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Article

Human Leukocyte Antigen Class I-Restricted Activation of CD8+ T Cells Provides the Immunogenetic Basis of a Systemic Drug Hypersensitivity

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SUMMARY

The basis for strong immunogenetic associations between particular human leukocyte antigen (HLA) class I allotypes and inflammatory conditions like Behçet's disease (HLA-B51) and ankylosing spondylitis (HLA-B27) remain mysterious. Recently, however, even stronger HLA associations are reported in drug hypersensitivities to the reverse-transcriptase inhibitor abacavir (HLA-B57), the gout prophylactic allopurinol (HLA-B58), and the antiepileptic carbamazepine (HLA-B*1502), providing a defined disease trigger and suggesting a general mechanism for these associations. We show that systemic reactions to abacavir were driven by drug-specific activation of cytokine-producing, cytotoxic CD8+ T cells. Recognition of abacavir required the transporter associated with antigen presentation and tapasin, was fixation sensitive, and was uniquely restricted by HLA-B*5701 and not closely related HLA allotypes with polymorphisms in the antigen-binding cleft. Hence, the strong association of HLA-B*5701 with abacavir hypersensitivity reflects specificity through creation of a unique ligand as well as HLA-restricted antigen presentation, suggesting a basis for the strong HLA class I-association with certain inflammatory disorders.

INTRODUCTION

Polymorphic human leukocyte antigen (HLA) class I molecules present pathogen-derived peptides to killer T cells mediating protective immunity (Margulies et al., 2008). However particular major histocompatibility complex-I (MHC-I) allotypes are much more strongly associated with inflammatory, autoimmune-like diseases than they are with protective immunity phenotypes in infectious disease (Margulies et al., 2008). The reason for this paradox is unclear, and the mechanisms by which certain MHC-I allotypes are associated with inflammatory diseases like ankylosing spondylitis, Behçet's disease, and birdshot retinopathy are not understood (Margulies et al., 2008). Recently, however, hypersensitivity to the reverse-transcriptase inhibitor abacavir has been strongly associated with HLA-B*5701 (Hetherington et al., 2002; Mallal et al., 2002; Martin et al., 2004), providing an opportunity to unravel the mechanism of at least this MHC-I disease association with the potential to understand other MHC-I associations. Multiorgan reactions to abacavir occur in approximately 2%–8% of patients with human immunodeficiency virus-I (HIV-1) infection (Cutrell et al., 2004). Abacavir hypersensitivity syndrome (AHS) manifests systemically as a combination of fever, rash, malaise, nausea, vomiting, and diarrhea and has even been associated with death in rechallenged individuals (Hetherington et al., 2001). This constellation of symptoms has some of the features of a graft versus host-like cellular reaction. Indeed, there is strong evidence for an immunological basis of AHS reflected in the presence of infiltrating CD8+ T cells in the skin of patients with a rash (Phillips et al., 2002) and the induction of elevated tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) in patient whole blood and/or mononuclear cells exposed to abacavir in vitro (Martin et al., 2007). Patients who have experienced AHS also have more active nonspecific cytokine production by T cells from peripheral blood (King et al., 2005). A cellular immune reaction to abacavir is further supported by the specificity of positive skin-patch tests for delayed-type hypersensitivity in patients with AHS (Phillips et al.,

2005). The association of AHS with the well-defined 57.1 major histocompatibility complex (MHC) haplotype encoding the MHC class I allotype (MHC-I), *HLA-B*5701*, is very strong (Hetherington et al., 2002; Mallal et al., 2002; Martin et al., 2004), especially when AHS is rigorously defined (Mallal et al., 2002; Mallal et al., 2008; Martin et al., 2004). Thus, for immunologically confirmed AHS in the PREDICT-1 clinical trial, *HLA-B*5701* was associated with a positive predictive value of 47.9% and a negative predictive value of 100% (Mallal et al., 2008). The prevalence of AHS varies according to the genetic background of the population, probably because of ethnic variation in the phenotypic frequency of HLA-B*5701 in some populations (Hughes et al., 2004a). For instance AHS is less common in African Americans, who are known to have higher frequency of HLA-B*5801, HLA-B*5702, and HLA-B*5703 subtypes and a correspondingly lower frequency of HLA-B*5701 than patients of European descent (Hughes et al., 2004a). Some population differences in the degree of association between AHS and HLA-B*5701 may arise because reliance upon the clinical definition of AHS leads to overassignment of the syndrome, thus confounding the association with HLA-B*5701, especially where this allotype is present at a low frequency (Hetherington et al., 2002; Mallal et al., 2008; Martin et al., 2004; Sun et al., 2007). Accordingly, it is an acknowledged best practice and is cost effective to carry out HLA-B*5701 genotyping to prevent abacavir hypersensitivity (Hughes et al., 2004b).

The mechanism of abacavir-induced hypersensitivity and the basis for its association with HLA-B*5701 remain unknown but are likely to be important in understanding the basis of other MHC-I-associated disorders such as ankylosing spondylitis (HLA-B27), Behçet's disease (HLA-B51), and birdshot retinopathy (HLA-A29). Abacavir is a prodrug that is converted to the active compound, leading us to hypothesize that this process may create a neoantigen that triggers $CD8^+$ T cell immunity in HLA-B*5701-positive individuals.

RESULTS

Abacavir-Specific, HLA-B*5701-Restricted $CD8^+$ T Responses in Patients with AHS

The peripheral-blood mononuclear cells (PBMCs) from two HIV-1-infected patients with a history of AHS and who were HLA-B*5701-positive, were stimulated with abacavir (10 μg/mL) for 11 to 14 days in vitro to test our hypothesis. This is the optimal culture period for expansion of alloreactive and antigen-specific memory $CD8^+$ T cells in cultures that are supplemented with Interleukin-2 (IL-2). At the completion of the culture period, the expanded T cells were restimulated for 6 hr in the presence and absence of abacavir with the HLA-B*5701-transfected, MHC-I-deficient B lymphoblastoid cell line C1R as antigen-presenting cells (APCs). Responding T cells were then examined for their phenotype and the accumulation of intracellular cytokines (as depicted schematically in Figure S1 available online).

$CD8^+$ T cells from patients with AHS proliferated in response to abacavir over 13 days and were specifically activated to produce IFNγ and TNFα, in the restimulation phase of the assay (Figure 1). Notably, it was the $CD8^+$ T cells that responded by making cytokines, whereas the $CD4^+$ T cells were not activated under the same conditions. Similar results were obtained when autologous

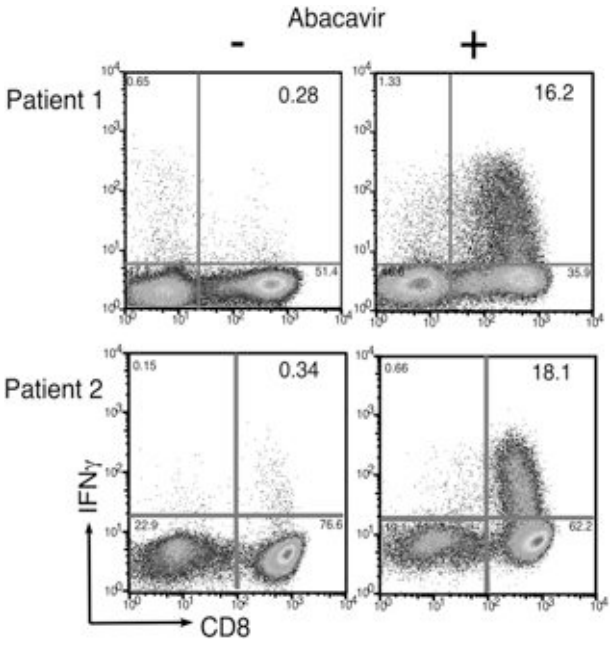

Figure 1. $CD8^+$ T Cells from Abacavir-Hypersensitive Patients with HIV-1 Proliferate and Produce Cytokines in Response to Abacavir Stimulation In Vitro

Flow-cytometry histograms of lymphocyte intracellular staining for IFNγ after abacavir stimulation. PBMCs from two HIV-1-infected patients with a history of AHS were stimulated with abacavir for 11 to 14 days in vitro after which the expanded T cells were then restimulated for 6 hr with the transfected lymphoblastoid cell line C1R expressing HLA-B*5701 in the presence (+) or absence (–) of abacavir (10 μg/mL). Cells were permeabilized and stained for intracellular IFNγ (y axis) and CD8 (x axis). Cells were gated as live lymphocytes. The percentage of total gated lymphocytes is shown in each quadrant.

PBMCs were used as APCs (not shown). These findings raised the likelihood that abacavir-specific $CD8^+$ T cells play a key role in the pathogenesis of AHS.

Abacavir-Specific Responses Can Be Primed In Vitro in Healthy Normal Donors

Expansion of abacavir-specific $CD8^+$ T cells from HIV-1-infected patients with AHS was comparable to the expansion observed for Epstein-Barr virus (EBV)-specific memory T cells or allogeneic stimulator cells (Mifsud et al., 2008), suggesting proliferation of a memory cell population. This was confirmed by ex vivo elispot studies on blood from patients with abacavir hypersensitivity syndrome in which abacavir-specific, IFNγ-producing cells were detected with frequencies of up to 700 spots per 10^6 PBMCs (Figure S2). In contrast to the presence of memory T cells in the circulation of abacavir-hypersensitive patients, we did not detect abacavir-specific IFNγ-producing cells in ex vivo Elispot analyses of PBMCs from abacavir-naive normal HLA-B*5701-positive blood donors. However, we also examined T cell responses from abacavir-naive, HLA-B*5701-positive blood donors after in vitro stimulation for 11–14 days by using the same protocol as in HIV-1-infected patients. $CD8^+$ T cells from these healthy individuals also proliferated in vitro and were specifically activated by abacavir to make IFNγ and TNFα (Figure 2A) over a pharmacological range of abacavir

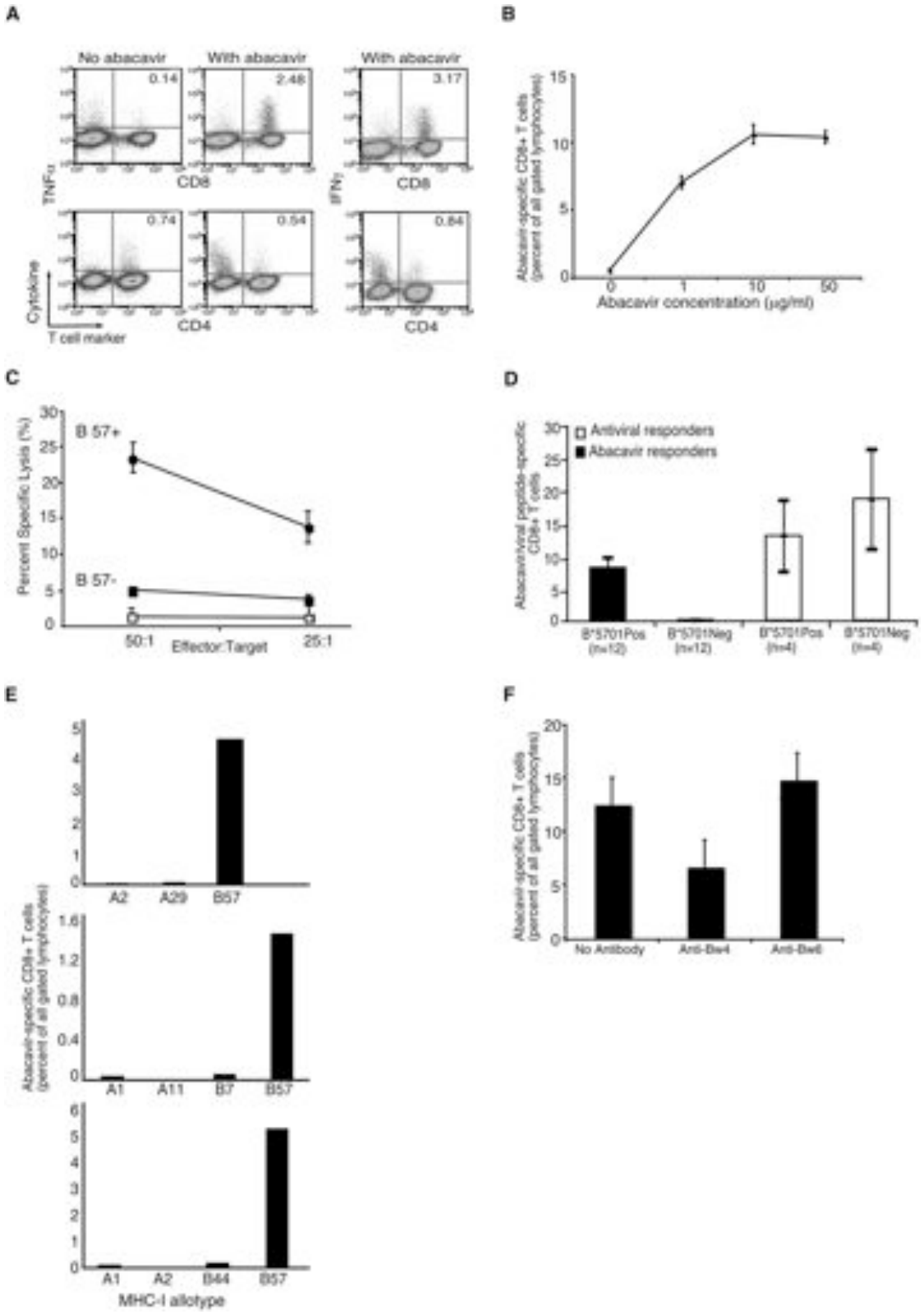

Figure 2. CD8$^+$ T Cells from HLA-B*5701-Positive Healthy Donors Specifically Proliferate and Make Cytokines in Response to Abacavir

(A) Flow-cytometry histograms of T cell intracellular staining for TNFα (left panels) and IFNγ (right panels) after abacavir stimulation. PBMCs from two healthy, HLA-B*5701-positive blood donors were stimulated with abacavir for 11 to 14 days in vitro after which the expanded T cells were then restimulated for 6 hr in

concentrations (0.5-10 μg/mL or 1.8-36 μM), (Figure 2B). Extensive washing of the APC after loading with abacavir preserved T cell recognition, demonstrating that the response was APC dependent and not a direct interaction between the T cell and the drug. Abacavir-reactive $CD8^+$ T cells from normal HLA-B*5701-positive donors were also cytotoxic (Figure 2C). Importantly, these responses were only observed in HLA-B*5701-positive individuals and could not be induced in individuals with any of the other MHC-I backgrounds we tested (Figures 2C and 2D). Nonetheless, EBV-specific $CD8^+$ T cell responses were readily elicited in HLA-B*5701-negative donors when a cocktail of known viral peptides was used to restimulate the T cells (right panel, Figure 2D). Abacavir-specific $CD8^+$ T cells were not activated by C1R.B57 loaded with a peptide derived from an EBV latent membrane protein (LMP), IALYLQQNW (IALY) (Duraiswamy et al., 2003) (not shown).

$CD8^+$ T responses within each individual were entirely restricted by the HLA-B*5701 allotype despite expression of non-HLA-B*5701 MHC-I allotypes in these donors, (Figure 2E and not shown). Thus, abacavir-reactive T cells from three different donors were rechallenged with abacavir-loaded APCs expressing their individual MHC-I allotypes (e.g., for donor 1, HLA A2, HLA A29, and HLA B57). T cell recognition of abacavir occurred only when the cells were stimulated with HLA-B*5701-postive APCs, (Figure 2E, randomized-block ANOVA $p < 0.001$).

In summary, abacavir-specific $CD8^+$ T responses were uniquely HLA-B*5701 restricted in HLA-B*5701- positive individuals, and these responses could not be elicited in HLA-B*5701-negative donors expressing seven common HLA-A allotypes (HLA-A1, 2, 3, 11, 24, 29, and 68) and nine common HLA-B allotypes (HLA-B7, 8, 18, 27, 35, 44, 45, 65, and 62). Moreover, abacavir-specific, HLA-B*5701-restricted $CD8^+$ T cells did not react with abacavir-loaded APCs expressing 11 different allogeneic MHC-I allotypes (not shown). Thus HLA-B*5701 restriction is a defining feature of abacavir-specific T cells isolated from abacavir-naive individuals. Abacavir-specific $CD8^+$ T cells interacted conventionally with HLA-B*5701 because cytokine responses were partially blocked by addition of a Bw4, HLA-B*5701-reactive mAb, but not when the non-HLA-B*5701-reactive Bw6 mAb was added to the T cell cultures (Figure 2F).

We observed that in vitro cultures from patients with AHS generated a greater proportion of specific $CD8^+$ T cells than similar cultures from naive donors, consistent with $CD8^+$ T cell memory in the patients. Nonetheless, abacavir-specific T cells from both groups were exclusively $CD8^+$, HLA-B*5701-restricted, produced TNFα and IFNγ, and were sensitive to abacavir over a dose-response range that includes the serum concentration of abacavir in treated patients. We conclude that the abacavir-specific, HLA-B*5701-restricted $CD8^+$ T cells generated in normal blood donors are similar in their specificity and nature to the abacavir-reactive T cells detected in patients with AHS. Therefore, we used responses from normal HLA-B*5701-positive donors to probe the biology and specificity of these abacavir-specific responses.

Abacavir Responses Require the Conventional MHC-I Antigen-Presentation Pathway

In the MHC-I-restricted antigen-presentation pathway, endogenous peptide ligands are created in the cytoplasm by proteolysis and then imported into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP) (McCluskey et al., 2004) where peptide loading is optimized by the chaperone, tapasin (Williams et al., 2002b). Functional loss of either TAP or tapasin leads to defective peptide loading and impaired antigen presentation (Williams et al., 2002a). However, the MHC-I antigen-presentation pathway can be bypassed by the addition of exogenous peptide ligands to either living or aldehyde-fixed APCs through direct loading of ligand-receptive

the presence or absence of abacavir (10 μg/mL) with the transfected lymphoblastoid cell line C1R expressing HLA-B*5701. Cells were permeabilized and stained for intracellular cytokine (y axis) and either CD8 (x axis, upper panels) or CD4 (x axis, lower panels). Cells were gated as live lymphocytes with percentages shown in each quadrant. The number of abacavir-specific, cytokine-producing T cells could be enriched by iterative in vitro restimulation in the presence of IL-2, but many cultures undergo exhaustion after 3 to 6 weeks in culture. However, in some donors the proportion of cytokine-producing T cells can be enriched to > 70% abacavir-specificity over 4 weeks in culture.

(B) Dose dependence of abacavir-specific $CD8^+$ T cell responses in HLA-B*5701-positive blood donors determined by the percentage of IFNγ-producing T cells detected at the end of the in vitro stimulation period as described above. Figure depicts the mean ± standard error of the mean (SEM) of abacavir-specific CD8 T cells elicited at each concentration of abacavir. Data are representative of three independent experiments on three different donors.

(C) T cells were first expanded from an HLA $B57^+$ and two $B57^-$ blood donors in the presence of abacavir. The expanded polyclonal T cells were then tested in a ^{51}chromium release assay for cytotoxicity of autologous PHA blast target cells loaded with abacavir. Only T cells from the HLA $B57^+$ donor lysed the abacavir-loaded PHA blast cells. Although abacavir-specific T cells cannot be cultured from PBMCs in non-B*5701 donors, the same number of viable T cells was added to the ^{51}Cr-labelled target cells in all cases. We assume that the viable T cells cultured from PBMCs in non-B*5701 donors are nonspecifically expanded in the presence of IL-2 in the cultures. There was negligible (<1%) cytotoxicity of PHA blasts in the absence of abacavir (not shown). Data represent the mean percentage of specific lysis ± SEM; $p < 0.02$ determined with a Student's t test.

(D) Abacavir-specific $CD8^+$ T cell responses are elicited in HLA-B*5701-positive but not HLA-B*5701-negative donors. PBMCs from HLA-B*5701-positive (Pos) and -negative (Neg) blood donors were stimulated with abacavir as described above, and the responding $CD8^+$ T cells ($IFN\gamma+$) are shown as a percentage of total gated lymphocytes (left histogram). $CD8^+$ T cells from HLA-B*5701-positive and -negative donors were also expanded by a cocktail of cognate Epstein-Barr viral peptides and then incubated with autologous APC loaded with the same peptides. Responses show the responding $IFN\gamma^+$ $CD8^+$ T cells as a percentage of total gated lymphocytes (right histogram). Data represent the mean abacavir-specific CD8 T cells ± SEM in each cohort. $p < 0.0005$ determined with a Student's pairwise t test in a two-tailed analysis.

(E) Abacavir-specific T cells expanded in vitro from three $B57^+$ individuals as described above were rechallenged with a panel of abacavir-pulsed (10 μg/mL) cell lines, either 721.221 or C1R, expressing the individual HLA-A and HLA-B allotypes expressed by each subject. The number of responding $IFN\gamma^+$ $CD8^+$ T cells with each APC is shown as a percentage of total gated lymphocytes.

(F) Activation of preformed abacavir-specific T cells by HLA-B*5701-positive APC was blocked by an anti-Bw4 monoclonal antibody (5 μg/mL) reactive with a conformational epitope located on the alpha-1 helix of HLA-B*5701 (left histograms). Blocking with the control non-HLA-B*5701-reactive monoclonal antibody anti-Bw6 (right histograms). The figure shows the mean ± SEM of the percentage of responding $IFN\gamma^+$ $CD8^+$ T cells as a proportion of total gated lymphocytes in three independent experiments; $p < 0.03$ determined with a Student's pairwise t test in a one-tailed analysis.

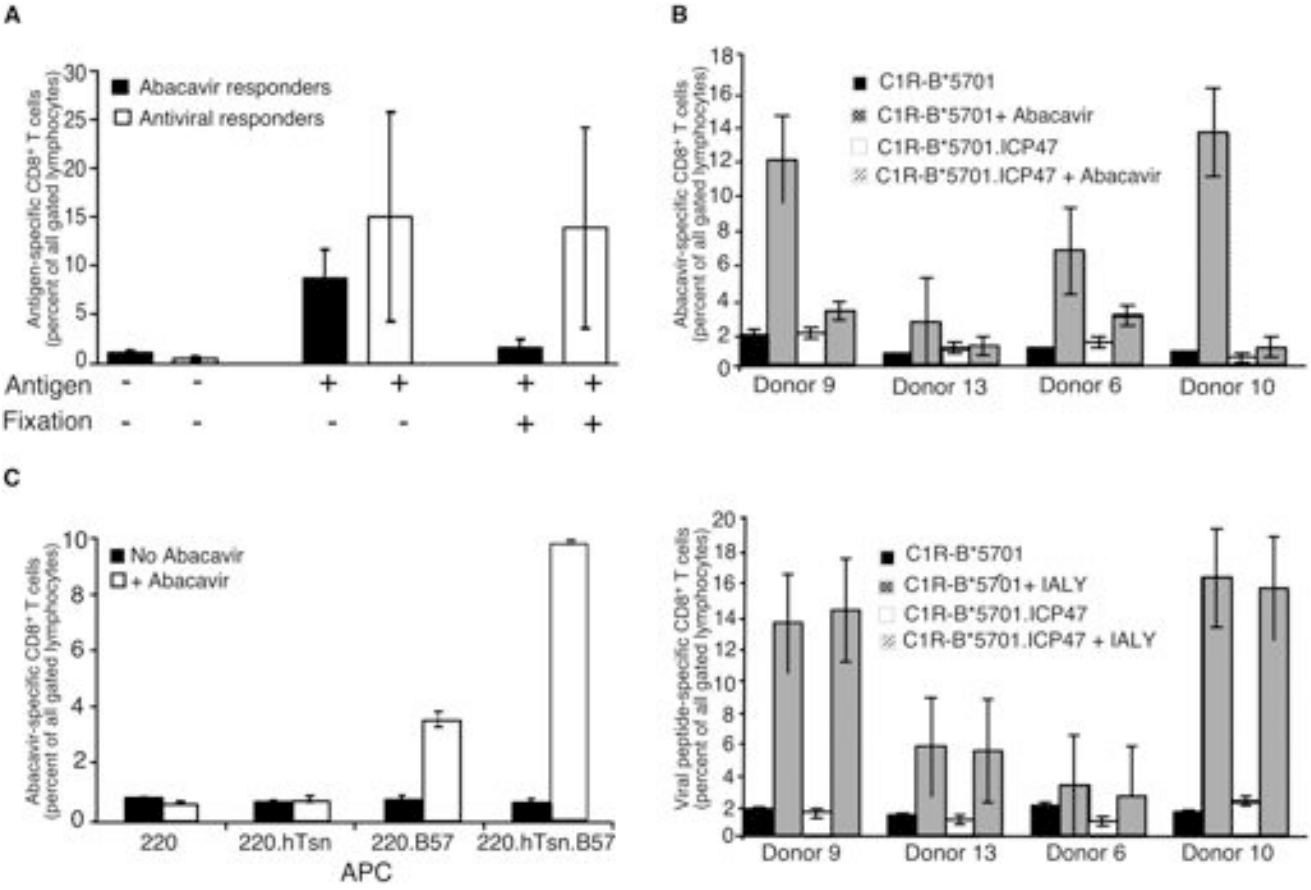

Figure 3. Recognition of Abacavir by HLA B*5701-Restricted, CD8$^+$ T Cells Requires Processing by APCs and Is TAP Dependent and Tapasin Dependent

(A) Antigen-specific T cells recognizing the HLA-B*5701-restricted viral peptide IALY (open boxes) or abacavir (closed boxes) were used to probe MHC-I recognition on unfixed (fixation −) and aldehyde-fixed (fixation +) APCs. After washing of the APCs, they were loaded with abacavir (10 μg/mL) or synthetic viral peptide (IALY, 1 μg/mL) and washed twice prior to a 2 hr incubation with specific CD8$^+$ T cells followed by addition of BFA and intracellular cytokine staining 4 hr later. Responses were compared in the presence (+) and absence (−) of antigen and shown as responding CD8$^+$ T cells as a percentage of gated lymphocytes.

(B) Abacavir-specific CD8$^+$ T cells were generated in four independent HLA-B*5701-positive donors and tested for abacavir recognition on normal HLA-B*5701-positive APC (C1R.B57) and HLA-B*5701-positive APCs expressing the viral TAP-inhibitor ICP47 (C1R.B57.ICP47). Responses were assayed in the presence (gray histograms) and absence (filled and open histograms) of abacavir (10 μg/mL) (upper panel). Surface expression of HLA-B*5701 was reduced 10-fold or more on ICP47 transfectants (not shown). For each donor, CD8$^+$ T cell responses were also generated toward the EBV peptide IALY and tested on the same APC but in the presence (gray histograms) and absence (filled and open histograms) of the IALY peptide (10 μg /mL) (lower panel). Responses show the responding IFNγ$^+$ CD8$^+$ T cells as a percentage of total gated lymphocytes.

(C) HLA-B*5701-restricted, abacavir-specific CD8$^+$ T cells were assayed for abacavir presentation in the presence (open boxes) or absence (closed boxes) of the drug on APC that included the tapasin-deficient parental cell line 721.220 (220), 721.220 transfected with a human tapasin gene (220.hTsn), 721.220 expressing HLA-B*5701 (220.B57), and 721.220 expressing human tapasin and HLA-B*5701 genes (220.hTsn.B57). Intracellular cytokine staining was carried out as described in the Experimental Procedures. Responses show the responding IFNγ$^+$ CD8$^+$ T cells as a percentage of total gated lymphocytes.

MHC-I molecules on the cell surface. Such complexes are capable of recognition by antigen-specific T cells (Figure 3A). However, when abacavir was added to aldehyde-fixed, HLA-B*5701-positive APCs, it did not reconstitute a ligand that could stimulate abacavir-specific CD8$^+$ T cells (Figure 3A), indicating a need for live APCs to mediate intracellular processing or modification of the drug to create a ligand (Figure 3A). This finding was reproducible in multiple experiments ($n = 7$) and statistically significant ($p < 0.001$, Tukey HSD multiple comparison following logarithmic transformation of the data). Mutant HLA-B*5701-positive APCs in which TAP function was impaired by coexpression of the viral TAP inhibitor ICP47 also did not present abacavir to specific CD8$^+$ T cells generated in four different donors (Figure 3B, upper panel). Exogenous loading of TAP-blocked APC with a HLA-B*5701-restricted EBV peptide resulted in efficient recognition by specific antiviral CD8$^+$ T cells in the same donors (Figure 3B, lower panel). Similarly, presentation of abacavir was substantially reduced by the mutant tapasin-deficient cell line 721.220 expressing HLA-B*5701 (Figure 3C). When tapasin expression was restored in these cells by gene transfection, presentation of abacavir was also restored (Figure 3C). Presentation of abacavir to CD8$^+$ T cells was also impaired by pretreatment of APC with the antifungal agent Brefeldin A, which disrupts egress of newly synthesized and antigen-loaded

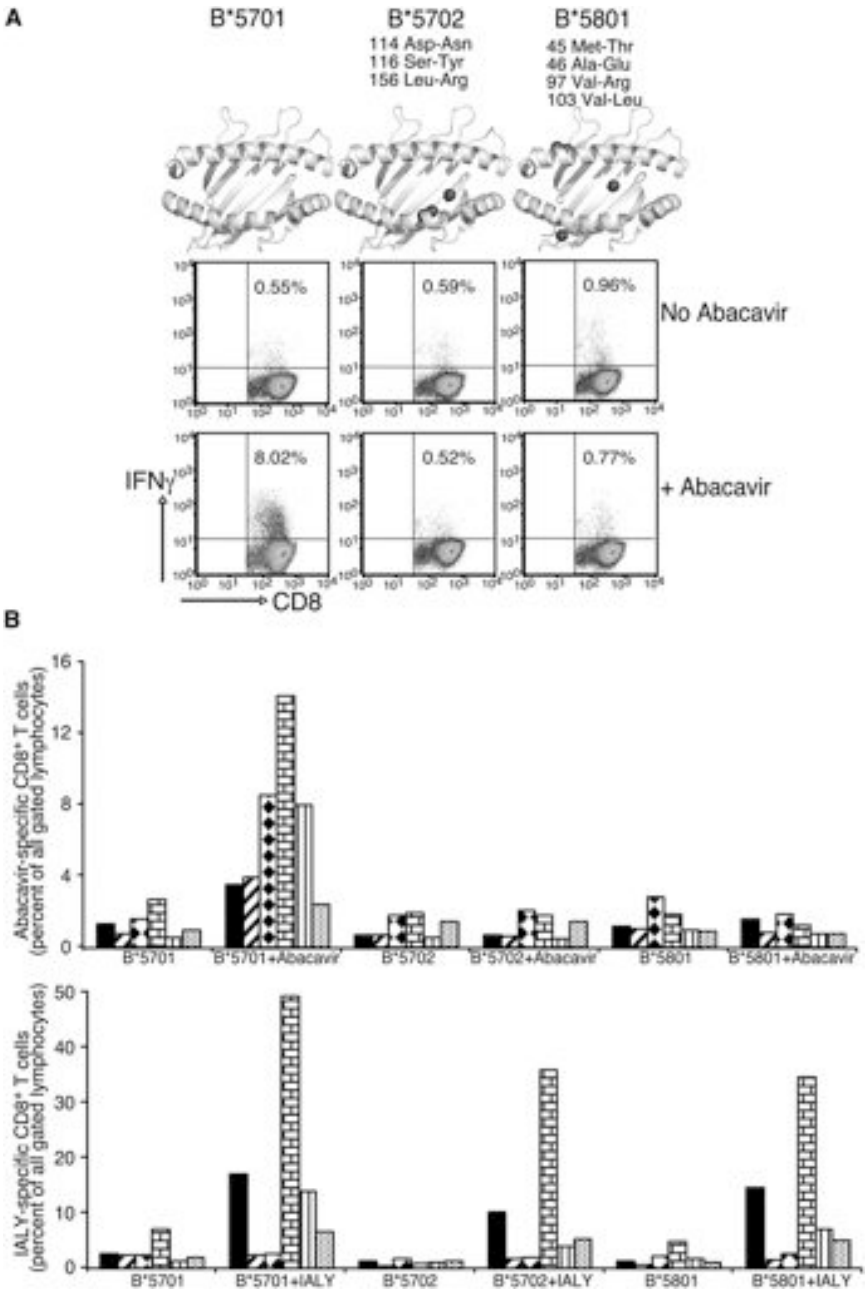

Figure 4. Abacavir-Specific CD8$^+$ T Cells Are Restricted by HLA-B*5701 and Not HLA-B*5702 or HLA-B*5801

(A) Abacavir-specific T cells were raised in vitro from a HLA-B*5701-positive donor and then re-stimulated with human lymphoblastoid cell lines expressing HLA-B*5701, HLA-B*5702, or HLA-B*5801 in the presence (upper histograms) or absence (lower histograms) of abacavir (10 μg/mL). Flow histograms of gated lymphocytes are shown stained for intracellular IFNγ (y axis) and CD8 (x axis). The percentage of responding IFNγ$^+$ CD8$^+$ T cells as a fraction of total gated lymphocytes is shown in the upper-right-hand quadrants. Differences between the peptide-binding clefts of HLA-B*5701, HLA-B*5702, and HLA-B*5801 are indicated with filled circles, and the amino acid substitutions from HLA-B*5701 are given above each illustration.

(B) Abacavir-specific T cells were raised in vitro from six independent HLA-B*5701-positive donors and used to probe abacavir presentation by APCs expressing HLA-B*5701, HLA-B*5702, or HLA-B*5801. T cell responses were assayed after stimulation in the absence of abacavir (B*5701, B*5702, and B*5801) and in the presence of abacavir (B*5701, B*5702, and B*5801+abacavir) (upper panel). Donor CD8$^+$ T cells were also raised in vitro against the EBV viral peptide IALY and assayed for intracellular IFNγ after stimulation in the absence of peptide (B*5701, B*5702, and B*5801) and in the presence of IALY 10 μg/mL peptide (B*5701, B*5702, and B*5801+IALY) (lower panel). Data from each donor are shown as individual shaded histograms indicating the responding IFNγ$^+$ CD8$^+$ T cells as a percentage of total gated lymphocytes.

MHC-I molecules from the endoplasmic reticulum to the cell surface (not shown).

Taken together, these findings indicated that abacavir recognition depends upon the conventional MHC-I antigen-presentation pathway and demonstrate that the unmodified prodrug is insufficient to form a T cell ligand by direct cell-surface MHC-I loading.

Specificity of Abacavir Recognition Maps to the MHC-I-Binding Cleft

HLA-B*5701 is part of the serologically defined HLA-B17 allotypic family that includes naturally occurring variants HLA-B*5702 and HLA-B*5801, which differ from HLA-B*5701 by only three and four amino acids, respectively (Figure 4A). Indeed, these closely related MHC-I allotypes have significant overlap in their peptide repertoire (Barber et al., 1997), and many virus-specific T cells are crossreactive with all three MHCp complexes (Duraiswamy et al., 2003). However, abacavir-loaded lymphoblastoid cell lines expressing these allotypes did not stimulate polyclonal abacavir-specific, HLA-B*5701-restricted CD8$^+$ T cells from six independent donors (Figures 4A and 4B). Nonetheless, in four of these donors, there was crossreactivity of HLA-B*5701-restricted antiviral CD8$^+$ T cells on peptide-loaded HLA-B*5702 and HLA-B*5801 APCs (Figure 4B). The mutations that distinguish HLA-B*5702 and HLA-B*5801 from HLA-B*5701 are predominantly located in the antigen-binding cleft (Figure 4A). Notably, HLA-B*5801 differs at residues 97 and 103 as well as residues 45 and 46, which form the B pocket anchor site. In contrast, HLA-B*5702 differs at position 156 and residues 114 and 116, the latter of which forms the F pocket anchor site (Figure 4A). Accordingly, the failure of HLA-B*5801 and HLA-B*5702 to stimulate CD8$^+$ T cells suggests the abacavir-related ligand is either presented in an altered conformation or is

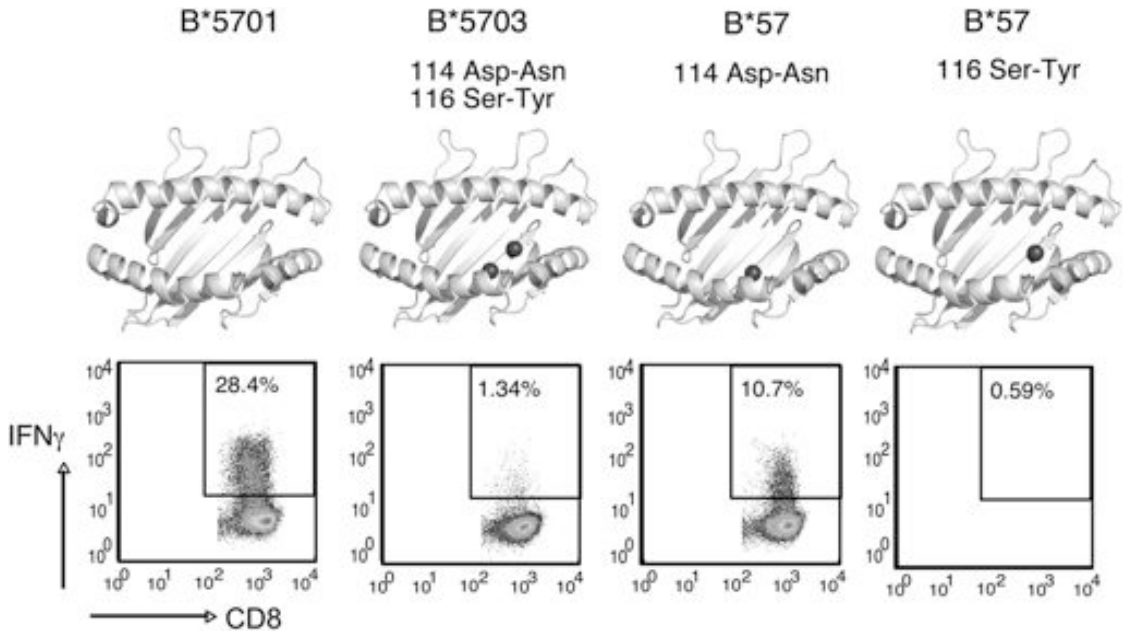

Figure 5. Fine Specificity of Abacavir-Specific CD8⁺ T Cells Determined by the Antigen-Binding F Pocket

Abacavir-specific T cells were raised in vitro from a HLA-B*5701-positive donor and then restimulated with the C1R cell lines expressing HLA-B*5701, HLA-B*5703, the HLA-B*5703 back mutant 114Asp→Asn, or the HLA-B*5703 back mutant 116Ser→Tyr. Flow histograms of gated lymphocytes are shown stained for intracellular I IFNγ (y axis) and CD8 (x axis). The percentage of responding IFNγ⁺ CD8⁺ T cells as a fraction of total gated lymphocytes is shown in the upper-right-hand quadrants. Differences between the peptide-binding clefts of HLA-B*5701, HLA-B*5703, and HLA-B*5703 back mutants are indicated with filled circles, and the amino acid substitutions from B*5701 are given above each illustration.

not presented at all by these MHC-I allotypes because of the altered chemical structure of their antigen-binding cleft. The latter seems more likely, given the lack of any association between AHS and these or any other MHC-I allotypes.

Despite the overlap in peptide repertoire of the B17 allotypic group, HLA-B*5701 prefers a lysine residue as the optimal residue of the bound peptide at position 3, whereas HLA-B*5702 prefers an aspartic acid in this position (Barber et al., 1997). Hence, the amino acid substitutions between HLA-B*5701 and HLA-B*5702 at positions 114 (D→N), 116 (S→Y), and 156 (L→R) could influence abacavir recognition via selection of amino acids at P3, the C terminus, or both. To determine whether the loss of abacavir presentation by HLA-B*5702 could arise entirely from the substitutions at 114 (D→N) and 116 (S→Y), we made these two substitutions by site-directed mutagenesis, thus creating the natural allotypic variant, HLA-B*5703. abacavir-loaded C1R cells expressing the HLA-B*5703 molecule did not stimulate abacavir-specific CD8⁺ T cells, indicating that substitution of F pocket residues 114 and 116 was sufficient to impair presentation of the abacavir-related ligand (Figure 5, second panel). The impact of individually changing residues 114 and 116 was then examined. Substitution of residue 114 from a negatively charged aspartate in HLA-B*5701 to a neutrally charged asparagine in HLA-B*5703 (D→N) resulted in a > 50% reduction in the number of IFNγ-producing CD8⁺ T cells and lower levels of IFNγ production by the remaining responding cells (Figure 5, third panel). Substitution of position 116, from a polar serine in HLA-B*5701 to the aromatic tyrosine residue of HLA-B*5703 (S→Y), completely abrogated recognition by abacavir-specific CD8⁺ T cells (Figure 5, fourth panel). Position 116 is particularly important in determining the structure of the F pocket and thus controls selection of the dominant anchor residue at the C terminus of bound peptides (Gomez et al., 2006; Zernich et al., 2004).

Distinct Architecture of the HLA-B*5701 and HLA-B*5703 F Pockets Specifies Antigen Selection

The repertoire of peptides bound by HLA- HLA-B*5701, HLA-B*5702, and HLA-B*5801 allotypes share a preference for tryptophan or a phenylalanine at position 9 in the F pocket (Barber et al., 1997). However, only HLA-B*5701 is reported to also accommodate a tyrosine in this position, indicating that the F pocket polymorphism results in selection of a different subset of endogenous ligands by HLA-B*5701 (Barber et al., 1997) as observed for other MHC-I allotypes (Kubo et al., 1998; Ramos et al., 2002; Zernich et al., 2004). To understand the impact of the F pocket polymorphism better, we directly solved the X-ray crystallographic structure of HLA-B*5701 complexed to a self-peptide (LSSPVTKSF, LF9) (see Supplemental Data) and compared this to the published structure of HLA-B*5703 bound to an HIV-1 peptide (KAFSPEVIPMF, KF11) (Stewart-Jones et al., 2005). The structure of the HLA-B*5701-LF9 complex confirmed that the two critical anchor residues at positions P2 (Ser) and P9 (Phe) bind in the B and F pockets of HLA-B*5701, respectively (not shown and Figure 6). Although HLA-B*5701 and HLA-B*5703 are complexed to two different peptides, the KF11 bound to HLA-B*5703 and the LF9 bound to HLA-B*5701 both have a Phe at the C terminus. Moreover, in the structures of HLA-B*5703 complexed with three different peptides, there was very little difference in the side-chain conformations of the F pocket amino acids. However, the H bonding of the Asp114

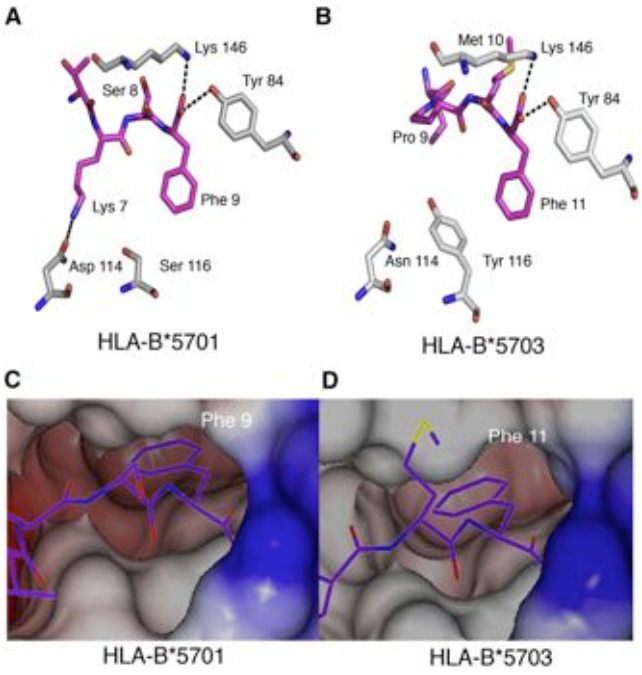

Figure 6. Distinct Chemical Architectures and Electrostatic Potential Characterizes the F Pocket of HLA-B*5701 Compared with HLA-B*5703

(A) The C-terminal residues of the self-peptide, LSSPVTKSF (LF9, purple), in the F pocket of HLA-B*5701 are shown, PDB ID code 2RFX. The residues at the polymorphic positions 114 and 116 are shown in gray.

(B) The C-terminal residues of the HIV-1 peptide, KAFSPEVIPMF (KF11, purple), in the F pocket of HLA-B*5703 are shown. The residues at positions 114 and 116, which are the polymorphic residues, are shown in gray.

(C) Surface representation of the F pocket of HLA-B*5701 and (D) HLA-B*5703, showing the electrostatics of the pocket (red, electronegative; blue, electropositive). The LF9 peptide is shown in purple. The F pocket is more electronegative in HLA-B*5701 compared to HLA-B*5703.

residue of HLA-B*5701 with LF9, as well as the small serine side chain of the F pocket residue 116 in HLA-B*5701 (Figure 6A), suggests that substitution of this residue to a large aromatic tyrosine found in HLA-B*5703 (Figure 6B) may abolish or alter binding of some peptides. Overall, the F pocket of HLA-B*5701 is more electronegative than in HLA-B*5703 (Figures 6C and 6D). These findings are consistent with the changes in F pocket architecture that alter immune recognition and peptide repertoire as observed between other closely related MHC-I allotypes (Hulsmeyer et al., 2002; Zernich et al., 2004).

Taken together, the findings indicate exquisite fine specificity in the HLA restriction of abacavir-specific $CD8^+$ T cell responses. This specificity can be mapped to the structure of the F pocket of the MHC-1 antigen-binding cleft, typical of many peptide-specific $CD8^+$ T cell responses. Moreover, in addition to this HLA restriction, abacavir-reactive T cells did not react with three compounds structurally related to abacavir, namely, didanosine, carbovir, and guanosine (Figure S3).

DISCUSSION

When skin-patch testing is combined with clinical diagnosis, the sensitivity of HLA-B*5701 testing in predicting AHS is ~100% and the specificity is 90%–100%, suggesting an exquisitely controlled mechanism involving the HLA-B*5701 molecule itself. Here we have shown that abacavir is highly immunogenic to $CD8^+$ T cells not only in patients with a history of AHS but also in healthy, abacavir-naive HLA-B*5701-positive blood donors. The stimulation of $CD8^+$ T cells is abacavir specific and only observed in individuals expressing HLA-B*5701. The T cells are HLA-B*5701 restricted and expanded to high frequencies (up to 25% of lymphocytes) after in vitro culture with IL-2. Abacavir-specific $CD8^+$ T cells secrete inflammatory mediators TNFα and IFNγ and are cytotoxic to abacavir-loaded APCs.

Our findings on the molecular mechanisms of abacavir presentation show that this is linked to the classical MHC-I pathway and shows fine specificity typical of most peptide antigens recognized by T cells. Abacavir is also a prodrug that undergoes deamination and phosphorylation to form an active drug, carbovir triphosphate (Ray et al., 2002), providing an opportunity for reactive groups on the drug to couple with enzyme side chains, or perhaps other cellular proteins, creating a neo-self-antigen. In preliminary experiments, we observed that abacavir-specific T cells did not react against three related compounds, carbovir, didanosine, and guanosine. The data implicate the 6-cyclopropylamino group on the main guanosyl ring as a possible reactive site because all three compounds lack this group whereas the primary amine group in the abacavir structure is also present in guanosine and carbovir. In other preliminary experiments, we have identified several fractions eluted from abacavir-loaded, HLA-B*5701-positive cell lines that have weak stimulatory activity when added back to abacavir-free HLA-B*5701-positive cells (not shown). Therefore, we propose that a derivative of abacavir forms a conjugate with one or more self-peptides that are uniquely presented by HLA-B*5701. Subtle alterations to self-peptides can be highly immunogenic, including accidental modifications that occur during peptide synthesis (Chen et al., 1996; Purcell et al., 1998), posttranslational modifications (Henderson et al., 2007; Molberg et al., 1998; Skipper et al., 1996), glycosylation, and chemical haptenation (Haurum et al., 1994; Mizushima et al., 1985).

The relationship between the in vitro responses observed in $CD8^+$ T cells and the development of AHS in vivo is compelling. $CD8^+$ T cells are abundant in the skin biopsies of patients with AHS and a rash. Skin-patch testing recalls a delayed-type-hypersensitivity reaction typical of prior cellular immunity. The time of onset of AHS after initiation of drug therapy (10–40 days) is consistent with the time needed to prime naive T cells. Moreover, the lack of any abacavir recognition using HLA-B*5702, HLA-B*5703, and HLA-B*5801 APCs suggests a failure of antigen presentation by these allotypes consistent with the lower prevalence of AHS in populations where HLA-B*5702, HLA-B*5703, or HLA-B*5801 are more common than HLA-B*5701. The detection of abacavir-reactive $CD8^+$ T cells from drug-naive, healthy normals suggests that HIV-1 infection does not play a specific role in AHS. However, the overall immune activation in HIV infection has been defined as a general risk factor for drug hypersensitivity and may amplify the likelihood of systemic reactions.

The expansion of T cell reactivities in vitro is generally observed where antigens have high precursor frequencies resulting from prior antigen exposure, or where very high precursor frequencies exist in the naive repertoire, such as T cells recognizing

allogeneic MHC molecules (Macdonald et al., 2003). Hence, the induction of abacavir-specific $CD8^+$ T cells in abacavir-naive, healthy blood donors is surprising. Moreover, many MHC-I specificities require T helper activity for their initial expansion and effector functions. We have not found any evidence that abacavir-specific $CD8^+$ T cells in abacavir-naive donors are drawn from a crossreactive antiviral memory population. TCR Vβ usage of abacavir-specific $CD8^+$ T cells is polyclonal and reactivity toward known HLA-B*5701-restricted viral peptides was negative (not shown). We consider that the expansion of these cells in vitro must reflect the potent immunogenicity of this drug. One reason for this may be that abacavir might have intrinsic costimulatory activity because it is known to induce changes in APC that might in turn help prime $CD8^+$ T cells in vitro overcoming the lack of a memory population in naive individuals (Martin et al., 2007).

Our findings demonstrate that abacavir hypersensitivity is an MHC-I-restricted cellular hypersensitivity mediated by $CD8^+$ T cell activation, in contrast to the more common nickel hypersensitivity and occupational lung disease, Berylliosis, which are MHC-II linked and elicit $CD4^+$ T cell delayed-type-hypersensitivity responses (Lombardi et al., 2001; Richeldi et al., 1993). The high frequency of reactive T cells observed in nickel hypersensitivity occurs because solvent-exposed amino acids on the MHC-peptide and TCR proteins jointly coordinate the nickel ions (Lu et al., 2003), directly linking the TCR-MHC-peptide-like weak superantigens (Gamerdinger et al., 2003). T cell responses to beryllium (Be^{++}) are exquisitely dependent upon glutamic acid (Glu) at position 69 in the HLA-DP β-chain, also suggesting specific metal coordination on a solvent-exposed interface involved in TCR ligation (Lombardi et al., 2001; Richeldi et al., 1993). Unlike the $CD4^+$ T cell hypersensitivity to Ni^{++} and Be^{++}, the fine specificity of abacavir recognition maps to the F pocket of HLA-B*5701 and is not accessible for TCR interaction and yet is crucial to the presentation of the abacavir ligand. The critical residue at position 116 that controls the difference in antigen presentation by HLA-B*5701 (Ser) and HLA-B*5703 (Tyr) could exert its influence on peptide binding through subtle differences in accommodation of C-terminal peptide side chains. It is also possible that abacavir itself, or a metabolite, is accommodated in the F pocket and that the smaller serine side chain is necessary for this binding. Alternatively, abacavir may be conjugated to a solvent-exposed region of an endogenously bound peptide, creating a haptenated self-peptide. Our study does not resolve this issue, nor can we say whether abacavir is bound covalently or noncovalently to an endogenous peptide ligand. Importantly, however, dramatic functional differences in HIV immunity are also observed in patients with HLA-B*5701 versus those with HLA-B*5703. Patients with these MHC-I allotypes make a dominant CTL response to the same HIV-1 Gag epitope, but the T cell repertoire and prevalence of HIV-specific epitope escape mutants differ dramatically in the two immune responses (Gillespie et al., 2006; Yu et al., 2007). Presumably, the conformation of the HIV peptide must be different in these two MHC-I allotypes.

It is also possible that the differences in the F pocket of HLA-B*5701 and HLA-B*5703 could alter the pathway of peptide acquisition as observed between the tapasin-dependent HLA-B*4402 (Asp116) and tapasin-independent HLA-B*4405 (Tyr116) molecules (Zernich et al., 2004). Although these allotypes only differ at position 116, they have different peptide repertoires and different modes of peptide acquisition that make them differentially resistant to viral evasion (Zernich et al., 2004).

The exquisite fine specificity of abacavir presentation coupled with the strong association of HLA-B*5701 with AHS has important implications for understanding other MHC-I associations with disease. Strong associations between particular MHC-I allotypes and protection from infection have been difficult to demonstrate. There are only a few examples, such as HIV-1 infection (Carrington and O'Brien, 2003), where the small viral genome and prolonged nature of the infection are probably crucial to revealing progressor and nonprogressor MHC-I associations. Another is protection against cerebral malaria associated with HLA-B53 (Hill et al., 1992). None of these associations are particularly strong compared with diagnostically useful strong association of HLA-B27 with ankylosing spondylitis or HLA-A29 and Birdshot retinopathy (Margulies et al., 2008). We propose that strong protective MHC-I phenotypes are not easily observed in immunity to infections because of the large number of potential peptide ligands available as signatures of pathogen recognition and the diversity of potential MHC-I allotypes expressed in the host (up to six distinct MHC-I allotypes). The mechanism of AHS suggests that two layers of specificity operate to single out HLA-B*5701 as a genetic determinant of this drug hypersensitivity. The first point of specificity occurs during drug targeting of endogenous proteins or peptides to create a unique ligand(s). This step is likely to considerably narrow the pool of potential ligands available for host class I molecules. The second layer of specificity occurs through selective binding and presentation of one or more of these ligands by particular MHC-I molecules as a result of the polymorphic nature of the antigen-binding cleft. Perhaps the strong association of ankylosing spondylitis with HLA-B27, Birdshot retinopathy with HLA-A29, or Behçet's disease with HLA-B51 also reflects more than one layer of specificity. Thus, pathogen-derived small molecules (or enzymes) may interact with, or modify, specific peptides that are then subject to further selection by particular MHC-I allotypes, creating pathogenic ligands of highly refined specificity.

Our findings explain the strong pharmacogenetic link between HLAB*5701 and abacavir hypersensitivity syndrome. They also point the way to understanding the basis for other HLA-linked drug reactions and implicate T cells in the pathogenesis of systemic reactions such as Stevens-Johnson syndrome. Further understanding of these life-threatening reactions should allow rational immunotherapeutic treatment with anti-T cells or anti-cytokine reagents.

EXPERIMENTAL PROCEDURES

Clinical samples came from the HIV clinics of Royal Perth Hospital (Wellington St., Perth, Western Australia) and St. Vincent's Hospital (Sydney, Australia). Normal blood donors were from the Australian Bone Marrow Donor Registry. Institutional ethics approvals were obtained for use of all clinical material. HLA-A and B loci were typed for each donor by DNA sequencing to obtain four-digit high-resolution genotyping of class I alleles (Victorian Transplantation and Immunogenetics Service [VTIS], Victoria, Australia).

Cell Lines and Culture

Peripheral-blood samples were collected in heparinized vacutainer tubes and PBMC were isolated via the standard Ficoll-Paque and density-gradient centrifugation method. Single-gene MHC-I transfectants of the HLA-class I-deficient lymphoblastoid cell lines C1R (Zemmour et al., 1992) or 721.221 (Shimizu

et al., 1988) were used as defined APCs to map HLA restriction of T cell responses at the conclusion of the period of in vitro stimulation with autologous PBMCs. Transfection was carried out as previously described (Peh et al., 1998; Zernich et al., 2004), or cell lines were obtained as gifts (Rajiv Khanna, Queensland Institute of Medical Research). Culture conditions are described in the Supplemental Data.

ICP47 and Tapasin Transfectants

C1R cells were transfected with a cDNA encoding the Herpes Simplex TAP inhibitor ICP47 by electroporation of plasmid DNA and drug selection of transformants as described (Zernich et al., 2004). HLA-B*5701 expression was reduced by 10-fold or more in the presence of ICP47. A cDNA encoding HLA-B*5701 was transfected into the tapasin-deficient cell line 721.220 as previously described (Zernich et al., 2004). Rescue of the impaired cell-surface expression of HLA-B*5701 (10- to 100-fold increase) was achieved by cotransfection of a cDNA encoding the human tapasin gene (Zernich et al., 2004). Expression of tapasin was confirmed by immunoblotting as previously described (Peh et al., 1998).

Lymphocyte Stimulation and Intracellular Cytokine Staining

T cell cultures were generated from approximately 10^7 responder PBMCs stimulated with 5×10^6 irradiated autologous PBMCs (Macdonald et al., 2003). Abacavir (Glaxo Smith Kline, Ziagen tablets) was present throughout the culture period at 10 μg/mL. Abacavir purity was confirmed by mass spectrometry (not shown). Culture medium was supplemented with 10 U/ml recombinant human IL-2 and changed every 2–3 days to maintain saturating levels of nutrients and growth factors. On day 11–14, $\sim 2 \times 10^5$ responders from T cell culture were harvested, washed, and then restimulated with 10^5 defined APC (either autologous PBMCs or defined cell lines) in the presence and absence of 10 μg/ml antigen resuspended in RPMI (peptide samples) or RPMI + 10% fetal calf serum (abacavir samples). After incubating for 1 hr at room temperature (RT), cells were washed twice in RPMI at 500 × g (5 min, RT) and resuspended in relevant media. After 2 hr of coincubation at 37°C/5% CO_2, 10 μg/ml Brefeldin A (Sigma) was added for a further 4 hr. Responder $CD8^+$ T cells were stained with anti-CD8 CyChrome and anti-CD4 phycoerythrin (Becton Dickinson Biosciences, San Jose, CA), fixed with 1% paraformaldehyde (ProSciTech, Queensland, Australia) and permeabilized with 0.3% Saponin (Sigma) containing anti-IFNγ FITC or anti-TNFα APC (Becton Dickinson). Samples were incubated at 4°C, in the dark for at least 40 min. Cells were washed and resuspended in 150 μl of PBS for analysis by flow cytometry within 48 hr. The percentage of gated lymphocytes producing IFNγ was determined by flow cytometry with FlowJo software (Tree Star Incorporated, CA). Abacavir-specific T cell responses were generated independently for most experiments and varied according to the particular responder and other variables intrinsic to cellular immunology.

For mAb blocking studies, after addition of antigen, purified anti-Bw4 antibody (5 μg/mL) was added to APC, mixed with responder T cells, and the assay was carried out as described above. Fixation, ^{51}chromium release assays, and statistical methods are described in the Supplemental Data. Structural methods are described elsewhere (Clements et al., 2002) with additional methods, data collection, and refinement statistics provided in Table S1.

ACCESSION NUMBERS

The HLA-B*5701^{LSSPVTKSF} structure has been deposited in the Protein Data Bank under code 2RFX.

SUPPLEMENTAL DATA

Additional Experimental Procedures, three figures, and one table are available at http://www.immunity.com/cgi/content/full/28/6/822/DC1/.

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