Brief Report: Enhanced Allogeneic Cellular Responses to Mismatched HLA-B Antigens Results in More Efficient Killing of HIV Infected Cells

Steffanie Sabbaj, University of Alabama Birmingham
Nicholas Scanlon, University of Alabama Birmingham
Victor Du, University of Alabama Birmingham
Yuge Wang, University of Alabama Birmingham
Jianming Tang, University of Alabama Birmingham
Eric Hunter, Emory University
Paul A. Goepfert, University of Alabama Birmingham

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Enhanced allogeneic cellular responses to mismatched HLA-B antigens results in more efficient killing of HIV infected cells

Steffanie SABBAJ, Ph.D. *,1, Nicholas SCANLON, B.S.1, Victor Y. DU, M.S.2, Yuge WANG, M.S.2, Jianming TANG, Ph.D.1, Eric HUNTER, Ph.D.3, and Paul A. GOEPFERT, M.D.1,2
1Department of Medicine, University of Alabama at Birmingham, Birmingham, AL
2Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL
3Emory Vaccine Center at Yerkes National Primate Research Center and Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia

Abstract
Epidemiologic studies have demonstrated that HIV-1 discordant couples who share HLA-B alleles were more likely to transmit HIV-1. These data lead us to hypothesize that individuals who match at both HLA-B alleles should have a reduced allogeneic response than those who are not matched. We observed diminished killing of CD4+ target cells only when HLA-B alleles were matched. We propose that for cell-associated HIV-1 transmission the ability of the recipient to eliminate infected cells from a donor partner may be enhanced when HLA-B alleles are different between partners. These findings suggest a novel mechanism for protection against HIV infection.

Keywords
HIV-1 transmission; T-cells; allogeneic responses; HLA-B; mixed lymphocyte reaction; Cytotoxic T-cells

INTRODUCTION
Epidemiologic studies demonstrated that HIV-1 discordant couples sharing HLA-B more likely transmit HIV-1, a finding not seen when sharing HLA-A or HLA-C alleles[1, 2]. Furthermore, mother and child HLA concordance increased risk of vertical transmission[3–6] and children sharing both HLA-B alleles with their mothers more likely acquired HIV-1 via vertical transmission[5]. Additional studies, demonstrated allo-immunization in humans elicited cellular responses that inhibited HIV-replication in vitro[7, 8]. More recently, it was demonstrated that monogamous partners practicing unprotected sex developed allogeneic CD8+ T-cell responses to HLA class I antigens that differed among partners. These

*Address correspondence to: Steffanie Sabbaj, Ph.D., 845 19th Street South, BBRB 554 Birmingham, AL 35294 Telephone: (205) 975-7612 Fax: (205) 975-6027, sabbaj@uab.edu.

AUTHOR CONTRIBUTIONS:
NS, YW, VYD and SS performed experiments; JT HLA-typed the samples, SS analyzed the data, PAG, EH & SS designed experiments and wrote the manuscript.

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responses inhibited HIV-1 infectivity in PBMC ex vivo in the heterosexual partners[9]. In vitro studies demonstrated allo-antigen stimulated anti-HIV activity in alloantigen-activated lymphocytes suppressing both heterologous and autologous HIV-infected cells, where effectors were obtained from seronegatives[10–12]. Further studies demonstrated HIV-1 suppressive CTLs from HIV-infected individuals mismatched at all HLA class I alleles[13]. These findings give merit to the idea that allogeneic responses confer some protection against HIV-infection, but do not explain the epidemiologic evidence that HLA-B sharing is most relevant. Therefore, we sought to determine a biological mechanism for this effect by asking if sharing of HLA-B but not HLA-A or HLA-C dampens the CD8 T-cell-mediated allogeneic response, benefiting cell-to-cell transmission of HIV-1. We propose that HLA-B mismatch between partners may impede cell-associated HIV transmission, as HIV-infected cells bearing mismatched HLA-B are recognized as “foreign” and targeted for elimination.

MATERIALS AND METHODS

Study Population

Cryopreserved PBMC from 36 HIV-seronegatives and 38 HIV-seropositives with HLA class I genotypes were used. HLA class I alleles were typed to four-digit specificity by PCR amplification using sequence-specific primers (Pel-Freez Clinical Systems, Brown Deer, WI) as previously described[14]. Informed consent was obtained under the guidelines of the Institutional Review Board of the University of Alabama at Birmingham.

CFSE based Mixed Lymphocyte Reaction (MLR) and Intracellular Cytokine Staining Assay (ICS)

We combined the ability to measure proliferation and cytokine production by flow cytometry for the detection of allo-stimulation in a mixed lymphocyte assay[15]. Briefly, PBMC stained with CFSE[16] were used as effectors (1 × 10⁶ cells) and incubated with irradiated (3000R) matched or mismatched stimulators (examples Table1) at a 1:1 ratio for 5 days, 37°C, 5% CO₂. On day 5 Brefeldin was added for 6 hrs. Surface staining with anti-CD3-Alexa780 (ebioscience), anti-CD4-PeCy7 and anti-CD8-PerCPCy5.5 for 20 minutes in the dark at RT followed. Washed cells were permeabilized with Cytofix/cytoperm reagent for 20 minutes at RT in the dark. ICS for the production of IFN-γ-Alexa700, Perforin-PE and Granzyme B-V450 followed. At least 100,000 CD3+ events were acquired using a LSRII cytometer (BD, San Jose, CA). Data was analyzed using FlowJo Version 9.4 (TreeStar, San Carlos, CA). Proliferation was measured off the CD3+CD8+ gate and the media control values were used to set gates. Our analysis measured the cytokine-secreting cells as a proportion of the total proliferating cell population (CFSElo). SEB was the positive control and unstimulated cells the negative control (data not shown). Antibodies were obtained from BD except where noted.

Killing Assay (p24)

CD8-depleted PBMCs from exclusively HIV-seronegative individuals were activated with PHA for 2 days prior to infection with HIV-1 NL4.3 (MOI=0.1) as previously described[17, 18]. During infection, CD8+ T-cells (effectors) from exclusively HIV-seronegative individuals were purified using CD8 untouched Dynal Beads (per
manufacturer’s instructions, Invitrogen). Purified CD8+ T-cells (100,000) were mixed at a 1:1 and/or 10:1 ratio with infected CD4+ T-cells from matched or mismatched individuals for HLA class I alleles (Table1). Cells were co-cultured in duplicate and incubated for 72 hrs at 37° C, 5% CO2. After incubation, cells were stained with surface markers (CD4-Pecy7, CD8-PercypCy5.5, and CD3-Pacific Blue) and permeabilized with Perm A reagent followed by intracellular staining with p24-FITC (Beckman-Coulter) in Perm B buffer (Caltag). The killing of targets was measured by p24 reduction by measuring the %p24. Gates were set using the uninfected targets as previously described\cite{18}. Percent killing is calculated as follows: 100- (%p24 with effectors/%p24 no effectors) × 100.

Cytotoxicity assay using 7-aminoactinomycin D (7-AAD) staining

Targets were CD8-depleted from isolated PBMCs of HLA class I matched HIV-seronegative donors as above. The targets were activated with PHA (5μg/mL) in the presence of IL-2 (100 U/mL) for 2 days. Activated matched or mismatched CD4 targets (5×10^5 cells) were co-cultured with appropriate HLA CD8 T-cells (Effectors) from HIV-seronegatives for 24 hrs at four effector to target (E/T) ratios. Cells were surface-stained with anti-CD3-Pacific Blue and anti-CD4-Alexa780 (eBioscience) followed by staining with 0.25μg 7-AAD solution (BD Biosciences) for 20 mins to detect dead cells. Dead targets were measured by gating on 7-AAD+CD4+ T-cell populations, and samples with no effectors were used to set the background gates. Target cell death was determined by comparing the net percentage of 7-AAD+CD4+ T-cells in the presence of effectors relative to no effectors. Percent killing is calculated as follows: (% 7-AAD targets with effectors - %7-AAD targets without effectors/100- %7-AAD targets without effectors) × 100\cite{19}. All flow cytometry data was analyzed using FlowJo Version 9.4 software (TreeStar, San Carlos, CA).

Statistics

Paired data used for p24 killing and statistical analyses were performed using the non-parametric Wilcoxon Signed Rank test. Area under the curve data was generated by comparing the % killing from 7-AAD for all E:T ratios. The p-values were calculated using a paired t-test. Differences were considered significant on the basis of 95% confidence intervals. Analyses were done with Graphpad Prism 5.0 for Mac.

RESULTS and DISCUSSION

Proliferation and effector molecule production by CD8+ T cells in a modified MLR assay

We hypothesized that individuals who match at both HLA-B alleles should have a reduced mixed lymphocyte reaction (MLR) than those who are not matched. To test this hypothesis, we performed a variation of the MLR by incubating pairs of matched (for both copies of any of the three HLA class I genes) and mismatched PBMC at all alleles (Table1). Paring was done to account for the variability between subjects in the MLR. For our analysis we measured the percent effector molecule secretion from the total proliferating population (CFSE^{lo}). Allogeneic CD8 T cells, matched at HLA-B plus one HLA-C allele (due to linkage disequilibrium), have significantly decreased percentage of proliferating CD8+ T cells producing IFN-γ, Perforin or Granzyme compared with cells that are mismatched at all class...
I alleles (Figure 1A–C). This effect was not seen when PBMC were mismatched for HLA-A, HLA-B or -C alleles alone (Figure 1A–C) or with CD4+ T cells or CD3− cells (non-T cells) (data not shown).

**Killing of allogeneic targets is diminished only when HLA-B class I is matched**

While proliferation is a traditional tool used to measure allogeneic responses, it is only a surrogate in terms of analyzing elimination of infected CD4 T cells. To experimentally demonstrate whether individuals who matched at HLA-B alleles have a decreased ability to kill allogeneic targets, we tracked the elimination of CD4+ targets by infection with HIV-1 in the p24 killing assay. We co-cultured matched or mismatched HIV-infected CD4+ targets with CD8+ T-cells from the same allogeneic uninfected individual and measured killing by p24+ CD4 T-cell diminution. Effector cell killing was compared among paired samples, such that the responses from the same effectors were measured to either matched or mismatched targets (Table 1). Only three pairs of HLA-C matched individuals were tested because it was difficult to find enough uninfected individuals who only matched at HLA-C alleles without additionally matching HLA-A or HLA-B alleles in our cohort. We noted a significant diminution in killing between individuals who matched both HLA-B alleles compared to those who mismatched for all alleles (Figure 1D).

While MLR is likely to be independent of HIV infection, especially since the samples used are from uninfected individuals, it is possible that these responses are skewed in the presence of HIV infection. This may especially be the case for the observation that we saw effects at the HLA-B allele but not the others[1, 20]. To ascertain whether HIV-infection itself is responsible for the observed differences, we used a 7-AAD killing assay, were killing is measured by increases in 7-AAD and no viral infection is needed (as in the p24 killing assay). PBMC from two individuals for each class I allele pair were used as targets, one matching and one mismatching all HLA class I alleles from the effector CD8 T-cells. Killing was measured by an increase in 7-AAD (Figure 1E). HLA-A and HLA-C alleles did not show differences among match and mismatch combinations. Taking the area under the curve for the 7-AAD data, we observed a significant increased killing when differences between matched and mismatched HLA-B allele experiments were compared. Again, this was not seen with HLA-A or HLA-C (Figure 1F). Lastly, when we compared the differences in the fold-increase in 7-AAD between match and mismatched samples, differences where only seen between samples matching at HLA-B and their paired mismatch samples (p=0.0005, data not shown).

In this report killing of CD4+ targets was diminished when HLA-B alleles were matched relative to paired allogeneic cells that are mismatched at all class I alleles. The fact that proliferation data demonstrating differences only if HLA-B plus one HLA-C allele were matched maybe due to the strong linkage disequilibrium between HLA-B and HLA-C alleles. Moreover, the killing assay is more reminiscent of the in vivo biology whereby the allogeneic effect is most likely to function via a killing mechanism than through a proliferative mechanism. Overall, these data support allogeneic responses as a possible mechanism for protection in HIV-discordant couples and mother and infants who do not share HLA-B alleles[1, 5].
While other immune factors such as anti-HLA antibodies\cite{21,22} have been shown to inhibit HIV-infection, these seem less likely to be playing a role in the increased susceptibility to infection since in mother-infant pairs these antibodies were not found to be associated with protection\cite{23,24}. Resistance to HIV-1 infection has also been shown in women alloimmunized with their partner’s lymphocytes. In these studies, down regulation of CCR5 on T-cells and an increase in the secretion of antiviral factors as a result of allogeneic responses were demonstrated\cite{7}. Although the down-regulation of CCR5 is clearly not playing a role in our studies due to the fact that we infected target CD4 T-cells prior to exposure to the effector CD8 T-cells, the secretion of anti-viral factors maybe playing a role in the differential killing observed. However, our assays cannot discern this effect. Other non-mutually exclusive mechanisms to explain HLA-mediated effects influencing HIV acquisition include HLA interactions with NK cell receptors that could mediate innate immunity and modulate HIV transmission\cite{25,26}. The percent NK cells in our cultures was <2.8% by measuring CD3-CD8+ cells (range 0.1–2.8%, mean=1%, median=0.9%) and therefore unlikely to be mediating this effect. Finally, viruses that have escaped T-cell recognition in a donor could be more easily transmitted to a recipient if HLA class I alleles are shared.

In our opinion these data best support the explanation that differences in HLA between partners could induce allogeneic CD8+ T-cell responses that eliminate incoming infected cells reducing transmission. The fact that allogeneic responses mediated by HLA-B are the only responses that significantly affect cell killing and proliferation can partly be explained by the increase in precursor frequency observed against HLA-B alleles relative to HLA-A demonstrated in former studies by others\cite{27,28}. Why HLA-B alleles have an increase in precursor frequency as shown by others and why this results in diminished killing of matched HLA-B targets is not clear. A further understanding of the unique aspects of these HLA-B-associated effects is warranted as a possible alternative HIV vaccine design based on allogeneic immunity.

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Figure 1. HLA-B mismatching is associated with reduced killing in a MLR assay
Percent production of effector molecules from proliferating cells: A. IFN-γ, B. Perforin, and C. Granzyme-B production by proliferating (CFSE<sup>lo</sup>) CD8<sup>+</sup> T cells in response to PBMC matched at HLA-B, -A, -C, -B + 1 A, or -B + 1 C locus and mismatched at all loci. Matched samples are open symbols, mismatches are shown in grey. D. The diminution of p24 was measured when CD8<sup>+</sup> T-cells from HIV seronegatives (HLA-A N=7, HLA-B N=13 and HLA-C N=3) were incubated with CD4 targets infected with HIV-1. Comparisons were made in a paired manner such that responses from the same effectors were compared to matched and mismatched targets. For example, responses from effectors from Vol#1: HLA-A*1101/3101, HLA-B*4002/4402, HLA-C*0202/0501 were co-cultured with targets from Vol#2: HLA-A*0201/2902, HLA-B*4001/4403, HLA-C*0304/1601 as an HLA-B match and from targets from Vol#3: HLA-A*2425/6901, HLA-B*3522/5501, HLA-C*0102/0401 as a mismatch. E. CD8<sup>+</sup> T-cells from seronegatives were incubated with CD4+ targets from seronegatives and labeled with 7-AAD and the % killing was measured. In this assay HIV-1 is not present. Two samples were tested for each locus, open symbols are matched, closed symbols mismatched F. Area under the curve was determined and the p values were calculated by comparing % killing from 7-AAD assay using a paired t test. All other statistically significant differences were determined by Wilcoxon Signed rank test. Graphpad Prism 5.0 for Mac was used for all statistical analysis. Differences were considered to be significant on the basis of 95% confidence intervals.
Table 1

Examples of matched and mismatched HLA allotypes for testing

<table>
<thead>
<tr>
<th></th>
<th>HLA-A1</th>
<th>HLA-A2</th>
<th>HLA-B1</th>
<th>HLA-B2</th>
<th>HLA-C1</th>
<th>HLA-C2</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Effectors:</td>
<td>Vol#1</td>
<td>*03;01</td>
<td>*11;01</td>
<td>*07;02</td>
<td>*55;01</td>
<td>*03;03</td>
</tr>
<tr>
<td>Targets:</td>
<td>Match</td>
<td>*03;01</td>
<td>*11;01</td>
<td>*35;04</td>
<td>*51;01</td>
<td>*04;01</td>
</tr>
<tr>
<td></td>
<td>Mismatch</td>
<td>*01;01</td>
<td>*24;02</td>
<td>*40;02</td>
<td>*40;02</td>
<td>*02;02</td>
</tr>
<tr>
<td><strong>HLA-B Match</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Effectors:</td>
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<td>*31;01</td>
<td>*40;02</td>
<td>*44;02</td>
<td>*02;02</td>
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<td>Match</td>
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<td>*29;02</td>
<td>*40;01</td>
<td>*44;03</td>
<td>*03;04</td>
</tr>
<tr>
<td></td>
<td>Mismatch</td>
<td>*24;25</td>
<td>*69;01</td>
<td>*35;22</td>
<td>*55;01</td>
<td>*01;02</td>
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<tr>
<td><strong>HLA-C Match</strong></td>
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<tr>
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<td>*07;02</td>
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<td>Targets:</td>
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<td>*68;02</td>
<td>*08;01</td>
<td>*14;02</td>
<td>*07;01</td>
</tr>
<tr>
<td></td>
<td>Mismatch</td>
<td>*03;01</td>
<td>*11;01</td>
<td>*35;04</td>
<td>*51;01</td>
<td>*04;01</td>
</tr>
<tr>
<td><strong>HLA-B Match plus one HLA-C match</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Effectors:</td>
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<td>*24;02</td>
<td>*08;01</td>
<td>*57;01</td>
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<td>*08;01</td>
<td>*57;02</td>
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</tr>
<tr>
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<td>*68;02</td>
<td>*15;03</td>
<td>*81;01</td>
<td>*02;01</td>
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