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Broad Cross- Reactive TCR Repertoires Recognizing Dissimilar Epstein-Barr and Influenza A Virus Epitopes


Memory T cells cross-reactive with epitopes encoded by related or even unrelated viruses may alter the immune response and pathogenesis of infection by a process known as heterologous immunity. Because a challenge virus epitope may react with only a subset of the T cell repertoire in a cross-reactive epitope-specific memory pool, the vigorous cross-reactive response may be narrowly focused, or oligoclonal. We show in this article, by examining human T cell cross-reactivity between the HLA-A2–restricted influenza A virus-encoded M158-66 epitope (GILGFVFTL) and the dissimilar Epstein-Barr virus-encoded BMLF1280–288 epitope (GLCTLVAML), that, under some conditions, heterologous immunity can lead to a significant broadening, rather than a narrowing, of the TCR repertoire. We suggest that dissimilar cross-reactive epitopes might generate a broad, rather than a narrow, T cell repertoire if there is a lack of dominant high-affinity clones; this hypothesis is supported by computer simulation. The Journal of Immunology, 2010, 185: 6753–6764.

The T cell response to viral infections consists of expanded clones of T cells arising from Ag-inexperienced naive populations of small clone size and from Ag-experienced memory populations of much larger clone size (1–3). The relative influence of the memory cells on the T cell response depends on the host’s previous exposure to pathogens, which has consequently shaped its memory T cell repertoire. Memory T cells cross-reactive with related or even unrelated viruses may change the immune response to a newly encountered virus and alter viral clearance and pathogenesis, which is referred to as heterologous immunity (4, 5). Cross-reactive memory cells have the capacity to dominate immune responses initiating from naive cells because the memory cells are present at much higher frequencies and because they are easier to activate than naive T cells (1–3). If a cross-reactive challenge epitope is not completely homologous with the epitope that initially generated the memory cell population, it is likely that only a subset of that memory T cell repertoire will react with the new epitope sufficiently to induce proliferation. The proliferation of a subset of the memory cell population would likely result in a vigorous response with a narrow oligoclonal TCR repertoire (6). This phenomenon has been demonstrated in mouse models of infection with different strains of influenza virus bearing a point mutation in a cross-reactive epitope and with lymphocytic choriomeningitis virus (LCMV) and Pichinde virus (PV), which have cross-reactive epitopes bearing six of eight amino acids in common (6–8). Further, narrowly focused hepatitis C virus T cell responses cross-reactive with influenza A virus (IAV) have correlated with fulminant hepatitis in humans (9). Narrow TCR repertoires have been observed with several human infections, including CMV, hepatitis C virus, and HIV, and this property may allow for the generation of epitope escape variants (10–13). These narrow repertoires may or may not be the consequence of heterologous immunity, but they are generally considered undesirable.

Although it seems intuitively obvious that expanding a subset of a T cell memory pool should result in a narrow oligoclonal response, it may not always be the case. One needs to consider the relative numbers and affinities of the naive T cell precursors in comparison with the cross-reactive memory T cell clones and consider what proportion of the memory cells specific to the immunizing epitope would be able to cross-react with the challenge epitope. We questioned whether narrowing of the T cell repertoire is always a consequence of heterologous immunity and show, by examining human T cell cross-reactivity between IAV and Epstein-Barr virus (EBV), that it clearly is not.

We previously revealed the existence of cross-reactive T cells with specificity for two immunodominant HLA-A2–presented epitopes, IAV M158-66 and EBV BMLF1280–288, which have relatively little sequence similarity in comparison with most previously reported cross-reactive epitopes (14). This cross-reactive T cell population was functionally heterogeneous with regard to cytokine production, perhaps due to differences in the quality or strength of the signal emanating from each TCR. Structural diversity occurs in M1- and BMLF1-specific TCR repertoires but in unique ways. M1-specific TCR repertoires primarily involve one Vβ family (Vβ17)...
and often express the CDR3β motif xRSx (15–18). However, within the Vβ17 family there exist hundreds of T cell clones that differ in their nucleotide sequence (19). In contrast, BMLF1-specific TCR repertoires include multiple Vβ families that are often composed of relatively few T cell clones (20–22). Although there are at least four common BMLF1-specific Vβ families (Vβ2, 4, 16, and 22), their combination and hierarchy differ between individuals. The present study demonstrates that the cross-reactive T cell population specific for these two dissimilar epitopes (IAV-M1 and EBV-BMLF1) includes a wide array of TCRs, with little evidence for clonal dominance and repertoire narrowing. Furthermore, similar to the mouse model of cross-reactivity, each cross-reactive TCR repertoire examined was unique to the individual, revealing no common or predictable cross-reactive TCR structure (6). These results suggest that the breadth of the cross-reactive T cell repertoire may depend on various factors, including the level of structural similarity between epitopes, which we hypothesize impacts clonal dominance and is supported by computer simulation of human immune responses.

Materials and Methods

Subjects
Five IAV-immune patients with acute EBV infection, between the ages of 18 and 23 y, were volunteers from the University of Massachusetts (UMass) Student Health Services (Amherst, MA). HLA typing and EBV serology were performed, as previously described (14). Positive staining with HLA-A2 tetramers loaded with influenza-M1 epitope was used as an indication that these individuals had been exposed to IAV. For this study, a 50-ml blood sample was provided from patients within a year after presentation with symptoms of infectious mononucleosis (IM). Four healthy donors between the ages of 42 and 50 y were volunteers from the research community at UMass Medical School. HLA status and immunity to EBV and IAV were assessed using an HLA-A2–specific mAb (BB7.2, BD Biosciences, San Jose, CA) and tetramer stains, respectively. EBV serology was further confirmed through the detection of IgG specific for the viral capsid protein (14). This study was approved by the Human Studies Committee at UMass Medical School.

Blood preparation and bulk T cell culture
PBMCs were isolated using Ficoll-Paque plus (Amersham Biosciences, Uppsala, Sweden). CD8+ cells were isolated using the Miltenyi Biotec (Auburn, CA) MACS system and were cultured using our published protocol (14). Briefly, CD8+ lymphocytes were plated at a 5:1 ratio with T2 cells (#CRL-1992; American Type Culture Collection, Manassas, VA) that had been pre-pulsed with 1 μM peptide, irradiated, and washed. These T2 cells were used to restimulate the T cell lines weekly.

HLA-A2–restricted peptides
EBV-BMLF1280–288 (GLCTLV AML) and IAV-M158–66 (GILGFVFTL) peptides were synthesized to >90% purity by BioSource International (Camarillo, CA).

MHC class I tetramers
A detailed description of the protocol used by the tetramer facility at UMass Medical School was published previously (23). Tetrators were assembled using the above peptide sequences and were conjugated to allophtocya- nin (Caltag Laboratories, Burlingame, CA) or Quantum Red (Sigma-Aldrich, St. Louis, MO).

Extracellular/intracellular staining and cell sorting
CD8+ T cells were plated at 10⁵ per well and washed with FACS buffer (PBS, 2% FCS, 1% sodium azide). Tetrators were incubated alone at room temperature for 20 min, followed by an additional 20 min of incubation with Vβ-specific mAbs (Beckman Coulter, Fullerton, CA). The TCR Vβ nomenclature used in this study was originally described by Arden et al. (24). Cells were fixed with FACS Lysing Solution (BD Biosciences) for 5 min at room temperature and then washed with FACS buffer. Cells stained for intracellular IFN-γ were incubated with peptide and brefeldin A for 5 h before being permeabilized with Cytofix/Cytoperm reagent (BD Biosciences). Cells were then incubated for 30 min at 4°C with anti-IFN-γ Ab (clone B27; BD Biosciences). Cells for sorting were incubated with tetramer for 40 min at room temperature in 2% FCS/PBS buffer and were immediately isolated using the MoFlo cell sorter (Beckman Coulter).

CDR3 spectratyping and sequencing
RNA was isolated from sorted CD8+ T cell populations using TRizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. We synthesized cDNA using a poly-T primer, as previously described (14). The cDNA was amplified using published primer sequences specific for TCR Vβ and Vα families with those specific to the Cβ and Cα regions, as previously described (14, 25, 26). The Vβ and Vα nomenclature used in this study was originally described by Arden et al. (24). For spectratyping analysis, formamide dye was added to the PCR products, which were boiled for 3 min and then put on ice before being loaded onto a 5% polyacrylamide gel and run at 2500 V. Gels were read and analyzed using a FluorImager 595 and ImageQuant software (both from Molecular Dynamics, Sunnyvale, CA), respectively. For subcloning and sequencing, clonotypes within a given Vβ or Vα family, PCR products were ligated into the pCR4-TOPO vector (Invitrogen), as previously described, and were sequenced at the UMass Nucleic Acid Facility (Worcester, MA) using universal primers (14).

Computer simulation
We used IMMSIM, an agent-based model of the immune system governed by probabilistic events. This program is available (http://www.immsim.org) and can be downloaded for the purpose of research and education. The IMMSIM model consists of epithelial cells in a grid of discrete interaction sites where T cells, B cells, and APCs of the immune system encounter each other and Ags. Cellular and humoral responses mount whenever a virus infects and expresses Ags in the target epithelial cells. The interactions are governed by affinity and chance encounters (via computer-generated random numbers). This model’s past applications are found in several reports (6, 27–30). For the present modeling, we started by creating a group of 26 virtual individuals containing 7500 CD8+ T lymphocytes from a theoretical repertoire of 65,536 different receptors. We injected the individuals with 70 virus particles sporting a single epitope (hex 0xF0) to obtain a stable representative primary response specific to this epitope that has a sufficiently wide TCR memory repertoire (50 clones) to be used by the adoptive-transfer feature (available on the recent versions of the simulator); this feature is used to apply successive cross-reactive challenges. To study the response of structurally similar “near” versus dissimilar (“far”) cross-reactive epitopes in a comparable way and to limit the number of memory CD8 T cells needed for the simulations, we started by selecting a single medium cross-reactive epitope (hex 0x10) that we used in the model for cross-reactive (to 0xF0) challenge simulations (6). We increased or decreased the affinity (to the single cross-reactive epitope 0x10) in two or three of the most abundant T cell clones found in the primary repertoire. The change in affinity was accomplished using the "more bits of mismatch - less affinity” rule of the simulator, in which we modified the TCR hexadeclial (hex) IDs by increasing the “bits of mismatch” (to 0x10) to decrease the affinity and vice versa to increase it. The ultimate result was that we obtained two slightly modified primary T cell repertoires: one acted as a similar (“near”) cross-reactive challenge (in which some of the abundant T cells in the primary response are cross-reactive at high affinity), and the other acted as a dissimilar (“far”) cross-reactive challenge (in which none of the abundant T cells in the primary response are cross-reactive at high affinity). These two T cell memory repertoires were then adoptively transferred into two groups of 26 virtual individuals, and all individuals were challenged with 1000 virus particles sporting the same cross-reactive epitope 0x10. A representative output for each group was selected for the plot, whereas a two-tailed t test was applied to all the results to determine the statistical significance.

Statistical analysis
Where indicated, the paired Student t test was used to assess differences between groups.

Results
Cross-reactive CD8 T cells recognizing the two dissimilar HLA-A2–presented IAV-M1 and EBV-BMLF1 epitopes were characterized in the peripheral blood of healthy immune donors and patients with EBV-associated acute IM by co-staining with M1- and BMLF1-loaded tetrators (14). These cross-reactive T cells could be expanded in vitro in the presence of M1, BMLF1, or both peptides simultaneously. These cultured T cells exist as a highly heterogeneous population with regard to their ability to functionally respond.

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by proliferation, cytotoxicity, or cytokine production to either peptide and in their ability to bind one or both peptide-specific tetramers. For instance, in the M1-specific lines, the cells were able to produce cytokines, such as MIP-1β, IFN-γ, and TNF-α, in response to the cross-reactive ligand BMLF1. However, there was a hierarchy in the cytokine responsiveness of the cells: MIP-1β > IFN-γ > TNF-α (14). MIP-1β is most readily secreted upon cross-reactive stimulation, for as much as 90% of an M1-stimulated line could produce MIP-1β in response to the BMLF1 peptide (14). Some subsets of functionally cross-reactive cells efficiently stained with only one tetramer type but produced cytokines or proliferated in response to the other peptide. In this article, we show such an example: a subset (1.5%) of a CD8 T cell line grown in the presence of only M1 peptide was more efficiently stained with a BMLF1-loaded tetramer than with an M1-loaded tetramer. However, the BMLF1-tetramer+ population proliferated in response to M1 peptide stimulation, and ~83% of the cells produced IFN-γ upon M1 stimulation (Fig. 1). In this study, we sought to characterize the cross-reactive TCR repertoire and compare it with non-cross-reactive repertoires, so we chose to include two cross-reactive populations defined by M1/BMLF1 double-tetramer+ staining (cross-reactive population 1 [CXR-1]) or, as demonstrated in Fig. 1, BMLF1 single-tetramer+ T cells that responded functionally to M1 peptide stimulation (cross-reactive population 2 [CXR-2]).

**T cell lines accurately reflect the Ag-specific TCR repertoire ex vivo**

To fully investigate the organization and structural diversity of cross-reactive TCR repertoires, we first ensured that our culturing conditions supported the growth of most Ag-specific T cell clones. Because the population of BMLF1-specific memory CD8 T cells in healthy donor D-002 was large enough to sort ex vivo, we compared the TCR Vβ usage of freshly isolated BMLF1-tetramer+ cells with those from the same healthy donor grown in the presence of BMLF1 peptide for 4 wk; the two repertoires were almost identical with regard to Vβ usage and individual clonotypes (Fig. 2). Directly ex vivo, Vβ14 and Vβ16 families predominated compared with the un gated CD8 T cell population (Supplemental Fig. 1) and were conserved during culture. Our culturing conditions also effectively supported the growth of low-frequency Vβ families, including Vβ17, Vβ18, and Vβ22. We then subclassed and sequenced the TCR CDR3β expressed by BMLF1-specific T cells, whereby every unique nucleotide sequence was considered a unique Vβ clonotype. We found BMLF1-specific clonotypes using high- and low-frequency Vβ families in ex vivo and in vitro repertoires (Fig. 2). The same dominant clonotype within each of the Vβ14, 17, 18, and 22 families was present ex vivo and in vitro. The dominant Vβ16+ clonotype ex vivo (ID: B16.2) became codominant in vitro with another Vβ16+ clonotype (ID: B16.1) that differed by only one of eight amino acid residues making up the CDR3β. These results suggested that TCR repertoire analyses of cultured T cell lines accurately reflect the Ag-specific repertoire present in vivo.

Extensive TCR repertoire analyses performed on BMLF1-specific T cell populations derived from healthy donor D-002 confirmed many of the general characteristics of BMLF1-specific TCR structure and repertoire organization previously established (20–22, 31). All four of the common Vβ families (Vβ2, 4, 16, 22) were represented in the BMLF1-specific TCR repertoire of healthy donor D-002 (Fig. 2). Furthermore, we confirmed the observation that a given BMLF1-specific Vβ family often consists of only one or two clonotypes (31). For donor D-002, one or two clonotypes accounted for >60% of each Vβ population investigated (Fig. 2). Where determined, the clonotypes expressed the conserved Jβ family and/or CDR3β amino acid motif specifically associated with their respective Vβ family. Vβ16+ clonotypes fit the CDR3β motif AS-SQPGGTQ-YF and had fewer restrictions on their Jβ usage, whereas Vβ22+ clonotypes were more restricted by the usage of Jβ2.1 or Jβ2.2 than by the amino acid sequence of their CDR3β loop (generally fitting the published motif AS-S*G*V*P*GELFF) (22) (Supplemental Table I). These data also revealed features not previously published, to the best of our knowledge, of the BMLF1-specific TCR repertoire, such as the usage of Vβ families 14, 17, and 18. All three Vβ families were composed of one dominant clonotype with a CDR3β sequence that may be conserved in other donors with BMLF1-specific repertoires that include these particular Vβ families (Fig. 2). Overall, the five Vβ families analyzed in this study (Vβ14, 16, 17, 18, 22) represented >80% of the BMLF1-specific repertoire of donor D-002. The total number of unique Vβ clonotypes identified out of the total number of sequences analyzed was as few as 18 of 201 isolated ex vivo and 21 of 160 isolated in vitro, suggesting that the BMLF1-specific TCR repertoire is relatively narrow or oligoclonal (Supplemental Table I), consistent with previous observations (22).

**Multiple Vβ families can be used to corecognize M1 and BMLF1**

Subsets of T cells cultured in vitro with M1 peptide were considered cross-reactive with BMLF1 by their ability to bind BMLF1-loaded tetramer simultaneously with M1-loaded tetramer (CXR-1) or exclusively while retaining the ability to proliferate and produce cytokines in response to M1 stimulation (CXR-2) (14) (Fig. 1). Both subsets of cross-reactive T cell populations were present within an M1-specific T cell line derived from IM patient E1101. CXR-1 cells cocultured with both tetramers (M1+ BMLF1+; 0.1% of the T cell line) and CXR-2 cells stained with only the BMLF1-loaded tetramer (M1+ BMLF1+; 2% of the T cell line) but remained functionally responsive to M1 stimulation (Fig. 3). We gated on these distinct cross-reactive T cell subsets and used Vβ-specific mAbs to determine their TCR usage (Fig. 3B). The M1+ BMLF1+ CXR-1 cells predominantly used Vβ17 (81%) but also used Vβ families 2 (8%) and 4 (12%). The M1+ BMLF1+ CXR-2 cells within an M1-specific T cell line were not dominated by one Vβ family but instead used four Vβ families: 2 (12%), 4 (18%), 16 (6%), and 22 (8%).

**FIGURE 1.** BMLF1-tetramer+ T cells can produce IFN-γ following M1 stimulation. CD8 T cells isolated from patient D-002 were grown in the presence of M1 peptide for 3 wk. After a 5-h stimulation with M1 peptide, the M1-specific T cell line was stained extracellularly with M1- or BMLF1-specific tetramer and intracellularly for IFN-γ. The percentage of the M1-specific T cell line that stained with the indicated tetramer (upper number) and the percentage of the tetramer+ population that costained with anti–IFN-γ (lower number) are shown. Not shown are control peptide EBV-BRLF1190 stimulation, whereby <0.06% of the BMLF1 tetramer+ population produced IFN-γ, and control tetramer staining, CMVpp65 < 0.001%.

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cross-reactive Vβ repertoires were clearly different from those of the tetramer− cell populations in these cultures (Supplemental Fig. 2). We performed the same Vβ analyses on the cross-reactive T cells derived from four additional IM patients and four healthy immune donors (Fig. 4A, 4B). Among these nine individuals, we determined that each cross-reactive TCR repertoire contained at least 1 of 12 Vβ families, including Vβ1, 2, 3, 4, 5, 7, 8, 12, 14, 16, 17, 21, and 22. Thus, a wide range of TCR structures appeared capable of recognizing the two dissimilar M1 and BMLF1 peptides. Overall, the two most common Vβ families detected among the cross-reactive repertoires were Vβ4 and 17, used by five and four of nine individuals, respectively. Although we were unable to screen for all of the known Vβ families, because mAbs to all known Vβ families are not available, the cross-reactive repertoire of two individuals was composed of as few as one Vβ family (IM patient E1178; Vβ1; healthy donor D-042; Vβ4), whereas the cross-reactive repertoire of two other individuals was composed of as many as six Vβ families (IM patient E1109: Vβ4, 5, 7, 12, 14, 17, 21, 3; IM patient E1106: Vβ1, 2, 3, 5, 17, 22). We found no discernable difference in the breadth of the cross-reactive repertoires of IM patients (median = 5, range: 1–6; n = 5) compared with healthy immune donors (median = 3, range: 1–4; n = 4). Rather, the breadth of the cross-reactive Vβ repertoire was significantly (p < 0.02) more extensive in individuals who had two distinct, tetramer-defined (CXR-1, M1+BMLF1+ and CXR-2, M1+BMLF1+) populations of cross-reactive T cells, such as IM patients E1101, E1106, and E1109 and healthy donor D-035 (median = 5.5, range: 3–6; n = 4) compared with those with only one cross-reactive population (CXR-2, M1+BMLF1+) (median = 2,
cells in a specific Vβ population. It seemed that the specific combination of BMLF1-loaded tetramer were considered a noncross-reactive BMLF1-specific T cell population, whereas T cells that grew in the presence of M1-loaded tetramer were considered a noncross-reactive M1-specific population. Following incubation with tetramers, cells in A were stained with Vβ-specific Abs. Each bar graph illustrates the percentage of cells within its respective tetramer-defined gate that use each Vβ family. C, CD8 T cells isolated from patient E1101 were cultured for 3 wk in the presence of BMLF1-pulsed T2 cells before being costained with M1- and BMLF1-specific tetramers. D, Following incubation with tetramers, cells in C were stained with Vβ-specific Abs. The bar graph illustrates the percentage of BMLF1-tetramer+ cells that use each Vβ family. Control tetramer CMVpp65 in M1 and BMLF1 lines was <0.001%.

FIGURE 3. Each tetramer-defined subpopulation of a T cell line has a distinct Vβ repertoire. A, CD8 T cells isolated from patient E1101 were cultured for 3 wk in the presence of M1-pulsed T2 cells before being costained with M1- and BMLF1-specific tetramers. B, Following incubation with tetramers, cells in A were stained with Vβ-specific Abs. Each bar graph illustrates the percentage of cells within its respective tetramer-defined gate that use each Vβ family. C, CD8 T cells isolated from patient E1101 were cultured for 3 wk in the presence of BMLF1-pulsed T2 cells before being costained with M1- and BMLF1-specific tetramers. D, Following incubation with tetramers, cells in C were stained with Vβ-specific Abs. The bar graph illustrates the percentage of BMLF1-tetramer+ cells that use each Vβ family. Control tetramer CMVpp65 in M1 and BMLF1 lines was <0.001%.

range: 1–4; n = 5). Most notably, the breadth of the cross-reactive Vβ repertoire, including both cross-reactive populations, was significantly more extensive than that of the M1+ or BMLF1+ noncross-reactive populations (Fig. 4E, Supplemental Fig. 3).

The cross-reactive TCR repertoire is unique to each individual

It was documented that M1- and BMLF1-specific TCR repertoires have features that are unique to each individual, such as the clonal composition of each individual’s M1-specific Vβ17+ repertoire or the Vβ hierarchy of each individual’s BMLF1-specific repertoire. This is consistent with the concept of a private specificity for each TCR repertoire (6, 18, 20, 22, 32–34). Thus, we predicted that this private specificity of each M1- and BMLF1-specific repertoire was responsible for the wide range of cross-reactive TCRs that we observed and the individual variability between cross-reactive repertoires (Fig. 4).

We compared the Vβ repertoires of cross-reactive and noncross-reactive T cell populations derived from each of the nine individuals included in our study. T cells that grew in the presence of M1 peptide and only stained with M1-loaded tetramer were considered a noncross-reactive M1-specific population, whereas T cells that grew in the presence of BMLF1 peptide and only stained with BMLF1-loaded tetramer were considered a noncross-reactive BMLF1-specific population. It seemed that the specific combination of BMLF1-specific Vβ families used by each individual influenced the specific combination of cross-reactive Vβ families used by that same individual. For example, the noncross-reactive BMLF1-specific repertoire of donor D-042 included Vβ4 and Vβ16 but not Vβ2 or Vβ22, and the cross-reactive repertoire of this donor reflected that by including one of the two Vβ families known to be used by the BMLF1-specific T cell population, Vβ4 (Fig. 4B, 4D). Thus, the composition of the noncross-reactive Vβ repertoire seemed to influence the organization of the cross-reactive Vβ repertoire. The individual variability associated with both BMLF1-specific and, through association, cross-reactive Vβ repertoires likely stems from the clonal composition of each individual’s precursor T cell population, which includes naive and memory T cell clones that can be activated by the BMLF1 epitope. A larger study cohort is necessary to reveal any common features of this cross-reactive repertoire that may be shared by multiple unrelated individuals.

Unique features of the cross-reactive TCR repertoire

Several features of the cross-reactive TCR repertoires made them unique. For instance, although the CXR-1 (M1+ BMLF1+) and noncross-reactive M1-specific populations derived from IM patient E1101 predominantly used Vβ17, the cross-reactive repertoire remained distinct by the inclusion of additional Vβ families (Vβ2 and 4) present at lower frequencies (Fig. 3B). Similarly, the CXR-2 (M1− BMLF1+) population within an M1-specific T cell line and the noncross-reactive BMLF1-specific population within a BMLF1-specific T cell line were derived from the same IM patient, and both used Vβ families 2, 4, and 22. However, the cross-reactive repertoire remained distinct through the increased usage of Vβ16 (Fig. 3B, 3D).

Selective gating on cross-reactive T cell populations seemed to enhance the detection of Vβ families used more frequently by cross-reactive T cells than by noncross-reactive T cells. Overall, the cross-reactive repertoire of seven of nine individuals included at least one Vβ family not detected within that individual’s noncross-reactive repertoires (Fig. 4). IM patient E1217 represented the most extreme
example of this, with the cross-reactive repertoire consisting entirely of two VB families (VB5.1 and VB21.3) that were not detected in this individual’s noncross-reactive repertoires. In some cases, the VB family strictly detected in one cross-reactive repertoire was an otherwise common component of the noncross-reactive repertoires of other individuals, such as VB families 4, 16, 17, and 22.

In other cases, the VB families detected only in a cross-reactive repertoire were rare, even among the noncross-reactive repertoires of other individuals, such as VB families 5.1, 7.2, and 21.3.

We also observed differences between cross-reactive and noncross-reactive repertoires on a clonal level. We isolated the tetramer-defined cross-reactive and noncross-reactive populations from T cell lines derived from one healthy donor, D-002 (Fig. 5A, 5B). Because the cross-reactive and noncross-reactive BMLF1-specific T cells predominantly used VB14 (Fig. 4B, 4D), we subcloned and sequenced the CDR3b loops of each VB14+ T cell population. We detected more VB14+ clonotypes in the cross-reactive repertoire (5 unique clonotypes of 23 total sequences analyzed) compared with the highly restricted noncross-reactive BMLF1-specific repertoire (1 unique clonotype of 10 total sequences analyzed) (Figs. 2B, 5C). Three of the four cross-reactive VB14+ clonotypes used an alternative Jb family (Jb2.1) and a longer CDR3b loop (10–11 aa length). Similarly, we subcloned and sequenced several cross-reactive VB17+ clonotypes, representing the dominant VB family of donor D-002’s noncross-reactive M1-specific repertoire. The majority of the cross-reactive VB17+ repertoire, 64% (27/42) of the unique clonotypes, consisted of newly identified clonotypes that were not previously detected in either noncross-reactive repertoire (Fig. 5D).

**Similarities between the cross-reactive and noncross-reactive TCR repertoires**

VB repertoire comparisons revealed that the CXR-1 (M1+ BMLF1+) T cells often expressed TCRs similar in structure to those expressed by noncross-reactive M1-specific T cells (Fig. 4A, 4C), whereas the CXR-2 (M1 M1 BMLF1) T cells found within M1-specific T cell lines more often expressed TCRs similar in structure to those expressed by noncross-reactive BMLF1-specific T cells (Fig. 4B, 4D). Based on IM patients E1101, E1106, and E1109 and healthy donor D-035, cross-reactive T cells that efficiently bound M1-loaded tetramer predominantly used VB17, perhaps indicative of a relatively high avidity for the M1 peptide (15). In contrast, cross-reactive T cells that preferentially bound BMLF1-loaded tetramer tended to express non-VB17 families that are commonly present in BMLF1-specific repertoires. Previous studies suggested that the BMLF1-specific TCR repertoires of most individuals include at least one of four common VB families (VB2, 4, 16, 22) (20–22, 31). Interestingly, each of these four VB families was represented at least once among the cross-reactive repertoires analyzed in this study (Fig. 4).
To distinguish any clonal differences between cross-reactive and noncross-reactive T cells of the same V\(\beta\) family, we subcloned and sequenced the CDR3\(\beta\) regions of sorted populations from T cell lines derived from healthy donor D-002 (Fig. 5A, 5B). This donor’s noncross-reactive BMLF1-specific population predominantly used V\(\beta\)14 (66%), which was used by 57% of the cross-reactive population (Fig. 4B, 4D). Although there was a greater number of unique clonotypes in the cross-reactive repertoire, the most frequently detected V\(\beta\)14\(^+\) clonotype in the cross-reactive repertoire (ID: B14.1, J\(\beta\)1.1, 7-aa length) was the same clonotype that dominated the noncross-reactive BMLF1-specific repertoire of this donor. Similar results were obtained when we subcloned the V\(\beta\)17 family, which was predominantly used by the noncross-reactive M1-specific repertoire population (81% V\(\beta\)17\(^+\)) and used at a low frequency by the cross-reactive population (<1% V\(\beta\)17\(^+\)) (Fig. 4B, 4C). This small population of V\(\beta\)17\(^+\) cross-reactive cells was surprisingly diverse at the clonal level. It consisted of 42 unique clonotypes of 55 total sequences analyzed, and the organizational pattern mimicked that typical of a memory M1-specific TCR repertoire in a healthy donor (Fig. 5D) (19, 36). For instance, there was no apparent skewing of the repertoire by one dominant clonotype or any alteration of the J\(\beta\) usage, which was previously observed during an acute EBV infection when a select population of cross-reactive clones may have proliferated in vivo (14). In agreement with the literature, IRSS was the dominant CDR3\(\beta\) loop sequence found within the noncross-reactive M1\(^+\) V\(\beta\)17\(^+\) repertoire (used by 25% [10/40] of the unique clonotypes) and remained dominant within the cross-reactive population (used by 31% [13/42] of the unique cross-reactive clonotypes), including the most frequently detected clonotype (ID: 17.3, frequency: 7% of total sequences) (Fig. 5D, 5E). Interestingly, one of the two most frequently detected clonotypes within the cross-reactive population was previously detected within the BMLF1-tetramer\(^+\) population isolated ex vivo, suggesting that the dominant V\(\beta\)17\(^+\) BMLF1-specific memory T cell clones may be those that are cross-reactive with influenza-M1 (Figs. 2A, 5D, Supplemental Table I). However, concurrent \(\alpha\)-chain analyses are required to determine whether the similarities in \(\beta\)-chain usage observed in

<table>
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**FIGURE 5.** The V\(\beta\) repertoire of cross-reactive cells is composed of multiple unique clonotypes using a combination of M1- and BMLF1-specific TCR elements. A. An M1-specific T cell line derived from donor D-002 was costained with M1- and BMLF1-specific tetramers. Two separate populations were sorted: M1\(^+\) BMLF1\(^+\) (CXR-2) and M1\(^+\) BMLF1\(^-\) (noncross-reactive M1). B. A BMLF1-specific T cell line derived from donor D-002 was costained with both tetramers and the BMLF1-tetramer\(^+\) (noncross-reactive BMLF1) cells were collected. C. The CDR3\(\beta\) regions of cross-reactive V\(\beta\)14\(^+\) clonotypes were sequenced. Based on a unique nucleotide sequence, clonotypes were assigned an ID, and their frequency is shown (i.e., the number of times that clonotype was detected among the total sequences analyzed). The length of the CDR3\(\beta\) region was determined, according to Chothia et al. (51), shown supported by two flanking framework regions. Bold type indicates that the clonotype was previously detected within BMLF1-specific cell populations. D. The CDR3\(\beta\) regions of cross-reactive V\(\beta\)17\(^+\) clonotypes were sequenced. Unique clonotypes are arranged based on their frequency among all sequences analyzed. E. The CDR3\(\beta\) regions of noncross-reactive M1\(^+\) V\(\beta\)17\(^+\) clonotypes were sequenced and arranged, as described above.
this study truly indicate that the same T cell clones dominate the cross-reactive and BMLF1-specific populations.

The Vα repertoire of cross-reactive cells consists of multiple Vα families and is distinct from that of noncross-reactive M1+ or BMLF1+ populations

The α-chain analyses of the same tetramer-defined cross-reactive and noncross-reactive T cell populations derived from healthy donor D-002 (Fig. 5A, 5B) required using only the PCR-based technique known as CDR3α spectratyping, because very few mAbs specific to Vα families are available. This technique allowed us to detect the presence of specific Vα families, as well as the presence of clonal T cell expansions within specific Vα families that were distinguished based on CDR3α length; however, it did not allow us to quantify the frequency of Vα family usage (25, 37). In an initial screen using primers specific for 24 Vα families, the cross-reactive repertoire included at least 9 Vα families (Vα1b, 2, 6, 10, 11, 15, 20, 23, 24) (Fig. 6A). Thus, just as was observed upon Vβ analysis, a wide range of TCR structures seemed capable of interacting with the two dissimilar peptides M1 and BMLF1.

When comparing the Vα repertoires of the cross-reactive and noncross-reactive populations, we noted several similarities, as well as some distinguishing features, between them. Noncross-reactive BMLF1-specific T cells used Vα2, 10, 11, 15, 22, and 23, along with some additional Vα families that may have been at the limit of detection using this protocol (Fig. 6B). All but Vα22 were also detected in the cross-reactive repertoire (Fig. 6A). The Vα repertoire of the noncross-reactive M1-specific population also included a wide range of Vα families, most consistently including Vα1b, 2, 10, and 24, but with many additional Vα families being detected, such as Vα6, 11, 15, 20, and 23 (Fig. 6C). All of these families were also detected in the cross-reactive repertoire (Fig. 6A). To compare the clonal composition of the cross-reactive and noncross-reactive populations that expressed the same Vα family, we ran the corresponding PCR reactions on the same polyacrylamide gel and compared the relative lengths of the CDR3α loops (Fig. 6C). CDR3 length differences were an indication of clonal differences between the three T cell populations analyzed in this study. Such differences were evident within the Vα11 family, in which cross-reactive T cells clearly expressed longer CDR3α loops than did noncross-reactive BMLF1- or M1-specific T cells (Fig. 6C). Similarly, Vα20+ cross-reactive T cells seemed to express a unique CDR3α loop length compared with either noncross-reactive T cell population. However, there were also cases where the cross-reactive and noncross-reactive T cells expressed similar CDR3α loop lengths. Cross-reactive T cells that expressed Vα2 or Vα15 had CDR3α lengths similar to BMLF1-specific T cells, whereas cross-reactive T cells that expressed Vα10 had CDR3α lengths similar to M1-specific T cells (Fig. 6C).

As reported in the literature, BMLF1-specific T cells often coexpress Vα15 with one of three common Vβ families (Vβ2, 4, 16), perhaps indicating an important role for this particular α-chain in binding the BMLF1 peptide (20–22). Because CDR3 spectratyping analysis revealed similarities between cross-reactive and noncross-reactive Vα15+ T cells, we sequenced and compared Vα15+ clonotypes isolated from both populations (Fig. 6D). The same Vα15+ clonotype (ID: B1) dominated the cross-reactive (70% of the sequences analyzed) and noncross-reactive (76% of the sequences analyzed) BMLF1-specific repertoires. However, the cross-reactive repertoire also included Vα15+ clonotypes that expressed unique Jα families (Jα11 and Jα13) and CDR3α loops 6 aa long, a length that was more frequent in the cross-reactive repertoire (18% of sequences analyzed) than in the noncross-reactive BMLF1-specific repertoire (2% of sequences analyzed). These unique cross-reactive Vα15+ clonotypes also did not resemble any of the noncross-reactive M1-specific Vα15+ clonotypes we identified (Fig. 6D).

Computer simulation: lack of high-affinity cross-reactive clones leads to a broader repertoire distribution

Using a murine model of viral infection, we previously identified a cross-reactive T cell population that recognized two distinct, but structurally similar, epitopes, differing by only two amino acids, derived from the nucleoprotein (NP) of two viruses: LCMV and PV (7). Following heterologous virus infection, TCR analyses of this cross-reactive T cell population revealed the development of a more restricted TCR repertoire compared with that prior to the infection or that seen in a naïve mouse infected with the second virus (6). Computer studies with virtual immune mice challenged with a cross-reactive epitope mimicked our experimental data, showing that T cell cross-reactivity can modulate clonal dominance and narrowing of the TCR repertoire. This system was further useful in predicting that repertoire narrowing could be a function of the proportion of high-avidity cross-reactive T cells (6).

However, in our current studies of cross-reactive T cells in human viral infections, the cross-reactive epitopes are structurally dissimilar, with only three amino acids in common and with two of them important for binding to the MHC molecule. In this case, we observed that the cross-reactive T cell repertoire was actually broader than the noncross-reactive repertoires. We were interested in using the virtual immune system to determine whether the dichotomy in the cross-reactive TCR repertoires observed in the human and mouse systems could be explained, in part, by the level of structural similarity between the epitopes involved. Based on our earlier observations, we hypothesized that if there are a few high-frequency memory clones specific to the primary infection pathogen that also have high affinity to the subsequent challenging pathogen, this type of clone will dominate the TCR repertoire to the challenge pathogen’s cross-reactive epitope and lead to a narrower repertoire. This is more likely to occur when epitopes are structurally similar (termed “near” cross-reactivity in Fig. 7). When epitopes are structurally dissimilar (termed “far” cross-reactivity in Fig. 7), this type of high-affinity clone may not exist. The resulting repertoire would be broader, lacking any dominant preexisting cross-reactive memory clones. For this investigation, we used the IMMSIM model, a stochastic agent-based computer simulator with which we can do experiments on “virtual” immune systems of mice or humans (6, 28–30).

Fig. 7A shows a computer-generated repertoire after a primary infection and shows higher-affinity clones to the primary pathogen epitope than to the cross-reactive epitope of the challenge pathogen. Fig. 7B shows what the repertoire would be to a cross-reactive epitope if we removed the three highest-frequency clones having high affinity for the cross-reactive epitope from the memory pool of the primary infection. Both cross-reactive TCR repertoire distributions were evaluated after challenging with the cross-reactive epitope-containing pathogen in a simulation of the classic “adoptive-transfer” technique, a feature introduced into IMMSIM’s code to allow these kinds of studies. This feature allowed us to analyze the diversity of secondary responses obtained from the same memory repertoire, but in different individual environments, with remarkable accuracy and with similarity to in vivo adoptive-transfer experiments. Upon challenging an individual with the unmodified memory TCR repertoire depicted in Fig. 7A with a structurally similar (“near”) cross-reactive epitope, the breadth of the cross-reactive repertoire shrunk, with notable skewing and domination of clone FFAF (Fig.
In contrast, upon challenging an individual with the modified memory TCR repertoire depicted in Fig. 7A with a structurally dissimilar (“far”) cross-reactive epitope, the breadth of the cross-reactive repertoire expanded (Fig. 7B). Note that the highest-frequency clone FFAE now represents a lower proportion of the response than does its counterpart in Fig. 7A. Interestingly, new unique clonotypes appeared in the repertoire during the latter experiment, which further increased diversity (FFEF, FFAF, FFEE, EF67). In Fig. 7A and 7B, after the secondary challenge, all of the higher-frequency clones had high affinity for the second epitope, because they were driven to expand by their cross-reactivity to the second epitope. Following secondary challenge with the “near” cross-reactive epitope, the domination of a few clones having higher affinity for the primary pathogen epitope and the cross-reactive epitope masked the detection of low-frequency clones and resulted in a more polarized repertoire (Fig. 7A). The dominance of these high-affinity memory clones is assured by their ability to outcompete the expansion of naive clones. In contrast, clones having lower affinity for the epitopes were present at lower frequencies upon challenge with the “far” cross-reactive epitope, allowing ample space for increased repertoire diversity (Fig. 7B). The data presented in Fig. 7 represent 1 experiment of 26 conducted. The TCR distributions presented in this article were confirmed with statistical analyses that showed during “near” cross-reactive epitope-challenge experiments, 75% of the responding repertoire was composed of a mean of 5.3 different clones (95% confidence interval: 4.9–5.6 clones) \(p, 0.0001; n=26\). Thus, these computer-simulation experiments conclude that the TCR repertoire responding to a structurally similar cross-reactive epitope is polarized by dominant high-affinity clones, whereas that responding to a structurally dissimilar epitope has a broad distribution of lower-affinity clones.

**Discussion**

Under conditions of heterologous immunity, reports suggested that a cross-reactive population in response to a relatively similar epitope can lead to a narrower T cell repertoire and strong clonal dominance (6). When examining cross-reactive repertoires in vitro that specifically recognize two dissimilar epitopes, IAV-M1 and EBV-BMLF1, we made a number of observations. First, upon validating the use of cultured cells in analysis of TCR repertoires, we showed that the
cross-reactive repertoires were broader, using as many as 12 Vβ families, and flatter, without selection of highly dominant clonotypes, compared with the noncross-reactive repertoire for each epitope. Second, spectratype analysis of the more-difficult-to-study TCR Vα repertoire revealed an equally broad distribution using nine Vα families. Third, the cross-reactive repertoires differed among nine individuals tested, consistent with private specificity. Finally, the cross-reactive repertoires were enriched in otherwise low-frequency T cell clones that sometimes expressed a TCR with a longer CDR3 loop length, often containing uncharged, nonbulky amino acid residues, such as glycines and serines (Fig. 5C). These features give TCRs added flexibility and, therefore, the ability to accommodate interactions with more than one epitope.

The mechanisms that shape T cell memory through α-TCR selection have been difficult to delineate because of the technical restraints associated with the lack of Vα-family-specific mAbs and the ability of T cells to coexpress two α-chains, with one usually being nonfunctional. We recently examined the α-TCR repertoires of memory CD8 T cells reactive against the influenza A viral epitope, M158–66, restricted by HLA-A2.1 (38). The M158–66-specific, clonally diverse Vβ17 T cells expressed α-chains encoded by multiple AV genes with different CDR3 sizes. A unique feature of these α-TCRs is the presence of poly-Gly/Ala runs in the CDR3, fitting to an AGA(Gn)GG-like amino acid motif much like those observed for the cross-reactive clones in this study. These nontemplate-encoded poly-Gly/Ala runs in the CDR3 of influenza A M1-specific memory pool are significantly enriched over that in naive thymocytes, suggesting that these clones are preferentially selected during peripheral Ag exposure. The presence of these poly-Gly/Ala runs in the CDR3 of α- and β-chains might provide enhanced TCR

FIGURE 7. Computer simulation of cross-reactive response: lack of high-affinity cross-reactive clones leads to a broader repertoire distribution. A, Frequency hierarchy of clones responding to the primary infection. The clonal affinity to the primary immunogen is represented on the left, and the affinity to the cross-reactive challenging pathogen is represented on the right. The bars are shaded from black to white; black represents clones having the highest affinity, whereas white represents clones having no affinity to the particular Ag. The individual clones are identified by a four-digit code. B, Frequency hierarchy of clones responding to the primary infection that has been modified to represent the distribution of clonotypes after the removal of the three highest-frequency clonotypes with high affinity to the cross-reacting pathogen, thus simulating responses to a cross-reactive infection that is structurally dissimilar from the original Ag. No high-frequency high-affinity clones to the primary epitope were cross-reactive with the challenging infection; clones FFOF, FFAF, and FF2F have been replaced. C, The resulting repertoire upon secondary challenge of an individual having a memory TCR repertoire, as depicted in A, with a “near” or similar cross-reactive epitope. D, The resulting repertoire upon secondary challenge of an individual having a modified memory TCR repertoire, as depicted in B, with a “far” or dissimilar cross-reactive epitope. For C and D, the clonal affinity to the cross-reactive challenging pathogen is represented on the left and the affinity to the primary pathogen is represented on the right. These results are representative of experiments repeated on two sets (near and far cross-reactive adoptive transfers) of 26 virtual individuals each.
flexibility and the ability to accommodate interaction with multiple epitopes (39, 40).

In this study, we hypothesized that the breadth of the cross-reactive T cell repertoire may depend on various factors, such as the level of similarity between the epitopes. We tested this by computer simulation using IMMISIM analysis, which is capable of recapitulating a virtual immune response to viruses in individuals. We made the assumption that if epitopes are very dissimilar, it is less likely that high affinity cross-reactive clones exist that would rapidly dominate the response. In fact, the resulting repertoires did become broader and flatter upon exposure to the cross-reactive ligand compared with the starting memory repertoire responding to either cognate ligand alone. Such a diverse array of possible cross-reactive TCRs enhances the probability that a cross-reactive T cell response will occur during EBV infection, as well as expands or maintains the pool of cross-reactive memory T cell clones with the potential to effectively control EBV replication or contribute to protection from other new infections.

A difference between our murine and human models of heterologous immunity is the structure of the epitopes involved. The sequences of LCMV-NP205 and PV-NP205 are very similar, having six of eight amino acid residues in common, all of which are available to interact with the TCR. The sequences of IAV-M1 and EBV-BMLF1260 are quite dissimilar, having only three of nine amino acid residues in common, with two of the amino acids important for binding to MHC. The cross-reactive repertoire of these two dissimilar epitopes seemed to include features of both Ag-specific TCR repertoires. M1-specific repertoires are polyclonal, composed of many unique Vβ17 clonotypes, whereas BMLF1-specific repertoires are often composed of multiple Vβ families. Thus, both Ag-specific TCR repertoires offered some level of structural diversity that, together, resulted in a cross-reactive TCR repertoire reduction. This is a circumstance that is likely when there is a great deal of structural similarity between the two epitopes. In contrast, if there are small numbers of memory clones with moderate to high affinity to the cross-reactive epitope, as might occur with more dissimilar cross-reactive epitopes, this allows for some limited expansion of most of them, as well as expansion of some new (unique) cross-reactive clones, leading to greater repertoire diversity.

Recent work using mutations in the H2Kb-restricted SIINFEKL epitope of OVA and OVA-specific transgenic T cells indicates that low-affinity T cells initially expand with kinetics similar to that of high-affinity T cells but leave the lymph node earlier and do not have the sustained expansion of higher-affinity T cell clones, which eventually outcompete the low-affinity clones and dominate the response (42). The same may also be true for low-affinity cross-reactive memory T cell clones that appear early during infection as a result of their higher starting frequency but eventually are diluted by higher-affinity less cross-reactive clones. In fact, we found the highest proportions of cross-reactive T cells during acute EBV infection and much lower proportions in the resting memory state (14). This editing of the lower-affinity clones as the infection progresses may tend to eliminate the dominance of these clones and lead to a broader, less skewed repertoire when analyzed. This might contrast with the mouse model of LCMV and PV, in which higher-affinity T cells responding to more similar cross-reactive NP205 epitopes dominate the immune response, during the acute and memory phase, upon PV infection of LCMV-immune mice (6). In conclusion, this study makes the point that a cross-reactive T cell response can be composed of a diverse array of T cell clones. With structural and functional diversity, a responding T cell repertoire may be in a better position to combat a viral infection. With age, the naive T cell population decreases (43–46), memory T cells to previous infections are also deleted with each new infection (47–50), and each individual becomes more dependent on the diversity and potential cross-reactivity of memory T cells for any new response. Thus, the selection of a broad array of potentially cross-reactive memory T cells at low frequencies may ultimately become beneficial, maintaining a more diverse repertoire as the immune system ages. Although there may be no current way to control the breadth of a T cell repertoire responding to a natural infection, one can aim to activate a broad T cell response during vaccination procedures.

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Disclosures
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References
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BROAD TCR REPERTOIRE WITH DISSIMILAR CROSS-REACTIVE EPITOPES