induction protocols of each cell; i.e., $CD8^+$ T cells, $CD4^+$ T cells and B cells, will need to be appropriately combined in the future to achieve the development of a new vaccine strategy.

When taking advantage of virus vector vaccination, pre-existing immunity, particularly neutralizing Ab, is known to suppress the induction of antigen-specific $CD8^+$ T cells. Although the use of re-viruses as vaccine vectors is potentially a powerful tool to combat infectious pathogens, its use will require significant precautions to avoid unexpected suppression of immune responses. Our current study suggests that the use of multiple virus vectors for the induction of antigen-specific $CD8^+$ T cells is feasible and that the pre-existing immunity against a specific virus could be overcome by the use of alternative virus vectors. In addition, since the $CD8^+$ T cell immune responses wane over time, the additional booster vaccination will be required by the use of alternative vectors to avoid the pre-existing immunity and to maintain the effective protective immunity for a longer period of time. In these cases, our study will be helpful to consider which vectors must be used when and in which order. Our study could also be beneficial for people who are not able to pay for multiple virus vector immunizations, since they could at least choose which virus vectors should be considered first for the priming vaccination. The selection of priming vectors in critical to avoid possible immune suppression by inappropriate combinations and inappropriate immunization orders of virus vectors.

Overall, the elucidation of immunological mechanisms to induce qualitative differences of $CD8^+$ T cells would be indispensable, since its clarification will complement the physiological and spatial restrictions, which make quantitative enhancement of $CD8^+$ T cell immunity in an unlimited manner impossible, and will lead to the future development of a clinically effective $CD8^+$ T cell vaccine strategy.

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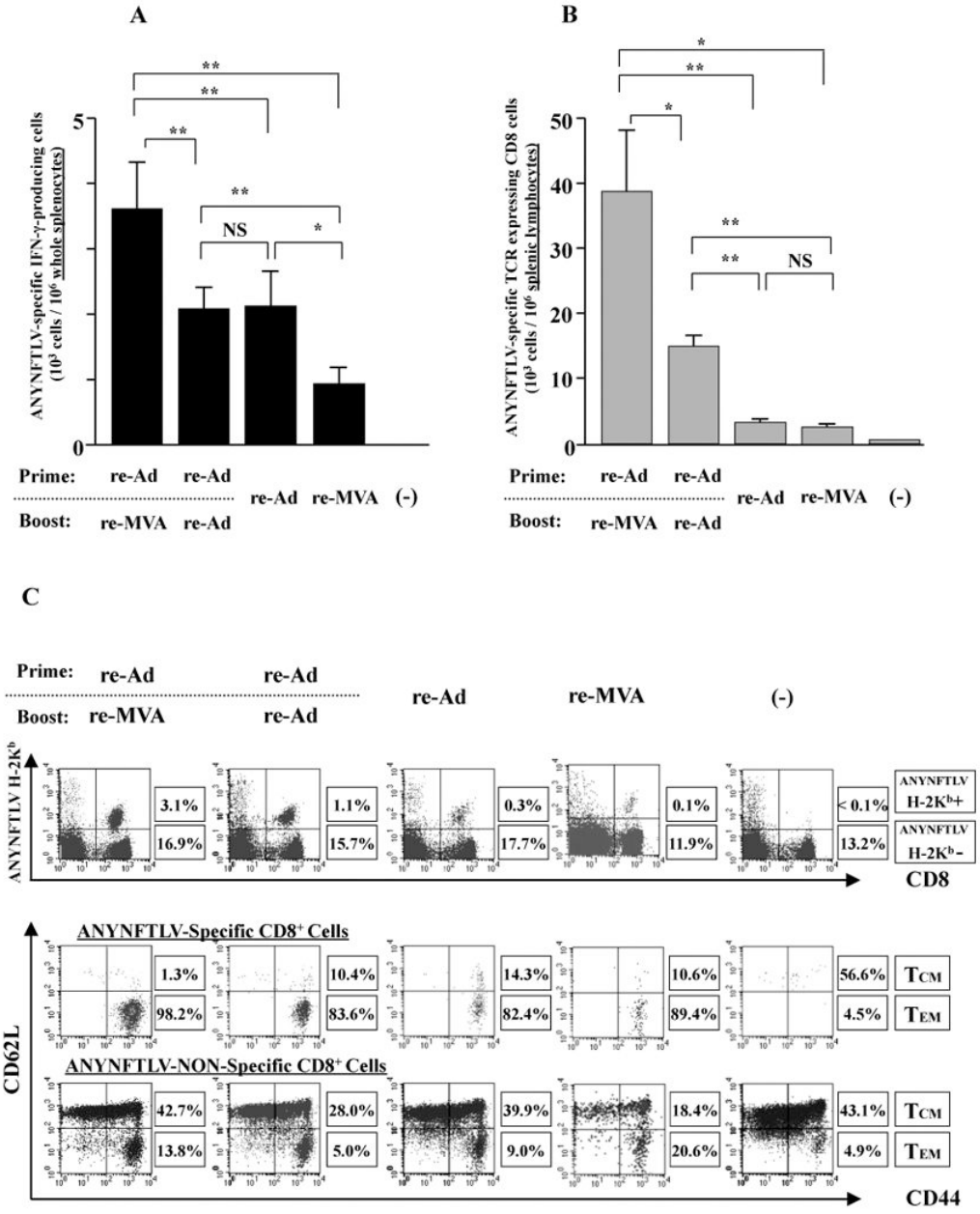


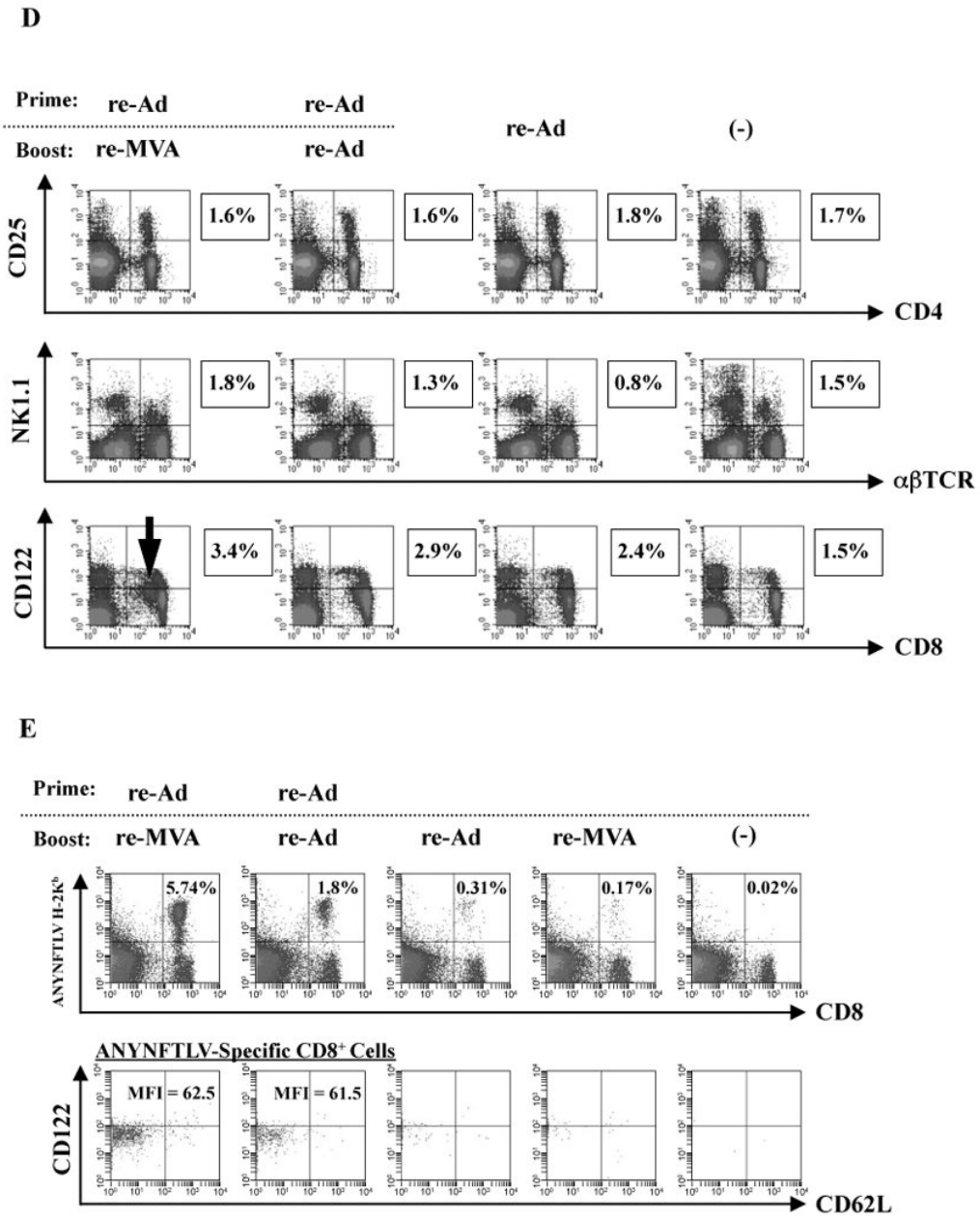


Fig. 1.

Phenotypic features of ANYNFTLV, a peptide derived from *Trypanosoma cruzi* surface antigen-specific $CD8^+$ T cells induced by the use of recombinant adenoma virus (re-Ad) and recombinant vaccinia virus (re-MVA). B6 mice were first primed with 5×10^7 plaque forming units (PFU) of re-Ad and then were given a booster immunization 14 days later with 5×10^7 PFU of either re-Ad or re-MVA. The single re-Ad immunized and non-immunized mice were included as controls. The mice were sacrificed 12 days after the last immunization and spleens were removed. The freshly-isolated splenocytes were subjected to immunological assays. (A) The 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 per 10^6 splenocytes was counted 22 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 ± S.D. of three to nine mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. “NS” means “not statistically significant”. (B) The splenocytes were subjected to flow cytometory (FCM) analyses for detecting K^b/ANYNFTLV pentamer-reactive $CD8^+$ T cells. The T-cell receptor (TCR) was stained by the phycoerythrin (PE)-conjugated K^b/ANYNFTLV pentamer, and the CD8 was stained by the PerCP-Cy5.5-conjugated anti-CD8 monoclonal antibody (mAb). The number of $ANYNFTLV^+$, $CD8^+$ T cells per 10^6 splenic lymphocytes was calculated. Data represent the mean ± standard deviation of three to nine mice in each group. *, $P < 0.05$ and **, $P < 0.01$ determined by the Student's *t*-test. (C) The representative data of the FACS analyses for detecting K^b/ANYNFTLV pentamer-reactive or non-reactive $CD8^+$ T cells. The $ANYNFTLV^+$, $CD8^+$ T cells or $ANYNFTLV^-$, $CD8^+$ T cells were stained with the APC-conjugated anti-CD44 mAb and the FITC-conjugated anti-CD62L mAb. T_{CM}: T cells of central memory phenotype, T_{EM}: T cells of effector memory phenotype. (D) The representative data of FACS analyses for detection of “regulatory” cell populations. After collecting immune splenocytes, the FITC-conjugated anti-TCRb chain mAb, the PE-conjugated anti-NK1.1 mAb, the PE-conjugated anti-CD122 mAb, the PerCP-Cy5.5-conjugated anti-CD4 mAb and the PerCP-Cy5.5-conjugated anti-CD8 mAb were used to stain immune cells for the identification of $CD4^+CD25^+$ T cells, natural killier (NK) T cells and $CD8^+CD122^+$ T cells. (E) The representative data of FACS analyses for detection of a K^b/ANYNFTLV pentamer-reactive, $CD122^{dull}$ cell population. The TCR was stained with the PE-conjugated K^b/ANYNFTLV pentamer, the PerCP-Cy5.5-conjugated anti-CD8 mAb, the APC-conjugated anti-CD122 mAb and the FITC-conjugated anti-CD62L mAb. The mean fluorescence intensity (MFI) of CD122 staining was calculated by gating the $ANYNFTLV^+$, $CD8^+$ cells. The single re-MVA immunized mice were also included for this experiment. All of the experiments were repeated at least twice for the confirmation of reproducibility. The use of control Ad and control MVA which are not expressing the epitope did not induce the ANYNFTLV-specific immune responses (data not shown).

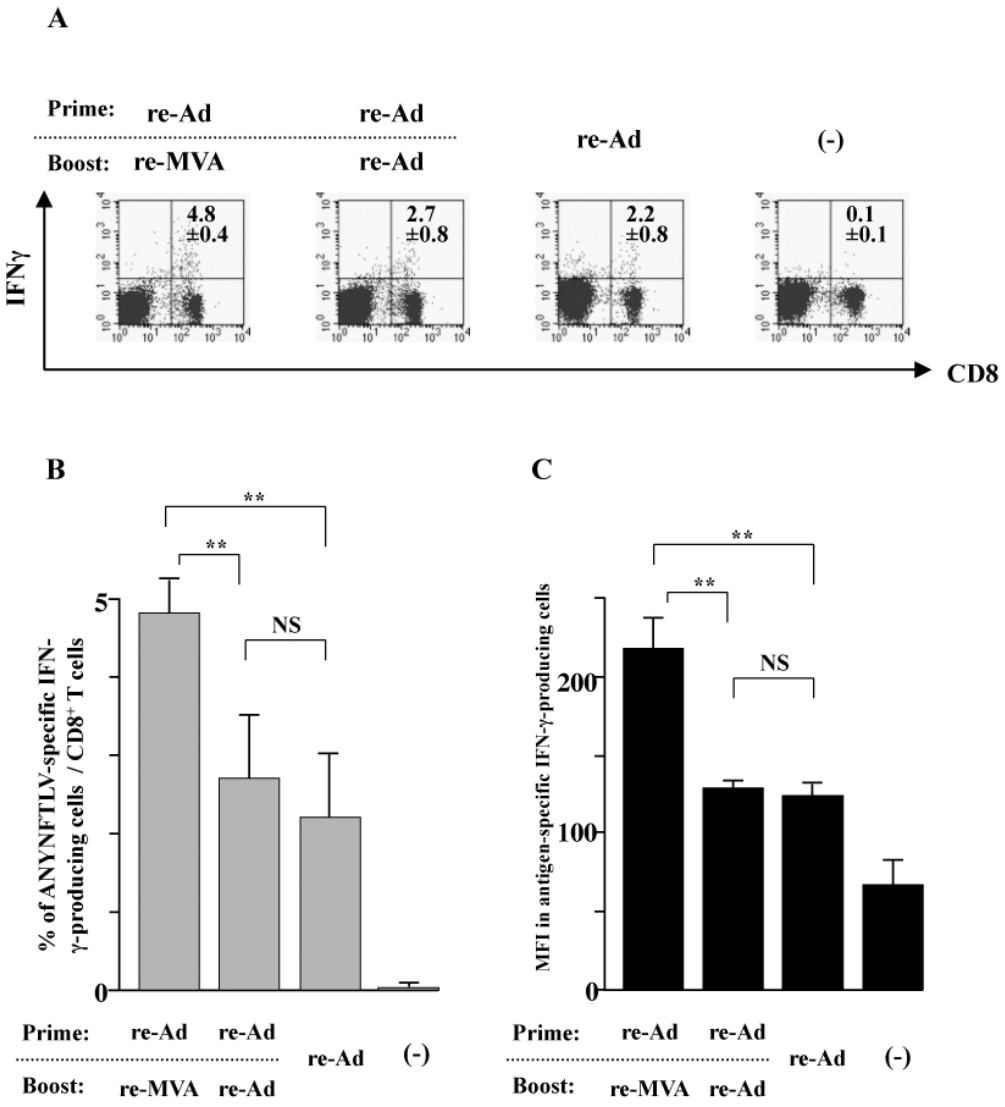
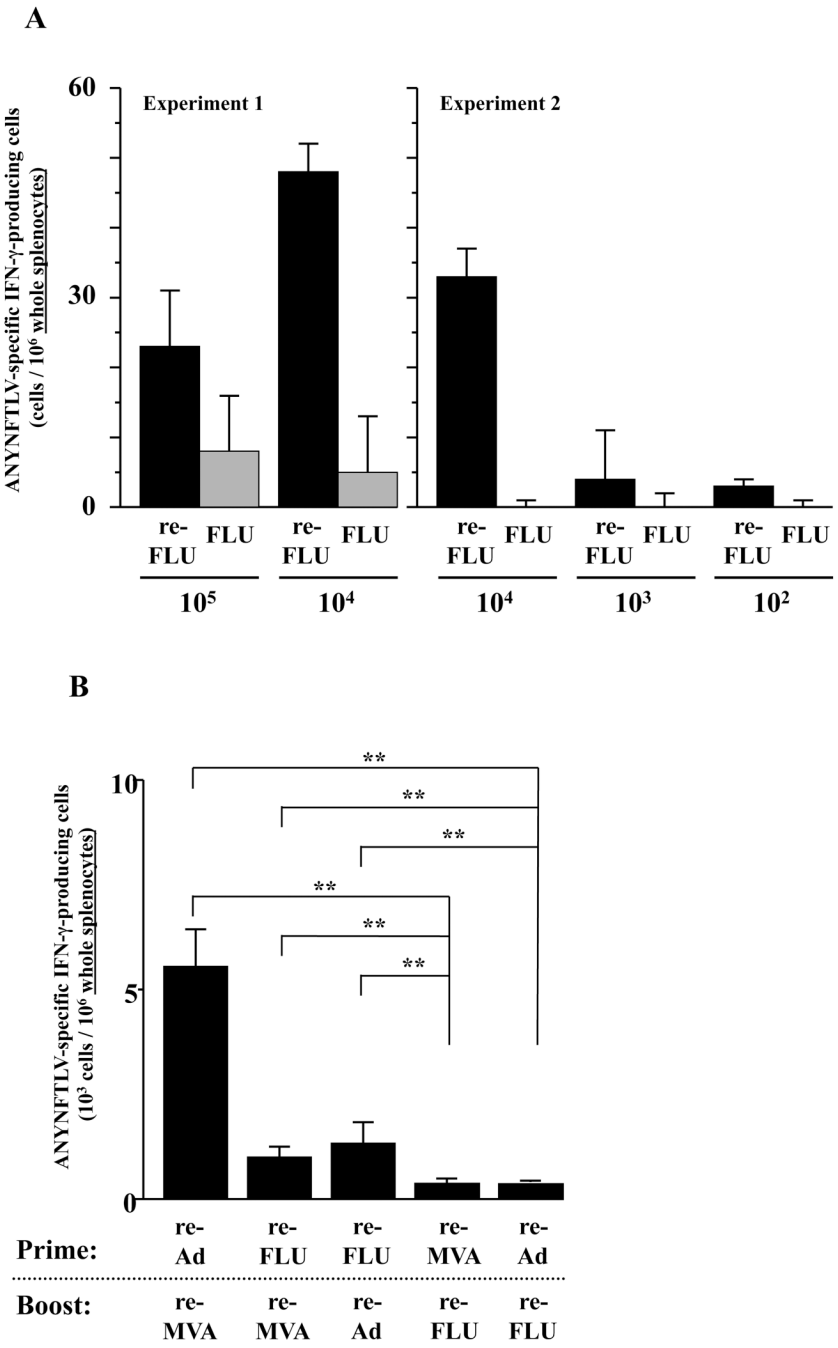


Fig. 2.

The intracellular IFN-γ staining of the ANYNFTLV, a peptide derived from *Trypanosoma cruzi* surface antigen-specific $CD8^+$ T cells induced by the use of recombinant adenovirus (re-Ad) and/or recombinant vaccinia virus (re-MVA) for vaccination. (A) The representative data of intracellular IFN-γ staining of ANYNFTLV-specific $CD8^+$ T cells derived from the re-Ad and / or re-MVA-immunized mice. B6 mice were first primed with 5×10^7 plaque forming units (PFU) of re-Ad and then were given a booster immunization 14 days later with 5×10^7 PFU of either re-Ad or re-MVA. The single re-Ad immunized and non-immunized mice were included as controls. The mice were sacrificed 12 days after the last immunization, and spleens were removed. The freshly-isolated splenocytes were subjected to the FACS analyses for the intracellular IFN-γ^+ staining in response to the ANYNFTLV-pulsed EL-4 cells. The cell culture condition for the assays was described in the Materials and methods. The number of IFN-γ^+ cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ^+ cells that appeared against peptide-pulsed EL-4. (B) The percentage of IFN-γ^+ cells among total $CD8^+$ T cells was calculated. Data represent the mean ± S.D. of three mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. “NS” means “not statistically significant”. (C) The mean fluorescence intensity (MFI) of ANYNFTLV-responding IFN-γ^+ cells was

calibrated by gating cells which were both IFN-γ-positive and CD8-positive. Data represent the mean ± S.D. of three mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. “NS” means “not statistically significant”.



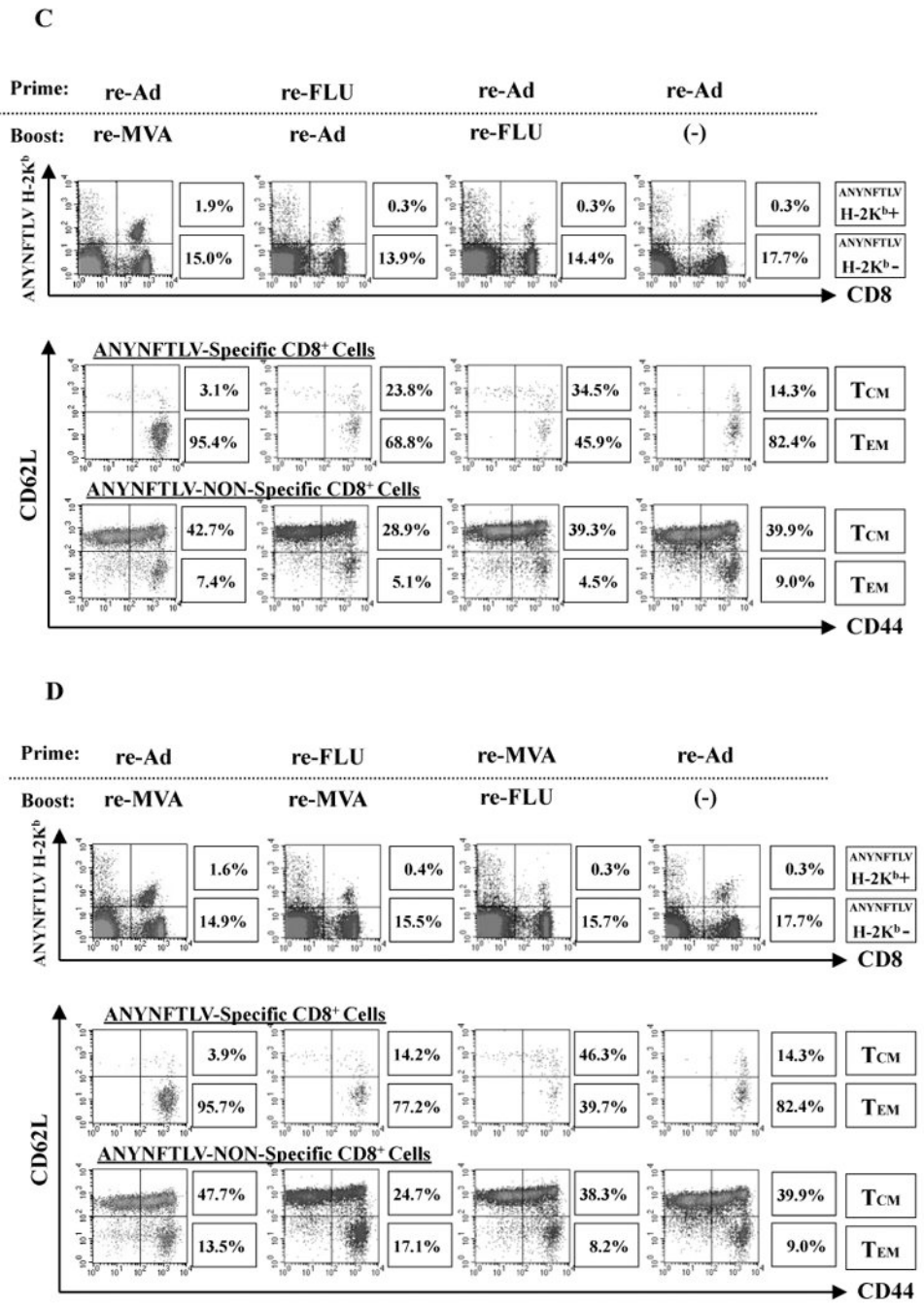
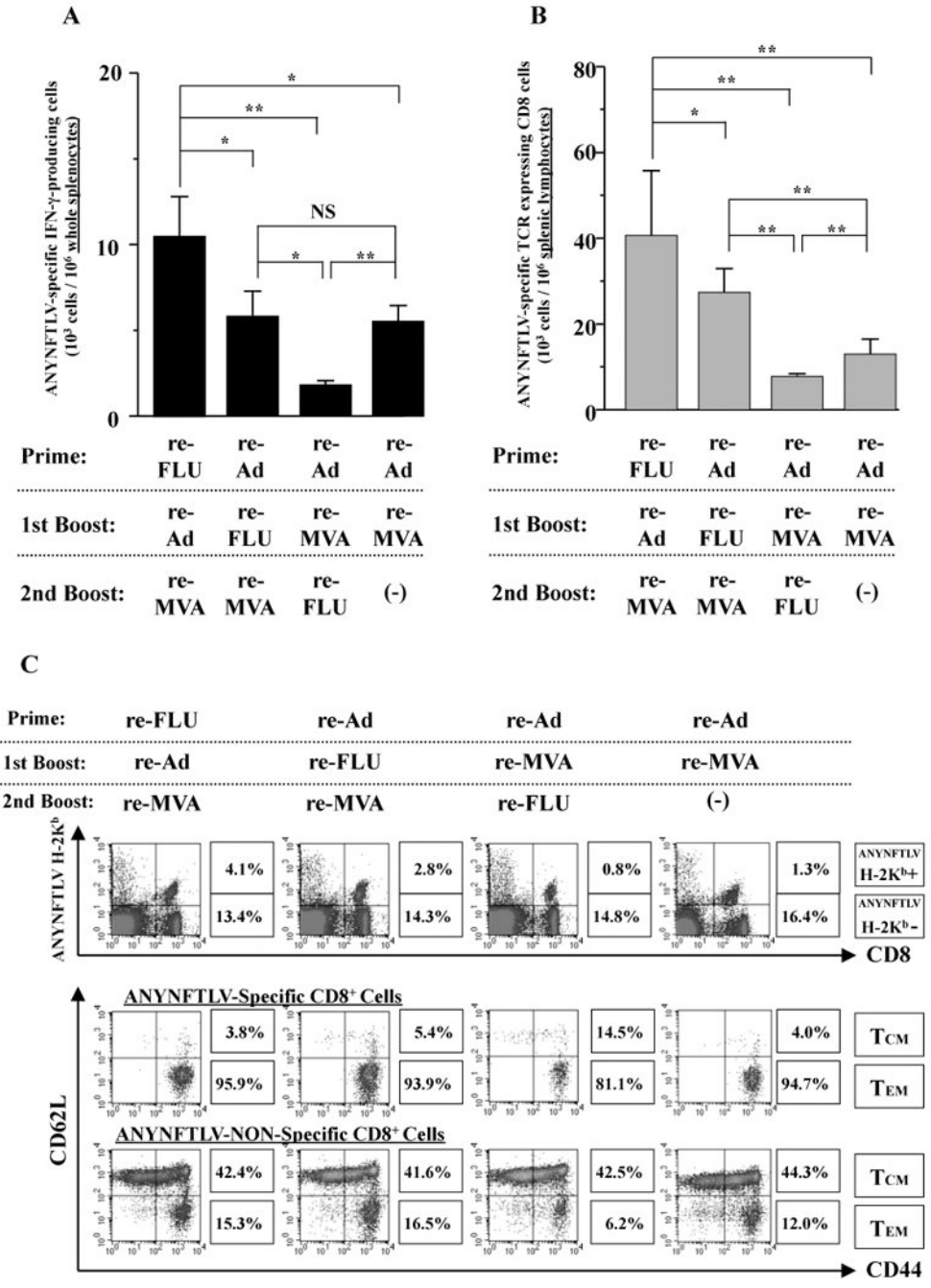


Fig. 3.

Characterization of recombinant influenza virus (re-FLU) for the induction of antigen-specific $CD8^+$ T cells. (A) B6 mice were immunized with either re-FLU or control FLU at four different immunizing plaque forming unit (PFU) doses. They were sacrificed 12 days (Experiment 1) or 14 days (Experiment 2) after the 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, a peptide derived from *Trypansoma cruzi* surface antigen, peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells × 10^6 was counted 22 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. (B) B6

mice were first primed with 1×10^4 PFU of re-FLU, 5×10^7 PFU of recombinant adenovirus (re-Ad) or 5×10^7 PFU of recombinant vaccinia virus (re-MVA). The same immunization doses of each virus were given to the mice as booster immunizations 14 days later. The mice were sacrificed 12 days after the last 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 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 ± S.D. of three mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. (C, D) The representative data of the FACS analyses for detection of K^b/ANYNFTLV pentamer-reactive or non-reactive $CD8^+$ T cells. Immune splenocytes described in (B) were subjected to the flow cytometory analyses for the detection of K^b/ANYNFTLV pentamer-reactive $CD8^+$ T cells. The T-cell receptor (TCR) was stained by the phycoerythrin (PE)-conjugated K^b/ANYNFTLV pentamer, and the CD8 was stained by the PerCP-Cy5.5-conjugated anti-CD8 monoclonal antibody (mAb). The ANYNFTLV$^+$, $CD8^+$ T cells or ANYNFTLV$^-$, $CD8^+$ T cells were stained with the APC-conjugated anti-CD44 mAb and the FITC-conjugated anti-CD62L mAb. T_{CM}: T cells of central memory phenotype, T_{EM}: T cells of effector memory phenotype. The use of control Ad, control MVA and control FLU which do not express the epitope did not induce the ANYNFTLV-specific immune responses (data not shown). All of the experiments were repeated at least twice for the confirmation of reproducibility.



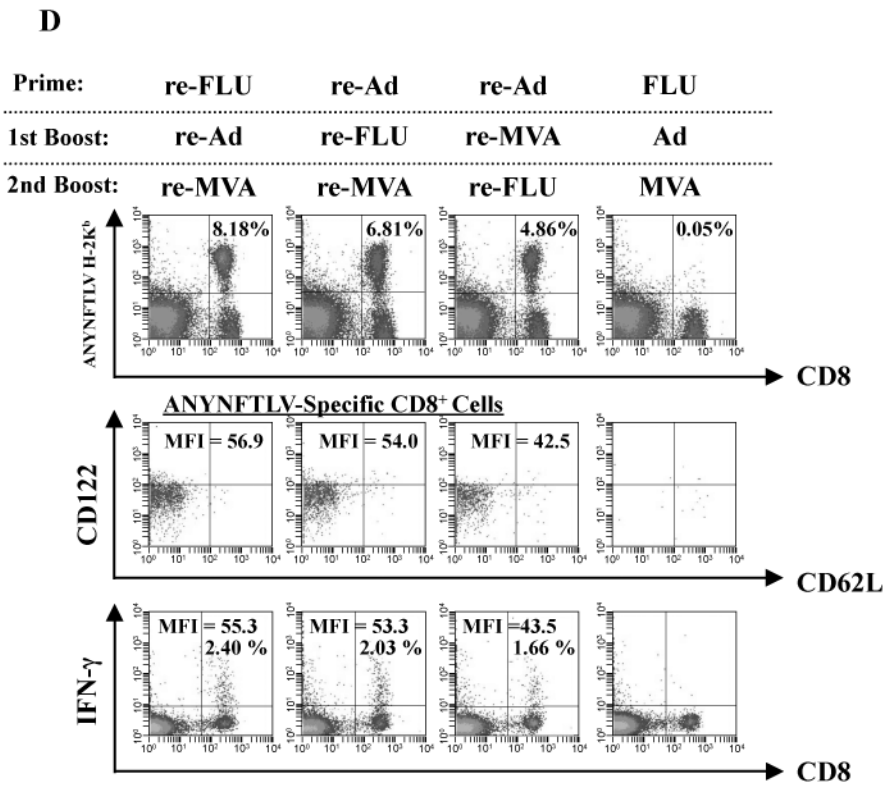


Fig. 4.

Phenotypical and immunological features of ANYNFTLV, a peptide derived from *Trypanosoma cruzi* surface antigen-specific $CD8^+$ T cells induced by triple virus vector immunization. B6 mice were first primed with 1×10^4 plaque forming units (PFU) of recombinant influenza virus (re-FLU) or 5×10^7 PFU of recombinant adenovirus (re-Ad), followed by booster immunizations 14 days later with 1×10^4 PFU of re-FLU, 5×10^7 PFU of re-Ad or 5×10^7 PFU of recombinant vaccinia virus (re-MVA). They were further given second booster immunizations 14 days later with 1×10^4 PFU of re-FLU, or 5×10^7 PFU of re-MVA. (A) The mice were sacrificed 12 days after the last immunization, and 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 $\times 10^6$ was counted 22 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 ± S.D. of three to nine mice in each group. *, $P < 0.05$ and **, $P < 0.01$ determined by the Student's *t*-test. “NS” means “not statistically significant”. (B) The same immune splenocytes in (A) were subjected to the flow cytometory (FCM) analyses for detection of K^b/ANYNFTLV pentamer-reactive $CD8^+$ T cells. The T-cell receptor (TCR) was stained by the phycoerythrin (PE)-conjugated K^b/ANYNFTLV pentamer, and the CD8 was stained by the PerCP-Cy5.5-conjugated anti-CD8 monoclonal antibody (mAb). The number of $ANYNFTLV^+$, $CD8^+$ T cells per 10^6 splenic lymphocytes was calculated. Data represent the mean ± S.D. of three to nine mice in each group. *, $P < 0.05$ and **, $P < 0.01$ determined by the Student's *t*-test. (C) The representative data of the FACS analyses for detection of K^b/ANYNFTLV pentamer-reactive or non-reactive $CD8^+$ T cells. The $ANYNFTLV^+$, $CD8^+$ T cells or $ANYNFTLV^-$, $CD8^+$ T cells were stained with the APC-conjugated anti-CD44 mAb and the FITC-conjugated anti-CD62L mAb. T_{CM}: T cells of central memory phenotype, T_{EM}: T cells of effector memory phenotype. (D) The mice were sacrificed 14 days after the last immunization, and spleens were removed. The representative data of the

FACS analyses showed the detection of K^b/ANYNFTLV pentamer-reactive, $CD122^{dull}$ cell population and the IFN-γ^+, $CD8^+$ T cell population. The TCRs were stained with the PE-conjugated K^b/ANYNFTLV pentamer, the PerCP-Cy5.5-conjugated anti-CD8 mAb, the APC-conjugated anti-CD122 mAb and the FITC-conjugated anti-CD62L mAb. The MFI of CD122 staining was calculated by gating the ANYNFTLV$^+$, $CD8^+$ cells. The same immune splenocytes were also subjected to the FACS analyses for the detection of intracellular IFN-γ^+ cells in response to the ANYNFTLV-pulsed EL-4 cells. The cell culture condition for the assays was described in the Materials and methods. The number of IFN-γ^+ cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ^+ cells that appeared against peptide-pulsed EL-4. The mean fluorescence intensity (MFI) of ANYNFTLV-responding IFN-γ^+ cells was calibrated by gating cells which were both IFN-γ-positive and CD8-positive. The use of control Ad, control MVA and control FLU which do not express the epitope did not induce the ANYNFTLV-specific immune responses (data not shown). All the experiments were repeated at least twice for the confirmation of reproducibility.

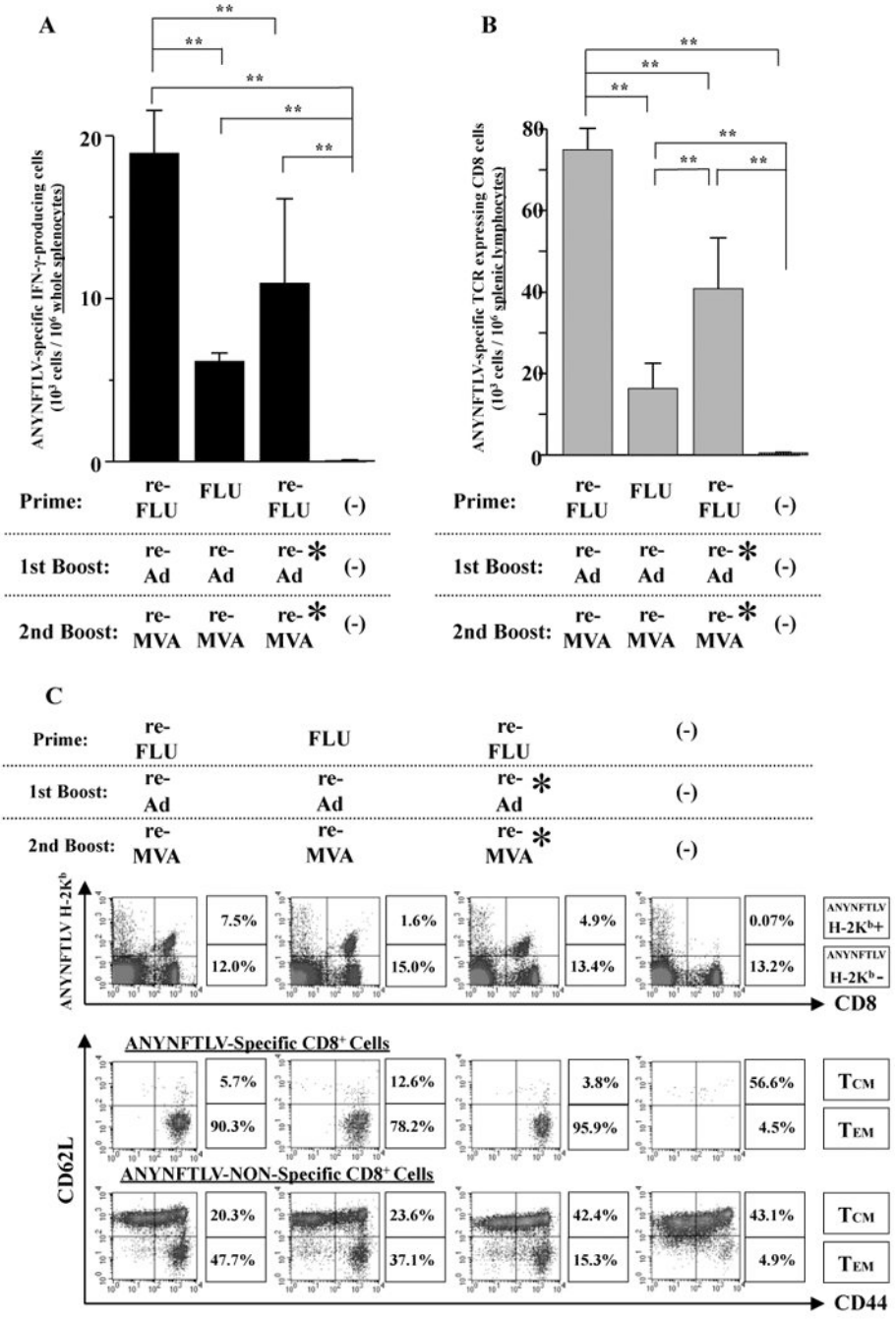
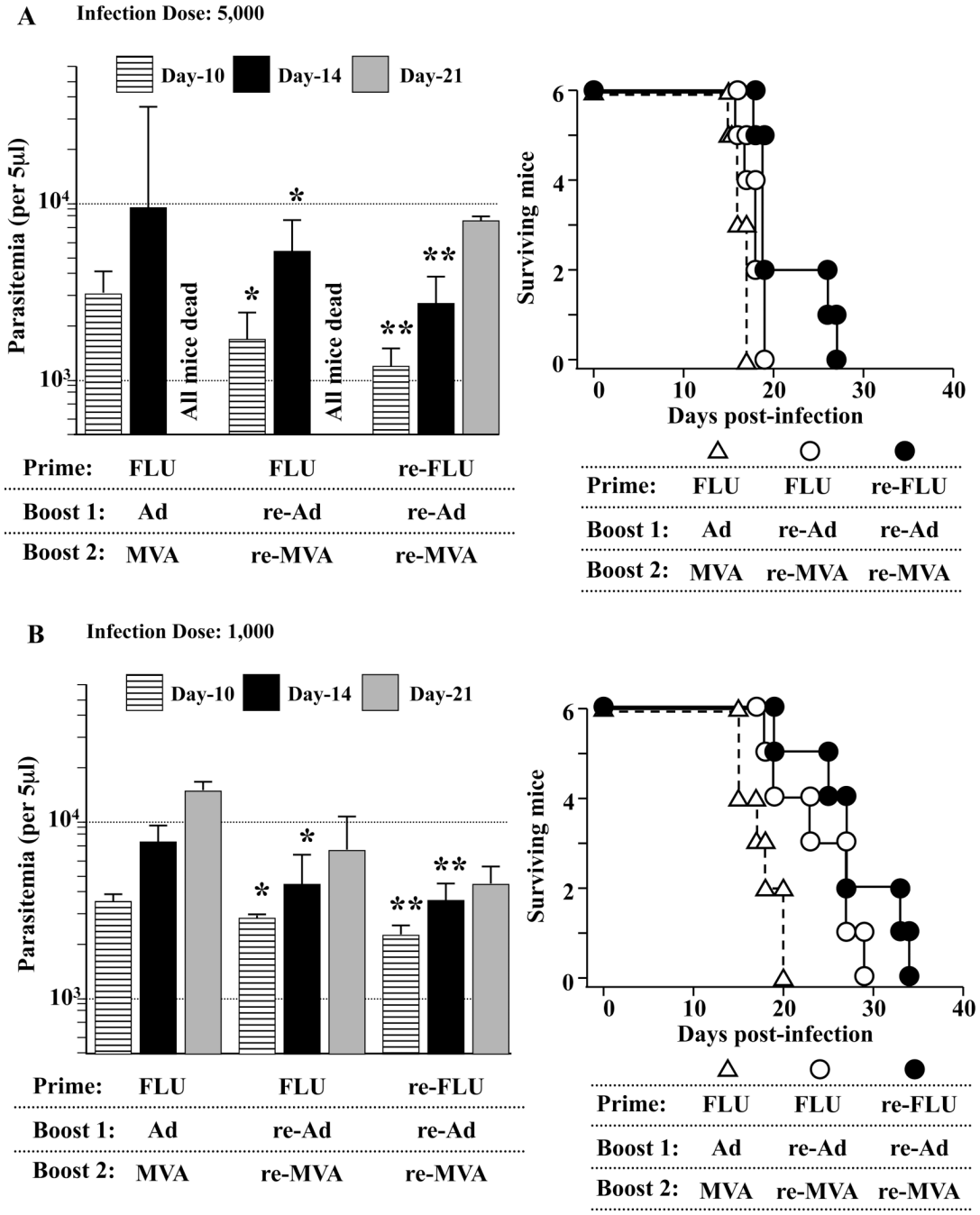


Fig. 5.

The impact of the increase in the number of immunizing doses of recombinant adenovirus (re-Ad) and recombinant vaccinia virus (re-MVA) on the induction of ANYNFTLV, a peptide derived from *Trypanosoma cruzi* surface antigen-specific $CD8^+$ T cells, both the quantity and their memory phenotype. B6 mice were first primed with 1×10^4 plaque forming units (PFU) of either recombinant influenza (re-FLU) or the control FLU, and then were given a booster immunization 14 days later with either 5×10^7 PFU of re-Ad (shown as re-Ad*) or 5×10^8 PFU of re-Ad. The mice were further immunized 14 days later with either 5×10^7 PFU of re-MVA (shown as re-MVA*) or 5×10^8 PFU of re-MVA. The mice were sacrificed 12 days after the last immunization and spleens were removed. (A) 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 was counted 22 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 ± S.D. of three to nine mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. (B) The splenocytes were also subjected to the flow cytometory (FCM) analyses for detection of K^b/ANYNFTLV pentamer-reactive $CD8^+$ T cells. The T-cell receptor (TCR) was stained by the phycoerythrin (PE)-conjugated K^b/ANYNFTLV pentamer, and the CD8 was stained by the PerCP-Cy5.5-conjugated anti-CD8 monoclonal antibody (mAb). The number of $ANYNFTLV^+$, $CD8^+$ T cells per 10^6 splenic lymphocytes was calculated. Data represent the mean ± S.D. of three to nine mice in each group. **, $P < 0.01$ determined by the Student's *t*-test. (C) The representative data of the FACS analyses for detectionn of K^b/ ANYNFTLV pentamer-reactive or non-reactive $CD8^+$ T cells. The $ANYNFTLV^+$, $CD8^+$ T cells or $ANYNFTLV^-$, $CD8^+$ T cells were stained with the APC-conjugated anti-CD44 mAb and the FITC-conjugated anti-CD62L mAb. T_{CM}: T cells of central memory phenotype, T_{EM}: T cells of effector memory phenotype. All the experiments were repeated at least twice for the confirmation of reproducibility.



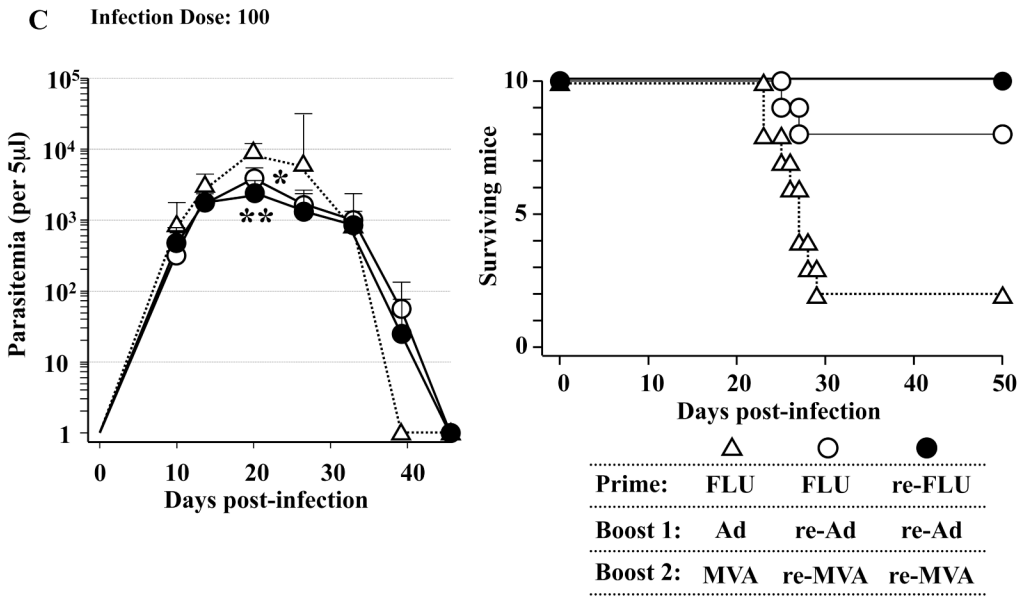


Fig. 6.

The immunological efficacy of a triple virus vector vaccination regimen using three different recombinant viruses. B6 mice were primed with 4×10^4 plaque forming units (PFU) of either recombinant influenza (re-FLU) or control FLU. Fourteen days later, the mice were immunized with 5×10^8 PFU of either recombinant adenovirus (re-Ad) or control Ad. Then, 14 days after the first booster immunization, mice were given a second booster immunzation with either 5×10^8 PFU of recombinant vaccinia virus (re-MVA) or 5×10^8 PFU of control MVA. Each experimental group includes six to 10 mice. They were infected with 5,000 (A), 1,000 (B) or 100 (C) of Tulahuen strain of *Trypanosoma cruzi* blood-form trypomastigotes 14 days (A) or 21 days (B, C) after the last immunization. The number of parasites in 5 µl of peripheral blood (parasitemia) was counted at 10, 14, 21, 28, 35, 42 and 49 days p.i. *, $P < 0.05$ and **, $P < 0.01$ determined by the Student's *t*-test compared with the parasitemia of FLU/Ad/MVA-immunized mice. Survival was monitored daily. (A) The survival of re-FLU/re-Ad/re-MVA-immunized mice (●) was significantly different compared with either the survival of FLU/re-Ad/re-MVA-immunized mice (○) ($P < 0.05$) or the survival of FLU/Ad/MVA-immunized mice (△) ($P < 0.01$). The survival of FLU/re-Ad/re-MVA-immunized mice (○) was significantly different compared with the survival of FLU/Ad/MVA-immunized mice (△) ($P < 0.05$). (B) The survival of re-FLU/re-Ad/re-MVA-immunized mice (●) was significantly different compared with the survival of FLU/Ad/MVA-immunized mice (△) ($P < 0.01$). The survival of FLU/re-Ad/re-MVA-immunized mice (○) was significantly different compared with the survival of FLU/Ad/MVA-immunized mice (△) ($P < 0.05$). (C) All of the re-FLU/re-Ad/re-MVA-immunized mice (●) survived the *T. cruzi* infection. The survival of FLU/re-Ad/re-MVA-immunized mice (○) was significantly different compared with the survival of FLU/Ad/MVA-immunized mice (△) ($P < 0.01$).