Persistent infection drives development of CD8+ T cells specific for late lytic infection antigens in lymphocryptovirus infected macaques and Epstein-Barr virus infected humans

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Abstract

We examined the CD8+ T cell repertoire against lytic infection antigens in rhesus macaques persistently infected with their Epstein-Barr virus (EBV)-related lymphocryptovirus (rhLCV). CD8+ T cells specific for late (L) antigens were detected at rates comparable to early antigens and associated with increasing duration of infection. L-specific CD8+ T cells were also readily detected in adult, EBV-positive humans. Thus, viral MHC class I immune evasion genes expressed during lytic LCV infection do not prevent L-specific CD8+ T cell development over time during persistent infection.
The repertoire of CD8+ T cells specific for Epstein-Barr virus (EBV) lytic infection antigens is believed to be biased away from cells specific for late (L) lytic infection antigens since they are rarely detected in humans with infectious mononucleosis (IM) (11). To begin addressing how lytic antigen-specific CD8+ T cells might affect EBV infection, we asked if the CD8+ T cell repertoire is also biased against L antigens in rhesus macaques since they are naturally infected with EBV-related herpesviruses in the same lymphocryptovirus (LCV) genus.

Rhesus LCV (rhLCV)-specific T cells were expanded from healthy, rhLCV-seropositive macaques by repetitive stimulation of peripheral blood mononuclear cells with autologous, rhLCV-immortalized, lymphoblastoid cell lines (rhLCV-LCL) (6). T cell lines contained predominantly CD8+ T cells (average 88% CD8+, range 56-99%, 11% CD4+, range 1-51%).

The repertoire of lytic infection antigens recognized by cell lines was determined by IFN-γ ELISPOT assay. Cells were stimulated with individual lytic infection antigens presented by autologous, baboon LCV immortalized LCL (baLCV-LCL) infected with recombinant vaccinia viruses (rVV) (6, 12). Lines stimulated with control rVV-infected baLCV-LCL routinely had background levels of <100 spot forming cells per million cells (SFC/10^6 cells). Cell lines from 13 naturally and 2 experimentally infected animals showed a total of 76 antigen-specific responses; 11, 36, and 29 responses against Immediate Early (IE), Early (E), and L antigens respectively (Figure 1).

To identify responses mediated by CD8+ T cells, T cell activity after CD4+ T cell immunodepletion (<0.3% CD4+ T cells remaining; range 0.02-2.38%) was compared to non-depleted lines. In 25 instances, CD4+ T cell depletion eliminated responses to undetectable levels, consistent with CD4+ T cells as the source of the responses (pre-depletion SFC in white boxes, figure 1). In 39 instances, CD4+ T cell depletion had no effect, or increased the response,
compared to non-depleted cells, consistent with responses from CD8+ T cells (SFC in black boxes). In 12 instances, CD4+ depletion decreased, but did not completely eliminate the response (range 15-90% of original activity). These results were interpreted as indeterminate since they were not definitively associated with CD4+ or CD8+ T cell responses (SFC in gray boxes). 6 of the indeterminate results were specific to L antigens, and intracellular cytokine staining (ICS) for IFN-γ and TNF-α was used to identify the responding T cells in these instances.

A representative ICS analysis for the Mm406-97 cell line is shown in figure 2A. Stimulation with the autologous rhLCV-LCL showed an increase in cytoplasmic IFN-γ (1.09%) and TNF-α (1.21%) in CD3+CD8+ cells, a 15 and 42 fold increase respectively compared to medium alone (0.071% and 0.029%). Responses to stimulation with control rVV infected baLCV-LCL were not significantly different from medium alone (<2 fold increase). Similarly, no significant ICS responses (<2 fold increase versus rVV control) were seen from cells stimulated with rVV-infected baLCV-LCL expressing lytic antigens that either induced no ELISPOT responses (rhBZLF1, rhBARF1, rhBVRF2) or ELISPOT responses assigned to CD4+ T cells (rhBALF5). ICS responses were seen in CD3+CD8+ T cells stimulated with rhBMRF1 or rhBNRF1, E and L antigens where strong CD8+ T cell responses were detected by ELISPOT (rhBMLF1: 10 fold increases; rhBNRF1: 4 fold increases). A clear CD8+ ICS response (4 and 3 fold increases) was also detected after rhBLLF1 stimulation, an indeterminate response by CD4+ depletion in ELISPOT assays.

ICS analyses from 10 cell lines are summarized in Figure 2B. In addition to Mm406-97, 4 other lines had indeterminate results for L antigens (marked superscript L in figure 2B; cells from the sixth animal with an indeterminate L antigen response were not available for further
study). In 3 animals, the indeterminate response to L antigens was confirmed as CD8+ T cells by ICS (figure 1, gray boxes annotated with black triangle). This included 2 animals with rhBNRF1-specific CD8+ T cell responses (297-07; 4 and 5 fold increase, and 246-04; 5 and 12 fold increase), and 1 animal with a rhBILF2 response (292-04; 3 fold increases). The one indeterminate response that did not test positive by CD8+ ICS is presumed to be from CD4+ T cells (Mm141-97, gray box with white triangle). Thus, 4 additional L-specific CD8+ T cell responses from 4 animals were identified by ICS.

Furthermore, ICS analysis confirmed the accuracy of CD4+ and CD8+ T cell interpretations from CD4+ T cell depletion in ELISPOT assays. In the 10 cell lines, every instance that was negative or assigned to CD4+ T cells by ELISPOT (n=28), also tested negative for CD8+ T cell responses by ICS, and every instance assigned to CD8+ T cells by ELISPOT (n=29), also tested positive for CD8+ T cell responses by ICS. In total, 8 of 9 indeterminate ELISPOT responses were confirmed by ICS as CD8+ T cell responses.

Overall, we detected 47 CD8+ T cell responses to lytic antigens (9IE, 21E, and 17L) in rhLCV-specific T cell lines derived from 15 rhesus macaques with persistent rhLCV infection. Each of the 15 animals had CD8+ T cell responses to at least one lytic infection antigen; 7/15 animals had CD8+ T cell responses to at least one IE antigen, 13/15 had responses to one or more E antigens, and 11/15 had responses to at least one L antigen. CD8+ T cells also target multiple L antigens (6 of 8) indicating that the repertoire was not skewed due to a single immunodominant L antigen. Since there were 30, 150, and 120 opportunities to detect IE-, E-, and L-specific T cell responses (2IE, 10E, and 8L antigens x 15 animals), this translates into a 30%, 14%, and 14% response rate to IE, E, and L antigens respectively for this study. The comparable response rates and number of animals positive for E- and L-specific CD8+ T cells
suggests that there is not a bias against L-specific CD8+ T cells in persistently infected rhesus macaques.

Our data also suggests that L-specific CD8+ T cells arise over time, during persistent infection. Animals are listed in figure 1 by increasing age at time of study (birth years are indicated by the final 2 digits). 11 L-specific responses were detected in older animals (6 were >7 years old; both experimental animals were infected with rhLCV by 3 years of age and studied >7 years later) versus 6 responses in the younger age animals (9 were <4 years old, p=0.0092, students t test).

If L-specific responses also increase over time in humans, then we predicted that L-specific CD8+ T cells should be readily detectable in EBV-infected adults, years, or decades, after acute infection. EBV-specific T cell lines were derived from 5 EBV-seropositive adult humans (7, 14) and tested for CD8+ T cells specific for selected EBV L antigens. IFN-γ ELISPOT analysis showed 12 L-specific responses. 5 were consistent with CD8+ T cells by CD4 depletion studies (Figure 3; black boxes). 2 responses, indeterminate by IFN-γ ELISPOT (rhBVRF2 for Donor11 and Donor12), were confirmed as CD8+ T cell responses by ICS (data not shown). The remaining 5 responses were eliminated by CD4 depletion. Thus, in EBV-specific T cell lines from 5 persistently infected humans a total of 7 L-specific CD8+ T cell responses were detected, compared to only 4 L-specific CD8+ T cell clones reported from 11 humans with acute IM (11). The different methodologies used (T cell lines versus T cell cloning) may slightly influence the rates of L-specific CD8+ T cell detection previously reported for acute infection (11) versus our studies in persistent infection. However, taken together these data support the model that L-specific CD8+ T cells may be less frequent during acute infection and arise over time during persistent infection. This contrasts with the initial expansion of IE and
E-specific CD8+ T cells during acute infection, followed by a contraction during persistent infection (2).

The bias in the lytic-specific CD8+ T cell response during acute infection is hypothesised to result from viral immune evasion mechanisms. Recent studies have shown that EBV encodes at least three proteins that inhibit the Class I Major Histocompatibility Complex (MHC-I) pathway; BNLF2a, BILF1, and BGLF5 (4, 9, 10, 17, 19, 20). rhLCV encodes homologues for all three (13), and conserved function demonstrated for rhBNLF2a (9) and rhBILF1 (19) suggests strong evolutionary selection for MHC-I inhibition.

Why do LCV make such a concerted effort to downregulate MHC-I and avoid L-specific CD8+ T cells? Preventing L-specific CD8+ T cell development may be critical during acute infection in order to allow virus replication and successful establishment of persistent LCV infection. Alternatively, evasion of L-specific CD8+ T cell recognition may be essential for viral reactivation, oral shedding, and transmission to new hosts. Studies with rhesus cytomegalovirus (rhCMV) showed that MHC-I inhibiting genes are dispensible for acute infection, but essential for rhCMV to superinfect hosts with pre-existing rhCMV-specific immunity (8).

It remains to be determined whether MHC-I inhibiting genes have evolved in EBV to enhance superinfection. The importance of EBV superinfection for virus survival within the host population is not understood. In immunocompromised hosts, co-infection with type 1 and 2 EBV can be frequently detected (15, 18). In immunocompetent hosts, type 1 and 2 coinfection can be detected in the oral cavity by PCR amplification in 14-20% of LCV-infected individuals (1, 3). PCR amplification for LMP1 sequence polymorphisms from oral secretions and peripheral blood suggested that many hosts harbor multiple EBV variants (16). Our data indicates that EBV-infected adult hosts frequently develop L-specific CD8+ T cells. MHC-I inhibiting genes
would enable EBV to more readily infect hosts with established L-specific CD8+ T cells. In this
case, MHCI inhibiting genes may not have evolved to prevent development of L-specific CD8+
T cells in the host, but were selected because they allow evasion of existing L-specific CD8+ T
cells during superinfection.

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individual EBV lytic antigens.
References


Figure 1. CD4+ and CD8+ T cell responses to recombinant rhLCV lytic infection antigens by IFN-γ ELISPOT assay. rhLCV-specific T cell lines were derived from 13 naturally and 2 experimentally (141-97 and 144-97) rhLCV-infected rhesus macaques. Antigen-specific T cell responses were measured by IFN-γ ELISPOT assay of T cell lines stimulated with rVV-infected, autologous baLCV-LCL for 16 hours. Numbers of spot forming cells (SFC) per million cells after subtraction of background values with control rVV infection are reported for positive results, and undetectable responses of <50 SFC are blank. Responses were assigned to CD4+ T cells if SFC were reduced to <50 SFC cells after CD4+ T cell depletion (white boxes), or CD8+ T cells if SFC were equal or increased after CD4+ T cell depletion (black boxes). Results that failed to fulfill these criteria after CD4+ T cell depletion were interpreted as indeterminate results (range = 15% to 90% of original ELISPOT response after CD4+ T cell depletion) and are shown as gray boxes. Animals with T cell lines studied by ICS are indicated by an asterisk. Indeterminate responses confirmed or not confirmed as CD8+ T cell responses by ICS are shown as gray boxes with black or white triangles respectively. Naturally infected animals are listed in order of increasing age at time of study, and experimentally infected animals were studied 7 years after inoculation.

Figure 2. Intracellular cytokine staining of T cell lines stimulated with recombinant lytic infection antigens. T cell lines were exposed to rhLCV-LCL as a positive control, autologous, vector control rVV-infected baLCV-LCL as a negative control, or baLCV-LCL infected with rVV expressing individual lytic infection antigens at a ratio of 10:1 for 12h. Cells were surface
stained for CD3 and CD8, permeabilized, and stained for intracellular IFN-γ and TNF-α as previously described (5). At least $10^6$ events were collected on a FACSCalibur (BD) and analysed by FlowJo (Treestar). (A) Representative ICS analysis for Mm406-97 T cell line for IFN-γ (top row) and TNF-α (bottom row). Samples were gated on CD3+ cells, and plots show cytokine (X axis) versus CD8+ T cells (Y axis). The percentage of cytokine positive CD8+ T cells for each analysis is shown at the top right. (B) Summary of ICS responses in rhLCV-specific T cell lines from 10 animals. Animals with indeterminate ELISPOT responses against E or L antigens are identified with a superscript letter. The percentage of cytokine positive CD8+ T cells is shown for each test performed (blank=not done) with positive ICS responses (greater than a 2 fold increase over controls) shown in black boxes and negative ICS responses (less than 2 fold increase over control) shown in white boxes.

Figure 3. CD4+ and CD8+ T cell responses to recombinant EBV late lytic infection antigens by IFN-γ ELISPOT assay. EBV-specific T cell lines were derived from 5 seropositive adult humans by repetitive stimulation with irradiated, autologous EBV-LCL. Antigen-specific T cell responses were measured by IFN-γ ELISPOT assay of T cell lines stimulated with rVV-infected PBMC for 16 hours. Numbers of spot forming cells (SFC) per million cells after subtraction of control values from vector control rVV infected PBMC are reported for positive results, and responses of <50 SFC are blank. Responses were assigned to CD4+ T cells if SFC were reduced to <50 SFC cells after CD4+ T cell depletion (white boxes), or CD8+ T cells if SFC stayed the same or increased after CD4+ T cell depletion (black boxes). Indeterminate results (range=57% to 94% of original ELISPOT response after CD4+ T cell depletion) are shown as gray boxes. All
indeterminate responses were confirmed as CD8+ T cell responses by ICS (data not shown), indicated by black triangles.
### Table

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### Diagram

[Diagram showing cytokine expression levels for various cell lines and conditions]