T Cell Cross-Reactivity between a Highly Immunogenic EBV Epitope and a Self-Peptide Naturally Presented by HLA-B*18:01 + Cells

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T Cell Cross-Reactivity between a Highly Immunogenic EBV Epitope and a Self-Peptide Naturally Presented by HLA-B*18:01+ Cells

Melissa J. Rist,*‡,1 Kelly M. Hibbert,‡,#,1 Nathan P. Croft,‡ Corey Smith,* Michelle A. Neller,* Jacqueline M. Burrows,* John J. Miles,*†,‡ Anthony W. Purcell,‡ Jamie Rossjohn,*‡,# Stephanie Gras,‡,# and Scott R. Burrows*‡†

T cell cross-reactivity underpins the molecular mimicry hypothesis in which microbial peptides sharing structural features with host self-peptides stimulate T cells that cross-react with self-peptides, thereby initiating and/or perpetuating autoimmune disease. EBV represents a potentially important factor in the pathogenesis of several T cell–mediated autoimmune disorders, with molecular mimicry a likely mechanism. In this study, we describe a human self-peptide (DELEIKAY) that is a homolog of a highly immunogenic EBV T cell epitope (SELEIKRY) presented by HLA-B*18:01. This self-peptide was shown to bind stably to HLA-B*18:01, and peptide elution/mass spectrometric studies showed it is naturally presented by this HLA molecule on the surface of human cells. A significant proportion of CD8+ T cells raised from some healthy individuals against this EBV epitope cross-reacted with the self-peptide. A diverse array of TCRs was expressed by the cross-reactive T cells, with variable functional avidity for the self-peptide, including some T cells that appeared to avoid auto-reactivity by a narrow margin, with only 10-fold more of the self-peptide required for equivalent activation as compared with the EBV peptide. Structural studies revealed that the self-peptide–HLA-B*18:01 complex is a structural mimic of the EBV peptide–HLA-B*18:01 complex, and that the strong antiviral T cell response is primarily dependent on the alanine/arginine mismatch at position 7. To our knowledge, this is the first report confirming the natural presentation of a self-peptide cross-recognized in the context of self-HLA by EBV-reactive CD8+ T cells. These results illustrate how aberrant immune responses and immunopathological diseases could be generated by EBV infection. The Journal of Immunology, 2015, 194: 000–000.

Self-peptides associated with MHC molecules, while positively selecting those with the potential to recognize antigenic peptide epitopes (2). Importantly, self or autoreactive T cells with a very low affinity/avidity can escape negative selection in the thymus and, under certain conditions, can facilitate a variety of autoimmune conditions in humans (3, 4).

Generated somatically through site-specific DNA recombination reactions, TCR diversity is a result of the random assembly of V(D)J gene segments. The TCR α-chain is comprised of V and J gene segments whereas V(D)J genes encode the TCR β-chain (5). Each chain has three CDRs that make the Ag-binding site of the TCR. The CDR3 loop is a critical component of the TCR, as it generally mediates contact with the antigenic peptide–MHC complex (6, 7). The mathematical estimation of potential TCR diversity is $\sim 10^{13}$ different TCRs, although after considering thymic positive and negative selection events, the size of the naive TCR repertoire is estimated to be $\sim 2 \times 10^7$ TCRs for each human (5).

As the binding sites of TCRs arise from random genetic mechanisms, these sites have the capacity to react not only with pathogens and environmental Ag but also with self-antigens. Indeed, autoimmune disease and allergies can occur when activation of the adaptive immune response is directed at Ags that are self or environmental. Forming the basis of the molecular mimicry hypothesis is T cell cross-reactivity, whereby it is postulated that T cells stimulated by an epitope derived from an infectious agent can cross-react with a self-antigen that may share sequence or structural homology with that epitope. This phenomenon may initiate and/or perpetuate autoimmune disease (8). Consistent with this theory, a number of animal studies have demonstrated that Ag mimicry can induce a T cell response in addition to autoimmune disease in the form of...
murine autoimmune oophoritis (9), and limited sequence homology can evoke a multiple sclerosis (MS)-like disease (10).

The lymphotropic γ-1 herpesvirus, EBV, is widespread in all human populations, infecting ~95% of the adult population worldwide (11, 12). EBV infection is associated with several autoimmune conditions, including MS, for which a molecular mimicry mechanism has been proposed (13). Recently, we investigated the CD8 T cell response to the immediate early, BZLF1 Ag of EBV and described an octamer peptide 175SELEIKRY180 (termed SEL) that is recognized by HLA-B*18:01^2^ individuals (14). In the present study, we have identified a human protein sequence (DELEIKAY, termed DEL) with sequence homology to the octamer EBV epitope.

T cell cultures

PBMCs were isolated by Ficoll-Hypaque centrifugation into RPMI 1640 medium supplemented with 10% FCS (R10 medium). Blood donors were healthy, EBV-seropositive individuals who had given written informed consent. Approval was obtained from the QIMR Berghofer Medical Research Institute Human Ethics Committee (Brisbane, QLD, Australia). T cell cultures were raised by culturing PBMCs (2 × 10^6^ per 2 ml well) with either irradiated autologous lymphoblastoid cell lines (2 × 10^6^ per 2 ml well) for EBV-specific cultures or PBMCs that had been precoated with the EBV-SEL peptide (1 µM for 1 h, responder/stimulator ratio of 2:1) for SEL-specific cultures. Cultures were supplemented with IL-2 (20 U/ml) from day 3 and analyzed on day 18. Synthetic EBV peptide SEL and self-peptide DEL were synthesized by use of the Merrifield solid-phase method and purchased from New England Peptides (Gardner, MA) (for SEL) and Mimotopes (Clayton, VIC, Australia) (for DEL).

ELISPOT assays

IFN-γ ELISPOT assays were performed using cytokine capture and detection reagents according to the manufacturer’s instructions (ELISPOT protocol for human IFN-γ, Mabtech, Stockholm, Sweden). Briefly, 96-well nitrocellulose plates precoated with anti–IFN-γ mAb were seeded with ~5 × 10^4^ EBV-specific T cells and SEL or DEL peptides at various concentrations. After incubation for 16 h at 37°C in 5% CO2, the cells were discarded, and captured IFN-γ was detected with a biotinylated anti–IFN-γ Ab, followed by development with an alkaline phosphatase substrate solution (BCIP/NBT-plus). Spots were counted using an automated plate counter.

Peptide–MHC multimer and TCR β-chain variable staining

T cell cultures (lymphoblastoid cell line– or peptide-stimulated) from healthy virus carriers were incubated for 30 min at 4°C with an SEL–HLA-B*18:01 allogeneic-cytokine-labeled tetramer (14). Cells were then washed and incubated with PerCP-Cy5.5-conjugated anti-human CD8 mAb (BioLegend, San Diego, CA) and one of the following PE- or FITC-labeled TCR β-chain–specific mAbs (Beckman Coulter): Vβ1 (TCR β-chain variable [TRBV9], Vβ2 (TRBV20-1), Vβ3 (TRBV28), Vβ4 (TRBV29), Vβ5.1 (TRBV5-1), Vβ5.2 (TRBV5-6), Vβ5.3 (TRBV5-5), Vβ6.7 (TRBV7-1), Vβ7 (TRBV4), Vβ7.2 (TRBV4-3), Vβ8 (TRBV12), Vβ9 (TRBV11), Vβ11 (TRBV25-1), Vβ12 (TRBV10), Vβ13.1 (TRBV6-9), Vβ13.2 (TRBV6-2), Vβ13.6 (TRBV6-5), Vβ14 (TRBV27), Vβ16 (TRBV14), Vβ17 (TRBV19), Vβ18 (TRBV18), Vβ20 (TRBV30), Vβ21.3 (TRBV11-1), Vβ22 (TRBV2), or Vβ23 (TRBV13). Cells were washed twice and fixed in 1% paraformaldehyde in PBS. These samples were analyzed on a BD LSRII Fortessa flow cytometer using FACS Diva software (BD Biosciences).

Intracellular cytokine staining

T cell lines from EBV-seropositive, HLA-B^*18:01^ donors, raised by stimulation of PBMCs with the SEL peptide, were incubated for 4 h at 37°C with either SEL or DEL peptide (1 µg/ml) in R10 medium supplemented with 5 µg/ml brefeldin A (BioLegend). These cells were then washed and incubated at 4°C for 30 min with fluorescein-labeled mAbs specific for cell surface markers (CD8-PerCP-Cy5.5, BioLegend) and CD45-Alexa Fluor 488 (BioLegend). Cells were washed and then fixed and permeabilized with Cytotox/Cytoperm fixation/permeabilization solution (BD Pharmingen) at 4°C for 20 min. Next, the cells were washed in Perm/Wash (BD Pharmingen), incubated with IFN-γ-PE (BD Pharmingen) at 4°C for 30 min, washed with Perm/Wash, resuspended in PBS, and analyzed on a BD LSRII Fortessa. In experiments evaluating the TRBV usage of DEL-reactive T cells, TRBV staining was performed prior to the fixation and permeabilization steps.

Protein expression, purification, and crystallization

HLA-B^*18:01^1,2,26 and β2-microglobulin were transformed in BL21-RIL Escherichia coli competent cells and isolated from inclusion bodies similarly as previously described (20). Thirty milligrams HLA-B^*18:01^ H chain, 20 mg β2-microglobulin, and 10 mg of each peptide (DEL or SEL) were dissolved in refolding buffer containing 100 mM Tris-HCl (pH 8.0), 400 mM l-arginine–HCl, 2 mM EDTA, 0.2 mM PMFS, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione. Two further 30-ml aliquots of the H chain were added to the refolding buffer, leaving at least 3 h between each injection. After 48 h, the protein was dialyzed three times against 10 l 10 mM Tris-HCl (pH 8.0) during a period of 24 h. The refolded protein was then purified by elution over anion exchange chromatography.

Crystals of the DEL–HLA-B^*18:01^ complex were grown by the hanging-drop, vapor-diffusion method at 20°C with a protein/reservoir drop ratio of 1:1, at a concentration of 10 mg/ml in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) using 14–20% polyethylene glycol (PEG) 4K, 0.2 M ammonium acetate, and 0.1 M sodium citrate (pH 5.6).
Data collection and structure determination

The DEL–HLA-B*18:01 crystals were soaked in a cryoprotectant solution containing mother liquor solution with a final PEG concentration of 30% (w/v) PEG 4K and then flash frozen in liquid nitrogen. The data were collected on the MX1 beamline at the Australian Synchrotron (Clayton, VIC, Australia) using the ADSC Quantum 210 CCD detector (at 100K). Data were processed using MOSFLM (21) and scaled using SCALA on CCP4 suite (22). The structure was determined by molecular replacement using the phaser molecular replacement program in PHENIX (23) with the SEL–HLA-B*18:01 (Protein Data Bank accession no. 4QJV; http://www.rcsb.org/pdb/home/home.do) (14) as a search model minus the peptide. The model was then refined using PHENIX (23) and manually built using COOT (24). The final model has been validated using the Protein Data Bank validation Web site and the final refinement statistics are summarized in Table I. Coordinates have been submitted to the Protein Data Bank database (code 4XXC). All molecular graphics representations were created using PyMol (25).

Thermal stability assay

To assess the difference in stability between HLA-B*18:01 bound to the DEL self-peptide and SEL EBV peptide, a thermal shift assay was performed. The fluorescent dye SYPRO orange was used to monitor the protein unfolding. The thermal stability assay was performed in a real-time detection system (Corbett Rotor-Gene 3000), originally designed for PCR. Data were acquired for the two peptide–HLA complexes at two concentrations (5 and 10 mM) in TBS buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl) in duplicate and temperature was increased at a rate of 1°C/min over a range of 29–90°C. The fluorescence intensity was measured with excitation at 530 nm and emission at 555 nm. The thermal melt point represents the temperature at which 50% of the protein is unfolded. The raw data were processed using GraphPad Prism software.

Results

Self-peptide homology to an EBV epitope presented by HLA-B*18:01

The BZLFl1 Ag of EBV includes a highly immunogenic octamer epitope 173SELEIKRY180 that is recognized by CD8+ T cells from HLA-B*18:01+ individuals (14). Database searches identified a human protein sequence (DELEIKAY) with sequence homology to this EBV epitope. This sequence is from CPSF3L, which is a component of the multiprotein integrator complex associated with the C-terminal domain of RNA polymerase II (26), as well as a member of a group of the zinc-dependent hydrolases called the metallo-β-lactamase family (27). To establish whether a peptide corresponding to this sequence can bind to HLA-B*18:01, the stability of this HLA molecule bound to the EBV or self-peptide was compared using a thermal stability assay. The two amino acid difference between the two peptides had little effect on the overall stability of the peptide–HLA-B*18:01 complexes, with the two peptides showing a similar thermal melting point of ~70°C (Fig. 1).

To determine whether this self-peptide is presented by cells bearing the HLA-B*18:01 allele, we screened mass spectrometry spectra from peptides eluted from C1R cells engineered to express HLA-B*18:01 (14). ProteinPilot-based searches detected the octamer DELEIKAY, although the confidence level assigned (~74%) was below the stringent false discovery rate cut-off that would normally be applied to refine the data from such searches. We also identified the C-terminally extended nonameter peptide DELEIKAYY at a confidence level of 99%. Reasoning that both peptides may be presented rather than just the nonamer, with the differential abundance potentially accounting for higher confidence scoring of the nonamer, we manually interrogated the spectra for the presence of precursor MSI ions from both peptides (Fig. 2). Doubly charged precursor ions were detected for both the octamer (theoretical m/z of 490.7514; Fig. 2A) and nonamer (theoretical m/z of 572.2821; Fig. 2C) sequences. MS2 fragmentation spectra (Fig. 2B, 2D) triggered from both of these precursors confirmed their correct sequence assignment and thus their presence on HLA-B*18:01+ cells. Note that the signal intensity for the MS1 and MS2 spectra for the octamer was ~10-fold lower than that observed for the nonamer, and the relatively low level of the former was likely to account for the lower confidence scoring by the ProteinPilot algorithm. These data also suggested that the octamer is presented at lower abundance than the nonamer, although without accounting for relative differences in ionization efficiency between the two peptides, it is not possible to state this conclusively without further investigation. We failed to detect either peptide from cells not expressing HLA-B*18:01 (data not shown). These experiments therefore confirm the natural presentation of the DELEIKAY self-peptide homolog of the HLA-B*18:01-restricted EBV T cell epitope on the surface of human cells.

Cross-recognition by EBV-reactive CD8+ T cells of a self-peptide presented by HLA-B*18:01

To determine whether the DEL self-peptide is cross-recognized by EBV-reactive T cells, polyclonal T cell lines were generated from the PBMCs of healthy EBV-exposed, HLA-B*18:01+ individuals by stimulation with either SEL peptide or autologous EBV-infected cells, followed by screening for recognition of the SEL and DEL peptides. IFN-γ ELISPOT assays using various concentrations of each peptide were conducted. All donors showed strong IFN-γ responses to the SEL EBV peptide, whereas cross-recognition of the human peptide was variable (Fig. 3). Only donors 1 and 2 recognized the DEL self-peptide, although in both cases higher concentrations of DEL were required for equivalent levels of recognition to those observed with the EBV peptide. Nonetheless, the EBV-reactive T cell repertoire of donor 1 appeared to avoid autoreactivity by a surprisingly narrow margin, with only 10-fold more of the self-peptide required for equivalent activation compared with the EBV peptide.

To determine the proportion of CD8+ T cells within the SEL-reactive repertoire that cross-recognizes the DEL self-peptide, T cell lines were raised from healthy EBV-exposed, HLA-B*18:01+ individuals by stimulation with SEL peptide and screened by intracellular cytokine staining for recognition of SEL and DEL (Fig. 4). Results showed variability in the percentage of SEL-reactive T cells cross-recognizing DEL, ranging from 60% in donor 1, 8% in donor 2, and 20% in donor 5. This experiment was repeated using a T cell culture from donor 2 that was generated by using stimulation with the autologous lymphoblastoid cell line and, although the frequency of SEL (17.0%)- and DEL (1.6%)-reactive T cells was lower, the percentage of SEL-reactive T cells cross-recognizing DEL (9%) was...
very similar to that measured in the T cell line from donor 2 generated by using SEL peptide stimulation (data not shown). These data therefore demonstrate that T cells with significant avidity for a naturally presented self-peptide are expanded and activated by EBV infection of some HLA-B*18:01+ individuals.

TCR repertoire of cells cross-recognizing the self-peptide and EBV epitope

To determine whether SEL/DEL cross-reactivity is mediated by a monoclonal, oligoclonal, or polyclonal population of T cells, the next set of experiments analyzed TRBV gene usage of the cross-reactive T cells from donors 1, 2, and 5. Before examining the cross-reactive subset, we first analyzed the total SEL-reactive population using flow cytometry with TRBV mAb in combination with an SEL–HLA-B*18:01 tetramer (Fig. 5A). Although the constraints imposed by tolerance to a self-peptide homolog could be expected to restrict the diversity of a T cell response, relatively diverse TRBV gene usage was observed. Bias toward particular TRBV genes was noted, however, with strong selection of TRBV10 in all three donors, TRBV4 in donors 2 and 5, TRBV6.5 in donor 2, and TRBV25 in donor 5. We next analyzed the DEL–cross-reactive subset; however, it was not possible to use a DEL–HLA-B*18:01 tetramer because preliminary experiments revealed that this tetramer failed to label T cells from all donors (data not shown), presumably due to the low avidity of some of these T cells for this self-peptide–HLA complex, as suggested by the peptide dose-response data shown in Fig. 3. Instead, the DEL-reactive T cells were assessed for intracellular IFN-γ following stimulation with this peptide. As shown in Fig. 5B, this DEL–cross-reactive subset was also surprisingly polyclonal, but with dominant usage of TRBV10 in donors 1 and 2, codominance of TRBV25 in donor 2, and strong usage of both TRBV11 and TRBV20 in donor 5, indicating that a diverse array of TCRs have the capacity to cross-recognize these two peptides.

The self-peptide structure is a mimic of the EBV epitope

To have a better understanding of the cross-reactivity observed for the SEL-specific T cells, we next crystallized the DEL self-peptide in complex with the HLA-B*18:01 molecule to compare it with the previously solved HLA-B*18:01–SEL complex structure (14). The structure of the HLA-B*18:01–DEL complex was solved at a resolution of 1.43 Å (Table I), revealing a clear electron density for the peptide (Fig. 6) in the cleft of HLA-B*18:01. We super-
posed the structures of the self and EBV peptides in complex with the HLA-B*18:01 (Fig. 7). The HLA structures were very similar, with a root mean square deviation on the Cα atoms of the α1–α2 domains of 0.14 Å between the two peptide–HLA complexes. The peptides also adopted a very similar conformation, with a root mean square deviation on the peptide Cα atoms of 0.17 Å. The major differences between the two structures occurred at P1 and P7 where the substitutions between the two peptides are located (Fig. 7B). Overall, the structure of HLA-B*18:01 in complex with the DEL self-peptide shows that the substitutions with the EBV epitope did not affect the structure of the peptide–HLA complex, and that the viral epitope was a structural mimic of the self-peptide.

We previously showed that the substitution of Ser with Ala at P1 of the EBV peptide did not affect recognition by a polyclonal T cell line, but that Ala substitution at P7 decreased T cell recognition of the EBV epitope (14). Because the self-peptide contains a P7-Ala, it is likely that this substitution is the basis for the lower TCR avidity implied by the dose-response curves. The P7-Arg is presumably the focus of the EBV-specific T cell response in healthy virus carriers to avoid self-reactivity.

Discussion

Although T cells have the capacity to discriminate between an enormous number of peptide–MHC complexes, there is a limit to their specificity due to the small size of the target epitope. A small, strongly immunogenic CDS8+ T cell epitope from EBV was found to share six of its eight residues with a sequence from the human CPSF3L protein, including the primary anchor residues, raising questions on the potential of this antiviral response to be “dangerous” or autoreactive under certain circumstances. Our investigations first showed that this self-peptide does bind well to HLA-B*18:01, and it is presented on human cells at levels detectable by liquid chromatography–tandem mass spectrometry. Furthermore, some T cells raised from healthy donors against this EBV peptide could recognize the human peptide. Although self-tolerance had ensured there was a window of differential sensitivity between T cell recognition of the viral versus self-peptide, in some cases only 10-fold more of the self-peptide was required for equivalent T cell activation to that observed using the EBV peptide—a surprisingly small buffer zone of functional avidity to prevent autoimmunity. The TCR repertoire for the EBV peptide was not highly restricted.
by the self-peptide homology, and many different TRBV genes were also expressed by the subset of T cells that cross-reacted with the self-peptide. This was unexpected, given that structural analysis showed that the viral peptide–HLA and self-peptide–HLA complexes were virtually indistinguishable, with the exception of the two mismatched peptide side-chains. This is the first report, to our knowledge, confirming the natural presentation on human cells of a self-peptide cross-recognized by EBV-reactive CD8+ T cells, for which the presenting HLA allele for the EBV and self-peptide is the same. Earlier work on alloantigen cross-reactivity by EBV-reactive CD8+ T cells demonstrated that molecular mimicry also plays a role in alloreactivity. In this case, EBV-reactive T cells were shown to cross-recognition an HLA-B*08:01–bound viral epitope and an HLA-B*44:02/5–bound peptide from a human ATP binding cassette protein (28). Previous studies have also described T cell cross-reactivity between EBV and peptides from the MS autoantigen, myelin basic protein (MBP). In one case, CD4+ T cells raised against EBV nuclear Ag 1 cross-reacted with MBP and other myelin proteins (29), and in another example, CD4+ T cells were shown to cross-react with an epitope from the DNA-polymerase protein of EBV (28). An increased seroprevalence of EBV has been noted in North Americans of different ethnicities with systemic lupus erythematosus (38). An increased seroprevalence of EBV has been noted in North Americans of different ethnicities with systemic lupus erythematosus (39, 40). Systemic lupus erythematosus patients also have increased EBV genome load in blood compared with healthy individuals (41), with viral loads peaking after initiation of disease flares (42). Importantly, in the context of the present study, systemic lupus erythematosus patients also have aberrant expression of the BZLF1 Ag in the blood (43). It is also relevant to highlight that EBV-reactive CD8+ T cells are enriched in or near the diseased organs of patients with rheumatoid arthritis (44, 45) and MS (46), including a high proportion of T cells specific for BZLF1 (47). This could reflect a local immune response against BZLF1 in the diseased organs, or nonspecific homing of virus-specific T cells to inflamed sites (48).

It is possible that autoreactivity by EBV-reactive T cells is partly responsible for the immunopathology associated with acute IM following primary EBV infection. BZLF1-specific T cells are known to be expanded to huge numbers during acute IM, with one report of T cells specific for a single BZLF1 epitope accounting for 44% of the CD8+ T cells in IM blood (49). Indeed, an earlier study by Misko et al. (50) demonstrated another example of T cell cross-reactivity between a BZLF1 octamer peptide and a self-peptide, both presented by HLA-B8, although in this case, the self-peptide was not shown to be naturally presented. Mouse studies certainly support the notion that autoimmunity following infection with pathogens is mediated by effector T cells with low avidity for self and which are not normally primed by endogenous amounts of self-antigen (4, 51).

As well as highlighting the potential danger of self-reactivity by T cells expanded in response to common viral infection, the present study also underscores the exquisite specificity of the immune system by showing that a strong T cell response, utilizing a variety of TCRs, can be mounted against a foreign peptide–HLA complex that is essentially identical to a self-peptide–HLA complex with the exception of one TCR-accessible peptide side-chain. Although...
immune tolerance ensures that the healthy subjects examined in this study are ignorant toward their own cells, increasing self-peptide levels above those naturally presented (by addition of exogenous self-peptide) leads to readily detectable T cell activation. The functional avidity of T cells from one healthy subject was only 10-fold higher for the viral epitope compared with the self-peptide. Such minor differences in T cell activation threshold are presumably sufficient to avoid autoreactivity in most individuals, as supported by previous studies showing that small avidity differences can have a major functional impact on T cell recognition (52, 53). It is difficult to establish the mechanisms behind the EBV/autoimmunity association because EBV does not infect animals commonly used in models of autoimmune diseases. However, it is notable that recent studies with a humanized mouse model of EBV infection reported the development of erosive arthritis resembling rheumatoid arthritis in most such mice (54). The present study has provided support for a molecular mimicry mechanism by demonstrating that EBV-reactive CD8+ T cell expansions can be identified in healthy people that can cross-react with a naturally presented self-peptide. The potential for self-reactivity by these T cells is presumably kept under rigorous control by normal self-tolerance mechanisms. However, these EBV/self cross-reactive T cells could pose a autoimmune threat if costimulatory adhesion molecules or HLA-B*18:01-DEL levels increased or the T cell activation threshold was reduced as a result of cytokine release during inflammation or acute infections (4). The cross-reactive T cell populations identified in the present study should be considered for their potential role in autoimmune diseases associated with HLA-B*18:01 such as type 1 diabetes (55).

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Disclosures
The authors have no financial conflicts of interest.

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