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The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells

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The cytidine deaminase APOBEC3G (A3G) enzyme exerts an intrinsic anti-human immunodeficiency virus (HIV) defense by introducing lethal G-to-A hypermutations in the viral genome. The HIV-1 viral infectivity factor (Vif) protein triggers degradation of A3G and counteracts this antiviral effect. The impact of A3G on the adaptive cellular immune response has not been characterized. We examined whether A3G-edited defective viruses, which are known to express truncated or misfolded viral proteins, activate HIV-1-specific (HS) CD8⁺ cytotoxic T lymphocytes (CTLs). To this end, we compared the immunogenicity of cells infected with wild-type or Vif-deleted viruses in the presence or absence of the cytidine deaminase. The inhibitory effect of A3G on HIV replication was associated with a strong activation of cocultivated HS-CTLs. CTL activation was particularly marked with Vif-deleted HIV and with viruses harboring A3G. Enzymatically inactive A3G mutants failed to enhance CTL activation. We also engineered proviruses bearing premature stop codons in their genome as scars of A3G editing. These viruses were not infectious but potently activated HS-CTLs. Therefore, the pool of defective viruses generated by A3G represents an underestimated source of viral antigens. Our results reveal a novel function for A3G, acting not only as an intrinsic antiviral factor but also as an inducer of the adaptive immune system.

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Abbreviations used: A3G, APOBEC3G; AID, activation-induced deaminase; APOBEC, apolipoprotein B editing complex; HS, HIV-1 specific; mA3, mouse APOBEC3; Vif, viral infectivity factor.

During the acute phase of HIV infection, a rapid immune response is required to counteract viral replication (Deeks and Walker, 2007). The innate immune system senses pathogens through PRRs (pattern-recognition receptors) and triggers the activation of antimicrobial defenses. PRR stimulation leads to the secretion of cytokines, e.g., IFNs, which increase the expression of intrinsic factors such as APOBEC3G (A3G), thus preventing viral replication and spread (Peng et al., 2006).

A3G belongs to the activation-induced deaminase (AID)/apolipoprotein B editing complex (APOBEC) family of cytidine deaminases. AID has important functions in adaptive immunity including B cell receptor editing and class switching (Rosenberg and Papavasiliou, 2007). The APOBEC3-A, -B, -H, -G, and -F deaminases inhibit the replication of a wide range of viruses such as HIV and endogenous retroviruses (Esnault et al., 2005; Goila-Gaur and Strelb,

2008; Vartanian et al., 2008). A3G expression in lymphocytes, macrophages, and DCs is regulated by cytokines such as IFN- α and IL-2 (Koning et al., 2009). A3G restricts HIV replication via at least two mechanisms. First, A3G is packaged into newly formed HIV particles and subsequently edits dC residues to dU in the nascent proviral minus strand (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). A3G-mediated editing is very efficient, with up to 20% of all minus strand dC residues being deaminated to dU, which ultimately results in incorporation of A residues in the plus strand and subsequent G-to-A hypermutations in the proviral genome. A large part of edited proviruses will be defective. Second, in resting CD4⁺ T cells, cellular A3G might act as a postentry antiviral factor via its RNA-binding properties rather than by its deaminase activity (Chiu et al., 2005). However, this issue is controversial (Kamata et al., 2009).

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The viral infectivity factor (Vif) from HIV-1 counteracts this deaminase-dependent inhibition of viral replication but has less effect on the RNA-binding block mediated by A3G. In infected cells, Vif targets A3G for proteasomal degradation, thus reducing the amount of A3G incorporated into the virions as well as the efficiency of viral RNA editing in the target cells (Mariani et al., 2003; Yu et al., 2003). In vivo, the action of Vif is not absolute and hypermutated viral genomes have been isolated from the PBMCs of HIV-1-positive individuals at different stages of infection (Kieffer et al., 2005; Kijak et al., 2008). Editing patterns are dominated by GG-to-AG hypermutations, leading to a high frequency of amino acid substitutions and to the introduction of premature STOP codons (Vartanian et al., 1991). These crippled proviruses express aberrant (misfolded/truncated) viral proteins and are unable to produce infectious particles (Simm et al., 1995).

Viral recognition by the innate immune system activates the adaptive immune response. HIV-1-specific (HS) CD8⁺ CTLs are involved in the decrease of viremia during acute infection and chronic stages of the disease (Goulder and Watkins, 2008). CTLs freshly isolated from the blood of infected individuals inhibit HIV-1 replication in autologous CD4⁺ T cells (Sáez-Cirión et al., 2007). Despite the presence of CTLs, most infected individuals control viremia poorly in the absence of antiviral treatment. Several cellular and viral factors contribute to the failure of the immune system to control HIV-1 (Deeks and Walker, 2007). The emerging concept is that the quality, rather than the magnitude, of T cell response is crucial for determining disease outcome of various infections, including HIV (Appay et al., 2008). Our understanding of CD8⁺ T cell efficacy in HIV-1 infection is limited. Multiple factors, including antigen processing and presentation by infected cells, contribute to the activation of effective T cell responses. CTLs recognize peptides derived from exogenous or endogenous sources and presented by MHC class I (MHC-I) molecules. Most endogenous peptides loaded onto MHC-I molecules are derived from nascent polypeptides. These polypeptides originate primarily from errors during transcription, translation, and/or folding and are degraded by the proteasome (Yewdell and Nichitta, 2006).

In this paper, we examined the role of A3G in the generation of MHC-I-restricted HIV antigens. We first studied the immunogenicity of WT and mutant HIV isolates (e.g., HIV_{Δvif}). We then analyzed the role of A3G editing in HS-CTL activation. Finally, we engineered defective viruses expressing truncated Gag proteins that induced a robust activation of HS-CTLs by mimicking A3G editing. Altogether, our results uncover a dissociation of the capacity of A3G-edited viruses to productively infect cells and to activate HS-CTL responses. We demonstrate that A3G editing enhances the ability of HIV-infected cells to activate HS-CTLs. Therefore, A3G cytidine deaminase not only acts as an intrinsic antiviral factor but also regulates the recognition of HIV-infected cells by the immune system.

RESULTS AND DISCUSSION

Vif-deficient HIV-1 is a potent activator of HS-CTLs

We first analyzed how T cells infected with WT or Δvif HIV stimulate HS-CTLs. To this end, primary CD4⁺ T cells harboring endogenous A3G were infected with HIV particles produced in the absence of A3G (Fig. 1 A). From the literature, we anticipated that after CD4⁺ T infection, A3G will be incorporated in the progeny virions, thus exerting its editing activity starting from the second cycle of replication. Viral propagation was monitored by flow cytometry (intracellular Gag staining; Fig. 1 B). As expected, replication of HIV_{Δvif} was markedly reduced compared with HIV, with 3 and 20% Gag⁺ cells at day 5 after infection, respectively. We compared the ability of HIV and HIV_{Δvif}-infected CD4⁺ T cells to activate EM40-F21, an HS CD8⁺ CTL clone (Fig. 1 C). EM40-F21 was derived from an HIV-infected patient and recognizes a well-characterized immunodominant epitope of Gag p17 (SL9) presented by HLA-A*0201 (Moris et al., 2004). Surprisingly, HIV_{Δvif}-infected CD4⁺ T cells activated EM40-F21 to a greater extent than cells infected with the WT virus (Fig. 1 C). Similar results were obtained with cells from three different donors infected with various HIV inocula (Fig. 1 D). When expressed as the number of IFN-γ-producing CTL/Gag⁺ CD4 cells, CD4⁺ T cells infected with HIV_{Δvif} were found to be two to three times more efficient at activating HS-CTLs than HIV-infected cells (Wilcoxon rank-sum test: P = 0.032; Fig. 1 D). These data strongly suggest that there is dissociation between the capacity of HIV_{Δvif} to infect CD4⁺ T cells and to activate HS-CTLs.

To escape CTL recognition, the HIV Nef protein down-regulates the surface expression of HLA class I molecules (Schwartz et al., 1996). We asked whether Nef might affect the immunogenicity of HIV_{Δvif}-infected T cells. To this end, we constructed a ΔnefΔvif double mutant (HIV_{ΔnefΔvif}). HIV_{Δnef} isolates replicate less efficiently than WT virus. To obtain sufficient numbers of infected cells, CD4⁺ T lymphocytes were exposed to VSV-G-pseudotyped HIV_{Δnef} and HIV_{ΔnefΔvif} (Fig. 1 E). HIV_{ΔnefΔvif} replication was reduced (three- to sixfold) compared with HIV_{Δnef} at 48 and 72 h after infection. Remarkably, at each time point, even if very few Gag⁺ cells were detected, HIV_{ΔnefΔvif} induced a robust activation of HS-CTLs (Fig. 1 F). As expected, Nef-deficient isolates were more efficient than WT virus in activating CTLs (Fig. 1, compare C and F). Similar results were obtained with cells from eight different donors (Fig. 1 G), demonstrating that cells infected with HIV_{ΔnefΔvif} were two to three times more efficient than HIV_{Δnef}-infected cells in activating HS-CTLs (Wilcoxon rank-sum test: P = 0.0005; Fig. 1 G). Therefore, the enhancing effect of Δvif on CTL activation is observed both in the context of WT and Δnef HIV.

CTLs secrete a panel of chemokine and cytokine upon activation (Price et al., 1998; Wagner et al., 1998; Appay et al., 2008). We performed a peptide titration experiment to identify cytokines other than IFN-γ that are secreted in an antigen-specific manner by EM40-F21. Primary CD4⁺ T cells were loaded with the SL9 peptide, co-cultured with

HS-CTLs, and lymphokine production was analyzed in culture supernatants using the luminex technology. As previously reported (Price et al., 1998; Wagner et al., 1998), we observed a dose-dependent secretion of MIP-1 α and MIP-1 β (Fig. 2, F and I). Thereafter, we examined the effect of Δ vif viruses on MIP-1 β and MIP-1 α secretions by EM40-F21. Primary T cells were infected with VSV-G-HIV $_{\Delta$ nef and -HIV $_{\Delta$ nef/vif $_{\Delta$ vif} monitored for Gag expression (12 and 1% of Gag $^{+}$ cells, respectively), and used to activate EM40-F21 (Fig. 2, A, B, D, and G). Using IFN- γ Elispot, we first confirmed the enhancing effect of Δ vif on IFN- γ secretion by EM40-F21

(Fig. 2, B and C). We then measured in co-culture supernatants the release of MIP-1 α and MIP-1 β . Upon co-culture with HIV $_{\Delta$ nef $^{-}$ and HIV $_{\Delta$ nef/vif $^{-}$ -infected targets (at a ratio of 1 CTL to 50 CD4 $^{+}$ target cells), EM40-F21 secreted similar levels of MIP-1 α (130 and 106 pg/ml, respectively; Fig. 2 D). HIV $_{\Delta$ nef $^{-}$ and HIV $_{\Delta$ nef/vif $^{-}$ -infected cells also allowed the secretion of MIP-1 β (292 and 167 pg/ml, respectively; Fig. 2 G). These levels were at least three times higher than background secretion induced by uninfected control cells (Fig. 2, D and G). HIV infection did not increase the background lymphokine production by target CD4 $^{+}$ T cells alone (unpublished

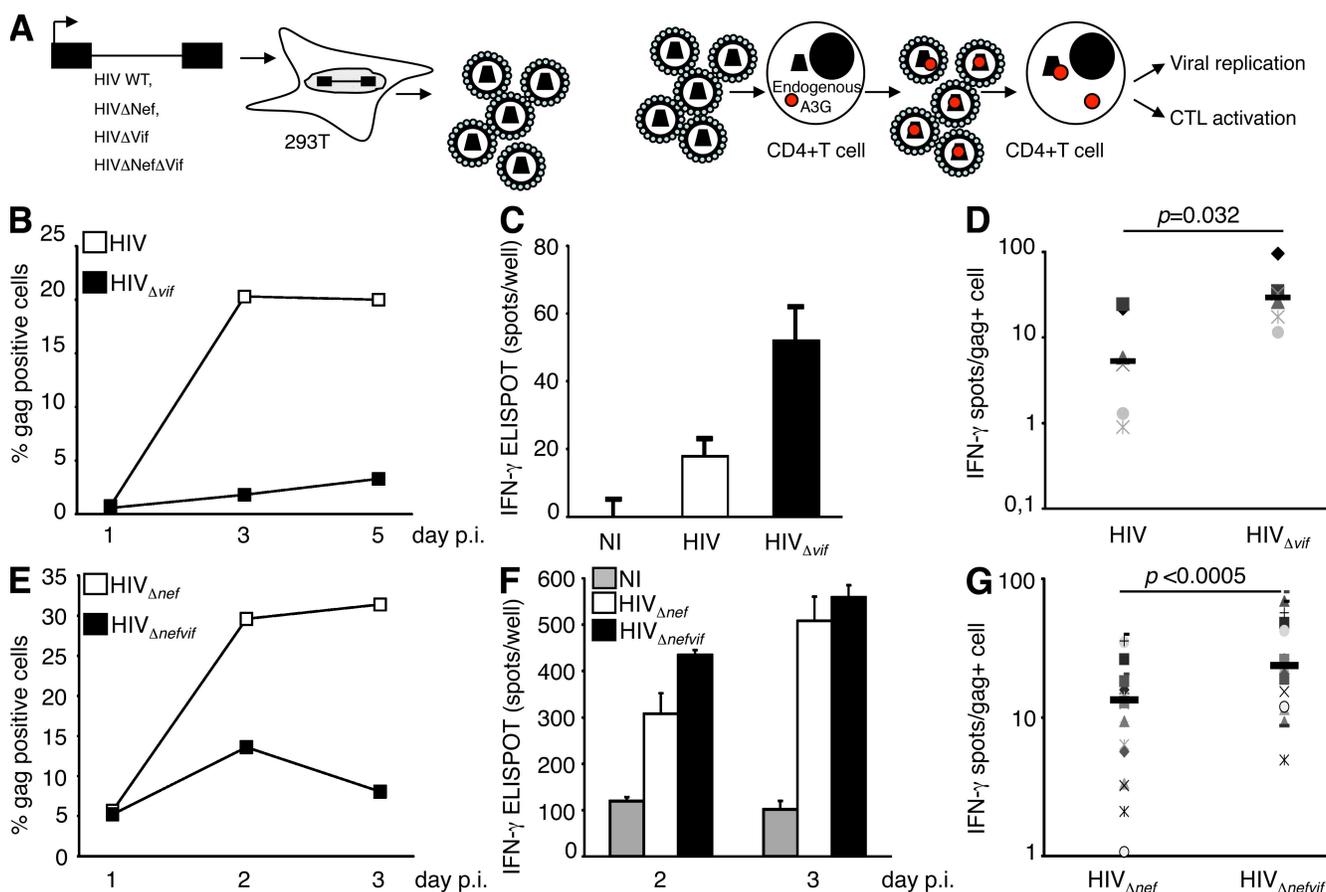


Figure 1. Vif-deficient HIV-1 is a potent activator of HS-CTLs. (A) Viruses were produced by transfection of 293T cells (that do not express endogenous A3G). Viruses were then used to infect PHA-activated primary CD4 $^{+}$ T cells. Endogenous A3G (red) is incorporated into the progeny virions and exerts its editing activity starting from the second cycle of replication. (B) Activated T cells were incubated with HIV $_{NL}$ or HIV $_{NL\Delta$ vif (100 ng/ml p24) and the kinetic of viral infection analyzed by intracellular Gagp24 FACS staining. (C) At day 5 after infection, infected cells were collected and used to stimulate HS-CTL clone EM40-F21 (2,500 clone/well) in an IFN- γ Elispot. Background IFN- γ production induced by uninfected cells is shown. Background IFN- γ productions by target cells alone have been subtracted and are at least three times lower than with HS-CTLs. Activation levels reached using SL9 peptide-loaded cells as positive control were \sim 600 IFN- γ^{+} spots/well (not depicted). Data are mean (\pm SD) of triplicates. (D) Data from six experiments performed as in B and C, using primary CD4 $^{+}$ cells from three donors, are presented as IFN- γ^{+} spots to percentage of Gag $^{+}$ cells (as measured by FACS) on a logarithmic scale. CD4 $^{+}$ T cells infected with Vif-deficient HIV-1 are more efficient than WT HIV-infected cells in activating HS-CTL ($P = 0.032$, Wilcoxon rank-sum test). Each symbol corresponds to an independent experiment. Horizontal bars indicate the mean IFN- γ /Gag $^{+}$ cell ratio based on six experiments. (E) Activated primary CD4 $^{+}$ T cells were incubated with VSV-pseudotyped HIV $_{NL\Delta$ nef or HIV $_{NL\Delta$ nef/vif and kinetic of viral infection monitored (as in A). (F) Infected cells were collected and used to stimulate EM40-F21 (as in C). Data are the mean (\pm SD) of triplicates. (G) Similar results were obtained with cells from eight donors infected at various inocula. Data are presented as IFN- γ^{+} spots to percentage of Gag $^{+}$ cells on a logarithmic scale. CD4 $^{+}$ T cells infected with Vif-deficient HIV $_{\Delta$ nef are more efficient than HIV $_{\Delta$ nef-infected cells in activating HS-CTLs ($P < 0.0005$, Wilcoxon rank-sum test). Horizontal bars indicate the mean IFN- γ /Gag $^{+}$ cell ratio. NI, noninfected cells; p.i., post infection.

data). We noticed that the secretion of MIP-1 β with $\Delta nefvif$ viruses was reduced as compared with Δnef (Fig. 2 G), suggesting that the effect of Δvif viruses might vary depending on the lymphokine. Nonetheless, even if very few Gag⁺ cells were detected, HIV _{$\Delta nefvif$} induced a potent secretion of both MIP-1 α and MIP-1 β by HS-CTLs (Fig. 2, E and H). To gain information on the magnitude of A3G-mediated effect, we used MIP-1 α and MIP-1 β SL9 peptide titration curves (Fig. 2, F and I) as standards to calculate the relative antigen presentation levels on the surface of infected (Gag⁺) target cells. Based on MIP-1 α secretion, HIV _{Δnef} - and HIV _{$\Delta nefvif$} -infected cells presented 6.6×10^{-15} and 4×10^{-14} exogenous peptide equivalent, respectively. Based on MIP-1 β secretion, the relative antigen presentation levels were 1.98×10^{-15} and 7.9×10^{-15} exogenous peptide equivalent for HIV _{Δnef} - and HIV _{$\Delta nefvif$} -Gag⁺ cells, respectively. Thereafter, cells infected

with HIV _{$\Delta nefvif$} viruses likely present four to six times more peptide antigen than cells infected with HIV _{Δnef} . This calculation was done using populations harboring different percentages of Gag⁺ cells. It would have also been informative to confirm these findings by calculating exogenous peptide equivalents in the presence of equal number of infected (Gag⁺) target cells. The low replicative capacity of HIV _{$\Delta nefvif$} in primary CD4⁺ T cells precluded this possibility. As expected, titrating down the amount of infected cells in the co-culture (1 CTL for 10 CD4⁺ targets) reduced the overall secretion levels and confirmed that in the absence of Vif, infected cells enhance cytokine production by HS-CTLs (Fig. S1). It is noteworthy that Nevirapine, a reverse transcription inhibitor of viral replication, blocked antigen presentation, indicating that activation of EM40-F21 was not a result of the presentation of incoming HIV antigens (Fig. 2, A and B).

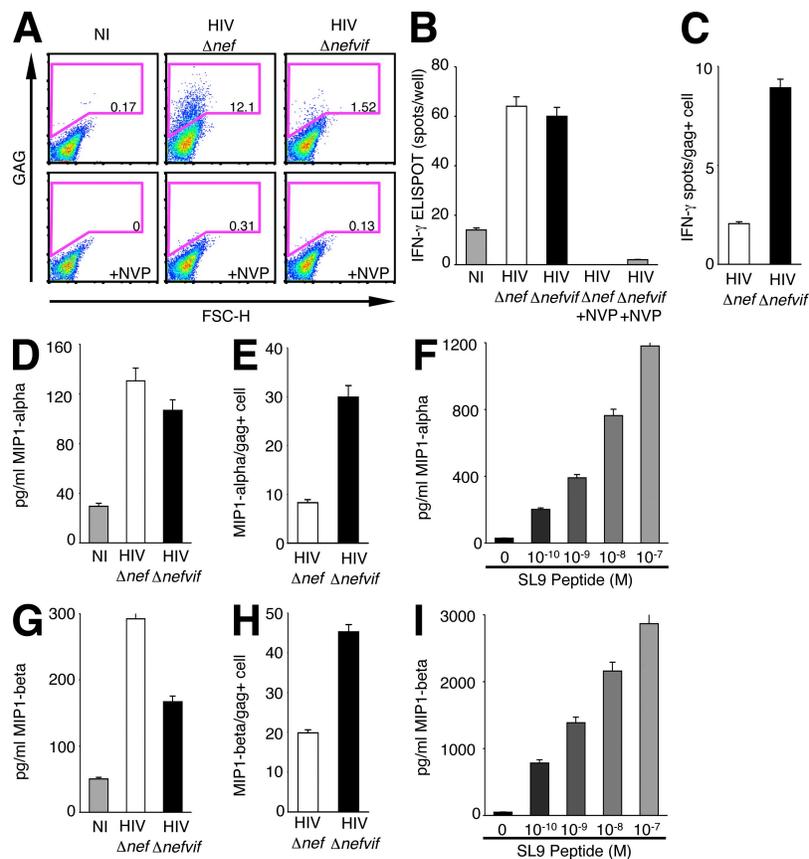


Figure 2. Vif-deficient HIV-1 stimulates chemokine secretion of HS-CTLs. (A) Activated primary CD4⁺ T cells were incubated with VSV-pseudotyped HIV_{NL Δnef} or HIV_{NL $\Delta nefvif$} in the presence or absence of Nevirapine (NVP). At day 4 after infection, viral replication was monitored by FACS. The percentage of infected (Gag⁺) cells is indicated. (B) Infected cells were then used to stimulate HS-CTL clone EM40-F21 in an IFN- γ Elispot (2,500 clones/well). Activation levels reached, using SL9 peptide-loaded cells as positive control, were ~ 600 IFN- γ spots/well (not depicted). Data are the mean (\pm SD) of triplicates and are representative of at least three independent experiments. (C) IFN- γ Elispot data are presented as IFN- γ spots to percentage of Gag⁺ cells. (D and G) 2.5×10^5 HIV_{NL Δnef} - and HIV_{NL $\Delta nefvif$} -infected cells were co-cultured for 24 h with 5×10^3 EM40-F21 (50/1 ratio). Culture supernatants were collected and the release of the indicated chemokine quantified by Luminex. Background secretions in the absence of HS-CTLs have been subtracted. HIV infection didn't induce an increase of the background lymphokine productions by target CD4⁺ T cell alone (not depicted). (E and H) Chemokine quantifications presented as concentration to percentage of Gag⁺ cells. (F and I) Activated T cells were loaded with SL9 peptide at the indicated concentrations, co-cultured for 24 h with EM40-F21 (50/1 ratio), and chemokine release was measured by Luminex. Luminex data are the mean (\pm SD) of duplicates and are representative of three independent experiments.

Collectively, our results demonstrate that Vif-deficient HIV-1 replicates less efficiently but is a potent activator of HS-CTLs, inducing the secretion of various lymphokines such as IFN- γ , MIP-1 α , and MIP-1 β . More specifically, Vif impacts the immunogenicity of HIV-infected T cells. In infected cells, Vif targets A3G for proteasomal degradation (Mariani et al., 2003; Yu et al., 2003); therefore, A3G may render HIV-infected cells more able to activate CTLs.

A3G-mediated viral restriction enhances HS-CTL activation

We further analyzed the contribution of A3G restriction in HS-CTL activation. We examined the effect of A3G, incorporated into incoming virions, on viral replication. Viral particles were produced in the presence or absence of A3G (Fig. 3 A; and Fig. S2, B and C; Gaddis et al., 2003). A3G expression was confirmed by Western blotting (Fig. S2 C). As expected, the amount of A3G was markedly reduced with Vif⁺ viruses (Fig. S2 C). In an assay of a single cycle of replication, A3G moderately reduced the infectivity of WT or HIV $_{\Delta nef}$ whereas HIV $_{\Delta vif}$ isolates were highly sensitive to A3G inhibition (Fig. S2 B).

CEM-A2, a CD4⁺ T cell line which does not express endogenous A3G (Gaddis et al., 2003), was infected with HIV $_{\Delta nef}$ produced in the presence (HIV $_{\Delta nef}$ + A3G) or absence of A3G (Fig. 3 A). The use of HIV $_{\Delta nef}$ enables better CTL activation (Fig. 1). We did not use a Δvif HIV in this setting because this mutant was not viable when produced with A3G (Fig. S2 B). Importantly, in this experimental system, A3G exerts its editing activity exclusively on the first cycle of infection (Fig. 3 A). An infection peak was reached 2 d after HIV $_{\Delta nef}$ and HIV $_{\Delta nef}$ + A3G infection with 78 and 26% Gag⁺ cells, respectively, thus confirming that A3G generates partly defective viruses (Fig. 3 B). Remarkably, HIV $_{\Delta nef}$ + A3G-infected cells induced a robust activation of the CTL clone EM40-F21 (Fig. 3 C). Similar data were obtained with two different HIV isolates (NL4-3 and SF2) used at various inocula (Fig. 3 D). Notably, when expressed as the number of IFN- γ -producing CTL/Gag⁺ CD4 T cells, HIV $_{\Delta nef}$ + A3G-infected cells were two to three times more efficient in activating HS-CTLs than HIV $_{\Delta nef}$ -infected cells (Wilcoxon rank-sum test: $P = 0.0005$; Fig. 3 D). To further highlight the role of A3G in enhancing CTL activation, we compared the capacity of CEM-A2 with similar levels of Gag⁺ cells to activate HS-CTLs. Data from six independent experiments are presented in Fig. 3 (E and F). In the presence of A3G, there was a stronger activation of EM40-F21 (Wilcoxon rank-sum test: $P = 0.015$; Fig. 3 F).

The CTL clone EM40-F21 is specific for a Gag-derived epitope presented by HLA-A2. We examined whether A3G also activates CTLs that recognize other viral epitopes. To this end, we generated a Nef-specific CTL line, restricted by HLA-B*07. As a negative control, we used CTLs raised against a CMV epitope. An HLA-B*07⁺ cell line, T1-B7, was used as target (Cardinaud et al., 2004). T1-B7 cells were infected with HIV or HIV + A3G. At 24 h after infection, cells were monitored for viral replication and used as targets in

Elispot assays. As expected, replication of HIV + A3G was reduced compared with HIV alone (unpublished data). CMV-specific control T cells did not react with HIV-infected cells (Fig. 3 H). T1-B7 cells infected with HIV + A3G induced a strong activation of Nef-specific CTLs (Fig. 3 G). Activation with HIV + A3G-infected cells was threefold higher than with HIV-infected cells (Fig. 3 G). When normalized to the amount of infected (Gag⁺) cells, HIV + A3G-infected cells activated Nef-specific CTLs ninefold better than HIV-infected cells (unpublished data). Overall, using two HIV isolates (NL4-3 and SF2) and CTLs specific for two different HIV proteins (Gag and Nef) presented by distinct HLA molecules (HLA-A2 and -B7), we demonstrated that A3G enhances recognition of HIV-infected cells by HS-CTLs.

The editing activity of A3G is required to promote HS-CTL activation

We then questioned whether the editing activity of A3G is necessary to promote HS-CTL stimulation. A3G possesses two consensus deaminase motifs, which is a hallmark of APOBEC protein family members. Mutations in the C-terminal deaminase motif (such as H257R and C288S) abrogate editing activity (Haché et al., 2005; Newman et al., 2005).

HIV $_{\Delta nef}$ particles were produced in the presence of WT or mutant (H257R and C288S) A3G. The amounts of WT and mutant A3G proteins were comparable (Fig. S2 C). CEM-A2⁺ cells were infected with two different doses of virus (Fig. 4 A). At 48 h after infection, the percentage of infected cells was reduced using HIV $_{\Delta nef}$ + A3G compared with HIV $_{\Delta nef}$ whereas HIV $_{\Delta nef}$ produced in the presence of A3G mutants H257R and C288S replicated to levels similar to those of HIV $_{\Delta nef}$. Infected cells were then used to stimulate HS-CTL EM40-F21 (Fig. 4 B). As already observed, cells infected with HIV $_{\Delta nef}$ + A3G activated HS-CTLs to a great extent, which was comparable to activation levels obtained with HIV $_{\Delta nef}$ -infected cells. Cells infected with HIV $_{\Delta nef}$ containing A3G mutants also induced strong IFN- γ secretion (Fig. 4 B). When expressed as IFN- γ ⁺ CTLs/Gag⁺ CD4 cells, HIV $_{\Delta nef}$ + A3G was twofold more efficient in activating HS-CTL than HIV $_{\Delta nef}$. Mutating the deaminase motif of A3G abrogated the capacity of A3G to enhance CTL activation (Fig. 4 C). Similar results were also obtained using another A3G mutant lacking both N- and C-terminal cytosine deaminase motifs (H65R/H257R; unpublished data). Thus, the editing activity of A3G is required to mediate its effect on HS-CTL-recognition.

Defective viruses with premature STOP codons are efficiently recognized by HS-CTLs

In HIV samples derived from patients, editing patterns are dominated by GG-to-AG hypermutations (Vartanian et al., 1991). On the protein level, this preferential editing leads to a high proportion of tryptophan (TGG) to STOP codon (e.g., TAG) substitutions.

We asked whether the expression of aberrant HIV proteins with premature STOP codons might be involved in the

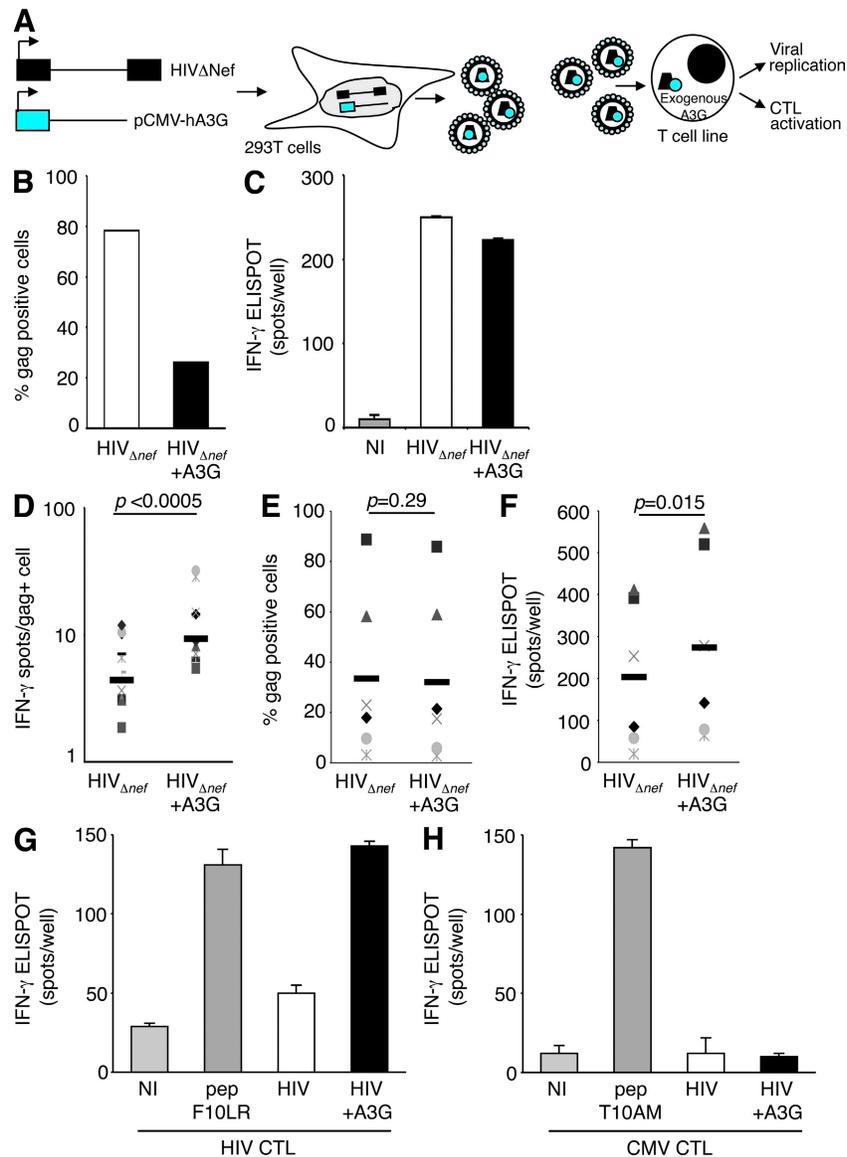


Figure 3. A3G-mediated viral restriction enhances HS-CTL activation. (A) Viruses were produced upon cotransfection in 293T cells of HIV genome and plasmids encoding for A3G. CEM-A2⁺ cells were then infected. CEM-A2⁺ cells do not express A3G (blue); hence, exogenous A3G exerts its editing activity exclusively during the first cycle of replication. (B) CEM-A2⁺ cells were incubated with HIV_{SF2 Δ nef} or HIV_{SF2 Δ nef} + A3G (100 ng/ml p24) and the kinetic of viral infection analyzed by p24 FACS staining. (C) 48 h after infection, infected cells were collected and used to stimulate HS-CTL clone EM40-F21 in an IFN- γ Elispot (10,000 clones/well). Background IFN- γ production induced by uninfected cells is shown. Background IFN- γ productions by CEM-A2⁺ cells alone were close to zero. Activation levels with SL9 peptide-loaded cells were around 600 IFN- γ ⁺ spots/well (not depicted). Data are the mean (\pm SD) of triplicates. (D) Data from nine independent experiments performed as in B and C using two HIV isolates (NL Δ nef and SF2 Δ nef) are presented as IFN- γ ⁺ spots to percentage of Gag⁺ cells on a logarithmic scale. CEM-A2⁺ cells infected with HIV Δ nef + A3G are more efficient than HIV Δ nef-infected cells in activating HS-CTL ($P < 0.0005$, Wilcoxon rank-sum test). Each symbol corresponds to an independent experiment. Horizontal bars indicate the mean IFN- γ /gag⁺ cell ratio based on the nine independent experiments. (E) To reach similar numbers of Gag⁺ cells, CEM-A2⁺ cells were infected with 5–10 \times more HIV_{SF2 Δ nef} + A3G than HIV_{SF2 Δ nef} (doses ranging from 20 to 100 ng p24/ml). The percentage of Gag⁺ cells achieved at 48 h after infection is indicated for six independent experiments. Using these experimental conditions, there is no difference in terms of gag⁺ cells between HIV_{SF2 Δ nef} or HIV_{SF2 Δ nef} + A3G-infected cells ($P = 0.29$, Wilcoxon rank-sum test). Horizontal bars indicate the mean percentage of Gag⁺ cells based on the six independent experiments. (F) Infected cells were then used in Elispot assays to stimulate EM40-F21 (10,000 clones/well). Results of six independent experiments are summarized. In the presence of A3G, there was a stronger CTL activation ($P = 0.015$, Wilcoxon rank-sum test). Each symbol in E and F corresponds to an independent experiment. Horizontal bars indicate the mean IFN- γ Elispot response based on the six independent experiments. (G) T1-B7 cells were incubated with HIV_{SF2} or HIV_{SF2} + A3G (100 ng/ml p24). 48 h after infection, cells were submitted to an IFN- γ Elispot assay to activate HIV Nef-specific CTLs restricted by HLA-B*0702 (10⁴ CTL/well). HIV Nef-specific CTL lines were generated in three different HLA-B*0702 transgenic mice and used in two independent experiments. One representative experiment with one CTL line is shown. (H) As in F, HIV-infected cells were used to activate a control CTL line specific for CMV. Data are the mean (\pm SD) of triplicates.

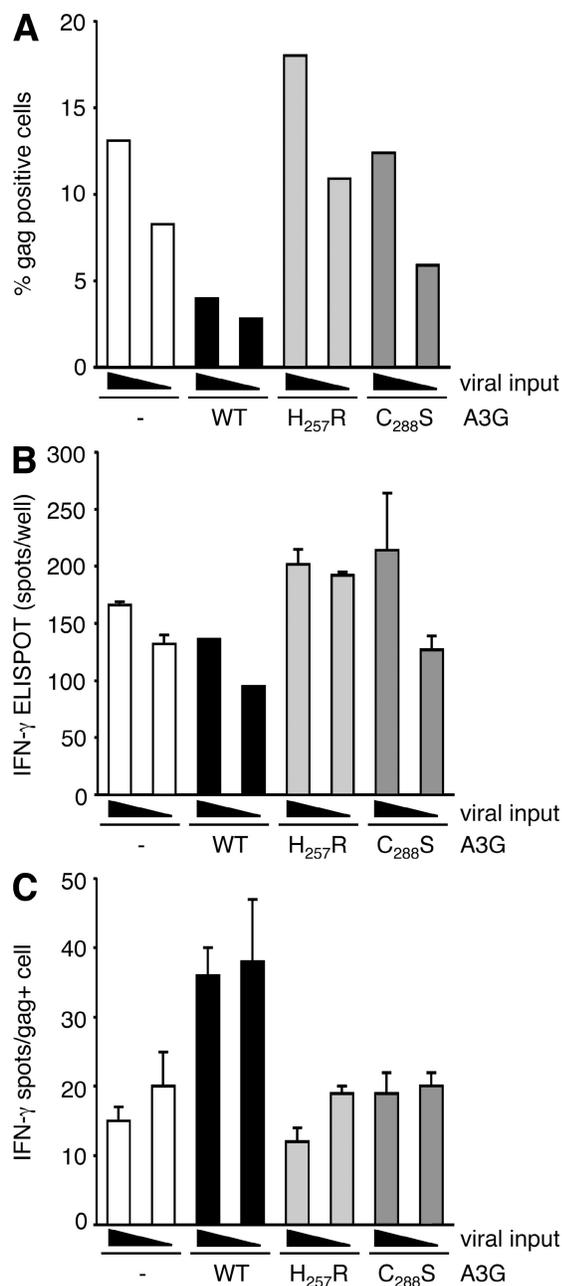


Figure 4. The editing activity of A3G is required to enhance HS-CTL activation. (A) CEM-A2⁺ cells were infected with HIV_{NLΔnef} produced without A3G or in the presence of WT and C-terminal domain mutants (H257R and C288S) of A3G (triangles, p24 doses: 20 and 10 ng/ml). 48 h after infection, viral infection was analyzed by Gagp24 staining (data are representative of four independent experiments) and infected cells were used to stimulate HS-CTL clone EM40-F21 in an IFN-γ Elispot (10,000 clones/well) (B). Background IFN-γ production induced by uninfected cells has been subtracted. Background IFN-γ productions by CEM-A2⁺ cells alone were close to zero. Activation levels reached using SL9 peptide-loaded cells as positive control were ~600 IFN-γ⁺ spots/well (not depicted). (C) Data are presented as a ratio of IFN-γ Elispot to percentage of Gag⁺ cells. Impairing the editing activity of A3G abrogates A3G-mediated enhancement of CTL recognition. Data are the mean (±SD) of triplicates and are representative of four independent experiments.

enhanced immunogenicity mediated by A3G. Downstream of the Gagp17 epitope SL9 recognized by EM40-F21, we identified two tryptophan codons (W₂₁₂ and W₂₄₉) that are potential targets for A3G editing. HIV proviruses with STOP codons at these positions have been previously isolated from PBMCs of infected individuals (Kijak et al., 2008). In addition, A3G edits these tryptophans into STOP codons in vitro (Armitage et al., 2008). We thus mimicked A3G editing by introducing STOP codons at these two positions, yielding HIV_{Δnef}P2-Stop and HIV_{Δnef}P3-Stop, respectively. Upon transfection, HIV_{Δnef}P2 and HIV_{Δnef}P3 led to the expression of truncated Gag proteins of 24 and 34 kD, respectively (Fig. 5 B). Viral particles produced were not infectious (not depicted) and lacked Gag p24 expression (Fig. 5 C). Transfected HeLa-HLA-A2⁺ cells expressing HIV_{Δnef}P2-Stop and HIV_{Δnef}P3-Stop were then used as targets to activate EM40-F21 (Fig. 5 D). Activation levels obtained with truncated P2 and P3 Gags were similar to or higher than those induced by cells producing infectious HIV (Fig. 5 D). CTL activation by infectious HIV, or by HIV-P2-Stop or -P3-Stop, was blocked by the proteasome inhibitor epoxomicin (Fig. 5 E), strongly suggesting that processing of full-length and truncated Gag-derived epitopes occurs through the classical MHC-I presentation pathway. Therefore, defective HIV strains bearing premature STOP codons are efficiently recognized by HS-CTLs.

Concluding remarks

AID/APOBEC family members are cytidine deaminases that exert different functions in the host defense against pathogens. In B cells, AID is necessary for antibody somatic hypermutation and class-switch recombination. AID provides the molecular flexibility that is crucial to establishing efficient humoral immune responses. APOBEC3 proteins offer a strong innate antiviral defense. Recent studies suggested that the adaptive and innate immune functions of APOBEC3/AID family members might overlap. AID protects the host against the oncogenic viruses Abelson murine leukemia virus, Epstein-Barr virus, and hepatitis B virus (Rosenberg and Papavasiliou, 2007). AID seems to act on the host genome through a mechanism that is not yet fully understood (Gourzi et al., 2007). Conversely, mouse APOBEC3 (mA3) is required to establish an efficient neutralizing antibody response against Friend virus (Santiago et al., 2008). Again the mechanism remains to be determined, however it has been proposed that mA3 might be involved in shaping the antibody repertoire. In the present study, we reveal a new role for A3G. In addition to restricting viral replication, A3G enhances the capacity of infected T cells to activate virus-specific CTLs. This phenomenon requires the editing activity of A3G.

Misfolded or truncated proteins represent a major source of peptides for the loading of MHC-I molecules (Yewdell and Nicchitta, 2006). This was demonstrated using several model antigens, such as influenza NP, OVA, or HIV-1 proteins. For instance, targeting HIV-1 Gag/Nef and Env to rapid proteasomal degradation by the addition of N-terminal degen signals (i.e., Arginine residue and/or ubiquitin fusion

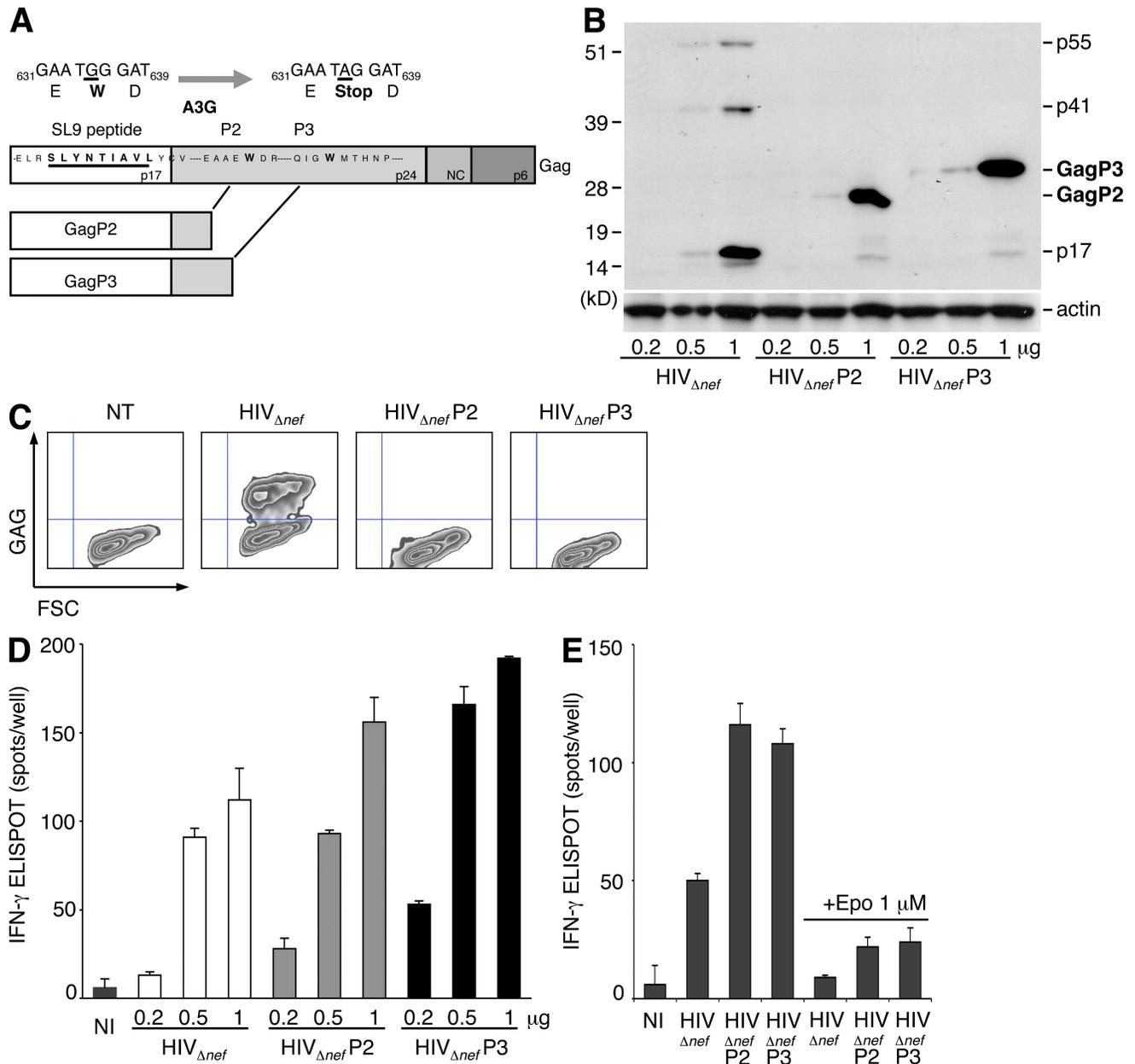


Figure 5. Defective viruses with premature STOP codons are efficiently recognized by HS-CTLs. (A) Schematic representation of the strategy used to construct defective HIV (HIV_{NLΔnef}P2 and HIV_{NLΔnef}P3) mimicking A3G-mediated editing. (Top) Nucleotide and amino acid sequences of a fraction of *gag* gene (*gag*P2) targeted for mutagenesis. The G-to-A transition introduced by mutagenesis is underlined and the resulting amino acid change (W₂₁₂ to STOP) is in bold. Nucleotide numbering is according to HIV_{LAI} sequence. (Bottom) Schematic representation of Gag full length and truncated Gag proteins (GagP2 and GagP3) generated by mutagenesis. The SL9 peptide amino acid sequence within p17 is indicated. HeLa-A2⁺ cells were transfected with HIV, HIV_{NLΔnef}P2, and HIV_{NLΔnef}P3 proviruses. (B and C) Gag expression profiles were analyzed 2 d after transfection using anti-p17 antibody and Western blotting (B) or anti-p24 antibody and FACS (C). Data are representative of three independent experiments. For Western blotting, transfected cells were pelleted, lysed, loaded on SDS-PAGE, and transferred to nitrocellulose. Membranes were then incubated with antibodies to p17 and actin. (D) HeLa-A2⁺ cells were transfected with the indicated doses of provirus. At 36 h after transfection, cells were used to activate HS-CTL clone EM40-F21 in an IFN- γ Elispot assay (10,000 clones/well). Data are the mean (\pm SD) of triplicates and are representative of three independent experiments. (E) HeLa-A2 cells transfected with 1 μ g of indicated HIV DNA were mock or Epoxinomycin treated (1 μ M) for 6 h prior to co-culture with HS-CTLs. Epoxinomycin, a proteasome inhibitor, diminishes CTL activation. Background IFN- γ production induced by untransfected cells is shown. Background IFN- γ productions by target cells alone have been subtracted and are at least three times lower than with HS-CTLs. Activation levels reached using SL9 peptide-loaded cells as positive control were \sim 400 IFN- γ ⁺ spots/well (not depicted). Data are mean (\pm SD) of triplicates and are representative of two independent experiments.

sequence) resulted in enhanced MHC-I presentation and increased activation of mouse CTLs (Tobery and Siliciano, 1997; Goldwisch et al., 2008). We extend these observations by showing that premature STOP codons in HIV genome, a hallmark of A3G editing, enhance CTL activation. We suggest that truncated/misfolded HIV polypeptides, generated by A3G, supply the pool of MHC-I-restricted HIV antigens. In addition to A3G, other mechanisms (e.g., viral recombination or errors during reverse transcription) generate crippled viruses. These defective viruses might also be an underestimated source of antigens.

HIV has developed multiple mechanisms for escaping CTLs. Nef, by modulating MHC-I expression, decreases the recognition and killing of infected cells by CTLs. The efficacy of Nef-mediated CTL escape is partial and depends on the kinetic of antigen expression and on the quantity of MHC-I-HIV epitope complexes (Fig. 1; Tomiyama et al., 2005). We show in this paper that A3G enhances the generation of HIV-derived epitopes, thus partially counteracting the action of Nef. Moreover, in primary CD4⁺ T cells, Vif reduced the capacity of infected cells to activate CTLs by degrading A3G. Recognition of HIV-infected cells by CTLs results from a balance between the efficacy of the generation of antigens enhanced by A3G editing and the immune-escape mechanisms mediated by Nef and Vif.

A3G has several genetic variants that might influence the progression to AIDS (An et al., 2004). It will be worth examining the contribution of A3G polymorphism in the induction of CTL responses. Simian immunodeficiency virus (SIV) might also be used to further characterize the role of A3G in CTL activation because Vif-deficient SIV replicates poorly but elicits an antibody response in macaques (Desrosiers et al., 1998).

In conclusion, cellular and viral factors determine the efficiency of antigen presentation that, in turn, influences the quality of T cell responses and thus the control of HIV infection (Appay et al., 2008). Our study demonstrates that A3G-mediated viral restriction contributes to the immunogenicity of HIV-infected cells and to CTL activation, thus linking innate and adaptive immunity. It is tempting to speculate that the function of A3G in enhancing CTL recognition occurs with other APOBEC3/AID family members and with other viruses sensitive to cytidine deamination.

MATERIALS AND METHODS

Cells and CTLs

PBMCs were isolated by ficoll density gradient from the blood of healthy donors and screened by FACS for the expression of HLA-A*02 using BB7.2 antibody. CD4⁺ T lymphocytes were then isolated from PBMCs using negative depletion (Miltenyi Biotec). HeLa-CHITA-HLA-A*0201⁺ and HUT-HLA-A*0201⁺ cell lines were generated by electroporation and selection using G418. CEM-HLA-A*0201⁺ cells were provided by K.L. Collins (University of Michigan, Ann Arbor, MI). T1 (174xCEM, CCR5⁺ LTR-GFP⁺) cells stably transfected with the HLA-B7^{mec3} construct were used as antigen-presenting cells (Cardinaud et al., 2004).

The HS EM40-F21 CTL clone is specific for Gag p17 (aa 77–85) presented by HLA-A*0201 and has been previously described (Moris et al., 2004). Human protocols were approved by the ethics committee of the Pasteur Institute. CTL lines specific for the HIV Nef (F10LR, Nef₆₈FPVT-

PQVPLR₇₇) and CMV (T10AM, pp65₄₁₇TPRVTTGGGAM₄₂₆) were derived from splenocytes of peptide-immunized HLA-B7^{mec3} transgenic mice (Cardinaud et al., 2004). Splenocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and stimulated every 10 d with peptide-pulsed syngeneic LPS lymphoblasts.

Virus and infection

The HIV strains used in the study were HIV_{NL4-3}, HIV_{SF2}, and their *Δnef* counterparts (Fackler et al., 2006). HIV and HIV(VSV) virions were produced and titrated as previously described (Moris et al., 2004). HIV virions containing A3G WT and H257R or C288S mutants were produced in 293T cells by cotransfection (1:1 DNA ratio) of HIV provirus with a pcDNA3-V5 plasmid encoding for WT or mutant A3G (A3G and mutant constructs provided by A.J. Hance [Hopital Bichat, Paris, France] and M.H. Malim [King's College, London, England, UK]). pNL4-3 XCS and pNL4-3_{Δnef} XCS (provided by A.J. Hance) were used to generate HIV_{NLΔnef} and HIV_{NLΔnefif} respectively. In brief within *nef*, a frame shift mutation of four bases was inserted at a unique XhoI site (Schwartz et al., 1996). HIV_{NLΔnef}P2 and HIV_{NLΔnef}P3 proviruses were generated by introducing in HIV_{NLΔnef} a STOP codon at positions 212 and 249 (amino acid sequence), respectively, using the primers for HIV_{NLΔnef}P2 (5'-GAGGAAGCTGCAGAATAGGATAG-ATTGCATCCAG-3') and HIV_{NLΔnef}P3 (5'-CAGGAACAAATAGGATAGATGACACATAATCCACCTATCC-3'); mutagenized nucleotides in bold) with QuikChange XL Site-Directed Mutagenesis kit (Stratagene). 48 h after transfection, viral supernatants were collected and p24 content was measured by ELISA (PerkinElmer). When stated, the infectivity was tested using HeLa-CD4⁺ cells (P4 cells), which carry the integrated HIV LTR-lacZ reporter cassette. P4 was infected with 5 ng p24 of the indicated viruses. 48 h later, β-galactosidase activity was measured in cell extracts.

5 × 10⁶ CEM-A2⁺, HeLa-A2⁺, HUT-A2⁺, and T1-B7 cells were infected with various amount of HIV (ranging from 10 to 100 ng p24/ml) for 3 h at 37°C in 10 mM Hepes, pH 7.4, and 2 μg/ml DEAE-dextran containing culture medium. Primary CD4⁺ T cells were PHA activated (1 μg/ml for 48 h; Abbot Laboratories), washed, and grown in IL-2-containing culture medium (50 IU/ml; Proleukin [Novartis]) for at least 10 d. Cells were then infected for 3 h at 37°C with 10–100 ng/ml HIV in the presence of DEAE-dextran and grown with IL-2. Viral replication was then followed using either p24 intracellular staining. Infected cells were then used as antigen presenting cells in Elispot assays.

Flow cytometry

Cells were fixed, permeabilized, and stained using standard procedures with anti-Gagp24 (KC57; Beckman Coulter) or an IgG1 isotype mAb as a negative control. Samples were analyzed using FACSCalibur (BD).

T cell activation assays

Elispot. Stimulator cells were co-cultured for at least 8 h with CD8⁺ T cell clones (ranging from 2,500 to 10⁴ EM40-F21/well and 2,000 to 10⁴ anti-Nef CTLs/well). IFN-γ production was then measured in an Elispot assay as previously described (Moris et al., 2004). As positive controls, stimulators were incubated with 1 μg/ml of cognate (SL9) peptides before addition of CTLs.

Chemokine quantification by Luminex. Infected or SL9 peptide-loaded stimulator cells were co-cultured in a 96-well plate with EM40-F21 at a 1/50 and 1/10 ratio (5,000 CTLs for 250,000 or 50,000 CD4⁺ T cells, respectively). Cell culture supernatants were collected after 24 h, and MIP-1α and MIP-1β secretion was measured according the manufacturer's instructions (Chemokine Human 5-plex Panel LHC0005; Invitrogen).

Western blots. 24 or 48 h after transfection, cells were lysed in PBS-1% Triton X-100 (Sigma-Aldrich) supplemented with protease inhibitors (Roche). Viral supernatants were ultracentrifuged (10⁵ rpm for 20 min) and resuspended in PBS containing 1% triton and protease inhibitors. Cell lysates and viral particles were analyzed by SDS-gel electrophoresis using 4–12% NuPAGE gels (Invitrogen). Anti-HIVp17, anti-V5 (both provided by

M.H. Malim), and anti-A3G (National Institutes of Health reagent 9968) monoclonal antibodies were used.

Statistical analysis

Data were analyzed by a Wilcoxon rank-sum test with StatView statistics program (Abacus Concepts).

Online supplemental material

Fig. S1 shows that Vif-deficient HIV-1 stimulates chemokine secretion of HS-CTLs at a 10:1 ratio. Fig. S2 shows the infectivity and phenotype of HIV particles produced in the presence or absence of A3G. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091933/DC1>.

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