

EBNA1-specific CD4⁺ T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function

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The Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA1) maintains the viral episome in all host cells infected with EBV. Recently, EBNA1 was found to be the main EBV latency antigen for CD4⁺ T cells and could be recognized in cultures from all donors tested. We now identify a polarized Th1 phenotype and obtain evidence for its presence *in vivo*. When T cells were stimulated with dendritic cells infected with vaccinia vectors expressing EBNA1, 18 of 19 donors secreted IFN- γ , whereas only two of 19 secreted IL-4. Magnetic selection was then used to isolate cells from fresh blood based on EBNA1-induced cytokine production. Specific IFN- γ CD4⁺ cell lines were established from six of six donors and IL-4 lines from three of six. Only the Th1 lines specifically lysed targets expressing three different sources of EBNA1 protein. When the IgG isotype of EBNA1 plasma Ab's was tested, most specific Ab's were IgG1 and of a high titer, confirming a Th1 response to EBNA1 *in vivo*. Ab's to other microbial antigens generally were not skewed toward IgG1. Given emerging evidence that Th1 CD4⁺ T cells have several critical roles in host defense to viral infection and tumors, we propose that EBNA1-specific CD4⁺ Th1 cells contribute to resistance to EBV and EBV-associated malignancies.

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Introduction

Epstein-Barr virus (EBV), after an initial lytic phase, establishes a life-long latent infection in resting memory B cells (1). Despite a relatively benign course in most carriers, EBV has growth-transforming capabilities (2) and is associated with several malignancies, e.g., endemic Burkitt's lymphoma, nasopharyngeal carcinoma, and approximately half the cases of Hodgkin's lymphoma (3). It is important to identify mechanisms whereby healthy carriers avoid development of EBV-associated malignancy.

In most EBV-seropositive adults, strong CD8⁺ cytotoxic T-lymphocyte (CTL) responses develop (4). However, these are preferentially directed toward the nuclear antigens, EBNA3A, EBNA3B, and EBNA3C (3, 5), which are not expressed in many EBV-associated malignancies. EBV-transformed cells exhibit one of three latency types distinguished from each other by the panel of expressed EBV antigens (3). In latency I, only EBNA1 is expressed, as in Burkitt's lymphoma. In latency II, exemplified by Hodgkin's lymphoma and nasopharyngeal carcinoma, LMP1 and LMP2, as well as EBNA1, are expressed. Only in latency III immunoblastic lymphomas are the highly immunogenic EBNA3 genes expressed. Therefore, many EBV-associated malignancies do not seem to provide good targets for the dominant human CD8⁺ T-cell response to EBV-latency gene products.

The nuclear antigen, EBNA1, is an optimal EBV-specific antigen because it must be expressed in all proliferating EBV-infected cells to maintain the viral episome. However, this protein contains an NH₂-terminal glycine-alanine repeat domain that blocks its own proteosomal processing and hence presentation on MHC class I molecules (6, 7). EBNA1-specific CD8⁺ CTLs have been identified, but these do not recognize EBV-transformed cells unless EBNA1 is added externally (8). Likewise, CD8⁺ T cells specific for LMP1 are infrequent and for LMP2 are observed only in some haplotypes (4, 5, 9).

Therefore, to understand how most healthy carriers of EBV avoid latency I and II malignancies, we turned our attention to CD4⁺ T cells. In mouse models, these T cells are important for resistance to cancer (10–12, and reviewed in ref. 13) and viruses (14–17), including virally induced malignancy (reviewed in refs. 13 and 18). Activated CD4⁺ T cells deliver survival and maturation stimuli to dendritic cells (DCs), which, in turn, are vital to the induction and expansion of antigen-specific CD8⁺ T lymphocytes (17, 19, 20). The type of activated CD4⁺ T cell also influences the outcome of the immune response (reviewed in ref. 21). Th1 CD4⁺ cells secrete IFN- γ and help in the development of cellular immunity, including the activation of macrophages. Th2 CD4⁺ cells secrete IL-4 and IL-5, thereby stimulating eosinophils and mucosal Ab production. Th1 CD4⁺

T cells differ from T_{H2} cells in other ways, such as the pattern of expressed chemokine receptors (22) and a capacity to kill targets via Fas-FasL interactions (reviewed in ref. 23).

Recently, we uncovered a consistent EBNA1-specific, CD4⁺ T-cell response in blood cells from healthy donors (24). To detect these CD4⁺ T cells, a 2-week stimulation culture was used in which DCs were the antigen-presenting cells and purified CD4⁺ T cells were the responders. The present study investigates whether EBNA1-specific CD4⁺ T cells are polarized toward a Th1 or Th2 phenotype and whether this response can be detected *ex vivo*. We show that EBNA1-specific CD4⁺ T cells are consistently Th1 and that these cells can be directly isolated from blood. Furthermore, EBNA1 Ab's consistently are of the opsonic and complement-fixing IgG1 subclass, reflecting Th1 polarization *in vivo*. We discuss the emerging evidence that Th1 cells are important for defense against infection with persistent viruses and tumors.

Methods

Dendritic cell and CD4⁺ T-cell preparations. PBMCs were obtained from leukocyte concentrates (New York Blood Center, New York, New York, USA) and blood from lab donors. After Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation, CD14⁺ cells were selected with monoclonal anti-CD14 conjugated to paramagnetic microbeads (Miltenyi Biotec, Gladbach, Germany). Cells were passed through an LS⁺/VS⁺ column attached to a MACS magnet (Miltenyi), washed three times with PBS containing 0.5% BSA (MACS buffer), and the column removed from the magnet. MACS buffer was then flushed through the column with a plunger to collect CD14⁺ cells. These were cultured at 2×10^6 per well in six-well plates in medium (RPMI-1640 with 1% human serum, glutamine, and gentamicin). rHuIL-4 (R&D Systems, Minneapolis, Minnesota, USA) and rHuGM-CSF (Immunex, Seattle, Washington, USA) were added each to 1,000 U/ml on days 0, 2, 4, and 6. On day 6, the nonadherent cells were transferred to a new six-well plate, and half of the medium was replaced with monocyte-conditioned medium for 2 days to mature the DCs as described (25).

In some experiments, a rEBNA1 protein or rPCNA control protein was added to the DC cell culture at the indicated concentrations with the maturation stimulus. In other experiments, 10 µg/ml of *Candida albicans* cell lysate (Allermed Laboratories, San Diego, California, USA), mumps skin antigen USP (Pasteur Merieux Connaught, Swiftwater, Pennsylvania, USA), or tetanus toxoid (Wyeth Lederle, Philadelphia, Pennsylvania, USA) was added to the wells with maturation stimulus on day 6 of DC culture. For use as target in ⁵¹Cr-release assays, DCs were pulsed with either 1 µg/ml of rEBNA1 protein or rPCNA control before use as targets. For positive selection of CD4⁺ T cells, CD14⁺ cells were treated with a monoclonal anti-human CD4

Ab conjugated to magnetic microbeads (Miltenyi Biotech). T cells and DCs were used fresh or after cryopreservation in FCS and 5% DMSO.

Cell lines. Autologous B-lymphocyte cell lines (B-LCLs) were generated on all lab donors by culturing CD14⁺ cells with the supernatant of the B95-8 marmoset cell line in RPMI-1640 fortified with 20% FCS plus gentamicin, glutamine, and 1 µg/ml cyclosporine A. Established B-LCLs were then maintained in RPMI-1640 plus 10% FCS plus gentamicin and glutamine. T2 cells (American Type Culture Collection, Manassas, Virginia, USA) were cultured in DMEM plus 10% FCS with gentamicin and glutamine.

DC infection with recombinant vaccinia viruses. Viruses were kindly provided by M. Kurilla as described elsewhere (5). Mature DCs were infected with recombinant vaccinia vectors (vTK⁻) as a negative control or vaccinia vectors expressing EBNA1 deleted of the glyala repeat domain (vEBNA1ΔGA) at an MOI of two for 1 hour at 37°C, and washed three times in medium with 5% human serum. Vaccinia infection was verified at 6–12 hours by intracellular staining as described previously (26) using VV1-6B6 Ab to a vaccinia early protein, followed by FACS analysis. Infection of DCs was 40–60%.

Expression and purification of recombinant EBNA1 and proliferating cell nuclear antigen control protein. EBNA1_{458–641} inserted in the expression vector pET15b (Novagen, Madison, Wisconsin, USA) was a gift from Dan Zhang and Michael O'Donnell (Rockefeller University, New York, New York, USA). The vector was transfected into *Escherichia coli* BL21 (DE3) pLysS cells. Proliferating cell nuclear antigen (PCNA) expressing *E. coli* BL21 (DE3) pLysS cells were a gift from Ming Guo (Weill Medical College of Cornell University, New York, New York, USA). Bacterial cultures were grown to an OD₅₉₅ of 0.8 at 37°C. Then EBNA1 or proliferating cell nuclear antigen (PCNA) expression was induced with 1 mM IPTG (GIBCO-BRL, Grand Island, New York, USA) for 3 hours. After harvesting by centrifugation, the cells were resuspended in 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole to a volume of 5 ml/g of cell pellet. Lysozyme was added to 1 mg/ml for 30 minutes on ice, and the suspension was sonicated for complete lysis. After centrifugation at 20,000 g for 30 minutes at 4°C, the cleared supernatant was filtered through a 0.45-µm filter and 1 ml of Ni-NTA agarose (QIAGEN Inc., Valencia, California, USA) per 10 ml lysate was added. The suspension was rotated for 1 hour at 4°C and packed into a column (Bio-Rad Laboratories Inc., Hercules, California, USA). The matrix was washed with 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole until the flow through OD₂₈₀ was less than 0.01. The recombinant proteins were then eluted with 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole. Protein-containing fractions were pooled and dialyzed overnight at 4°C against PBS. The protein concentration was determined at OD₂₈₀, purity determined by SDS-PAGE, and identity determined by Western blot analysis with

EBNA1-specific Ab (MAB8173; Chemicon International, Temecula, California, USA) or the 6xH-specific Ab AD1.1.10 (R&D Systems).

Enzyme-linked immunospot assay for IFN- γ and IL-4 secreting cells. Enzyme-linked immunospot (ELISPOT) assays were performed as described previously (26). Briefly, 96-well multiscreen plates (Millipore Corp., Bedford, Massachusetts, USA) were coated overnight at 4°C with 10 μ g/ml of IFN- γ Ab, 1-DIK, or IL-4 Ab, 82.4 (Mabtech AB, Nacka, Sweden). Plates were blocked with RPMI with 5% pooled human serum (PHS) 1 hour at 37°C. T cells (10⁵ per well) were stimulated in duplicate or triplicate with 3,000 DCs for 18 hours. To develop the ELISPOTs, the plates were treated with a second biotinylated 7-B6-1 IFN- γ or 12.1 IL-4 Ab (MABTECH), followed by avidin-bound biotinylated horseradish peroxidase (HRP) (Vectastain ABC kit; Vector Laboratories, Burlingame, California, USA). Spots were developed for 5 minutes in stable 3,3-diaminobenzidine (DAB; Research Genetics, Huntsville, Alabama, USA), washed with sterile water, and air-dried before counting using a stereomicroscope. A vvEBNA1 Δ GA response was considered significant if it was ten spots greater than the negative control (vvTK⁻) and at least twice that of the negative control.

Expansion of ELISPOT-producing CD4⁺ T cells. Positively selected CD4⁺ T cells were expanded for 7 days in medium supplemented with 5% PHS, with vvEBNA1 Δ GA-infected DCs or vvTK-infected DCs at a DC/T-cell ratio of 1:30. In some experiments we added 5 μ g/ml of W6/32 anti-MHC class I or L243 anti-MHC class II blocking Ab on days 0, 3, and 7. At day 7, expanded cells were restimulated with DCs pulsed with 1 μ g/ml rEBNA1 or control protein, rPCNA, and assayed for IFN- γ or IL-4 ELISPOTs. To compare the response to EBNA1 with other antigens, 10⁵ positively selected CD4 lymphocytes were expanded for 7 days with DCs alone or DCs pulsed with 10 μ g/ml of *C. albicans* cell lysate, tetanus toxoid, mumps skin antigen, rPCNA control protein, or rEBNA1 protein in a 96-well plate with a DC/T-cell ratio of 1:30. On day 7, the supernatant was replaced with fresh media, and wells were split 1:2, with half the well restimulated with DCs pulsed with appropriate antigen and half the well stimulated with either appropriate control antigen or DCs pulsed with nothing and assayed for IFN- γ or IL-4 secretion by ELISPOT.

Generation of EBNA1-specific cell lines by cytokine secretion in freshly stimulated PBMCs. PBMCs (75 \times 10⁶) were stimulated with autologous vvEBNA1 Δ GA-infected DCs at a ratio of 30:1 in medium fortified with 5% PHS for either 7 hours (IFN- γ) or 18 hours (IL-4). Then the cells were washed with MACS buffer, centrifuged 10 minutes at 1,800 rpm, and resuspended in cold RPMI containing 10% FCS (R10) at a concentration of 10⁷ cells per 80 μ l of media. A primary anti-CD45 Ab, conjugated to either anti-IFN- γ or anti-IL-4 (Miltenyi Biotec) was then added at a ratio of 10 μ l of Ab per 10⁷ cells. The cells were then placed on ice for 5 minutes,

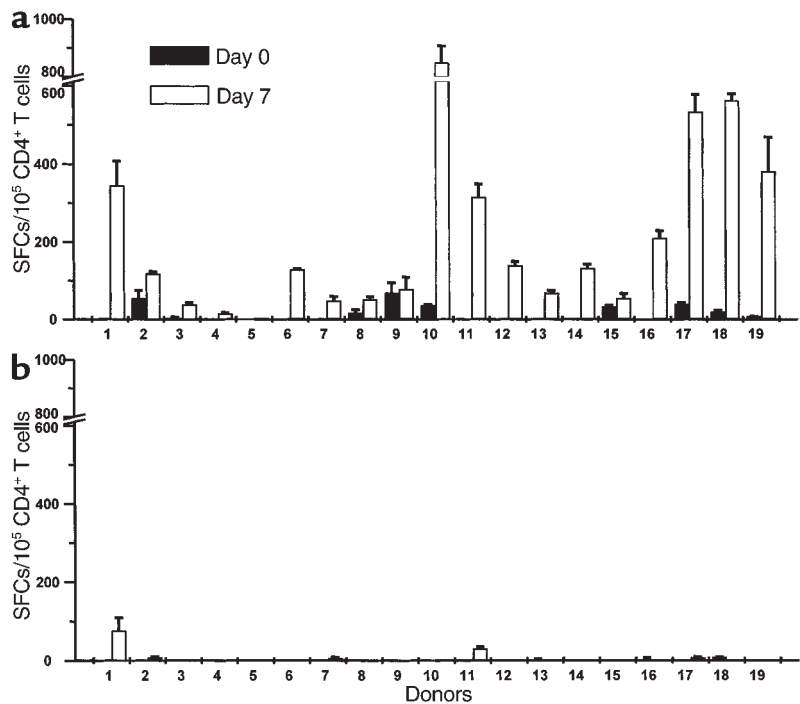
then by the addition of warm R10 to a concentration of 5 \times 10⁶ cells per milliliter, and incubated for 45 minutes under continuous rotation at 37°C. After this incubation, cells were washed with MACS buffer and centrifuged. The pellet was resuspended in 80 μ l of MACS buffer per 10⁷ cells and 10 μ l/10⁷ cells of secondary Ab to either IFN- γ or IL-4 labeled with phycoerythrin (PE; Miltenyi Biotec), placed on ice for a 10 minutes, followed by washing with MACS buffer and centrifugation. A final anti-PE Ab labeled with a paramagnetic microbead (Miltenyi Biotec) was added at a ratio of 10 μ l/10⁷ cells for 15 minutes at 4°C. Magnetic separation was performed as above and repeated to increase purity of the recovered cells. Cells were then centrifuged and cultured in medium with 5% PHS and 10⁵ irradiated CD14⁻ feeder cells in one well of a 96-well plate. The cells were restimulated weekly; alternating vvEBNA1 Δ GA-infected DCs with DCs pulsed with 2 μ g/ml rEBNA1 at the time of the maturation stimulus (days 5–7). After 3 weeks, 10 U/ml IL-2 (Lymphocult; Biotest, Minneapolis, Minnesota, USA) was added. In this way, we were able to set up lines from cells isolated from fresh blood that secreted IFN- γ and IL-4 in response to EBNA1-pulsed DCs.

FACS and functional analysis of IFN- γ and IL-4 cell lines. Cells (10⁴), 3 weeks after initiation of the lines as above, were stained 15 minutes on ice with Simultest (CD4 FITC and CD8 PE), or isotype controls (IgG1 FITC and IgG2a PE; PharMingen, San Diego, California, USA), or PE-labeled CD56 Ab, or PE-labeled isotype control (PharMingen) at a ratio of 1:50. After three washes and fixation with 4% paraformaldehyde, cells were analyzed on a FACScan (Becton Dickinson, San Diego, California, USA). For cytotoxic activity, the cell lines in triplicate were added to 10⁴ targets at the indicated effector/target ratios for 5 or 24 hours. The targets were labeled with 50 μ Ci Na₂⁵¹CrO₄ for 1 hour at 37°C and washed three times with R10. To measure cytotoxicity, 50 μ l of culture supernatant was added to 100 μ l of scintillation fluid (Wallac, Turku, Finland) in a 96-well sample plate, and radioactivity was measured in a gamma counter (1450 Microbeta counter; Wallac). Percentage of specific lysis was calculated by the following formula: [(cpm experimental well – cpm spontaneous release) / (cpm total release – cpm spontaneous release)] \times 100%. Spontaneous release was determined by incubating labeled targets in medium alone and total release by incubating targets with 1% Triton X-100.

ELISA for IgG subclasses. Ninety-six-well polystyrene plates (Nalgene Nunc International, Rochester, New York, USA) were coated with 1 μ g/well of rEBNA1 protein, mumps skin antigen USP (Pasteur Merieux Connaught), tetanus toxoid (Wyeth Lederle), *C. albicans* cell lysate (Allermed Laboratories) in PBS or PBS alone overnight at 4°C. Plates were blocked with 50 μ l/well 3% nonfat milk powder for 30 minutes, followed by 30 minutes in PBS containing 3% BSA. Test plasma samples, diluted 1:10 or 1:100 in 3% BSA, were added for

Figure 1

EBNA1-specific T-cell responses are dominated by Th1-cytokine secretion. CD4⁺ T cells were stimulated with DCs infected with vEBNA1ΔGA or vTK⁻ (negative control) and tested for their secretion of IFN-γ (a) or IL-4 (b) on the day of T-cell isolation (day 0) and after 1-week expansion (day 7). Values shown are the mean of triplicates and were derived by subtracting the negative control from the number of EBNA1-specific spots. A vEBNA1ΔGA response was considered significant if it were at least ten spots above the vTK⁻ control (usually less than 20 spots) and at least twice this negative control. SFCs, spot-forming cells.



20 minutes at room temperature. Plates were washed three times with TBST (10 mM Tris, 140 mM NaCl, 0.05% Tween 20). Biotin mouse anti-human IgG1, IgG2, IgG3, IgG4 Ab's (PharMingen) were added at 1:1000 in TBST for 20 minutes at room temperature. After plates were washed three times in TBST, avidin-bound biotinylated HRP was added for 20 minutes at room temperature, followed by TMB substrate (R&D Systems) to develop the reaction for 10 minutes at room temperature and 1 M H₂(SO₄) to stop the reaction. Plates were read in a microplate reader (Dy nex Technologies, Chantilly, Virginia, USA).

Results

CD4⁺ T-cell responses to EBNA1 primarily involve IFN-γ/Th1 cells. In a previous study (24), we identified EBNA1-specific, IFN-γ-secreting, CD4⁺ T cells using two, week-long stimulations by DCs infected with recombinant vaccinia EBNA1 virus (vEBNA1ΔGA). Here, we assessed if this response could be detected at the time of T-cell isolation (day 0) and after 1 week of expansion (day 7), and we enumerated both IFN-γ- and IL-4-secreting cells. At both time points, no EBNA1-dependent T cells could be detected in cultures stimulated with DCs infected with control vaccinia virus (vTK⁻). With DCs expressing vEBNA1ΔGA, we found Th1 cells in seven of 19 normal donors in day-0 cultures, but Th2 in none ($P = 0.011$) (Figure 1, a and b). In 1-week cultures, 18 of 19 donors demonstrated an expansion of IFN-γ cells, but only two of 19 had EBNA1-dependent IL-4 secretors ($P < 0.001$) (Figure 1, a and b). In these two individuals who made detectable IL-4, the number of IFN-γ ELISPOTs was nevertheless three times greater. Therefore, in most donors,

EBNA1-responsive CD4⁺ T cells have a Th1 phenotype, secreting IFN-γ and not IL-4.

To verify the MHC II restriction of these CD4⁺ T-cell responses, we stimulated T cells with vEBNA1ΔGA-infected DCs for 1 week in the absence or presence of blocking Ab's to MHC class I (W6/32) or HLA-DR (L243). When IFN-γ-secreting cells were enumerated by ELISPOT, only the L243 mAb decreased responses by a range of 88–100% in three experiments (data not shown). We conclude that CD4⁺ T-cell responses to EBNA1 are primarily MHC II restricted and of the Th1 type.

In addition to vaccinia vectors as a source of EBNA1 protein, we tested the efficacy in an ELISPOT assay of a purified recombinant EBNA1 protein consisting of amino acids 458–641, with rPCNA as a control. These proteins, checked for purity and specificity by SDS PAGE and Western blot analysis (Figure 2a), were pulsed in graded doses onto DCs and used to read IFN-γ ELISPOTs after a week's expansion with vEBNA1ΔGA-infected DCs. Figure 2b shows the dose response of rEBNA1 compared with rPCNA control. The inset compares responses of vEBNA1ΔGA-expanded cells restimulated with vTK⁻ or vEBNA1ΔGA. A dose of only 1 μg/ml of rEBNA1 protein pulsed onto DCs gave a response that was comparable to that of the recombinant vaccinia EBNA1. This result demonstrates that EBNA1-specific Th1 cells are capable of responding to very low doses of EBNA1.

EBNA1-specific, cytokine-secreting cells isolated directly ex vivo are primarily CD4⁺. Since the EBNA1-specific ELISPOT responses from most donors required a week's culture of CD4⁺ T cells with DCs, which make high levels of the Th1-skewing cytokine IL-12 (27, 28),

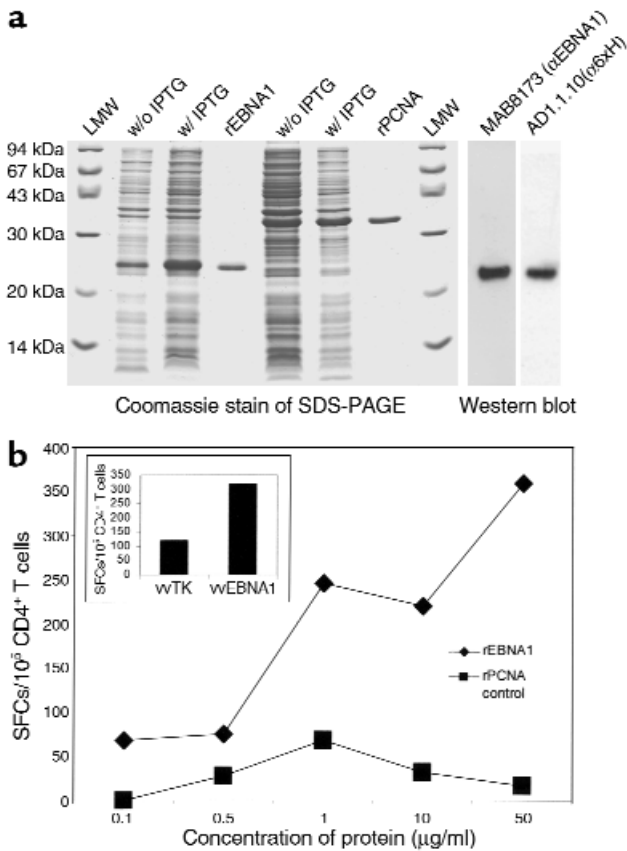


Figure 2 EBNA1-specific responses can be detected at very low doses of antigen. A recombinant EBNA1 protein or control PCNA protein was eluted from *E. coli*-expressing vectors. Proteins were dialyzed overnight and tested for purity with SDS PAGE (left) (a). The recovered rEBNA1 protein was tested for specificity by Western blot analysis using an anti-EBNA1 Ab MAB8173 (right). The Ab AD1.1.10 recognizes a histidine tag contained in the rEBNA1 protein. The wEBNA1ΔGA-infected DCs were used to expand CD4⁺ T cells in a 1-week culture. The expanded T cells were restimulated using DCs pulsed with the indicated concentration of rEBNA1 protein or rPCNA control protein and read out using ELISPOT (b). The rEBNA1 protein was added to the DCs during the maturation phase (days 6–8) of the DC culture. The inset graph shows the ELISPOT results of CD4⁺ T cells expanded with wEBNA1ΔGA-infected DCs for 1 week and restimulated with either wEBNA1ΔGA-infected DCs or wTK-control. LMW, low molecular weight marker.

we isolated EBNA1-specific cells directly ex vivo. EBNA1-specific Th1 and Th2 cell lines were isolated and expanded from six donors (see Methods).

EBNA1-specific, IFN-γ-secreting cell lines were established in all six donors, and IL-4-secreting lines in three of six donors. When the lines were analyzed by FACS (for CD56, CD4, or CD8), both IFN-γ and IL-4 lines consisted primarily of CD4⁺ cells. Figure 3 shows representative IFN-γ-secreting lines from three donors demonstrating that greater than 90% of the cells expressed CD4 and less than 2% expressed CD8 or CD56 (data not shown). Likewise, three EBNA1-specific, IL-4-secreting lines consisted of greater than 90%

CD4 and less than 2% CD8 or CD56 cells (data not shown). We conclude that EBNA1-specific, cytokine-secreting CD4⁺ T cells in healthy adults are already differentiated in vivo (Figure 3), but these cells typically must be expanded for 1 week with autologous DCs in vitro to be detected in ELISPOT assays (Figure 1).

EBNA1-specific Th1 and not Th2 CD4 T cells are able to lyse EBNA1-expressing DCs. We had shown previously that EBNA1-specific T-cell lines could kill autologous B-LCLs and DCs pulsed with different sources of EBNA1 protein (24). We assessed if this function was primarily expressed by Th1 lines, as has been observed with CD4⁺ T cells specific for other antigens (29–31). IFN-γ cell lines from five donors and IL-4 lines from three donors were tested simultaneously in cytotoxicity and ELISPOT assays. The cell lines maintained a Th1 or Th2 polarity after weeks of stimulation in the absence of exogenous IL-12 or IL-4 (Figure 4a). When we tested lysis of wEBNA1ΔGA-infected DCs, DCs loaded with rEBNA1 protein, or autologous B-LCL by ⁵¹Cr-release assay, only Th1 lines lysed EBNA1-expressing targets over the controls, though the Th2 cell lines recognized EBNA1-expressing DCs with the secretion of IL-4. Figure 4b shows data at graded effector/target (E/T) ratios, while Figure 4c summarizes killing at an E/T ratio of 10:1 for several lines. Th1 but not Th2 cell lines had cytolytic activity.

Polarized secretion of IFN-γ by expanded CD4⁺ T cells after 1-week culture is not seen with other microbial antigens. To compare EBNA1 with other proteins under our stimulation conditions, we cultured purified CD4⁺ T cells from five donors with DCs that had been pulsed with nothing, rEBNA1, rPCNA control protein, *Candida* cell lysate, mumps skin antigen, or tetanus toxoid. Most adults are known to be primed to these latter three antigens. We then tested for IFN-γ and IL-4 cytokine

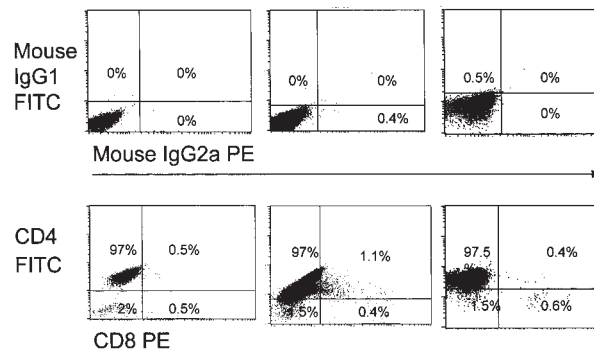
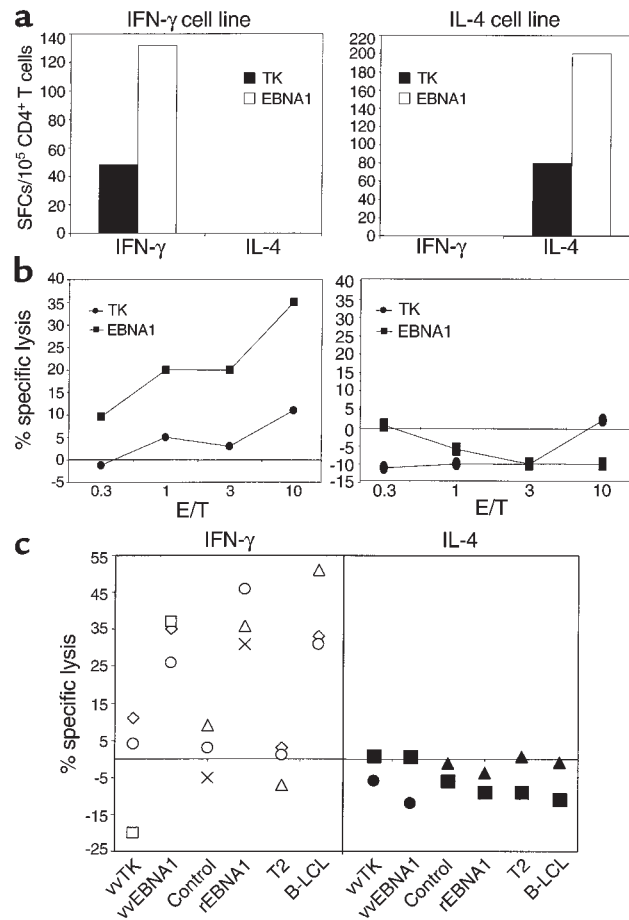


Figure 3 EBNA1-specific, cytokine-producing cells from fresh blood are CD4⁺ T cells. Freshly isolated PBMCs were stimulated with wEBNA1ΔGA-infected DCs for either 7 hours for maximal production of IFN-γ or 18 hours for IL-4. They were then positively selected based on the secretion of either IFN-γ or IL-4. IFN-γ cell lines were established in six of six donors and IL-4 cell lines in three of six donors. Shown here are CD4 versus CD8 stains of three IFN-γ lines analyzed by FACS. Gates were set on the lymphocyte population and on living cells as determined by propidium iodide staining.

Figure 4

EBNA1-specific Th1 cells, but not Th2 cells, lyse EBNA1-expressing DCs. Cell lines were isolated from PBMCs stimulated with wEBNA1ΔGA-infected DCs for either 7 hours (IFN-γ) or 18 hours (IL-4). The cells were then positively selected based on the secretion of IFN-γ or IL-4. EBNA1-specific IFN-γ- and IL-4-producing cells were isolated from six of six donors and three of six donors, respectively. These cells were expanded with weekly restimulations of irradiated wEBNA1ΔGA-infected DCs alternating with DCs pulsed with a rEBNA1 protein. (a) Cells were restimulated with wEBNA1ΔGA-infected or wTK-infected DCs and tested for either IFN-γ or IL-4 secretion after 3 weeks of expansion. The IFN-γ-secreting cell line isolated from donor 3 representative of all Th1 cell lines is shown in the left panel, and a representative EBNA1-specific IL-4-secreting cell line from donor 1 is illustrated in the right panel. (b) Cells were then tested for their ability to lyse wEBNA1ΔGA-infected DCs by ⁵¹Cr-release assay. The results of the ⁵¹Cr release from cell line from donor 3 are shown with graded effector-to-target ratios and are representative of all six established IFN-γ cell lines (left panel). Results shown are from a cell line established from donor 1 and are representative of all IL-4-secreting cell lines isolated. (c) CTL assay results at an effector-to-target ratio of 10:1 are shown for five IFN-γ cell lines and three IL-4 lines for three sources of EBNA1 antigen (wEBNA1ΔGA, rEBNA1, or the physiologically expressed protein in B-LCL) as compared with controls (wTK, rPCNA control protein, or T2 cells, respectively). Different symbols are representative of each cell line, with Th1 cell lines as open symbols and Th2 cell lines as filled symbols. Th1 and Th2 cell lines isolated from the same donor share symbol shapes.



secretion with ELISPOT at day 0 and day 7. In 1-day cultures, two of five donors demonstrated a significant EBNA1-specific IFN-γ response, but no donor demonstrated IL-4 secretion (Figure 5a). One of five donors responded to mumps and tetanus toxoid with the secretion of IFN-γ, but in contrast to EBNA1, three of five donors had IL-4-secreting antigen-specific T cells to *Candida* and mumps. IL-4 secretion was also detected on day 0 for two of five donors in response to stimulation with tetanus toxoid. In 7-day cultures, all five donors expanded IFN-γ-secreting EBNA1-specific T cells, but none had IL-4-positive ELISPOT results (Figure 5b). In contrast, an expansion of IL-4-secreting T cells specific for *Candida* and tetanus toxoid in 1-week culture was seen in all donors. Expansions of IFN-γ-secreting cells were also seen in some donors. For these other antigens, the presence of IFN-γ-secreting Th1 cells in culture did not prohibit simultaneous growth of IL-4-secreting cells. Furthermore, the use of DCs as antigen-presenting cells did not polarize to Th1 in the control antigens. The polarized Th1 response seen for EBNA1 in 1-week cultures was therefore distinct among the microbial antigens tested.

EBNA1-specific IgG subclasses in vivo reflect Th1 immunity. To assess the relative activity of EBNA1-specific Th1 and Th2 cells in vivo, we monitored the IgG subclass of the Ab response. In humans, it is believed that Th1

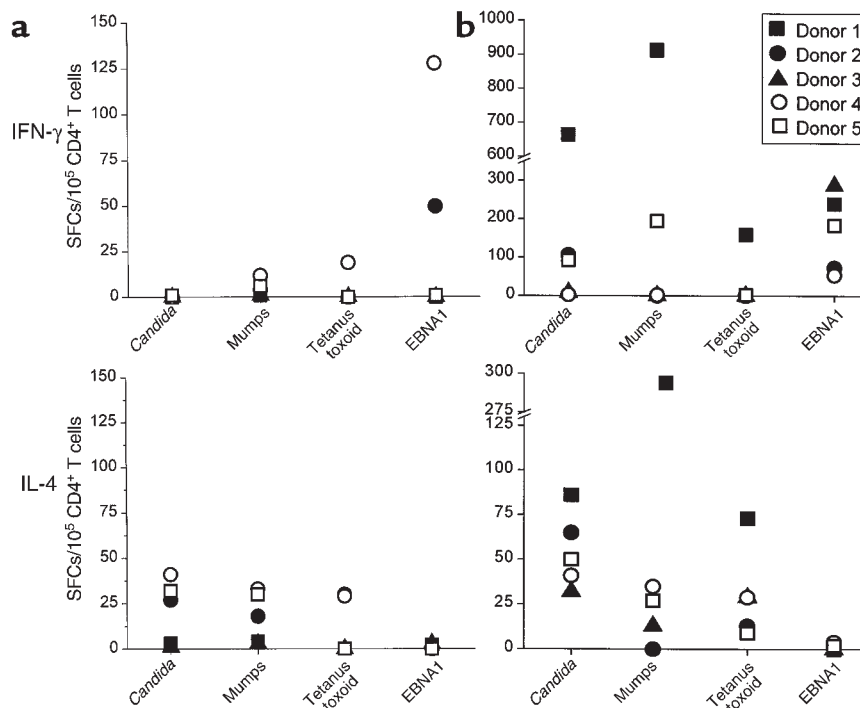
cytokines skew Ab responses toward the IgG1 opsonic and complement-fixing subclass and Th2 toward the allergic IgG4 subclass (32–34). However, the data are based on observations of IgG subclass in the context of disease states thought to be Th1 mediated, such as Lyme borreliosis and tuberculoid leprosy. An ELISA assay for IgG subclasses was used to describe the isotype of EBNA1-specific IgG Ab's and Ab's to other antigens. In all 20 donors tested, EBNA1-specific IgG1 Ab's were more abundant than all other IgG subclasses (Figure 6a; $P < 0.001$). In addition, the mean OD for EBNA1-specific IgG1 was significantly higher than the mean OD for all the other antigen-specific IgG1 responses ($P < 0.0001$ for all antigens). The Ab data, coupled with the ready detection of IFN-γ-secreting T cells in fresh and 1-week cultures of CD4⁺ T cells, indicates that the response to EBNA1 in vivo in healthy EBV carriers is consistently Th1 in type.

Discussion

How might the EBNA1-specific CD4⁺ T-cell response be skewed toward Th1 in vivo? The new data in this study indicate that healthy EBV carriers consistently make a Th1-type response to EBNA1 in vivo. These cells can be expanded directly from blood and likely explain the observed skewing of the anti-EBNA1 Ab response to the IgG1 isotype. Since B cells and B-cell lines are not known to actively

Figure 5

IFN- γ -secreting cells dominate the response to EBNA1, but not other antigens. Positively selected CD4⁺ T cells (10⁵) were stimulated with 3,000 DCs pulsed with 10 μ g/ml of rEBNA1 protein, mumps skin antigen, candidal cell lysate, or tetanus toxoid. IFN- γ and IL-4 secretion was measured on the day of T-cell isolation (a) and after 7-day expansion (b). CD4⁺ T cells stimulated with unpulsed DCs served as a negative control for control antigens, and rPCNA-pulsed DCs served as a control for rEBNA1. Results shown have the negative controls subtracted from the total. Control wells containing T cells stimulated with unpulsed DCs and rPCNA DCs had 0–30 spots. Results were considered positive if there were more than ten spots and twice that of the negative control.



produce IL-12 or to bias the CD4⁺ T-cell response toward Th1, we suspect that DCs, which are high IL-12 producers (27, 28), are responsible for Th1 skewing. It is now known that human DCs efficiently cross-present EBNA1 from dying EBV-infected B cells (24) and that mouse DCs skew T cells toward the Th1 type in vivo (35, 36). Perhaps this cross-priming occurs during acute infection when there is death of B cells undergoing lytic infection with EBV. Therefore, we would suggest that DCs, rather than infected B cells, are the direct inducers of the Th1 response to EBNA1 in healthy carriers.

The importance of CD4⁺ T cells in resistance to persistent viral infections. In HIV-1 infection, a minority of patients have high CD4⁺ T-cell counts and low viral loads without antiretroviral therapy (37). These long-term non-progressors are distinct in their vigorous CD4⁺ T-cell proliferative responses to HIV p24 and gp160 protein. The role of CD4⁺ T cells in chronic viral infection has been more directly assessed in mice (16). Primary infection with the murine γ -herpesvirus, MHV-68, resolves similarly in MHC class II^{-/-} and MHC class II^{+/+} mice. However, 3 weeks after the initial infection, the virus recrudesces in MHC II knock-out mice, which then develop wasting, and within 4 months the majority die. This occurs despite the fact that initial viral clearance takes place, apparently through CD8⁺ T-cell cytotoxicity (16). Taken together, these results are part of an emerging consensus that CD4⁺ T cells maintain effective CD8⁺ T-cell function against viruses (reviewed in ref. 14) and tumors (reviewed in ref. 13).

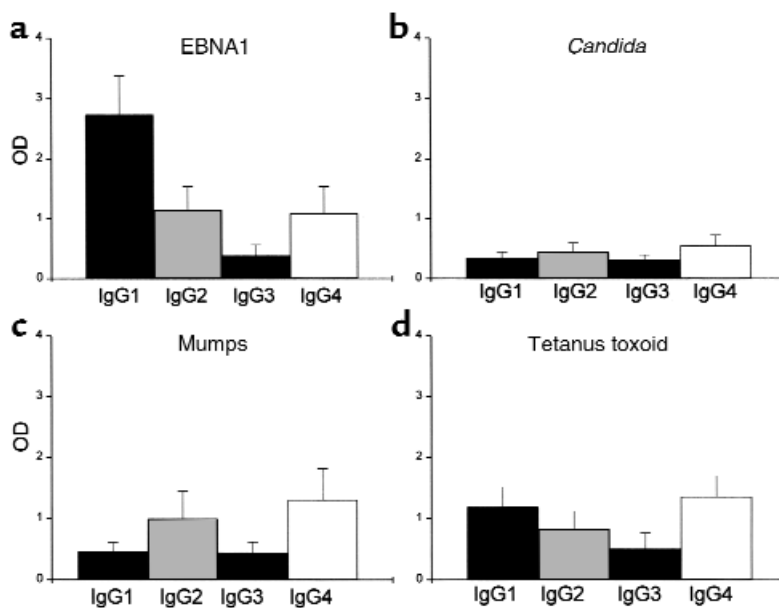
The mechanism underlying this role for CD4⁺ T cells could reflect improved function of antigen-presenting cells, especially DCs (17, 19, 20). CD40L, which is expressed more abundantly on activated CD4⁺ than

CD8⁺ T cells, is strongly implicated as the stimulus for DCs. CD40 is abundant on DCs, and its ligation mediates several critical steps in DC development and function. This includes their generation from CD34⁺ progenitors (38), mobilization from peripheral tissues (39), maturation (40), survival (41), and cytokine secretion, particularly IL-12 (27, 28).

The Th1 subset of CD4⁺ T cells is more important than Th2 in resistance to viruses and tumors. Cytomegalovirus-seronegative (CMV-seronegative) recipients of CMV-positive kidneys were monitored for CMV-specific immune responses after transplantation and a polarized Th1 response was observed (42). These patients recovered from acute infection without signs of chronic CMV disease, despite immunosuppressive therapy. Mouse models more directly illustrate the importance of Th1 CD4⁺ cells in immunological resistance to persistent antigens. When ovalbumin was expressed in tumors as a surrogate antigen, adoptive transfer of ovalbumin-specific Th1 cells led to stronger CD8⁺ T-cell memory than adoptive transfer of Th2 cells with the identical T-cell receptor for ovalbumin (31). In another study, neonatal mice immunized with an influenza subunit vaccine, in combination with IL-12, exhibited enhanced Th1 cytokine expression and demonstrated a 100% survival after challenge with influenza virus in comparison with a 55% survival rate among neonatal mice immunized with influenza subunit vaccine alone (43). A recent study employed adoptive transfer of Th1 and Th2 cells expressing an identical TCR transgene specific for the vesicular stomatitis virus (VSV) glycoprotein into TCR β ^{-/-} δ ^{-/-} mice (44). Both Th1 and Th2 T cells conferred systemic protection against VSV infection,

Figure 6

EBNA1-specific Ab's are predominantly IgG1 in contrast to Ab's to three other microbial antigens. Values shown are the mean of 20 donors with the plasma diluted 1:10 with 3% BSA. The background levels in the absence of antigen for each IgG subclass were subtracted from the results with the microbial proteins. IgG subclass distribution of Ab's specific to a rEBNA1 protein are shown (a). IgG subclass distribution to other proteins, mumps, *Candida*, and tetanus toxoid were also determined (b-d).



most likely through the elaboration of neutralizing anti-envelope Ab's. However, when the mice were challenged with a vaccinia-VSV recombinant virus, only Th1 CD4⁺ T cells to VSV glycoprotein protected the mice against the recombinant virus. Furthermore, only the Th1 cells provoked a delayed-type hypersensitivity (DTH) response and protected against lethal intranasal infection.

Several possible mechanisms for the protective function of Th1 CD4⁺ T cells. Th1 CD4⁺ T cells could control viral infections through cytotoxicity. In the current study, only Th1 cells could lyse EBNA1-expressing targets (Figure 4). The restriction of cytotoxicity to Th1 cells has been described previously (29–31). Likewise, in a model in which ovalbumin-specific Th1 and Th2 cells were generated from TCR-transgenic mice and tested for their ability to lyse murine tumor cells expressing ovalbumin, only the IFN- γ -secreting CD4⁺ T cells lysed tumor cells (31).

Th1 and Th2 cells also differ in their ability to home to sites of infection (45, 46). Th1 cells, but not Th2 cells, migrate in response to the chemokines, MCP-1, Mig, and IP-10, which are produced in infected tissues (44). This is due to differential expression of chemokine receptors, Th1 cells expressing CCR2 and CXCR3 and Th2 cells, CCR3 and CCR4.

As mentioned above, in a murine model of tumor immunity, adoptive transfer of either Th1 or Th2 cells eradicated tumors, but the mechanisms of tumor eradication appeared very different between the two types of helper cells. In mice receiving Th1 cells, the tumor was infiltrated mainly by lymphocytes. Conversely, the mice that received Th2 cells demonstrated a tumor infiltrate marked by eosinophils and neutrophils. Interestingly, only Th1 cells generated immunological memory to tumor rechallenge (31). Perhaps Th1 cells are better able to induce IL-15, a known product of DCs (47, 48) and a key factor in maintaining CD8⁺ T-cell memory (49).

The isotype of IgG Ab's to microbial antigens. In mice, IgG subclass switching is strongly influenced by the type of T-helper cytokines secreted (reviewed in ref. 50).

IFN- γ selects for IgG2a Ab's and IL-4 switches the response toward allergic IgG1 and IgE isotypes. In humans, increased levels of IL-4 direct secretion of IgG4 (51, 52), while certain disease states that are thought to be Th1 mediated show an increase in either IgG1 (32, 34, 53, 54) or an elevated IgG2/IgG3 ratio (55). However, these studies have not correlated the IgG subclass of human Ab's with cytokine secretion of the antigen-specific T-helper cells.

The current study compares Ab isotypes and T-cell cytokine secretion for several microbial antigens (Figures 5 and 6). EBNA1-specific Ab was primarily of the IgG1 type, and this correlated with a consistently polarized IFN- γ secretion by EBNA1-specific T cells. The other microbial antigens elicited secretion of both IL-4 and IFN- γ from CD4⁺ lymphocytes, and the IgG Ab's to these antigens were distributed across the different subclasses.

Elevations of IgG1 have been reported for other chronic viral infections, such as hepatitis B (53, 56) and CMV (54). The ability of IgG1 immunoglobulins to fix complement and bind to macrophage Fc receptors is thought to be important in the neutralization of free viral particles (57). We conclude that the observed polarization of EBNA1-specific IgG subclasses is most likely a consequence of a predominant Th1 phenotype of responding CD4⁺ lymphocytes and provides evidence for the polarization of the T-cell response to Th1 in vivo.

Implications. Since the CD8⁺ T-cell arm of the EBNA1 response is blocked at the level of antigen presentation on MHC class I, the CD4⁺ response becomes the principal T-cell mechanism to recognize EBNA1, which is the sole latency antigen known to be expressed in all forms of EBV-associated cancers. Because the EBNA1 response is of the Th1 type that conveys resistance to viruses and tumors, this protein provides a new focus

for vaccination and immunotherapy. Reciprocally, a loss of the Th1 phenotype of the EBNA1 response may allow for the development of EBV-associated malignancies. It will be important to determine if both the quality and quantity of EBNA1 immunity is altered in these conditions. It is known that Reed-Sternberg cells in Hodgkin's lymphoma make large amounts of IL-13 (58). Like IL-4, IL-13 can skew the CD4 response to the Th2 type, thereby reducing T cell-mediated resistance, as suggested by the literature, to other persistent viruses and tumors discussed above. We would suggest that vaccines and therapies against EBV and EBV-associated malignancies should include a means to elicit a polarized Th1 CD4⁺ response to EBNA1, most likely by targeting EBNA1 to mature immunogenic DCs.

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