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CD8 T Cell Cross-Reactivity Networks Mediate Heterologous Immunity in Human EBV and Murine Vaccinia Virus Infections


In this study, we demonstrate complex networks of CD8 T cell cross-reactivities between influenza A virus and EBV in humans and between lymphocytic choriomeningitis virus and vaccinia virus in mice. We also show directly that cross-reactive T cells mediate protective heterologous immunity in mice. Subsets of T cell populations reactive with one epitope cross-reacted with either of several other epitopes encoded by the same or the heterologous virus. Human T cells specific to EBV-encoded BMLF1 280–288 could be cross-reactive with two influenza A virus or two other EBV epitopes. Mouse T cells specific to the vaccinia virus-encoded a11r 198–205 could be cross-reactive with three different lymphocytic choriomeningitis virus, one Pichinde virus, or one other vaccinia virus epitope. Patterns of cross-reactivity differed among individuals, reflecting the private specificities of the host’s immune repertoire and divergence in the abilities of T cell populations to mediate protective immunity. Defining such cross-reactive networks between commonly encountered human pathogens may facilitate the design of vaccines.

E stablished memory T cell responses to a previously encountered pathogen can have a major impact on the course and outcome of a subsequent infection with an unrelated pathogen. This phenomenon, known as heterologous immunity, is dependent on the sequence of virus infections and can be either beneficial or detrimental to the host (1–6). Some HLA-A2+ patients with EBV-associated infectious mononucleosis (IM) have T cell responses cross-reactive between the influenza A virus (IAV) M1 54–66 epitope and the immunodominant EBV-BMLF1 280–288 epitope, suggesting that the CD8 T cell response in IM includes memory T cells cross-reactive with previously encountered viruses (5). Fulminant hepatitis during hepatitis C virus infection has been associated with T cell responses dominated by cross-reactivity between hepatitis C virus and a second influenza epitope, NA 231–239 (4).

There are numerous examples of heterologous immunity in mouse models. For example, a history of lymphocytic choriomeningitis virus (LCMV) infection predisposes to both protective immunity and altered immunopathology upon challenge with vaccinia virus (VV) (7, 8). Some of these mice develop panniculitis in their visceral fat, with a pathology resembling human erythema nodosum, a form of panniculitis that has been reported following human smallpox vaccination. During VV infection of LCMV-immune mice, there are proliferative expansions of T cells specific to any one of three different LCMV epitopes, NP 205–212, GP 34–41, or GP 118–125 (9). Adoptive transfer of these LCMV-immune splenocytes from one mouse into three naive congenic hosts resulted in all three mice expanding the same LCMV-epitope–specific population upon VV infection, thereby demonstrating private specificity in the cross-reactive memory T cell repertoire in each individual donor. Using the concept of molecular mimicry as a premise for identifying potential cross-reactive epitopes between LCMV and VV (10), we found two H-2Kb-restricted VV epitopes that had 50% sequence similarity to LCMV-NP 205–212, VV-e7r130–137, and VV-a11r 198–205 (11). Evidence to date is consistent with the concept that reactivated cross-reactive memory T cells mediate heterologous immunity, but this has yet to be definitively established (7, 12).

Many reports have described epitope-specific T cell responses that can cross-react with another single epitope (3–5, 12–15). In this study, we demonstrate, first within human and then within murine CD8 memory T cell pools, that one epitope can stimulate T cell populations that contain subsets that cross-react with either of several different epitopes encoded by the same or heterologous viruses. Each individual has a cross-reactivity network that is, in part, determined by the history of prior infections and the resultant changes in private specificities of their memory TCR repertoires. In the murine infection model, which is more amenable to manipulation, we demonstrate how the sequence of virus infection influences cross-reactive patterns and also for the first time demonstrate that cross-reactive T cells can mediate protective heterologous immunity.

Materials and Methods

Mice
C57BL/6 (B6, H-2b) and B6.SJL-�ptrc” (LY5.1) congenic male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY), respectively. Mice were used at 2–8 mo of age. All of the mice were maintained under specific pathogen-free conditions at Massachusetts Medical School, Worcester, MA 01655; Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany; and Discovery Research, Medarex, Milpitas, CA 95035

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The online version of this article contains supplemental material.

Abbreviations used in this paper: EBNA3A, EBV-encoded nuclear Ag 3A; IAV, influenza A virus; ICS, intracellular cytokine staining; IM, infectious mononucleosis; LCMV, lymphocytic choriomeningitis virus; PV, Pichinde virus; VV, vaccinia virus.


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the Department of Animal Medicine, University of Massachusetts Medical School.

Cell lines

American Type Culture Collection Vero cells were used in plaque assays. The TAP-2-deficient B6-derived T lymphoma cell line (RNA-S), provided by H.-G. Ljunggren (Karolinska Institute, Stockholm, Sweden), was grown in RPMI 1640. As stimulators for CD8 T cell lines, RMA-S cells were incubated with 1 μM peptide for 1 h at 37°C and then irradiated (3000 rad) and washed before being added to the culture. All cell lines were supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM l-glutamine, 10 mM HEPES, and 10% heat-inactivated (56°C, 30 min) FBS (Sigma-Aldrich, St. Louis, MO). All of the cell lines were cultured in MEM unless otherwise stated (Invitrogen, Carlsbad, CA).

 Viruses

The Western Reserve strain of VV, a DNA virus in the orthopoxvirus family, was propagated in L929 cells (16). LCMV (Armstrong strain) and Pichinde virus (PV) (AN7379 strain), viruses in the Old World and New World arenavirus families, respectively, were propagated in BHK21 baby hamster kidney cells (16). The mouse-adapted IAV A/P/8/34 (H1N1), a RNA virus in the orthomyxovirus family, was grown in the allantoic fluid of 10-d-old embryonated chicken eggs (SPAFAS, Preston, CT) (17).

Infection protocols

Mice were infected i.p. with 5 × 10⁶ PFU LCMV, 10⁶ PFU VV, and 2 × 10⁶ PFU PV, and metofane-anesthetized mice were challenged intranasally with 70 PFU IAV A/P/8/34 (H1N1). To control for culture contaminants, VV and PV stocks were purified through a sucrose gradient and diluted in HBSS (Invitrogen), and LCMV was diluted >40-fold in HBSS. Mice were considered immune 6 wk or later postinfection. Control naïve mice were either left unoinoculated or inoculated with tissue culture media or HBSS. The control mice always were age-matched to the experimental group and housed exactly the same in pathogen-free conditions. Viruses

VV titers in 10% tissue homogenate from each organ (fat pads and testes) were determined by plaque assays on American Type Culture Collection Vero cells, as described elsewhere (8).

Identification and screening for potential cross-reactive VV epitopes

We searched for VV epitopes that could generate cross-reactive T cell responses with the H-2Kb–restricted LCMV-NP205 epitope (YTVKYPNL) (10, 11). The search of the VV genome using the DNA/RNA and protein analysis software DNAvis (Hitachi Software Engineering Company, Tokyo, Japan) to 8mers that maintained the H-2Kb binding motif and had ≥30% sequence similarity to LCMV-NP205. These studies are described in more detail elsewhere (11). Two H-2Kb-restricted epitopes were identified, one in the VV protein c7r, positions 130–137 (STLNLNFLN), and the second in the VV protein a11r, positions 198–205 (ALVNYANL). In addition, LCMV epitopes NP366 Db (ASNENMETM) (22), and OVA epitope OVA257–264, Kb (SIINFEKL) were used. These synthetic peptides were obtained from Biocytogen, Cambridge, MA (18)–21), IA V (Invitrogen). LCMV (Armstrong strain) and New World arenavirus families were propagated in BHK21 cells (16). LCMV was diluted >40-fold in HBSS. Mice were considered immune 6 wk or later postinfection. Fat pads, testes, or both were analyzed for VV titers, because these organs had the highest titers for the longest time (11). Peritoneal exudates were analyzed for immune responses and for division of transferred T cells. Surface staining was performed as described above with fluorochrome-labeled Abs, PerCP-anti-CD45.2 (Ly5.2, clone 104) and allophycocyanin–anti-CD8 (clone 53-6.7). Transferred donor cells were identified as positive for CD45.2 and CD8 when congenic mice were used.

SMART-RAE PCR analysis of the CD8 repertoire

Cells from the CD8 T cell lines were revived from cryopreservation and washed in complete media. To extract RNA, 10⁶ live cells were subjected to the TRIzol method of RNA extraction according to manufacturer’s protocol (Invitrogen). cDNA was synthesized using the SMART RACE (Clonetics Labs, Mountain View, CA) cDNA amplification kit from BD Clontech (Palo Alto, CA) using a modified manufacturer’s protocol. In place of PowerCycler II (MJ Research, Waltham, MA) cDNA synthesis, Superscript III (Invitrogen) was used. To amplify the TCR-β–specific genes, the Advantage2 system was used according to the manufacturer’s protocol (BD Clontech). For the 5’ forward primer, the Universal Primer Mix from the SMART RACE cDNA amplification kit was used. To amplify the genes of interest, a 3’ reverse primer specific to the TCR-β region was used and reported previously (23). The PCR amplification program used was provided in the manufacturer’s protocol. The resulting PCR product of appropriate size (~500–700 bp) was gel-purified using the NucleoSpin Extract II kit according to the manufacturer’s protocol (BD Clontech). Purified PCR product was ligated into pCR4 vector from the TOPO TA cloning kit (Invitrogen) according to the manufacturer’s protocol and transformed into DH5α Escherichia coli from One Shot TOP10 chemically competent cells (Invitrogen) using the manufacturer’s procedures. Colonies were selected and amplified overnight for sequencing. Amplified colonies were then preserved in 20% glycerol on dry ice and sent to Agencourt Bioscience (Beverly, MA) for sequencing. Resulting sequences were aligned using Sequencher and analyzed using IMGT/TVQUEST (www.imgt.org) (24, 25).

Human subjects

IAV-immune patients with acute EBV infection between the ages of 18 and 23 were volunteers from the University of Massachusetts Student Health Services (Amherst, MA). HLA typing was performed using the Lymphotype Class I system (Biotest Diagnostics, Rockaway, NJ) and an Olerup SSP kit (Olerup, Solna, Sweden). Acute EBV infection was determined by a monospot test and the detection of capsid-specific IgM in patient sera. Positive staining with HLA-A2 tetramers loaded with influenza-M1 was used as an indication that these individuals had been exposed to IAV in the
past. Patients provided up to eight blood samples (50 ml each) starting at presentation with IM (day 0), then weekly for the following 6 wk, and then again at 1 yr. Healthy donors between the ages of 24 and 50 were volunteers from the research community at the University of Massachusetts Medical School (Worcester, MA). HLA status and immunity to EBV and IAV were assessed using mAb (BB7.2; BD Biosciences) and tetramer stains, respectively. Previous exposure to EBV was confirmed by the detection of capsid-specific IgG in donor sera. Donors provided up to three blood samples (60 ml each). This study was approved by the human studies committee at the University of Massachusetts Medical School.

**Blood preparation and bulk human T cell culture**

PBMCs were isolated and cultured as described previously (5). Briefly, PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) and were stained with anti-CD8 microbeads before being positively selected using the Miltenyi Biotech (Auburn, CA) MACS system. CD8+ lymphocytes were plated at a concentration of 2.5 × 10^5 cells per milliliter together with peptide-pulsed (1 μM) irradiated (3000 rad) T2 cells (American Type Culture Collection CRL-1992) at a concentration of 5 × 10^5 cells per milliliter in 4 ml total volume per well of a 12-well plate. T cell lines were fed media (AIM-V [Life Technologies, Carlsbad, CA] supplemented with 1% human AB serum [Nabi Biopharmaceuticals, Rockville, MD], 16% MLA-144 supernatant [26], 10 U/ml IL-2 [BD Biosciences], 1% t-glutamine [Life Technologies], 0.5% 2-ME [Sigma-Aldrich], and 1% HEPES [Hyclone, Logan, UT]) every 3–4 d and were restimulated with T2 cells weekly. We did most of our in vitro studies after only three to four stimulations, because in this range we found the T cell repertoire of our Ag-specific population to closely resemble the in vivo repertoire (data not shown). After six stimulations, there is more skewing of the Ag-specific repertoires for some Ags but not all. Our design was to come as close to an approximation of the Ag-specific TCR repertoire in vivo prior to stimulation. Because we only do three to four doses, the culture does not become 100% tetramer-positive for high-affinity cells. There most likely is a polyclonal population that has a range of affinities to the stimulating ligand. Frequently, cells in the culture will produce cytokines such as MIP1β in response to cognate ligand and yet not bind the cognate tetramer (5).

**HLA-A2–restricted peptides**

The following peptides were synthesized to >90% purity by BioSource International: EBV-BMLF1 (219–228; GLCLTVAML), IAV-M1 (27–35; GILGFVFTL), tyrosinase (YMNGTMSQY), EBV-BRLF1 (1190–1198; YVLHLIVY), EBV-LMP2 (320–337; LLWTLVLL), IAV-NP (95–99; GLKFNYQMM) (5, 27–31).

**Human MHC class I tetramers**

A detailed description of the protocol used by the tetramer facility at the University of Massachusetts Medical School has been published previously (5). Tetramers were assembled using the above peptide sequences for EBV-BMLF1 and IAV-M1 and were conjugated to PE (Sigma-Aldrich), allophycocyanin (Caltag Laboratories, Carlsbad, CA), or Quantum Red (Sigma-Aldrich). Tetramers assembled with tyrosinase (Immunomics, Gaithersburg, MD) were used as a negative control, and nonspecific staining was not observed.

**Human extracellular and intracellular staining**

A total of 10^6 cells per well were plated at and washed with staining buffer (PBS [Sigma-Aldrich] supplemented with 2% FCS [Gemini Bio-Products, West Sacramento, CA] and 1% sodium azide [Sigma-Aldrich]). Tetramers were added to the cells, incubated at room temperature for 40 min, and washed off, and the cells were fixed with FACS Lysing Solution (BD Biosciences). For intracellular cytokine staining (ICS), cells first were incubated at 37°C with 5 μM peptide and brefeldin A (Goji Plugs; BD Biosciences) for 5 h before being stained with tetramer as described above. Cells were permeabilized using BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions and were stained with anti-IFN-γ (B27; BD Biosciences) mAb for 30 min at 4°C. Isotype control Abs did not stain positive. Samples were read on FACSCalibur (BD Biosciences).

**Statistical analysis**

Descriptive statistics are expressed as mean values ± SEs of the mean. Comparisons between groups were performed with the unpaired t test (two-tailed).

**Results**

**Cross-reactive T cell networks in humans: T cell populations specific to three unrelated Ags can cross-react with a single EBV epitope**

The demonstration that some individuals expand cross-reactive T cell populations specific to three unrelated Ags can cross-react with a single EBV epitope provides insight into the mechanisms underlying cross-reactive T cell responses. Cross-reactive T cell networks were defined as T cell populations that expand in response to cognate ligand and yet not bind the cognate tetramer (5). In this study, we used tetramer staining of freshly isolated PBMCs to identify these new cross-reactive T cell responses (5). We define a pattern of cross-reactivities involving several EBV and IAV epitopes (Table I). Our studies focused on the EBV-BMLF1219 epitope, against which 100% of HLA-A2+ individuals mount a CD8 T cell response during the acute phase of EBV infection (27). T cell lines derived from 5 out of 16 donors

Table I. CD8 T cell epitopes that participate in cross-reactive responses

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Amino Acid Sequence and Position</th>
<th>% of Amino Acids in Common with EBV-BMLF1219</th>
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<tbody>
<tr>
<td>EBV-BMLF1219</td>
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<tr>
<td>EBV-BRLF1</td>
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<td>IAV-M1</td>
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<td>IAV-NP</td>
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**Epitope Amino Acid Sequence and Position % of Amino Acids in Common with EBV-BMLF1219**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Amino Acid Sequence and Position</th>
<th>% of Amino Acids in Common with VV-a11r198</th>
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<tbody>
<tr>
<td>H-2Kb–restricted murine CD8 T cell epitopes that generate cross-reactive responses to VV-a11r198</td>
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<tr>
<td>VV-a11r198</td>
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<td>LCMV-GP</td>
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<td>LCMV-NP</td>
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<td>PV-NP</td>
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<tr>
<td>VV-e7T150</td>
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<tr>
<td>OVAa2</td>
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</table>

**Epitope Amino Acid Sequence and Position % of Amino Acids in Common with VV-a11r198**

Boldface amino acids are common with EBV-BMLF1-228 or VV-a11r198.
and cultured with either IAV-M158 or EBV-BMLF1280 peptide recognized both IAV-M158 and EBV-BMLF1280 epitopes with relatively equal estimated avidity (5). Such cross-reactivity was not originally predicted, because IAV-M158 shares only three of nine amino acids in common with the EBV-BMLF1 epitope (Table I). This confirms our earlier report (5), but in this paper we show that EBV-BMLF1–specific T cells also could recognize other epitopes. EBV-BMLF1–specific T cell lines derived from IM patients could contain a discrete population of T cells that also recognized the IAV-NP85 epitope. Fig. 1A demonstrates the results for one of these individuals, where 2% of the CD8 T cells produced IFN-γ in response to stimulation with the subdominant IAV-NP85 epitope (Fig. 1Ai). This IAV-NP85–responding population costained with EBV-BMLF1280 tetramer (Fig. 1Aii). Control T cell lines, derived from the same donors and grown in the absence of the EBV-BMLF1280 peptide or stimulated with IAV-M158, did not include a similar subset of IAV-NP85–responsive cells; this suggests that the growth of these cross-reactive T cells in culture was dependent on the EBV-BMLF1280 peptide.

Some donors mounted cross-reactive T cell responses not only to heterologous virus epitopes but also to other EBV epitopes. EBV-BMLF1280–specific T cell lines derived from IM donors could contain a population of T cells that also recognized a latent EBV epitope, LMP2329. Fig. 1B shows the results for one of these individuals, where 34% of an EBV-BMLF1280–specific line produced IFN-γ in response to stimulation with the EBV-LMP2329 peptide (Fig. 1Bi). Costaining studies demonstrated that 50% of the EBV-BMLF1280 tetramer-positive population in this line produced IFN-γ in response to stimulation with EBV-LMP2329 peptide (Fig. 1Bii). Control T cell lines derived from this individual and grown in the absence of the EBV-BMLF1280 peptide or stimulated with IAV-M158 peptide had <1% IFN-γ–producing CD8 cells following

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**FIGURE 1.** Cross-reactive T cell responses in humans. A, Cross-reactive T cells with specificity for EBV-BMLF1280 and IAV-NP85. CD8 T cells derived from IM patient E1109 were cultured with BMLF1 peptide-pulsed T2 cells for 4–5 wk. i, The Ag specificity of the cell line was tested in a standard ICS assay, where the cell line was restimulated for 5 h with a variety of different peptides. The percentage represents the proportion of the T cell line producing IFN-γ. ii, A similar ICS assay was combined with extracellular tetramer staining to demonstrate that a portion of T cells bound to BMLF1 tetramer could also produce IFN-γ following restimulation with IAV-NP85. Tyrosinase peptide served as a nonspecific stimulation control. The percentage represents the proportion of the T cell line binding BMLF1280 tetramer and producing IFN-γ. Note that the TCR was significantly downregulated upon BMLF1 restimulation; the total percentage of IFN-γ–producing cells would all presumably bind BMLF1 tetramer. B, Cross-reactive T cells with specificity for EBV-BMLF1280 and EBV-LMP2329. BMLF1–specific T cell lines were derived from healthy donor D002 and were cultured as described in A. i, Standard intracellular IFN-γ assays are shown, where T cell lines were restimulated with EBV-LMP2329 or a nonspecific peptide, tyrosinase. Positive and negative controls also are provided, BMLF1280 or no restimulation respectively. The percentage of the T cell line producing IFN-γ is shown. ii, A intracellular IFN-γ stain was combined with an extracellular BMLF1280 tetramer stain, where EBV-encoded nuclear Ag 3A (EBNA3A) peptide served as a nonspecific stimulation control. The percentage of the cell line producing IFN-γ is shown. ii, An intracellular IFN-γ stain was combined with an extracellular BMLF1280 tetramer stain, where EBV-encoded nuclear Ag 3A (EBNA3A) peptide served as a nonspecific stimulation control. The percentage of the cell line producing IFN-γ is shown.
EBV-LMP2329 stimulation, and this small population did not contain BMLF1280 tetramer, further suggesting that the cross-reactive T cell population responding to EBV-LMP2329 was growing in response to the EBV-BMLF1280 peptide.

In freshly isolated PBLs (Fig. 1C) and in T cell lines (data not shown) of some IM patients, we detected cross-reactive T cell populations that recognized both EBV-BMLF1280 and EBV-BRLF1190, an epitope derived from an immediate-early EBV protein (18). Fig. 1C shows ex vivo peripheral blood data demonstrating this cross-reactivity in two patients at the indicated times after presentation with IM. We observed that 0.3% of CD8 T cells from IM patient E1232 costained with both EBV-BMLF1280 and EBV-BRLF1190-loaded tetramers simultaneously 13 d after presentation with IM (Fig. 1Ci). In this patient at this time point, there was no evidence for cross-reactivity by tetramer staining between EBV-BMLF1280 and IAV-M138 (Fig. 1Ci).

In some of these studies, the binding of one tetramer competed with another, suggesting that they were recognizing the same TCR. For instance, in patient E1205, at the time of presentation with IM (day 0), there was not a clear separate population that costained with both EBV-BMLF1280 and EBV-BRLF1190-loaded tetramers in freshly isolated CD8 T cells (Fig. 1Ci). However, 1.0% of the BRLF1190 tetramer staining population also was dimly positive for BMLF1280 tetramer, consistent with this being a cross-reactive T cell population. Also, in the presence of EBV-BRLF1190-loaded tetramer, one third (0.9%) of the EBV-BMLF1280-specific cells did not stain with BMLF1 tetramer, as their frequency declined to 0.7% single-tetramer-positive and 1.0% dim double-tetramer-positive, in contrast to a frequency of 2.6% in the presence of the control IAV-M138-loaded tetramer (Fig. 1Ci ). A similar observation also was made a second time in this patient 19 d after presentation with IM; in the presence of EBV-BRLF1190-loaded tetramer, 36% (0.4%) of EBV-BMLF1280-specific cells failed to stain with BMLF1 tetramer, because their frequency declined to 0.7%, in contrast to a frequency of 1.1% in the presence of the control IAV-M138-loaded tetramer. Because EBV-BRLF1190 but not IAV-M138 tetramer appears to block the binding of EBV-BMLF1280 tetramer and because the EBV-BMLF1 tetramer staining in the double-tetramer-positive population is dim compared with that of the EBV-BRLF1 tetramer, there may be cross-reactive T cells in patient E1205 that have a higher avidity for the EBV-BRLF1190 epitope than the EBV-BMLF1280 epitope.

Table I shows 0–50% amino acid homology between EBV-BMLF1280 and IAV-M138, IAV-NP85, EBV-LMP2329, and EBV-BRLF1190. Thus, these cross-reactive patterns could not easily be predicted based on epitope sequence similarity. Also, the networks of cross-reactivity within T cell populations of different individuals varied, as summarized in the diagram in Fig. 2A. For instance, the IAV-M138 and EBV-BMLF1280 epitopes are highly conserved targets for HLA-A2–restricted T cells, yet cross-reactive T cell responses specific for these two epitopes were only observed in 30% of HLA-A2+ individuals (5). This individual variation in cross-reactive networks is consistent with the concept that the private specificity of each epitope-specific memory T cell repertoire influences which cross-reactive pattern emerges.

Cross-reactive T cell networks in mice: one VV epitope can activate three different LCMV-specific memory populations

We questioned whether cross-reactive T cell networks occurred in the murine model of heterologous virus infection using LCMV-immune mice infected with VV, because the mouse model allows for greater experimental manipulation of the phenomenon. We focused these studies on the H-2Kb-restricted VV-a11r198 epitope identified by 50% sequence similarity to LCMV-NP205 (Table I). T cells specific to the VV-a11r198 peptide can activate CD8 T cell populations specific for four different peptides of rather dissimilar sequence derived from two different viruses, IAV and EBV. In the diagram of the cross-reactive T cell responses focused on BMLF1280, the fractions in the figure indicate the number of individuals of the total tested that demonstrated the indicated cross-reactive response. The epitopes in gray or white boxes are EBV- and IAV-specific, respectively. Cross-reactive responses were detected using both ex vivo and in vitro tetramer and ICS assays.

LCMV-NP205 epitope proliferates in ~50% of LCMV-immune mice challenged with VV (9, 11). The VV-a11r peptide stimulated strong growth of CD8 T cell lines from LCMV-immune mouse splenocytes that had never previously been exposed to this ligand in vivo (Fig. 3, Table I). As specificity controls, VV-a1r did not stimulate the outgrowth of T cells from nonimmune mice, and the non-cross-reactive VV epitope e7r130 did not stimulate T cells from LCMV-immune mice (11). When the VV-a1r–stimulated lines were screened for their reactivity to LCMV epitopes, we expected to see cross-reactivity to LCMV-NP205. However, cross-reactivity was generated against three different LCMV H2Kb-restricted epitopes, GP34, GP118, and NP205 (Table I), as demonstrated by ICS assays (Fig. 3, Table I), tetramer staining (Fig. 4A, 4C), and cytotoxicity in [51Cr]-release assays (data not shown). For instance, 85–92% of the CD8 cells from three VV-a1r198–stimulated T cell lines originating from different LCMV-immune mice produced IFN-γ in response to their cognate ligand, VV-a1r198, and also to LCMV peptides (Fig. 3A–C, Table I). However, each line contained T cell populations with different patterns of cross-reactivity. In one line 84% of the cells responded to LCMV-GP34 (Fig. 3A), in another 91% responded to LCMV-GP118 (Fig. 3B), and the third...
had some responsiveness to all three LCMV peptides (Fig. 3C). We had observed previously that VV infection of LCMV-immune mice can result in the activation of several LCMV epitope-specific memory populations, depending on the private specificity of the host TCR repertoires (9). However, we did not expect that one VV epitope could activate three (GP34, GP118, and NP205) distinct LCMV-specific memory populations, because these LCMV epitopes do not seem to generate cross-reactive T cell responses against each other during LCMV infection. For example, we examined many LCMV-NP205-stimulated lines derived from LCMV-immune mice, and such lines did not demonstrate cross-reactivity with other LCMV epitopes.

Although the cross-reactive epitope specificity of the CD8 T cell populations varied among VV-a11r198–specific lines derived from different individual LCMV-immune mice (Fig. 3A–C, Table II), they were highly reproducible among separate lines derived from the same LCMV-immune mouse. Fig. 4Ai–iii shows that each of three separate lines had a similar pattern of cross-reactivity when costaining with VV-a11r198 and LCMV-GP118 tetramers. In contrast, in a total of 10 lines, five VV-a11r198–specific CD8 T cell lines derived from different mice showed dominant cross-reactivity with LCMV-GP118, corresponding to >84% of each line responding to both peptides (Figs. 3A, 4A, Table II). However, the other five T cell lines showed strong cross-reactivity with LCMV-GP118, and three of these were also moderately reactive with LCMV-NP205 (Fig. 3B, 3C, Table II), and three of these were also moderately reactive with LCMV-NP205 (Fig. 3B, 3C, Table II). The CD8 T cells cross-reactive with VV-a11r198 and LCMV-GP118 were also directly detected in cells freshly isolated from LCMV-immune mice challenged with VV (Fig. 4B). CD8 T cell frequencies detected by tetramer staining directly ex vivo for VV-specific and cross-reactive responses were lower than those in cell lines, but still detectable, indicating the normal presence of cross-reactive cells in vivo. This is analogous to the generally low frequencies observed in vivo in most human virus-specific responses (Fig. 1C). Cell lines, however, provide a useful technique in both humans and mice for amplifying and characterizing these cross-reactive responses.

Costaining with two tetramers to define cross-reactive populations was not always a useful technique to demonstrate cross-reactive responses (Fig. 4Ci). For instance, in some cases the majority of the T cells in lines from LCMV-immune mice stimulated with VV-a11r stained with either VV-a11r198 (70%) or LCMV-GP118 tetramers (72%), indicating that many of the T cells were cross-reactive. However, when costaining with both tetramers simultaneously or even sequentially in this same culture, the LCMV-GP118 tetramer was noted to completely block VV-a11r198 tetramer binding, which declined from 70 to 0% (Fig. 4Ci). These results suggest that in this mouse these VV-a11r–specific cells are higher avidity to the original ligand, LCMV-GP118, which they first saw in vivo during LCMV infection, than to the stimulating VV peptide. In this same cell line, the LCMV-NP205 tetramer detected a low frequency of cross-reactive cells with VV-a11r but did not block the binding of the VV-a11r tetramer. In other VV-a11r–specific T cell lines generated from LCMV-immune mice, there was partial blocking of VV-a11r tetramer binding. For instance, costaining with both tetramers simultaneously showed that the LCMV-GP118 tetramer blocked ~5% of the VV-a11r tetramer binding (Fig. 3Ci). This competition between tetramers for TCR binding suggests that in these two particular cell lines the cross-reactive populations may bind the epitope (LCMV-GP118) specific for the first virus encountered with
higher avidity than the cross-reactive epitope (VV-a11r198) that reactivated some part of this memory population.

**History of virus infection influences pattern of VV-a11r198 cross-reactivity and reveals that VV intraviral cross-reactive T cell responses only become apparent in VV-immune but not in LCMV + VV-immune mice**

The private specificity of epitope-specific memory T cell repertoire development is proposed to be influenced by the stochastic processes of T cell selection in the thymus and by the random encounters of T cells with their ligands in the periphery (32). These processes also influence the pattern of VV-a11r–specific cross-reactive responses that predominate in each individual LCMV-immune host upon VV infection (9). The specific sequential order of virus infections is a third factor that could influence the pattern of VV-a11r198–specific cross-reactive responses. For example, cross-reactive T cell responses to the three LCMV epitopes were very low or absent in VV-a11r–specific cell lines generated from splenocytes of VV-immune mice (Fig. 3a). This indicates that the majority of VV-a11r–specific T cells generated during acute VV infection of naïve mice are not cross-reactive with LCMV epitopes and suggests that acute VV infection generates a different T cell repertoire in naïve mice compared with LCMV-immune mice. Having an enhanced precursor frequency of these cross-reactive clones in the LCMV-specific memory pool was apparently necessary for their amplification and detection during acute VV infection.

### Table II. Summary of adoptive transfer of cross-reactive (a11r198) and non-cross-reactive (e7r130) VV-specific CD8 T cell lines that enhanced VV clearance

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specificity</th>
<th>VV Titer (log PFU/ml) ± SEM (Organ)</th>
<th>% Reduction</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>a11r (98%), GP118 (89%), GP34 (15%), NP205 (0.5%)</td>
<td>2.56 ± 0.18 (testes) day 4</td>
<td>86.2</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>a11r (75%), GP118 (45%), GP34 (23%)</td>
<td>2.36 ± 0.33 (testes) day 4</td>
<td>91.3</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>a11r (98%), GP118 (89%), GP34 (15%), NP205 (0.5%)</td>
<td>3.42 ± 0.26 (testes) day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a11r (75%), GP118 (45%), GP34 (23%)</td>
<td>3.67 ± 0.10 (fat pads) day 4</td>
<td>84.2</td>
<td>0.049</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>a11r (98%), GP118 (89%), GP34 (15%), NP205 (0.5%)</td>
<td>3.44 ± 0.25 (fat pads) day 4</td>
<td>90.7</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>a11r (75%), GP118 (45%), GP34 (23%)</td>
<td>4.47 ± 0.20 (fat pads) day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 3</td>
<td>a11r (92%), GP118 (91%), GP118 (3%)</td>
<td>2.28 ± 0.38 (testes) day 4</td>
<td>90.5</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>a11r (85%), GP118 (76%), GP118 (9%), NP205 (3%)</td>
<td>3.07 ± 0.43 (testes) day 4</td>
<td>41.1</td>
<td>0.214</td>
</tr>
<tr>
<td></td>
<td>a11r (92%), GP118 (92%), NP205 (16%), GP34 (1%), NP366 (92%)</td>
<td>3.30 ± 0.22 (testes) day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 4</td>
<td>a11r (72%), GP118 (72%), GP118 (1%), NP205 (1%)</td>
<td>3.68 ± 0.04 (testes) day 4</td>
<td>91.3</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>a11r (92%), GP118 (92%), NP205 (16%), GP34 (1%), NP366 (92%)</td>
<td>4.74 ± 0.40 (testes) day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 5</td>
<td>c7r (93%)</td>
<td>2.88 ± 0.32 (testes) day 4</td>
<td>97.6</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>a11r (96%), GP118 (92%), NP205 (16%), GP34 (1%), NP366 (92%)</td>
<td>4.00 ± 0.39 (testes) day 4</td>
<td>67.6</td>
<td>0.376</td>
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<tr>
<td></td>
<td>IV/NP366-2 (IAT NP366-specific T cell line) n = 3</td>
<td>4.49 ± 0.07 (testes) day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 6</td>
<td>c7r (95%)</td>
<td>4.21 ± 0.20 (fat pads) day 3</td>
<td>92.6</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>NP366 (92%)</td>
<td>5.34 ± 0.17 (fat pads) day 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 7</td>
<td>e7r (92%)</td>
<td>3.58 ± 0.18 (fat pads) day 3</td>
<td>98.3</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>PBS n = 5</td>
<td>5.36 ± 0.14 (fat pads) day 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 8</td>
<td>c7r (95%)</td>
<td>4.16 ± 0.21 (testes) day 3</td>
<td>83.0</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>PBS n = 5</td>
<td>4.92 ± 0.36 (testes) day 3</td>
<td></td>
<td></td>
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</tbody>
</table>

*VV/VV/E7R (VV-e7r30), L/VV/A11R (a11r198), and LV/VV/A11R (a11r198) lines were generated from VV-immune, LCMV-immune or LCMV + VV-immune splenocytes, respectively, and were adoptively transferred into recipient mice and challenged with VV as described in Materials and Methods.*

*Fat pads or testes were harvested 3–4 d after VV infection and analyzed for VV titers by plaque assays.*

*Percentage reduction of VV load in the experimental groups as compared with the control group.*

*Specificity of each CD8 T cell line was determined in tetramer staining.*

*Specificity of each CD8 T cell line was determined in ICS assays.*
FIGURE 4. A, Pattern of cross-reactivity is similar among three different cultures from one individual mouse. Three individual VV-a11r198 cell lines (i, ii, iii) were generated from one LCMV-immune mouse and were stained with either one or two tetramers simultaneously. B, Presence of cross-reactive CD8 T cells ex vivo during VV infection (day 6) of LCMV-immune mice. Double-tetramer staining demonstrates the presence of cross-reactive CD8 T cells that recognize both VV-a11r198 and LCMV-GP34 as well as VV-a11r198 and LCMV-NP205 (gated on CD8 cells). C, Double-tetramer staining of two VV-a11r198-specific T cell lines generated from two different LCMV-immune mice. i, The majority of this 3 wk culture of a VV-a11r-specific T cell line bound either LCMV-GP34 or VV-a11r198 tetramers when stained separately. Costaining with both tetramers resulted in the LCMV-GP34 tetramer blocking binding of the VV-a11r tetramer. ii, In a second VV-a11r-specific T cell line, after a short-term 10 d culture, costaining experiments with LCMV-GP34 tetramer blocked ~5% of the VV-a11r tetramer binding (gated on CD8).
The VV-e7r130 epitope was not cross-reactive with LCMV, and VV-e7r–specific T cell lines could not be established by stimulating LCMV-immune splenocytes with VV-e7r130. Interestingly, some T cells in VV-a11r–specific lines generated from VV-immune splenocytes were cross-reactive with VV-e7r130 (Fig. 3F). Eighty-eight percent of a cell line cultured with VV-a11r198 peptide also responded to the VV-e7r130 peptide (Fig. 3E). This cross-reactivity was not completely reciprocal, because only 19% of a cell line derived from the same VV-immune mouse but cultured with VV-e7r130 peptide also responded to the VV-a11r198 peptide (Fig. 3F). As expected, the VV-e7r130 cell line generated from this VV-immune mouse did not react with any LCMV epitopes (Fig. 3F). By comparison, two different VV-a11r–stimulated cell lines derived from the splenocytes of mice that were immune to both LCMV and VV, given sequentially in that order, did not cross-react with VV-e7r130 but did cross-react with the three LCMV epitopes, GP34, GP118, and NP205 (Fig. 3D, Table II, LV/a11r-3 line). These data suggest that the VV-a11r198 memory repertoire resulting from the activation of cross-reactive LCMV-specific memory cells during acute VV infection of LCMV-immune mice did not include T cells cross-reactive with the VV-e7r130 epitope. Thus, T cell cross-reactivity between VV and LCMV is highly plastic, where VV-a11r–specific T populations contain subsets of cells with the potential to cross-react with five different epitopes derived from three viruses (LCMV, PV, and VV) and where the cross-reactive network that emerges in a given individual is influenced by the history of sequential virus infections, as diagrammed in Fig. 2B.

Cross-reactive CD8 T cell lines reduce VV titer in vivo

Immunity to LCMV is known to mediate partial protection against VV infection, and adoptive transfer studies using splenocytes from LCMV-immune mice demonstrated that T cells were mediating this effect (8). Despite a strong correlation between activation of cross-reactive memory CD8 T cells and protective heterologous immunity (7, 8), direct evidence that cross-reactive CD8 T cells proliferate in vivo in response to the heterologous virus and mediate protective heterologous immunity has been lacking. We therefore tested cross-reactive cell lines in vivo for their proliferation and manifestation of protective heterologous immunity. VV-a11r–specific cell lines generated from LCMV-immune splenocytes (Fig. 5) were labeled with CFSE and adoptively transferred i.p. into naive congenic mice, which then were infected with VV. These VV-a11r–specific CD8 T cells proliferated in response to both LCMV and VV infection but not to PV infection in vivo (Fig. 5B). Next, we tested VV-a11r–specific cell lines generated from different mice for their ability to reduce VV load. Regardless of whether they were generated from an LCMV-immune mouse (L/a11r-2, L/a11r-4, and L/a11r-6) or a mouse that had been infected with VV (LV/a11r-3), four of six VV-a11r–specific

**FIGURE 5.** Cross-reactive VV-a11r–specific CD8 T cells proliferate in vivo after LCMV or VV infection and reduce VV load. A, This is a representative VV-a11r–specific CD8 T cell line (L/a11r-4) derived from LCMV-immune splenocytes; it was predominantly cross-reactive with LCMV-GP34 but also recognized GP118 in an IFN-γ ICS assay. B, Cross-reactive VV-a11r–specific cell line proliferates in response to LCMV and VV but not to PV-infected or PBS-treated controls, as assessed by loss of CFSE label by day 3 after the simultaneous adoptive transfer and infection of congenic LY5.1 mice. C, Adoptive transfer of cross-reactive VV-a11r–specific CD8 T cell lines derived from an LCMV + VV-immune mouse (filled triangles, LV/a11r-3) or an LCMV-immune mouse (open triangles, L/a11r-2) led to a significant VV reduction compared with PBS controls (gray triangles). VV titer in the testes were assayed on day 4 post-infection (LV/a11r-3 versus PBS, p < 0.01; L/a11r-2 versus PBS, p < 0.05). D, Differential effect on VV titer upon the use of different cross-reactive VV-a11r–specific CD8 T cell lines, L/a11r-4 and L/a11r-5. There was a greater reduction of VV titer in testes day 4 postinfection in mice injected with L/a11r-4 (gray circles) compared with L/a11r-5 (open circles) (p < 0.08, n = 4) or PBS (filled circles) (p < 0.08, n = 4). E, Time course of weight loss after VV infection. Adoptive transfer of the T cell line L/a11r-4 resulted in a 50% inhibition of weight loss (day 2, 2.4 ± 0.6% versus 4.8 ± 0.7% weight loss, n = 4, p = 0.06; day 3, 2.3 ± 0.5% versus 4.5 ± 0.7% weight loss, n = 4, p = 0.08) after VV infection, whereas T cell line L/a11r-5 did not affect weight loss when compared with the control, PBS-injected mice (p > 0.3).
lines with high levels of cross-reactivity to LCMV-GP118 or LCMV-GP34 significantly decreased VV titers by an average of 89 ± 1% upon adoptive transfer into naive hosts (Fig. 5C, 5D, Table II). This level of protection was consistent, as demonstrated by using the same cell lines (L/a11r-2 and L/a11r-3) twice, in two separate experiments (Table II). Furthermore, these four cross-reactive VV-a11r–specific cell lines generated from LCMV-immune mice were found to be as effective as four putatively non-cross-reactive VV-e7r–specific cell lines generated from VV-immune mice (Table II).

Qualitative characteristics of a cross-reactive response may determine its effectiveness against VV

These well-characterized cross-reactive cell lines were useful for asking whether there may be qualitative differences in cross-reactive responses. For instance, two of the six VV-a11r–specific CD8 T cell lines, L/a11r-5 and L/a11r-7, generated from different individual LCMV-immune mice were poorly efficient at mediating protective immunity (Fig. 5D, Table II). Adoptive transfer of the line L/a11r-5 resulted in no protection from weight loss and only a 41% reduction in VV titers when compared with controls. This is in contrast to adoptive transfer of a protective line such as L/a11r-4, which resulted in inhibition of weight loss (Fig. 5E) and a significant 90% reduction in VV titers at day 4 of VV infection (Fig. 5D).

Variations in the degree of protective immunity against VV mediated by different T cell lines suggest that cross-reactive responses can be qualitatively different. We characterized the cross-reactive profiles of two cell lines by 1) estimating TCR avidity to VV-a11r198 and to the alternate LCMV epitopes using peptide titration in an intracellular cytokine assay and 2) assessing differences in TCR repertoire (Fig. 6). The two cell lines differed in their cross-reactive pattern, because the protective L/a11r-4 line was dominated by a VV-a11r198–specific CD8 T cell population that predominantly recognized LCMV-GP34 (91%), with 3% of the population recognizing GP 118, whereas the nonprotective L/a11r-5 line had broader cross-reactive specificity in that it recognized LCMV GP34 (76%), GP118 (9%), and NP205 (3%).

**FIGURE 6.** Two VV-a11r–specific cross-reactive CD8 T cell lines show different patterns of avidity to cross-reactive epitopes and different TCR repertoires. A and B, Titration of peptide concentrations in ICS assays using VV-a11r–specific lines L/a11r-4 and L/a11r-5. The indicated concentrations of VV-a11r198, LCMV-GP34, LCMV-GP118, and LCMV-NP205 peptides were used in a 4 h ICS assay to stimulate the production of IFN-γ and TNF-α by the VV-a11r–specific T cell lines L/a11r-4 or L/a11r-5, which were generated from two different LCMV-immune mice. The percentage of IFN-γ or IFN-γ”TNF-α” cells of total CD8+ cells is shown above each plot. The numbers above the upper quadrants represent the percent cytokine production (gated on CD8). The percentage of maximum cytokine response to each peptide concentration is graphed in B, C. Private specificity in TCR Vβ repertoire. TCR Vβ mRNA expression of the L/a11r-4 and L/a11r-5 T cell lines demonstrates different VV-a11r–specific Vβ repertoires in lines derived from two different LCMV-immune mice.
Cross-reactivity alters the immunodominance hierarchy during VV infection

The advantage of using a murine model is the ability to manipulate the sequence of virus infections in vivo and track the effects that cross-reactive responses have on both immunodominance hierarchies and protection. For instance, the hierarchy of VV epitope-specific CD8 T cell responses typically observed during VV infection of naive mice was altered in the presence of cross-reactive LCMV memory T cell responses. All of the naive mice infected with VV generated a VV-a11r198 response higher than that to VV-a11r98, and all examined LCMV-immune mice generated a weak response to VV-a11r198 but no response to VV-e7r130. Fig. 7A shows a representative experiment with an LCMV-immune mouse that had a change in immunodominance as reflected by an enhanced response to the VV-a11r198 epitope upon VV infection compared with a naive mouse. A similar change in the VV epitope immunodominance hierarchy was observed in 33% (three of nine) of the LCMV-immune mice examined in this experiment (Fig. 7B). In 8 of 21 mice examined overall in several experiments, there was enhanced expansion of VV-a11r198–specific cells. Thus, just as T cell lines derived from each mouse showed different patterns of cross-reactivity, individual mice showed a different pattern of cross-reactive proliferation in vivo. This altered the VV epitope immunodominance hierarchy and was apparently influenced by the private cross-reactive repertoire of the host.

Discussion

Cross-reactive CD8 T cell responses have been reported previously during viral infection, but their commonality and importance have not been appreciated (3–5, 12). The studies in this paper demonstrate that it is highly likely that T cell cross-reactivity may be the rule rather than the exception. We demonstrate, in both humans and mice, that cross-reactive T cell responses are so common that we can track populations of cells within complex well-defined networks where subsets of T cell populations reactive with one epitope cross-react with either of several other epitopes encoded by the same or heterologous viruses (Fig. 2A, 2B). Our data suggest that...
the specific Ags that are corecognized depend on a number of factors that include 1) the inherent structural characteristics of the epitopes, 2) the private specificity of the individual’s naive TCR repertoire, 3) the sequential order of infections, and 4) the resulting alterations in the private specificity of memory TCR repertoire with each new infection. Using a mouse model, we were able to study the direct impact of these cross-reactive responses in vivo. Immunodominance hierarchies could be altered in the presence of cross-reactive T cell responses (Fig. 7), and cross-reactive responses could impact disease outcome by enhancing viral clearance (Fig. 5, Table II). Our data also suggest that the qualitative characteristics of the cross-reactive T cell responses may relate to their effectiveness at providing protective immunity to future infections. This needs further study and could lead to a better understanding of which qualitative characteristics of T cell cross-reactivity (i.e., avidity, specific Vβ usage, and functional response to the ligands) maximize the effectiveness of cell-mediated immunity while minimizing immunopathology. This information would be particularly useful in designing vaccines. Of interest is that a large trial of a T cell-based vaccine against HIV used an adenovirus vector, and the individuals with prior immunity to adenovirus developed an increased incidence of HIV infection (34, 35). It is possible that heterologous immunity played some role in mediating this adverse outcome.

In this study, we demonstrated human cross-reactive T cell responses where subsets of T cells specific to one EBV epitope, BMLF1280-288, could cross-react with up to two IAV (M158 and NP58) and two other intraviral EBV epitopes (BRLF1190 and LMP2329) (Fig. 2A). In mice, we showed that subsets of T cell populations specific to the typically subdominant VV epitope, a11r198, had the potential to cross-react with five different immunogenic epitopes, LCMV-GP118, LCMV-GP34, LCMV-NP120, PV-NP120, and VV-e7F130, derived from three different viruses, LCMV, PV, and VV (Fig. 2B). We have no evidence that a single VV-a11r–specific CD8 T cell can recognize all five epitopes, because most of our data suggest that a single T cell may recognize two epitopes or three epitopes in the case where two of the epitopes are highly homologous like PV-NP120 and LCMV-NP120. Structural similarity does play a role in cross-reactive T cell responses. Although all five of these epitopes have ≤50% sequence similarity to VV-a11r198, they do share amino acids at positions 4, 6, and 7 (Table I). Structural studies of three different H2-Kβ/peptide interactions with TCRs have shown that side chains of amino acids at positions 4, 6, and 7 of the peptide are crucial for interaction with the TCR (36). These studies, plus the knowledge that an individual TCR can tolerate certain amino acid substitutions in the peptide sequence and still become activated (37), help explain why VV-a11r–specific CD8 T cells could potentially cross-react with five alternate epitopes. The fact that TCRs are reportedly able to undergo significant conformational changes upon binding to Ag gives them the capability to interact with diverse structures and enhances the occurrence of cross-reactive responses (38). Also a recent report systematically examined many known cross-reactive T cell responses, including all of our reported epitopes, and suggested that sequence similarity based on biochemically similar and not necessarily identical amino acids directly correlated with an increased likelihood of cross-reactivity (39). This may help to explain why epitopes with much less sequence similarity, such as EBV-BMLF1280 and IAV-M158 or EBV-BRLF1190 (Table I), can also stimulate cross-reactive T cell responses.

The ability to track and predict cross-reactive T cell responses could be useful in designing vaccines, in determining the order and combination of vaccines in children, and in predicting the outcome of disease postinfection in any particular individual. However, to achieve this there are other contributing factors that are identified in this study that increase the complexity and difficulty in predicting cross-reactive responses. This includes the diversity (40) and the private specificity of Ag-specific TCR repertoires (9, 23, 32, 41–43) as well as the resulting qualitative differences in cross-reactive responses depending on the cross-reactive clone. Individual LCMV-immune mice mounted complicated VV-a11r198 responses that cross-reacted with LCMV epitopes (GP118, GP118, and NP205) to varying extents (Fig. 3A–C). Isolating and identifying highly reproducible dominant cross-reactive responses thus far has not been common, although not many studies have directly focused on identifying cross-reactive responses as was done in the current study in the context of sequential heterologous virus infections. Awareness of the variability in the sequence of virus infections and the private specificity of an individual’s TCR repertoire, which plays a major role in determining cross-reactive patterns, can assist investigators in tracking dominant cross-reactive T cell responses, as we have done in both mice and humans (Fig. 2A, 2B). It should be noted that functionally relevant cross-reactive responses are more easily detected within memory T cell populations compared with naive, because memory T cells are present at a much higher frequency and exist in an enhanced activation state (44–46). Thus, for all of these reasons trying to study T cell cross-reactivity using the conventional technique of deriving high-avidity T cell clones that are highly selected with repeated stimulation with one Ag may underestimate the prevalence of cross-reactive T cell responses.

To best control for variables caused by private specificity patterns and the fact that we may be only observing a subset of potential cross-reactivity patterns, we decided that well-characterized cell lines were the best approach to directly test whether cross-reactive CD8 T cell responses could mediate protective immunity. When we transferred LCMV-immune splenocytes from one mouse into three naive congenic hosts, all three mice expand the same LCMV epitope-specific population upon VV infection, but patterns of expansion differ among donors. Consistent with the in vivo adoptive transfer model, cultures from 10 different mice showed different patterns of cross-reactivity whereas three cultures from the same mouse were similar (9). Thus, by transferring the expanded well-characterized cross-reactive T cell lines into a host, we can monitor their proliferation and influence on virus infection in vivo. In this study, we directly demonstrate for the first time that cross-reactive T cell lines can enhance viral clearance (Fig. 5, Table II).

In general, the efficiency of viral clearance by activated T cells is at least partially dependent on the avidity of the TCR interaction with its ligand. Studies with altered peptide ligands, an artificial model of cross-reactivity, show that high- and low-potency ligands differ in the length of time that the TCR interacts with MHC/ligand (i.e., TCR avidity) and that this can modify the functional potential of individual clones (47, 48). Thus, a low-avidity TCR interaction with an alternate ligand may induce different cytokines or a different hierarchy of cytokine production that results in less efficient killing and proliferation than T cells interacting with their original ligand with presumably higher avidity. This may help to explain why a cross-reactive T cell line (Lα11r-5) that interacted with a VV epitope with lower avidity had an altered cytokine profile killing and proliferation than T cells interacting with their original ligand. Studies with altered peptide ligands, an artificial model of cross-reactivity, show that high- and low-potency ligands differ in the length of time that the TCR interacts with MHC/ligand (i.e., TCR avidity) and that this can modify the functional potential of individual clones (47, 48). Thus, a low-avidity TCR interaction with an alternate ligand may induce different cytokines or a different hierarchy of cytokine production that results in less efficient killing and proliferation than T cells interacting with their original ligand with presumably higher avidity. This may help to explain why a cross-reactive T cell line (Lα11r-5) that interacted with a VV epitope with lower avidity had an altered cytokine profile and did not protect as well against a heterologous VV challenge (Figs. 5, 6) or why heterologous immunity may not be as effective at mediating protective immunity as homologous immunity.

It is highly likely that just the existence of a cross-reactive T cell response does not lead to protective effects. In some cases, cross-reactive responses may lead to immunopathology, as shown in murine models (7, 8) and also observed in human infections (4). Many factors, such as TCR avidity and the functional response (e.g.,
cytokine production or cytotoxic capability) to alternate epitopes, influence the effectiveness of a cross-reactive response. This is a subject about which we still have a great deal to learn and suggest that future studies should focus on identifying the characteristics of cross-reactive T cells that lead to better protective immunity. T cell responses can sometimes be detrimental to the host and mediate significant immunopathology. LCMV is a classic example where the same T cells responsible for viral clearance mediate severe leptomeningitis if the virus is replicating in the brain (49). Whether disease outcome is subclinical or severely symptomatic with as-leptomeningitis if the virus is replicating in the brain (49). Whether disease outcome is subclinical or severely symptomatic with as-leptomeningitis if the virus is replicating in the brain (49). Whether disease outcome is subclinical or severely symptomatic with as-leptomeningitis if the virus is replicating in the brain (49). Whether disease outcome is subclinical or severely symptomatic with as-leptomeningitis if the virus is replicating in the brain (49). Whether disease outcome is subclinical or severely symptomatic with a host’s unique immune repertoire, and the complex plasticity of T cell cross-reactivity described in this paper highlights the pervasiveness of this phenomenon and our ability to trace these patterns. Ultimately, our ability to track and characterize these flexible cross-reactive networks within and between individuals may help us to design useful vaccine strategies and perhaps even predict and prevent immunopathology during viral infection.

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