

HLA-B*35-Px-mediated acceleration of HIV-1 infection by increased inhibitory immunoregulatory impulses

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A subset of HLA-B*35 alleles, B*35-Px, are strongly associated with accelerated HIV-1 disease progression for reasons that are not understood. Interestingly, the alternative set of B*35 subtypes, B*35-PY, have no detectable impact on HIV-1 disease outcomes, even though they can present identical HIV-1 epitopes as B*35-Px molecules. Thus, the differential impact of these alleles on HIV-1 disease progression may be unrelated to interactions with HIV-1-specific CD8⁺ T cells. Here, we show that the B*35-Px molecule B*3503 binds with greater affinity to immunoglobulin-like transcript 4 (ILT4), an inhibitory MHC class I receptor expressed on dendritic cells, than does the B*35-PY molecule B*3501, even though these two B*35 molecules differ by only one amino acid and present identical HIV-1 epitopes. The preferential recognition of B*3503 by ILT4 was associated with significantly stronger dendritic cell dysfunction in *in vitro* functional assays. Moreover, HIV-1-infected carriers of B*3503 had poor dendritic cell functional properties in *ex vivo* assessments when compared with carriers of the B*3501 allele. Differential interactions between HLA class I allele subtypes and immunoregulatory MHC class I receptors on dendritic cells thus provide a novel perspective for the understanding of MHC class I associations with HIV-1 disease progression and for the manipulation of host immunity against HIV-1.

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Abbreviations used: CTL, cytotoxic T lymphocyte; ILT4, immunoglobulin-like transcript 4; LILR, leukocyte immunoglobulin-like receptor; MDDC, monocyte-derived dendritic cell; MLR, mixed lymphocyte reaction; SPR, surface plasmon resonance.

Specific HLA class I alleles are strongly associated with HIV-1 disease outcomes, and the identification of mechanisms accounting for their impact on HIV-1 disease progression provides a premier opportunity to analyze components of protective immunity against HIV-1 and how the immune system can be effectively manipulated in a therapeutic manner (Carrington and O'Brien, 2003). Remarkably, prior studies (Gao et al., 2001) have found that HLA-B*35-Px subtypes (HLA-B*3502, B*3503, B*3504, and B*5301) are associated with accelerated HIV-1 disease courses, in contrast to HLA-B*35-PY (HLA-B*3501 and B*3508) subtypes, which do not have any detectable impact on

HIV-1 disease progression, even though B*35-PY and B*35-Px subtypes can differ by as few as one amino acid. Because HLA class I alleles restrict cytotoxic T lymphocyte (CTL) epitopes, the differential disease progression between the B*35-PY and B*35-Px groups was proposed to depend on divergent CTL responses with a potential skewing of B*35-Px-restricted HIV-1-specific CTL responses toward nonfunctional (decoy) epitopes. Recent studies, however,

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found no positive evidence for this (Jin et al., 2002; Streeck et al., 2007), and the mechanisms accounting for the differential influence of HLA-B*35 subtypes on HIV-1 disease progression and the specific negative impact of B*35-Px subtypes remain unknown (Goulder and Watkins, 2008). Notably, HLA-B*35-Px and -PY subtypes can present identical HIV-1 CTL epitopes, and thus provide a unique model to study HLA class I-mediated immune activity against HIV-1 independently of the presented peptides.

In addition to their role as immunogens for the generation of antigen-specific CTLs, peptide-MHC class I complexes have important regulatory functions that are mediated by binding to immunomodulatory MHC class I receptors such as killer immunoglobulin-like receptors (KIRs; Lanier, 1998) or leukocyte immunoglobulin-like receptors (LILRs; Brown et al., 2004). LILRB2, also termed immunoglobulin-like transcript 4 (ILT4), is a prominent inhibitory myelomonocytic MHC class I receptor (Colonna et al., 1998) that is expressed primarily on professional antigen-presenting cells, such as monocytes and dendritic cells, and is strongly up-regulated during chronic HIV-1 infection (Vlad et al., 2003). Recent data suggest that interactions between ILT4 and peptide-MHC class I complexes can critically depend on the presented antigenic peptide or the respective presenting MHC class I molecule (Shiroishi et al., 2006; Lichterfeld et al., 2007), raising the possibility that HLA class I alleles could impact HIV-1 disease progression by differentially affecting ILT4-mediated immunomodulatory properties of dendritic cells.

In the present study, we tested this hypothesis by determining the binding interactions between ILT4 and HLA-B*3503 (a B*35-Px molecule) as well as -B*3501 (a B*35-PY molecule) in the context of identical CTL epitopes that are presented by both HLA-B*35 molecules. We found that B*3503 binds ILT4 significantly stronger than does the B*3501 molecule, independently of the presented epitopes. This corresponded to higher degrees of ILT4-mediated dendritic cell dysfunction mediated by B*3503 *in vitro*, and a striking functional impairment of dendritic cells in HIV-1-infected carriers of the B*3503 allele in *ex vivo* assessments. Overall, these data suggest that allele-specific interactions between HLA class I molecules and their receptors on dendritic cells may significantly impact HIV-1 disease outcomes and thus provide a novel perspective for the understanding of immunoregulatory functions of HLA class I alleles in the pathogenesis of HIV-1 infection.

RESULTS AND DISCUSSION

To test whether HIV-1 CTL epitopes presented by alternative B*35 subtypes are differentially recognized by the inhibitory myelomonocytic receptor ILT4 on dendritic cells, we focused on two CTL epitopes that are both targeted in HIV-1-infected carriers of HLA-B*35-Px or -B*35-PY subtypes: the NY9 epitope (NPDIVIYQY) in RT and the PY9 epitope (PIIPVGDIIY) in Gag. We used recombinant, fluorophore-labeled HLA-B*3501 (PY) and -B*3503 (Px) tetramers refolded with the respective epitopes to stain peripheral blood

Lin⁻HLA-DR⁺CD11c⁺ dendritic cells from untreated HIV-1-infected individuals with chronic progressive HIV-1 infection. As determined by flow-cytometric studies, we found that B*3503 (Px) complexes have significantly higher binding intensities to dendritic cells, compared with the respective B*3501 (PY) complexes refolded with identical epitopes (Fig. 1, A and B). These binding interactions were almost completely abrogated after incubation with polyclonal antibodies that specifically block ILT4 and have no detectable cross-reactivity with alternative receptors from the LILR family (Fig. 1, A and B). In contrast, antibodies blocking ILT2, an alternative HLA class I receptor expressed on dendritic cells (Colonna et al., 1997), had no effects on tetramer binding to dendritic cells (unpublished data). To confirm these flow-cytometric findings, we investigated the binding interactions between the recombinant tetramers (two independently produced sets obtained from the National Institutes of Health Tetramer Core Facility) and recombinant ILT4 directly by cell- and label-free surface plasmon resonance (SPR) analysis. These experiments indicated that the binding affinity between B*3503 (Px)-NY9 tetramers and ILT4 was higher compared with the corresponding interaction between ILT4 and B*3501 (PY)-NY9 tetramers. Notably, SPR experiments also showed that the PY9 epitope bound to ILT4 only when presented by the HLA-B*3503 molecule (Fig. 1 C), consistent with our flow cytometry data indicating no detectable ILT4-mediated binding of the B*3501-PY9 tetramer to dendritic cells (Fig. 1, A and B). To test if alternative HLA-B*35-Px subtypes also mediate enhanced binding to ILT4, we used a recombinant B*3502 tetramer refolded with the described PY9 epitope. Similar to the B*3503-PY9 tetramer, this molecule had significantly increased binding intensity to ILT4 compared with the B*3501-PY9 tetramer, as determined by flow cytometric and SPR experiments (Fig. S1, A and B). Overall, these data indicate that although presenting identical epitopes as B*3501 (PY), B*3503/02 (Px) molecules have preferential binding properties to ILT4, and may thus cause increased inhibitory impulses on dendritic cells.

To determine if the preferential binding of B*3503 (Px) complexes to ILT4 translates into a functional inhibition of dendritic cells, we used *in vitro* mixed lymphocyte reactions (MLRs) with monocyte-derived dendritic cells (MDDCs) that had previously been matured in the presence of the respective B*3503 (Px) or B*3501 (PY) tetramers refolded with identical epitopic peptides. ILT4 surface expression during this maturation process was similar in both groups, irrespective of whether B*3503 or B*3501 tetramers had been added. Using MDDCs from 11 donors, these studies showed that after dendritic cell maturation in the presence of HLA-B*3503 tetramers, proliferation of allogeneic CD4⁺ and CD8⁺ T cells was significantly impaired, compared with experiments in which MDDCs were matured while exposed to B*3501 tetramers refolded with the identical epitopes (Fig. 2, A and D). Notably, after specific down-regulation of ILT4 surface expression on MDDCs using targeted siRNA-mediated

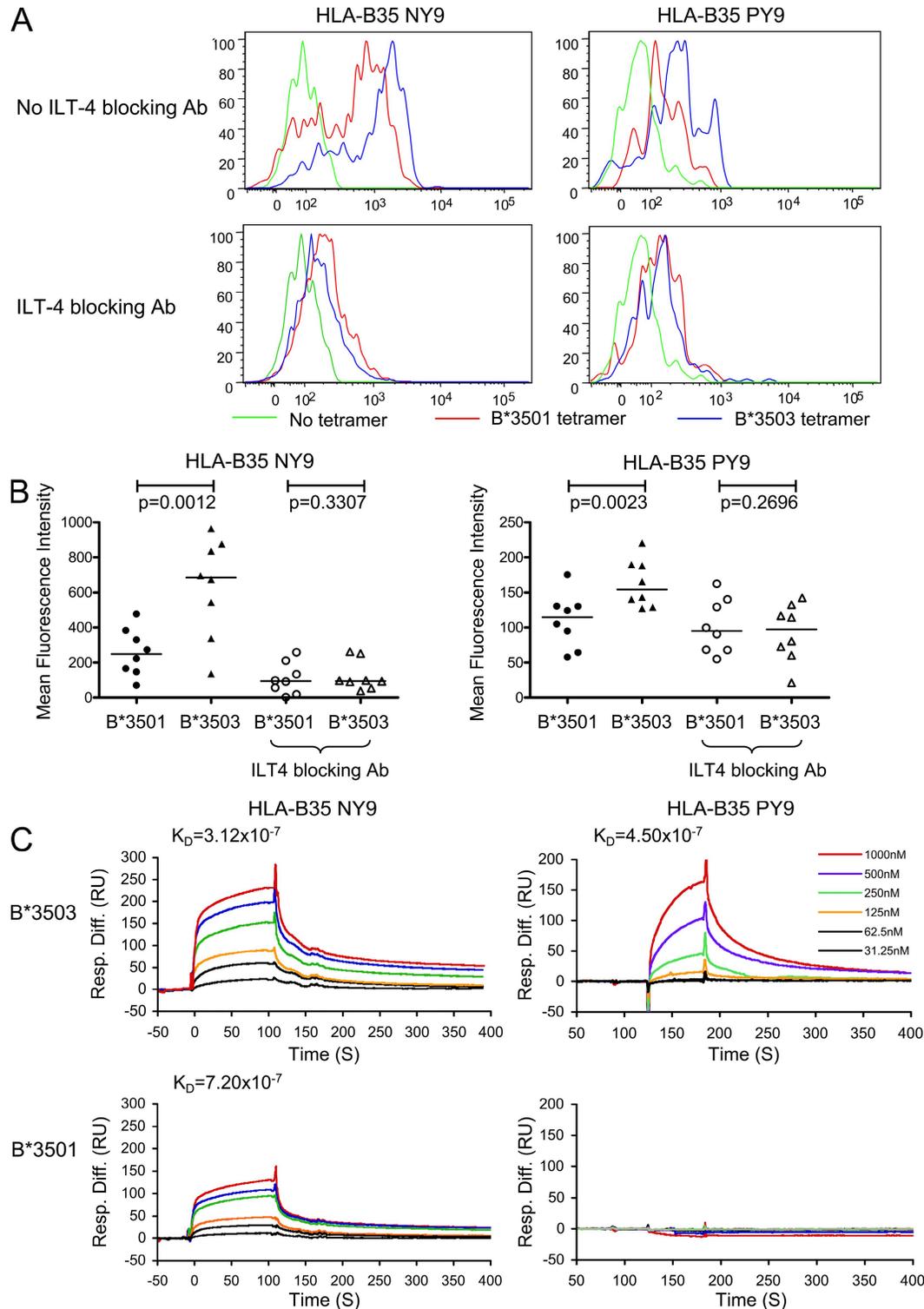


Figure 1. Preferential recognition of HIV-1 CTL epitope/HLA-B*3503 (Px) complexes by the inhibitory myelomonocytic receptor ILT4.

(A and B) Binding of HLA-B*3503 (Px) and -B*3501 (PY) tetramers refolded with two different CTL epitopes to PBMCs, as determined by flow cytometry. ILT4-specific antibodies were able to fully abrogate tetramer binding to dendritic cells. (A) Histograms for one representative example are shown.

(B) Cumulative flow cytometry data from HIV-1-infected patients ($n = 8$) (two-tailed paired Student's t test). (C) SPR sensograms reflecting the control surface-subtracted interactions between recombinant ILT4 and immobilized HLA-B*3503 or -B*3501 tetramers refolded with the indicated epitope. ILT4 was injected in five serial twofold dilutions from a starting concentration of $1 \mu\text{M}$. The respective apparent steady-state equilibrium constant (K_D) is indicated. One representative experiment out of three is shown.

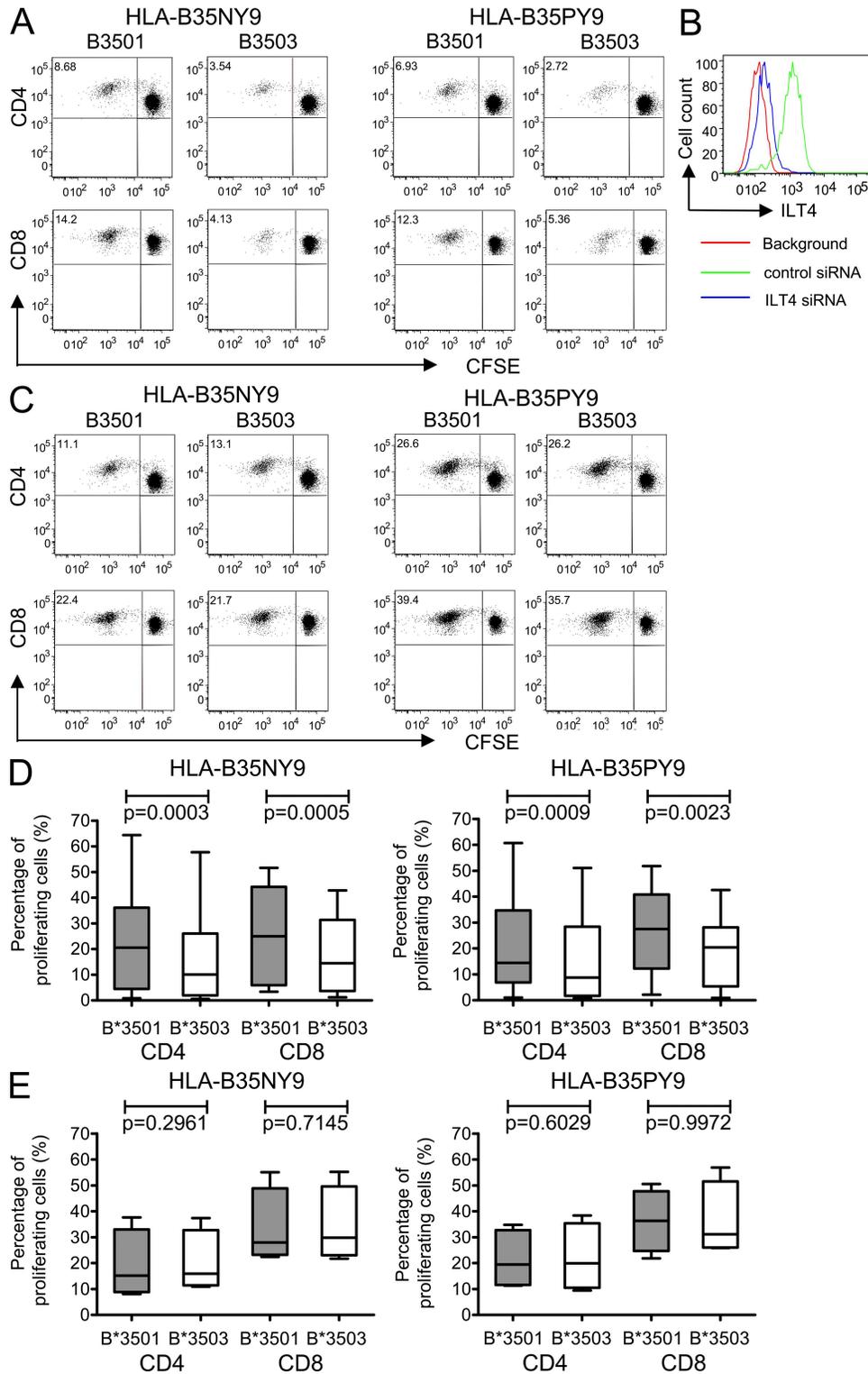


Figure 2. Allostimulatory dysfunction of MDDCs mediated by interactions between HLA-B*35-Px complexes and ILT4. (A and D) Allogeneic CD4⁺ and CD8⁺ T cell proliferation after in vitro stimulation with MDDCs matured in the presence of HLA-B*3503 (Px) or -B*3501 (PY) tetramers refolded with the indicated epitopes at a DC/T cell ratio of 1:50. Dot plots from one representative experiment are shown in A; cumulative data from $n = 11$ study subjects are shown in D (two-tailed paired Student's t test). (B) ILT4 surface expression on MDDCs after electroporation with ILT4-specific or unspecific control siRNA. (C and E): Allogeneic T cell proliferation after stimulation with MDDCs matured with the indicated HLA-B*3503 or -B*3501 tetramers in the presence of siRNA-mediated down-regulation of ILT4. Dot plots from one representative experiment are shown in C; cumulative data from $n = 4$ study subjects are shown in E (two-tailed paired Student's t test).

gene knockout (Fig. 2 B), these inhibitory effects were abrogated and no difference was observed between the functional properties of MDDCs exposed to either the B*3503 or B*3501 tetramers (Fig. 2, C and E). Importantly, decreased allostimulatory properties of dendritic cells were also observed after dendritic cell exposure to the alternative HLA-B*35-Px tetramer, B*3502-PY9 (Fig. S1 C).

In addition to allostimulatory activities, we also assessed cytokine secretion and phenotypic characteristics of dendritic cells induced by the HLA-B*3503 (Px) and -B*3501 (PY) tetramers. These experiments showed that dendritic cell maturation in the presence of HLA-B*3503 (Px) tetramers resulted in reduced expression of the costimulatory molecule CD86 and the maturation marker HLA-DR, as well as in decreased secretion of IL-12p70 and IL-6, two cytokines

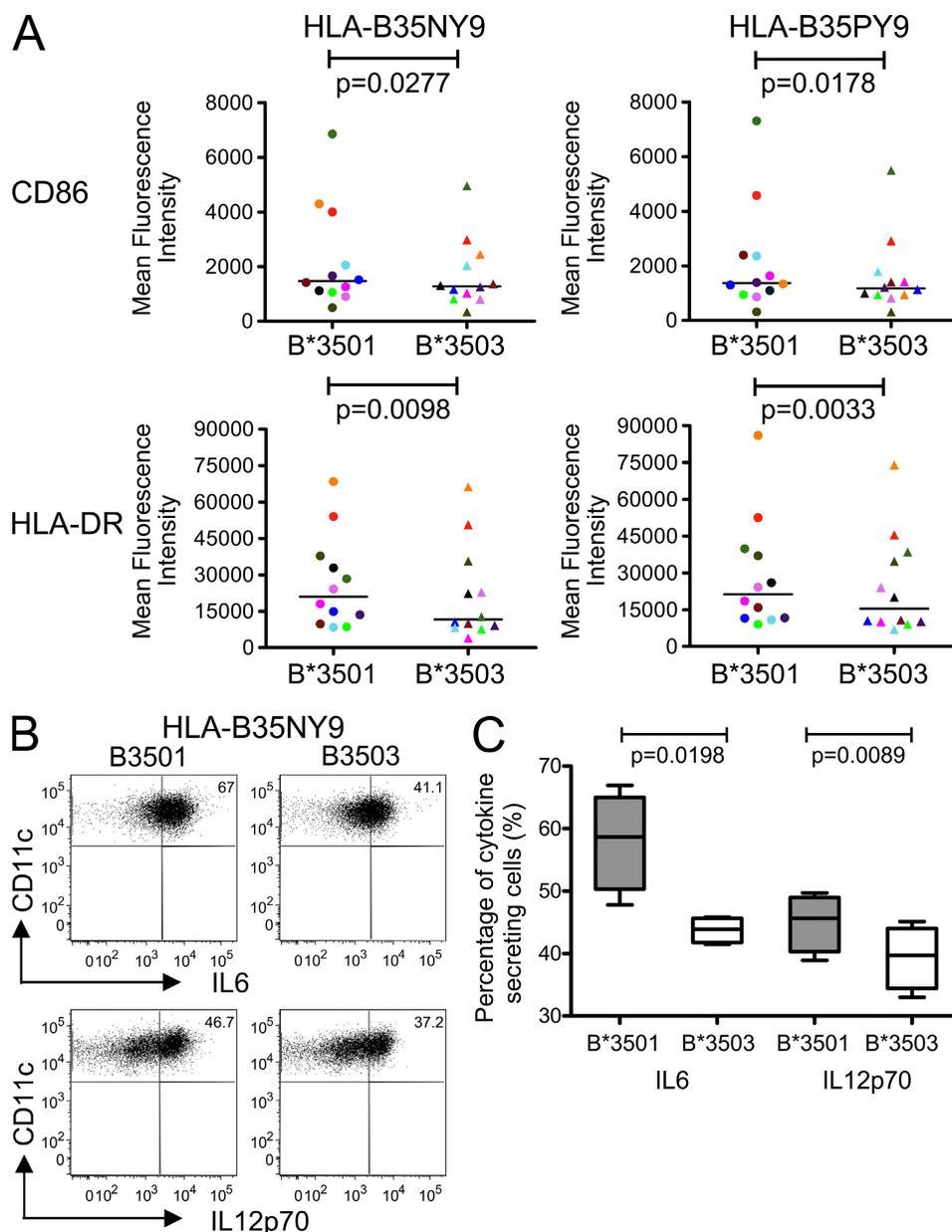


Figure 3. Decreased maturation and cytokine production of MDDC mediated by interactions between HLA-B*3503 complexes and ILT4.

(A) Expression of the costimulatory molecule CD86 and the maturation marker HLA-DR on MDDC after maturation in the presence of HLA-B*3503 (Px) or -B*3501 (PY) tetramers refolded with the indicated epitopes. Cumulative data from $n = 12$ study subjects are shown (two-tailed paired Student's *t* test). Colors reflect samples identity. (B and C) Cytokine production of MDDC after maturation and exposure to HLA-B*3503 or -B*3501 tetramers refolded with NY9 epitope. Dot plots from one representative experiment are shown in B; cumulative data from $n = 4$ study subjects are shown in C (two-tailed paired Student's *t* test).

involved in dendritic cell maturation (Fig. 3). Collectively, these experiments show that stronger interactions between B*35-Px subtypes and ILT4 can lead to functional impairment of dendritic cells.

Based on these *in vitro* findings, we next tested for *in vivo* functional differences between dendritic cells from carriers of HLA-B*3503 (Px) or -B*3501 (PY). For this purpose, we conducted MLRs with dendritic cells isolated from the peripheral blood of HAART-naive HIV-1-infected carriers of HLA-B*3503 or -B*3501 recruited from a multicenter cohort of hemophilic seroconverters (Goedert et al., 1989). HIV-1 viral loads (mean of 52,335 vs. 115,535 copies/ml, $P = 0.39$) and CD4 cell counts (mean of 21 vs. 15%, $P = 0.23$) did not differ significantly between carriers of HLA-B*3501 (PY) and -B*3503 (Px), despite a longer duration of HIV-1 infection in the HLA-B*3501 (PY) group (mean of 13 vs. 10 yr). Except for the expression of different HLA-B*35 subtypes, these patients did not differ significantly in their patterns of expressed HLA class I molecules. Using MLR assays, we observed a statistically significant defect of allogeneic T cell proliferation with myeloid DCs from carriers of B*3503 as compared with carriers of B*3501 (Fig. 4, A and B). This difference increased with escalating DC/T cell ratios (Fig. 4 B) and was highly reproducible with allogeneic T cells from an alternative T cell donor (inter-assay correlation $R = 0.9015$, $P = 0.0004$). Notably, the difference between dendritic cell function in these patients with long histories of HIV-1 infection was substantially more pronounced than the differential function of dendritic cells achieved by B*3503 (Px) and B*3501 (PY) tetramers in our short-term tissue culture experiments.

Of all HLA class I alleles, the B*35-Px grouping of alleles plays a unique role in HIV-1 immunopathogenesis, as it represents the strongest identified predictor of an accelerated HIV-1 disease progression (Carrington et al., 1999; Gao et al., 2001; O'Brien et al., 2001). The reason for this negative impact on HIV-1 disease courses has been a matter of intense debate, but how an HLA class I subtype can substantially worsen HIV-1 disease outcomes has remained unclear thus far. Notably, HIV-1-infected carriers of B*35-Px subtypes progress to AIDS faster than individuals with homozygous HLA-B alleles (Gao et al., 2001, 2005), and these subtypes are therefore likely to actively mediate a negative effect in HIV-1 pathogenesis, rather than simply representing the functional equivalent of a null allele at the HLA-B locus. Prior studies have related HLA class I-mediated effects on HIV-1 disease outcomes to the restriction of specific viral epitopes putatively mediating higher susceptibility to host immune activity (Frahm et al., 2005; Stewart-Jones et al., 2005; Kiepiela et al., 2007; Loffredo et al., 2009). However, it appears that specific HLA class I alleles associated with divergent clinical outcomes of HIV-1 infection can restrict CTL epitopes in viral regions that are overlapping or immediately adjacent to one another (Brander and Goulder, 2000). This is particularly true in the setting of B*35-Px and -PY subtypes, which can present identical viral epitopes despite

their opposing association with HIV-1 disease progression. Overall, this suggests that HLA class I alleles can affect immune activity against HIV-1 by mechanisms other than CTL epitope presentation and interactions with T cell receptors (TCRs) on HIV-1-specific CD8⁺ T cells.

The data presented here add a novel dimension to this concept by highlighting previously unrecognized, CTL epitope-independent immunoregulatory effects of HLA class I alleles that are mediated by their interactions with the myelomonocytic MHC class I receptor ILT4 and lead to functional changes of dendritic cells. Our data indicate that inhibitory impulses resulting from preferential recognition of B*3503 (Px) subtypes by ILT4 may lead to substantial dendritic cell dysfunction, and are thus likely to play a key role within the molecular

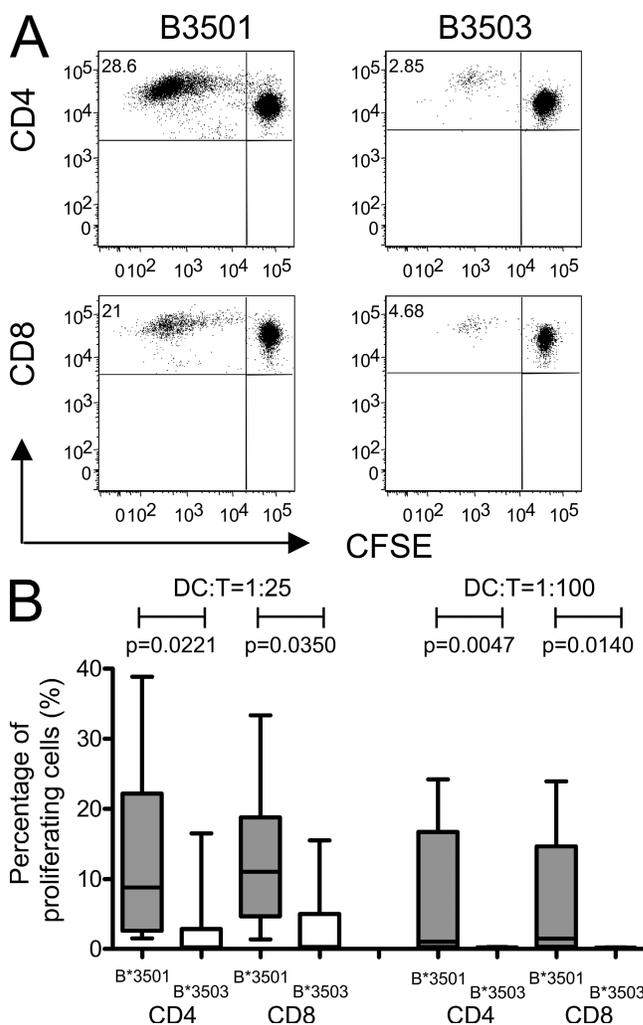


Figure 4. Impaired dendritic cell function in HIV-1-infected carriers of HLA-B*3503 (Px) alleles. Data show allogeneic T cell proliferation after stimulation with dendritic cells isolated from peripheral blood of HAART-naive HIV-1-infected carriers of HLA-B*3503 (Px) or -B*3501 (PY) at the indicated DC/T cell ratios. (A) Data from one representative experiment; (B) summarized data from $n = 6$ B*3501 carriers and $n = 7$ B*3503 carriers (two-tailed Mann-Whitney U test).

processes leading to the accelerated disease progression observed in carriers of B*35-Px subtypes. Interestingly, the accelerated course of HIV-1 infection mediated by B*35-Px subtypes starts during primary HIV-1 infection but remains continuously detectable during the entire disease course (Gao et al., 2005), which is more consistent with a negative effect mediated by interfering with immunoregulatory mechanisms rather than with the targeting of specific CTL epitopes or the evolution of characteristic viral escape variants. Moreover, a worsening of HIV-1 disease outcomes by activation of inhibitory immunoreceptor signaling is well in line with the recent recognition of the significant roles of other inhibitory immunoregulatory receptors, such as PD-1 (Day et al., 2006; Trautmann et al., 2006), in HIV-1 pathogenesis.

In a recent study we have shown that in the setting of a shared HLA class I molecule, recognition of peptide-MHC class I complexes by ILT4 can critically depend on the sequence of the presented CTL epitopes (Lichterfeld et al., 2007). Other investigators have recently extended this finding by identifying lipid antigen-specific interactions between CD1d and ILT4 (Li et al., 2009). Here, we show that presentation of identical CTL epitopes by closely related HLA subtypes can similarly have an important influence on the binding affinity of peptide-MHC class I complexes to ILT4, and on the functional profile of dendritic cells. Collectively, these data show that both viral and host genetics can impact dendritic cell function through interactions with the immunoregulatory receptor ILT4 and provide a new perspective to understanding the molecular mechanisms by which HLA class I alleles can impact HIV-1 disease progression. The specific manipulation of interactions between peptide-MHC class I complexes and myelomonocytic MHC class I receptors on dendritic cells may represent an attractive therapeutic strategy to improve HIV-1 disease outcomes in clinical settings. Moreover, ILT4-mediated immunoregulatory properties of peptide-MHC class I complexes need to be considered in the design of HIV-1 vaccines.

MATERIALS AND METHODS

Patients. HIV-1-infected individuals with chronic HIV-1 infection were recruited from the Massachusetts General Hospital in Boston. Hemophilic HIV-1-infected carriers of HLA-B*35 alleles were recruited from the Multi-center Hemophilia Cohort Study. All subjects gave written informed consent to participate, and the study was approved by the Institutional Review Boards of the Massachusetts General Hospital and the National Cancer Institute.

Flow cytometry. PBMCs were stained with tetramers (obtained from the National Institutes of Health Tetramer Core Facility) for 5 min at room temperature before being incubated with PE-Cy7-labeled HLA-DR antibodies, APC-labeled CD11c antibodies, and a cocktail of PE-labeled lineage antibodies. For ILT4 blocking assays, cells were incubated with a polyclonal ILT4-specific antibody (R&D Systems) for 30 min at 37°C before being stained with tetramers. Cells were then fixed and acquired on a flow cytometer (model LSR II; BD).

Functional dendritic cell assays. MDDCs were prepared as described previously (Lichterfeld et al., 2007). 2×10^6 PBMCs were plated in 5% pooled human serum medium, and incubated for 60 min at 37°C to adhere monocytes. Adherent monocytes were propagated in the presence of 50 µg/ml

GM-CSF (Amgen) for 5 d in 1% human plasma medium. On d 5, immature myelomonocytic cells were harvested using Hanks-based Cell-disassociation buffer (Invitrogen), incubated with HLA-B*35-Px or -PY tetramers for 4 h, and then matured using a previously described cytokine cocktail containing IL-1β, TNF-α, PGE-2, and IL-6 (Kavanagh et al., 2006). After 16 h, cells were either stained with surface antibodies against CD86 and HLA-DR and processed to immediate flow cytometric analysis for phenotypic characterization, or mixed with allogenic CFSE-labeled T cells for mixed lymphocyte reactions. After 6 d of culture, cells were stained with CD4 and CD8 antibodies and processed to flow cytometric analysis using the LSR II flow cytometer. For MLRs with ex vivo-collected cells from carriers of HLA-B*35 alleles, CD1c⁺ and CD141⁺ myeloid dendritic cells were isolated from PBMCs using immunomagnetic anti-BDCA-1 and anti-BDCA-3 beads (Miltenyi Biotec) and immediately mixed with CFSE-labeled allogenic T cells as described above. To analyze cytokine secretion, immature MDDCs were prepared and treated with tetramers as described above and then matured using 5 µg/ml CL097 (InvivoGen) in the presence of 5 µg/ml brefeldin A. After 20 h, cells were fixed and permeabilized, stained with antibodies recognizing intracellular IL-12p70 and IL-6, and processed to flow cytometric acquisition.

ILT4 knockdown. siRNA pools specific for ILT4 message (LILRB2 ON-TARGETplus SMARTpool; ThermoFisher Scientific) or unspecific control siRNA message (ThermoFisher Scientific) were used at concentrations of 1 nmol/million cells. 10^6 MDDCs were suspended in 300 µl Opti-mem in the presence of siRNA and transferred to a 4-mm electroporation cuvette (Bio-Rad Laboratories). Cells were left on ice for 5 min, electroporated (900 V, 0.75 msec square wave; Genepulser Xcell; Bio-Rad Laboratories), and transferred back to culture medium for another 16 h before analysis.

SPR experiments. SPR experiments were performed using a BIAcore 3000 instrument (BIAcore). Recombinant tetramers at a concentration of 2mg/ml were individually immobilized (600 resonance units) to a CM5 sensor chip (BIAcore) using standard amine coupling methods. Toxic shock syndrome toxin 1 (TSST-1) in an equivalent surface density was used as the control surface. Serial twofold dilutions of a recombinant protein dimer containing two extracellular domains of human ILT4 fused to the Fc part of human IgG (R&D Systems) were injected over the sensor chip. After subtraction of background binding between ILT4 and TSST-1, the apparent steady-state equilibrium constant (K_D) was determined by nonlinear regression analysis of SPR responses to the various concentrations of injected ILT4 using the BiaEvaluation 4.1 software program (BIAcore).

Online supplemental materials. Fig. S1: Interactions between HLA-B*3502 (Px) complexes and the inhibitory myelomonocytic receptor ILT4. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091386/DC1>.

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SUPPLEMENTAL MATERIAL

Huang et al., <http://www.jem.org/cgi/content/full/jem.20091386/DC1>

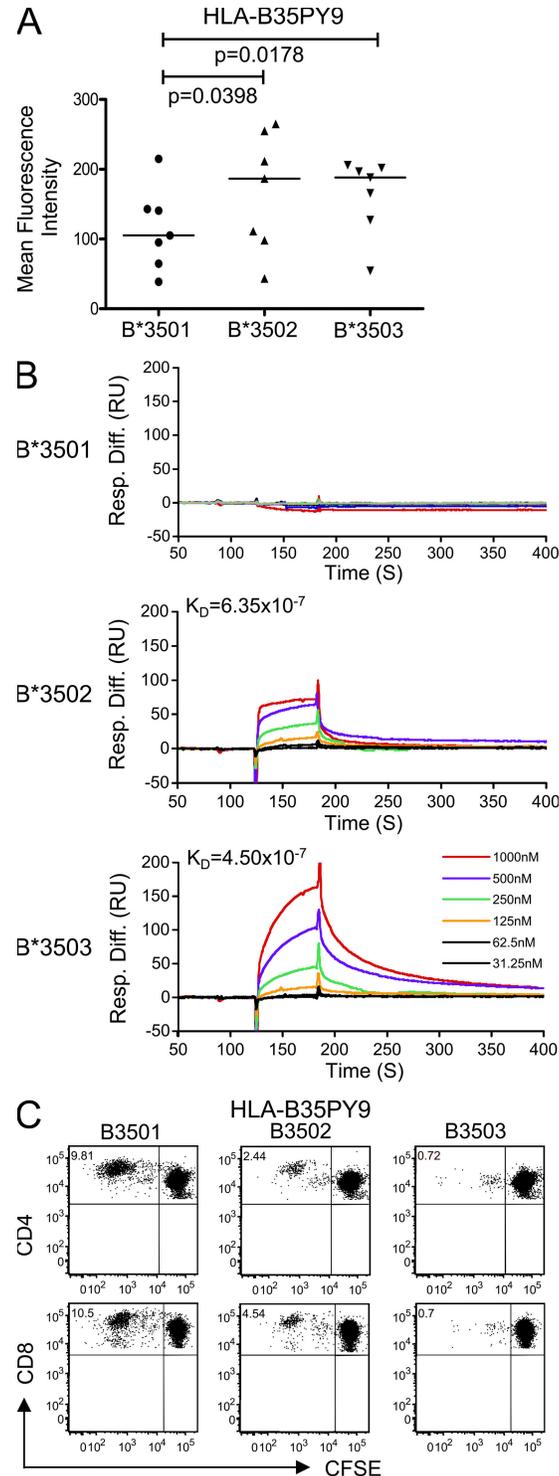


Figure S1. Interactions between HLA-B*3502 (Px) complexes and the inhibitory myelomonocytic receptor ILT4. (A) Binding of HLA-B*3502 (Px), -B*3503 (Px), and -B*3501 (PY) tetramers refolded with the PY9 epitope to peripheral blood myeloid dendritic cells, as determined by flow cytometry. Cumulative flow cytometry data from HIV-1-infected patients ($n = 7$) are shown (two-tailed paired t test). (B) SPR sensograms reflecting the control surface-subtracted interactions between recombinant ILT4 and immobilized HLA-B*3503 (Px), -B*3502 (Px), or -B*3501 tetramers refolded with the PY9 epitope. ILT4 was injected in five serial twofold dilutions from a starting concentration of $1 \mu\text{M}$. One representative experiment out of two is shown. (C) Allogeneic CD4⁺ and CD8⁺ T cell proliferation after in vitro stimulation with MDDC matured in the presence of HLA-B*3502 (Px), -B*3503 (Px), or -B*3501 (PY) tetramers refolded with the PY9 epitope at a DC/T cell ratio of 1:100.