JCI The Journal of Clinical Investigation

Cross-reactive influenza virus–specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus–associated infectious mononucleosis

Shalyn C. Clute, … , Raymond M. Welsh, Liisa K. Selin

J Clin Invest. 2005;115(12):3602-3612. https://doi.org/10.1172/JCI25078.

Research Article **Immunology**

The marked proliferation of activated $CD8^+$ T cells is pathognomonic of EBV-associated infectious mononucleosis (IM), common in young adults. Since the diversity and size of the memory $CD8^+$ T cell population increase with age, we questioned whether IM was mediated by the reactivation of memory $CD8^+$ T cells specific to previously encountered pathogens but cross-reactive with EBV. Of 8 HLA-$A2^+$ IM patients, 5 had activated T cells specific to another common virus, as evidenced by a significantly higher number of peripheral blood influenza A virus $M1_{58–66}$–specific T cells compared with healthy immune donors. Two patients with an augmented M1 response had tetramer-defined cross-reactive cells recognizing influenza M1 and EBV-$BMLF1_{280–288}$, which accounted for up to one-third of their BMLF1-specific population and likely contributed to a skewed M1-specific T cell receptor repertoire. These epitopes, with only 33% sequence similarity, mediated differential effects on the function of the cross-reactive T cells, which may contribute to alterations in disease outcome. EBV could potentially encode an extensive pool of T cell epitopes that activate other cross-reactive memory T cells. Our results support the concept that cross-reactive memory $CD8^+$ T cells activated by EBV contribute to the characteristic lymphoproliferation of IM.

Cross-reactive influenza virus–specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus–associated infectious mononucleosis

Shalyn C. Clute,[1,2] Levi B. Watkin,[1,2] Markus Cornberg,[1] Yuri N. Naumov,[1] John L. Sullivan,[3,4] Katherine Luzuriaga,[3,4] Raymond M. Welsh,[1] and Liisa K. Selin[1]

[1]Department of Pathology, [2]Program in Immunology and Virology, [3]Department of Pediatrics, and [4]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

The marked proliferation of activated CD8+ T cells is pathognomonic of EBV-associated infectious mononucleosis (IM), common in young adults. Since the diversity and size of the memory CD8+ T cell population increase with age, we questioned whether IM was mediated by the reactivation of memory CD8+ T cells specific to previously encountered pathogens but cross-reactive with EBV. Of 8 HLA-A2+ IM patients, 5 had activated T cells specific to another common virus, as evidenced by a significantly higher number of peripheral blood influenza A virus $M1_{58–66}$–specific T cells compared with healthy immune donors. Two patients with an augmented M1 response had tetramer-defined cross-reactive cells recognizing influenza M1 and EBV-$BMLF1_{280–288}$, which accounted for up to one-third of their BMLF1-specific population and likely contributed to a skewed M1-specific T cell receptor repertoire. These epitopes, with only 33% sequence similarity, mediated differential effects on the function of the cross-reactive T cells, which may contribute to alterations in disease outcome. EBV could potentially encode an extensive pool of T cell epitopes that activate other cross-reactive memory T cells. Our results support the concept that cross-reactive memory CD8+ T cells activated by EBV contribute to the characteristic lymphoproliferation of IM.

Introduction

There is a high degree of individual variation in disease severity associated with human virus infections, and age is one of many factors that can contribute to such variation. Childhood infection with EBV is often subclinical while the same infection is frequently symptomatic in adolescents and adults and presents as infectious mononucleosis (IM). The 3 classic criteria for IM diagnosis are the following: (a) lymphocytosis, the marked expansion of lymphocyte numbers in the peripheral blood caused by the proliferation of EBV-specific CD8+ T cells; (b) clinical symptoms that include fever, pharyngitis, and lymphadenopathy; and (c) a positive serologic test (1, 2). IM can vary in duration from a few weeks to 6 months, and the symptoms can vary in severity (3). Complications, such as pneumonia and fulminant hepatitis, are more common in older adults and have been linked to the infiltration of activated T cells and EBV-infected B cells into these tissues (4–6). When comparing IM and asymptomatic cases of acute EBV infection, Silins et al. found that the magnitude of the CD8+ T cell response, not viral load, correlated with the presence of disease (7). Furthermore, treatment of IM patients with antiviral drugs, although decreasing viral load, did not have any effect on the disease course (8, 9). These data suggest that a massive CD8+ T cell response can be counterproductive and mediate the disease pathology. It is still unclear why this massive CD8+ T cell proliferation occurs more frequently in older individuals.

Based on animal models of heterologous immunity that showed that T cells specific to a previously encountered virus may enhance immunopathology during a second, unrelated virus infection and based on the increasing number of reports documenting virus-specific CD8+ T cell cross-reactivity, we hypothesized that cross-reactive memory T cells specific to previously encountered pathogens contribute to the lymphoproliferation characteristic of EBV-associated IM (10–15). In support of this, there is well-documented evidence that at least a proportion of the CD8+ T cells activated by EBV can have alternative specificities for allogeneic MHC molecules, self peptides, and bacterial antigens (16–19). Testing this hypothesis is challenging due to individual variation in HLA allele expression, the history of infections, and the private specificity of the responding T cell repertoire (20–22).

In order to optimize our chances of detecting whether cross-reactive T cells were contributing to the EBV-induced CD8+ T cell response during IM, we focused our studies on individuals with a common HLA allele, A*0201, and their responses to a commonly encountered virus, influenza A. The majority of the world's population, starting at a young age, are repeatedly infected with influenza virus A. In almost all HLA-A2+ individuals, the CD8+ T cell response to influenza A virus is focused on an immunodominant epitope, $M1_{58–66}$, which is derived from the matrix protein (23). This protein is well conserved among different virus strains, ensuring the maintenance of M1-specific T cells in an individual's mem-

Nonstandard abbreviations used: Cβ, constant region of TCR β-chain; CDR3, complementarity-determining region 3; EC_{50}, effective concentration eliciting 50% of the maximum response; IM, infectious mononucleosis; Jβ, joining region of TCR β-chain; LCMV, lymphocytic choriomeningitis virus; MIP-1β, macrophage inflammatory protein-1β; Vβ, variable region of TCR β-chain; VV, vaccinia virus.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **115**:3602–3612 (2005). doi:10.1172/JCI25078.

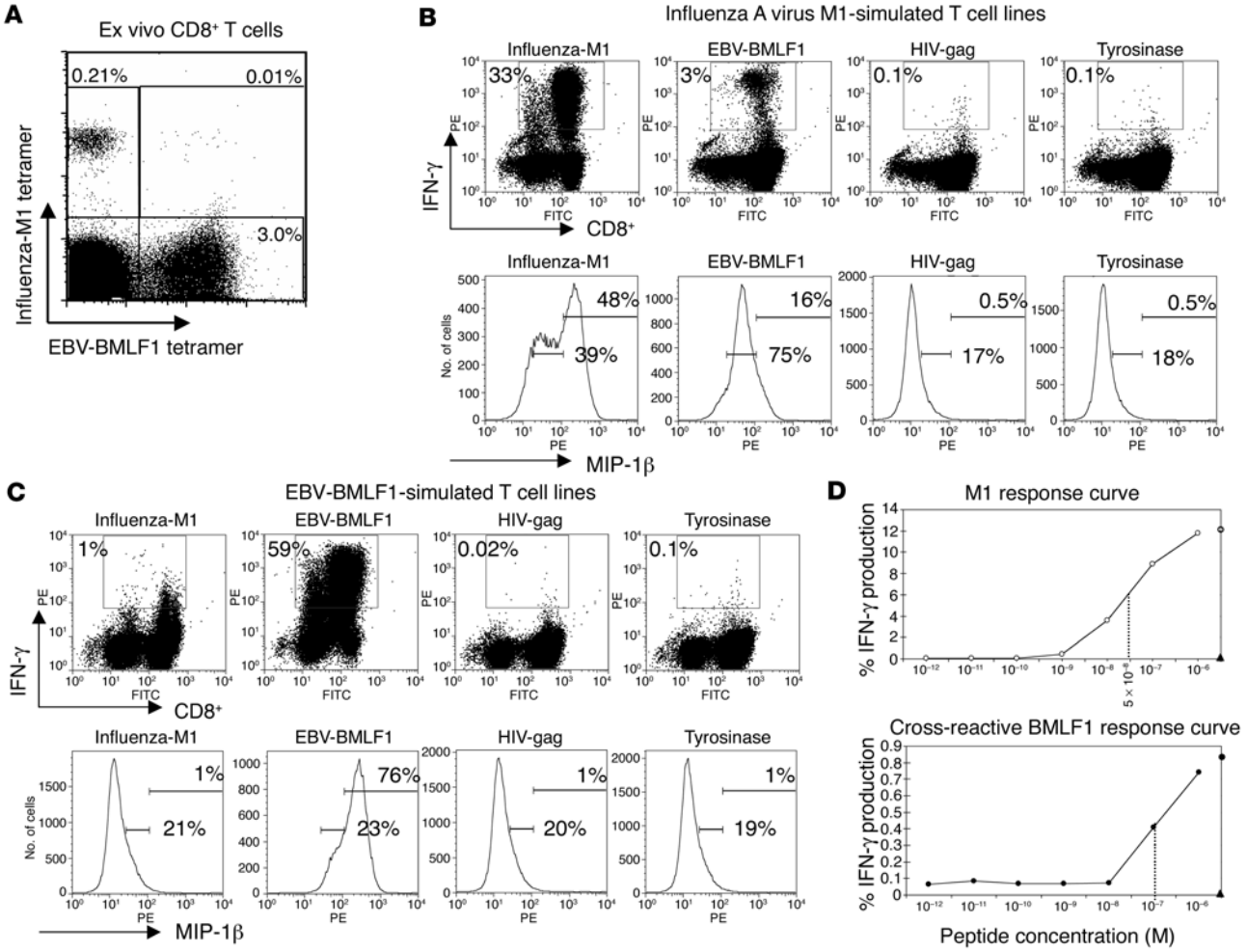

Figure 1

T cell lines grown in the presence of 1 peptide can respond to stimulation with a second unrelated peptide. (**A**) CD8+ T cells were isolated ex vivo from healthy donor D-002 and costained with M1- and BMLF1-loaded tetramers; 10^6 events were collected. (**B** and **C**) Fresh CD8+ T cell lines derived from donor D-002 were grown for 3–4 weeks in the presence of (**B**) M1 peptide–pulsed or (**C**) BMLF1 peptide–pulsed T2 cells and then stained intracellularly for the production of IFN-γ or MIP-1β following 5 hours of stimulation with various HLA-A2–restricted peptides at a 5 μM final concentration. Percentages of CD8+ T cells producing each cytokine are shown. (**D**) Titration of peptide concentrations in an intracellular IFN-γ assay using an M1-specific T cell line derived from donor D-002 demonstrated a slight difference in avidity for M1 versus BMLF1. Filled triangles, tyrosinase; open circles, M1; and filled circles, BMLF1 stimulation.

ory pool. While there is significant individual variation regarding which EBV antigen(s) drive a dominant CD8+ T cell response, we focused on the response to $BMLF1_{280–288}$, an immunodominant epitope within an early lytic protein that is also consistently recognized by all HLA-A2+ individuals during the acute response (24–26). In this study, we detected a cross-reactive response with specificity for these 2 dissimilar epitopes, influenza M1 and EBV-BMLF1, in bulk T cell cultures and at the clonal level, and we demonstrate that these cross-reactive cells participate in the characteristic CD8+ T cell–mediated pathology observed during acute IM.

Results

Maintenance of cross-reactive CD8+ T cells in the memory pools of healthy donors. Using ex vivo tetramer staining, we found a small subset of T cells in a healthy immune donor that recognized both influenza M1 (GILGFVFTL) and EBV-BMLF1 (GLCTLVAML) epitopes (Figure 1A). In order to further verify this observation and enhance the detection of cross-reactive T cells, we cultured CD8+ T cell lines in the presence of either M1 or BMLF1 peptide, and their specificity was then tested with an intracellular stain for the production of IFN-γ. As expected, a large proportion (33%) of an M1-specific T cell line derived from donor D-002 was able to produce IFN-γ following M1 stimulation (Figure 1B). However, a subset (3%) of this same M1-specific line were able to produce a high level of IFN-γ following BMLF1 stimulation (Figure 1B). The IFN-γ production in response to BMLF1 was considered antigen specific because stimulation with HLA-A2–presented peptides derived from HIV-gag or tyrosinase, a self antigen, resulted in very little IFN-γ production. In the case of donor D-002, a putatively cross-reactive subset of cells was also detected within the BMLF1-specific CD8+ T cell line (Figure 1C). Not only did the majority (59%) of the line produce IFN-γ following BMLF1 stimulation, but at least 1% of the

line produced a low level of IFN-γ following M1 stimulation. Table 1 summarizes the results of several intracellular IFN-γ stains on both M1- and BMLF1-specific T cell lines derived from 8 healthy donors with previous exposure to EBV and, presumably, influenza A virus. Using this technique, we were able to detect cytokine production to the heterologous peptide over background levels in cell lines from 3 out of 8 healthy donors.

The observation that a greater proportion of the M1-specific population compared with the BMLF1-specific population were cross-reactive T cells in the blood (5% of M1-tetramer$^+$ cells versus 0.3% of BMLF1-tetramer$^+$ cells, Figure 1A) and in culture (3% of an M1-specific line versus 1% of a BMLF1-specific line, Figure 1, B and C) prompted us to compare the relative avidities of the cross-reactive interaction with M1 versus BMLF1. To estimate TCR avidity, we performed an intracellular IFN-γ assay, using a peptide titration, on an M1-specific T cell line derived from donor D-002. A concentration of 5×10^{-8} M of M1 peptide compared with 10^{-7} M of BMLF1 peptide resulted in about half of the maximum amount of IFN-γ produced by this M1-specific T cell line (effective concentration eliciting 50% of the maximum response [EC_{50}]) (Figure 1D). Thus, the avidity for these 2 epitopes was slightly different, but both were within the avidity range previously reported for M1-specific and BMLF1-specific T cell clones using IFN-γ production or cytotoxicity as the readout (27, 28). Although the stronger M1

Table 1
CD8$^+$ T cell lines from multiple healthy donors responding to M1 and BMLF1 stimulation

Donor	PMA+ionomycin	M1 (influenza)	BMLF1 (EBV)	gag (HIV)	Tyrosinase	No peptide
Intracellular IFN-γ production						
Influenza-M1–stimulated T cell lines[A]						
D-002	56.9	33.2	**2.8**	0.1	0.1	0.1
D-012	78.9	23.5	**1.1**	ND	0.5	0.1
D-042	94.9	17.1	**1.2**	0.5	0.6	0.4
D-035	45.0	11.9	0.6	ND	0.6	0.5
D-044	70.2	2.4	1.9	0.2	0.2	0.1
D-045	8.6	9.2	1.3	ND	1.7	0.8
D-046	72.4	38.0	0.2	0.1	0.1	0.2
D-048	16.1	7.5	0.3	0.6	0.5	0.2
EBV-BMLF1–stimulated T cell lines						
D-002	76.3	**1.0**	59.0	0.0	0.1	0.0
D-012	76.9	**0.4**	35.7	ND	0.1	0.1
D-042	70.4	**5.5**	63.8	0.2	0.3	0.1
D-035	18.5	0.6	20.2	ND	0.3	0.2
D-044	93.8	0.6	67.6	0.2	0.6	0.5
D-045	NA	NA	NA	NA	NA	NA
D-046	81.1	0.2	38.5	0.1	0.1	0.1
D-048	27.0	0.4	14.0	0.5	0.5	0.4
Control T cell lines grown with unpulsed T2 cells						
D-002	50.2	0.1	0.8	0.1	0.1	0.0
D-012	94.9	0.0	0.0	ND	ND	0.0
D-042	95.9	0.2	0.2	0.3	0.3	0.2
D-035	23.6	1.6	0.2	ND	0.2	0.2
D-044	64.5	0.3	1.9	0.1	0.1	0.0
D-045	29.1	0.2	0.2	ND	0.2	0.1
D-046	78.5	0.1	0.3	0.1	0.1	0.0
D-048	7.5	0.1	0.1	0.1	0.0	0.1
Intracellular MIP-1β production						
Influenza-M1–stimulated T cell lines[A]						
D-002	73.9	48.1	**15.9**	0.5	0.5	0.9
D-046	68.5	43.4	6.0	4.9	5.8	8.9
D-048	14.2	9.0	3.3	4.4	3.7	3.3
EBV-BMLF1–stimulated T cell lines						
D-002	87.4	1.3	76.3	1.2	1.2	1.6
D-046	76.4	5.1	50.4	3.5	4.1	6.3
D-048	28.8	3.1	20.4	4.4	4.3	3.4
Control T cell lines grown with unpulsed T2 cells						
D-002	72.4	0.3	0.3	0.4	0.4	0.3
D-046	70.1	2.6	2.5	2.3	2.5	3.1
D-048	6.6	0.5	0.5	0.7	0.6	0.5

[A]T cell lines were grown for a minimum of 3 weeks. Numbers indicate the percentage of CD8$^+$ T cells that produce cytokine in response to the respective stimuli. ND, not determined; NA, no cell line available. Bold numbers indicate that the percentage of a given T cell line, cultured with peptide, responding to an unrelated peptide stimulation was greater than either the percentage of that same T cell line responding to negative control peptides or the percentage of the control T cell line, cultured without peptide, responding to the same unrelated peptide stimulation.

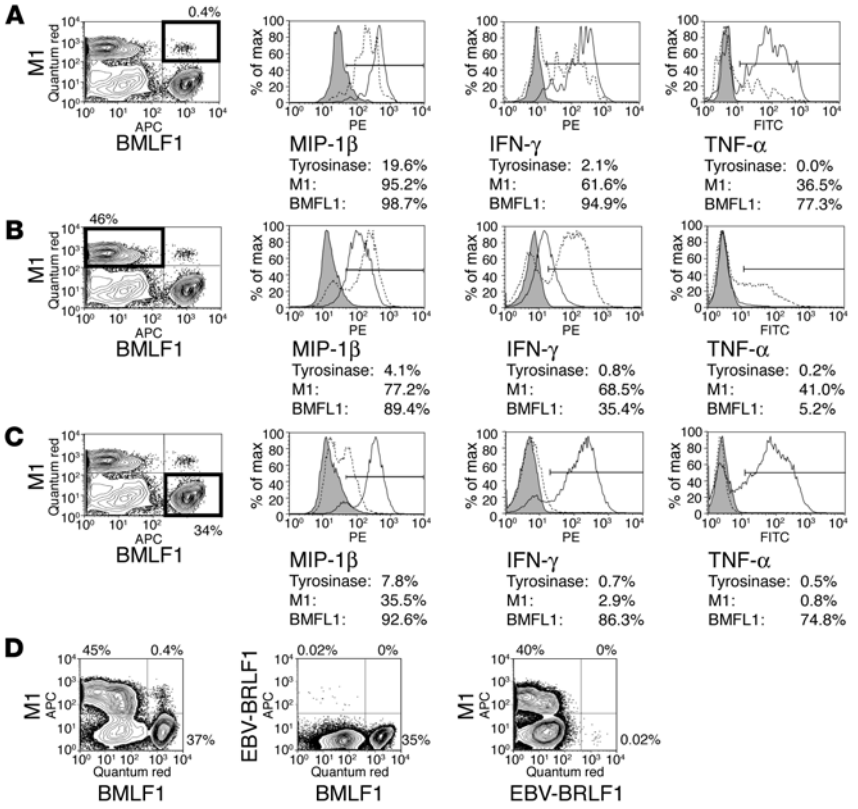

Figure 2

Culturing with M1 and BMLF1 peptides simultaneously promotes the growth of cross-reactive cells. A CD8+ T cell line derived from healthy donor D-002 was grown for 4 weeks in the presence of both M1 and BMLF1 peptide–pulsed T2 cells. Cells were stimulated for 5 hours with various peptides and then stained extracellularly with tetramers and intracellularly for the production of MIP-1β, IFN-γ, and TNF-α. We gated (indicated by a bold box) on (**A**) the percentage of CD8+ T cells that costained with both M1- and BMLF1-loaded tetramers, (**B**) the percentage of CD8+ T cells that stained with only M1-loaded tetramer, and (**C**) the percentage of CD8+ T cells that stained with only BMLF1-loaded tetramer. We then assessed the cytokine production of those cells in response to the following peptide stimulations: tyrosinase (gray profiles), M1 (dotted lines), and BMLF1 (solid lines). The percentage of CD8+ T cells producing each cytokine within the positive gate (horizontal lines) drawn is shown below each of the corresponding histograms. (**D**) This T cell line was stained extracellularly with EBV-BRLF1–loaded tetramer as a control.

stimulus may have been more efficient at promoting the growth of cross-reactive cells, it is likely that the cross-reactive population growing in the M1-specific T cell line is composed of a distinctly different subset of T cell clones than that growing in a BMLF1-specific T cell line. In support of this, the same peptide titration assay was performed using the BMLF1-specific T cell line, and this resulted in a cross-reactive population with a much greater discrepancy between the avidities for these 2 epitopes. Interestingly, the EC_{50} in response to the cross-reactive M1 stimulation was 10^{-9} M compared with 5×10^{-7} M in response to BMLF1 stimulation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI25078DS1). Thus, the cross-reactive cells grown in the presence of only BMLF1 peptide had an even higher avidity for the M1 epitope than did the cross-reactive cells grown in the presence of M1 peptide. The stimulating antigen used in culture appeared to drive differences in the clonal composition of M1- and BMLF1-specific T cell lines derived from the same donor, and this likely explains the lack of reciprocity in the frequency of the cross-reactive cells found within them.

Cross-reactive stimulation also resulted in the production of macrophage inflammatory protein-1β (MIP-1β), an antiviral C-C (β) chemokine. Nearly every cell of an M1-specific line derived from donor D-002 was able to produce some level of MIP-1β following stimulation with BMLF1 but, importantly, not in response to stimulation with HIV-gag or tyrosinase (Figure 1B). The production of MIP-1β, therefore, appeared to be more sensitive than the production of IFN-γ for the measurement of cross-reactivity in lines that had IFN-γ–producing cross-reactive T cells. In cell lines that did not have IFN-γ–producing cross-reactive T cells, no MIP-1β was produced in response to cross-reactive stimulation, suggesting that the protocol used here did not support the outgrowth of cross-reactive cells from the CD8+ T cell populations of donors D-046 and D-048 and that the production of MIP-1β was specifically induced by TCR engagement with antigen (Table 1). Thus, MIP-1β production was a more sensitive measurement of cross-reactivity than IFN-γ production, but this required cross-reactive cells to be present. There was no detectable cross-reactive MIP-1β production in the BMLF1-specific line derived from donor D-002, which again likely reflects differences in the clonotypic composition of this BMLF1-driven T cell line compared with the M1-driven T cell line (Figure 1C).

Breadth and quality of cross-reactivity revealed through alternative techniques. The growth of cross-reactive cells specific to both M1 and BMLF1 improved when we cultured CD8+ T cells with both peptides simultaneously. The frequency of cells that costained with both M1- and BMLF1-loaded tetramers increased to a range of 0.3–1.1%. The cross-reactive cells that bound both tetramers were also able to respond functionally to both epitopes. They produced MIP-1β, IFN-γ, and TNF-α specifically following either M1 or BMLF1 stimulation, but BMLF1 stimulation appeared to result in a more robust production of all 3 cytokines (Figure 2A). However, a peptide titration assay revealed that these $M1^+$ $BMLF1^+$ cross-reactive T cells actually had a slightly higher avidity for the M1 epitope. The EC_{50} of the cross-reactive response to the M1 peptide was 10^{-8} M compared with 10^{-7} M in response to the BMLF1 peptide (Figure 3A). The more robust functional response to BMLF1 initially observed using a 5 μM concentration of peptide appeared to be an effect of significant TCR downregulation, which decreased the sensitivity of M1-tetramer binding.

The tetramer-based frequency of cross-reactive cells within this T cell line was lower than the frequency based on function. The subset of cells only able to bind the M1-loaded tetramer produced MIP-1β and IFN-γ but very little TNF-α in response to BMLF1

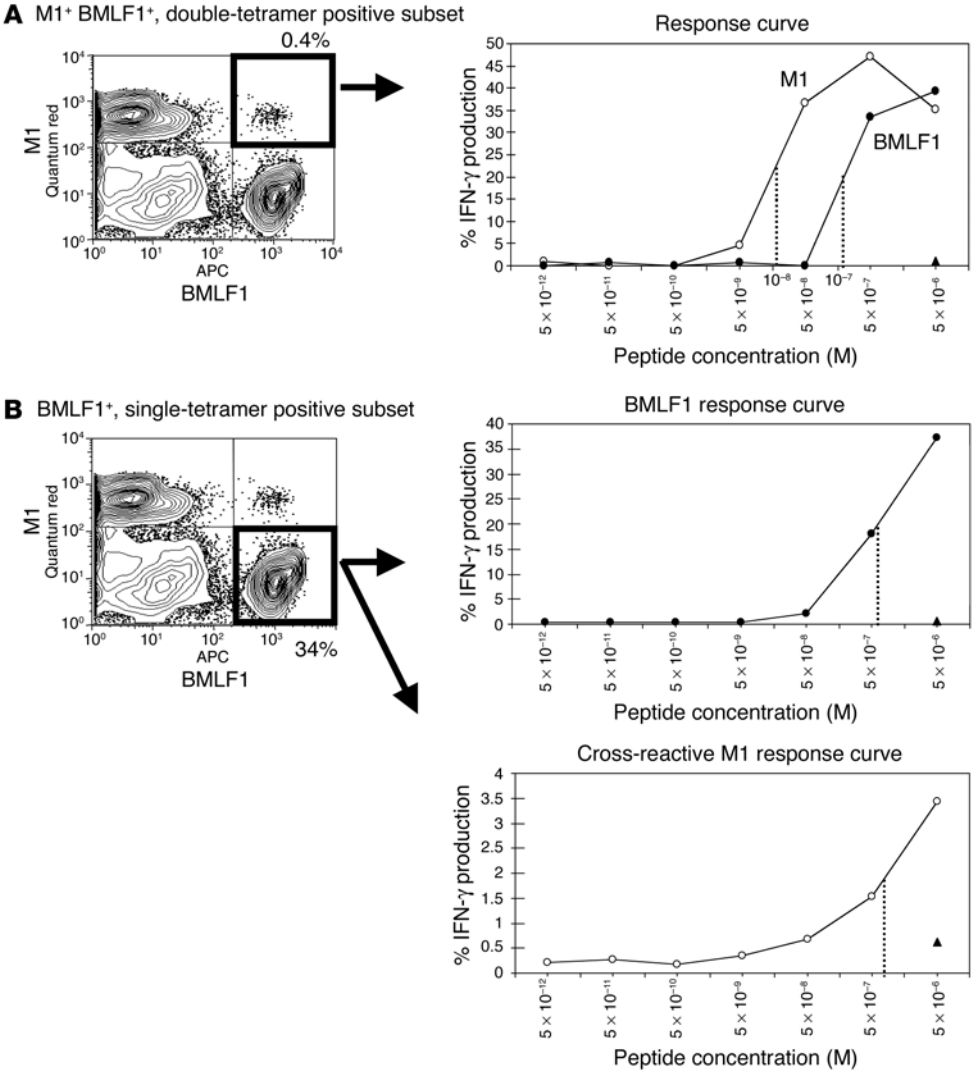

Figure 3
Tetramer-defined subsets of cross-reactive T cells differ in their avidity for the 2 epitopes. A similar intracellular IFN-γ assay was performed on the same T cell line described in Figure 2, which had been grown in the presence of M1 and BMLF1 peptides for 4 weeks, using a titration of peptide concentrations. Filled triangles, tyrosinase; open circles, M1; and filled circles, BMLF1 stimulation. We assessed the IFN-γ production of gated, tetramer-defined subsets of the T cell line: (**A**) M1+ BMLF1+ and (**B**) M1− BMLF1+.

stimulation (Figure 2B). The cells that bound only BMLF1-loaded tetramer also showed some degree of functional cross-reactivity. At least 35% of the BMLF1+ cells produced a low level of MIP-1β in response to M1 stimulation although this cross-reactive stimulation was not as efficient at inducing IFN-γ or TNF-α production (Figure 2C). A separate peptide titration experiment on this single BMLF1 tetramer–positive subset revealed that the cross-reactive cells present in this subpopulation had a similar avidity for the M1 and BMLF1 epitopes, where the EC_{50} was 5×10^{-7} M of either peptide (Figure 3). Overall, it would appear that how a cross-reactive T cell interacts with its alternative ligand is highly variable and that cross-reactive T cell populations are indeed heterogeneous. Hence, multiple techniques are required to detect T cell cross-reactivity, including tetramer staining and different functional assays. As shown here, TCR avidity is an important factor to consider when detecting cross-reactive T cell responses. An interaction between a cross-reactive T cell and its alternative ligand may be too weak to stably bind tetramer but may still be sufficient to induce a distinct hierarchy of cytokine production. This is analogous to the observation that certain non–cross-reactive influenza M1–specific clones were unable to bind M1-loaded tetramers but produced IFN-γ following M1 peptide stimulation (29).

Cross-reactivity at a clonal level. We next sought to clone these cross-reactive cells from a polyclonal T cell line, using the experimental design outlined in Figure 4A. Briefly, we allocated single T cells that costained with both M1- and BMLF1-loaded tetramers into microwells. The single cells were propagated for 2 weeks (referred to as clones from here on) and then assessed for functional specificity. As expected from the T cell line data, there was tremendous variability in the functional characteristics of each clone in response to either antigen. Of all the clones that grew, 8% produced IFN-γ following stimulation with either M1 or BMLF1 (Figure 4B). Similarly, 11% of the different clones analyzed killed both M1- and BMLF1-pulsed target cells in a ^{51}chromium release cytotoxicity assay (Figure 4C). The number of functionally cross-reactive clones varied with the technique used for their detection and reflected a similar ratio of BMLF1-responders to M1-responders (4:1) as seen in the assessment of IFN-γ production by the double-tetramer+ population within a polyclonal T cell line (1.5:1) (Figure 2A). These results definitively show that individual T cell clones can recognize and respond to both M1 and BMLF1, 2 epitopes that share little sequence similarity.

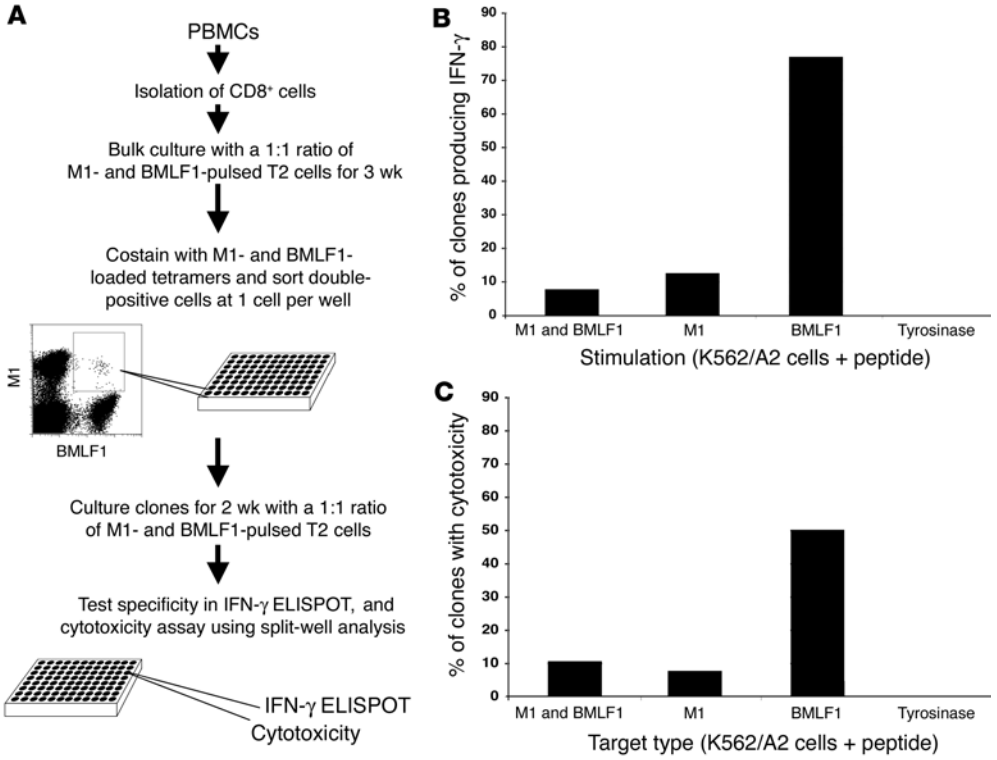

Figure 4
Cross-reactive clones are heterogeneous in their response to M1 versus BMLF1. (**A**) An outline of the experimental design used to clone cross-reactive T cells from healthy donor D-002 is shown. (**B**) $CD8^+$ T cells incubated for 20 hours with either M1-, BMLF1-, or tyrosinase-pulsed K562/HLA-A2 cells. The percentages shown represent the number of wells harboring cells that produced IFN-γ following stimulation with both M1 and BMLF1 or following stimulation with only 1 of the peptides. (**C**) $CD8^+$ T cells incubated for 8 hours with either M1-, BMLF1-, or tyrosinase-pulsed K562/A2 cells. The percentages shown represent the number of wells harboring cells that killed both M1- and BMLF1-pulsed target cells or that killed only 1 target cell type in a ^{51}chromium release assay. Data presented here accurately represent the trends observed in 3 separate experiments.

Cross-reactive cells participate in the lymphoproliferation that defines IM syndrome. Since we were able to detect cross-reactive cells that recognize M1 and BMLF1 in bulk culture and at a clonal level, we next determined whether these cross-reactive cells participated in the overzealous $CD8^+$ T cell response that defines EBV-associated IM. We noticed that, despite the large expansion of EBV-specific cells, the frequency (as percentage of $CD8^+$ T cells) of M1-specific cells in IM patients (mean, 0.20%; range, 0.02–0.49%) was similar to that in healthy influenza-immune donors (mean, 0.26%; range, 0.09–0.79%). The maintenance of a resting-state frequency suggested that at least a subset of M1-specific cells were proliferating in response to infection because non–cross-reactive memory cells should be diluted out by the proliferation of virus-specific cells. In fact, the average number of M1-specific cells (per ml of blood) was a very significant 4-fold higher in IM patients (0.004×10^6 per ml) compared with healthy donors (0.001×10^6 per ml) ($P = 0.02$) (Figure 5A). Of 8 IM patients, 5 had a higher than average number of M1-specific cells at presentation, and this number decreased over the course of the infection with contraction kinetics similar to the BMLF1 response and overall lymphoproliferation (Figure 5, B–D). These observations suggested that the M1-specific population within each of these 5 IM patients contained T cells that were cross-reactive with an EBV-derived antigen.

We next costained freshly isolated $CD8^+$ T cells from IM patients with M1- and BMLF1-loaded tetramers. Double-positive cross-reactive cells were prominent in 2 patients, E1101 and E1178 (Figure 6B and Supplemental Figure 2). The percentage as well as the total number of cross-reactive cells shifted with this active infection, including a considerable increase at day 22 (0.003×10^6 $M1^+$ $BMLF1^+$ cells per ml of blood) after presentation of patient E1101's clinical symptoms (Figure 6, A and B). This translated to as many as 1/3 of the T cells specific to the immunodominant BMLF1 epitope being cross-reactive with M1. Fewer tetramer-defined cross-reactive T cells were detected during patient E1178's infection, but there was a discernable increase in frequency at days 12 and 34 (0.0002×10^6 $M1^+$ $BMLF1^+$ cells per ml of blood) after presentation with IM (Supplemental Figure 2). These data support our hypothesis that cross-reactive T cells contribute to lymphoproliferation during IM. Although the BMLF1-specific T cell population represented only a minor proportion (0.4–1.9%) of the total $CD8^+$ T cell pool in the blood, it remains possible that additional EBV-derived antigens simultaneously activate cross-reactive memory T cells with specificity for antigens other than influenza M1. Interestingly, both of these patients presented with symptoms of IM but with differences in severity. Patient E1101, who had the higher frequency of $M1^+$ $BMLF1^+$ cross-reactive cells, presented with severe (grade 5 on a scale of 1–5) symptoms and signs of IM, including fatigue, sweats, chills, sore throat, nausea, myalgia, lymphadenopathy, pharyngitis, and stomatitis. Notably, reversion of the CD4:$CD8^+$ T cell ratio, a hallmark of an active viral infection, was not evident until day 34 after presentation. In contrast, patient E1178, who had a lower frequency of cross-reactive cells, presented with only moderate (grade 2–3) symptoms and signs of IM, including fatigue, loss of appetite, and only mild hepatosplenomegaly. For this patient, CD4:$CD8^+$ reversion was observed on days 0 and 6, with a second episode of reversion again at day 27 after presentation, suggesting fluctuations in disease course. More patients will need to be studied to determine whether the correlation of cross-reactivity with M1 and disease severity will hold, but here we document high levels of cross-reactive T cells associated with severe IM pathology.

Skewing of the M1-specific Vβ17$^+$ TCR repertoire during IM. The M1-specific memory TCR repertoire is organized in a conserved pat-

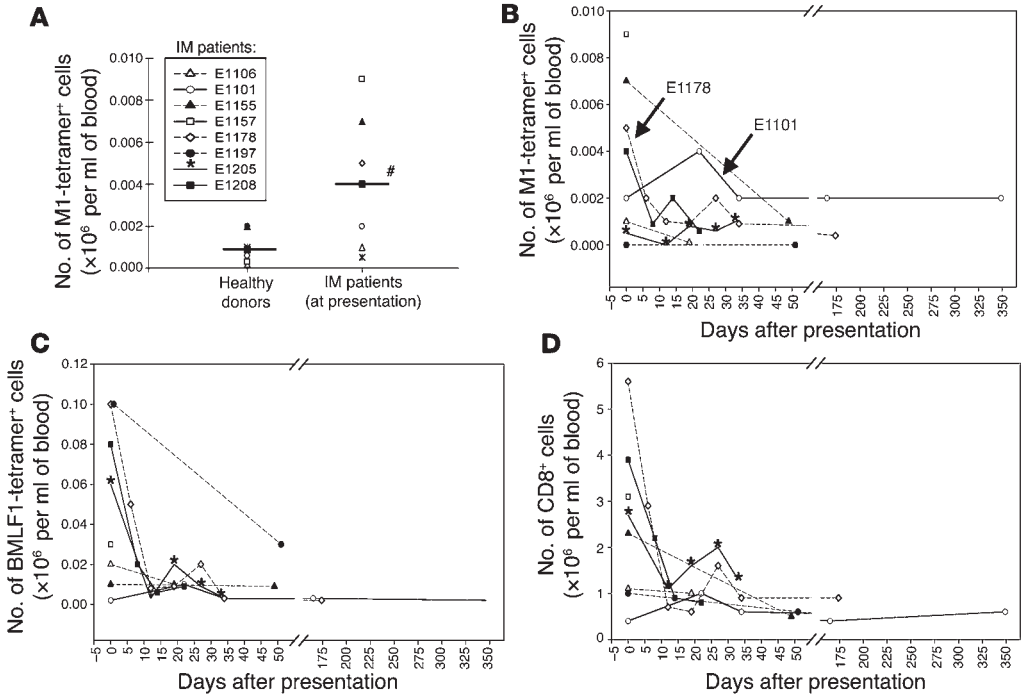

Figure 5

IM patients have an augmented number of M1-specific cells in their bloodstream. PBMCs were isolated from 8 healthy donors or from 8 patients experiencing IM. Blood from IM patients was collected at various points after presentation with symptoms of IM. Please note that the number of data points for each patient is variable. (**A**–**C**) $CD8^+$ cells were first isolated from PBMCs and then immediately stained with tetramer. The percentages of tetramer-positive cells were used to calculate the total number of either M1- or BMLF1-specific cells per ml of blood. (**A**) The difference between the means of the 2 subject groups was determined to be statistically significant using an unpaired, 2-tailed Student's *t* test. $^{\#}P = 0.02$. Patient E1197 had an M1-specific memory population that grew out in culture but was undetectable ex vivo; therefore, this patient was excluded from calculation of the mean. (**D**) PBMCs were used to costain with CD3 and $CD8^+$ antibodies and calculate the total number of $CD8^+$ T cells per ml of blood.

tern, as unique TCR subclones using the variable (Vβ) gene family Vβ17 can be ordered in a distinct hierarchy based on their joining gene (Jβ) usage, where Jβ2.7 dominates (used by 55–62% of subclones), followed by Jβ2.3 (used by 10–20% of subclones), and then, often, Jβ2.1, Jβ2.5, Jβ1.1, or Jβ1.2 at lower, more variable frequencies (Y.N. Naumov et al., unpublished observations). We investigated whether the M1-specific repertoires of E1101 and E1178 were skewed from this pattern and thereby reflective of cross-reactive TCR-mediated clonal expansions. Due to limited blood samples, we were unable to sort and sequence the TCRs of the M1-specific cells directly ex vivo. Rather, we generated M1-specific T cell lines from these IM patients, and Vβ analyses indicated that their M1-specific repertoires were focused on the Vβ17 family, similar to that previously described for healthy individuals (data not shown) (30–33). However, when we sequenced the $V\beta17^+$ subclones within the cell lines derived from both patients, they did not follow the highly conserved organizational pattern observed in healthy influenza-immune donors. At day 22 after presentation, the time point when the number of cross-reactive T cells was highest ex vivo, the Jβ2.3 family was overrepresented (30%) while the normally dominant Jβ2.7 family was vastly underrepresented (10%) within the $M1^+$ $V\beta17^+$ repertoire of patient E1101 (Figure 6, B and C). The skewing of the repertoire was even more pronounced at day 165 (Jβ2.3, 37%; Jβ2.7, 3%), again a time when cross-reactive cells could easily be detected ex vivo (Figure 6, B and C). When the M1-specific population was presumably more stable at 1 year after presentation, the repertoire was still skewed from that typical of a resting memory state but appeared to be slowly reverting, as the number of subclones using Jβ2.3 (25%) declined and those using Jβ2.7 (14%) were better represented (Figure 6C). Similarly, the M1-specific repertoire of patient E1178 was skewed during the acute phase of the immune response to EBV but in a way that was different from that of patient E1101. In the case of patient E1178, we found that the Jβ1.2 family was overrepresented within the $M1^+$ $V\beta17^+$ repertoire (50% at days 19 and 174), illustrating the point that a crossreactive TCR repertoire may be unique to each individual and therefore not easily predicted (Supplemental Figure 2). This perturbation in the influenza M1–specific TCR repertoire of both patients with IM is highly consistent with the concept that cross-reactive M1-specific T cells are expanded in the host during acute EBV infection in an antigen-driven manner by EBV-derived epitopes such as BMLF1.

Discussion

We show here that cross-reactive T cells specific to a previously encountered virus could be major contributors to the overzealous $CD8^+$ T cell response that defines IM. In 5 out of 8 patients, influenza M1–specific $CD8^+$ T cells participated in EBV-induced lymphoproliferation. Of these 5 patients, 2 had dramatically skewed M1-specific TCR repertoires and increased levels of clearly identifiable, tetramer-defined, cross-reactive $CD8^+$ T cells capable of recognizing the 2 dissimilar epitopes influenza M1 and EBV-BMLF1. Based on our ability to culture these cross-reactive cells from 3 out

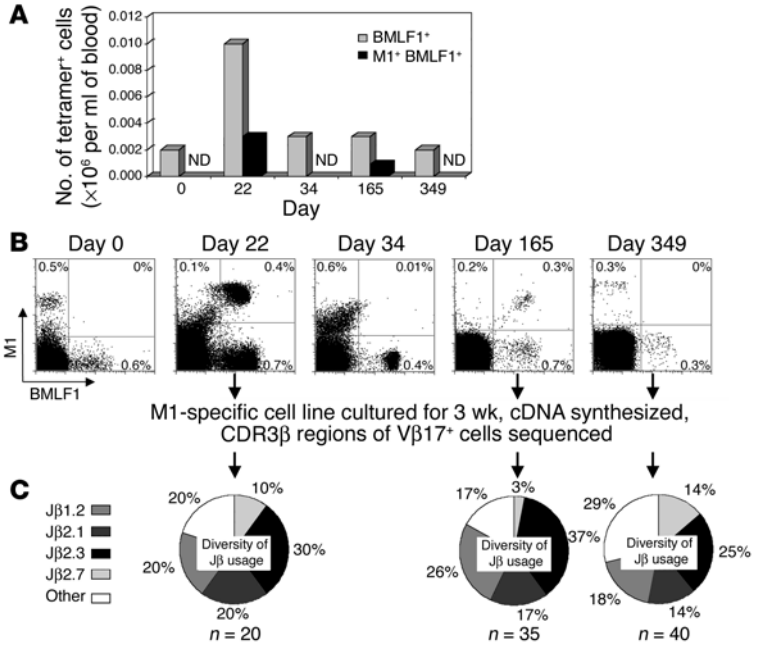

Figure 6
Acute EBV infection augments the number of cross-reactive cells that recognize M1 and BMLF1. CD8+ T cells were isolated ex vivo from patient E1101 at various time points after presentation with symptoms of IM. (**A**) The total number of antigen-specific T cells per ml of blood was calculated using the frequencies of tetramer-positive cells. BMLF1+, tetramer positive; M1+ BMLF1+, double-tetramer positive; ND, not determined because the frequency was below the limit of detection using this technique. (**B**) The percentages of CD8+ T cells staining positive when costained with M1- and BMLF1-loaded tetramers are shown. The number of events shown is variable because the maximum number possible was collected for each sample. (**C**) CD8+ T cells isolated at days 22, 165, and 349, were cultured for 3 weeks in the presence of M1 peptide–pulsed T2 cells. Following the RNA isolation and cDNA synthesis of those T cell lines, the CDR3β region of Vβ17+ subclones was sequenced. The pie charts illustrate the percentages of unique Vβ17+ subclones using each Jβ family, where *n* = the total number of unique subclones. The complete CDR3 sequences of all the subclones analyzed are displayed in Supplemental Table 1, structured according to Chothia et al. (57).

of 8 healthy donors with previous exposure to both viruses, these cross-reactive cells are maintained in memory and their functional responses to either antigen can include cytotoxicity and the production of MIP-1β, IFN-γ, and TNF-α. Cross-reactive T cells may play a major role in the development of IM, and the diversity of their functions may contribute to the severity of the syndrome. These studies examined only one cross-reactive population while it is likely that infection with EBV, a virus with the potential to encode hundreds of epitopes, could reactivate many memory T cell populations yet undefined. As demonstrated here, the identification of cross-reactive T cells can be complicated by their ability to recognize alternative peptides having little sequence similarity to their native ligand, their strict growth requirements in vitro, and the sensitivity of the different techniques used to detect them. These are all challenges for future elucidations of individual cross-reactive T cell responses and their potential impact on the outcome of IM.

Our work suggests that acute EBV infection activated influenza M1–specific CD8+ T cells. These cross-reactive M1-specific T cells are most likely memory cells for the following reasons: (a) by this age, everyone is immune to influenza A virus; (b) almost all HLA-A2+ individuals develop an M1-specific response; and (c) costaining with M1- and BMLF1-loaded tetramers showed that these cross-reactive cells brightly stained with the M1-loaded tetramer, suggestive of a high avidity interaction typical of antigen-specific memory. The activation of cross-reactive memory cells coincident with the development of IM disease pathology is highly analogous to the examples of heterologous immunity we have observed in mouse models, where memory T cell responses to a prior infection with an unrelated virus altered the host's immune response to a subsequent infection and caused a marked deviation in disease course (34).

It has recently been shown that acute HIV infection can upregulate the expression of activation markers such as CD38, HLA-DR, and Ki67 on memory cells specific to influenza A virus, EBV, and CMV, but the role of the TCR in this activation was not determined (35). Our work suggests that acute EBV infection can activate influenza-specific memory cells through a TCR-dependent mechanism. The expansion of M1-specific memory cells was evident in only 5 of 8 patients with IM despite the fact that all probably had memory to M1 and all would have been influenced by any cytokine-mediated, or bystander, activation. When possible, we also looked for the expansion of a second memory T cell population, specific for CMV-pp65. Only 2 IM patients proved to be CMV seropositive, E1155 and recent enrollee E1238. The frequency of pp65-specific T cells in patients E1155 and E1238 was low during massive, EBV-induced lymphoproliferation. At day 0, E1155 and E1238 had a pp65-specific T cell frequency of 0.2% and 0.7% respectively while, by 41–50 days after presentation, those frequencies climbed to 0.6% and 1.1% respectively. These data would suggest that the pp65-specific memory populations of these 2 patients did not contain T cell clones cross-reactive with EBV and were therefore initially diluted out by the extensive proliferation of EBV-specific T cells.

For 2 of the 5 patients with higher M1 frequencies (E1101 and E1178), we determined that BMLF1 was at least 1 of the EBV-derived antigens recognized by cross-reactive T cells. Although both patients shared this particular pattern of cross-reactivity, their responses remained unique. Vβ17+ subclones using Jβ2.3 preferentially expanded in patient E1101 while those using Jβ1.2 preferentially expanded in patient E1178. Thus, the M1-specific TCR repertoire of both patients was notably skewed from that known to be conserved among healthy HLA-A2+ individuals (Y.N. Naumov et al., unpublished observations). These data are further suggestive of antigen-driven clonal expansions because a bystander activation mechanism would drive the expansion of all clones and would maintain the conserved repertoire organization. The fact that cross-reactive T cells specific for M1 and BMLF1 were observed in 2 but not all patients is probably reflective of these clonal differences, known as the private specificity of each individual TCR repertoire. In support of this, cross-reactive T cell responses involving lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV) showed that only 50% of VV-challenged LCMV-immune mice mounted a strong response to a specific

LCMV epitope, $NP_{205–213}$, and this was a function of the private specificity of the LCMV memory TCR repertoire of each mouse (22). The existence of T cell responses unique to the individual adds to the complexity in resolving the importance of cross-reactive responses in human infections.

Previous studies showed that the extent of T cell activation and proliferation correlated with the severity of IM (7, 36). We suggest that the magnitude of the T cell response represents the combined efforts of cross-reactive memory and primary T cells. Our limited clinical data indicate that the number of cross-reactive cells specific for M1 and BMLF1 may correlate with disease severity, but further investigation is necessary to confirm this observation. The severity of IM might be influenced by both the number of activated cross-reactive cells and their effectiveness at clearing virus. There is recent evidence for the exacerbation of human disease by the activation of cross-reactive influenza-specific memory $CD8^+$ T cells during acute HCV infection. Like EBV, acute HCV infection is often asymptomatic, but when clinical symptoms manifest, they are likely caused by the immune response. HCV encodes an epitope, $NS3_{1073–1081}$, that can activate influenza $NA_{231–239}$–specific memory cells (14). However, unlike EBV-BMLF1 and influenza M1, which are only 33% similar in sequence, HCV-NS3 and influenza NA are 78% similar in sequence. In congruence with our observations during EBV infection, the activation of these cross-reactive NA-specific memory cells enhanced the magnitude of the $CD8^+$ T cell response to HCV and resulted in severe disease pathology (37). Despite strength in numbers, these HCV-specific T cells were unable to sufficiently clear the virus, and the patients developed persistent HCV infections (37). Cross-reactive cells may be inefficient at interacting with infected host cells due to a low avidity for the alternative ligand, but they may still produce pathology-generating cytokines. Their presence may interfere with the primary T cell response by preventing access to the infected cells or changing patterns of T cell immunodominance, thereby prolonging resolution of the infection.

Our work in animal models has suggested that cross-reactive T cells can induce pathological conditions despite their ability to clear virus. LCMV-specific memory cells lower the titer of VV delivered intranasally but in doing so also alter the disease pathology from pulmonary edema to bronchiolitis obliterans (34, 38, 39). The cytokines, notably IFN-γ, secreted by these activated memory cells may have played a major role in the development of this immunopathology. In the present study, we showed that cross-reactive cells specific to M1 and BMLF1 secreted several cytokines in a hierarchal pattern whereby most secreted MIP-1β, fewer secreted IFN-γ, and even fewer secreted TNF-α. Similar functional hierarchies were observed in studies with freshly isolated HIV- or CMV-specific $CD8^+$ T cells from healthy donors with these persistent infections as well as during the primary immune response to EBV in IM patients (40–42). The mechanism behind T cell functional heterogeneity has been extensively studied by varying the quantity of TCRs engaged and by varying the quality of ligands used to engage them (28, 43–45). Our work presents an opportunity to apply this knowledge to T cell cross-reactivity involving 2 natural viral epitopes in order to understand how cross-reactive T cells mediate the development and severity of IM. Interestingly, both MIP-1β and TNF-α levels are known to be elevated in the serum and tonsils of IM patients compared with healthy controls (46, 47). MIP-1β can be readily secreted because it is preformed and stored within human $CD8^+$ T cells (48). Even a low avidity interaction may stimulate its release from the cell. This is logical because MIP-1β broadens the immune response by recruiting other immune cells to the site of infection (49). The increase in number of activated immune cells may enhance IM severity. Fewer of the cross-reactive cells secreted TNF-α following peptide stimulation, suggesting that a higher avidity interaction may be required to initiate its production. However, an overall increase in the number of responding T cells capable of secreting TNF-α at the site of infection could be harmful to the host and promote the clinical symptoms of IM (50, 51). In fact, the high production of TNF-α, possibly by cross-reactive memory cells, has been implicated in the immunopathogenesis associated with dengue hemorrhagic fever (52). Thus, depending on the cross-reactive specificity pattern and private specificity of the TCR repertoire, cross-reactive memory T cells activated by EBV may function and modulate the disease outcome of each individual very differently. Identifying these cross-reactive patterns will be a challenge for the future.

In conclusion, our data suggest that cross-reactive memory T cells participate in the massive lymphoproliferation that characterizes EBV-associated IM and may influence disease severity. For the purposes of this study, we focused on detection and activation of M1-specific memory cells, but EBV, a virus with a large genome that encodes numerous different proteins, has the potential to generate epitopes reactive with many different memory T cell populations. The cross-reactive pattern that emerges is influenced both by an individual's unique history of infection and by the private specificity of the TCR repertoire responding to each of those infections. Overall, this demonstration of cross-reactivity involving 2 immunodominant epitopes derived from 2 of the most common human viruses among people that share the most common MHC class I haplotype in North America highlights the potential importance of cross-reactive T cells in human disease states.

Methods

Subjects. Influenza A virus–immune patients between the ages of 18 and 23 with acute EBV infection were volunteers from the University of Massachusetts (UMass) Student Health Services (Amherst, Massachusetts, USA). HLA typing was performed using the Lymphotype Class I system (Biotest) and an Olerup SSP kit (GenoVision). Acute EBV infection was confirmed 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 influenza A virus in the past. Patients provided up to 8 blood samples (50 ml each) starting at presentation with IM (day 0), then weekly for the following 6 weeks, and then again at 1 year. Healthy donors between the ages of 24 and 50 were volunteers from the research community at UMass Medical School (Worcester, Massachusetts, USA). HLA status and immunity to EBV and influenza A virus were assessed using monoclonal antibody (BB7.2; BD) and tetramer stains, respectively. Previous exposure to EBV was confirmed by the detection of capsid-specific IgG in donor sera. Donors provided up to 3 blood samples (60 ml each). This study was approved by the Human Studies Committee at UMass Medical School, and all subjects participating in our study gave informed consent.

Blood preparation and bulk T cell culture. PBMCs were isolated using Ficoll-Paque plus (Amersham Biosciences) and were stained with anti-$CD8^+$ microbeads before being positively selected using the Miltenyi Biotech MACS system. $CD8^+$ lymphocytes were plated at 2.5×10^5 per ml together with peptide-pulsed (1 μM), irradiated (30 Gy) T2 cells (CRL-1992; ATCC) at 5×10^4 per ml in 4 ml total volume per well of a 12-well plate. T cell lines

were fed media (AIM-V [Gibco; Invitrogen Corp.] supplemented with 14% human AB serum [Nabi Biopharmaceuticals], 16% MLA-144 supernatant [ref. 53], 10 U/ml rIL-2 [BD], 1% L-glutamine [Gibco; Invitrogen Corp.], 0.5% β-mercaptoethanol [Sigma-Aldrich], and 1% HEPES [HyClone]) every 3–4 days and were restimulated with T2 cells weekly.

HLA-A2–restricted peptides. The following peptides were synthesized to greater than 90% purity by Biosource: EBV-BMLF1$_{280–288}$ (GLCTLVAML), influenza A virus-M1$_{58–66}$ (GILGFVFTL), tyrosinase (YMNGTMSQV), and HIV-gag$_{77–85}$ (SLYNTVATL).

MHC class I tetramers. A detailed description of the protocol used by the tetramer facility at UMass Medical School has been previously published (25). Tetramers were assembled using the above peptide sequences for EBV-BMLF1 and influenza M1 and were conjugated to PE (Sigma-Aldrich), APC (CALTAG Laboratories), or Quantum Red (Sigma-Aldrich). Tetramers assembled with HIV-gag or tyrosinase (Immunomics) were used as negative controls, and nonspecific staining was never observed.

Extracellular and intracellular staining. Cells were plated at 10^6 per well and washed with staining buffer (PBS, 2% FCS, and 1% sodium azide). Tetramers were incubated at room temperature for 40 minutes and were washed off. Cells were either fixed with FACS Lysing Solution (BD) or permeabilized using Cytofix/Cytoperm (BD) according to the manufacturer's instructions. The following monoclonal antibodies were used: anti–IFN-γ (B27; BD), anti–MIP-1β (D21-1351; BD), and anti–TNF-α (mAb11, eBioscience). Isotype control antibodies did not stain positive.

T cell cloning. T cell lines were costained in 2% FCS/PBS with tetramers as described above. Double tetramer-positive cells were sorted using BD Vantage into 96-well plates at 1 cell per well. Each well contained 10^5 irradiated donor-specific CD4$^+$ T cell blasts and 2×10^3 of a 1:1 mixture of irradiated T2 cells pulsed with BMLF1 or M1 peptides. Clones were fed media every 3–4 days and restimulated with T2 cells at day 7; their specificity was tested on day 14.

IFN-γ ELISPOT. Our protocol was adapted from a previously published method using Mabtech reagents (54). The APCs were K562 cells stably transfected with HLA-A2.1 and pulsed with 50 μM peptide for 3 hours at 37°C, then washed of free peptide. Clones were tested using split-well analysis; 5 μl of each clone was loaded per well per peptide-loaded cell type. Plates were stained with streptavidin-horseradish peroxidase (Mabtech), diluted 1:100, and Nova Red Substrate (Vector Laboratories) according to the manufacturers' instructions, then read manually.

^{51}Chromium release assay. K562/HLA-A2.1 cells (54) served as the targets and were pulsed with 100 μM peptide for 1 hour at 37°C and then for an additional hour with 100 μCi ^{51}Cr per 10^6 cells. Targets were washed 3 times and plated at 5×10^3 per well. Clones were tested using split-well analysis; 30 μl of each clone was loaded per well per target type. Plates were incubated for 8 hours at 37°C, and the supernatants were analyzed with a MicroBeta TriLux scintillation counter (PerkinElmer).

*Sequencing the TCR CDR3*β. RNA was isolated from 10^6 cells derived from T cell lines using TRIzol (Invitrogen Corp.) according to the manufacturer's instructions. Using 0.5–1 μg of RNA, cDNA synthesis was performed with 2 μM poly-T (Integrated DNA Technologies) and Superscript III reverse transcriptase (Invitrogen Corp.) according to manufacturer's protocol. The cDNA was amplified with 1.6 μM each of Vβ17-specific (nomenclature of Arden et al.; ref. 55) and constant gene–specific (Cβ-specific) primers (56) using the protocol recommended by Applied Biosystems with AmpliTaq DNA polymerase. PCR products were ligated into the pCR4-TOPO vector (Invitrogen Corp.) and were used to transform TOP10 chemically competent cells (Invitrogen Corp.) according to the manufacturer's protocol. Individual colonies were picked for overnight cultures. DNA was isolated with QIAprep miniprep kits (QIAGEN), and complementarity-determining region 3β (CDR3β) was sequenced at the UMass Nucleic Acid Facility (Worcester, Massachusetts, USA) using universal primers.

Acknowledgments

We thank Rose Cicarelli, Robin Brody, and Joyce Pepe for their technical assistance. This work is supported by a Worcester Foundation grant, Deutsche Forschungsgemeinschaft fellowship CO 310/1-1, and NIH grants AI-49320, AI-42845, and DR-32520 and immunology training grant 5 T32 AI-07349-16. The contents of this publication are solely the responsibility of the authors and do not represent the official view of the NIH.

Received for publication March 18, 2005, and accepted in revised form October 4, 2005.

Address correspondence to: Liisa K. Selin, Department of Pathology, Room S2-238, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, USA. Phone: (508) 856-3039; Fax: (508) 856-0019; E-mail: liisa.selin@umassmed.edu.

1. Hoagland, R.J. 1975. Infectious mononucleosis. *Prim. Care.* **2**:295–307.
2. Young, L.S., and Rickinson, A.B. 2004. Epstein-Barr virus: 40 years on. *Nat. Rev. Cancer.* **4**:757–768.
3. Rea, T.D., Russo, J.E., Katon, W., Ashley, R.L., and Buchwald, D.S. 2001. Prospective study of the natural history of infectious mononucleosis caused by Epstein-Barr virus. *J. Am. Board Fam. Pract.* **14**:234–242.
4. Auwaerter, P.G. 1999. Infectious mononucleosis in middle age. *JAMA.* **281**:454–459.
5. Axelrod, P., and Finestone, A.J. 1990. Infectious mononucleosis in older adults. *Am. Fam. Physician.* **42**:1599–1606.
6. Rickinson, A.B., and Kieff, E. 2001. Epstein-Barr virus. In *Fields virology*. D.M. Knipe and P.M. Howley, editors. Lippincott Williams & Wilkins. Philadelphia, Pennsylvania, USA. 2575–2627.
7. Silins, S.L., et al. 2001. Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load. *Blood.* **98**:3739–3744.
8. Andersson, J., et al. 1987. Acyclovir treatment in infectious mononucleosis: a clinical and virological study. *Infection.* **15**(Suppl. 1):S14–S20.
9. Torre, D., and Tambini, R. 1999. Acyclovir for treatment of infectious mononucleosis: a meta-analysis. *Scand. J. Infect. Dis.* **31**:543–547.
10. Brehm, M.A., et al. 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat. Immunol.* **3**:627–634.
11. Mongkolsapaya, J., et al. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.* **9**:921–927.
12. Nilges, K., et al. 2003. Human papillomavirus type 16 E7 peptide-directed CD8+ T cells from patients with cervical cancer are cross-reactive with the coronavirus NS2 protein. *J. Virol.* **77**:5464–5474.
13. Selin, L.K., Nahill, S.R., and Welsh, R.M. 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. *J. Exp. Med.* **179**:1933–1943.
14. Wedemeyer, H., Mizukoshi, E., Davis, A.R., Bennink, J.R., and Rehermann, B. 2001. Cross-reactivity between hepatitis C virus and influenza A virus determinant-specific cytotoxic T cells. *J. Virol.* **75**:11392–11400.
15. Welsh, R.M., Selin, L.K., and Szomolanyi-Tsuda, E. 2004. Immunological memory to viral infections. *Annu. Rev. Immunol.* **22**:711–743.
16. Burrows, S.R., Khanna, R., Burrows, J.M., and Moss, D.J. 1994. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J. Exp. Med.* **179**:1155–1161.
17. Misko, I.S., et al. 1999. Crossreactive recognition of viral, self, and bacterial peptide ligands by human class I-restricted cytotoxic T lymphocyte clonotypes: implications for molecular mimicry in autoimmune disease. *Proc. Natl. Acad. Sci. U. S. A.* **96**:2279–2284.
18. Strang, G., and Rickinson, A.B. 1987. Multiple HLA class I-dependent cytotoxicities constitute the "non-HLA-restricted" response in infectious mononucleosis. *Eur. J. Immunol.* **17**:1007–1013.
19. Tomkinson, B.E., Maziarz, R., and Sullivan, J.L. 1989. Characterization of the T cell-mediated cellular cytotoxicity during acute infectious mononucleosis. *J. Immunol.* **143**:660–670.
20. Bousso, P., et al. 1998. Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. *Immunity.* **9**:169–178.
21. Cibotti, R., et al. 1994. Public and private V beta T cell receptor repertoires against hen egg white

lysozyme (HEL) in nontransgenic versus HEL transgenic mice. *J. Exp. Med.* **180**:861–872.
22. Kim, S.K., et al. 2005. Private specificities of CD8 T cell responses control patterns of heterologous immunity. *J. Exp. Med.* **201**:523–533.
23. Gotch, F., Rothbard, J., Howland, K., Townsend, A., and McMichael, A. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature.* **326**:881–882.
24. Callan, M.F. 2003. The evolution of antigen-specific CD8+ T cell responses after natural primary infection of humans with Epstein-Barr virus. *Viral Immunol.* **16**:3–16.
25. Catalina, M.D., Sullivan, J.L., Bak, K.R., and Luzuriaga, K. 2001. Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *J. Immunol.* **167**:4450–4457.
26. Steven, N.M., et al. 1997. Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *J. Exp. Med.* **185**:1605–1617.
27. Trautmann, L., et al. 2002. Dominant TCR V alpha usage by virus and tumor-reactive T cells with wide affinity ranges for their specific antigens. *Eur. J. Immunol.* **32**:3181–3190.
28. Valitutti, S., Muller, S., Dessing, M., and Lanzavecchia, A. 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med.* **183**:1917–1921.
29. Lawson, T.M., et al. 2001. Functional differences between influenza A-specific cytotoxic T lymphocyte clones expressing dominant and subdominant TCR. *Int. Immunol.* **13**:1383–1390.
30. Lawson, T.M., et al. 2001. Influenza A antigen exposure selects dominant Vbeta17+ TCR in human CD8+ cytotoxic T cell responses. *Int. Immunol.* **13**:1373–1381.
31. Lehner, P.J., et al. 1995. Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment. *J. Exp. Med.* **181**:79–91.
32. Moss, P.A., et al. 1991. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. *Proc. Natl. Acad. Sci. U. S. A.* **88**:8987–8990.
33. Naumov, Y.N., Naumova, E.N., Hogan, K.T., Selin, L.K., and Gorski, J. 2003. A fractal clonotype distribution in the CD8+ memory T cell repertoire could optimize potential for immune responses. *J. Immunol.* **170**:3994–4001.
34. Welsh, R.M., and Selin, L.K. 2002. No one is naive: the significance of heterologous T-cell immunity. *Nat. Rev. Immunol.* **2**:417–426.
35. Doisne, J.M., et al. 2004. CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. *J. Immunol.* **173**:2410–2418.
36. Williams, H., et al. 2004. Analysis of immune activation and clinical events in acute infectious mononucleosis. *J. Infect. Dis.* **190**:63–71.
37. Urbani, S., et al. 2005. Heterologous T cell immunity in severe hepatitis C virus infection. *J. Exp. Med.* **201**:675–680.
38. Chen, H.D., et al. 2001. Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. *Nat. Immunol.* **2**:1067–1076.
39. Selin, L.K., Varga, S.M., Wong, I.C., and Welsh, R.M. 1998. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J. Exp. Med.* **188**:1705–1715.
40. Appay, V., et al. 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* **192**:63–75.
41. Callan, M.F., et al. 2000. CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *J. Clin. Invest.* **106**:1251–1261.
42. Gillespie, G.M., et al. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J. Virol.* **74**:8140–8150.
43. Betts, M.R., et al. 2004. The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. *J. Immunol.* **172**:6407–6417.
44. Madrenas, J., and Germain, R.N. 1996. Variant TCR ligands: new insights into the molecular basis of antigen-dependent signal transduction and T-cell activation. *Semin. Immunol.* **8**:83–101.
45. Sloan-Lancaster, J., and Allen, P.M. 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* **14**:1–27.
46. Foss, H.D., et al. 1994. Patterns of cytokine gene expression in infectious mononucleosis. *Blood.* **83**:707–712.
47. Nakayama, T., et al. 2004. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein-Barr virus. *J. Virol.* **78**:1665–1674.
48. Zaitseva, M., et al. 2001. Human peripheral blood T cells, monocytes, and macrophages secrete macrophage inflammatory proteins 1alpha and 1beta following stimulation with heat-inactivated Brucella abortus. *Infect. Immun.* **69**:3817–3826.
49. De Rosa, S.C., et al. 2004. Vaccination in humans generates broad T cell cytokine responses. *J. Immunol.* **173**:5372–5380.
50. Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol.* **10**:411–452.
51. Xu, L., et al. 2004. Cutting edge: pulmonary immunopathology mediated by antigen-specific expression of TNF-alpha by antiviral CD8+ T cells. *J. Immunol.* **173**:721–725.
52. Rothman, A.L., and Ennis, F.A. 1999. Immunopathogenesis of Dengue hemorrhagic fever. *Virology.* **257**:1–6.
53. Rabin, H., et al. 1981. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor T cells. *J. Immunol.* **127**:1852–1856.
54. Britten, C.M., et al. 2002. The use of HLA-A*0201-transfected K562 as standard antigen-presenting cells for CD8(+) T lymphocytes in IFN-gamma ELISPOT assays. *J. Immunol. Methods.* **259**:95–110.
55. Arden, B., Clark, S.P., Kabelitz, D., and Mak, T.W. 1995. Human T-cell receptor variable gene segment families. *Immunogenetics.* **42**:455–500.
56. Maslanka, K., Piatek, T., Gorski, J., Yassai, M., and Gorski, J. 1995. Molecular analysis of T cell repertoires. Spectratypes generated by multiplex polymerase chain reaction and evaluated by radioactivity or fluorescence. *Hum. Immunol.* **44**:28–34.
57. Chothia, C., Boswell, D.R., and Lesk, A.M. 1988. The outline structure of the T-cell alpha beta receptor. *EMBO J.* **7**:3745–3755.