



Research Article

Potent T cell Responses Induced by Single DNA Vaccine Boosted with Recombinant Vaccinia Vaccine*

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Plasmid DNA, an effective vaccine vector, can induce both cellular and humoral immune responses. However, plasmid DNA raises issues concerning potential genomic integration after injection. This issue should be considered in preclinical studies. Tiantan vaccinia virus (TV) has been most widely utilized in eradicating smallpox in China. This virus has also been considered as a successful vaccine vector against a few infectious diseases. Potent T cell responses through T-cell receptor (TCR) could be induced by three injections of the DNA prime vaccine followed by a single injection of recombinant vaccinia vaccine. To develop a safer immunization strategy, a single DNA prime followed by a single recombinant Tiantan vaccinia (rTV) AIDS vaccine was used to immunize mice. Our data demonstrated that one DNA prime/rTV boost regimen induced mature TCR activation with high functional avidity, preferential T cell V β receptor usage and high sensitivity to anti-CD3 antibody stimulation. No differences in T cell responses were observed among one, two or three DNA prime/rTV boost regimens. This study shows that one DNA prime/rTV boost regimen is sufficient to induce potent T cell responses against HIV.

HIV; Vaccine; T cell responses; Prime-boost regimen

The prime-boost strategies are promising AIDS vaccine methods for inducing potent immune responses (Excler J L, et al., 1997; Ramshaw I A, et al., 2000). Recently, this strategy has shown modest efficacy in preventing HIV infection by 31.2% compared with placebo in Thailand among more than 16,000 volunteers (Rerks-Ngarm S, et al., 2009). Novel vaccination approaches, such as DNA vaccines and recombinant viral vector-based vaccines,

have further expanded the scope of prime/boost strategies (Schneider J, et al., 1998; Hel Z, et al., 2001; Shiver J W, et al., 2002).

Wolff et al. first demonstrated that naked DNA plasmids can be taken up and expressed in mouse skeletal muscle cells (Wolff J A, et al., 1990). Since then, DNA injection has become a useful technique for expressing foreign proteins in mammalian cells. DNA vaccines are advantageous for their stability, *in vivo* expression which ensures that protein more closely resembles normal eukaryotic structure, with accompanying post-translational modification (Alarcon J B, et al., 1999), and antigen presentation by both MHC class I and MHC class II molecules (Montgomery D L, et al., 1997; Robinson H L, et al., 2000). The main safety concern for DNA vaccines is plasmid integration. To date, few studies have been undertaken on the subject and most have concluded that there were no plasmid genomic integrations (Martin T,

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et al., 1999; Ledwith B J, et al., 2000; Vilalta A, et al., 2005). To detect rare DNA integration events, repeat-anchored integration capture (RAIC)-PCR had been developed (Wang Z, et al., 2004). Random integrations of plasmid DNA into genomic DNA were detected (17 copies/ μg genomic DNA, 50 μg plasmid inoculation) after intramuscular injection (Wang Z, et al., 2004). Therefore, efforts should still be made to enhance the safety of DNA vaccines in preclinical studies, such as reducing the frequency of immunization.

Recombinant vaccinia is considered as one of the most promising viral vectors because of its ability to induce long-lasting cellular responses against foreign antigen (Schneider J, et al., 1998; Hel Z, et al., 2001; Shiver J W, et al., 2002). In China, Tiantan vaccinia virus had most widely been utilized to eradicate smallpox (Zhao K, et al., 1995). Recombinant Tiantan vaccinia virus (rTV) has also been used as a vaccine vector against Epstein-Barr Virus (EBV) (Gu S, et al., 1991) and Hepatitis A virus (HAV) (Guo K J, et al., 1992) in human trials.

The DNA prime/rTV boost regimen reported previously consisted of a series of three vaccinations with DNA and a single boost with rTV (Ren L, et al., 2006; Huang X, et al., 2006). In this study, we explored a safer and effective DNA prime/rTV boost system, single DNA priming followed by a single rTV booster, for potential clinical trial as an AIDS vaccine.

MATERIALS AND METHODS

Immunization of BALB/c mice

Female BALB/c mice (H-2d, 6 weeks old, 18-22 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College. 100 μg of purified plasmid DNA (SV1.0- cn_{54}gag or SV1.0) was inoculated once, twice or three times intramuscularly into the tibialis anterior as scheduled (Fig. 1A). The SV1.0 vector (pDRVISV1.0) carries a kanamycin resistance gene and a 72-bp element of the SV40 enhancer. Thereafter, all mice were immunized intramuscularly with 1×10^7 pfu recombinant Tiantan Vaccinia-vector vaccines (rTV- cn_{54}gag or rTV) at three weeks after the last DNA inoculation. The mice were sacrificed at three weeks after the last inoculation. Splenocytes were freshly isolated for Elispot, cell surface and intracellular staining. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the China CDC animal facility and were performed in accordance with relevant

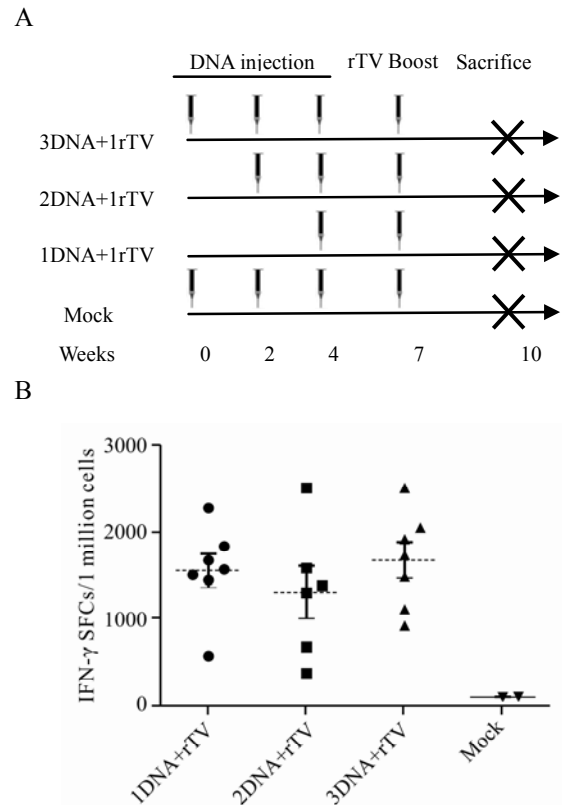


Fig. 1. A: T cell immune responses induced by DNA prime/rTV boost regimens. All BALB/c mice were divided into four groups. A single DNA prime followed by a single rTV boost (1DNA+rTV) were injected at week 4 and week 7; two DNA primes followed by a single rTV boost (2DNA+rTV) were injected at week 2, 4 and 7; three DNA primes followed by a single rTV boost (3DNA+rTV) and Mock (SV1.0+752-1) were injected at week 0, 2, 4 and 7. All mice were sacrificed three weeks after the last inoculation. B: The T cell immune response was quantified with the IFN- γ ELISPOT assay with HIV-1 cn_{54} Gag peptide AMQMLKETI. Spot-forming cells (SFCs) were defined as the average amount of spots in duplicate wells per 10^6 cells. Cells from the mock-immunized mice generated <100 SFCs/ 10^6 splenocytes. n.s. not significant ($p > 0.05$).

guidelines and regulations.

IFN- γ ELISPOT assay and the functional avidity assay

IFN- γ ELISPOT plates (BD Biosciences) were coated with purified anti-mouse IFN- γ at a concentration of 5 $\mu\text{g}/\text{mL}$, incubated at 4 $^{\circ}\text{C}$ overnight, then washed once with RPMI1640 containing 10% FBS and 1% Penicillin-Streptomycin-L-glutamine and finally blocked for two hours at room temperature. Mouse splenocytes (5×10^5) were added to wells in duplicate. Cells were stimulated with 5 $\mu\text{g}/\text{mL}$ of HIV-1 cn_{54} Gag peptide (Dd-restricted CTL epitope AMQMLKETI). This peptide (Gag 49) was selected by peptides pools using HIV-1 consensus C peptide complete

sets and induced greater than 15% of the T-cell immune response induced by the whole Gag peptide pool in BALB/c mice (Xu J, et al., 2006). For the functional avidity assay, T cells were stimulated with a ten-fold serial dilution of antigenic peptide. The positive control was stimulated with PMA (50 ng/mL) and Ionomycin (1 μ g/mL). Plates were washed three times with PBS-T prior to one-hour incubation with biotinylated anti-mouse IFN- γ antibody, followed by the addition of streptavidin-HRP for one hour at 37 °C. Plates were washed again and developed with 100 μ L of AEC substrate solution for 20 min. IFN- γ spots were analyzed by an automated ELISPOT plate reader (ImmunoSpot, C.T.L). Spot-forming cells (SFCs) were defined as the average number of spots in duplicate wells per 10^6 cells. The extent of T cell functional avidity was calculated by comparing the value of SFCs at varying peptide concentrations to the amount of SFCs at 5 μ mol/L peptide concentration.

T cell surface and intracellular staining

To detect the affinity of MHC-I HIV peptides and CD8+ T cells, two million splenocytes were stained for 10 min on ice with 10 μ L of APC conjugated Pentamer bound to the HIV-1 Gag p24 peptide AMQILKDTI (ProImmune, Oxford, UK). After washing, cells were incubated for 10 min at room temperature with CD3 PE and CD8 PerCP (BD Pharmingen).

The TCR V β usage of CD8+ cells was measured by staining stimulated cells with CD3 PerCP, CD8 APC, IFN- γ PE and a panel of 15 FITC conjugated V β antibodies (BD Pharmingen). Brefeldin A (Sigma) was added during the last four hours of incubation (10 μ g/mL).

T cell sensitivity to anti-CD3 antibody were measured by staining cells with Fluo-3, AM and Pluronic F-127 (Biotium) for 30min at 37 °C. Cells were labeled with CD3 PerCP and CD8 APC (BD Pharmingen) for another 10 min at room temperature. The stained cells were stimulated by 10-fold diluted purified anti-mouse CD3e (BD Pharmingen). The concentration of all antibodies was titrated prior to use.

All samples were acquired on a FACSCalibur (BD). Data analysis was performed with FCS Express version 3.

Statistical Analysis

Comparisons between immunization groups were analyzed using the Graphpad Prism software (GraphPad Software, San Diego, CA). The significance of immune responses was calculated with the Student's t test (two tailed, confidence intervals 95%), as indicated by the P

value. Differences with a *P* value < 0.05 were considered to be significant.

RESULTS

HIV-1-specific T cell immune responses

BALB/c mice were immunized with DNA vaccine SV1.0, SV1.0-_{cn54}gag at two-week intervals and boosted with rTV vaccines (Fig. 1A). To determine the HIV-1_{cn54} Gag-specific T cell immune response, splenocytes were stimulated in duplicate with HIV-1_{cn54} Gag peptide AMQMLKETI. T cell immune responses were determined by the IFN- γ -based ELISPOT assay after stimulation. All the results were subtracted from the background resulting from stimulation with no peptide. Results showed that inoculations with a single SV1.0-_{cn54}gag and boosted with a single rTV-_{cn54}gag (1DNA+rTV group) mounted vigorous T cell immune responses (1553 \pm 195 SFCs/ 10^6 splenocytes, N=7), which was comparable to what was induced in the 2DNA+rTV group (1303 \pm 306 SFCs/ 10^6 splenocytes, N=6) (*p*=0.49, Fig. 1B). It was also comparable to what was elicited in the 3DNA+rTV group (1675 \pm 210 SFCs/ 10^6 splenocytes, N=7) (*p*=0.68, Fig. 1B). All responses from HIV Gag immunized groups were higher than those from the mock controls where only marginal SFCs were observed.

Cytotoxic T lymphocyte functional avidity

To determine the functional avidity of cytotoxic T lymphocytes (CTL), splenocytes were stimulated with serial dilutions of HIV-1 CTL epitope AMQMLKETI. The extent of T cell functional avidity was calculated by comparing the value of SFCs at varying peptide concentrations to the amount of SFCs at 5 μ mol/L peptide concentration. The cut-off value was set at 50% of the maximum response at 5 μ mol/L peptide concentration. In 1DNA+rTV immunized mice, five of seven mice had CTL with high functional avidity (\leq 100 nmol/L peptide, Fig. 2B). In other Gag immunized groups, four of seven mice had high CTL functional avidity. In all Gag immunized groups, three of seven mice had very high CTL functional avidity (\leq 50 nmol/L peptide, Fig. 2B). In the mock-immunized group, the SFCs were too low to analyze.

HIV-1-specific MHC-TCR affinity

To determine the HIV-1-specific TCR affinity of T cells induced by vaccination, splenocytes were labeled with MHC-pentamer-HIV-1 epitope AMQMLKETI. No significant difference in percentage of labeled CD8+ cells was detected among three immunized groups (1DNA+rTV 0.33%, 2DNA+rTV

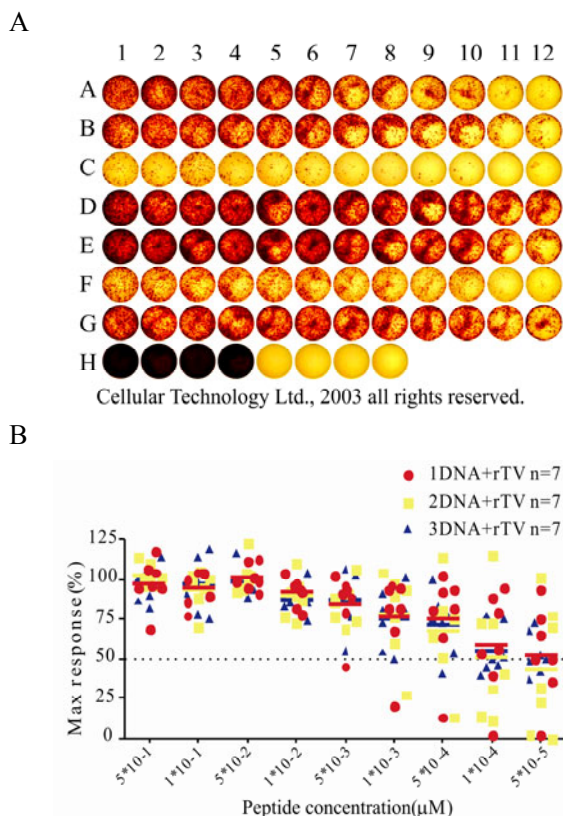


Fig. 2. T cell functional avidity. T cells for functional avidity assay were stimulated with a ten-fold serial dilution of HIV-1 Gag peptide AMQMLKETI. The positive control was stimulated with PMA (50 ng/mL) and Ionomycin (1 μ g/mL). A: A1-G12, Elispot results in different immunized groups; H1-4, Positive control; H5-8, Mock control. B: The extent of T cell functional avidity was calculated by comparing the value of SFCs at varying peptide concentration to the amount of SFCs at 5 μ mol/L peptide concentration. The dotted line in the figure indicates 50% of the maximum responses at 5 μ mol/L peptide concentration. No significant differences were observed in the different immunized groups.

0.41%, 3DNA+rTV 0.39%, Fig. 3). No HIV- specific T cell affinity was observed in the mock- immunized mice.

TCR V β receptor usage

To determine whether different vaccination regimens induced various preferential usages of T cell receptors on T cells, T cells were stained with CD3, CD8, IFN- γ , and a panel of TCR V β receptor antibodies. A very similar preferential TCR receptor usage was observed among the three groups. All immunized mice preferentially used three TCR V β receptors instead of a dozen receptors (Fig. 4B). T cell receptor V β 8.1/8.2 was most preferentially used by greater than 20% in each group (1DNA+rTV, 23.3%; 2DNA+rTV, 21.8%; 3DNA+rTV, 26.8%). T cell receptor V β 8.3 was also preferentially used in each group (1DNA+rTV, 18.2%; 2DNA+rTV, 14.1%; 3DNA+rTV, 15.2%). Furthermore,

receptor V β 3 was used in all three immunized groups (1DNA+rTV, 12.0%; 2DNA+rTV, 11.4%; 3DNA+rTV, 10.5%). All other TCR were used less than 10% (Fig. 4B).

T cell sensitivity to anti-CD3 antibody stimulation

To determine T cell sensitivity to anti-CD3 antibody, splenocytes were stained with Fluo-3, AM and Pluronic F-127. The stained cells were stimulated by 10-fold diluted purified anti-mouse CD3e antibodies. All the T cells isolated from mice immunized with HIV-1 Gag were sensitive to anti-CD3 antibody at a concentration of 5×10^{-1} to 5×10^{-4} μ g/mL. The secretion of calcium lasted longer (75 sec) in 1DNA+rTV group compared to other groups. Only a marginal response was observed in mock controls.

DISCUSSION

Despite many years of ongoing research, no single effective approach has been approved to control HIV transmission. It is believed that an effective HIV vaccine is the only solution for preventing the HIV epidemic. However, the first human trial of a HIV vaccine designed to induce potent cellular responses has shown no protection against infection (Buchinder S P, et al., 2008). Moreover, the vaccine appeared to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector. This failed HIV vaccine trial does not suggest that the HIV vaccines designed to induce T cell responses should be put on hold, because the elite control of HIV infection has been associated with dominant T cell response targeting of Gag and increased functionality of CD4⁺ and CD8⁺ T cells (Betts M R, et al., 2006; Saez-Cirion A, et al., 2007; Potter S J, et al., 2007; Saez-Cirion A, et al., 2009). All these studies suggest that a more thorough assessment of vaccine-induced T cell responses should be considered.

DNA vectors and rTV have shown limited comparative efficacy as a stand-alone platform in primates. In most HIV vaccine studies, DNA plasmids were boosted with viral vectors or recombinant protein, and DNA plasmids were immunized more than once. In our previous study, DNA prime/rTV boost also induced effective T cell responses (Xu J, et al., 2006). To develop a safer DNA prime/rTV boost system, mice were immunized with a single DNA prime followed by a single rTV boost and compared to two or three DNA primes. To evaluate the whole profile of T cell responses induced by these vaccine regimens, cytotoxic T lymphocyte response targeting Gag, functional avidity, MHC affinity, T cell V β receptor usage and sensitivity to anti-CD3 antibody stimulation of T cells separated from

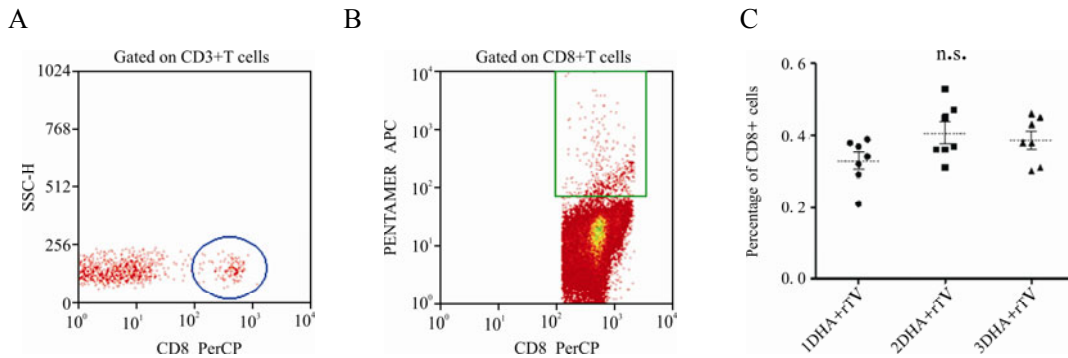


Fig. 3. HIV specific MHC-TCR affinity. A: CD8+ T cells are defined by CD8+ cells from CD3+ splenocytes (Gated on CD3+ T cells). B: The affinity of HIV specific CD8+ T cells was monitored by staining cells with MHC Pentamer - HIV-1 Gag p24 peptide AMQILKDTI. Samples were collected by a FACSCalibur (BD). C: No HIV Gag-specific T cell responses were observed in mock controls.

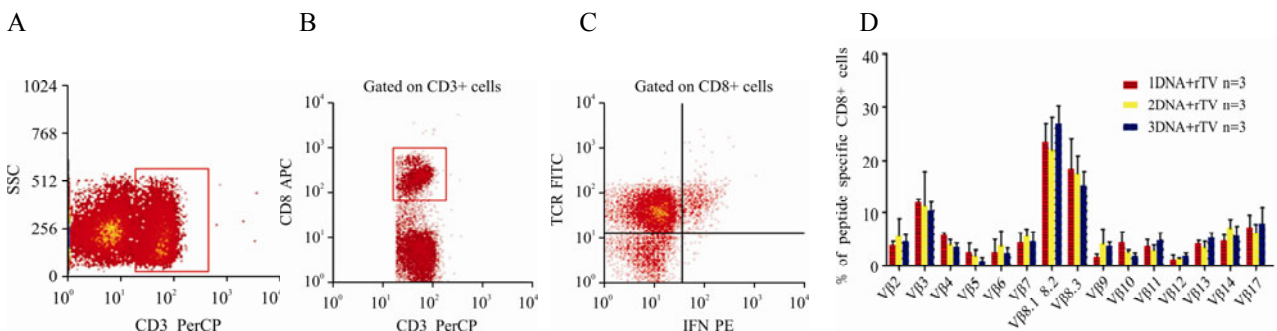


Fig. 4. TCR V beta Receptor usage. The preferential T cell receptor usages were measured after vaccination. A: Splenocytes were stimulated by HIV-1 Gag peptides and stained with anti-CD3. B: CD8+ T cells are CD8+ cells from CD3+ cells (Gated on CD3+ cells). C: IFN- γ and a panel of TCR V β receptor antibodies were used to measure the TCR usage of CD8+ cells. The percentage of usage was calculated by comparing the number of TCR V β +IFN- γ + CD8+ cells to the number of all CD3+CD8+ cells. D: The percentage of 15 TCR V β usages of CD8+ cells in 1DNA+rTV, 2DNA+rTV, and 3DNA+rTV immunized mice (n=3). No significant differences in TCR usage were observed.

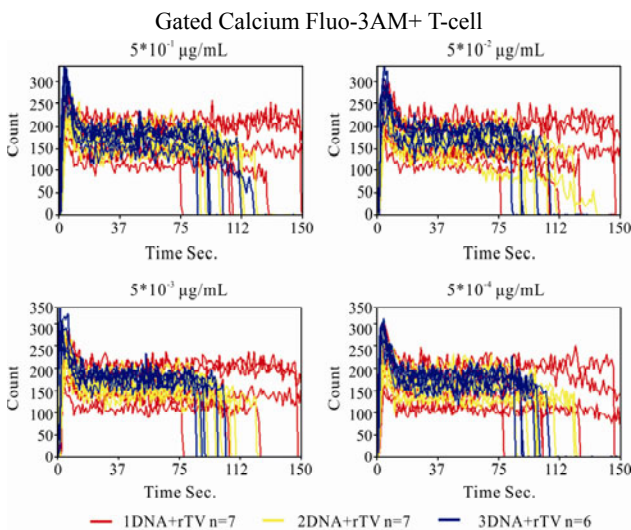


Fig. 5. Ca²⁺ release in Anti-CD3+ Antibody stimulated T cells. Sensitivity of Gag-specific T cells to 10-fold diluted anti-CD3e antibodies. Labeled CD8+ T cells were stained with Fluo-3, AM and Pluronic F-127. The stained cells were stimulated by purified anti-CD3 antibodies. The length of calcium release was measured after stimulation. The response from mock controls was close to the baseline.

immunized mice were measured.

The anti-HIV activity of T cells correlates strongly with the activity of HIV Gag-specific CD8+ T cells in HIV controllers (Saez-Cirion A, et al., 2009). In our study, the cytotoxic T lymphocyte response targeting Gag induced by a single SV1.0_{-cn54gag} and boosted with a single rTV_{-cn54gag} (1DNA+rTV group) was comparable to the response induced by 2 DNA or 3 DNA prime-boost regimens (Fig. 1B). The functional avidity of Gag-specific T cell response was also measured in this study. In all three Gag-immunized groups, 50 nmol/L peptide was adequate to stimulate T cell responses to HIV epitopes (Fig. 2B). These data show that high avidity and activated T cell responses were induced by a single DNA prime/rTV boost regimen. Our data also demonstrate that comparable HIV specific T cell affinity was detected in a single DNA prime/rTV boost regimen in immunized mice (Fig. 3). To evaluate the status of T cells in all Gag immunized mice, the usage of T cell receptors was measured. T cell Receptor V β 8 was the most preferentially used receptor in all three Gag immunized groups. Similar preferential usages

of T cell receptors were demonstrated with different regimens in immunized mice (Fig. 4). T cells activation through TCR results in inducing the release of calcium in T cells. In this study, all T cells from Gag immunized mice were highly sensitive to anti-CD3 antibody stimulation. The sensitivity of T cells to anti-CD3 antibody in the single DNA prime regimen immunized group was more variable and long lasting compared to others (Fig. 5). In all, our results demonstrate that potent and highly qualified T cell immune responses could be induced by one DNA prime/rTV boost regimen. This study improves the safety of DNA vaccines and maintains potent T cell responses induced by vaccines.

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