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Antigen-Driven Expansion and Contraction of CD8⁺-Activated T Cells in Primary EBV Infection

Yo Hoshino,*† Tsuneo Morishima, † Hiroshi Kimura, † Kazuo Nishikawa, † Tatsuya Tsurumi,* and Kiyotaka Kuzushima² *

The origin of the increased numbers of CD8⁺ atypical lymphocytes, expressing activated markers such as HLA-DR or CD45RO, in the peripheral blood of patients with infectious mononucleosis (IM) has been debated. Using a recently developed assay to detect intracellular accumulation of IFN-γ in EBV-reactive T cells by FACS, we have demonstrated that 34–54% of HLA-DR⁺/CD8⁺ and 34–60% of CD45RO⁺/CD8⁺ T cells in the PBMCs of febrile patients suffering from IM are EBV-specific. The EBV-specific CD8⁺ T cell counts in the PBMCs of four febrile patients suffering from IM ranged between 2260 and 8200/µl, decreasing to 5.1% and 7.9% of the counts in the first samples over 10 days in two donors. The decline of CD8⁺ T cell subpopulations, namely HLA-DR⁺, CD45RO⁺, and EBV-specific T cells, was in parallel with the drop in the EBV genome load. These data indicate that the Ag-driven expansion of CD8⁺ T cells and subsequent contraction with the Ag decline in vivo in humans is effective for clearing virus-infected cells with minimal disturbance of the homeostasis of the immune system. The Journal of Immunology, 1999, 163: 5735–5740.

Epstein-Barr virus is a ubiquitous gamma herpes associated with several malignant diseases, including a proportion of Hodgkin’s lymphomas, nasopharyngeal carcinomas, Burkitt’s lymphomas, and immunoblastic lymphomas seen in immunocompromised hosts (1). Primary infection is usually asymptomatic, although some individuals may suffer from infectious mononucleosis (IM), followed by a strong HLA class I-restricted, virus-specific CD8⁺ CTL response (1, 2). This response is believed to play an important role in controlling the virus both during primary infection and in the long-term carrier state, whereby EBV persists for life in a subset of B cells (1). Both lytic and latent cycle EBV proteins are known to be recognized by EBV-specific CTLs targeting short peptides presented on the cell surface by HLA class I molecules (2, 3). Proteins synthesized in the cytosol of target cells are degraded, primarily by the proteasome system, and the resultant peptides are translocated into the endoplasmic reticulum by TAPs. The binding of high-affinity peptides to nascent HLA class I molecules generates stable MHC class I-β₂-microglobulin-peptide trimer complexes that then move via the endoplasmic reticulum-Golgi network to the cell surface for presentation to the relevant CD8⁺ CTLs (4).

In the PBMCs of IM patients, there are large numbers of atypical lymphocytes, the majority being activated CD8⁺ T cells expressing HLA-DR and CD45RO Ags (5–7). Such T cell proliferation during viral infections in vivo could theoretically result by several mechanisms: 1) Ag-driven expansion of specific T cells, 2) stimulation of cell division by cross-reactive Ags, or 3) cytokine-mediated bystander activation (8–11). Recent work using mice challenged with lymphocytic choriomeningitis virus (LCMV) revealed that 50–70% of the activated CD8⁺ T cells in the primary infection were virus-specific (12). This unexpectedly high Ag-specific CD8⁺ T cell frequency was disclosed by means of sensitive assays, such as binding to tetrameric MHC class I molecule-peptide complexes or measuring IFN-γ production at the single-cell level, whereas a classical limiting dilution analysis (LDA) to quantify virus-specific CTLs indicated that only a small fraction (1–5% most) of the activated CD8⁺ T cells were Ag-specific at the peak of the primary response (13–15). The differential may result from in vitro outgrowth underestimating the true magnitude of the Ag-induced primary response, because only cells that are capable of dividing and surviving the 2-week in vitro culture period will score as positive (12).

As a natural situation to study the development of the CD8⁺ T cells immune response and their fate in vivo in humans, EBV-specific T cells in the case of IM have been analyzed (3, 15–17). Callan et al.(16) demonstrated biased TCR Vβ usage in most of the IM patients studied. In selected individuals, ≤25% of CD8⁺ T cells expressed a single Vβ chain, suggesting Ag-driven expansion (16). The authors also demonstrated, using tetrameric MHC-peptide complexes, that T cells specific for a single peptide derived from an EBV lytic protein comprised 44% of the total CD8⁺ T cells in the PBMCs of an HLA-B8⁺ patient (17). Their data strongly support the notion that a massive expansion of CD8⁺ T cells in IM is Ag-driven. However due to the HLA-restricted reagents used to detect Ag-specific T cells, there is still debate regarding whether such expansion is restricted to some HLA alleles or happens in unselected IM patients.

Recently we have developed an efficient method for the detection of EBV-specific class I-restricted CD8⁺ T cells in PBMCs irrespective of HLA typing (18). During IM, CD8⁺ T cells are directed not only to EBV latent proteins but also to lytic cycle...
proteins (3, 15, 17). Here we applied autologous lymphoblastoid cell lines (LCLs), which are B cell lines that have been transformed with a laboratory EBV strain, as APCs, because a small portion of LCLs is known to express lytic cycle proteins and effectively stimulate T cells specific for such proteins (3, 19) in addition to latent proteins. PBMCs of the patients are thereby incubated with autologous LCLs in the presence of an intracellular transport blocker, brefeldin A (BFA), for 6 h, and the accumulation of IFN-γ is detected by multiparameter FACS analysis. The results demonstrate that 34–54% of HLA-DR+/CD8+ and 34–60% of CD45RO+/CD8+ T cells in the PBMCs of unselected patients suffering with IM are EBV-specific. The decline of the subpopulations of CD8+ cells, namely HLA-DR+ , CD45RO+, and EBV-specific T cells, is in parallel with reduction of the EBV genome load in PBMCs. Our data clearly point to Ag-driven expansion of the activated CD8+ T cell population and its subsequent rapid contraction by a mechanism termed activation-induced cell death (AICD) (13) in the natural course of primary EBV infections.

Materials and Methods

Blood donors

A diagnosis of IM was made on the basis of typical clinical manifestations and a positive IgM Ab titer for EBV capsid Ag (1). The study design and purpose, which had been approved by the Institutional Review Board, were fully explained to all donors. Peripheral blood was only sampled after informed consent was obtained.

Preparation of PBMCs and LCLs

PBMCs were separated by centrifuging heparinized blood over Ficoll/ Hypaque (Pharmacia Biotech, Uppsala, Sweden). Aliquots of PBMCs were frozen at −80°C until use. LCLs were prepared by transforming PBMCs with B95-8 cell culture supernatant as described previously (20). LCLs were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 × 10−8 M 2-ME, and 10% heat-inactivated FCS (HyClone, Logan, UT) (referred to as culture medium).

Detection of IFN-γ-producing CD8+ T cells in response to LCLs by FACS

For determination of the CD8+ Ag-specific T lymphocyte frequency, an intracellular cytokine assessment using FACS was performed as described previously with slight modifications (18). Briefly, thawed PBMCs were incubated over night in culture medium containing rIL-2 (100 U/ml). On the following day, cells were resuspended at a concentration of 4 × 106/ml in the same medium. Autologous LCLs were resuspended at a concentration of 1.2 × 107/ml, and 0.5-ml aliquots along with PBMCs (0.5 ml) were mixed in 16 × 125 mm culture tubes and incubated in a humidified 5% CO2 incubator at 37°C for 1 h. BFA (Sigma, St. Louis, MO) was added at 10 μg/ml and the cells were cultured for an additional 5 h. For blocking experiments, an anti-HLA class I mAb (clone W6/32, Cedarlane, Ontario, Canada) or an isotype-matched mAb (D) at a responder to stimulator ratio of 1:4. A, Unstimulated PBMCs were also incubated, fixed, and subsequently mixed with autologous LCLs before staining. After fixation and permeabilization, the cells were stained for CD8, CD45RO, and IFN-γ. The CD8high subset of T cells was gated and analyzed by flow cytometry. The frequency of CD8+ T cells producing IFN-γ is shown as a percentage of the total CD45RO+ cells.

Results

Class-I restricted production of IFN-γ in EBV-specific CD8+ T cells

We applied a strategy using tricolor analysis for the detection of EBV-specific IFN-γ-producing T cells. First, the PE-cyanin-5.1-labeled anti-CD8 mAb was used to gate the population. Second, the PE-labeled anti-CD45RO or anti-HLA-DR mAb was employed to segregate the CD8+ population. Fig. 1 shows the results using PBMCs of a donor. IFN-γ production was observed exclusively in the CD45RO+ subpopulation. Sixty percent of CD45RO+ /CD8+ T cells produced IFN-γ upon stimulation with autologous LCLs (Fig. 1B), indicating their EBV specificity. As an unstimulated control, we used responder cells and LCLs that had been separately incubated in the same medium with BFA and mixed and stained after fixation (Fig. 1A). Only 0.47% of the unstimulated CD45RO+/CD8+ T cells produced IFN-γ. To confirm that the IFN-γ production was class I-restricted, the same responder cells were stimulated with autologous LCLs in the presence of anti-class I mAb (Fig. 1C), or with isotype-matched mouse mAb (D) as a controls. The IFN-γ-producing population was drastically reduced (4.1%) with anti-class I mAb but not with control mAb (57%). These results indicate that the IFN-γ in the CD8+
T cells is produced through authentic recognition of the Ags presented by class I molecules but not stimulated by putative superantigens on LCLs (22) or by IL-12, an IFN-\(\gamma\)-inducing factor secreted by LCLs (23).

EBV-specific IFN-\(\gamma\)-producing T cells reside in the CD45RO\(^+\) fraction of CD8\(^+\) cells

It has been reported that there are large numbers of CD45RO\(^+\)/CD8\(^+\) T cells that are prone to apoptosis in the PBMCs of IM patients (6, 7). Blood samples were obtained from donors 1–4 in febrile periods. After 6 h of stimulation with autologous LCLs, the cells were stained for CD8, CD45RO, and IFN-\(\gamma\). The CD8\(^{high}\) subset of T cells was gated and analyzed by FACS. The frequency of CD8\(^+\) T cells producing IFN-\(\gamma\) is shown as a percentage of the total CD45RO\(^+\) cells.

at 10 days after the first sample was obtained, and the percentages of EBV-specific CD8\(^+\)/CD45RO\(^+\) T cells were found to be reduced to 34% and 41%, respectively (Fig. 2, D and F). The first sample from donor 5 was obtained 2 wk after the symptoms of IM had resolved (G); the second sample was obtained 1 mo after the first sampling (H). Thawed PBMCs were stimulated with autologous LCLs at a responder to stimulator ratio of 1:3. After fixation and permeabilization, the cells were stained for CD8, HLA-DR, and IFN-\(\gamma\). The CD8\(^{high}\) subset of T cells was gated and analyzed by flow cytometry. The frequency of CD8\(^+\) T cells producing IFN-\(\gamma\) is shown as a percentage of the total HLA-DR\(^+\) cells.

FIGURE 2. EBV-specific IFN-\(\gamma\)-producing T cells residing in the CD45RO\(^+\) fraction of CD8\(^+\) cells in the PBMCs of donors 1 (A), 2 (B), 3 (C and D), 4 (E and F), and 5 (G and H) suffering from IM. Samples from donors 1–4 were obtained in the febrile period (A–C and E). Second samples were obtained 10 days after the first sampling from donors 3 and 4 (D and F). The first sample from donor 5 was obtained 2 wk after the symptoms of IM had resolved (G); the second sample was obtained 1 mo after the first sampling (H). Thawed PBMCs were stimulated with autologous LCLs at a responder to stimulator ratio of 1:3. After fixation and permeabilization, the cells were stained for CD8, CD45RO, and IFN-\(\gamma\). The CD8\(^{high}\) subset of T cells was gated and analyzed by flow cytometry. The frequency of CD8\(^+\) T cells producing IFN-\(\gamma\) is shown as a percentage of the total CD45RO\(^+\) cells.

FIGURE 3. EBV-specific IFN-\(\gamma\)-producing CD8\(^+\)/HLA-DR\(^+\) T cells in the PBMCs of donors 1 (A), 2 (B), 3 (C and D), 4 (E and F), and 5 (G and H) suffering from IM. Samples from donors 1–4 were obtained in the febrile period (A–C and E). Second samples were obtained 10 days after the first sampling from donors 3 and 4 (D and F). The first sample from donor 5 was obtained 2 wk after the symptoms of IM had resolved (G); the second sample was obtained 1 mo after the first sampling (H). Thawed PBMCs were stimulated with autologous LCLs at a responder to stimulator ratio of 1:3. After fixation and permeabilization, the cells were stained for CD8, HLA-DR, and IFN-\(\gamma\). The CD8\(^{high}\) subset of T cells was gated and analyzed by flow cytometry. The frequency of CD8\(^+\) T cells producing IFN-\(\gamma\) is shown as a percentage of the total HLA-DR\(^+\) cells.
of IM were HLA-DR$^+$, indicating their activation status (Fig. 3, A–G). In contrast, 6 wk after the symptoms had resolved, the EBV-specific CD8$^+$ T cells resided in the HLA-DR$^+$ population, indicating their entry into the resting or memory fraction (Fig. 3H).

Decline of CD8$^+$ EBV-specific T cells parallels that of EBV genome load

The absolute numbers of lymphocytes, CD8$^+$ T cells, and their subpopulations of the samples are shown in Table I. In the PBMCs of four febrile patients, the absolute cell counts of EBV-specific CD8$^+$, HLA-DR$^+$/CD8$^+$, and CD45RO$^+$/CD8$^+$ T cells ranged between 2,260 and 8,200/µl, 3,380 and 13,100/µl, and 3,240 and 14,500/µl, respectively. The cell counts of the three subpopulations decreased to 51.1%, 5.9%, and 8.3% of those in the first samples by 10 days in donor 3. They also decreased to 7.9%, 6.2%, and 11.8% of those in the first samples by 10 days in donor 4 (Table I). In donor 5, the same values decreased to 18%, 6.3%, and 16.6% of those in the first samples over 1 mo. Thus, the three subpopulations expanded and contracted in parallel. Finally, the kinetics of EBV-specific CD8$^+$ T cells of the three paired samples are shown in Fig. 4, along with quantitative data for EBV genome load. The decline of EBV-specific CD8$^+$ T cells paralleled that of the EBV genome load.

**Discussion**

During IM, there are increased numbers of CD8$^+$ T cells expressing activated markers such as HLA-DR or CD45RO in PBMCs; in addition, absolute cell numbers of CD8$^+$/HLA-DR$^+$ T cells during IM are 35–300 times larger than those in control subjects (5, 6). There has been a long-standing question regarding what is the driving force that promotes the massive expansion of activated CD8$^+$ T cells in primary infections with viruses, including EBV (12, 13). Some CD3$^+$/CD8$^+$ T cells in IM have cytotoxicity to HLA-mismatched LCLs as well as autologous LCLs (24). LDA to quantify EBV-specific CTLs has shown that the rates of T cells that are specific for a single EBV epitope vary from 1:100 to 1:500 during IM (15). These observations may lead to the hypothesis that the CD8$^+$ T cell expansion is generally not Ag-specific but rather represents bystander activation (10, 11) and/or cross-reactive stimulation of nonspecific cells (8, 9). However, a study analyzing TCR usage by CD8$^+$ T cells during IM pointed to the presence of unusually large T cell expansions that can constitute 25% of the total CD8$^+$ pool and whose monoclonal or oligoclonal population of TCR usage implies that they are Ag-driven (16). The conflict of the data between LDA and TCR usage is partially due to the fact that LDA appears to underestimate the true frequency of effector cells, which are highly activated and thus sensitive to AICD (12, 13). In addition to differences due to AICD, it is also possible that the vast majority of EBV-specific CD8$^+$ T cells can secrete IFN-γ, but that only a subset of these cells can differentiate into CTLs.

One additional complexity of studying the CD8$^+$ T cell response to EBV is that EBV has two types of replication cycles, namely lytic infection, in which infectious virions are produced, and latent infection, which is represented by the majority of LCLs. Some proteins expressed in either cycle are well recognized by CD8$^+$ T cells, which expand in IM (3, 17). Because ≤5% of LCLs have been found to express lytic cycle proteins (3, 19), CTL

Table I. Absolute cell counts of lymphocyte subpopulations in PBMCs of IM patients

<table>
<thead>
<tr>
<th>Donor</th>
<th>Lymphocytes</th>
<th>CD8$^+$ T Cells</th>
<th>CD8$^+$ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EBV-Specific</td>
</tr>
<tr>
<td>1</td>
<td>12,500$^a$</td>
<td>6,370</td>
<td>2,260</td>
</tr>
<tr>
<td>2</td>
<td>12,200</td>
<td>8,420</td>
<td>2,470</td>
</tr>
<tr>
<td>3</td>
<td>8,930$^b$</td>
<td>6,040</td>
<td>3,120</td>
</tr>
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<td>4</td>
<td>4,240</td>
<td>880</td>
<td>160</td>
</tr>
<tr>
<td>5</td>
<td>3,560</td>
<td>2,210</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>2,060</td>
<td>680</td>
<td>90</td>
</tr>
</tbody>
</table>

$^a$ Numbers are the absolute cell counts per microliter of the indicated populations.

$^b$ Second samples were obtained 10 days after the first sampling from donors 3 and 4. The first sample from donor 5 was obtained 2 wk after the symptoms of IM had resolved; the second sample was obtained 1 mo after the first sampling.

**FIGURE 4.** EBV-specific CD8$^+$ T cell counts in the PBMCs of donors 3 (A), 4 (B), and 5 (C) with titers of the EBV genome load in PBMCs (C), 1st; the first samples, 2nd; the second samples. The first samples from donors 3 and 4 were obtained in the febrile period; the second samples were obtained 10 days after the first sampling (A and B). The first sample from donor 5 was obtained 2 wk after the symptoms of IM had resolved; the second sample was obtained 1 mo after the first sampling (C).
precursors specific to the lytic proteins may be stimulated and may expand in limiting dilution cultures. However, at the end of the culture, the wells containing such effector cells should be scored negative because a CTL assay is insensitive if <10% of the target cells (in this case, the same LCLs used for stimulation) express the relevant Ags. In contrast, in assays using cytokine production or the proliferation of T cells as a marker of Ag recognition, LCLs have been shown to work effectively as APCs for CD8+ T cells specific to EBV lytic cycle proteins (3, 19). In the current study and in previous studies (18), we applied autologous LCLs as APCs for polyclonal CD8+ EBV-specific T cells with the aim of introducing as many epitopes as possible at the same time. Thus in our system, both T cells specific for latent proteins and those specific for lytic proteins should have contributed to the production of IFN-γ, their relative proportions being unknown. Besides, we could not deny the possibility that our assay may underestimate the frequencies of T cells specific for lytic proteins, which are expressed by small populations of LCLs.

We demonstrated here that 34–54% (mean: 48.5) of HLA-DR+/CD8+ and 34–60% (mean: 51.5) of CD45RO+/CD8+ T cells in the PBMCs of febrile patients suffering from IM were EBV-specific using an efficient method developed recently in our laboratory for the detection of EBV-specific class I-restricted CD8+ T cells in PBMCs irrespective of HLAs typing (18). Callan et al. have similarly demonstrated, using tetrameric MHC-peptide complexes, that T cells specific for a single peptide derived from an EBV lytic protein comprised 44% of the total CD8+ T cells in the PBMCs of an HLA-B8+ patient (17). Thus, the data strongly support the notion that the massive expansion of the CD8+ T cells in IM is Ag-driven, not only in cases with restricted HLA alleles but also in unselected IM patients. The appearance of atypical lymphocytes in peripheral blood, a central diagnostic criteria of IM, would be an expected result of morphological changes in such Ag-specific T cells. We have shown previously that EBV-specific T cell frequencies in the CD8+ PBMCs of seronegative individuals contribute no more than 0.03% of total CD8+ T cells as determined by the same assay (K. Kuzushima, unpublished observations). As demonstrated here, the EBV-specific T cell frequencies among the total CD8+ PBMCs of IM patients at the febrile period ranged from 29% to 54% (Table I). Taking into consideration the finding that the number of absolute CD8+ T cells in PBMCs during IM is four to eight times larger than that of control subjects (5, 6, 25), a 3,000- to 14,000-fold expansion of EBV-specific CD8+ T cells may occur during the primary infection. This is comparable with the 2,300-fold increase in the LCMV nucleoprotein-specific CD8+ T cells of mice with a primary infection during the first 8 days after virus challenge (12).

Activated CD8+ T cells expressing HLA-DR+ and CD45RO+ in PBMCs during IM have been shown to rapidly undergo apoptosis upon incubation in vitro in the absence of inhibitory cytokines such as IL-2 (6, 7). In LCMV-infected mice, it has been shown that there is a precipitous drop in the number of Ag-specific CD8+ T cells after viral clearance (26). We have demonstrated a decline of EBV-specific CD8+ T cells along with a drop in the EBV genome load in PBMCs. Because EBV-specific CD8+ T cell frequencies in the PBMCs of long-term EBV carriers are ~1% as determined by our assay (18), >99% of the EBV-specific CD8+ T cells generated during IM should disappear before entering the memory state to maintain homeostasis (13). Apoptosis due to Fas-Fas ligand interactions (27) and TNF (28) have been implicated in such an AICD-associated disappearance.

Our current data allow the dynamics of CD8+ Ag-specific T cells during virus challenge in IM to be outlined as a model of primary virus infection in vivo in humans. The rapid Ag-driven expansion of CD8+ T cells is followed by a subsequent decline due to AICD as an effective mechanism for clearing virus-infected cells with minimal disturbance of homeostasis of the immune system. Understanding the CD8+ T cell response from the primary EBV challenge towards recovery is essential for designing effective CTL therapies for various EBV-associated malignancies, such as immunoblastic B cell lymphomas seen in immunocompromised patients (29, 30), Hodgkin’s disease (31), nasopharyngeal carcinomas (2), and EBV-associated gastric carcinomas (32), in which some EBV Ags recognized by CTLs are expressed. In addition, our methodology to study the dynamics of quantitative viral load and the CD8+ Ag-specific T cell response should provide a useful approach to assess the pathogenesis of the diseases for which impaired cell-mediated immunity to EBV is postulated, such as EBV-associated hemophagocytic syndrome (33) and chronic active EBV infection (20, 34, 35).

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