T cell Epitope Clustering in the Highly Immunogenic

BZLF1 Antigen of Epstein-Barr virus

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Abstract

Polymorphism in the human leukocyte antigen (HLA) loci ensures that the CD8\(^+\) T cell response to viruses is directed against a diverse range of antigenic epitopes, thereby minimizing the impact of virus escape mutation across the population. The BZLF1 antigen of Epstein-Barr virus is an immunodominant target for CD8\(^+\) T cells but the response has only been characterized in the context of a limited number of HLA molecules due to incomplete epitope mapping. We have now greatly expanded the number of defined CD8\(^+\) T cell epitopes from BZLF1, allowing the response to be evaluated in a much larger proportion of the population. Some regions of the antigen fail to be recognized by CD8\(^+\) T cells while others include clusters of overlapping epitopes presented by different HLA molecules. These highly immunogenic regions of BZLF1 include polymorphic sequences, such that up to four overlapping epitopes are impacted by a single amino acid variation common in different regions of the world. This focusing of the immune response to limited regions of the viral protein could be due to sequence similarity to human proteins creating “immune blind spots” through self-tolerance. This study significantly enhances the understanding of the immune response to BZLF1, and the precisely mapped T cell epitopes may be directly exploited in vaccine development and adoptive immunotherapy.
Importance

Epstein-Barr virus (EBV) is an important human pathogen, associated with several malignancies including nasopharyngeal carcinoma and Hodgkin lymphoma. T lymphocytes are critical for virus control, and clinical trials aimed at manipulating this arm of the immune system have demonstrated efficacy in treating these EBV-associated diseases. These trials have utilized information on the precise location of viral epitopes for T cell recognition, for either measuring or enhancing responses. In this study, we have characterized the T cell response to the highly immunogenic BZLF1 antigen of EBV by greatly expanding the number of defined T cell epitopes. An unusual clustering of epitopes was identified, highlighting a small region of BZLF1 that is targeted by the immune response of a high proportion of the world’s population. This focussing of the immune response could be utilized in developing vaccines/therapies with wide coverage, or it could potentially be exploited by the virus to escape the immune response.
Introduction

Epstein-Barr virus (EBV), a lymphotropic gamma-1 herpesvirus, is widespread in all human populations, infecting around 95% of the adult population worldwide. Transmission of this lifelong persistent virus is via the oral route. After close contact, viral particles present in the saliva of infected individuals enter into the oral cavity of the naïve individual, and the virus is amplified by replicative (lytic) infection in permissive cells in the oropharynx. This lytic infection results in vast amounts of virus shedding into the throat. Simultaneously, infection of mucosal B cells occurs and the virus initiates latent, persistent infection of the B cell pool (1-3). In developing countries, EBV is associated with an asymptomatic infection, usually occurring in infancy or early childhood. In developed countries, however, acute infection is often delayed until adolescence or early adulthood and, in around 25% of cases, can result in the self-limiting lymphoproliferative disorder, infectious mononucleosis (IM). IM was etiologically linked to EBV in 1968 by Henle et al. Symptoms associated with IM range from fever and sore throat to lymphadenopathy and splenomegaly (3-5).

Around 80 EBV proteins are expressed during lytic viral replication, which involves the sequential expression of immediate early, early and late proteins. Lytic cycle activation is initiated by the expression of two immediate early proteins BZLF1 (Zta) and BRLF1 (Rta). Cox et al. noted that Rta and Zta appeared to regulate gene expression at the level of transcription and that these transactivators work in concert to facilitate reactivation (6). Our protein of interest, BZLF1 or ZEBRA (Z EBV replication activator) is a member of the basic leucine zipper family which binds to the AP1-like Z response elements in EBV early promoters (7) and exhibits sequence similarity to c-Fos (8). In addition to the transactivation domain (aa 1 - 166), BZLF1 contains a basic DNA recognition domain (aa 178 - 194 ) and a coiled-coil dimerization domain (aa 198 - 225) (9), (10).
The adaptive immune response is vital in controlling EBV infected B-cell proliferation and is of immense importance during persistent infection (1). CD8+ T cells recognise peptides derived from viral proteins associated with MHC molecules. Results from Pudney et al. suggest a focusing of CD8+ T cell responses toward epitopes from immediate early and early proteins, and a hierarchy of immunodominance amongst the EBV lytic cycle antigens (11). The lytic protein BZLF1 includes a number of defined CD8+ T cell epitopes presented by HLA-B and C alleles. The most widely studied is the HLA-B8 restricted RAKFQQLL epitope. Tan et al. demonstrated that up to 5.5% of CD8+ T cells in the peripheral circulation of healthy virus carriers are specific for this epitope (12). Other CD8+ BZLF1 epitopes include the highly immunogenic 13-mer LPEPLPQQLTAY, presented by HLA-B*3508, which overlaps with the HLA-B*3501 restricted EPLPQQLTAY epitope (13). Recently, we defined two novel overlapping epitopes from BZLF1: an HLA-B*1801 restricted octamer, SELEIKRY, which is encompassed by the HLA-B*4403-restricted EECDSELEIKRY dodecamer (14).

In the present study, we further investigated the CD8+ T cell response to the BZLF1 protein, identifying 11 novel epitopes, many presented by common HLA alleles. Interestingly, these epitopes appear to be clustered within certain domains of the protein, with many overlapping sequences.
Materials and Methods

Generation of T cell lines
PBMCs were isolated by Ficoll-Hypaque centrifugation into RPMI 1640 medium supplemented with 10% FCS (R10). Blood donors were healthy, EBV-seropositive individuals who had given written informed consent. Approval for this research was obtained from the QIMR Berghofer Medical Research Institute Human Ethics Committee (Brisbane, Australia). EBV-specific T cell cultures were raised by culturing PBMCs (2x10^6/2ml well) with irradiated autologous lymphoblastoid cell lines (LCLs) transformed with the B95-8 strain of EBV (2x10^5/2ml well). Cultures were supplemented with rIL-2 (120 IU/ml) from day 3 and analyzed on day 18.

Peptides
Forty-six overlapping peptides (20-mers overlapping by 15 amino acids) covering the entire length of BZLF1 were designed based on the sequence of the B95.8 strain of EBV. Synthetic peptides were purchased from either New England Peptides (Gardner, MA) or GL Biochem (Shanghai, China) and dissolved in DMSO.

ELISpot assays
IFN-γ ELISpot assays were performed using cytokine capture and detection reagents according to the manufacturer’s instructions (ELISpotPRO for human IFN-γ, Mabtech, Stockholm, Sweden). Briefly, 96-well nitrocellulose plates pre-coated with anti-IFN-γ mAb were seeded with approximately 5 x 10^4 EBV-specific T cells and peptide at various concentrations. After incubation for 16 hours at 37°C in 5% CO2, the cells were discarded and captured IFN-γ was detected with a biotinylated anti-IFNγ Ab, followed by development with an alkaline phosphatase substrate solution (BCIP/NBT-plus). All samples were tested in duplicate and spots were counted using an automated plate counter.
**Tetramer labelling**

PBMCs or T cell lines were labeled with an allophycocyanin (APC)-conjugated HLA-Cw*06-CRAKFKQLL tetramer (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) by incubation for 30 minutes at 4°C. Cells were then washed and incubated with Cy5.5-PerCP conjugated anti-human CD8 mAb (BioLegend, San Diego, CA), Cy7-PE conjugated anti-human CD3 mAb (eBioscience, San Diego, CA) and Alexa Fluor 700-conjugated anti-human CD4 (BD PharMingen, San Diego, CA) for 30 minutes at 4°C. Cells were washed and analysed on a BD LSR Fortessa flow cytometer using FACSDiva software (BD Biosciences).

**Intracellular cytokine staining**

EBV-specific T cell lines raised by in vitro stimulation with an irradiated autologous LCL were incubated in R10 containing various stimuli for 4 hours at 37°C, in the presence of 5 µg/ml Brefeldin A (BioLegend). T cell lines were stimulated with either peptide-pulsed (incubation with 1 µg/ml peptide for 1 hour) HLA-deficient T2 cell lines stably transfected to express HLA-A3, A24 or B7, or serially diluted peptide (0.0001–10 µg/ml). The cells were then washed and incubated at 4°C for 30 minutes with LIVE/DEAD fixable aqua dead cell stain (Life Technologies, Grand Island, NY) and fluorescently-labelled mAb specific for cell surface markers (CD4-FITC, BD PharMingen; CD8-Cy5.5-PerCP, BioLegend). Cells were washed, then fixed and permeabilized with Cytofix/Cytoperm fixation/permeabilization solution (BD PharMingen) at 4°C for 20 minutes. Next, the cells were washed with Perm/Wash buffer (BD PharMingen), incubated with IFN-γ-APC mAb (BD PharMingen) at 4°C for 30 minutes, and then washed again with Perm/Wash. Cells were resuspended in PBS for analysis on a BD FACSCanto II flow cytometer (BD Biosciences).
Results

**BZLF1 is a rich source of CD8+ T cell epitopes**

IFN-γ ELISpot assays were used to screen overlapping 20-aa peptides corresponding to the BZLF1 protein sequence for recognition by T cell lines. These T cells were raised by *in vitro* stimulation of PBMCs from 36 healthy EBV-seropositive individuals with their autologous LCLs (Table 1). Results for all individuals screened with this overlapping peptide set are shown in Fig. 1A-C. Confirming the importance of this small antigen as a target for T cell recognition, only 8% (3/36) of screened individuals showed no response to any of the BZLF1 overlapping peptides. It is notable that these three individuals also showed no responses to overlapping peptides from several other EBV antigens that were screened alongside the BZLF1 peptides (data not shown), so this may reflect a general weakness in the EBV-specific T cell response in these three donors. It is also clear that the T cell response was biased towards restricted regions of this antigen, with a strong focus on residues 160-220.

We next aimed to map the epitopes more precisely within the BZLF1 20mer sequences. Several individuals responded to the overlapping peptides 56LPQGQLTAYHVSTAPTGSWF75 and 61LTAYHVSTAPTGSWSFAPQP80, and so smaller peptides from the overlapping region were tested for recognition by T cell lines from three of these donors. Fig. 2A–C shows results from donors B4 (HLA-A26, A32, B40, B4402, Cw1, Cw3), B5 (HLA-A11, A24, B1501, B35, Cw3, Cw4) and JC33 (HLA-A3, A25, B18, B44). Since the pattern of recognition was quite distinct between B4/B5 and JC33, it appeared that more than one epitope was localized in this region. Subsequent peptide dose-response IFN-γ ELISpot assays (Fig. 2, D and E) confirmed the presence of two overlapping epitopes. The data indicate that the minimal sequence recognized by donor B4 was the 67STAPTGSWF75 nonamer peptide. This novel epitope conforms to the binding motif of HLA-Cw3, an HLA
allele shared by donors B4 and B5 (Fig. 2D) (15). However, we cannot rule out the possibility that the $^{66}$VSTAPTGSWF$^{75}$ sequence is also an epitope for this donor because the dose-response curves for these two sequences are very similar. In contrast, T cells from donor JC33 most efficiently recognized the overlapping $^{65}$HVSTAPTGSW$^{74}$ decamer, which conforms to the HLA-A25 binding motif (Fig. 2E) (16). Interestingly, Abbott et al. recently published data mapping an epitope to the $^{66}$VSTAPTGSWF$^{75}$ sequence, with an ascribed HLA restriction of HLA-B*58:01 (17); however, it is notable that the responding donor in this study was also HLA-Cw3$^+$. Subtle differences in the recognition of the overlapping 20-mers corresponding to the extreme N-terminus of BZLF1 were also noted between donors B2 and B25. While donor B2 showed strong responses towards peptides 11–30 and 16–35, donor B25 responded to peptides 6–25 and 11–30 (Fig. 1A). To investigate the basis for these differences, truncated versions of these peptides were assessed for T cell recognition, and distinct epitopes were mapped for each donor. As shown in Fig. 3A, $^{14}$TPDPYQVPFV$^{23}$ is a strongly recognized target epitope for T cells from donor B25, with a likely HLA restriction of HLA-B51 based on the published peptide binding motif for this HLA molecule (18). In contrast, the overlapping $^{18}$YQVPFVQAF$^{26}$ nonamer is the more potent target epitope for donor B2, with HLA-Cw4 the likely restricting molecule due to its preference for binding peptides with the primary anchor residue Phe at the C-terminus (Fig. 3B) (15). With a total of six epitopes mapped to the first 75 amino acids of the BZLF1 protein (Table 2), this N-terminal region is clearly an important target for the T cell response.

T cell recognition for some donors was also focussed on a central region of the antigen, between residues 106 and 130 (Fig. 1). T cells from donors MR67 and DP55 were found to recognize the nonamer $^{122}$VQTAAVVF$^{130}$ (Fig. 4, A and B) which conforms to the binding motif of HLA-B62 (24), an allele shared by both donors. This epitope encompasses
another T cell determinant, recognized by donor B26; an octamer (\textsuperscript{123}QTAAAVVF\textsuperscript{130}) which conforms to the HLA-B58 binding motif (25) (Fig. 4C). Abbott and colleagues noted that the decamer \textsuperscript{121}TVQTAANVVF\textsuperscript{130} stimulated T cell responses in an HLA-B62\textsuperscript{*} individual, supporting our conclusion that this region of BZLF1 includes an HLA-B62-restricted epitope (17).

“Hotspot” of T cell epitopes between residues 161-225 of BZLF1

In 1995, Bogedain et al. first described the highly immunogenic HLA-B8-restricted \textsuperscript{190}RAKFKQLL\textsuperscript{197} epitope and, in the same study, an HLA-Cw6-restricted response was localized within the sequence \textsuperscript{187}RKCRAKFKQQHLYR\textsuperscript{201} (22). Our results confirm the presence of an HLA-Cw6-restricted response in this region of BZLF1, and demonstrate the minimal sequence to be \textsuperscript{189}CRAKFKQLL\textsuperscript{197} which encompasses the HLA-B8-binding octamer epitope (Fig. 5A & 5C). T cell lines from three HLA-Cw6\textsuperscript{+} and HLA-B8\textsuperscript{−} donors demonstrated IFN-\(\gamma\) responses to almost all overlapping peptides that included the nonamer \textsuperscript{189}CRAKFKQLL\textsuperscript{197} (Fig. 5A). In addition, flow cytometric analysis using APC-conjugated HLA-Cw*06-CRAKFKQLL tetramer confirmed that HLA-Cw6\textsuperscript{+} PBMCs and EBV-specific T cell lines included significant populations of cells that recognized the novel nonamer peptide (Fig. 5C).

An additional epitope from BZLF1 that overlaps with \textsuperscript{190}RAKFKQLL\textsuperscript{197} and \textsuperscript{189}CRAKFKQLL\textsuperscript{197} was discovered from a donor who was negative for both HLA-B8 and Cw6. The results from donor B20 show that all overlapping peptides that included the nonamer \textsuperscript{188}KCRAKFKQL\textsuperscript{196} stimulated T cell IFN-\(\gamma\) production (Fig. 5B). Since this sequence did not conform to the peptide binding motif of any of the HLA alleles expressed by this donor, we utilized the HLA-deficient T2 cell line that was transfected to express either HLA-A3, A24 or B7, as peptide-presenting cells in order to determine the HLA restriction of
this response. Intracellular cytokine staining showed that a T cell line from donor B20, which was raised by in vitro stimulation with an autologous LCL, recognized the peptide when presented by T2-HLA-B7, but not other antigen-presenting cells (Fig. 5D).

Since these three epitopes differed by just one or two amino acids, experiments were conducted to assess if T cells have the capacity to cross-react between them, or if the responses are distinct and peptide-specific. As shown in Fig. 6A-C, EBV-specific T cell populations from individuals expressing either HLA-B8, Cw6 or B7 were specific for 189\text{RAKFKQLL} \text{\cite{197}}, 189\text{CRAKFKQLL} \text{\cite{197}} or 188\text{KCRAKFKQLL} \text{\cite{196}}, respectively, although the HLA-B8\textsuperscript+ T cells also recognized 189\text{CRAKFKQLL} \text{\cite{197}} at relatively high concentrations (Fig. 6A). These data indicate that distinct T cell receptor repertoires are utilized against each peptide-HLA complex.

The Gln residue at position 195 of BZLF1 is mutated to a charged His residue in Type-2 EBV strains and the dominant Type-1 EBV strains in China. To investigate the impact of this sequence polymorphism on the HLA-Cw6-restricted response, the T cells from donor MR67 were tested for recognition of various concentrations of the 189\text{CRAKFKQLL} \text{\cite{197}} epitope versus the 189\text{CRAKFKQHLL} \text{\cite{197}} variant. As shown in Fig. 6D, this single amino acid polymorphism had a significant impact on T cell recognition, with approximately 10 fold higher concentrations of the variant peptide required for equivalent T cell recognition.

The intense immunogenicity of this small region of BZLF1 was further highlighted by the mapping of a fourth epitope overlapping with these three T cell determinants. The HLA-B8\textsuperscript+ donor B9 not only responded to the 190\text{RAKFKQLL} \text{\cite{197}} epitope (data not shown) but also a distinct overlapping determinant further towards the protein C-terminus. Fig. 7 displays data from donor B9, screened for T cell recognition of 8-, 9-, 10- and 11-aa peptides from this region.
region. The best recognized peptides in the initial screen (FKQLLQHYR, QLLQHYREV and KFKQLLQHY) were further examined by peptide titration ELISpot assays confirming the nonamer \textsuperscript{192}KFKQLLQHY\textsuperscript{200} as the minimal epitope, with a likely restriction of HLA-A30 based on the published peptide binding motif for this HLA molecule (26).

The final epitope to be mapped was from the only HLA-A31\textsuperscript{+} donor included in the study. Donor B10 responded to this region of BZLF1 (peptides 171–190 and 176–195; Fig. 1), and the target epitope was mapped to the nonamer \textsuperscript{179}RYKNRVASR\textsuperscript{187}. This epitope is likely to be presented by HLA-A31 based on the published motif which has an Arg primary anchor at the C-terminus, and Tyr and Val as secondary anchors at the second and sixth residue, respectively (Fig. 7C) (27). This epitope lies directly adjacent to the \textsuperscript{188}KCRAKFQL\textsuperscript{196} epitope, confirming the intense focusing of CD8\textsuperscript{+} T cell epitopes in this C-terminal region of BZLF1.
The BZLF1 antigen of EBV is an essential component of the viral life cycle as it plays an
indispensable part in the switch from latent to lytic gene expression. This present study
explored the T cell response to this protein by testing a large cohort of individuals against an
overlapping peptide library covering the entire antigen, and using T cell lines raised against
autologous LCLs to optimize the sensitivity of the ELISpot assays. Our data provides an
extensive list of novel epitopes within this antigen, with evidence of epitope clustering and
responses restricted through a large number of HLA alleles (Fig. 8).

BZLF1 is clearly an important target for the T cell response to EBV and should
therefore be considered in epitope-based vaccine strategies. Of the nine previously defined
BZLF1 CD8+ T cell epitopes (Table 2), restriction was limited to seven HLA alleles,
including both HLA-B and C alleles but not HLA-A alleles. In this study we have defined a
further 11 BZLF1 epitopes (Table 2), including epitopes restricted through HLA-A as well as
HLA-B and C alleles, thereby significantly increasing the population coverage. Indeed, eight
out of the 36 individuals tested in this study showed responses directed towards these novel
epitopes but not previously defined epitopes.

Woodberry and colleagues showed that BZLF1 responses are significant in acute
infectious mononucleosis as well as during persistent EBV infection (28). Considering that
the BZLF1 protein is only 245 amino acids in length, it is notable that over half of these
amino acids (128 or 52%) are included in epitopes. The transactivation domain (1–166)
includes 10 epitopes, six of which were defined in this study. Furthermore, in a region of just
37 aa between residues 169 and 205, up to eight epitopes are present. This region includes the
dNA recognition domain (178–194) and part of the coiled-coil dimerization domain. Located
within the coiled-coil dimerization domain is the previously mapped HLA-B60 restricted
nonamer epitope \textsuperscript{209}SENDRLRLL\textsuperscript{217} (23). Interestingly, we have also found an HLA-B60-
negative individual responding to this epitope (data not shown). This individual (donor B8; 
HLA-A2, A66, B4402, B49, Cw7) expressed HLA-B49 which is likely to be the restricting 
allele based on the binding motif of HLA-B49 which includes anchor residues Glu at position 
2 and Leu at the C-terminus. It is notable that McDonald and colleagues found the motif 
\textsuperscript{209}SENDRLR\textsuperscript{215}, located midway along the coiled-coil dimerization region, to play a key role 
in the BZLF1 structure and is required for EBV DNA replication (29). In addition, the 
leucine at residue 217 is suggested to be important for dimerization, as it is positioned 
adjacent to the interacting faces of the helices (10). This presumably explains why this 
sequence and the nonamer epitope are conserved in both Type-1 and -2 EBV strains.

Why are some regions of the BZLF1 sequence so rich in CD8\textsuperscript{+} T cell epitopes while 
other areas are not targeted at all? One possibility is that sequence similarity with human 
protein sequences results in immune tolerance to parts of the BZLF1 antigen. Several 
previous studies have shown the phenomenon of epitope clustering. Examples range from 
bacteria and tumor antigens to HIV. Kim and DeMars found that the major outer membrane 
protein of \textit{Chlamydia trachomatis} displays a co-clustering of class I and class II epitopes 
within a 20-mer region of the protein (30). Furthermore, Valmori and colleagues identified 
clusters of closely overlapping epitopes in the NY-ESO-1 protein (expressed in several types 
of tumors) (31). HIV studies have shown epitope clustering in the Nef and Gag proteins, 
corresponding to areas of protein hydrophobicity. Interestingly, CD8\textsuperscript{+} T cell epitopes are 
predicted to be quite evenly distributed throughout the BZLF1 sequence according to the 
Immune Epitope Analysis Resource for ten common HLA class I alleles (data not shown), 
which uses predictors of proteasomal processing, TAP transport, and MHC binding to 
produce an overall score for each peptide's intrinsic potential of being a T cell epitope.
HLA polymorphism has evolved to ensure that a wide range of antigenic epitopes are presented across the population in order to reduce the impact of amino acid mutation within T cell epitopes. The clustering of epitopes and the sharing of amino acids between multiple epitopes has the potentially dangerous consequence that viral escape mutants may impact on a large proportion of the population. An example of this is the Gln residue at position 195 of BZLF1 which is included within four epitopes restricted by either HLA-A30, -B7, -B8 or -Cw6. Importantly, the Gln residue is mutated to a charged His residue in Type-2 EBV strains and the dominant Type-1 EBV strains in China (32). Since HLA-Cw6 is expressed at a high frequency in parts of China, we examined the impact of this sequence polymorphism on recognition by HLA-Cw6-restricted T cells raised against the ^CRAKFKQLL^ epitope. T cell recognition was significantly reduced by the Q\textsubscript{195} to H\textsubscript{195} variation, raising the interesting possibility that this variant may have arisen in the Chinese population as an escape mutant.

Our work adds significantly to the understanding of the immune response to BZLF1, the most highly immunogenic T cell target antigen from EBV. Precisely mapped T cell epitopes have become a critical tool for analysing the immune response to pathogens through, for example, HLA-peptide multimers. Furthermore, they are also being used directly in vaccine development and adoptive immunotherapy, particularly for tumor-associated viruses such as EBV from which individual proteins are potentially oncogenic (33). We have greatly expanded the number of defined CD8\textsuperscript{+} T cell epitopes from BZLF1, and highlighted their clustered distribution along the length of the sequence. This information is likely to be widely utilized in future studies aimed at manipulating the immune system to treat or prevent EBV-associated diseases.
Acknowledgements

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References


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TABLE 2  Summary of BZLF1 CD8+ T cell epitopes

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<th>Epitope Coordinates</th>
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<th>Likely HLA Restriction</th>
<th>Reference</th>
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<td>YQVPFVQAF</td>
<td>Cw3</td>
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<td>B35.08</td>
<td>(13)</td>
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<td>SENDRLRLL</td>
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**Figure Legends**

**FIGURE 1.** Initial screening of T cells from EBV seropositive individuals against overlapping peptides spanning the BZLF1 antigen. EBV-specific T cell lines from 36 individuals, raised by in vitro stimulation with irradiated autologous LCLs, were tested by IFN-γ ELISpot assays for recognition of overlapping peptides (10 μg/ml) corresponding to the lytic EBV BZLF1 antigen.

**FIGURE 2.** Overlapping length-variant epitopes mapped from the BZLF1 antigen of EBV. (A–C) EBV-specific T cell lines from three individuals, raised by in vitro stimulation with irradiated autologous LCLs, were tested in IFN-γ ELISpot assays for recognition of overlapping peptides (1 μg/ml) corresponding to a region of the BZLF1 protein sequence. EBV-specific T cell lines from (D) donor B4 and (E) donor JC33, tested with IFN-γ ELISpot assays to precisely map the minimal epitopes. Cells were tested with various concentrations of the indicated peptides. The experiments described in Fig. 2C, 2D & 2E were each performed with one other donor expressing the relevant HLA allele, with similar results.

**FIGURE 3.** Identification of overlapping minimal epitopes from the BZLF1 antigen of EBV. EBV-specific T cell lines were screened with various concentrations of the indicated peptides by IFN-γ ELISpot assays. Results shown are from (A) donor B25 and (B) donor B2. These experiments were each performed with one other donor expressing the relevant HLA allele, with similar results.

**FIGURE 4.** Mapping of minimal epitopes from the central region of the BZLF1 protein. IFN-γ ELISpot assays were used to define minimal epitopes, by screening EBV-specific T cell lines from HLA-B62+ individuals, (A) donor MR67, and (B) donor DP55, against truncated peptides at 1 μg/ml, and (C) a T cell line from the HLA-B58+ individual, donor B26, against various concentrations of the indicated peptides. The experiment described in...
Fig. 4C was performed with one other donor expressing the relevant HLA allele, with similar results.

**FIGURE 5.** Overlapping epitopes in the coiled-coil dimerization domain of the BZLF1 EBV antigen. EBV-specific T cells from (A) three HLA-Cw6+ individuals and (B) an HLA-B7+ individual were tested in IFN-γ ELISpot assays against overlapping peptides (1 μg/ml) corresponding to a region of the BZLF1 protein sequence. (C) PBMCs and EBV-specific T cell lines from HLA-Cw6+ individuals were analyzed by flow cytometry with the HLA-Cw6-CRAFKQQLL tetramer. (D) HLA-deficient T2 cell lines transfected to express either HLA-A3, A24 or B7 were presensitized with 188KCRAFKQQLL196 peptide (0.1 μg/ml) or left uncoated and then washed and exposed to EBV-specific T cells from donor B20. After incubation, these T cells were analyzed for IFN-γ production by intracellular cytokine staining.

**FIGURE 6.** T cell recognition of the clustered overlapping epitopes between residues 188 and 197. EBV-specific T cell populations from individuals expressing either (A) HLA-B8, (B) Cw6 or (C) B7 were tested for recognition of the peptides 190RAKFKQLL197, 189CRAFKQQLL197 or 188KCRAFKQQL196 at various concentrations by intracellular cytokine staining. (D) T cells from the HLA-Cw6+ donor MR67 were also tested in an ELISpot assay for recognition of the variant peptide 189CRAFKKHL197 in comparison to the 188CRAFKQQLL197 epitope.

**FIGURE 7.** Identification of two HLA-A restricted epitopes in the BZLF1 EBV antigen. (A) EBV-specific T cells from the HLA-A30+ individual, donor B9 were tested by IFN-γ ELISpot assays against overlapping peptides (1 μg/ml) corresponding to a region of the BZLF1 antigen. (B) Subsequent IFN-γ ELISpot assays mapped the minimal epitope by screening the indicated peptides at various concentrations. (C) EBV-specific T cells from the
HLA-A31+ individual, donor B10 were tested by IFN-γ ELISpot assays with overlapping peptides (1 μg/ml) as shown. The experiment described in Fig. 7A was performed with one other donor expressing the relevant HLA allele, with similar results.

FIGURE 8. Clustered distribution of CD8+ T cell epitopes within BZLF1. The 245 amino acid sequence of BZLF1 is derived from the B95-8 strain of EBV. Regions that are recognized by CD8+ T cells are shaded in gray and represent 105 amino acids (42.9% of the protein). Epitopes are listed below the full sequence, and the likely HLA restriction of each epitope is shown on the right of the figure.
Figure 2

A) Donor B4: HLA-A26, A32, B40, B44, Cw1, Cw3

B) Donor B5: HLA-A11, A24, B62, B35, Cw3, Cw4

C) Donor JC33: HLA-A3, A25, B18, B44

Peptide:
- STAPTGSW
- STAPTGSWF
- VSTAPTGS
- VSTAPTGSW
- VSTAPTGSWF
- HVSTAPTG
- HVSTAPTGS
- HVSTAPTGSW
- HVSTAPTGSWF

Spots per million cells

D) Donor B4: HLA-A26, A32, B40, B44, Cw1, Cw3

E) Donor JC33: HLA-A3, A25, B18, B44

Peptide Concentration (μg/ml)
Figure 3

A. Donor B25: HLA-A1, A2, B35, B51, Cw4, Cw14

B. Donor B2: HLA-A1, A11, B40, B52, Cw3, Cw4
Figure 4

A

Donor MR67: HLA-A2, B57, B62, Cw3, Cw6

Peptide:

GEAPQPGDNSTVQTAAPAVF
EAPQPGDNSTVQTAAPAVF
APQPGDNSTVQTAAPAVF
QPQPGDNSTVQTAAPAVF
QPGDNSTVQTAAPAVF
PQDNSTVQTAAPAVF
GDNSTVQTAAPAVF
DGDNSTVQTAAPAVF
DNSTVQTAAPAVF
NSTVQTAAPAVF
STVQTAAPAVF
TVQTAAPAVF
VQTAAPAVF
QTAAAPAVF

Spots per million cells

0 250 500 0 200 400

B

Donor DP55: HLA-A2, B18, B62, Cw1, Cw5

C

Donor B26: HLA-A2, A11, B35, B58, Cw4, Cw7

Peptide:

GeAPQPGDNSTVQTAAPAVF
EAPQPGDNSTVQTAAPAVF
APQPGDNSTVQTAAPAVF
PQPGDNSTVQTAAPAVF
QPGDNSTVQTAAPAVF
PGDNSTVQTAAPAVF
GDNSTVQTAAPAVF
DNSTVQTAAPAVF
NSTVQTAAPAVF
STVQTAAPAVF
TVQTAAPAVF
VQTAAPAVF
QTAAAPAVF

Spots per million cells

0 250

Peptide Conc. (μg/ml)

0.000001 0.00001 0.0001 0.001 0.01 0.1 1 10

Peptide:

TVQTAAPAVF
VQTAAPAVF
QTAAAPAVF
Figure 5

A

Donor MR67:
HLA-A2, A23
B57, B62, B*4402, B53
Cw3, Cw5, Cw6

Donor B28:
HLA-A2, A23
B7, B57
Cw5, Cw6

Donor B38:
HLA-A2, A3
B7, B57
Cw0602, Cw0702

Peptide:
SRKCRAKFKQLLQHY
SRKCRAKFKQLLQH
SRKCRAKFKQLLQ
SRKCRAKFKQL
SRKCRAKFKQ
RKCRAKFKQLLQHY
KCRAKFKQLLQHY
CRAKFKQLLQHY
RAFKQLLQHY
AKFKQLLQHY
KFQQLLQHY
FKQQLLQHY
RVASRCRAKFKQLL
VASRCRAKFKQLL
ASRCRAKFKQLL

Spots per well
0 1000
0 1000
0 600

B

Donor B20:
HLA-A3, A24
B7, B57

Peptide:
SRKCRAKFKQLLQHY
SRKCRAKFKQLLQH
SRKCRAKFKQLLQ
SRKCRAKFKQL
SRKCRAKFKQ
RKCRAKFKQLLQHY
KCRAKFKQLLQHY
CRAKFKQLLQHY
RAFKQLLQHY
AKFKQLLQHY
KFQQLLQHY
FKQQLLQHY
RVASRCRAKFKQLL
VASRCRAKFKQLL
ASRCRAKFKQLL

Spots per well
0 1000
0 600

C

Autologous LCL-stimulated T cell lines

Donor MR67
0.38%
0.38%
7.22%
1.52%

Donor B3
0.38%
2.70%

Donor B13
5.81%

Donor B28
2.53%

PBMCs

HLA-Cw6-CRAKFKQL tetramer

Donor MR67

Donor B3

Donor B13

Donor B28

D

Donor B20:
HLA-A3, A24
B7, B57

Antigen presenting cells

Nil

T2

T2-HLA-A3

T2-HLA-A24

T2-HLA-B7

% IFN-γ+ of CD8+ cells

0 0.8 1.6 2.4 3.2
Donor B40: HLA-A1, A32, B8, B62, Cw5, Cw7

Donor MR67: HLA-A2, B57, B62, Cw3, Cw6

Donor B20: HLA-A3, A24, B7, Cw7

Donor MR67: HLA A2, B57, B62, Cw6

Figure 6
Figure 7

A  Donor B9: HLA-A1, A30, B8, B18, Cw5, Cw7

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B  Donor B9: HLA-A1, A30, B8, B18, Cw5, Cw7

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C  Donor B10: HLA-A11, A31, B7, B51, Cw7, Cw15

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Fig. 8

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**HLA**

MDPNSSTSED

TPDPYQVPFVQAF

TPDPYQVPFVQAF

**HLA**

TVTAAAVVFACPGANQQGQQLDIGQFAPFVAPFVTRKQPQPS1ECDSELEIKRYVRHVASRKRAFFQQLQYREVAAAKSSENDRELLLQKXPSLDVTSSIFRTFVDLHEDLLNF

TVTAAAVVF

Cw6

**HLA**

B60/B49

**HLA**

B*44:03

**HLA**

B*31

**HLA**

B*08

**HLA**

Cw6