**Human CD4⁺ T Lymphocytes Consistently Respond to the Latent Epstein-Barr Virus Nuclear Antigen EBNA1**

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**Abstract**

The Epstein-Barr virus (EBV)-encoded nuclear antigen EBNA1 is critical for the persistence of the viral episome in replicating EBV-transformed human B cells. Therefore, all EBV-induced tumors express this foreign antigen. However, EBNA1 is invisible to CD8⁺ cytotoxic T lymphocytes because its Gly/Ala repeat domain prevents proteasome-dependent processing for presentation on major histocompatibility complex (MHC) class I. We now describe that CD4⁺ T cells from healthy adults are primed to EBNA1. In fact, among latent EBV antigens that stimulate CD4⁺ T cells, EBNA1 is preferentially recognized. We present evidence that the CD4⁺ response may provide a protective role, including interferon-γ secretion and direct cytolyis after encounter of transformed B lymphocyte cell lines (B-LCLs). Dendritic cells (DCs) process EBNA1 from purified protein and from MHC class II–mismatched, EBNA1-expressing cells including B-LCLs. In contrast, B-LCLs and Burkitt's lymphoma lines likely present EBNA1 after endogenous processing, as their capacity to cross-present from exogenous sources is weak or undetectable. By limiting dilution, there is a tight correlation between the capacity of CD4⁺ T cell lines to recognize autologous B-LCL-expressing EBNA1 and DCs that have captured EBNA1. Therefore, CD4⁺ T cells can respond to the EBNA1 protein that is crucial for EBV persistence. We suggest that this immune response is initiated in vivo by DCs that present EBV-infected B cells, and that EBNA1-specific CD4⁺ T cell immunity be enhanced to prevent and treat EBV-associated malignancies.

**Key words**: Epstein-Barr virus • Epstein-Barr virus nuclear antigen 1 • CD4⁺ T cell • cross-presentation • dendritic cells

**Introduction**

EBV is a human gamma herpesvirus with a tropism for B lymphocytes (1). More than 95% of the adult population carries EBV as a lifelong asymptomatic infection. Nevertheless, EBV has strong growth-transforming capacities (2). Each of its three latency programs gives rise to specific tumors originating from B cells or other cell types. As exemplified by EBV-transformed B cells (B lymphocyte cell lines [B-LCLs])¹ or lymphoproliferative disease, the latency III phenotype is characterized by the expression of nine gene products: six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP) and three latent membrane proteins (LMP1, LMP2A, and LMP2B) (3). In latency II, EBV-associated malignancies like Hodgkin's lymphoma, nasopharyngeal carcinoma, T cell lymphoma, gastric carcinoma, and uterine leiomyosarcoma, three specific EBV genes, EBNA1, LMP1, and LMP2, are

¹Abbreviations used in this paper: B-LCL, B lymphocyte cell line; DC, dendritic cell; EBNA, EBV nuclear antigen; ELISPOT, enzyme-linked immunospot; GA, Gly/Ala; HS, human serum; LMP, latent membrane protein; MOI, multiplicity of infection; MP, matrix protein; NS1, nonstructural protein 1; SFC, spot-forming cell; SLAM, signalling lymphocyte activation molecule; vv, vaccinia virus

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maintained (3). Burkitt’s lymphoma exemplifies the EBV latency I phenotype. Only the EBNA1 protein is expressed in these transformed B cells.

EBNA1, LMP1, and LMP2 are probably critical for tumorigenesis, inducing cell proliferation as well as resistance to apoptosis (4). EBNA1 binds as a dimer to the viral origin of replication and ensures episomal replication during B cell growth (5–8). The C O O H-terminal part of LMP1 can act as a direct oncoprotein (9–11) by mimicking CD40-mediated B cell activation (12–15). Thus, LMP1 engages signaling proteins for the T NF receptor family (16, 17) and Janus kinase 3 (18), ultimately leading to nuclear factor κB (17) and AP-1 (jun/jun or jun/Fos) (19) induction. In addition, LMP1 expression protects against apoptosis by induction of bcl-2 (20, 21). Instead, LMP2 mimics B cell receptor signaling, constitutively engaging σκ and lyn, protein tyrosine kinases (22). In Burkitt’s lymphoma, only EBNA1 seems to be required, as transformation is achieved by additional mechanisms probably involving c-myc uncoupling through chromosomal translocation (2). However, the increased incidence of B cell neoplasia in EBNA1 transgenic mice argues for an involvement of this EBV product in transformation even in latency I malignancies (23).

How then is transformation to latency I and II malignancies avoided in most carriers of EBV? Immunity to EBNA1 a priori could provide resistance to transformed cells, but it has proven difficult to detect specific T cell responses to this essential protein for EBV persistence. In fact, EBNA1 blocks its own processing for MHC class I presentation (24). This has been attributed to a defect in proteasomal processing, caused by the NH2-terminal Gly/Ala (GA) repeat domain (25, 26). A similar GA stretch prevents IκBα degradation by the proteasome (27). Other EBV latency gene products are the focus of a strong MHC class I-restricted CTL response, especially EBNA3A, EBNA3B, and EBNA3C (28). However, EBNA3 proteins are not expressed in most of the EBV-associated tumors mentioned above, and instead are expressed in transformed cultured lines (B-LCLs) and lymphoproliferative syndromes in immunosuppressed patients. CD8+ CTL responses to tumor-associated LMP1 (29) and LMP2 (30) proteins have only occasionally been detected.

It has been repeatedly apparent that the development (31–33) and maintenance (34–37) of effective CD8+ CTLs are dependent on CD4+ T cell help. Recognition of EBV products by CD4+ T cells has not been investigated in the same detail as the CD8+ T cell response (38). Only two EBV-specific CD4+ T cell clones have been described before (39, 54). While the EBNA2-specific CD4+ T cell clone recognized HLA-DQ-matched B-LCLs, the EBNA1-specific CD4+ T cell clone only killed targets after exogenous loading with recombinant EBNA1 protein. Therefore, it was suggested that EBNA1 prevents its own endogenous presentation onto MHC class II, and that CD4+ T cell recognition of EBNA1 does not mediate protective immunity against EBV-associated malignancies.

Dendritic cells (DCs) are potent APCs for CD4+ and CD8+ T cell immunity (40, 41). Therefore, we used DCs to search for CD4+ T cell responses to individual latent EBV products. For EBNA1, we delivered the antigen as recombinant protein (42–45), in recombinant vaccinia virus (vv) constructs, and by coculturing with B-LCLs. We have uncovered a strong CD4+ T cell response to EBNA1, as monitored by T cell activation and proliferation, IFN-γ secretion, and CTL activity. Paralleling the above results with one EBNA2-specific CD4+ T cell clone, we demonstrate that the EBNA1-specific CD4 responses—generated routinely from adult blood samples—recognize HLA-DR–matched B-LCLs and therefore could provide resistance to EBV infection and EBV-associated malignancy.

Materials and Methods

Cell Lines. The EBV-transformed B cell lines LR M (HLA-A2, -B44, -DRB1*0401, -DQA1*03, -DQB1*0301, and -DP4) (46), LG2 (HLA-DR B1*0101, -DQA1*0101, -DQB1*0105, -DPA1*0101, and -DPB1*0201) (47), and newly generated B-LCL and the Burkitt’s lymphoma lines Ramos, EBV+ , and Dauid, reverted to latency III (obtained from American Type Culture Collection), were cultured in RPMI 1640/10% FCS/glutamin/gentamicin. LCL-JT (HLA-DR B1*0301 and DRB1*1301), LCL-BM (HLA-A1, -A3, -B7, -B8, -Cw6, -Cw7, -DR4, -DRw14, -DRw52, -DRw53, and -DQw3), LCL-DC (HLA-A2, -A24, -B38, -B46, -Cw1, -Cw7, -DR B1*1502, -DRB1*0901, -DRB4*01, -DRB5*0101, -DQB1*0502, and -DQB1*0303), LCL-BC (HLA-DR B1*0401, -DRB1*0701, DRB4*01, -DQB1*0302, and -DQB1*0201), and buffy coat–derived B-LCLs were generated by culturing PBMCs of healthy donors with supernatant of the marmoset cell line B95.8 in RPMI 1640/20% FCS/glutamin/gentamicin/1 μg/ml cyclosporin A. The rabbit RK13 and monkey BSC40 kidney cell line was grown in DMEM/15% FCS/glutamin/gentamicin.

DC and PBMC Preparations. Leukocyte concentrates (buffy coats) from the New York Blood Center, as well as whole blood from lab donors, served as sources of PBMCs isolated by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). DCs were generated from CD2+ PBMCs as described (47). Vaccinia Virus Stock Generation and Infection of DCs. The EBV-transformed B cell lines LR M (HLA-A2, -B44, -DRB1*0401, -DQA1*03, -DQB1*0301, and -DP4) (46), LG2 (HLA-DR B1*0101, -DQA1*0101, -DQB1*0105, -DPA1*0101, and -DPB1*0201) (47), and newly generated B-LCL and the Burkitt’s lymphoma lines Ramos, EBV+, and Dauid, reverted to latency III (obtained from American Type Culture Collection), were cultured in RPMI 1640/10% FCS/glutamin/gentamicin. LCL-JT (HLA-DR B1*0301 and DRB1*1301), LCL-BM (HLA-A1, -A3, -B7, -B8, -Cw6, -Cw7, -DR4, -DRw14, -DRw52, -DRw53, and -DQw3), LCL-DC (HLA-A2, -A24, -B38, -B46, -Cw1, -Cw7, -DR B1*1502, -DRB1*0901, -DRB4*01, -DRB5*0101, -DQB1*0502, and -DQB1*0303), LCL-BC (HLA-DR B1*0401, -DRB1*0701, DRB4*01, -DQB1*0302, and -DQB1*0201), and buffy coat–derived B-LCLs were generated by culturing PBMCs of healthy donors with supernatant of the marmoset cell line B95.8 in RPMI 1640/20% FCS/glutamin/gentamicin/1 μg/ml cyclosporin A. The rabbit RK13 and monkey BSC40 kidney cell line was grown in DMEM/15% FCS/glutamin/gentamicin.

10650 CD4+ T Cells from Healthy Adults Consistently Respond to EBNA1
Table I. Percentages of Blasting CD4+ T Lymphocytes upon Stimulation with DCs Infected with Recombinant Vaccinia-EV B Viruses

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>vvTK-</th>
<th>vvEBNA1</th>
<th>vvEBNA3A</th>
<th>vvEBNA3B</th>
<th>vvEBNA3C</th>
<th>vvLM P1</th>
<th>vvLM P2A</th>
<th>vvBM LF1</th>
<th>Influenza</th>
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<tbody>
<tr>
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<td>4 (1)</td>
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*Percentages of blasting CD4+ T lymphocytes of MNCs incubated with 10^5 vv-infected autologous mature DCs (mean values of triplicates are shown).

Table II. Number of IFN-γ-producing CD4+ T Lymphocytes upon Stimulation with DCs Infected with Recombinant Vaccinia-EV B Viruses

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<thead>
<tr>
<th>Donor no.</th>
<th>vvTK-</th>
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<td>4*</td>
<td>17 (2)</td>
<td>22 (8)</td>
<td>11 (3)</td>
<td>N D</td>
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<td>8 (4)</td>
<td>3 (3)</td>
<td>17 (6)</td>
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<td>4 (1)</td>
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<td>25 (3)</td>
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<th>vvLM P1</th>
<th>vvLM P2A</th>
<th>vvBM LF1</th>
<th>Influenza</th>
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<td>14 ± 1</td>
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<td>225 ± 5</td>
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</table>

*Percentages of blasting CD4+ T lymphocytes of MNCs incubated with 10^5 vv-infected autologous mature DCs (mean values of triplicates are shown).
the restimulation to a final concentration of 10 U/ml (Lyophil.
cult). Microcultures were tested in split well 51Cr-release assays
against autologous DCs infected with vvEBNA1, or vvTK−,
autologous B-LCLs, or LCL721.221. As <30% of the wells
developed CTLs or IFN-γ-secreting cells, it is >90% probable
that the responding wells represent clones (50). The BC cell
line was generated separating EBNA1-specific CD4+ T cells after stimula-
tion with vvEBNA1GA-infected DCs using the IFN-γ-secretion
assay according to the manufacturer's instructions (Miltenyi
Biotec).

FA C5 κ Analysis of Stimulated CD 4+ T Cell Populations and
PBMCs Mature DCs were infected with recombinant vv at an
MOI of 2, or with influenza virus (PR8, Puerto Rico/8/34;
Spafas, Inc.) at an MOI of 0.5 for 1 h at 37°C in RPMI 1640 HS.
DCs were washed twice, and 3 × 105 were added to 108 CD8−
CD28−PBMCs in 96-well plates for 7 d. The cultures were restim-
ulated with irradiated (3,000 rads) 105 PBMCs and 3 × 104 DCs
per well and incubated for an additional 7 d. At day 14, cultures
were stained for 30 min on ice with 1 μl Simulset CD4-FITC/CD8−PE
(Becton Dickinson) and analyzed on a FACScan™
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negative control (vvTK-) were weak (Fig. 1 E) in all but one donor, excluded from the Tables. All donors responded to influenza-infected DCs as a positive control (Fig. 1 F). CD4 T cell responses by the 10 donors to the other vvEBV constructs were detected less consistently: EBNA3B (5/10), EBNA3A (1/10), EBNA3C (1/10), and LM P1 (6/10) (Table I). To ensure that all the recombinant vv infected a comparable proportion of the mature DCs, the intracellular expression of the 29-kD vaccinia early protein was measured by FACS®. Reproducibly, 40–60% of DCs were infected with the different vv (data not shown). The reliability of the CD4+ recognition of EBNA1 was also evident in an ELISPOT assay for IFN-γ secretion, where EBNA1 was the EBV latency gene most frequently recognized (Table II).

We regard these CD4+ T cell responses to EBNA1 to reflect priming by EBV infection of the blood donors in vivo, as we did not see blastogenesis in 2 wk if we stimulated neonatal T cells from cord blood specimens with EBNA1 (Fig. 2). The fetal CD4+ T cells performed similarly to adult CD4+ T cells in MLR proliferation assays, and the fetal DCs were likewise capable of eliciting strong MLR proliferation of adult CD4+ T cells (Fig. 2 A). However, none of the fetal samples were able to recognize EBV or influenza products in IFN-γ ELISPOT assays (Fig. 2). CD4+ T cells from adult controls recognized vvEBNA1ΔGA, vvEBNA3B, vvLMP1, and influenza-infected autologous DCs in the same assay (Fig. 2).

CD4+ T cells recognize EBNA1 in an MHC class II-restricted fashion. We verified that our donors showed HLA class II diversity, as only two expressed HLA-DR4 (data not shown). To establish that MHC class II products were presenting EBNA1, we generated T cell lines, initially, from an HLA-DR4+ donor. We then assessed reactivity of the lines with DCs infected with recombinant vv expressing EBNA1 or pulsed with soluble EBNA1 protein. One line, CM 171198, was derived from CD8+ CD2+ PBMCs stimulated alternatively with autologous DCs infected with vvEBNA1ΔGA or the DR4-matched B-LCL LRM. The vvEBNA1 construct was deleted of the GA repeat that blocks MHC class I presentation and also reduces expression of EBNA1. Another line, CM 110199, was stimulated with DCs charged during their final maturation with recombinant EBNA1 protein expressed either in E. coli (EBNA1) or in a baculovirus/insect cell system (EBNA1). After 1 mo of culture, both lines were predominantly CD4+ T cells, 90% in CM 171198 and 76% in CM 110199, with CD56+ NK cells being the main contaminant (not shown).

The CD4+ T cell lines recognized DCs that were infected with vvEBNA1ΔGA or exposed to recombinant EBNA1 (Fig. 3, A and B). Reactivity could be measured as IFN-γ secretion (ELISPOT assays, Fig. 3 A) or by proliferation (Fig. 3 B). The T cell responses were blocked by an anti–HLA-DR antibody, L243, but not an anti–HLA class I antibody, B-H9 (Fig. 3 A). In addition to DCs charged with EBNA1, the CM 171198 cell line recognized EBV-transformed B-LCLs without further addition of antigen (Fig. 3, A and B, bottom). In B-LCLs, only full-length EBNA1 is expressed at detectable levels (24). This implies that full-length EBNA1, as expressed endogenously by B-LCLs, can be processed on MHC class II molecules for...
CD4+ T cell recognition. The B-LCLs had to be matched at the DR 4 allele to trigger T cell function. Thus, DR 4+ B-LCLs (LRM and LCL-BM) induced proliferation, but DR 4- cells (LG2 and LCL-DC) did not (Fig. 3, A and B, bottom).

**EBNA1-specific CD4+ T Cells Kill B-LCLs.** To determine if EBNA1 was an antigen for CD4+ CTLs, we stimulated CD8-depleted PBMCs with an HLA-DR4+ donor, JT, with irradiated autologous B-LCLs (expressing all known latent EBV antigens [3]) for 2 wk. In parallel, the B-LCLs were used to stimulate bulk CD25- and CD4+CD2+ T cells. The content of the stimulated T cell populations was determined by FACSCalibur®. CD8-depleted responders were enriched for CD4+ cells, CD4-depleted responders were enriched for CD8+ cells, and the bulk T cells had a CD4+/CD8+ ratio of 1:2 (see Fig. 5, top). All contained ∼25% CD56+ NK cells (not shown).

51Cr-release assays showed that the three populations of stimulated T cells killed autologous B-LCLs, with less recognition of the T2 cell line (Fig. 4, bottom left). As expected, the killing of autologous B-LCLs was completely blocked by L243 anti–HLA-DR antibody when CD4+ enriched populations, but not CD8+-enriched cultures, were tested (Fig. 4, bottom left). However, the CD8+-enriched cultures repeatedly developed stronger cytolytic activity than CD4-enriched cultures. Killing by the bulk T cells was partially inhibited by the L243 antibody, but the block was ≤50% in our three experiments.

EBNA1-specific CTL function was also assessed using DC targets that had been infected with vvEBNA1ΔGA or recombinant eEBNA1 and bEBNA1 proteins (Fig. 4). CD4+ enriched T cells lysed EBNA1-pulsed DCs. In contrast, CD8+-enriched cultures and bulk T cells were able to kill LCLs, but not the EBNA1-pulsed DCs (Fig. 4, bottom right).

To begin to determine if individual CD4+ T cells could lyse DCs pulsed with EBNA1, as well as B-LCLs expressing EBNA1 endogenously, we used autologous B-LCLs to isolate CD4+ CTLs by limiting dilution from cryopreserved T cells. The DCs, T cells, and autologous B-LCLs were derived from leukocyte concentrates. All 11 limiting dilution sublines that killed DCs in an EBNA1-dependent fashion also killed autologous B-LCLs, and we did not find any clone that killed the DCs and not the B-LCLs (Fig. 5). This indicates a tight correlation between recognition of epitopes expressed by B-LCLs and recognition of DCs that have been pulsed with EBNA1. As we were studying cells obtained under limiting dilution conditions, with <30% recognizing B-LCLs, it is >90% likely that individual clones were responsible for killing DCs and B-LCLs, although formal cloning experiments will be required (50).

Recognition of DCs infected with the vvTK- control vector or LCL721.221, an HLA class I-negative NK target, was poor in all 11 sublines (Fig. 5). Therefore, CD4+ T cells can lyse autologous B-LCLs, and one target very likely is EBNA1.

EBNA1 is recognized on B-LCLs after Endogenous Processing. As we were observing EBNA1-specific CD4+ cells that make Th1 cytokines and exert cytolytic activity upon encountering transformed B-LCLs, it was important to establish that this new and potentially protective mechanism would operate on EBNA1 that was processed endogenously by transformed B cells. The alternative would be that in our cultures some cells were dying and were being reprocessed via an exogenous or endocytic pathway in a fraction of LCLs. We are not aware of inhibitors that efficiently and selectively block processing of EBNA1 from an endogenous or exogenous route.

Therefore, we first compared the capacity of DCs (as a positive control) and the EBV- Burkitt’s lymphoma cell line, Ramos, to present EBNA1 through an exogenous pathway, either rEBNA1 protein or EBNA1 expressed by...
allogeneic B-LCLs (Fig. 6 A). Because of MHC class II mismatching, the allogeneic B-LCLs could not directly present EBNA1 to T cell lines that had been selected for IFN-γ secretion upon stimulation with vvEBNA1ΔGA-infected autologous DCs. The Ramos cell line as well as the autologous DCs could present vvEBNA1ΔGA to an EBNA1-specific CD4+ T cell line from a donor matched at HLA-DR7 to Ramos (Fig. 6 A). In contrast, only the DCs, and not the Ramos Burkitt’s lymphoma cells, presented exogenous rEBNA1 and EBNA1 from allogeneic LCLs (having ~20% trypan blue–positive or dead cells; Fig. 6 A).

DCs also presented EBNA1 from additional exogenous sources. In Fig. 6 B, DCs presented EBNA1 from four different alloimmune DC preparations infected with vvEBNA1ΔGA, presumably because infection with vv is cytotoxic for some of the infected DCs (49). As vv-infected DCs cannot produce virus particles (49, 53), coinfection could not be responsible for the observed EBNA1 transfer from one DC to another. A fifth semiallogeneic DC preparation presented vvEBNA1ΔGA directly to the T cell line (Fig. 6 B).

Although there is already evidence in the literature that Burkitt’s lymphoma cells are comparably efficient to B-LCLs in presenting soluble antigen on MHC class II (54), we wanted to show that both types of lymphoma also share the inability to cross-present antigen from cocultured cells. Therefore, we infected mismatched DCs and EBV latency III type Daudi cells with recombinant vv expressing influenza NS1 or MP, and then looked for cross-presentation of these influenza products by autologous B-LCLs or DCs, to CD4+ T cells of an influenza-reactive donor. Again, the DCs could present antigen from the allogeneic vvNS1- or vvMP-infected cells, but the B-LCLs could not (Fig. 7). As a positive control, we showed that autologous B-LCLs could present NS1 (Fig. 7 A) and MP (Fig. 7 B) to CD4+ T cells when directly infected with vvNS1 or vvMP, respectively. Coculturing B-LCLs with allogeneic infected B-LCLs for 2 d apparently did not transfer sufficient antigen amounts to the matched B-LCLs to trigger IFN-γ secretion by CD4+ T cells. In contrast NS1- or MP-expressing allogeneic B-LCLs could be efficiently processed by DCs and presented to CD4+ T cells.

This series of experiments indicates that DCs efficiently cross-present on MHC class II antigens (EBNA1, NS1, and MP) from allogeneic DCs or B-LCLs, but that transformed B cells (B-LCLs or Burkitt’s lymphoma cells) are weak or inactive in this respect. The findings are consistent with prior publications showing that B-LCLs can present soluble proteins that bind to their Ig receptor, but not by a nonspecific exogenous route (55). Therefore, CD4 T cell recognition of EBNA1 in B-LCLs very likely represents recognition of the endogenously processed protein that is critical for EBV-induced transformation.

Discussion

EBNA1-specific CD4+ T Cell Immunity Exists in All Healthy Adults. In an effort to define the CD4+ T cell repertoire for EBV latency gene products, we have uncovered a consistent CD4+ T cell response to EBNA1 presented by DCs and by autologous B-LCLs. The EBNA1-specific CD4+ T cells proliferate, secrete IFN-γ, kill targets, and can be readily propagated as EBNA1-specific, MHC class II-restricted lines. Other latency antigens (LMPI, LMP2, EBNA3A, EBNA3B, and EBNA3C; Tables I and II) can be recognized by CD4+ T cells, but less consistently. The CD4+ T cells described here are found in
T Cells from Healthy Adults Consistently Respond to EBNA1

Bulk cultures and kill B-LCLs without further addition of EBNA1. The results are in marked contrast to the prior literature that has described only a single EBNA1-specific CD4+ T cell clone, and it only killed B-LCLs under limiting dilution. Then the wells were split and tested in 35Cr-release assays against vvEBNA1ΔGA- or vvTK-infected DCs as well as autologous B-LCLs and LCL721,221, an HLA class I NK target. The dot plot shows for the first experiment, as an example, the CTL specificities that could be detected above three times the SD (3×SD) of the unspecific targets. Only CTLs exclusively recognizing autologous B-LCLs or recognizing autologous B-LCLs and vvEBNA1ΔGA-infected autologous DCs could be detected. All 11 sublines that recognized EBNA1-expressing DCs also demonstrated specific killing of autologous EBV-transformed B cells.

Figure 5. EBNA1-specific CD4+ CTL sublines kill B-LCLs. B-LCLs were generated from two leukocyte concentrates. Cryopreserved CD8+CD2+ PBMCs were then stimulated for 2 wk with the autologous B-LCLs under limiting dilution. Then the wells were split and tested in 35Cr-release assays against vvEBNA1ΔGA- or vvTK-infected DCs as well as autologous B-LCLs and LCL721,221, an HLA class I NK target. The dot plot shows for the first experiment, as an example, the CTL specificities that could be detected above three times the SD (3×SD) of the unspecific targets. Only CTLs exclusively recognizing autologous B-LCLs or recognizing autologous B-LCLs and vvEBNA1ΔGA-infected autologous DCs could be detected. All 11 sublines that recognized EBNA1-expressing DCs also demonstrated specific killing of autologous EBV-transformed B cells.

Figure 6. DCs are efficient, and B-LCLs inefficient, in presenting EBNA1 by an exogenous pathway. (A) The EBNA1-specific HLA-DRB*0701 CD4+ T cell line BC, selected for IFN-γ secretion upon stimulation with vvEBNA1ΔGA-infected autologous DCs, responds comparably to vvEBNA1ΔGA-infected autologous DCs and vvEBNA1ΔGA-infected HLA-DRB*0701 Ramos cells. DCs, but not Ramos cells, present recombinant EBNA1 proteins and allogeneic LCL-J TT. (B) The EBNA1-specific CD4+ T cell line 090199.6 recognized autologous DCs either infected with vvEBNA1ΔGA, or cocultured with infected allogeneic (allo) DCs. Semiallogeneic (semiallo) DCs were recognized irrespective of coculturing with autologous DCs.

CD4+ T Cells from Healthy Adults Consistently Respond to EBNA1
**Figure 7.** Influenza antigens are efficiently cross-presented by DCs, but not B-LCLs. Allogeneic DCs (alloDC2) and the latency III type Burkitt’s lymphoma cell line Daudi were infected with recombinant vv expressing either (A) NS1 (vvNS1), or (B) influenza MP (vvMP). Then DCs and B-LCLs were cultured with maturing DCs or B-LCLs derived from another healthy adult donor for 2 d at a ratio of vv-infected cells to presenting cells of 2:1 (DC+/B-LCL+). CD2+CD8+ PBMCs autologous to the presenting cells were tested in IFN-γ ELISPOT assays. As controls, the presenting DCs or B-LCLs were also directly infected with vvNS1 and vvMP or vvTK as control (DCvvTK+/DCvvNS1/DCCvvMP or B-LCLvvTK+/B-LCLvvNS1).

**MHC class I by DCs (56).** It is likely that the observed EBNA1-specific CD8+ T cells are primed by DCs that process B-LCLs onto MHC class I. However, because of the Gly/Ala repeat, such CD8+ T cells would not see EBNA1 expressed by EBV-infected cells.

Evidence that endogenous processing and presentation of Physiological Epitope Levels on B-LCLs are Sufficient to elicit EBNA1-specific CD4+ T cell Immunity. The EBNA1-specific CD4+ T cells described in this study recognize autologous B-LCLs by proliferation, IFN-γ secretion, and cytolyis. As these responses are MHC class II restricted, and because EBNA1 is expressed endogenously in B-LCLs, two routes of processing can be envisioned. Either EBNA1 is processed directly by the EBV-infected cell itself (endogenous pathway), or dying EBNA1-expressing B-LCLs are endocytosed and presented (indirect or exogenous or cross-presentation pathway). This does not necessarily mean that the processing machinery differs, but that in the first endogenous case the antigen is processed in the cell that synthesizes EBNA1, making it a more reliable target for protective immunity in humans. From our experiments, exogenous processing is an unlikely explanation for presentation of EBNA1 on B-LCLs, as antigen transfer from HLA-mismatched cells was not observed (Fig. 6). B-LCLs were previously shown to be poor APCs for exogenous proteins (55), 100–300 times less efficient than DCs. Similarly we found that the Ramos Burkitt’s lymphoma line inefficiently processed rEBNA1 and cross-presented EBNA1 from cocultured B-LCLs to CD4+ effector cells (Fig. 6 A). Cross-presentation of influenza NS1 and MP by B-LCLs from vvNS1- and vvMP-infected DCs or EBV latency III type Daudi cells was also inefficient (Fig. 7). In contrast, DCs cross-presented antigen from B-LCLs or vvEBNA1ΔGA-infected DCs, and processed rEBNA1 (Fig. 6, A and B). This was not restricted to EBNA1, as DCs also presented NS1 and MP from cocultured vvNS1- and vvMP-infected DCs and Daudi cells (Fig. 7). As a positive control for the competence of the lymphoma cells as APCs, we showed that direct infection of Ramos or B-LCL cells with recombinant EBNA1 or influenza vv led to CD4+ T cell responses. This implies that in healthy carriers, the DCs that are known initiators of immune responses (41) cross-present B-LCL-derived EBNA1 for priming of specific CD4+ T cells. Once activated, these CD4+ T cells may attack B cells that process EBNA1 endogenously and contribute to EBV-specific immunity.

The Gly/Ala Repeat Domain Does Not Influence MHC Class II Processing of EBNA1. Our experiments confirm that EBNA1 can be processed onto MHC class II irrespective of the presence or absence of its Gly/Ala repeat. This domain inhibits proteasome-dependent processing for MHC class I (25, 26). B-LCLs, the only reliable source of full-length EBNA1 (24), readily present EBNA1 to specific CD4+ T cells (Figs. 2–4). Moreover, B-LCLs with full-length EBNA1 can be cross-presented by DCs (Fig. 5 B). Therefore, the proposal that EBNA1 prevents its endogenous MHC class II presentation (39) cannot be supported by our findings.

Evidence that CD4+ T cell Immunity could contribute to the control of EBV. EBNA1-specific CD4+ T cells could provide direct resistance to EBV-transformed cells, through their cytokines and lytic function or by sustaining the CD8+ CTL response to other lymphoma-related EBV products such as LMP1 and LMP2. A good deal of circumstantial evidence for CD4+ T cell protection against gamma herpesviruses in vivo exists. (a) CTLs to EBV in the cottontop tamarin Sanguinis oedipus (57) are to a large extent MHC class II restricted (58). MHC class I–restricted, EBV-specific CTMs have yet to be found in this New World monkey, and this species lacks classical MHC class I (although it does express homologues of nonclassical class I genes like HLA-G and HLA-F; 59). (b) Gamma herpesvi-
CD4+ T cells from healthy adults consistently respond to EBNA1

References


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