tion of single $Gag_{206-216}$ epitope-specific CTL responses, $Gag_{206-216}$-specific CTL responses were induced dominantly but $Gag_{241-249}$-specific CTL responses were undetectable at week 2. In contrast, $Gag_{241-249}$-specific CTL responses were induced dominantly at week 2 in group III. Both groups showed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 6. It may be difficult to compare these results with those in group II animals inducing whole Gag antigen-specific CTL and $CD4^+$ T-cell responses before challenge; the group II animals elicited $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 2. Our results indicate that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses and may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as nonvaccine antigens) after viral exposure.

A significant difference between groups III and IV is the pattern of selection of CTL escape mutation. All group IV animals showed rapid selection of a $Gag_{206-216}$-specific CTL escape mutation, while most group III animals showed no *gag* mutation at week 5 but selection of the $Gag_{206-216}$-specific CTL escape mutation later, at week 12. Thus, prophylactic vaccination may affect the patterns of viral genome diversification, possibly accelerating selection of CTL escape mutations. Interestingly, $Gag_{241-249}$-specific CTL mutations were not detected even at week 12 in group III animals, although a previous study observed not only the $Gag_{206-216}$-specific CTL escape mutation (GagL216S), but also a $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in the chronic phase of SIV infection in *90-120-Ia*-positive macaques (9). These results indicate that delayed, naive-derived $Gag_{206-216}$-specific CTL responses, as well as preceding $Gag_{241-249}$-specific CTL responses, exert strong suppressive pressure on SIV replication in group III animals, implying cooperation between vaccine antigen-specific and non-vaccine antigen-specific CTL responses for virus control.

Rapid selection of the $Gag_{206-216}$-specific CTL escape mutation (GagL216S) in group II and delayed selection of this mutation without a detectable $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in group III suggest that the virus with GagL216S (SIVmac239Gag216S) replicates more efficiently than the virus with GagD244E (SIVmac239Gag244E) under both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses. Our previous competition assay did not find a significant difference in viral fitness between these mutant viruses. Possibly, escape of SIVmac239Gag216S from $Gag_{206-216}$-specific CTL pressure may be more efficient than that of SIVmac239Gag244E from $Gag_{241-249}$-specific CTL pressure.

Our analysis revealed that the decline of plasma viral loads from week 3 to week 5 in group II+IV with rapid selection of the GagL216S mutation was significantly less than that in group III without the mutation at week 5, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the former groups around weeks 3 to 5. Even the comparison between groups II and III, both showing dominant $Gag_{241-249}$-specific CTL responses at week 2, revealed a significantly sharper decline in the latter ($P = 0.0087$). Thus, our results suggest three patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL cooperation for virus control after SIVmac239 challenge. First, as observed in group II, dominantly induced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses both work against wild-type SIV replication around week 2, but then a mutant virus escaping from the former CTL responses is selected, and the responses work against this mutant virus replication. Second, as observed in group III, dominantly induced $Gag_{241-249}$-specific CTL responses work against wild-type SIV replication around week 2 and then contribute to virus control, together with delayed, naive-derived $Gag_{206-216}$-specific CTL responses. Third, as observed in group IV, dominantly induced $Gag_{206-216}$-specific CTL responses work against wild-type SIV replication around week 2, but then a mutant virus escaping from $Gag_{206-216}$-specific CTL responses is selected, and delayed, naive-derived $Gag_{241-249}$-specific CTL responses instead work against this mutant virus replication. Viral loads at week 3 in group III looked higher than those in group IV, implying that $Gag_{206-216}$-specific CTL responses may exert a stronger suppressive effect on SIV replication in the acute phase than $Gag_{241-249}$-specific CTL responses. However, viral loads at week 5 in group III looked lower than those in group IV, and the comparison between the two groups showed significantly less decline in the latter ($P = 0.0303$). It is speculated that the third pattern observed in group IV is prone to failure in virus control. Indeed, two of five animals in group IV failed to control SIV replication. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CTLs generated, only several epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (18), and cooperation of these vaccine antigen-specific and non-vaccine antigen-specific CTL responses would be required for viral control. Thus, our results may imply a rationale of inducing escape-resistant, epitope-specific CTL memory by prophylactic AIDS vaccines.

In summary, this study showed dominant induction of vaccine antigen-specific CTL responses and delay in non-vaccine antigen-specific CTL responses in the acute phase of SIV infection, clearly describing the impact of prophylactic vaccination on CTL immunodominance and cooperation after virus exposure. Our results indicate that the patterns of cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses affect virus control and selection of CTL escape mutations. These findings provide great insights into antigen design in the development of a CTL-inducing AIDS vaccine.

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CTL escape and viral fitness in HIV/SIV infection

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Cytotoxic T lymphocyte (CTL) responses exert a suppressive effect on HIV and simian immunodeficiency virus (SIV) replication. Under the CTL pressure, viral CTL escape mutations are frequently selected with viral fitness costs. Viruses with such CTL escape mutations often need additional viral genome mutations for recovery of viral fitness. Persistent HIV/SIV infection sometimes shows replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness. Thus, multiple viral genome changes under CTL pressure are observed in the chronic phase of HIV/SIV infection. HIV/SIV transmission to HLA/MHC-mismatched hosts drives further viral genome changes including additional CTL escape mutations and reversions under different CTL pressure. Understanding of viral structure/function and host CTL responses would contribute to prediction of HIV evolution and control of HIV prevalence.

Keywords: HIV, SIV, MHC, cytotoxic T lymphocyte, escape mutation, viral fitness, capsid

INTRODUCTION

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of HIV and simian immunodeficiency virus (SIV) replication (Borrow et al., 1994; Koup et al., 1994; Matano et al., 1998; Jin et al., 1999; Schmitz et al., 1999; Goulder and Watkins, 2008). CTLs recognize viral antigen-derived peptides (epitopes) presented by major histocompatibility class I (MHC-I) molecules on the surface of viral-infected cells. Under the CTL pressure, viral mutations in and around epitope-coding regions which result in viral escape from CTL recognition are frequently selected with the cost of viral fitness (Phillips et al., 1991; Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). Thus, analysis of structural and functional constraints in viral proteins could facilitate determination of effective CTLs that can limit viral escape options, contributing to immunogen design in development of CTL-inducing AIDS vaccines.

We previously developed an AIDS vaccine using a Sendai virus vector expressing Gag (SeV-Gag), which induces Gag-specific CTL responses efficiently. Our analysis showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* (Matano et al., 2004; Kawada et al., 2008). $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL responses exert a suppressive effect on SIV replication and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag capsid (CA) with viral fitness costs (Kobayashi et al., 2005). Our studies starting with this finding revealed viral genome changes in persistent SIV infection, providing insights into HIV/SIV evolution.

LOSS OF VIRAL FITNESS BY ESCAPE MUTATIONS AND ITS RECOVERY BY COMPENSATORY MUTATIONS

In contrast to the SIVmac239 challenge experiment, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 (Hirsch et al., 1997), which has the same $Gag_{206–216}$ amino acid sequence with SIVmac239. SIVsmE543-3 has a different amino acid (glutamate [E]) from SIVmac239 (aspartate [D]) at Gag residue 205, and this GagD205E change resulted in escape from $Gag_{206–216}$-specific CTL recognition, leading to failure in control of SIVsmE543-3 replication in *90-120-Ia*-positive vaccinees (Moriya et al., 2008).

Theoretically, $Gag_{206–216}$-specific CTL responses can select for either GagD205E or GagL216S mutation. SIVmac239-infected *90-120-Ia*-positive macaques, however, select the latter GagL216S mutation but not GagD205E in a year postchallenge. This suggests a possibility that the GagD205E substitution in SIVmac239 results in larger reduction of viral fitness than GagL216S. Indeed, our analysis *in vitro* revealed much lower replicative ability of the virus with this GagD205E substitution, SIVmac239Gag205E, compared to the wild-type SIVmac239 (Inagaki et al., 2010). On LuSIV cells, which contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, indicating suppression of the early phase of this mutant virus replication.

Further passage of SIVmac239Gag205E-infected culture supernatants *in vitro* found an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag. Interestingly, SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. SIVmac239Gag205E340M showed similar replication kinetics with wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution. Thus, CTL escape mutations resulting in loss of viral fitness could be selected with compensatory mutations. **Figure 1** is a schema indicating the interaction between escape and compensatory mutations.

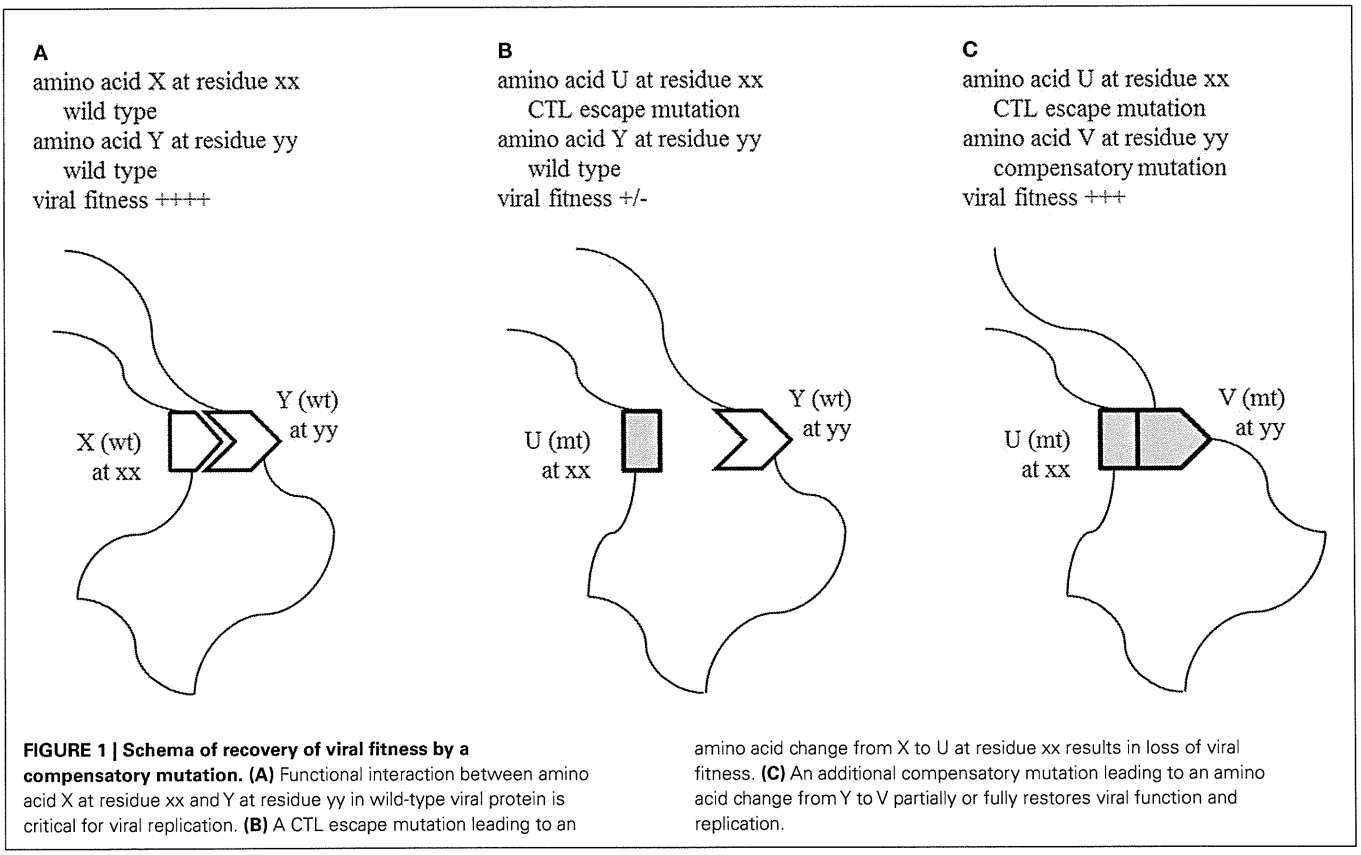

FIGURE 1 | Schema of recovery of viral fitness by a compensatory mutation. (A) Functional interaction between amino acid X at residue xx and Y at residue yy in wild-type viral protein is critical for viral replication. **(B)** A CTL escape mutation leading to an amino acid change from X to U at residue xx results in loss of viral fitness. **(C)** An additional compensatory mutation leading to an amino acid change from Y to V partially or fully restores viral function and replication.

GAG CA INTERMOLECULAR INTERACTION

The Gag CA is comprised of the N-terminal (NTD) and the C-terminal domains (CTD) (Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999). Modeling of CA monomer structure showed that the Gag 205th residue is located in the helix 4 of CA NTD and the 340th is in the loop between helices 10 and 11 of CTD. A possibility of intramolecular contact between Gag residues 205 and 340 is not supported by this modeling. However, CA molecules are known to form hexamer lattice in mature virions (Ganser et al., 1999; Li et al., 2000; Ganser-Pornillos et al., 2007, 2008; Pornillos et al., 2009). Modeling of CA hexamer structure revealed that the Gag 205th residue is located in close proximity to the 340th of the adjacent CA molecule. The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which appeared compensated by GagV340M substitution. Thus, there may be intermolecular interaction between Gag residues 205 and 340 in CA hexamers. This is consistent with our results obtained by viral core stability assay. The core stability was reduced by the GagD205E substitution but recovered by the GagV340M substitution. Loss of viral fitness by GagD205E and its recovery by GagV340M implies a structural constraint for functional interaction between CA NTD and CTD involved in the formation of CA hexamers. In addition to previous reports on intramolecular compensation for loss of viral fitness by CTL escape mutations (Friedrich et al., 2004a; Crawford et al., 2007), our results present evidence indicating intermolecular compensation.

REPLACEMENT OF A CTL ESCAPE MUTATION WITH AN ALTERNATIVE ESCAPE MUTATION TOWARD HIGHER VIRAL FITNESS

As stated above, SIVmac239-infected *90-120-Ia*-positive macaques usually select the $Gag_{206-216}$-specific CTL escape mutation, GagL216S, but not GagD205E in a year postchallenge. After that, however, we found that the GagD205E mutation together with GagV340M became dominant instead of GagL216S in a *90-120-Ia*-positive macaque (Inagaki et al., 2010). In this macaque, neither GagD205E nor GagV340M was detected until week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant until week 123 was undetectable at week 150. Thus, in this animal, SIVmac239Gag216S, whose replicative ability is lower than wild-type SIVmac239 but higher than SIVmac239Gag205E, became dominant under $Gag_{206-216}$-specific CTL pressure in the early phase, while in the later phase, this mutant virus was replaced with SIVmac239Gag205E340M, whose replicative ability is similar with the wild-type. This indicates replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness in the chronic phase, implying persistent $Gag_{206-216}$-specific CTL pressure for more than 2 years after selection of the CTL escape mutation.

MULTIPLE VIRAL GENOME CHANGES UNDER CTL PRESSURE

In another study (Kawada et al., 2006), we observed accumulation of multiple CTL escape mutations in viral genomes in SIV-infected macaques. SeV-Gag-vaccinated animals possessing

MHC-I haplotype *90-120-Ia* elicited $Gag_{206-216}$-specific CTL responses and controlled viral replication with rapid selection of the GagL216S mutation after SIVmac239 challenge. Among these SIV controllers, two animals (V3 and V5) accumulated additional *gag* mutations and showed reappearance of plasma viremia around week 60 postchallenge. Both animals first selected a $Gag_{241-249}$ epitope-specific CTL escape mutation leading to a GagD244E (aspartic acid [D] to glutamic acid [E] at the 244th aa in Gag) substitution, and then, a $Gag_{373-380}$ epitope-specific CTL escape mutation leading to a GagA373T (alanine [A] to threonine [T] at the 373rd) or GagP376S (proline [P] to S at the 376th) substitution during the period of viral control. At the viremia reappearance, SIVmac239Gag216S244E247L312V373T with five *gag* mutations, L216S, D244E, I247L (isoleucine [I] to L at the 247th), A312V (A to V at the 312th), and A373T, became dominant in one of them (V5), and SIVmac239Gag145A216S244E376S with four *gag* mutations leading to V145A (V to A at the 145th), L216S, D244E, and P376S became dominant in the other (V3). These viruses with multiple *gag* mutations showed lower replicative ability *in vitro* than SIVmac239Gag216S carrying single GagL216S mutation. Indeed, SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations had lower replicative ability *in vitro* compared to SIVmac239Gag216S244E373T carrying three *gag* mutations. These results suggest that selection of CTL escape mutations even with viral fitness costs could be advantageous for viral replication *in vivo* under CTL pressure.

SIV TRANSMISSION INTO MHC-MISMATCHED HOSTS DRIVES FURTHER VIRAL GENOME CHANGES

Previous studies (Friedrich et al., 2004b; Kobayashi et al., 2005; Loh et al., 2007) reported reversion of CTL escape mutations in the absence of CTL pressure by transmission of SIVs carrying single escape mutations between MHC-mismatched hosts. SIVs carrying CTL escape *gag* mutations selected in *90-120-Ia*-positive macaques showed lower replicative ability *in vitro*. We then examined *in vivo* replicative ability of those SIVs carrying CTL escape mutations in *90-120-Ia*-negative macaques (Seki et al., 2008). Coinoculation of macaques with SIVmac239GagL216S and SIVmac239Gag216S244E373T resulted in rapid selection of the former; i.e., D244E and A373T mutations were undetectable even in the acute phase, indicating lower replicative ability *in vivo* of the latter carrying three escape mutations than the former. Reversion of L216S was observed in a few months, confirming lower replicative ability *in vivo* of SIVmac239Gag216S than wild-type SIVmac239. Further competition indicated lower replicative ability *in vivo* of SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations than SIVmac239Gag216S244E373T carrying three.

We next examined viral genome changes after challenge of *90-120-Ia*-negative macaques with SIVs carrying multiple CTL escape mutations selected in *90-120-Ia*-positive macaques. Challenge with SIVs carrying five *gag* mutations, L216S, D244E, I247L, A312V, and A373T, resulted in persistent viremia in all four *90-120-Ia*-negative macaques. Two animals exhibited higher viral

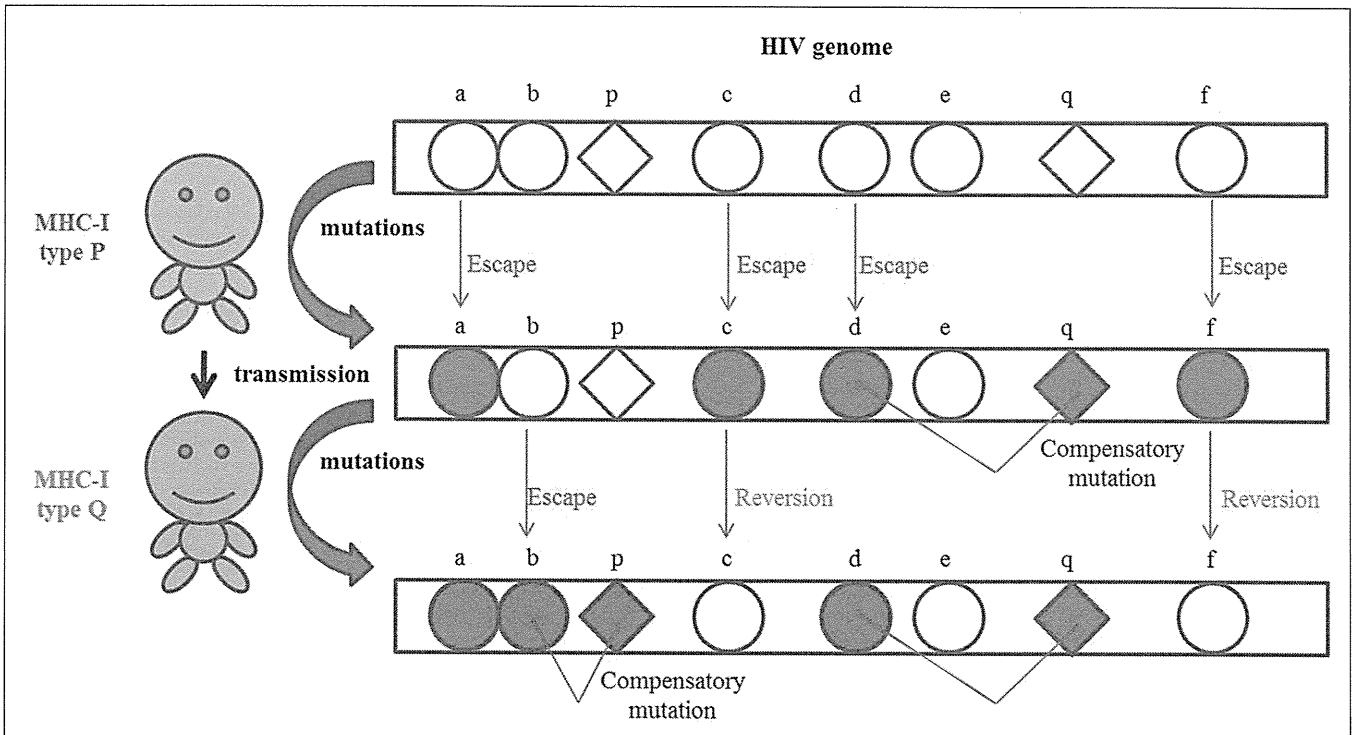

FIGURE 2 | Schema of HIV/SIV transmission resulting in accumulation of multiple viral mutations. Multiple CTL escape mutations resulting in viral fitness costs do not always revert rapidly even in the absence of CTL pressure after their transmission into HLA/MHC-mismatched hosts and such mutants can be transmitted further to other hosts. New escape mutations and compensatory mutations are also observed with transmissions. Thus, CTL affects HIV/SIV evolution in individuals with divergent HLA/MHC polymorphisms.

loads. One of them rapidly developed AIDS at week 18 while the other developed AIDS 2 years postchallenge. The former showed reversion of I247L and A312V but still had three CTL escape mutations, L216S, D244E, and A373T at AIDS onset. The latter showed reversion of four mutations in a year postchallenge, but the A373T mutation remained dominant without reversion until AIDS onset. In the remaining two animals that exhibited lower viral loads, multiple *gag* mutations including L216S and D244E were still dominant without reversion 1 year after challenge.

Thus, in the experiment of challenge with SIVs carrying multiple CTL escape mutations, the reversion of all the mutations was not required for AIDS onset, while transmission with SIVs carrying single CTL escape mutations showed their rapid reversion. This suggests that even HIVs accumulating multiple CTL escape mutations with viral fitness costs can induce persistent viral infection leading to AIDS progression after their transmission into HLA/MHC-mismatched individuals.

The reversion of the L216S mutation was delayed or not observed after challenge with SIVs carrying multiple *gag* mutations, whereas challenge with SIVmac239Gag216S resulted in its reversion in a few months. This may be due to the predominant selection of the reversion of other mutations, compensatory mutations, or to lower viral replication efficiency in the former case. Our results suggest that CTL escape mutations resulting in viral fitness costs may not always revert rapidly after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts, driving further viral genome changes with accumulation of mutations (**Figure 2**). These results provide an important insight into HIV evolution in human individuals with divergent HLA/MHC polymorphisms.

CONCLUDING REMARKS

Cytotoxic T lymphocyte responses exert strong selective pressure on HIV and play a central role in viral evolution (Kaslow et al., 1996; Brander and Walker, 2003; Kiepiela et al., 2004; O'Connor et al., 2004). Correlation of frequencies of viral epitope variants with prevalence of restricting HLA alleles has been shown, indicating HIV adaptation to HLA polymorphisms at a population level (Kawashima et al., 2009). Loss of viral fitness by CTL escape mutations may contribute to HIV control (Martinez-Picado et al., 2006; Schneidewind et al., 2007), but our results indicate the potential of even such HIVs with lower viral fitness to induce AIDS progression. Elucidation of structural constraints of viral antigens for viral function would lead to determination of conserved, escape-resistant epitopes whose mutations largely diminish viral replicative ability (Dahirel et al., 2011), contributing to immunogen design in development of CTL-inducing AIDS vaccines.

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Fluorescent reporter signals, EGFP, and DsRed, encoded in HIV-1 facilitate the detection of productively infected cells and cell-associated viral replication levels

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Flow cytometric analysis is a reliable and convenient method for investigating molecules at the single cell level. Previously, recombinant human immunodeficiency virus type 1 (HIV-1) strains were constructed that express a fluorescent reporter, either enhanced green fluorescent protein, or DsRed, which allow the monitoring of HIV-1-infected cells by flow cytometry. The present study further investigated the potential of these recombinant viruses in terms of whether the HIV-1 fluorescent reporters would be helpful in evaluating viral replication based on fluorescence intensity. When primary CD4+ T cells were infected with recombinant viruses, the fluorescent reporter intensity measured by flow cytometry was associated with the level of CD4 downmodulation and Gag p24 expression in infected cells. Interestingly, some HIV-1-infected cells, in which CD4 was only moderately downmodulated, were reporter-positive but Gag p24-negative. Furthermore, when the activation status of primary CD4+ T cells was modulated by T cell receptor-mediated stimulation, we confirmed the preferential viral production upon strong stimulation and showed that the intensity of the fluorescent reporter within a proportion of HIV-1-infected cells was correlated with the viral replication level. These findings indicate that a fluorescent reporter encoded within HIV-1 is useful for the sensitive detection of productively infected cells at different stages of infection and for evaluating cell-associated viral replication at the single cell level.

Keywords: HIV-1, flow cytometry, EGFP, DsRed, Gag, productive infection

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) interacts with its primary receptor, CD4, and a co-receptor, usually CCR5 or CXCR4, to infect T cells, macrophages, and dendritic cells (Mcclure et al., 1987; Berger et al., 1999; Tsunetsugu-Yokota, 2008). Single cell analysis of HIV-1-infected cells is an essential approach to investigate the differential dynamics of HIV-1 infection and the cellular consequences for each of the HIV-1-targeted cell populations. To monitor HIV-1 infection, a recombinant HIV-1 encoding a reporter luciferase (Luc) gene, or indicator cells transduced with enzymatic reporters such as Luc, β-galactosidase, alkaline phosphatase, and chloramphenicol acetyl transferase, incorporated downstream of the HIV-1 long terminal repeats (LTR) have been widely used (Kar-Roy et al., 2000). However, these reporters require additional substrates or co-factors, and lysis or fixation of cells is required to show reporter activity, which makes the experimental process more complex. In addition, it is difficult to distinguish infected cells from uninfected cells using these reporter assays.

An alternative molecule, green fluorescent protein (GFP) and/or its derivatives, is a powerful reporter that does not require any substrates and co-factors to generate a reporter signal (Chalfie, 1995; Cubitt et al., 1995; Heim et al., 1995). Page et al. (1997) first used a GFP derivative, called enhanced green fluorescent protein (EGFP), as a fluorescent reporter molecule for HIV-1 and showed that infected cells were detectable and, more importantly, distinguishable from uninfected cells using flow cytometry. Furthermore, a red fluorescent protein, DsRed, has been used as an HIV-1 fluorescent reporter (Weber et al., 2006). The main benefit of such recombinant HIV-1 molecules is that the targeted cells do not require any modulation (e.g., transfection) of exogenous reporter genes and, therefore, they allow the characterization of intact HIV-1-infected cells. In most cases of previous recombinant HIV-1 strains, the *nef* gene was replaced with a reporter gene. Therefore, we previously constructed *nef*-intact, replication-competent, recombinant HIV-1 strains encoding either EGFP or DsRed, and showed that CXCR4-tropic X4 and CCR5-tropic R5 viruses replicate differently in CD4+ T cells simultaneously infected with X4 HIV-1 encoding EGFP and R5 HIV-1 encoding

DsRed (Yamamoto et al., 2009). Such recombinant HIV-1 strains encoding a fluorescent reporter gene will be even more valuable because of recent advances in multicolor flow cytometry, which permit more detailed characterization of HIV-1-infected cells.

Flow cytometry is a reliable and convenient method for analysis at the single cell level. Because the transcriptional activity of HIV-1 can be quantitatively monitored in indicator cells according to the fluorescence intensity of an EGFP reporter driven by the HIV-1 LTR (Dorsky et al., 1996; Gervaix et al., 1997; Kar-Roy et al., 2000), we investigated whether the HIV-1-expressing fluorescent reporters EGFP and DsRed would allow the quantitative evaluation of viral replication using a flow cytometer. The results show that a fluorescent reporter signal generated by recombinant HIV-1 strains enables the detection of infected cells at various stages of the viral life cycle.

MATERIALS AND METHODS

CELL PREPARATION

Human peripheral blood samples were collected from healthy donors after written informed consent. Sample collection was approved by the Institutional Ethical Committee of the National Institute of Infectious Diseases (NIID; Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll–Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan) and $CD4^+$ T cells were negatively selected from the PBMCs using an EasySep Human $CD4^+$ T cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada).

CEM cells stably expressing human CCR5 (CEM–CCR5) were established by transducing CEM cells with the human *ccr5* gene using a conventional mouse retrovirus system. CEM–CCR5 cells were maintained in complete RPMI medium (10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) supplemented with 1 μg/ml puromycin at 37°C.

PREPARATION OF HIV-1 VIRUS STOCKS

We previously constructed pNL432-based proviral clones encoding EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-$1_{NL\text{-}E}$ or HIV-$1_{NL\text{-}D}$, respectively, and pNLAD8-based proviral clones encoding EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8\text{-}E}$ or HIV-$1_{NLAD8\text{-}D}$, respectively (Yamamoto et al., 2009; **Figure 1**). To prepare the HIV-1 viral stocks, the human embryonic kidney cell line 293T was transfected with pNL-E, pNL-D, pNLAD8-E, or pNLAD8-D using the calcium phosphate precipitation method and then incubated for 48 h. Culture supernatants were filtered and frozen at −80°C. The amount of virus in each culture supernatant was measured using an in-house HIV-1 Gag p24 enzyme-linked immunosorbent assay (ELISA; Tsunetsugu-Yokota et al., 1995).

STIMULATION OF T CELL RECEPTORS

T cell receptors (TCR) were stimulated as described previously (Yamamoto et al., 2009) with some modifications. In brief, primary $CD4^+$ T cells were suspended in complete RPMI medium supplemented with 5% human plasma and stimulated with 5 μg/ml of immobilized anti-human CD3 monoclonal antibody (mAb; eBioscience, San Diego, CA) and 1 μg/ml of soluble anti-human CD28 mAb (eBioscience) in U-bottom, 96-well plates at 37°C for 4 (weak stimulation) or 24 h (strong stimulation).

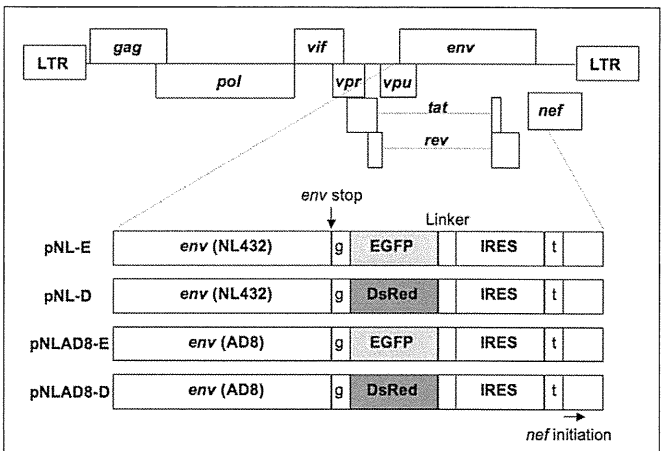

FIGURE 1 | Structure of the proviral DNA. The pNL432-based proviral clones encoded EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-$1_{NL\text{-}E}$ or HIV-$1_{NL\text{-}D}$, respectively, and the pNLAD8-based proviral clones encoded EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8\text{-}E}$ or HIV-$1_{NLAD8\text{-}D}$, respectively. EGFP or DsRed was not expressed as a fusion protein with Env due to the insertion of a single base after the Env stop codon. Nef was also independently expressed under the control of IRES. t (Thymine) and g (guanine) are additional DNA sequences.

HIV-1 INFECTION AND CELL CULTURE

Primary $CD4^+$ T cells (either unstimulated or pre-TCR-stimulated) or CEM–CCR5 cells were infected with 200 ng of p24-measured amounts of HIV-$1_{NL\text{-}E}$, HIV-$1_{NL\text{-}D}$, HIV-$1_{NLAD8\text{-}E}$, or HIV-$1_{NLAD8\text{-}D}$ per 1×10^6 cells by spinoculation at $1200 \times g$ for 2 h at 25 (conventional conditions) or 4°C (for CEM–CCR5 cells), as described previously (O'doherty et al., 2000; Dai et al., 2009). After spinoculation, cells were washed three times with PBS. Primary $CD4^+$ T cells were then suspended in complete RPMI medium supplemented with 5% human plasma. The cell suspensions derived from unstimulated or pre-TCR-stimulated $CD4^+$ T cells were settled onto U-bottom, 96-well plates with or without TCR-stimulation, respectively, at 37°C for 24 h. After the 24 h culture, cells were washed three times with PBS, suspended in complete RPMI medium supplemented with 5% human plasma and 50 U/ml recombinant interleukin-2, and cultured in U-bottom, 96-well plates at 37°C for up to 4 days.

FLOW CYTOMETRY

Cells were stained with fluorescence-conjugated mAbs as described previously (Yamamoto et al., 2009). The following mAbs were used for flow cytometry in various combinations: Pacific Blue-conjugated anti-human CD3 mAb (BioLegend, San Diego, CA, USA), phycoerythrin Cy7-conjugated anti-human CD4 mAb (BioLegend), and Alexa Fluor 700-conjugated anti-human CD8a mAb (BioLegend); and Nu24 mAb specific for HIV-1 Gag p24 (kindly provided by Dr. T. Sata, NIID, Tokyo, Japan) and conjugated to Alexa Fluor 647 using an Alexa Fluor 647 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). Dead cells were stained with propidium iodide or a LIVE/DEAD Fixable Dead Cell Stain

Kit (L34957; Invitrogen, Carlsbad, CA, USA). Intracellular staining (ICS) by Nu24 mAb was performed using a FIX and PERM Fixation and Permeabilization Kit (Invitrogen). Data collection was performed using a FACSCanto II (BD Bioscience, San Diego, CA, USA) and the data were analyzed using FACSDiva software (BD Bioscience) and FlowJo software (Tree Star, San Carlos, CA, USA).

QUANTIFICATION OF REPLICATED HIV-1 IN CELL CULTURE SUPERNATANTS

Human immunodeficiency virus type 1 replication was quantified in cell culture supernatants by ELISA and real-time RT-PCR. Gag p24 was measured using a RETRO-TEK HIV-1 p24 Antigen ELISA (ZeptoMetrix Corporation, Buffalo, NY, USA). For real-time RT-PCR, viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) and subjected to real-time RT-PCR using a SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), a set of HIV-1 *gag*-targeted primers, and a TaqMan probe as previously described (Saito et al., 2010). PCR was performed in an Mx3000P (Stratagene, La Jolla, CA, USA).

RESULTS

CD4 DOWNMODULATION IS ASSOCIATED WITH HIV-1 FLUORESCENT REPORTER INTENSITY

The cell surface CD4 molecule is downmodulated in HIV-1-infected cells in response to the HIV-1 components Env, Nef, and Vpu (Malim and Emerman, 2008). Therefore, to investigate the correlation between the level of CD4 downmodulation and the HIV-1 fluorescent reporter intensity, primary $CD4^+$ T cells infected with HIV-$1_{NL\text{-}E}$, HIV-$1_{NL\text{-}D}$, HIV-$1_{NLAD8\text{-}E}$, or HIV-$1_{NLAD8\text{-}D}$ followed by TCR-stimulation for 1 day and cultivation for a further 4 days were analyzed by flow cytometry. As shown in **Figure 2** (left panels), HIV-1-infected cells expressing a fluorescent reporter signal, EGFP, or DsRed, were detected, although the numbers varied between individual donors ($n = 3$–4): about 10–30% for X4-tropic HIV-$1_{NL\text{-}E}$-infected and HIV-$1_{NL\text{-}D}$-infected cells and 1–10% for R5-tropic HIV-$1_{NLAD8\text{-}E}$-infected and HIV-$1_{NLAD8\text{-}D}$-infected cells. However, the number of HIV-1^+ cells was comparable between HIV-$1_{NL\text{-}E}$ and HIV-$1_{NL\text{-}D}$ (X4-tropic), and between HIV-$1_{NLAD8\text{-}E}$ and HIV-$1_{NLAD8\text{-}D}$ (R5-tropic) within each donor, showing that the fluorescent reporter genes encoded within the HIV-1 proviral genome did not affect HIV-1 infectivity as described previously (Yamamoto et al., 2009). When we categorized $CD3^+CD8^-$ T cells into three fractions (HIV-1-negative, -dull, and -high) based on the fluorescence intensity of EGFP and DsRed, we found that CD4 was strongly downmodulated in the HIV-1 high fraction in all the HIV-1 strains (**Figure 2**, right panels). Interestingly, CD4 was also downmodulated in the HIV-1 dull fraction, but the level was modest compared with that in the HIV-1 high fraction (**Figure 2**, right panels). These results indicate that the level of CD4 downmodulation is associated with HIV-1 fluorescent reporter intensity.

FIXATION/PERMEABILIZATION WEAKENS THE HIV-1 FLUORESCENT REPORTER SIGNAL

To investigate the correlation between HIV-1 fluorescent reporter intensity and viral replication levels, we attempted to perform ICS for Gag p24 in HIV-1-infected cells prepared as described above. When we observed X4-tropic HIV-$1_{NL\text{-}E}$-infected and HIV-$1_{NL\text{-}D}$-infected cells from three donors by flow cytometry, we noticed that fixation/permeabilization, an essential step for ICS, weakened the fluorescent reporter signal for both EGFP and DsRed. **Figure 3** shows the flow cytometry profiles obtained for EGFP and DsRed at identical photomultiplier tube (PMT) voltages between intact (untreated) cells and fixed/permeabilized cells to visualize the differences in fluorescent reporter intensity. $DsRed^+$ cells were not properly separated from $DsRed^-$ cells within the population treated by fixation/permeabilization; the frequency of $DsRed^+$ cells was, therefore, markedly decreased. No adjustment of the flow cytometer settings, including PMT voltage and compensation, improved the blunted fluorescent reporter signal generated after fixation/permeabilization. Nevertheless, the number of $EGFP^+$ cells within the intact cell and fixed/permeabilized cell populations was comparable. Similar results were obtained for R5-tropic HIV-$1_{NLAD8\text{-}E}$ and HIV-$1_{NLAD8\text{-}D}$ (data not shown). Taken together, these results indicate that it is preferable to use an EGFP reporter when the fixation/permeabilization of cells is required.

HIV-1 FLUORESCENT REPORTER SIGNALS RELIABLY DETECT PRODUCTIVELY INFECTED CELLS SHOWING DIFFERENT VIRAL REPLICATION LEVELS

Following the results shown in **Figure 3**, we next assessed viral replication levels in the HIV-$1_{NL\text{-}E}$ infection group (5 days culture) from six donors using Gag p24 ICS (**Figure 4**). A representative flow cytometric analysis showed that not all $EGFP^+$ cells were Gag^+ and *vice versa*. When CD4 expression levels were compared in each of the four cell fractions based on the expression patterns of EGFP and Gag p24 ($EGFP^+Gag^+$, $EGFP^+Gag^-$, $EGFP^-Gag^+$, and $EGFP^-Gag^-$), the strongest downmodulation of CD4 was observed in $EGFP^+Gag^+$ cells (red fraction). CD4 downmodulation was moderate in $EGFP^+Gag^-$ cells (green fraction). However, CD4 was not downmodulated at all in $EGFP^-Gag^+$ cells (blue fraction) and the expression level of CD4 was the same as that in $EGFP^-Gag^-$ cells (black fraction). We further divided the $EGFP^+Gag^+$ cells (red fraction) into Gag^{hi} (brown fraction) and Gag^{lo} cells (pink fraction) and compared the expression levels of EGFP and CD4 with those of Gag p24. Gag^{hi} cells (brown fraction) showed the strongest expression of EGFP and the strongest downmodulation of CD4. Gag^{lo} cells (pink fraction) showed an intermediate level of EGFP expression [between that of Gag^{hi} cells (brown fraction) and that of $EGFP^+Gag^-$ cells (green fraction)] and CD4 expression [between that of Gag^{hi} cells (brown fraction) and $EGFP^-Gag^-$ cells (black fraction)]. These results indicate that the expression level of EGFP correlates with that of Gag p24 in HIV-1-infected cells in which CD4 is downmodulated.

HIV-1-BOUND OR -INTERNALIZED CELLS ARE ALSO DETECTED BY Gag p24 ICS

Because CD4 downmodulation was not observed in $EGFP^-Gag^+$ cells (**Figure 4**; blue fraction), it is possible that these cells may still be bound by or have internalized HIV-1 but have not produced virions. Therefore, we next investigated the kinetics of $EGFP^-Gag^+$ cells during 5 days post-infection. Primary $CD4^+$

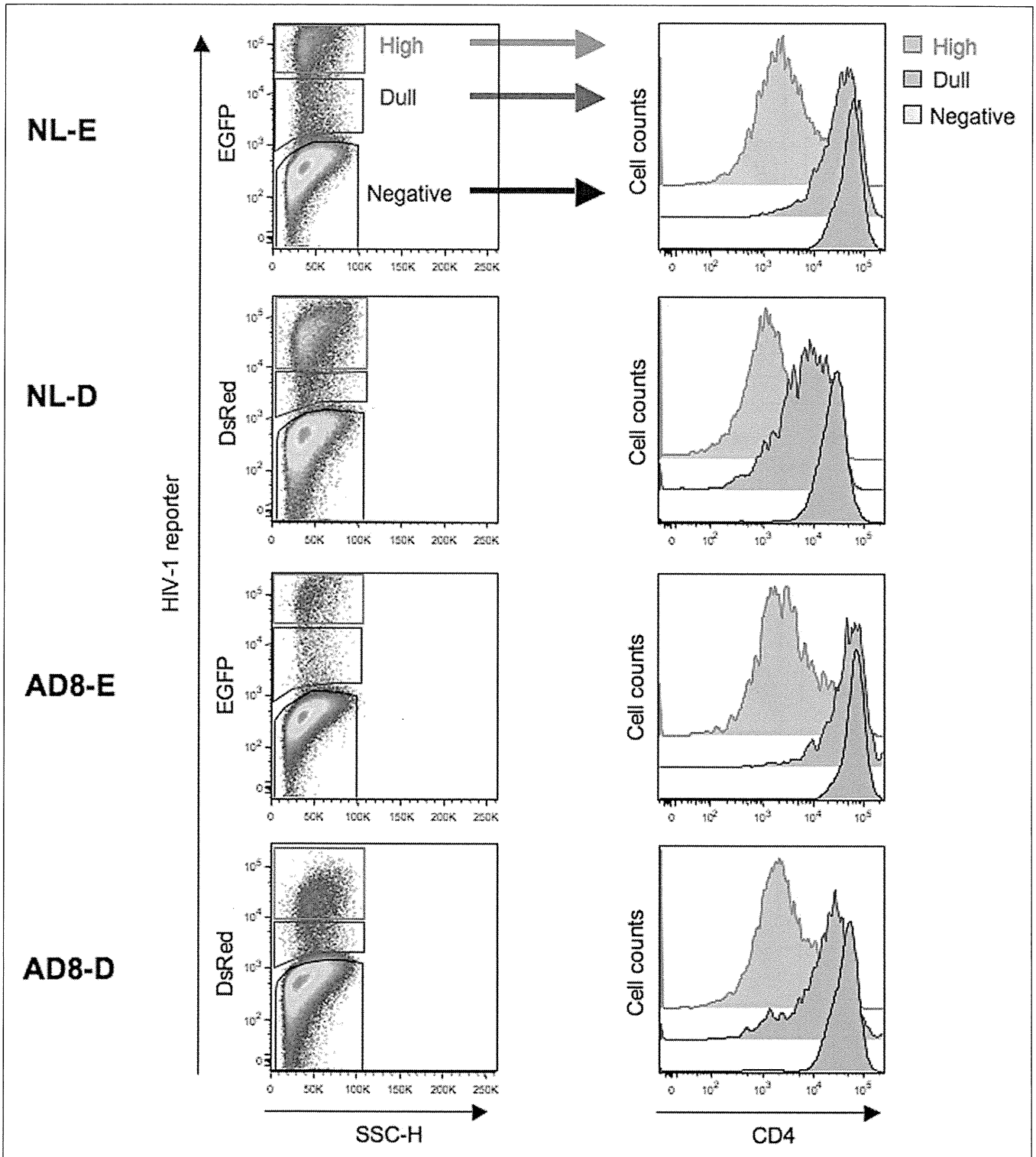

FIGURE 2 | Flow cytometry analysis of HIV-1-infected cells. Unstimulated CD4$^+$ T cells were infected with HIV-1_{NL-E}, HIV-1_{NL-D}, HIV-$1_{NLAD8-E}$, or HIV-$1_{NLAD8-D}$ and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). (Left panels) representative pseudo-color plot profiles for the Dead$^-$/CD3$^+$/CD8$^-$-gated cell fractions from three donors. Cells were classified as high (red), dull (blue), and negative (black) based on the HIV-1 reporter intensity. (Right panels) histograms of CD4 expression by the high (red), dull (blue), and negative (black) fractions defined.

T cells from three donors were infected with HIV-1_{NL-E} followed by TCR-stimulation for 1 day and cultivation for a further 4 days. **Figure 5A** shows a representative flow cytometric analysis. At 1 day post-infection, 17.6% of Gag p24$^+$ cells were observed,

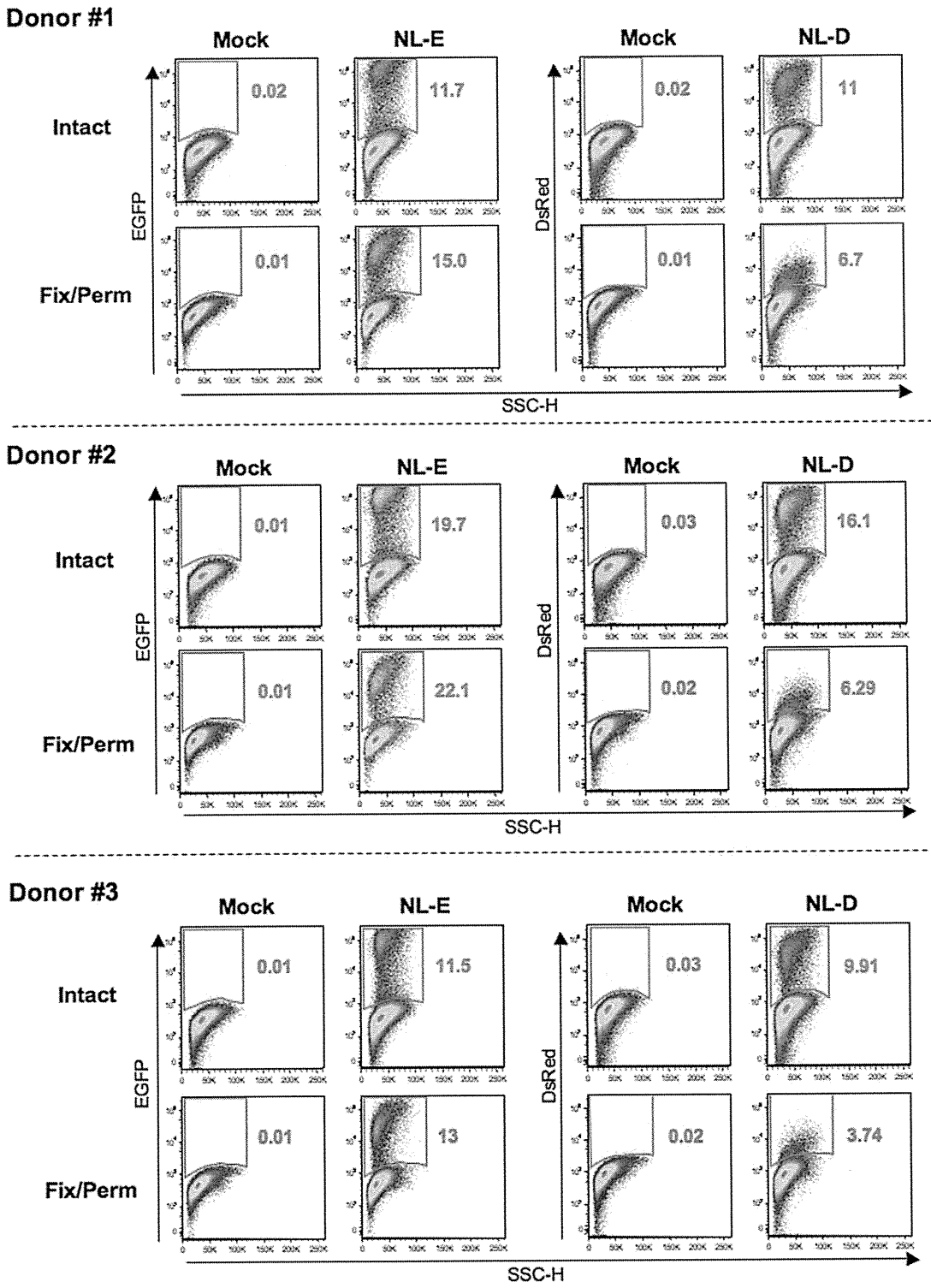

FIGURE 3 | Influence of fixation/permeabilization treatment on HIV-1 fluorescent reporter signals. Pseudo-color plot profiles for the Dead$^-$/CD3$^+$/CD8$^-$-gated cell fractions from the mock, HIV-1$_{NL\text{-}E}$ infection (NL-E), and HIV-1$_{NL\text{-}D}$ infection (NL-D) groups from all three donors tested. Analyzed cells were prepared as outlined in the legend to **Figure 2** and then either fixed/permeabilized (fix/perm) or not (intact).

despite the fact that no EGFP$^+$ cells were detected. At 2 days post-infection, the proportion of EGFP$^-$Gag$^+$ cells was decreased and EGFP$^+$ cells including Gag p24$^+$ and Gag p24$^-$ cells became to be observed, suggesting that initially infecting HIV-1 was

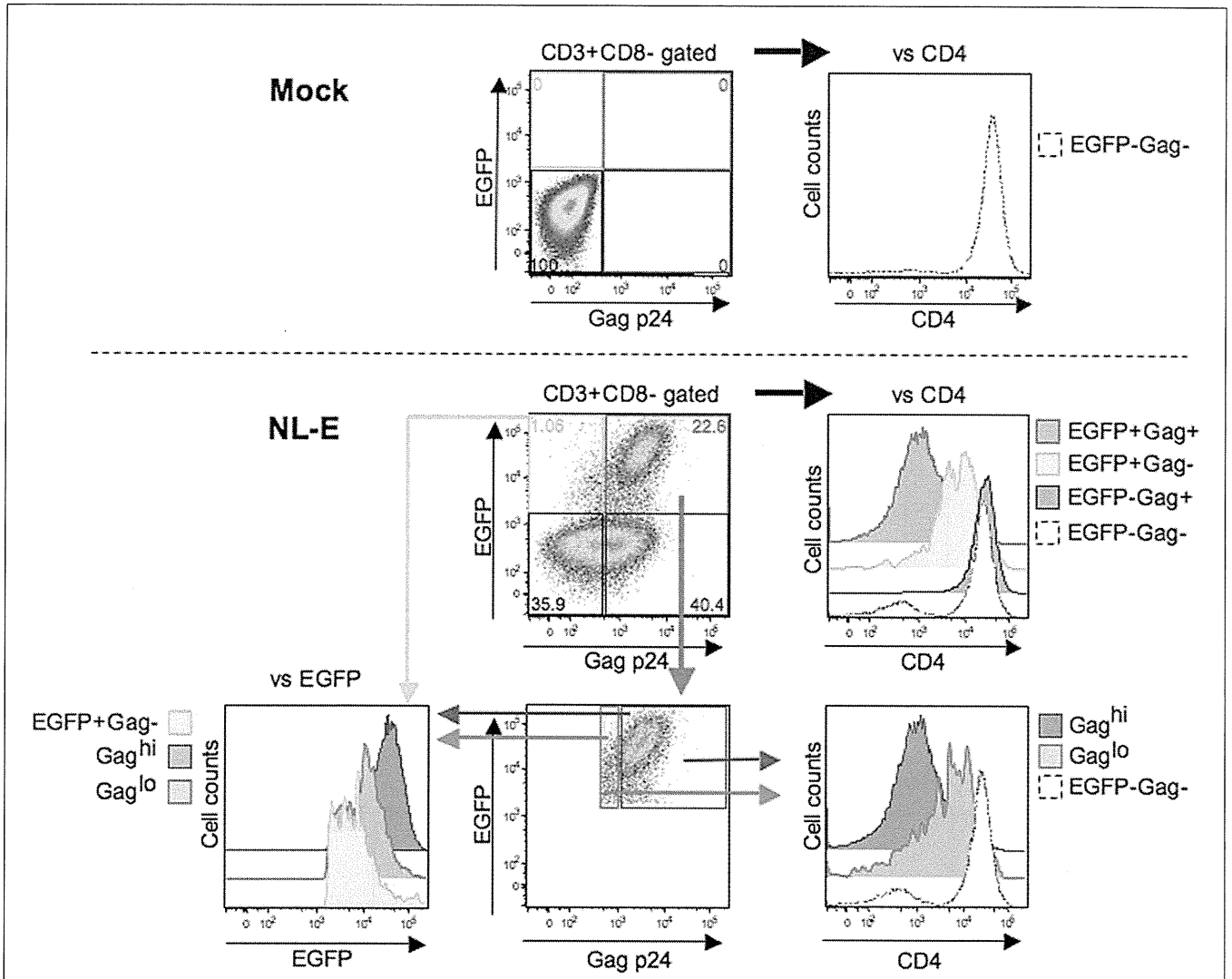

FIGURE 4 | Correlation between HIV-1 fluorescent reporter signal and Gag p24 expression. Representative flow cytometric analyses of the mock and HIV-1_{NL-E} infection groups from six donors. These infection groups were prepared as outlined in the legend to **Figure 2**. The $EGFP^+Gag^+$ (red), $EGFP^+Gag^-$ (green), $EGFP^-Gag^+$ (blue), $EGFP^-Gag^-$ (black), Gag^{hi} (brown), and Gag^{lo} (pink) cell fractions were categorized based on the expression patterns of EGFP and Gag p24. The expression levels of CD4 and EGFP in each cell fraction were compared according to their histogram profiles.

degraded and/or replaced with replication-competent proviruses. After 3 days post-infection, $EGFP^+$ cells were clearly visible and the proportion of $EGFP^-Gag^+$ cells turned to be increased, suggesting that progeny virus infection occurred. Because the CD4 expression levels were identical between $EGFP^-Gag^+$ cells and $EGFP^-Gag^-$ cells throughout the culture period, Gag p24 ICS must have detected cells that had bound or internalized HIV-1.

CEM–CCR5 cells, which are almost as susceptible to X4 and R5 HIV-1 fusion (data not shown), were used to confirm that Gag p24 ICS did indeed detect HIV-1-bound cells. Also, because it has been suggested that spinoculation at 25°C may induce HIV-1 fusion to the targeted cells (Dai et al., 2009), we tested Gag p24 ICS using CEM–CCR5 cells immediately after spinoculation with X4-tropic HIV-1_{NL-E} or R5-tropic HIV-1_{AD8-E} at 4°C. When cells were not fixed/permeabilized, no Gag $p24^+$ cells were detected by flow cytometry (**Figure 5B**, upper panels); however, when cells were fixed/permeabilized, a substantial proportion of Gag^+ cells was detectable in both the HIV-1_{NL-E} and HIV-1_{AD8-E} infection groups (**Figure 5B**, lower panels). Taken together, these results indicate that cells that have bound or internalized HIV-1 can be detected using flow cytometry for Gag p24 ICS.

THE INTENSITY OF THE HIV-1 FLUORESCENT REPORTER SIGNAL DEPENDS ON TCR-MEDIATED ACTIVATION LEVELS

T cell receptors-mediated activation of HIV-1-infected $CD4^+$ T cells increased productive viral replication, although the signaling pathway responsible may be different for X4 and R5 HIV-1 (Popik and Pitha, 2000). We investigated whether the intensity of the HIV-1 fluorescent reporter signal was affected by TCR-mediated

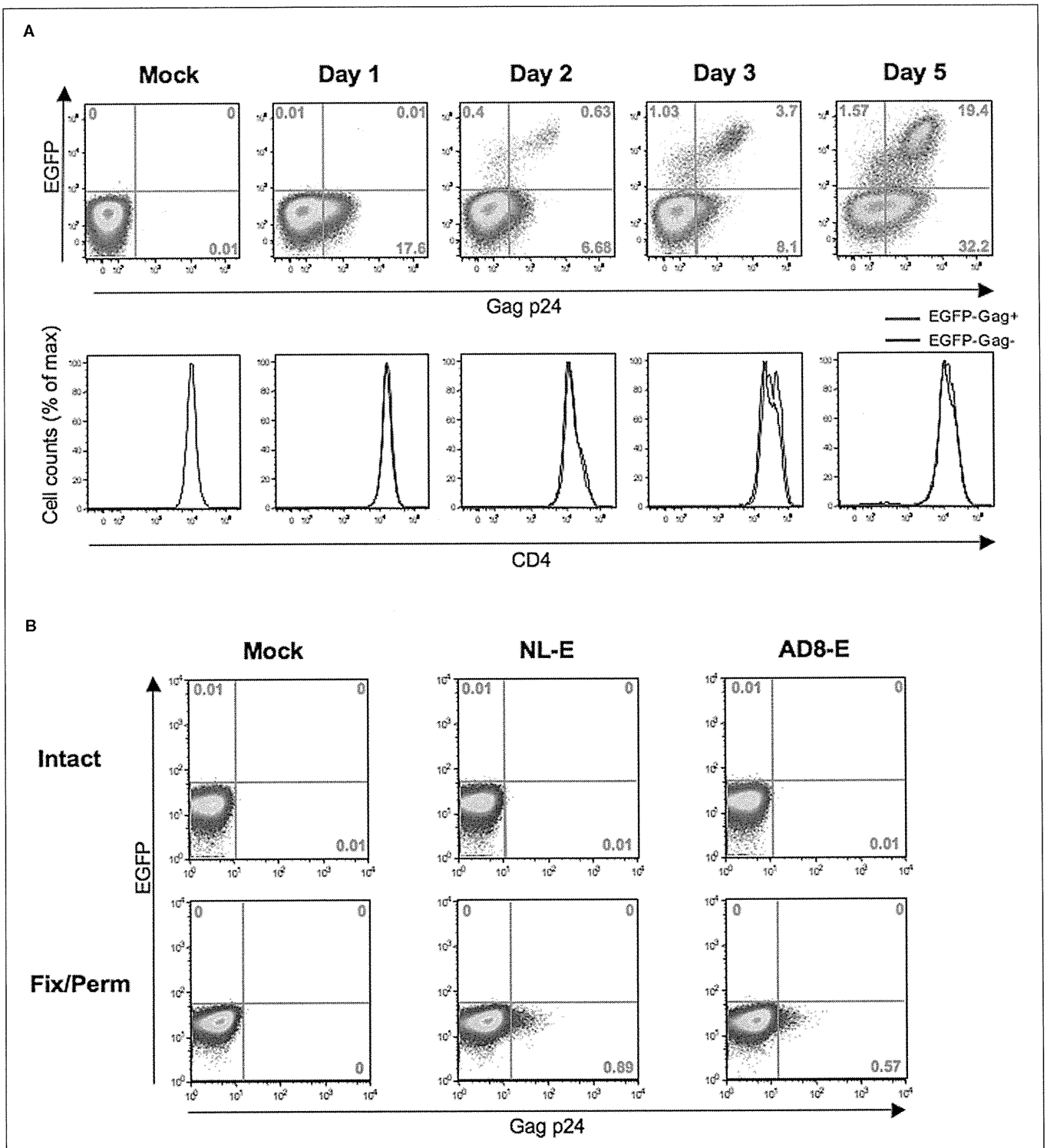

FIGURE 5 | Evaluation of Gag p24 ICS for HIV-1-internalized and -bound cells. (A) Representative flow cytometric analyses of the HIV-1_{NL-E} infection (NL-E) and mock (Mock) groups from three donors in which unstimulated primary $CD4^+$ T cells were infected with HIV-1_{NL-E} or not, respectively, and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). The expression patterns of EGFP and Gag p24 are indicated in the pseudo-color plot profiles for the $Dead^-/CD3^+/CD8^-$-gated cell fractions (upper panels). The expression level of CD4 in $EGFP^-Gag^+$ and $EGFP^-Gag^-$ cells is indicated in the histograms by the blue and black lines, respectively (lower panels). **(B)** Representative flow cytometric analyses of Gag p24 staining. CEM–CCR5 cells were infected with HIV-1_{NL-E} (NL-E), HIV-1_{AD8-E} (AD8-E), or not (Mock). Immediately after spinoculation at 4°C, the cells were washed and fixed/permeabilized (fix/perm) or not (intact) prior to Gag p24 staining.

activation levels. In this experiment, primary $CD4^+$ T cells from four donors were individually pre-stimulated via the TCR for 4 (weak stimulation) or 24 h (strong stimulation), infected with HIV-1_{NL-E}, and then cultured for a further 3 days. First, we confirmed that this experimental protocol allowed the preferential production of HIV-1_{NL-E} upon strong stimulation in all donors by examining the cell culture supernatants by ELISA (**Figure 6A**) and real-time RT-PCR (**Figure 6B**). Flow cytometric analysis of intact cells showed that HIV-$1_{NL-E}{}^+$ ($EGFP^+$) cells were more prevalent after strong stimulation than after weak stimulation, although the proportion of HIV-$1_{NL-E}{}^+$ cells varied among individuals (**Figure 6C**, upper and middle panels). The PMT voltage was optimized for EGFP to prevent excessive EGFP signaling (**Figures 2** and **3**). Of note, EGFP expression by HIV-$1_{NL-E}{}^+$ cells was lower in the weak stimulation group than in the strong stimulation group (as observed in donors #4 and #5), and EGFP expression in the weak stimulation group approached that in the strong stimulation group in parallel with the increase in the number of HIV-$1_{NL-E}{}^+$ cells (as observed in donors #6 and #7; **Figure 6C**, lower panels). Taken together, these results show that the intensity of the fluorescent reporter is highly correlated with the viral replication level.

DISCUSSION

Flow cytometric analysis is a reliable and convenient method for detecting HIV-1-infected cells at a single cell level. Here, we studied the potential usefulness of several HIV-1 fluorescent reporters that have been published previously (Yamamoto et al., 2009). We examined whether they would be helpful for evaluating viral replication levels based on their fluorescence intensity. In this study, we used recombinant HIV-1 encoding either EGFP or DsRed to show that the fluorescence intensity of the EGFP and DsRed reporters was associated with the level of CD4 downmodulation (**Figure 2**). Furthermore, we showed that EGFP intensity was associated with the expression level of Gag p24 (**Figure 4**). These findings clearly indicate that fluorescent reporter intensity is useful for evaluating viral replication levels. To confirm this argument, we further compared the fluorescent reporter intensity of HIV-1-infected cells that were strongly or weakly stimulated via the TCR. As expected, higher levels of HIV-1 replication/production occurred in strongly stimulated cells from all the donors tested (**Figure 6A,B**). Although the proportion and EGFP intensity of the HIV-1-infected cells varied among individuals, this might be due to differing susceptibility to HIV-1 and/or TCR-stimulation. Thus, the variability in EGFP expression is rather favorable to our argument, as increased EGFP intensity was associated with an increase in the number of HIV-1-infected cells after weak stimulation (**Figure 6C**).

Although Gag p24 ICS is usually used for flow cytometric analysis of other markers, we showed that it can also be used to detect cells that have internalized or bound HIV-1 (**Figure 5A,B**). However, Gag p24 ICS did not appear sensitive enough to detect HIV-1-infected cells because some HIV-1-infected cells in which CD4 was moderately downmodulated were identified as positive for EGFP but negative for Gag p24 (**Figure 4**). Bosque and Planelles (2009) also identified a small population of such reporter-positive but Gag p24-negative cells by flow cytometry when $CD4^+$ T cells were infected with EGFP-encoded DHIV incorporating a small out-of-frame deletion in the gp120-encoding area and pseudotyped with X4-tropic HIV-1_{LAI}, and assumed that these cells were at an early stage of the infection process and did not display late viral proteins. Therefore, our own findings indicate that it is the HIV-1 fluorescent reporter, rather than Gag p24 staining, that reliably detects HIV-1-infected cells at different stages of infection in flow cytometry experiments.

It is known that maturation of DsRed for coloration is usually slower compared with EGFP (Bevis and Glick, 2002; Maruyama et al., 2004). When we focused on the HIV-1 dull fraction in **Figure 2**, we found that CD4 downmodulation was stronger in $DsRed^+$ cells than in $EGFP^+$ cells. These results suggest that the EGFP reporter is preferable to the DsRed reporter for detection of earlier stage of infection. Furthermore, the detrimental effect of fixation/permeabilization on fluorescent reporter intensity, particularly when using the DsRed reporter, should be noted (**Figure 3**). Although the detailed mechanism remains obscure, this may result from the lower fluorescence intensity of DsRed compared with EGFP. A similar phenomenon was described regarding fixation with 3% paraformaldehyde, which significantly decreases the fluorescence intensity of DsRed, although specific data were not provided (Weber et al., 2006). Regardless of the weakened signal, the EGFP reporter is still compatible with fixation/permeabilization because the proportion of $EGFP^+$ cells was comparable between intact cells and fixated/permeabilized cells (**Figure 3**). Therefore, the EGFP reporter still maintains an advantage for analyses of cytokine/chemokine production and proliferation assays based on Ki-67 expression, for which ICS is necessary.

The HIV-1 fluorescent reporter has a potential application in molecular biology. In general, ICS-treated cells are not suitable for analysis using molecular biology techniques, since formaldehyde-based fixation (required for ICS) makes RNA extraction and reverse transcription and quantification problematic (Farragher et al., 2008) because of chemical cross-linking of proteins and nucleic acids (Kuykendall and Bogdanffy, 1992; Finke et al., 1993; Park et al., 1996), degradation of RNA (Bresters et al., 1994), and covalent modification of RNA via the addition of monomethylol groups to the bases (Masuda et al., 1999); therefore, by using the HIV-1 fluorescent reporter, HIV-1-infected cells can be sorted/purified without the need for fixation, allowing further characterization at a molecular level.

Given the usefulness of the HIV-1 fluorescent reporter shown here, it would also be very useful for investigating the mechanisms involved in the selective replication of R5 HIV-1 over X4 HIV-1 during the acute phase *in vivo* (Wolinsky et al., 1992; Zhu et al., 1993; van't Wout et al., 1994) and in cell culture systems *in vitro* (Schweighardt et al., 2004; Roy et al., 2005). We previously developed an *in vitro* dual infection model using EGFP-encoded X4 HIV-1 (HIV-1_{NL-E}) and DsRed-encoded R5 HIV-1 (HIV-1_{AD8-D}) and showed that the increase in the proportion of X4 HIV-1-infected cells is dependent upon their activation level (Yamamoto et al., 2009). Furthermore, the results of the present study show that the fluorescence intensity of the reporter molecule

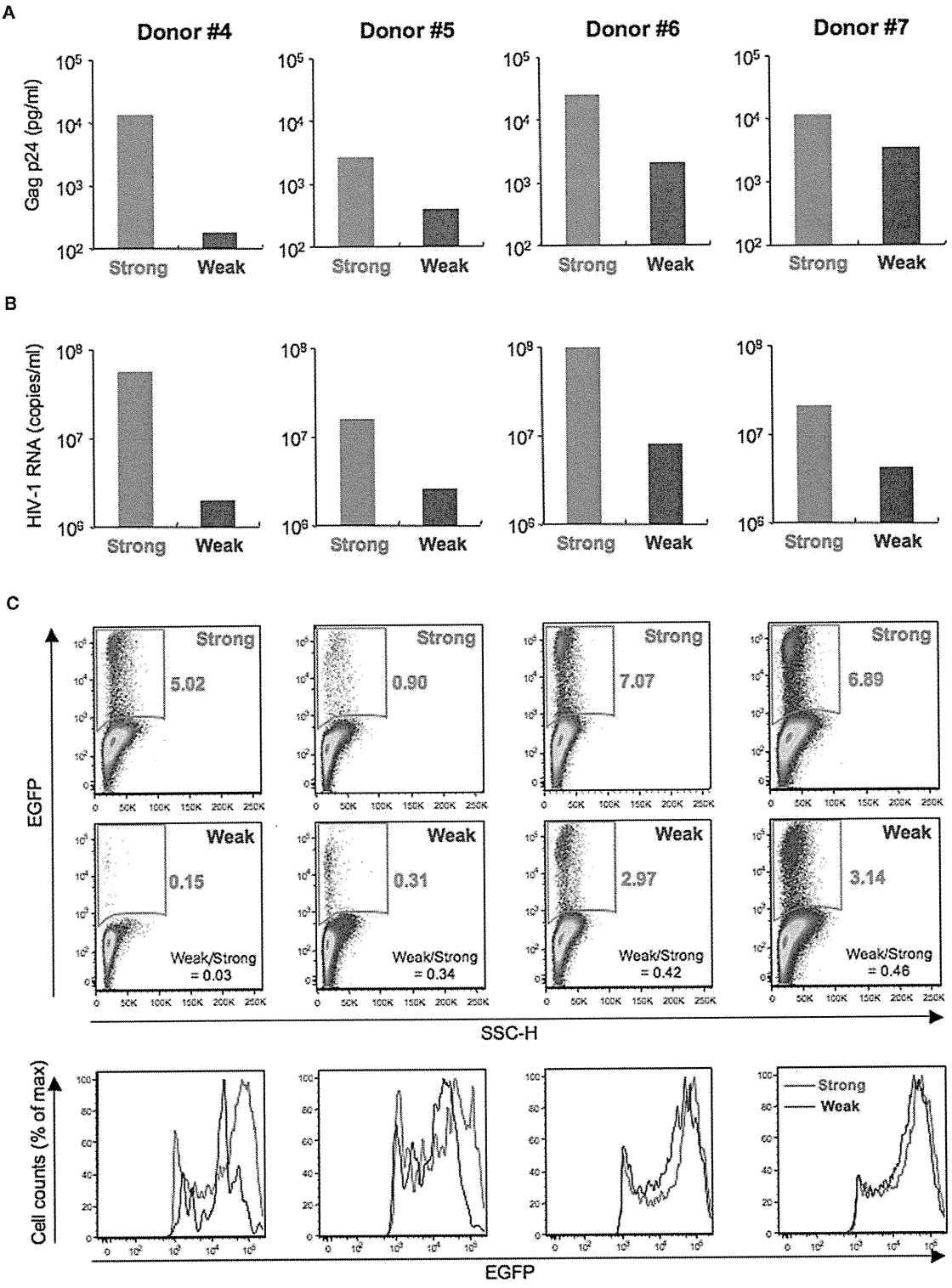

FIGURE 6 | Differences of HIV-1 replication according to TCR-mediated activation levels. Primary CD4+ T cells from four donors (Donor #4–7) were pre-stimulated via TCR for 4 (Weak; blue) or 24 h (Strong; red) and then infected with HIV-1_{NL-E} and cultured for 3 days. **(A)** ELISA for HIV-1 Gag p24 in the culture supernatants. **(B)** Quantitative real-time RT-PCR for HIV-1 RNA in the culture supernatants. **(C)** Flow cytometric analysis of intact cells showing pseudo-color plot profiles ($Dead^-/CD3^+/CD8^-$-gated cell fraction; upper and middle panels) and histogram profiles ($Dead^-/CD3^+/CD8^-/EGFP^+$-gated cell fraction; lower panels).

can be used to assess the level of viral replication in infected cells; therefore, by focusing on the HIV-1-infected cells and the fluorescent reporter intensity in the dual infection model, the detailed mechanism(s) of HIV-1 infection/pathogenesis can be clarified. In this regard, we have been investigating the dynamics of HIV-1 infection *in vivo* using humanized mice infected simultaneously with EGFP-encoded X4 HIV-1 (HIV-1_{NL-E}) and DsRed-encoded R5 HIV-1 (HIV-1_{AD8-D}; Ishige et al., in preparation). We believe that the advantages of the recombinant HIV-1 fluorescent reporter will contribute to the further understanding of HIV-1 infection/pathogenesis.

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HIV-1 Nef impairs multiple T-cell functions in antigen-specific immune response in mice

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Abstract

The viral protein Nef is a key element for the progression of HIV disease. Previous *in vitro* studies suggested that Nef expression in T-cell lines enhanced TCR signaling pathways upon stimulation with TCR cross-linking, leading to the proposal that Nef lowers the threshold of T-cell activation, thus increasing susceptibility to viral replication in immune response. Likewise, the *in vivo* effects of Nef transgenic mouse models supported T-cell hyperresponse by Nef. However, the interpretation is complicated by Nef expression early in the development of T cells in these animal models. Here, we analyzed the consequence of Nef expression in ovalbumin-specific/CD4$^+$ peripheral T cells by using a novel mouse model and demonstrate that Nef inhibits antigen-specific T-cell proliferation and multiple functions required for immune response *in vivo*, which includes T-cell helper activity for the primary and memory B-cell response. However, Nef does not completely abrogate T-cell activity, as defined by low levels of cytokine production, which may afford the virus a replicative advantage. These results support a model, in which Nef expression does not cause T-cell hyperresponse in immune reaction, but instead reduces the T-cell activity, that may contribute to a low level of virus spread without viral cytopathic effects.

Keywords: AIDS, acquired immunity, humoral response

Introduction

The Nef protein of the primate lentiviruses HIV-1/2 and the simian immunodeficiency virus (SIV) is expressed from the earliest stage of viral gene expression (reviewed in ref. 1). Nef-defective viruses cause a slow progression of clinical disease with reduced viral loads in humans and rhesus macaques with HIV-1/2 and SIV infection, respectively, indicating that Nef plays a crucial role in viral pathogenesis in human and non-human primates (reviewed in ref. 1). Nef associates with host cell membranes through N-terminal myristoylation and functions as an adaptor bringing together a large number of proteins in host cells, mainly protein kinases and components of endocytic trafficking machinery (reviewed in ref. 1; refs 2–7).

Nef reduces surface level receptors, including CD4, the primary receptor for HIV and SIV and MHC class I and class II complex, facilitating HIV immune evasion and thus increases viral pathogenesis (reviewed in ref. 1). Additionally, extensive *in vitro* studies, mostly carried out by using human T-cell lines, have suggested that Nef expression enhances TCR-mediated signaling pathways and transcriptional activation (reviewed in ref. 1; refs 2–5). Such alterations in signaling events may lower the TCR activation threshold in CD4$^+$

T cells and help more responsive to T-cell activation signals, a process that could support higher virus production upon stimuli mediated via the TCR (reviewed in ref. 1; refs 2–5). Moreover, Nef may alter host cell death pathways to prevent apoptosis of infected cells, thereby fostering their longevity (reviewed in ref. 1) These observations have led to a model in which Nef reorganizes the host cell activity so as to optimize viral propagation and cell survival, thus facilitating immune evasion and participating in virus spread.

The consequence of Nef expression in primary cells has been examined by using Nef transgenic (Tg) mice, in which Nef was constitutively or transiently expressed under control of a T-cell-specific promoter–enhancer element (8, 9). In this model system, Nef promotes T-cell activation, however, interpretation of these findings is complicated by the fact that expression of Nef early in the development of T cells results in wholesale depletion of thymocytes and peripheral T cells. Moreover, it remains obscure whether the T-cell activation seen in Nef Tg mice is mediated by lymphopenia-induced mechanisms rather than by an intrinsic effect of Nef expression on T-cell activation and proliferation (9, 10).

In the present study, to examine the consequence of Nef expression in primary cells, we established a double transgenic mouse (dTg), which expresses human coxsackie/adenovirus receptor (CAR) (11) and an ovalbumin (OVA)-specific TCR that recognizes the OVA peptide on antigen-presenting cell (APC) with high affinity under MHC Class II I-A^d-restriction. This system allowed us to analyze the effect of Nef on antigen-specific peripheral T-cell function by transfer of the *nef* gene into peripheral T cells using an adenovirus vector. The present study demonstrates that Nef expression does not cause T-cell hyperresponse but instead impairs T-cell functions required for immune response.

Methods

Mice

BALB/c and CB17-scid mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and Clea Japan, Inc. (Tokyo, Japan), respectively. Tg mice expressing the CAR under the control of the Lck proximal promoter (CAR Tg mice) on the BALB/c background have been described previously (11). DO11.10 mice express a transgenic TCR with specificity for OVA peptide residues 323–339 ($OVA_{323-339}$) restricted by I-A^d on the BALB/c background (12). All mice used in this study were maintained under specific pathogen-free conditions and used at 6–12 weeks of age in accordance with the guidelines of the Institutional Animal Care and Use Committee, National Institute of Infectious Diseases.

Adenovirus vector

Recombinant adenovirus vectors were generated using the AdEasy Adenoviral Vector System (Stratagene) according to the manufacturer's instructions. In order to express the *nef* gene under the CAG promoter, the pShuttle vector was digested with *Kpn*I, blunt-ended with T4 polymerase and then, the CAG promoter DNA was ligated (pShuttle-CAG). Next, an *Xho*I–*Xba*I fragment of pIRES2-EGFP (Invitrogen) was inserted into the *Xho*I–*Xba*I site of pShuttle-CAG, which was designated as pShuttle-CAG-I2-EGFP. HIV-1 NL4-3 nef wild-type and a mutant ($^{57}W^{58}L$ to $^{57}A^{58}A$) were PCR amplified from pNL432 and pNL-n57/2A proviral DNA, respectively, using specific primers containing *Eco*RI sites at both ends and then subcloned into pBluscript KS$^+$ (Stratagene). The *Eco*RI fragment containing wild-type or mutant nef was inserted into the *Eco*RI site of pShuttle-CAG-I2-EGFP. These shuttle vectors were linearized and co-transformed into *Escherichia coli* strain BJ5183-AD-1, which contains the pAdEasy vector, to induce homologous recombination (Supplementary Figure 1 is available at *International Immunology* Online). Recombinant adenoviral plasmids were selected and transfected into 293 cells to produce recombinant adenovirus particles. Recombinant adenovirus were purified by two rounds of Cesium chloride density gradient centrifugation as described previously (13). The concentrated virus was dialyzed against PBS containing 10% glycerol. The titer of the virus stock was determined by a plaque formation assay using 293 cells.

T-cell purification and recombinant adenovirus infection

For recombinant adenovirus infection, CD4$^+$ T cells were enriched by negative selection on a MACS column (Miltenyi Biotec GmbH, Gladbach, Germany) as previously described (14). Briefly, cells were blocked with anti-FcγRII/III (2.4G2; BD PharMingen, San Diego, CA, USA) and incubated with biotinylated mAbs against B220(RA3-6B2), IgM(II/41), IgD(11-26), Gr1(RB6-8C5), CD11c(N418), CD49b(DX5), CD11b(M1/70) and CD8(53–6.7) (eBioscience, San Diego, CA, USA), followed by incubation with streptavidin-coated microbeads (Miltenyi Biotec GmbH). Purified CD4$^+$ T cells (>95%) were infected with recombinant adenovirus vector at a multiplicity of infection of 10 (MOI 10) for 2 days in 24-well plates at a concentration of 2×10^6 per well in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 5×10^5 M 2-mercaptoethanol, L-glutamine, antibiotics and IL-7 (20 ng ml^{-1}; PeproTech, London, UK) at 37°C in an atmosphere of 5% CO_2.

Proliferation assays and ELISA

Sorted CD4$^+$ GFP$^+$ T cells were cultured in microtiter wells at a concentration of 4×10^4 cells per well in the presence of $OVA_{323-339}$ peptide and 5×10^5 irradiated T-depleted spleen cells. DNA synthesis of cultured cells in triplicate was estimated by the incorporation of [^{3}H] thymidine (0.5 μCi) added 12 h prior to cell harvest. The level of IFN-γ and IL-2 in the culture supernatants was measured by a Ready-Set-Go! ELISA assay kit (eBioscience), according to the manufacturer's instruction. In some experiments, CD4$^+$ GFP$^+$ T cells (2×10^6) were cultured for 2–3 days in 96-well plates immobilized with anti-TCR mAb (5 μg ml^{-1}) and anti-CD28 mAb (1 μg ml^{-1}) (BD PharMingen).

Chemotaxis assay

Chemotaxis assays were performed in Transwell (Corning Coster, Coring, NY, USA) with polycarbonate filters (5 μm pore size) as described previously (15). Briefly, purified CD4$^+$ GFP$^+$ T cells were suspended at 5×10^6 cells ml^{-1} in RPMI 1640 medium containing 1% FBS and 25 mM HEPES. One