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# Protective HIV-specific CD8+ T cells evade $T_{reg}$ cell suppression

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## Abstract

Specific human leukocyte antigens (HLAs), notably *HLA-B*27* and *HLA-B*57* allele groups, have long been associated with control of HIV-1. Although the majority of HIV-specific $CD8^+$ T cells lose proliferative capacity during chronic infection, T cells restricted by *HLA-B*27* or *HLAB*57* allele groups do not. Here we show that $CD8^+$ T cells restricted by 'protective' HLA allele groups are not suppressed by $T_{ref}$ cells, whereas, within the same individual, T cells restricted by 'nonprotective' alleles are highly suppressed *ex vivo*. This differential sensitivity of HIV-specific $CD8^+$ T cells to $T_{ref}$ cell–mediated suppression correlates with their expression of the inhibitory receptor T cell immunoglobulin domain and mucin domain 3 (Tim-3) after stimulation with their cognate epitopes. Furthermore, we show that HLA-B*27– and HLA-B*57–restricted effectors also evade $T_{ref}$ cell–mediated suppression by directly killing $T_{ref}$ cells they encounter in a granzyme B (GzmB)-dependent manner. This study uncovers a previously unknown explanation for why *HLA-B*27* and *HLA-B*57* allele groups are associated with delayed HIV-1 disease progression.

## INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are crucial in controlling HIV-1 infection (reviewed in ref. 1), which is emphasized by the influence of human leukocyte antigen (HLA) class I alleles on the rate of progression to AIDS[2, 3]. Long-term nonprogressors (LTNPs) are a rare, heterogeneous population of chronically infected individuals with low viral loads and sustained $CD4^+$ T cell counts who are relatively spared from disease progression even without antiretroviral therapy. The mechanisms allowing for such control of HIV-1 are not known, but a number of specific HLA allele groups, notably *HLA-B*27* and *HLA-B*57*, are

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**AUTHOR CONTRIBUTIONS**

S.E. designed and performed all the experiments and wrote part of the manuscript. N.L. assisted S.E. to perform some of the experiments. W.L.D. performed statistical analysis and graphing design. K.J.L. performed epitope mapping for individuals infected with HSV. D.M.K. advised on the HSV experiment. D.M.K., K.J.L., M.J.M. and A.C.C. supplied samples from subjects. H.H. designed and supervised all of the research and wrote the manuscript. All authors revised and edited the manuscript.

highly enriched in LTNP populations[4]. Possession of HIV-1–specific $CD8^+$ CTLs restricted by HLA-B*27 and HLA-B*57 during early HIV-1 infection correlates with longer AIDS-free survival[5] and better defines disease progression compared to HLA genotype alone, suggesting that HIV-specific $CD8^+$ CTLs, and not the presence of a particular HLA allele, determines disease progression[5].

LTNPs possess HIV-specific $CD8^+$ CTLs restricted by HLA-B*27 or HLA-B*57 that can continue to proliferate throughout chronic infection, whereas the majority of HIV-specific $CD8^+$ CTLs restricted by other HLA alleles lose their proliferative capacity[6, 7, 8]. Proliferative ability is linked to upregulation of perforin and is, therefore, associated with the cytotoxic capabilities of virus-specific $CD8^+$ CTLs[6]. We show here that HLA-B*27– and HLA-B*57–restricted HIV-specific $CD8^+$ CTLs possess an additional feature in that they evade suppression mediated by $T_{ref}$ cells. $T_{ref}$ cells, $CD4^+$ (or $CD8^+$) T cells with suppressive activity, have a key role in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory responses (reviewed in refs. 9,10). However, they may also have detrimental effects by suppressing effective antigen-specific immune responses. Several mechanisms are used by $T_{ref}$ cells to exert immunosuppressive function; however, our observation of differential suppression of $CD8^+$ CTLs restricted by distinct HLA alleles has not been previously described to our knowledge.

Tim-3 negatively regulates T helper type 1 responses upon interaction with its ligand, galectin-9 (Gal-9, encoded by *LGALS9*) expressed on lymphocytes and other cell types[11]. In humans, defects in Tim-3 expression contribute to multiple sclerosis pathology[12], suggesting that expression of Tim-3 on effector T cells is involved in inducing and maintaining peripheral tolerance of these T cells. $T_{ref}$ cells constitutively express Gal-9 (ref. 13) and, thus, could be providing the ligand for inducing tolerance in Tim-3–expressing effectors. Furthermore, a recent study demonstrated that Tim-3 expression defines a distinct population of dysfunctional effector T cells in progressing HIV-1–infected individuals[14].

Here we show that $T_{ref}$ cells suppress proliferation of nonprotective HIV-specific $CD8^+$ CTLs through Tim-3–Gal-9 interactions during chronic infection, but they do not suppress proliferation of HIV-specific $CD8^+$ CTLs restricted by HLA-B*27 or HLA-B*57. These HLA-B*27– or HLA-B*57–restricted CTLs can continue to proliferate and kill infected targets during chronic infection, which may account for delayed disease progression in persons with *HLA-B*27* and *HLA-B*57* allele groups. In addition, this finding may explain why the *HLA-B*27* allele group is also associated with resolution of other chronic infections (for example, hepatitis C virus) and why both *HLA-B*27* and *HLA-B*57* allele groups are associated with autoimmunity[15, 16, 17].

## RESULTS

### $T_{ref}$ cells disparately suppress CTLs based on HLA allele

To assess the susceptibility of HIV-1–specific CTLs to $T_{ref}$ cell–mediated suppression, we measured proliferation of numerous HIV-1 epitope–specific $CD8^+$ CTLs from LTNPs (individual clinical data are listed in Supplementary Table 1; epitope responses measured are listed in Supplementary Table 2) by measuring carboxyfluorescein succinimidyl ester (CFSE) dilution in the presence or absence of $T_{ref}$ cells[8, 18] *in vitro*. $T_{ref}$ cells differentially suppressed proliferation of $CD8^+$ CTLs restricted by different HLA alleles (Fig. 1). Epitope-specific $CD8^+$ CTLs restricted by the protective HLA allele groups (HLA-B*27 and HLA-B*57) were not suppressed by $T_{ref}$ cells ($P = 0.9$ and $P = 0.12$, respectively; median percentage $T_{ref}$ cell suppression −1.0 (indicating that effector T cells proliferated more in the presence of $T_{ref}$ cells than in their absence) and 13.3, respectively), whereas proliferation of epitope-specific $CD8^+$ CTLs restricted by other HLA-A and HLA-B alleles (control

alleles) was significantly ($P = 0.0005$) suppressed (Fig. 1a,c). The control allele groups were *HLA-A*02*, *HLA-A*24* and *HLAB*08*, allele groups with at least three epitope responses found in at least two HIV-1 infected individuals. Of note, responses were differentially susceptible to suppression, with CTLs restricted by HLA-A*03 being particularly susceptible ($P = 0.0005$; Fig. 1a; median percentage $T_{ref}$ cell suppression 92.8). Of note, $T_{ref}$ cells suppressed interferon-γ (IFN-γ) secretion from all $CD8^+$ CTLs (Fig. 1b,d; $P = 0.02$ for HLA-B*27–restricted responses and $P = 0.005$ for HLAB*57, HLA-A*03 and control allele group). This suggests that only the proliferative ability of effector $CD8^+$ CTLs is differentially suppressed by $T_{ref}$ cells, whereas cytokine secretion is always suppressed, regardless of the HLA restriction.

We also observed differential suppression of HIV-specific CTLs within individuals (for example, subjects NP02 and NP41, Fig. 1e). Thus HIV-specific CTLs restricted by HLA-B*27 or HLAB*57 were not suppressed by $T_{ref}$ cells, whereas HIV-specific CTLs restricted by other alleles from the same individual were suppressed, suggesting that the suppression was due to a difference in the CTLs (for example, in the strength of T cell receptor (TCR) signaling) rather than the $T_{ref}$ cells.

Although many individuals with *HLA-B*57* allele group have longer AIDS-free survival than individuals with other HLA alleles, the majority will eventually progress to disease. Indeed, it has been shown that *HLA-B*57*$^+$ LTNPs who eventually progress to disease have HLA-B*57–restricted T cells with lower proliferative capacity when compared to *HLA-B*57*$^+$ LTNPs who have not progressed[8]. This reduced proliferation correlated with an increased susceptibility to $T_{ref}$ cell–mediated suppression when we compared HIV-specific, HLA-B*57–restricted $CD8^+$ CTLs in *HLA-B*57*$^+$ LTNPs versus *HLA-B*57*$^+$ delayed progressors ($P = 0.0002$; Fig. 1f). Longitudinal analyses before and after progression in three individuals confirmed that when clinical progression to disease occurred, previously $T_{ref}$ cell–resistant HLA-B*57–restricted CTLs became susceptible to $T_{ref}$ cell-mediated suppression ($P = 0.004$; Fig. 1g).

## Differential suppression is independent of CTL frequency

HLA-B*27– and HLA-B*57–restricted $CD8^+$ CTLs proliferate more than HIV-specific $CD8^+$ CTLs restricted by other HLAs[8, 19]. Thus, cells capable of high proliferation may escape $T_{ref}$ cell–mediated suppression regardless of their allele restriction. We examined whether percentage suppression by $T_{ref}$ cells (defined in Methods) correlated with either the initial precursor frequency of epitope-specific $CD8^+$ CTLs (measured by IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay) or with magnitude of proliferation. Only about 10% of the data could be explained by either initial precursor frequency ($r^2 = 0.1$, $P = 0.01$; Supplementary Fig. 1a) or magnitude of proliferation ($r^2 = 0.13$, $P = 0.003$; Supplementary Fig. 1b) suggesting that neither initial precursor frequency nor magnitude of proliferation has a substantial role in determining the susceptibility of the CTLs to $T_{ref}$ cell–mediated suppression.

Because all of the HLA-A*03–restricted responses were of low magnitude (below 3% $CFSE^{lo}CD8^+$ CTLs) (Fig. 1a), we extended these analyses to look at only low-frequency responses below 3%. Even then, only 21% of the data variations were explained by the magnitude of proliferation ($r^2 = 0.21$, $P = 0.0004$; Supplementary Fig. 1c), and this weak correlation seemed to be mainly driven by HLA-A*03–restricted responses ($n = 11$; $r^2 = 0.82$, $P < 0.0005$), as responses restricted by HLA-B*57 were not correlated with their ability to be suppressed ($n = 12$; $P = 0.2$). These data suggested that for some low-frequency responses (for example, restricted by HLA-A*03), the magnitude of proliferation might explain susceptibility to $T_{ref}$ cell–mediated suppression, with lower frequency responses

being more susceptible. However, this does not explain why HLA-B*27– and HLA-B*57–restricted T cells are resistant to $T_{ref}$ cell–mediated suppression.

## $T_{ref}$ cells mediate differential suppression via Gal-9–Tim-3

Upon activation with their cognate epitopes, effector $CD8^+$ CTLs upregulate many inhibitory receptors (for example, programmed death-1 (PD-1) and Tim-3) as a means of self-limiting the inflammatory response. $T_{ref}$ cells constitutively express the ligands for Tim-3 (Gal-9) and PD-1[20, 21]. We hypothesized that the differential sensitivity to $T_{ref}$ cell–mediated suppression could be due to differential upregulation of inhibitory receptors on $CD8^+$ CTLs restricted by HLAB*27 and HLA-B*57 versus $CD8^+$ CTLs restricted by other HLA alleles. Tim-3 was of particular interest, as Tim-3–knockout mice are resistant to peripheral tolerance[22], and elevated frequencies of dysfunctional $Tim\text{-}3^+CD8^+$ CTLs are found in HIV-infected individuals with progressive disease[14]. Furthermore, although in recent years PD-1 has been the primary marker for 'exhausted' T cells[23], new data have shown that Tim-3 expression correlates better with dysfunctional $CD8^+$ CTLs than expression of PD-1 (ref. 24).

Peripheral blood mononuclear cells (PBMCs) from LTNPs are not exhausted and do not constitutively express PD-1 or Tim-3. However, they do upregulate these inhibitory receptors after antigenic stimulation. We stimulated PBMCs with HIV-1 epitopes bound by various HLAs and determined the frequency of Tim-3 expression on antigen-specific $CD8^+$ CTLs (as measured by either tetramer staining (Fig. 2a) or CD137 upregulation[25] (Fig. 2b)). As predicted, HIV-specific $CD8^+$ CTLs restricted by HLA-B*27 or HLA-B*57 allele groups upregulated significantly less Tim-3 after stimulation than HIV-specific $CD8^+$ CTLs restricted by other HLA alleles (Fig. 2c, $P = 0.005$), suggesting that $T_{ref}$ cells may mediate differential suppression of effector $CD8^+$ CTLs via Gal-9–Tim-3 interactions. To test this, we masked Gal-9 on $T_{ref}$ cells by adding lactose (Fig. 3), which is a ligand of the galectin family of lectins, to which Gal-9 belongs. Addition of lactose during *in vitro* proliferation assays[26] prevented $T_{ref}$ cell–mediated suppression of proliferation of HIV-specific $CD8^+$ CTLs restricted by any HLA alleles other than the HLA-B*27 or HLA-B*57 allele groups but had no effect on proliferation of HIV-specific $CD8^+$ CTLs restricted by HLA-B*27 or HLA-B*57 (Fig. 3a,c). Because lactose is not specific for Gal-9, but rather binds all lectins, we also isolated $T_{ref}$ cells from PBMCs, silenced *LGALS9* using siRNA (Supplementary Fig. 2) and then added the cells back to $T_{ref}$ cell–depleted PBMCs, followed by stimulation with their cognate HIV-1 epitopes. In agreement with the lactose studies, knockdown of *LGALS9* in $T_{ref}$ cells prevented their suppression of proliferation of HIV-specific $CD8^+$ CTLs restricted by any non–HLA-B*27 or non–HLA-B*57 allele groups but had no effect on their suppression of proliferation of HIV-specific $CD8^+$ CTLs restricted by HLAB*27 or HLA-B*57 (Fig. 3b,d). Therefore, differential suppression of proliferation of HIV-specific $CD8^+$ CTLs by $T_{ref}$ cells is mediated through Gal-9–Tim-3 interactions; HLA-B*27– or HLA-B*57–restricted HIV-specific $CD8^+$ CTLs upregulate less Tim-3 when they encounter their cognate epitopes than HIV-specific CTLs restricted by other HLAs, and, thus, they are less susceptible to $T_{ref}$ cell–mediated suppression.

## Protective CTLs kill $T_{ref}$ cells via granzyme B

A recent study showed that HIV-specific HLA-B*27– and HLA-B*57–restricted $CD8^+$ CTLs upregulate more granzyme B (GzmB, encoded by *GZMB*) on a per-cell basis than HIV-specific $CD8^+$ CTLs restricted by other HLAs[19]. This upregulation is independent of their enhanced proliferative ability. Moreover, it has also been shown that $CD4^+$ effectors that express high amounts of GzmB escape $T_{ref}$ cell–mediated suppression by killing $T_{ref}$ cells that they encounter[27]. This mechanism of $T_{ref}$ cell evasion has not been observed for human $CD8^+$ effector cells. To determine whether this mechanism could also be involved in

the observed allele-specific differential suppression by $T_{ref}$ cells, we performed epitope-specific CFSE dilution assays in the presence of z-AAD-CMK (z-Ala-Ala-Asp(OMe)-chloromethyl ketone), a potent and selective GzmB peptide inhibitor. When we preincubated $CD8^+$ T cells with z-AAD-CMK, $CD8^+$ CTLs restricted by HLA-B*27 and HLA-B*57 became susceptible to $T_{ref}$ cell suppression (Fig. 4a and Supplementary Fig. 3). Similarly, if we silenced *GZMB* in $CD8^+$ CTLs with *GZMB* siRNA (Supplementary Fig. 2b) before stimulation with cognate epitope in the presence or absence of $T_{ref}$ cells, the HLA-B*27– and HLA-B*57–restricted $CD8^+$ CTLs were rendered susceptible to $T_{ref}$ cell–mediated suppression, whereas $CD8^+$ CTLs restricted by HLA-B*27 and HLA-B*57 treated with an irrelevant siRNA were not susceptible to $T_{ref}$ cell suppression (Fig. 4b and Supplementary Fig. 3b). This suggests that either expression of Tim-3 or lack of expression of GzmB in $CD8^+$ effector CTLs may increase their susceptibility to $T_{ref}$ cell–mediated suppression. Furthermore, the resistance of HLA-B*27– and HLA-B*57–restricted $CD8^+$ CTLs to $T_{ref}$ cells resulted from their directly killing $T_{ref}$ cells when they were stimulated with their cognate epitopes, whereas we observed low killing of $T_{ref}$ cells in cultures stimulated with non–HLA-B*27 and non–HLA-B*57 epitopes (Fig. 5a). When we blocked GzmB activity with z-AAD-CMK, the frequency of apoptotic $T_{ref}$ cells was reduced greatly in cultures stimulated with HLA-B*27– and HLA-B*57–restricted epitopes compared with the absence of GzmB inhibitor (Fig. 5a). In contrast, blockade of GzmB activity in cultures stimulated with non–HLA-B*27 and non–HLA-B*57 epitopes had minimal effect on the frequency of apoptotic $T_{ref}$ cells. Thus, HLA-B*27– and HLA-B*57–restricted effector $CD8^+$ CTLs killed the $T_{ref}$ cells they encountered in a GzmB-dependent manner. Killing mediated by HLA-B*27– and HLAB*57–restricted HIV-specific $CD8^+$ CTLs was not limited to $T_{ref}$ cells, as they also killed non-$T_{ref}$ $CD4^+$ cells (Supplementary Fig. 4). HLA-B*27 and HLA-B*57–restricted CTLs killed considerably more $T_{ref}$ cells than non–HLA-B*27 and HLA-B*57–restricted CTLs (Fig. 5a). Notably, both HLA-B*27 or HLA-B*57–restricted effectors and non–HLA-B*27 or HLAB*57–restricted effectors induced more apoptosis of $T_{ref}$ cells than of non-$T_{ref}$ cells ($P < 0.0001$ for HLA-B*27 or HLA-B*57–restricted effectors and $P = 0.001$ for non–HLA-B*27 or HLAB*57–restricted effectors; Supplementary Fig. 4). Thus, $T_{ref}$ cells are not seen as '$T_{ref}$ cells' by protective $CD8^+$ CTLs but rather as any other $CD4^+$ T cell presenting their epitope.

Maximal expression of lytic effector molecules (perforin and GzmB) requires many days of proliferation[6, 19]. This explains why $T_{ref}$ cells were able to suppress the IFN-γ secretion capabilities of HLA-B*27– and HLA-B*57–restricted T cells, as there would be insufficient GzmB upregulation to kill $T_{ref}$ cells during this short assay (24 h). In agreement with this, we did not observe killing of $T_{ref}$ cells in cultures stimulated for 24 h with HLA-B*27 and HLA-B*57 epitopes (Fig. 5b). However, after 72 h, we observed a substantial increase in the killing of $T_{ref}$ cells by HLA-B*27–restricted $CD8^+$ CTLs (Fig. 5c).

In agreement with the data showing that HLA-B*57– restricted $CD8^+$ CTLs became susceptible to $T_{ref}$ cell–mediated suppression of proliferation upon disease progression, HIV-specific HLAB*57–restricted $CD8^+$ CTLs from delayed progressors failed to kill $T_{ref}$ cells ($P = 0.0003$; Fig. 5d). Analyses before and after progression in *HLA-B*57*+ individuals confirmed that when clinical progression to disease occurred, HLA-B*57–restricted HIV-specific CTLs lost their ability to kill $T_{ref}$ cells ($P = 0.008$; Fig. 5e,f).

The fact that stimulated HLA-B*27– and HLA-B*57–restricted $CD8^+$ CTLs kill $T_{ref}$ cells suggested that there should be fewer $T_{ref}$ cells in individuals who express these HLA alleles. Indeed, we found a significant decrease ($P = 0.0003$) in $T_{ref}$ cell frequency in *HLA-B*27*$^+$ or *HLA-B*57*$^+$ LTNPs compared with non–*HLA-B*27*$^+$ and non–*HLA-B*57*$^+$ LTNPs and seronegative individuals (Fig. 5g), suggesting that deletion of $T_{ref}$ cells occurs *in vivo* in

*HLAB* 27*$^+$ and *HLA-B*57*$^+$ individuals despite their maintenance of normal CD4$^+$ T cell counts.

Our data show that HLA-B*27– and HLA-B*57–restricted CTLs are resistant to $T_{ref}$ cell–mediated suppression irrespective of the epitopes recognized by the T cells (Fig. 1a and Supplementary Fig. 5), suggesting that resistance to $T_{ref}$ cell–mediated peripheral tolerance is related to allele restriction rather than epitope specificity. To determine whether this occurs only during HIV infection, we assessed $T_{ref}$ cell susceptibility of HLA-B*27–restricted versus HLA-A*02– or HLA-B*07–restricted herpes simplex virus (HSV-2) or Epstein Barr virus (EBV) epitopes in HIV-seronegative individuals. HSV- and EBV-specific HLA-B*27–restricted CD8$^+$ CTLs were also not susceptible to $T_{ref}$ cell–mediated suppression, whereas HSV-2– and EBV-specific CD8$^+$ CTLs restricted by other alleles were susceptible (Supplementary Fig. 6), proving that ability to evade $T_{ref}$ cells is due to the allele restriction of the CTL and not to the specific infection.

## DISCUSSION

Although the role of human $T_{ref}$ cells in preservation of self-tolerance is well documented, less is known about their influence on chronic viral infection. Several studies have shown that $T_{ref}$ cell frequencies are either increased[28, 29] or decreased in HIV infection[30, 31]. Despite the conflicting data, some recent *in vivo* studies suggest that high frequencies of $T_{ref}$ cells during HIV infection are detrimental. Specifically, a recent *in vivo* phase 3 trial showed that IL-2 therapy preferentially expands $T_{ref}$ cells in infected individuals and that individuals with the greatest expansion are more likely to progress to disease[32, 33].

We show that there are two divergent outcomes for HIV-specific CD8$^+$ CTLs during chronic infection: the majority of HIV-specific CTLs upregulate Tim-3 when they encounter their cognate epitopes and are subsequently suppressed by $T_{ref}$ cells; however, CD8$^+$ CTLs restricted by protective HLA allele groups upregulate less Tim-3 but more GzmB upon recognition of their cognate epitopes. They are subsequently less susceptible to $T_{ref}$ cell–mediated suppression, instead killing $T_{ref}$ cells they encounter. Thus, our data suggest a previously unknown model of how HLA-B*27– and HLA-B*57–restricted, HIV-specific CD8$^+$ CTLs may evade $T_{ref}$ cells and subsequently control HIV replication (Fig. 6). Moreover, we provide data showing that HIV-specific, HLA-B*57–restricted CD8$^+$ CTLs can be suppressed by $T_{ref}$ cells once progression to disease occurs. However, we cannot at present determine whether disease progression in these individuals is a cause or effect of loss of the ability to evade $T_{ref}$ cells.

The mechanisms accounting for why HLA-B*27– and HLA-B*57–restricted T cells upregulate less Tim-3 and more GzmB than other HIV-specific T cells upon recognition of their cognate epitopes are not known. Understanding these differences in TCR signaling will be crucial for determining potential therapeutic interventions that 'switch on' virus-specific CTLs that are restricted by other HLAs. Such mechanisms may include a higher-avidity TCR on CD8$^+$ CTLs restricted by HLA-B*27 and HLA-B*57 (ref. 34). In addition, HLA-B*27 is an unusual allele group in that it can form heavy-chain homodimers and trimers, which may lead to alternative TCR interactions[35].

The observation that $T_{ref}$ cells are killed more efficiently than non-$T_{ref}$ CD4$^+$ T cells suggests that, during HIV infection, $T_{ref}$ cells are more susceptible to effector-induced apoptosis. This is consistent with studies demonstrating that $T_{ref}$ cells have increased turnover and higher expression of caspase-3 during chronic infection[36]. Increased intracellular levels of caspase-3 have been shown to increase susceptibility of T cells to apoptosis[37]. Peripheral $T_{ref}$ cell frequencies are similar between *HLA-B*27*$^+$ or *HLA-B*57*$^+$

LTNPs and *HLA-B*27*$^+$ or *HLAB*57*$^+$ uninfected subjects. Most of the LTNPs studied here have low or undetectable viral load in their peripheral blood, so there is minimal antigen to stimulate killing of $T_{ref}$ cells by HLA-B*27 or HLA-B*57–restricted CTLs. We presume that HLA-B*27 or HLA-B*57–restricted CTLs will kill $T_{ref}$ cells where antigen is present, so it is not surprising that we did not observe significant differences in $T_{ref}$ cell frequencies in the periphery between uninfected and infected individuals. However, in lymph nodes and gut we would expect to see fewer $T_{ref}$ cells in infected individuals with *HLA-B*27* or *HLA-B*57*. This is supported by data from another group showing fewer $T_{ref}$ cells in lymph nodes from LTNPs than in those from infected individuals with progressive infection[29]. Therefore, it is possible that loss of effector cell evasion of $T_{ref}$ cells results in accumulation of $T_{ref}$ cells in lymph nodes and disease progression.

Our study indicates that CD8$^+$ CTLs are differentially susceptible to $T_{ref}$ cell–mediated suppression of proliferation dependent on HLA restriction but independent of the epitope specificity of the suppressed cell. Thus, HIV-specific CTLs restricted by HLA-B*27 and HLAB*57 are resistant to $T_{ref}$ cells, allowing them to continue to function maximally during chronic infection. This may explain why these HLAs are associated with delayed progression to disease. Resistance of HLA-B*27–restricted CTLs to $T_{ref}$ cell–mediated suppression may also explain why this allele group is associated with better clinical outcomes of other chronic infections, such as hepatitis C virus[15]. Both *HLA-B*27* and *HLA-B*57* allele groups are highly associated with autoimmunity: *HLA-B*27* allele groups with ankylosing spondylitis[16] and *HLA-B*57* allele groups with psoriasis[38]. Thus, possession of these allele groups seems to be a double-edged sword. Because T cells restricted by them cannot be tolerized, these allele groups are beneficial in chronic infection but detrimental in autoimmunity. Therefore, we feel this work has far-reaching implications for both control of chronic infection and autoimmunity.

## METHODS

### Study group

LTNPs were enrolled through the HIV Vaccine Trial Unit (M.J.M.). They were defined as HIV infected for more than 11 years, with repeated CD4$^+$ T cell counts over 500 cells per μl or CD4% over 28% and viral load <10,000 copies per ml in the absence of antiretroviral therapy. Clinical data and HLA genotypes are shown in Supplementary Table 1. An additional subject was recruited through the University of Washington Primary Infection Clinic (A.C.C.), meeting early criteria of an elite controller (viral load 58 copies per ml), infected for about ten years but recently progressed. We studied 17 LTNPs and 7 LTNPs who eventually progressed (delayed progressors), defined as HIV infected with viral load >10,000 RNA (copies per ml) or declining CD4$^+$ T cell count in the absence of antiretroviral therapy. All subjects were males of European descent except subject NP14 who reported African-American ethnicity. HIV-1 seronegative–individuals were recruited at Seattle BioMed.

Fourteen HIV-seronegative individuals (seven males and seven females) who were seropositive for HSV-2 were recruited and HLA-typed as described[39] (Supplementary Table 1). They were assumed to be EBV seropositive if they had an IFN-γ response to known EBV CD8$^+$ epitopes because most adults are EBV seropositive.

The appropriate Institutional Review Boards at the University of Washington, Fred Hutchinson Cancer Research Center and Seattle BioMed approved the studies, and all individuals provided written informed consent.

### Epitope mapping

HIV epitope specificities were mapped in each $HIV^+$ individual as previously described[8]. Some LTNPs were mapped using potential T cell epitopes provided by The National Institutes of Health AIDS Research and Reference Reagent Program. The epitope-specific responses assessed for each individual are shown in Supplementary Table 2. $HSV\text{-}2^+$ individuals were mapped as previously described[39]. HSV and EBV epitope-specific responses assessed for each individual are shown in Supplementary Table 2.

### Cell separation and flow cytometry

We purified $T_{ref}$ cells by magnetic separation (STEMCELL Technology) and assessed purity by flow cytometry using a LSRII flow cytometer (BD Biosciences). We analyzed flow data with FlowJo software v7.2.2 (Tree Star). To determine levels of $T_{ref}$ cell apoptosis, we labeled epitope-stimulated PBMCs with annexin V (BD Bioscience).

### *In vitro* cytokine measurements

We measured cytokine responses by performing ELISPOT or intracellular cytokine secretion (ICS) assays[40, 41]. In brief, we cultured $2 \times 10^5$ (ELISPOT) and $1 \times 10^6$ (ICS) cells per well and stimulated them with 2 μg $ml^{-1}$ of their cognate epitopes. For ELISPOT we called a response positive when the number of spot-forming cells was twice the background level and there were at least 50 spot-forming cells per $1 \times 10^6$ PBMCs. For ICS, we called a response positive when the percentage of bright $cytokine^+CD8^+$ T cells was twice that of the negative control.

### Proliferation assays

We performed CFSE dilution assays as previously described[8]. Where indicated, we added $T_{ref}$ cells (1:4) to PBMCs or $CD8^+$ T cell cultures, although we obtained similar data when we used a ratio as low as 1:16 $T_{ref}$ cells:PBMCs (that is, an approximate $T_{ref}$ cell frequency of 6%, which we have shown is in the physiological range of $T_{ref}$ cell frequencies *in vivo*; Supplementary Fig. 5). We performed some assays in the presence of lactose (Sigma-Aldrich; 30 mM) or the GzmB inhibitor z-AAD-CMK (Calbiochem; 10 μg $ml^{-1}$).

### Small interfering RNA transduction

For silencing of *LGALS9*, we performed RNA interference experiments on isolated $T_{ref}$ cells according to the manufacturer's protocol (Santa Cruz Biotechnology). Knockdown of *LGALS9* in $T_{ref}$ cells is shown in Supplementary Figure 2a. Control $T_{ref}$ cells received a scrambled I duplex RNA. Seven hours after transfection, we replaced the transfection medium with complete medium and cultured $T_{ref}$ cells for another 24 h before using them in CFSE proliferation assays. For silencing of *GZMB*, we electroporated PBMCs or $CD8^+$ T cells with GZMB siRNA (Applied Biosystems/Ambion) following the manufacturer's guidelines. We rested the cells for 36 h before using them in CFSE proliferation assays. Expression of intracellular GzmB after electroporation is shown in Supplementary Figure 2b.

### Statistical analyses

We calculated percentage suppression of proliferation by the formula (percentage $CFSE^{lo}CD8^+$ without $T_{ref}$ cells − percentage $CFSE^{lo}CD8^+$ with $T_{ref}$ cells) / percentage $CFSE^{lo}CD8^+$ without $T_{ref}$ cells × 100, grouped according to HLA restricting allele. Two responses with $CFSE^{lo}CD8^+$ cells without $T_{ref}$ cells were zero, and the lowest measured response (0.012) was substituted to prevent a division by zero error. We similarly calculated percentage suppression by lactose and *LGALS9* siRNA by substituting the differential suppression via these compounds. We calculated percentage suppression of cytokine

secretion by the formula (spot-forming cells per million PBMCs without $T_{ref}$ cells–spot-forming cells per million PBMCs with $T_{ref}$ cells) / spot-forming cells per million PBMCs without $T_{ref}$ cells × 100. We used signed Wilcoxon tests to compare paired data with and without the suppressing agent ($T_{ref}$ cells, lactose, siRNA). For comparisons of grouped data, we used Kruskal-Wallis tests with a *post hoc* Dunn's test for subgroup comparisons. We used Spearman rank tests for correlation analyses. All tests were two-tailed, with a *P* value of less than 0.05 considered statistically significant. We conducted statistical analyses and graphing with GraphPad Prism 5.02 (GraphPad) and Adobe Illustrator Creative Suite 3, 13.0.2 (Adobe Systems).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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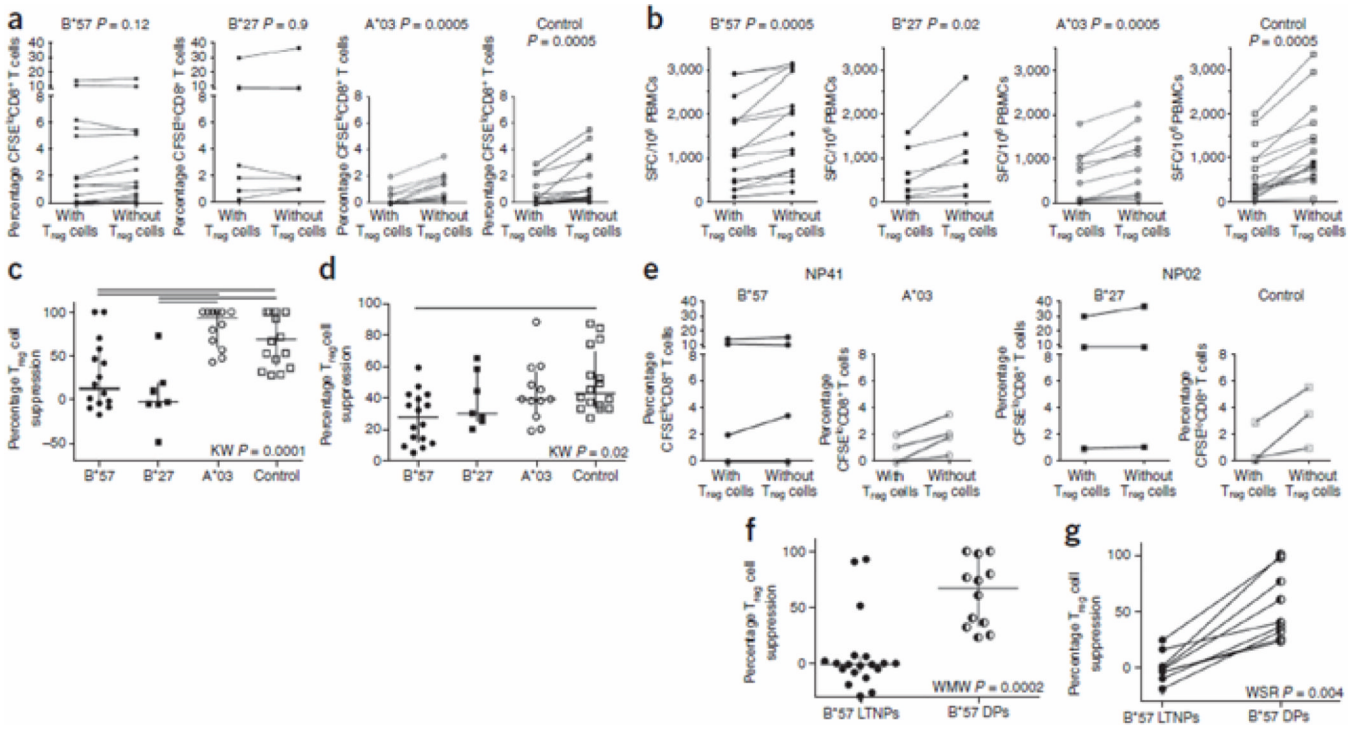


**Figure 1.**

Treg cell suppression of *in vitro* proliferative ability or cytokine secretion of CD8+ T cells restricted by HLA-B*57, HLA-B*27, HLA-A*03 and control HLAs (HLA-A*02, HLA-A*24 and HLA-B*08). (**a**) Background-subtracted percentage CFSEloCD8+ T cells in PBMCs with or without $T_{reg}$ cells when cultured in the presence of HIV-1 epitopes recognized by CD8+ T cells restricted by various HLA alleles. (**b**) Background-subtracted HIV-specific IFN-γ ELISPOT responses in the presence and absence of Treg cells. HLA-B*27 or HLA-B*57–restricted and non–HLA-B*27– and HLA-B*57–restricted responses are shown after stimulation with their cognate epitopes. SFC, spot-forming cell. In **a** and **b**, Wilcoxon signed-rank (WSR) test was used. (**c**) Percentage suppression of proliferation grouped according to HLA restricting allele. (**d**) Percentage suppression of cytokine secretion grouped according to HLA restricting allele. In **c** and **d**, Kruskal-Wallis (KW) test was used for grouped comparisons with a *post hoc* Dunn's test showing significant subgroup comparisons with horizontal lines. (**e**) Differential suppression of proliferation of HLA-B*27– and HLA-B*57– versus HLA-A*03– and control HLA–restricted HIV-specific CD8+ CTLs within the same person. NP02 and NP41 are two LTNPs. (**f**) Percentage suppression of proliferation by Treg cells of HLA-B*57–restricted CD8+ CTLs in *HLAB*57*+ LTNP versus *HLA-B*57*+ delayed progressors (DP). (**g**) Longitudinal analyses of percentage suppression of proliferation by Treg cells of HLA-B*57–restricted CD8+ CTLs before and after progression in *HLA-B*57*+ individuals.

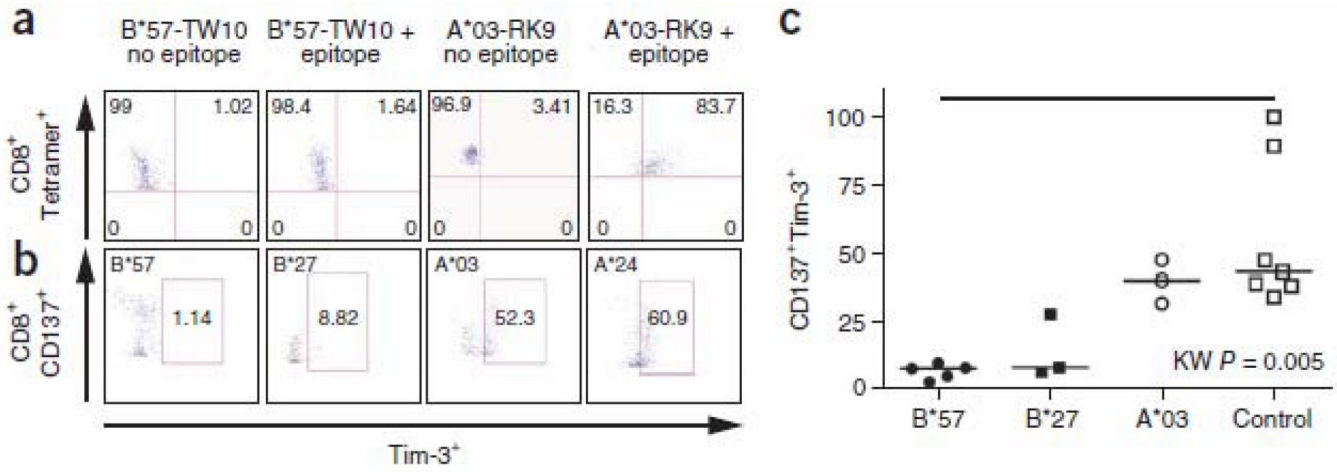


**Figure 2.**

Frequency of CD8+Tim-3+ T cells following stimulation with their cognate epitopes. (**a**) Percentage of Tim-3+ CD8+ T cells using allophycocyanin-labeled HLA-A*03–RLRPGGKKK tetramer or phycoerythrin-labeled HLA-B*57-TSTLQEQIGW tetramer staining of PBMCs before and after stimulation with their cognate epitopes. Top right quadrant shows percentage of Tim-3+tetramer+ CD8+ CTLs. (**b**) Percentage of Tim-3+ on CD8+ T cells using CD137 to identify antigen-specific T cells after stimulation with their cognate epitopes. (**c**) Percentage of CD137+Tim-3+ T cells after stimulation of PBMCs from different individuals with their corresponding epitopes.

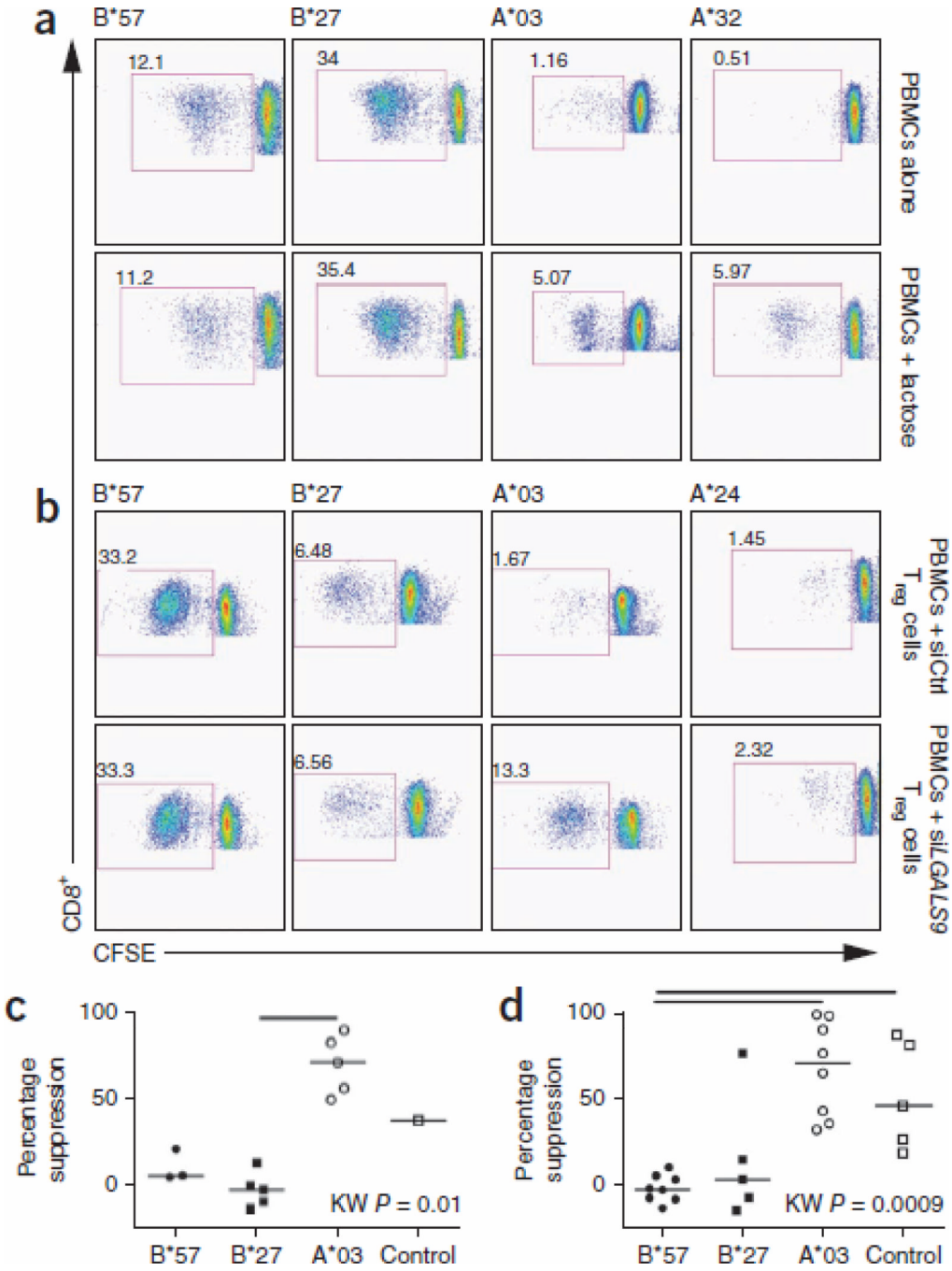


**Figure 3.**

CFSE dilution data showing inhibition of Gal-9–Tim-3 interactions by lactose and siRNA. (**a**) Examples of proliferation of PBMCs stimulated with their corresponding epitopes, showing percentage CFSEloCD8+ T cells in the absence or presence of lactose. (**b**) Examples of proliferation of CFSE-labeled, Treg cell–depleted PBMCs stimulated with their cognate epitopes in the presence of Treg cells treated with either *LGALS9* siRNA or siControl (at 1:0.25 ratio). The measures of coculture suppression by Treg cells in the presence or absence of lactose or siRNA are shown for a representative experiment from three repeat experiments for each approach. (**c**) Percentage of Treg cell suppression calculated after stimulation of CD8+ T cells with their corresponding epitopes in the

presence of lactose. (**d**) Percentage of Treg cell suppression calculated after stimulation of CD8+ T cells with their cognate epitopes in the presence of *LGALS9* siRNA–treated Treg cells (at 1:0.25 ratio).

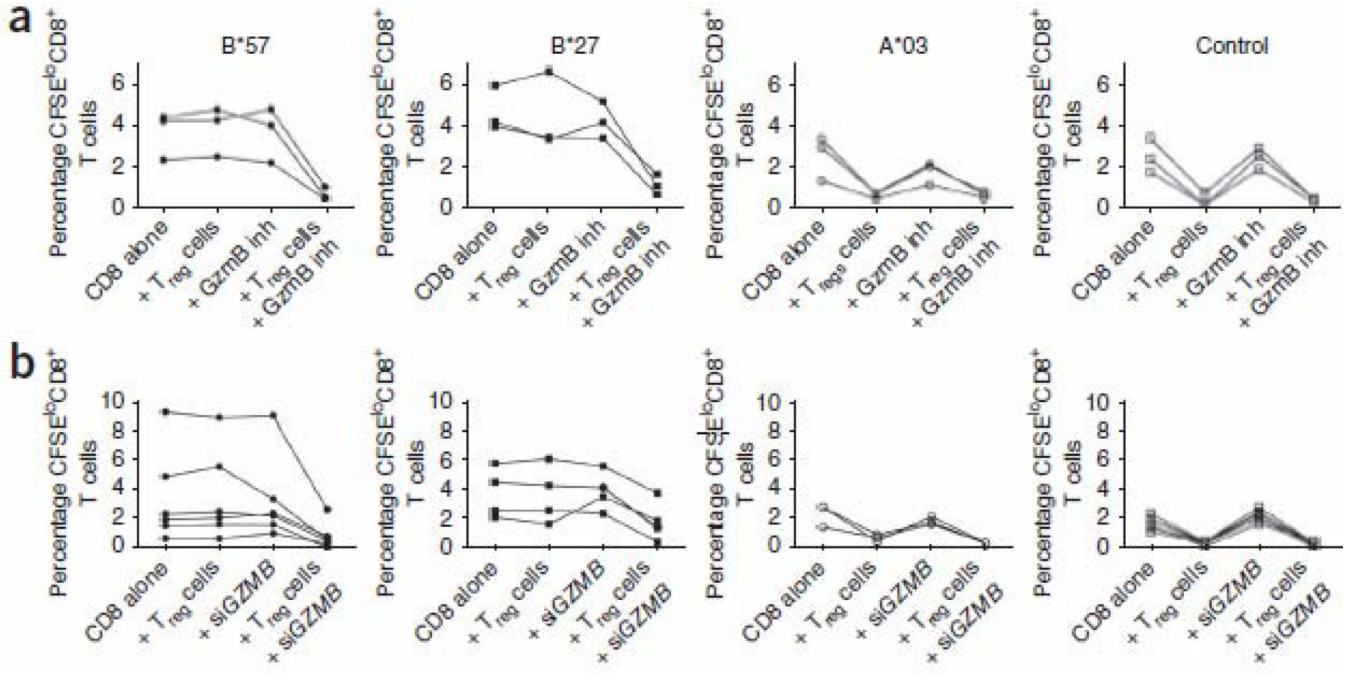


**Figure 4.**

CFSE dilution data showing CD8+ T cells restricted by HLA-B*57 and HLA-B*27 resist Treg cell-mediated suppression in a GzmB dependent manner. (**a**) Percentage CFSEloCD8+ T cells after CFSE-labeled isolated CD8+ T cells were stimulated with their corresponding epitopes alone or together with Treg cells (at 1:0.25 ratio), and also in the presence or absence of a GzmB peptide inhibitor (z-AAD-CMK). Examples of flow data are shown in Supplementary Figure 3a. (**b**) Percentage CFSEloCD8+ T cells after electroporation with *GZMB* siRNA or nonhybridizing negative control (siControl) siRNA oligonucleotides and stimulation with their cognate epitopes alone or with Treg cells (at 1:0.25 ratio). Examples of flow data are shown in Supplementary Figure 3b.

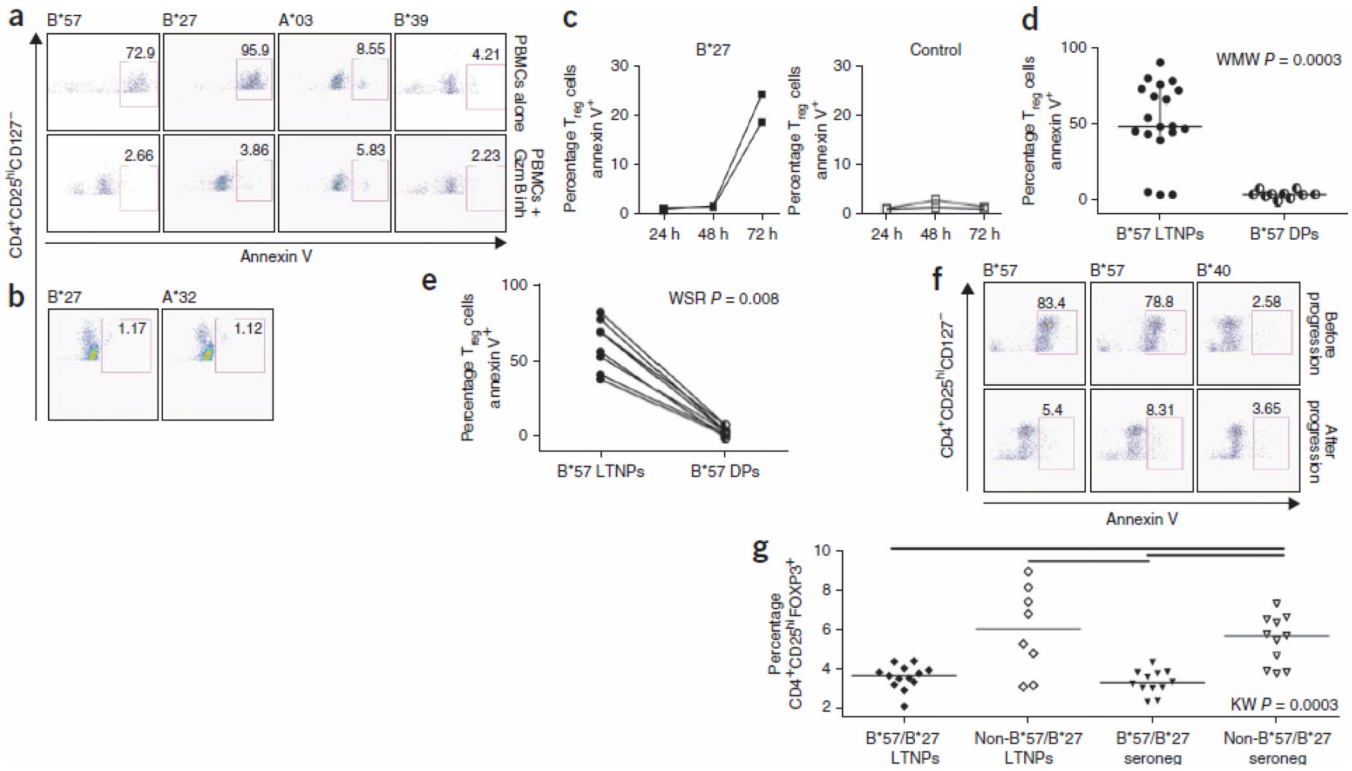


**Figure 5.**

CD8+ T cells restricted by HLA-B*27 and HLA-B*57 induce Treg apoptosis in a GzmB-dependent manner. (**a–c**) Percentage annexin V+ Treg cells (CD3+CD4+CD25hiCD127lo) in PBMCs stimulated for 4 d (**a**), 24 h (**b**) or 24–72 h (**c**) with HLA-B*27–, HLA-B*57–, HLA-B*39– or HLA-A*03–restricted epitopes in the presence or absence of GzmB peptide inhibitor. These data are representative of three separate experiments from different LTNPs. (**d**) Percentage annexin V+ Treg cells in PBMCs stimulated with HLA-B*57–restricted epitopes from *HLA-B*57*+ LTNP versus *HLA-B*57*+ DPs. (**e,f**) Percentage annexin V+ Treg cells in PBMCs stimulated with HLA-B*57–restricted epitopes before and after progression to disease. (**g**) Treg cell frequencies in HIV-1–seronegative individuals versus HIV-1 infected *HLA-B*27*+ or *HLA-B*57*+ and *HLA-B*27*− or *HLA-B*57*− LTNPs. Percentages of CD4+CD25hiFOXP3+ Treg cells are shown in PBMCs from 12 HIV-seronegative HLA-B*27+ or HLA-B*57+ individuals, 12 HLA-B*27– or HLA-B*-57– individuals, 13 *HLA-B*27*+ or *HLA-B*57*+ LTNPs and 8 *HLA-B*27*− or *HLA-B*57*− LTNPs.

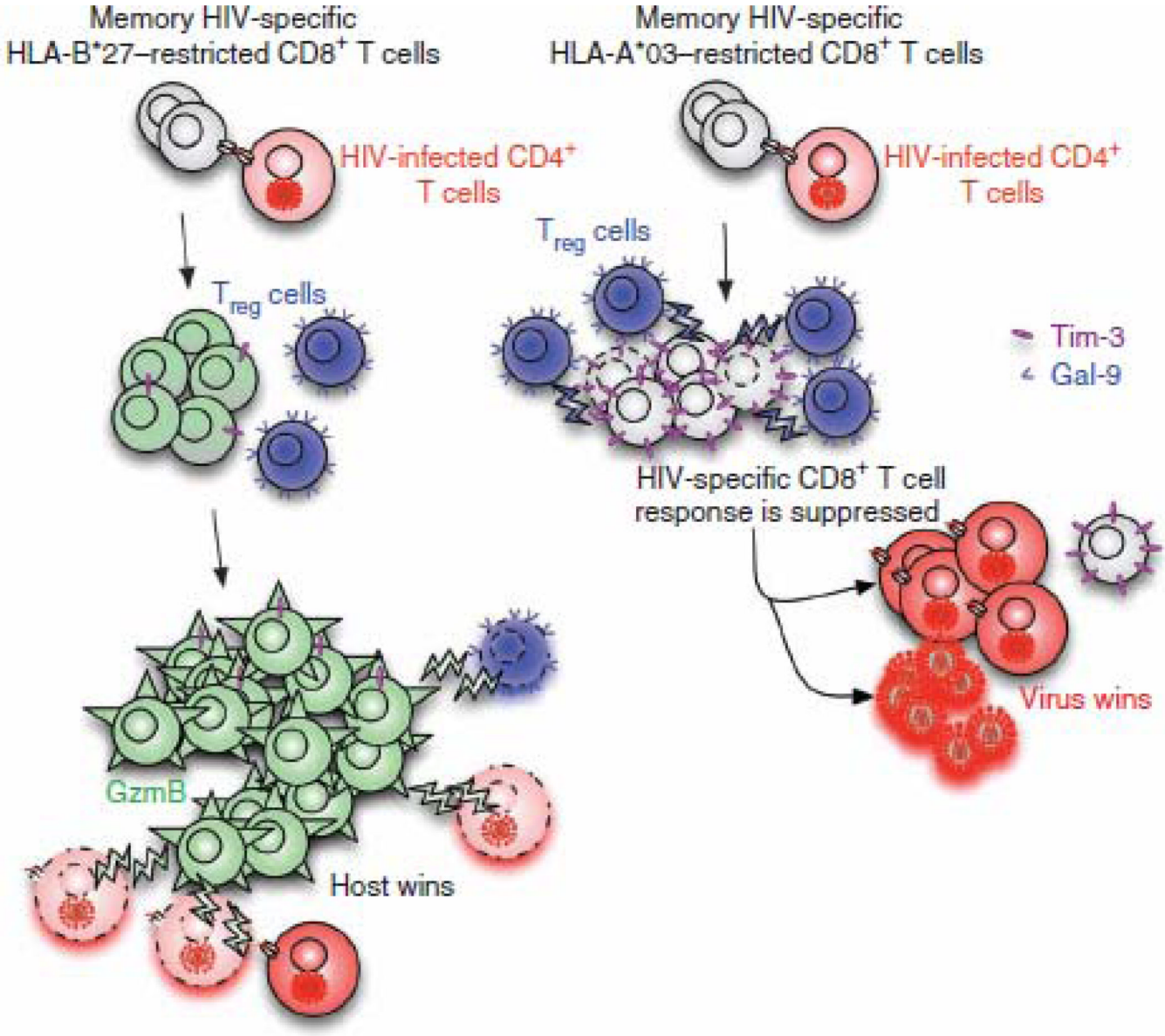


**Figure 6.**

Model depicting how HLA-B*27– or HLA-B*57–restricted HIV-specific CD8+ T cells evade Treg cell suppression and subsequently control HIV replication. HIV-specific, HLA-B*27–restricted CD8+ T cells do not upregulate surface expression of Tim-3 upon recognition of their cognate epitopes on HIV-infected CD4+ T cells, whereas HIV-specific, HLA-A*03–restricted CD8+ T cells upregulate high surface expression of Tim-3. Treg cells suppress HLA-A*03–restricted CD8+ T cells owing to their high expression of Tim-3 but cannot suppress proliferation of HLA-B*27–restricted CD8+ T cells. Highly proliferating HLA-B*27–restricted CD8+ T cells upregulate high levels of GzmB and kill not only infected CD4+ T cells but also infected Treg cells that they encounter. Thus, HLA-B*27–restricted CD8+ T cells can control HIV replication during chronic infection, whereas HLA-A*03–restricted CD8+ T cells cannot.