Antiviral CD8(+) T Cells Restricted by Human Leukocyte Antigen Class II Exist during Natural HIV Infection and Exhibit Clonal Expansion.

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Highlights

- CD8+ T cells restricted by HLA-DRB1 exist in a small number of HIV-infected persons
- These CD8+ T cells exhibit potent antiviral functions against HIV-infected cells
- TCRβ usage patterns indicate clonal expansion of class II-restricted CD8+ T cells
- CD8+ T cells that violate immunologic paradigms may contribute to viral control

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In Brief

MHC class II-restricted CD8+ T cells have been reported in Cd4-deficient mice and an SIV vaccine trial. Ranasinghe et al. demonstrate that HLA class II-restricted CD8+ T cells also exist in a small percentage of HIV-infected individuals, show potent antiviral functions against HIV-infected cells, and exhibit atypical TCR usage patterns.
Antiviral CD8+ T Cells Restricted by Human Leukocyte Antigen Class II Exist during Natural HIV Infection and Exhibit Clonal Expansion

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SUMMARY

CD8+ T cell recognition of virus-infected cells is characteristically restricted by major histocompatibility complex (MHC) class I, although rare examples of MHC class II restriction have been reported in Cd4-deficient mice and a macaque SIV vaccine trial using a recombinant cytomegalovirus vector. Here, we demonstrate the presence of human leukocyte antigen (HLA) class II-restricted CD8+ T cell responses with antiviral properties in a small subset of HIV-infected individuals. In these individuals, T cell receptor (TCR) analysis revealed that class II-restricted CD8+ T cells underwent clonal expansion and mediated killing of HIV-infected cells. In one case, these cells comprised 12% of circulating CD8+ T cells, and TCRα analysis revealed two distinct co-expressed TCRα chains, with only one contributing to binding of the class II HLA-peptide complex. These data indicate that class II-restricted CD8+ T cell responses can exist in a chronic human viral infection, and may contribute to immune control.

INTRODUCTION

Antigen-specific CD8+ T cells are a major defense against invading viruses, killing infected cells harboring non-self proteins. The first step in this process involves T cell receptor (TCR) recognition of virus-derived peptides presented on the surface of infected cells by molecules of the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) in humans. The dominant paradigm of T cell recognition dictates that CD8+ T cells recognize viral peptides of 8–11 amino acids in length bound to MHC class I molecules, whereas CD4+ T cells recognize peptides of 12 or more amino acids restricted by MHC class II (Neefjes et al., 2011). During thymic selection, double-positive T cells undergo a maturation process where they are selected as either CD8+ T cells or CD4+ T cells depending on TCR binding to peptide complexed with MHC class I or class II, respectively. However, in certain cases, T cells with atypical modes of MHC restriction have been reported. CD8+ T cell responses restricted by MHC class II have been described in approximately a dozen published reports over the past two decades. These responses have been observed in Cd4-deficient mice, where the complete absence of CD4+ T cells led to the unexpected expansion of CD8+ T cells restricted by class II (Matechak et al., 1996; Mizuochi et al., 1988; Pearce et al., 2004; Shimizu and Takeda, 1997; Suzuki et al., 1994; Tynnik et al., 2004), and in mouse models of transplantation (Hirosawa et al., 2011). In humans, reports have also described alloreactive CD8+ T cell responses that cross-recognize HLA class I and II (Heemskerk et al., 2001; Rist et al., 2009), indicating that unconventional HLA and TCR interactions may be an important feature of allorecognition.

Remarkably, virus-specific CD8+ T cells restricted by MHC class II were recently found to be the immunodominant responses detected in the context of immunization with a...
recombinant rhesus cytomegalovirus (RhCMV) vector in a macaque simian immunodeficiency virus (SIV) vaccine model (Hansen et al., 2013), which also induced non-classical MHC-E-restricted CD8+ T cell responses (Hansen et al., 2016). In this setting, the induction of CD8+ T cells with unconventional MHC restriction was predominantly under the genetic control of the strain 68-1 RhCMV vector and the epitopes targeted by these responses did not overlap with traditional MHC class I-restricted responses. These studies demonstrate that unconventional CD8+ T cell responses can be elicited by engineered RhCMV vaccine vectors and suggest that these responses may contribute to the control of viral replication (Ranasinghe and Walker, 2013).

The extent to which HLA class II-restricted CD8+ T cell responses play a role in natural human immunity is unclear. To address this in a chronic human viral infection, we screened a large cohort of HIV-1-infected persons, including HIV controllers who spontaneously control virus in the absence of antiretroviral therapy. In these HIV controllers, we and others have shown that class I-restricted CD8+ T cell responses, particularly HLA-B-restricted responses targeting epitopes within the highly conserved Gag protein, are associated with enhanced control of viral replication (Emu et al., 2008; Fellay et al., 2007; Kaslow et al., 1996; Kiepiela et al., 2004, 2007; Migueles et al., 2000; Persley et al., 2010). We now provide data showing induction of class II HLA-DR-restricted CD8+ T cell responses in 3 of 101 HIV controllers tested. These class II-restricted CD8+ T cells exhibited potent antiviral functions and were characterized by a single dominant TCRβ clonotype. In one individual, the HLA-DR-restricted CD8+ T cells were immunodominant, comprising 12% of circulating CD8+ T cells. These data reveal the rare presence of atypical CD8+ T cells restricted by HLA-DR in a human viral infection and indicate that T cells that violate immunologic paradigms may shape human antiviral responses.

RESULTS

CD8+ T Cell Responses Restricted by HLA-DRB1 Exist in Natural HIV Infection

Virus-specific CD8+ T cells typically recognize infected cells through presentation of processed viral peptides on HLA class I molecules, but class II-restricted responses have been detected in some experimental systems and in the context of CMV vector immunization in a macaque SIV vaccine model (Hansen et al., 2013). In order to determine whether such responses exist in a chronic human viral infection, we screened 129 people with untreated HIV-1 infection, all of whom expressed common class II DRB1 alleles (Table S1). These included 101 untreated HIV controllers who maintained viral loads of less than 2,000 RNA copies/mL plasma, as well as 28 individuals with untreated chronic HIV infection exhibiting high viral loads.

Purified CD8+ T cells isolated from the peripheral blood mononuclear cells (PBMCs) of each subject were co-cultured with mouse lymphoblastoid cell lines (LCL) stably expressing a single recombinant human DRB1 molecule (Ranasinghe et al., 2013; Southwood et al., 1998) matched to the donor, which had been pulsed with individual overlapping peptides spanning the HIV Gag protein. In initial screening, we identified an HIV controller, subject 474723, in whom a single Gag peptide presented by class II HLA DRB1*11:01 resulted in a robust interferon gamma (IFN-γ) Elispot response mediated by CD8+ T cells (Figure 1A). This targeted peptide, Gag41 (YVDRFYKTLRAEQASQEV, aa 164–181), is also known to be presented by DRB1*11:01 for CD4+ T cell recognition in HIV-infected persons (Ranasinghe et al., 2013). N- and C-terminal truncations indicated that presentation of the FYKTLRAEQA peptide contained within the larger peptide probably represented the core residues sufficient to elicit a response (Figure 1B), with phenylalanine or tyrosine likely to be the P1 anchor residue. Of the truncated peptides tested, the most robust response was to the 16 amino acid peptide DRFYKTLRAEQAQSEV, which is expected to extend out of the open class II binding groove. Class II restriction of the Gag41-specific CD8+ T cell response was verified using an anti-HLA-DR blocking antibody that inhibited IFN-γ production in a dose-dependent manner (Figure 1C).

To further confirm that this response was indeed mediated by class II-restricted CD8+ T cells, and to more precisely define the magnitude of the response, we constructed class II tetramers with a truncated version of the Gag41 peptide (DRFYKTLRAEQA) that elicited the strongest Elispot response. More than 12% of circulating CD8+ T cells were DR11-Gag41 tetramer positive directly ex vivo (Figure 2A), which was further confirmed by dual staining with both allophyococyanin (APC)- and phycoerythrin (PE)-conjugated versions of the tetramer (Figure 2B).

Assessment of subjects by IFN-γ Elispot revealed that this was the only person in whom a class II-restricted CD8+ T cell response could be detected to the Gag41 epitope, and this was further confirmed by staining PBMCs of 76 persons expressing HLA DRB1*11:01 (including the 39 initially screened by IFN-γ Elispot) with DR11-Gag41 tetramers (Table S1). However, extended IFN-γ Elispot screening and subsequent construction of class II tetramers revealed two additional subjects, 270245 and 388031, in whom class II-restricted CD8+ T cell responses were detectable, both targeting a peptide designated Gag37 (LNKIVRMYSPTSILD, aa 136–151) restricted by HLA-DRB1*01:01. Although these responses were present at much lower frequencies than the DR11-Gag41-restricted response, dual APC and PE tetramer staining of the CD8+ T cell populations using a DR01-Gag37 tetramer confirmed these were indeed class II restricted (Figure 2B). All class II tetramers showed minimal non-specific staining when tested on HLA-matched HIV-negative and HIV-positive subjects (Figure S1). Table S2 shows HLA genotyping and clinical characteristics of these three subjects with class II-restricted CD8+ T cell responses.

We next compared the magnitude and specificity of the class II-restricted CD8+ T cells with the most immunodominant class I-restricted Gag-specific CD8+ T cells in each of the three subjects, using HLA tetramers (Figure 2C) and Elispot assays (Figure S2). In terms of magnitude, for subject 474723 the DR11-Gag41-restricted response was immunodominant over all class I-restricted Gag-specific responses tested, at 12% frequency compared to a class I B57-KF11+ tetramer population of 2% frequency ex vivo. In terms of specificity, there was minimal overlap between HLA class I and class II epitopes to which responses were detected (Figure S3A). However, in the other two subjects, the unconventional responses were subdominant. The DR01-Gag37 epitope was adjacent to epitope B08-EI8 in subject 270245 and partially overlapped with immunodominant epitope
Figure 1. Elucidation of a HLA Class II DRB1-Restricted CD8+ T Cell Response in HIV Controller 474723

(A) Representative IFN-γ HLA-DR Elispot using magnetically enriched CD8+ T cells (FACS plot insert) co-cultured with an LCL stably transfected with DRB1*11:01 (matching the HLA type of subject) pulsed with 66 overlapping peptides (OLPs) spanning the HIV Gag protein. Error bar represents SD.

(B) Representative IFN-γ HLA-DR Elispot performed with N- and C-terminal peptide truncations presented on DRB1*11:01 LCL. Epitope FA10 within Gag41 is highlighted in red.

(C) Representative flow cytometric intracellular cytokine secretion (ICS) assay with anti-HLA-DR antibody performed on whole PBMCs co-cultured with DRB1*11:01 LCL.

See also Figures S1–S3 and Table S1.
B27-KK10 in subject 388031, yet each population was distinct by class I and II tetramer staining (Figures S3B and S3C).

We next investigated the phenotypic characteristics of the subject-derived class II tetramer-positive CD8\(^+\) T cell populations using flow cytometry (Figure 3A) and single-cell RNA sequencing (scRNA-seq) (Figure 3B). We found that both CD8\(^+\) and CD8\(^{+}\) were expressed at the protein and transcript levels. The class II-restricted CD8\(^{+}\) T cells did not co-express CD4 protein or mRNA. Next, we assessed the memory phenotype of these responses. All three subjects exhibited antigen-experienced effector memory phenotypes, although the degree of differentiation was different for each subject. In 474723, the immunodominant DR11-Gag41-positive CD8\(^{+}\) T cells manifested an unusual differentiation for HIV-specific CD8\(^+\) T cells: all tetramer-positive cells exhibited a highly differentiated effector memory (Temra) phenotype (CCR7\(^-\)CD45RA\(^+\)) (Figure 3C). In contrast, the DR01-Gag37-positive CD8\(^+\) T cells, which were subdominant in vivo in both individuals, predominantly exhibited an effector memory (Tem) phenotype (CCR7\(^+\)CD45RA\(^-\)). PD-1 expression was found to be consistent with their memory phenotype (Figure S4).

Collectively, these data indicate that virus-specific CD8\(^{+}\) T cell responses restricted by HLA-DRB1 exist in the setting of natural HIV infection, and although this is a rare event, such responses can represent an immunodominant HIV-specific CD8\(^{+}\) T cell response.

**HLA Class II DRB1-Restricted CD8\(^{+}\) T Cells Lyse Autologous HIV-Infected Targets Ex Vivo**

Little is known about the function of CD8\(^+\) T cells restricted by HLA class II, and particularly whether these cells have antiviral properties. Therefore, we examined the expression of granzyme B in class II-restricted CD8\(^{+}\) T cells by intracellular staining and flow cytometry. The majority of class II tetramer\(^+\) CD8\(^{+}\) T cells from each subject was granzymeB\(^{+}\) when compared to bulk CD8\(^{+}\) T cells (Figure 4A). Additionally, transcriptional profiling conducted by scRNA-seq on tetramer-sorted single cells confirmed that class II-restricted CD8\(^{+}\) T cells from all three subjects expressed perforin, granzyme B, granzyme H, MIP-1\(\beta\), and RANTES (Figure 4B).

Previous studies have shown proliferation in response to epitope recognition to be associated with antiviral function (Horton et al., 2006; Riou et al., 2014), so we next compared the proliferative capacity of class II-restricted CD8\(^{+}\) T cells with the most immunodominant class I-restricted Gag-specific CD8\(^{+}\) T cell response in Autologous HIV-Infected Targets Ex Vivo.
Figure 3. HLA Class II-Restricted CD8αβ T Cells in Three HIV Controllers Exhibit Heterogeneous Memory Phenotypes

(A) Representative FACS plots denoting surface expression of CD8α and CD8β. Tetramer-positive expression is shown in red. Bulk CD3+CD4+ T cells are shown in gray. CD3+CD4- T cells are shown in black.

(B) Violin plots denoting the distribution of single-cell expression levels of CD8α (CD8A), CD8β (CD8B), and CD4 RNA transcripts in unstimulated class II tetramer-sorted CD8+ and CD4+ T cells estimated from single-cell RNA-seq. Abbreviation: TPM, transcripts per kilobase million. Statistical significance was determined with Mann-Whitney-Wilcoxon test (***p < 0.001).

(C) Representative FACS plots show memory phenotype of tetramer-positive HLA class I- and class II-restricted CD8+ T cells. All populations shown are gated on tetramer+ CD3+CD8+CD4-CD19-CD14-CD56- live lymphocyte singlets. See also Figure S4.
Figure 4. HLA Class II-Restricted CD8+ T Cells Exhibit Cytolytic Properties and Proliferative Capacity

(A) Representative FACS plots gated on unstimulated CD8+ T cells expressing intracellular granzyme B and tetramer in three HIV controllers. Tetramer populations are gated on tetramer+CD3+CD8+CD4−/C0 CD19−/C0 CD14−/C0 CD56− live lymphocyte singlets.

(B) Violin plots denoting the distribution of single-cell levels of Perforin, Granzyme B, Granzyme H, MIP-1b, and RANTES RNA transcripts in unstimulated class II tetramer-sorted CD8+ T cells from three HIV controllers by scRNA-seq.

(C) Representative FACS plots gated on CD8+ T cells expressing CFSE and tetramer at day 7 after stimulation of bulk PBMCs from HIV controllers 474723 and 270245. Abbreviations: TPM, transcripts per kilobase million; PHA, phytohaemagglutinin.
subjects 474723 and 270245, for whom sufficient samples were available. The class II tetramer-positive cells demonstrated specific proliferation upon stimulation with cognate peptide (Figure 4C). Of note, class I tetramer-positive cells from subject 270245 showed populations that were carboxyfluorescein diacetate succinimidyl ester (CFSE\(^+\)) positive yet tetramer negative, possibly due to TCR downregulation or non-specific proliferation induced by activation. These data demonstrate that DR11-Gag41-positive CD8\(^+\) Temra cells and DR01-Gag37-positive CD8\(^+\) Tem cells have substantial proliferative capacity.

We next generated class II-restricted CD8\(^+\) T cell clones from subjects 474723 and 270245. These clones demonstrated specific, potent killing of autologous Epstein-Barr virus (EBV)-transformed B cells (BCL) pulsed with the cognate class II peptide. Additionally, the class II-restricted clones showed substantial cytolytic activity against autologous CD4\(^+\) T cells super-infected with HIV-1 NL4.3 as well as against CD14\(^+\) monocyte-derived macrophages super-infected with vesicular stomatitis virus G-protein (VSV-G) pseudotyped HIV-1 NL4.3-expressing GFP (Figure 5A).

Thereafter, we tested the ability of class II-restricted CD8\(^+\) T cells to kill directly ex vivo and compared their antiviral efficacy with the class I-restricted CD8\(^+\) T cell population (Figure 5B). BCLs, used as target cells for this assessment, displayed equivalent surface expression of HLA-ABC and HLA-DR (Figure S5). The DR11-Gag41\(^+\) and B57-KF11\(^+\) populations from subject 474723, as well as the DR01-Gag37\(^+\) and the B08-EI8\(^+\) populations from subject 270245, were tetramer sorted from fresh blood and then co-cultured with autologous BCL in a chromium release assay. The class II-restricted CD8\(^+\) T cells from both subjects demonstrated direct ex vivo specific killing of peptide-pulsed BCL akin to that of the immunodominant class I-restricted CD8\(^+\) T cell responses, indicating that these populations exhibit a similar killing efficiency (Figure 5B). Longer incubation of Gag41-loaded target cells with either tetramer-sorted CD8\(^+\) T cells or bulk CD8\(^+\) T cells from subject 474723 in a modified in Vitro Technique for Assessing Lysis (VITAL) assay resulted in greater than 98% elimination of target cells over a 36 hr incubation. The percentage of lysed target cells was similar to that achieved with bulk CD8\(^+\) T cells co-cultured with KF11-loaded target cells, whereas target cells loaded with a control peptide were not eliminated (Figure S6).

We subsequently evaluated the ability of ex vivo isolated class II-restricted CD8\(^+\) T cells to recognize and kill HIV-infected cells displaying naturally processed HIV antigen resulting from productive infection. Due to limited sample availability from subject 474723, we focused on autologous monocyte-derived macrophage targets that express naturally high levels of surface HLA-DR. The monocyte-derived macrophages were super-infected with a VSV-G-pseudotyped HIV-1 NL4.3 prior to co-culture with freshly isolated tetramer-sorted DR11-Gag41\(^+\)CD8\(^+\) T cells. We observed that effectors lysed HIV-infected macrophages directly ex vivo, with 19% mean specific lysis compared to 4% mean specific lysis in the control (p = 0.049) (Figure 5C). Infection of viable macrophages, measured by GFP expression or p24 staining, was >92% (Figure S5). These data demonstrate that HIV-infected macrophages effectively process and present naturally derived HIV peptide on the cell surface for recognition by class II-restricted CD8\(^+\) T cells.

As a further measure of potential CD8\(^+\) T cell-mediated antiviral function, we examined the sequence of the HLA-DR-restricted epitope for evidence of immune selection pressure. More than 80% of sequences in subject 474723 showed homology to the Gag41 peptide sequence. However, almost 20% of sequences exhibited a Q308H mutation within the Gag41 epitope (Figure 5D). Although the DR11-Gag41\(^+\) effectors and the bulk CD8\(^+\) T cells effectively lysed BCL pulsed with wild-type peptide, they showed no detectable lysis of the Q308H peptide, consistent with this being a putative escape mutation. There was no detectable CD8\(^+\) T cell response to B57-QW9 in this subject and prior sequencing of B57 controllers has not detected any Q308H mutants (Migueles et al., 2000), indicating that Gag41-specific T cells probably drive Q308H escape. These data suggest that the DR11-Gag41-restricted T cells may be capable of exerting immune selection pressure.

Taken together, our data assessing the cytolytic marker expression, epitope-specific proliferative capacity, target cell lysis, and immune selection pressure indicate that class II-restricted CD8\(^+\) T cells demonstrate potent antiviral properties.

**HLA Class II-Restricted CD8\(^+\) T Cells Are Constituted by One Dominant TCR\(^{\beta}\) Clonotype**

To further define these HLA class II-restricted CD8\(^+\) T cell responses, we computationally reconstructed TCR using scRNA-seq data from single tetramer-sorted class II-restricted CD8\(^+\) T cells from each of the three subjects. In each, we found that the class II-restricted CD8\(^+\) T cell response was characterized by expansion of a single TCR\(^{\beta}\) clonotype (Figure 6).

The HLA class II-restricted CD8\(^+\) T cell responses identified here are also immunodominant epitopes normally seen by conventional class II-restricted CD4\(^+\) T cell responses (Ranasinghe et al., 2012, 2013), so we next analyzed whether both CD4\(^+\) and CD8\(^+\) T cells could bind the class II tetramers. The same class II tetramers that recognized CD8\(^+\) T cells also stained ex vivo CD4\(^+\) T cells, albeit very weakly in two of the three subjects (Figure 7A). These tetramer-positive CD4\(^+\) T cells were within the expected range for an epitope-specific response (Scriba et al., 2005), yet the frequency of the class II-restricted CD8\(^+\) T cell population intra-patient was dramatically larger. Together, these data reveal an immunological phenomenon in which conventional HLA class II-restricted CD4\(^+\) and unconventional class II-restricted CD8\(^+\) T cells can bind the same peptide–HLA complex.

We next examined whether CD8\(^+\) and CD4\(^+\) T cells targeting the same peptide–HLA had similar TCR rearrangements. Due to sample availability and tetramer-positive CD4\(^+\) cell number constraints, we were limited to analysis of subject 474723. Because we had already identified the TCR\(^{\beta}\) V gene for the DR11-Gag41\(^+\) restricted CD8\(^+\) T cells, we used the class II tetramers and a TRBV2-specific fluorescent antibody to analyze both CD8\(^+\) and CD4\(^+\) populations (Figure 7B). Consistent with the sequencing results, we found that the class II-restricted CD8\(^+\) population was comprised of 99.9% TRBV2-positive cells, and that 73.9% of the tetramer-positive CD4\(^+\) response was also TRBV2 positive.

We also computationally reconstructed TCRs using scRNA-seq data from single DR11-Gag41 tetramer-sorted CD4\(^+\) T cells. We identified 22 TCR\(^{\beta}\) sequences for the DR11-Gag41-restricted CD4\(^+\) T cells. In contrast to the DR11-Gag41-restricted CD8\(^+\), where there was only one dominant clonotype,
we observed a more diverse response comprised of 16 different clonotypes for the CD4+ T cells. Many clonotypes (13/22) used the same TRBV2 gene but had different rearrangements, highlighted in green in Figure 6. Thus in subject 474723, we found that TRBV2 is preferentially selected by both CD8+ and CD4+ T cell responses that target this class II HLA-peptide complex.

From characterization of the TCRα gene usages of both DR11-Gag41 CD4+ and CD8+ T cells using computational reconstruction from the scRNA-seq data, we obtained TCRβ sequences from 71 and 17 single cells of the CD8+ and CD4+ populations, respectively. We found that the CD8+ T cell repertoire had only one TCRβ clonotype comprised of gene segments TRBV2, TRBJ2-7, and TRBC2, but two distinct TCRα clonotypes. One was comprised of gene segments TRAV6, TRAJ39, and TRAC and the second was comprised of TRAV26-1, TRAJ16, and TRAC. However, the CD4+ repertoire had ten different TCRβ

Figure 5. HLA Class II-Restricted CD8+ T Cells Lyse Autologous HIV-Infected Targets Ex Vivo and Exert Putative Immune Selection Pressure In Vivo

(A) Summary data assessing specific lysis of target cells by class II-restricted CD8+ T cell clones from subjects 474723 and 270245 in a standard 6 hr chromium release assay. Autologous targets included EBV-transformed BCL pulsed with peptide or no peptide, CD4+ T cells infected with the HIV strain NL4.3, and monocyte-derived macrophages infected with VSV-G pseu
dotyped HIV NL4.3 encoding GFP, all at an effector to target cell ratio (E:T) of 1:1. Statistical significance was determined using a paired t test.

(B) Summary data assessing specific lysis of target cells by ex vivo tetramer+ sorted HLA class I- and class II-restricted CD8+ T cells in a standard 6 hr chromium release assay at multiple E:T ratios. Ex vivo effector cells were derived from fresh blood and tetramer sorted within 12 hr of phlebotomy. As target cells, autologous EBV-transformed BCL from subjects 474723 and 270245 were pulsed with cognate peptide or no peptide. Statistical significance was determined using a paired t test.

(C) Summary data assessing specific lysis of autologous monocyte-derived macrophages pulsed with the cognate peptide Gag41 or infected with VSV-G pseu
dotyped HIV NL4.3 encoding GFP by ex vivo DR11-Gag41 tetramer+ sorted CD8+ T cells from subject 474723 in a standard 6 hr chromium release assay at an E:T ratio of 1:1.

(D) Deep sequencing of autologous virus in subject 474723. Mutation Q308H within the Gag41 epitope is boxed. Specific lysis of EBV-transformed BCL pulsed with wild-type Gag41 peptide or the Q308H mutant peptide was tested in a standard 6 hr chromium release assay with ex vivo tetramer-sorted DR11-Gag41 tetramer+ sorted CD8+ T cells or bulk CD8+ T cells at a 1:1 E:T ratio.

All data points for each graph represent biological replicates in a single experiment. Error bars represent SD. See also Figure S6.
clonotypes (Figure 6). Because this analysis was performed from sorted DR11-Gag41+ T cells at a single cell per well for downstream RNA-seq and TCR reconstruction, we could identify that the two different TCRα chains, TRAV6-TRAJ39 and TRAV26-TRAJ16, were co-expressed within the same CD8+ T cell, consistent with incomplete allelic exclusion of TCRα during thymic selection (Figure 7C).

Both TRAV26 and TRAV6 sequences in the class II-restricted CD8+ T cells were productively rearranged. Therefore, to test for functionality, we cloned the TCR constructs and generated two TCR hybridomas that expressed either TRAV6 with TRBV2 or TRAV26 with TRBV2. Both TRAV26 and TRAV6 were able to refold properly to pair with the β, TRBV2, and be expressed on the surface of cells. However, we found that only TRAV6 (shown in red), but not TRAV26 (shown in blue), bound to the DR11-Gag41 complex when paired with TRBV2 (Figure 7D). To evaluate the functionality of the hybridomas, we measured interleukin 2 (IL-2) secretion after stimulation with the Gag41 peptide. We found that TRAV6 with TRBV2, but not TRAV26 with TRBV2, was able to produce IL-2 in a dose-dependent manner in response to peptide (Figure 7E). Both assays showed that only the TRAV6 with TRBV2 pair was functional, confirming that TRAV6 was necessary for TCR recognition of DR11-Gag41 complex. Next, we produced soluble TCR of TRAV6 with TRBV2 to assess the binding kinetics to DR11-Gag41 using surface plasmon resonance. The interaction of TRAV6 and TRBV2 with DR11-Gag41 showed an overall dissociation affinity constant (K_D) of 8.1 μM, which is typical for MHCII-peptide-specific TCRs (van der Merwe and Davis, 2003). Collectively, these data indicate that class II-restricted CD8+ T cells demonstrated atypical patterns of TCR usage that challenge the current paradigm of T cell recognition. Collectively, our data from sequencing the TCRβ revealed that class II-restricted CD8+ T cells are monoclonal in all three subjects. Additionally, in subject 474723, a specific TRBV is preferentially selected and shared by CD8+ and CD4+ T cells targeting the same DR11-Gag41 complex. Finally, we showed that DR11-Gag41-specific CD8+ express two distinct TCRα chains, with only one contributing to the binding of this class II HLA-peptide complex.

**DISCUSSION**

CD8+ T cells play a critical role in control of viremia, typically through the recognition and killing of infected cells presenting pathogen-derived peptides on HLA class I molecules. Yet, whether CD8+ T cells restricted by HLA class II exist in natural human viral infections and exert antiviral functions is unclear. Here, we report the existence of CD8+ T cells that recognized HIV Gag peptides presented on HLA class II. Although these were rare events—found in 3% of the HIV controller population in this study—in one individual the class II-restricted CD8+ T cells were the most immunodominant CD8+ response detected, encompassing 12% of circulating CD8+ T cells. These cells exhibited high proliferative capacity and potent lysis of target cells and may have imposed selection pressure for the generation of viral escape mutants, comparable to the well-characterized antiviral efficacy of class I-restricted CD8+ T cells. These data illustrate that paradigm-violating HLA class II-restricted CD8+ T cells can be elicited in a chronic human viral infection. Moreover, our findings reveal an unexpected flexibility in CD8+ T cell recognition and demonstrate that the paradigm of CD8+ T cell restriction by HLA class I molecules is not absolute in human anti-viral immune responses.
the class II-restricted CD8+ T cell response was characterized by an expansion of a single TCRβ clonotype, rather than the typically oligoclonal TCR repertoires observed for epitope-specific class I-restricted CD8+ T cells (Almeida et al., 2007; Chen et al., 2012; Mendoza et al., 2012). Second, we observed a phenomenon in which class II-restricted CD8+ T cells targeted the same HLA-peptide complex as conventional CD4+ T cells. Our data indicated that, in one subject, this was associated with preferential selection of TRBV2 usage in all CD8+ T cells and most CD4+ T cells targeting the DR11-Gag41 complex. Although other studies have demonstrated TCRβ “public clonotypes” among virus-specific CD8+ (Iglesias et al., 2011) or CD4+ (Benati et al., 2016) T cells in unrelated individuals, here TCR sharing occurred between antigen-specific CD8+ and CD4+ T cells within an individual. Lastly, analysis of the TCRα chain revealed expression of two α chains. However, only TRAV6 in combination with TRBV2 was able to bind DR11-Gag41. We hypothesize that the second α chain (TRAV26) in combination with TRBV2 may have interacted with a class I molecule occupied by a self peptide or foreign peptide at sufficient affinity, leading to its positive selection resulting in differentiation into a CD8+ single-positive T cell in the thymus with subsequent migration into the
periphery. Subsequent HIV infection may have then fortuitously selected this peripheral CD8+ T cell clone in the context of the DR11-Gag41 peptide (via its TRAV6 TCR specificity), resulting in an expanded population of memory cells with potent antiviral function. Taken together, our data imply that these unconventional CD8+ T cells exhibit distinctive TCR characteristics and suggest a mechanistic explanation as to how class II-restricted CD8+ T cells can be selected; this phenomenon may also in part explain the rarity of these responses.

Our data revealed that Gag-specific CD8+ T cells restricted by HLA-DR had a cytotoxic T lymphocyte-like phenotype and effectively killed autologous HIV-infected cells. HLA-DR-restricted CD8+ T cells may confer multiple advantages in the context of HIV infection. Unconventional restriction may allow CD8+ T cells to exert antiviral effector functions on infected macrophages and activated CD4+ T cells that typically express high levels of HLA-DR. Cytotoxic CD8+ T cells restricted by HLA-DR may also have an advantage in settings where HIV Nef-mediated class I downregulation may impair recognition (Schwartz et al., 1996).

Furthermore, targeting of the Gag-37 and -41 peptides may allow HLA-DR-restricted CD8+ T cells to target a virus that has already escaped within epitopes restricted by conventional CD8+ T cells. Yet, it is difficult to assess the contribution of these unconventional CD8+ T cells to immune control. Subjects 474723 and 388031 express class I alleles B*57:03 and B*27:05, respectively, which are strongly associated with HIV-1 control (Carrington and Walker, 2012). Subject 270245 lacks these “protective” class I alleles and exhibits a DR01-Gag37-restricted CD8+ T cell response with demonstrable antiviral efficacy ex vivo. This raises the possibility that DR01-Gag37-restricted CD8+ T cell antiviral functions, in addition to CD8+ T cell responses restricted by “non-protective” class I alleles, may contribute to immune control in this individual. Further studies aimed at inducing unconventional CD8+ T cell responses in healthy humans would be required to determine their in vivo antiviral efficacy and delineate their overall contribution to control of viral replication.

The potential relevance of class II-restricted CD8+ T cell responses is underscored by results from an SIV vaccine trial. In monkeys immunized with strain 68-1 RhCMV vector and challenged with pathogenic SIV, two-thirds of the CD8+ T cell responses recognized a wide breadth of SIV Gag epitopes bound to class II molecules (Hansen et al., 2013). Induction of class II-restricted CD8+ T cells, which occurs in every immunized animal, is a consequence of the absence of two viral genes (Rh157.5 and Rh157.6) in the strain 68-1 vector, as indicated by the fact that repair of these two genes reverts CD8+ T cell responses back to class I restriction (Hansen et al., 2013). The Rh157.5 and Rh157.4 gene products are part of a RhCMV receptor for non-fibroblasts and their absence changes the cellular tropism of the vector, making it more fibroblast-tropic, which in turn is thought to change the priming environment to favor generation of class II-restricted CD8+ T cells. As the modified vector does not change the priming environment to favor generation of class II-restricted CD8+ T cells, which occurs in every immunized animal, our data suggested a mechanistic explanation as to how class II-restricted CD8+ T cells can be selected; this phenomenon may also in part explain the rarity of these responses.

Although we showed that class II-restricted CD8+ T cells can exist in natural HIV infection, we note a number of limitations in this study. We detected only a single Gag-specific CD8+ T cell response restricted to HLA-DRB1 in each of three HIV controller individuals and in none of the HIV chronic progressors. The low number of responses detected may be due to the method of screening, a modified IFN-γ Elispot using LCL stably expressing a single recombinant HLA-DR molecule. Arguably, the reliance on IFN-γ detection may thwart detection of unconventional CD8+ T cell responses if they do not secrete this cytokine. To circumvent this limitation, we also screened HIV-infected individuals with class II tetramers, but CD8+ T cell responses were found only in the aforementioned three individuals, confirming that the modified Elispot is unlikely to have missed low-level responses. Because the macaque studies evaluated only SIV Gag-specific CD8+ T cell responses restricted by MuMu-DRB, we focused this study on HIV Gag-specific CD8+ T cell responses restricted by common HLA-DRB1 alleles. We did not test for class II-restricted CD8+ T cell responses to other HIV proteins, or to class II DRB4, DRB5, DP, or DQ. Another constraint in our study was limited sample availability and low numbers of tetramer-positive cells, so in some parts of this study, we primarily focused on the characterization of subject 474723. This subject demonstrated potent killing of target cells ex vivo, showed putative evidence of viral escape in vivo, and exhibited unique TCR features. However, given the rarity of these unconventional CD8+ T cell responses, it is not clear whether we can make generalizations between class I- and class II-restricted CD8+ T cells. Indeed, further work will be required to determine whether these unconventional responses represent a distinct subset of HIV-responsive cells or represent class I-restricted CD8+ T cells that simply happen to bear TCR that cross-react with Gag peptide presented by class II. Finally, whether these results can be extrapolated to unconventional T cells in other pathogen infections or vaccine settings will require additional study.

In summary, these data reveal rare class II-restricted CD8+ T cell responses with potent antiviral properties and clonal expansion in the setting of a natural human viral infection, challenging current paradigms of T cell recognition and restriction. Our findings suggest greater flexibility in CD8+ T cell recognition and restriction, which is likely modulated by TCR cross-reactivity and which may be important for immunological outcomes. Thus, these data not only enhance our understanding of the basic immunology of TCR-peptide-HLA interactions, but also may be important for future T cell-based vaccine design and immunotherapeutic interventions, where induction of unconventional class II-restricted CD8+ T cells that show antiviral efficacy may be beneficial.
EXPERIMENTAL PROCEDURES

Subjects
A total of 129 HIV-infected individuals were recruited from Massachusetts General Hospital after providing informed consent. From those, 101 individuals were defined as “HIV controllers:” HIV-infected individuals who spontaneously control HIV infection in the absence of antiretroviral therapy for greater than 1 year. Additionally, 28 treatment-naive HIV progressors with plasma viral loads of greater than 2,000 HIV RNA copies/mL were utilized (see also Tables S1 and S2 and Supplemental Experimental Procedures).

HLA-DR CD8* Elispot
Screening for class II-restricted HIV-specific CD8* T cell responses and epitope fine mapping was conducted by enzyme-linked immunospot (Elispot) assay, using CD8* T cells enriched by CD8 MACS MicroBeads selection (Miltenyi). Antigen-presenting cells expressing the HLA-DRB1 of the subject consisted of mouse lymphoblastoid cell line (LCL) fibroblasts stably transfected with a plasmid encoding a single recombinant human HLA-DRB1 variant spanning common Caucasian alleles: DRB1*01:01, 03:01, 04:01, 07:01, 11:01, 13:01, and 15:01. LCLs were split across 70 wells of a V-bottom 96-well plate and pulsed with 10 µg/mL peptide. We used 66 individual overlapping peptides (OLPs) spanning HIV Gag protein (clade B 2001 consensus-sequence) tested in singlet and had 4 negative control wells without peptide. Plates were incubated at 37°C and 5% CO2 for 90 min and washed 6× to remove any unbound peptide. We then cultured 20,000 LCL with 100,000 CD8* T cells per well on a pre-coated interferon gamma (IFN-γ) plate. As a positive control, phytohemagglutinin (Sigma) was added at 1.8 µg/mL. The plates were incubated overnight at 37°C and 5% CO2 and processed as previously described (Fナンシングエトアル、2013). We used the AID Elispot reader (Autoimmun Diagnostika GmbH) to determine the number of spot-forming cells per 100,000 CD8* T cells. A HLA-DR restriction was considered positive only if ≥3 times the mean background and also ≥3 times the standard deviation of negative control wells. A caveat is that each OLP was tested only once and some responses may be below the threshold for a “positive” result. To rectify this, any responses deemed above or close to the positive threshold were independently re-tested in quadruplicate.

HLA Class I and II Tetramers
Class II tetramers custom-manufactured by MBL International in collaboration with Dr. James Moon included DR11-Gag41 (DRB1*11:01-DRFYKTRLAEQASQEV) and DR01-Gag37 (DRB1*01:01-LNKIVRMYSPTSLD) conjugated to either PE or APC. Class I tetramers were made in collaboration with Dr. Soren Buus as described (Leisner et al., 2008). The class I tetramers included B57-KF11 (KAFSEPVIIFM), B08-E1B (EYIKWRII), and B27-KK10 (KRWILGLNK) in either PE or APC. Tetramers were incubated with whole PBMCs (25 µL at 37°C, 5% CO2) and then stained for viability and surface markers prior to fixation. Tetramer staining of hybridomas was conducted with the same protocol. Intracellular staining was used for Granzyme B and IFN-γ using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions. All fixed samples were analyzed on a LSRII flow cytometer (BD Biosciences) with FlowJo software (Treestar).

Functional Characterization of Class II-Restricted CD8* T Cells
To determine HIV-specific cytokine secretion in response to peptide-HLA-DR stimulus, whole PBMCs were incubated with 2 µg/mL of peptide pulsed onto LCL or left unstimulated in the presence of anti-HLA-DR (azide-free clone L243, Biologend) to efficiently block HLA class II recognition. BFA and monensin were added to prevent cytokine secretion. IFN-γ secretion in response to peptide stimulus was measured from CD8* T cells. To confirm the restriction of the CD8* T cells, class I and class II tetramers were utilized. To determine proliferative capacity, PBMCs were stained with carboxylfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Life Technologies) for 7 min at 37°C, then washed. Appropriate class I and class II peptides were added at 0.1 µg/mL to CFSE-labeled whole PBMCs for 7 days in RPMI 1640 medium in the absence of IL-2 at 37°C, 5% CO2. PHA was used as a positive control and the absence of peptide stimulation was used as a negative control. After 7 days, cells were labeled with appropriate tetramer in APC together with antibodies to CD3, CD4, CD8, and CD25 and analyzed on a LSRII flow cytometer (BD Biosciences).

Chromium Release Assay
Chromium release assays were conducted with autologous targets (EBV-transformed B cell lines, CD4+ T cells, and monocyte-derived macrophages), as described in Supplemental Experimental Procedures. Activated autologous CD4+ T cells were infected with HIV NL4-3 by spinoculation at 800 µg/mL for 1 hr at 37°C and cultured for 48 hr at 37°C and 5% CO2. Autologous monocyte-derived macrophages were plated at 30,000 per well and VSV-G-pseudotyped SIV mac251 VLPs were added 3 hr prior to HIV challenge to abrogate host restriction factors and subsequently increase HIV infectivity. Macrophages were then infected with VSV-G-pseudotyped HIV NL4.3 expressing GFP by spinoculation at 800 µg/mL for 1 hr at 37°C and cultured for 48 hr at 37°C and 5% CO2. The CXCR4-utilizing HIV-1 laboratory strain NL-43 and its VSV-G-pseudotyped version were obtained from the AIDS Reference and Research Reagent Program, Division of AIDS, NIAID, NIH.

Single-Cell RNA-Seq
Whole-transcriptome amplification of single cells in 96-well plates was performed with a modified SMART-Seq2 protocol, as described previously (Trombetta et al., 2014). Samples were sequenced on an Illumina NextSeq 500 instrument using either 30- or 150-bp single-end reads. RNA-seq reads were first trimmed using Trimmomatic (Bolger et al., 2014) and then aligned to the RefSeq hg38 transcriptome and genome using RSEM and TopHat. Considering only single-cell libraries in which we could reconstruct a productive TCR alignment, we excluded from further analysis genes and libraries with poor performance or coverage, leaving 205 cells and 3,274 genes. Out of the 205 cells with reconstructed TCR sequences, 30 were CD4+ and 175 were CD8+ by flow cytometry gating. The TPM expression of CD4, CD8A, and CD8B transcripts between the CD4+ and CD8+ cells was compared using Mann-Whitney-Wilcoxon test.

TCR α and β Chain Sequencing
In order to reconstruct CDR3 sequences from single-cell RNA-sequencing data, we developed TrapeS (TCR Reconstruction Algorithm for Paired-End Single Cells), a software package for reconstruction of TCR sequences using short (~25 bp) single-cell paired-end RNA sequencing based on TopHat genomic alignments (Supplemental Experimental Procedures). TrapeS is available upon request.

TCR-Expressing T Cell Hybridomas
As previously described (White et al., 2000), an MSCV-derived retrovirus encoding CD8 and the common TRBV V-domain fused to mouse Cα and either the TRAV6 or TRAV26 domain fused mouse Cε was prepared. The virus was used to transduce a TCRαβ variant of the mouse T cell hybridoma that had been previously transduced to express human CD8α chain. T cells expressing high levels of TCR and CD8 were single cell cloned by FACs. 104 transduced T cell hybridomas were placed in 250 µL culture wells that had been either previously coated with an anti-mouse TCR Cα Mab (H57-597) or contained 105 HLA-DR11-bearing LCL cells and various concentrations of the Gag41 peptide. After overnight culture at 37°C, the culture supernatants were assayed for IL-2 using the IL2-dependent cell line HT2.

Statistical Analysis
Paired t tests and Mann-Whitney-Wilcoxon test were used to compare different conditions, when each condition was tested in triplicate or greater. All p values are two-sided and p < 0.05 was considered significant. Statistical analysis and graphing were performed using GraphPad Prism 5.0 or R.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.09.015.
S.R. was responsible for the concept and conduct of the study; S.R., P.A.L., and B.D.W. contributed to the experimental design and data analysis; S.R., P.A.L., D.Z.S., R.B.J., F.D., C.N., and P.J. performed the experiments; S.W.K. and A.K.S. conducted single-cell RNA-seq; M.B.C. and N.Y. computationally reconstructed TCR; G.M.C., F.C., J.W., A.M., and J.W.K. constructed TCR hybridomas and performed SPR; K.P. and T.M.A. conducted viral sequencing; H.S., D.E.K., and L.J.P. provided intellectual input and editorial comments; B.D.W. provided clinical samples and oversight of the project; S.R., P.A.L., and B.D.W. wrote the manuscript; and all authors contributed to revisions.

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