Type 2 Cytokines Predominate in the Human CD4\(^+\) T-Lymphocyte Response to Epstein-Barr Virus Nuclear Antigen 1

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Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that persistently infects 85% of the adult population worldwide. In this report, we examine the proliferative response and cytokine secretion profile of CD4\(^+\) T lymphocytes from a panel of unrelated EBV-positive donors against two EBV latent antigens, EBNA1 and EBNA3C. Substantial proliferative responses by CD4\(^+\) lymphocytes were demonstrated to both antigens in multiple, randomly selected donors. Surprisingly, we observed a striking and consistent difference in cytokine response to EBNA1 and EBNA3C. EBNA1-specific CD4\(^+\) T lymphocytes from multiple unrelated donors preferentially produced type 2-like cytokines in response to antigenic stimulation, while the response to EBNA3C was a characteristic type 1 response. The implications of these findings for EBV persistence and the development of EBV-associated malignancies are discussed.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects more than 85% of the adult population worldwide and establishes a persistent infection for the lifetime of the host. The EBV carrier state (latency) is characterized by expression of a limited set of EBV genes and by sporadic reactivation of the lytic cycle in latently infected cells, leading to viral replication. Several lines of evidence implicate latently infected B lymphocytes as the major EBV reservoir. Upon reactivation in tonsillar B lymphocytes, EBV can productively infect oropharyngeal epithelium, resulting in infectious virus production and transmission (29, 49).

The importance of EBV as a human pathogen is evinced by its etiologic role in the infectious mononucleosis syndrome. The potential significance of EBV in malignancy is suggested by its B-cell-transforming ability in vitro and by its strong association with a number of human malignancies, including Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, and immunoblastic lymphomas in immunocompromised patients. Despite the oncogenic potential of the virus, the vast majority of EBV-infected individuals remain asymptomatic. A considerable amount of evidence suggests that cell-mediated immune response, particularly CD8\(^+\) cytotoxic T-lymphocyte (CTL) recognition of the EBV latent proteins in persistently infected cells, is critical in suppressing EBV replication in latently infected individuals (reviewed in references 49 and 50).

Studies of EBV latent gene expression in persistently infected cells and EBV-positive tumors including both spontaneous and induced EBV-transformed B-lymphoblastoid cell lines (LCL) suggest that up to eight EBV proteins including six nuclear antigens (i.e., EBV nuclear antigen 1 [EBNA1], -2, -3A, -3B, -3C, and -LP) and two membrane proteins (latent membrane protein 1 [LMP1] and 2 [LMP2]) may be differentially expressed in cells exhibiting various forms of EBV latency. Significantly, EBNA1 is the only EBV latent antigen consistently expressed in all patterns of EBV latent gene expression, including EBV-positive malignancies; this reflects its essential function in maintaining the viral genome on latently infected cells. However, the dominant major histocompatibility complex (MHC) class I-restricted CTL responses identified in latently infected EBV carriers are directed to the EBNA3 family of latent gene products. Subdominant reactivities to LMP1 and LMP2, as well as to EBNA2 and EBNALP, have also been found in several individuals (50). By contrast, with rare exceptions (9), MHC class I-restricted CTL responses have not been detected against EBNA1 in EBV-seropositive carriers in spite of the requirement for EBNA1 expression during latent infection. The likely mechanism responsible for this absence of MHC class I-restricted CTL responses against EBNA1 in latently infected cells is cis-acting interference with proteosomal processing of EBNA1 mediated by the internal glycine-alanine repeat domain of EBNA1 (32, 33, 55). This finding led to speculation that inhibition by EBNA1 of its presentation to CD8\(^+\) MHC class I-restricted T lymphocytes may account for the lack of elimination of latently infected B lymphocytes in healthy carriers, and hence for EBV persistence and latent infection in healthy carriers (26, 30, 35, 42).

In contrast to the large body of data on the response of MHC class I-restricted CD8\(^+\) T lymphocytes to EBV latent proteins, minimal data are available on the response of MHC class II-restricted CD4\(^+\) T lymphocytes to these EBV gene products. In view of the importance of EBNA1 in EBV latency and persistence and the lack of a significant MHC class I-restricted CD8\(^+\) T-cell response to this latent antigen, we examined the response of CD4\(^+\) T lymphocytes to this essential EBV antigen to establish the contribution of EBNA1-specific MHC class II-restricted T lymphocytes to the control of EBV latency. In parallel with the analysis of EBNA1-specific T lymphocytes, we simultaneously examined the response of peripheral blood mononuclear cells (PBMC) from EBV-positive donors to another EBV latent antigen, EBNA3C. In contrast to EBNA1, the EBNA3C antigen represents a dominant target of the vigorous EBV-specific MHC class I-restricted cytolytic CD8\(^+\) T-cell response detected in healthy viral carriers (10, 26, 42, 50, 51).

In this report, we demonstrate that EBNA1-specific CD4\(^+\) T lymphocytes from the peripheral blood of a panel of unrelated healthy donors mount a vigorous proliferative response and preferentially secrete type 2 cytokines in response to the
and EBNA3C at a 1:200 dilution according to standard protocol. From left to right, the lanes represent prokaryotically expressed GST control antigen (GST), EBNA3C-specific CD4$^+$ T lymphocytes from these donors exhibit a characteristic type 1 cytokine response to this antigen. The significance of this unique cytokine response to EBNA1 for the maintenance of EBV latency and the development of EBV-related malignancies is discussed.

MATERIALS AND METHODS

Subject selection. Blood samples were collected from randomly selected, healthy adults, including three (MK, TB, and CH) with a history of infectious mononucleosis, for isolation of PBMC by Ficol-Hypaque (Ficoll-Plus [Pharmacia] or Histopaque 1077 [Sigma]) density gradient centrifugation. For donors MK and TB, EBV seropositivity was confirmed by immunoblotting. In some experiments, the buffy coat fractions of blood units obtained from Virginia Blood Services were used as an additional unselected source of PBMC from individuals likely to be EBV infected (in this instance, the lack of donor confidentiality arrangements prevented us from obtaining serum samples for direct confirmation of donor EBV status). PBMC isolated from buffy coats were individually aliquoted and cryopreserved in liquid N$_2$ until needed as a source of responding lymphocytes and/or autologous antigen-presenting cells (APC). All HLA typing for MHC class II alleles was performed by DNA typing through the generous efforts of Mary Carrington (National Cancer Institute, Frederick, Md.) and Karen Siegrist (Clinical Laboratory, University of Virginia [UVA]). Because of the high frequency of EBV seropositivity in the general adult population, cord blood samples isolated from neonatal donors, obtained through the UVA Obstetrics Department, were used to represent EBV-seronegative subjects in initial assays.

EBV preparation. EBV-transformed LCL were generated from individual donors by exogenous virus transformation of PBMC with the prototype EBV isolate B95.8 (63, 64) following established protocols (65). Briefly, approximately $10^7$ PBMC were cultured at a density of 10$^6$/ml in standard RPMI culture medium (RPMI 1640, 2 mM glutamine, 100 IU of penicillin/ml, 100 $\mu$g of streptomycin/ml, 10% fetal bovine serum) supplemented with 0.5 $\mu$g of cyclosporin A/ml following infection with B95.8-containing supernatants. Cultures were maintained at 37°C and 5% CO$_2$ with weekly (fresh medium) feedings until evidence of transformation. Therefore, established EBV-transformed LCL were cultured in standard RPMI medium or unsupplemented commercial serum-free medium designed for lymphocytes (ASM-V; Gibco, Gaithersburg, Md.).

Production and purification of prokaryotic EBNA1 and EBNA3C. Toxicity of full-length EBNA1 containing a large-interval Gly-Ala repeat sequence limits the expression of this antigen in both eukaryotic and prokaryotic expression systems. To overcome this limitation for eukaryotic expression of EBNA1 in insect cells, an EBNA1 deletion mutant (E1GA [Fig. 1]) lacking the Gly-Ala repeat was constructed by PCR deletional mutagenesis as described elsewhere (9). The E1GA deletion construct was fused with glutathione S-transferase (GST), introduced into a recombinant baculovirus produced in the laboratory of R. Ambinder (Johns Hopkins University), and expressed in SF9 cells. Lysates of baculovirus GST-E1GA and GST control recombinant-expressing insect cells, obtained from Kemp Biotechnologies (Frederick, Md.), were clarified by centrifugation and incubated with glutathione-Sepharose beads (Pharmacia, Piscataway, N.J.) at 4°C on a rotator overnight. Resin containing bound protein was then loaded into a column, washed extensively with sterile phosphate-buffered saline until the wash optical density at 280 nm (OD$_{280}$) was $<0.010$, and eluted as described below for bacterially expressed GST fusion proteins.

Production of prokaryotic EBNA1 and EBNA3C. The truncated EBNA1 construct (E1NX [Fig. 1]) was made by digesting pBS:E1, containing full-length EBNA1 (5), with NcoI and XcmI, repairing the ends with T4 DNA ligase, and cloning into the pBS vector using T4 DNA ligase. Tox-
resulting E1AB PCR fragment was cloned into the Smal site of the pGEX-2T bacterial GST fusion protein expression vector, producing an in-frame fusion of GST with E1AB (EBNA1 aa 1 to 93) with a predicted molecular mass of 36.1 kDa.

To enable efficient prokaryotic expression, plasmid pBS:EBNAC3, containing the coding sequence for full-length EBNA3C (992 aa), was cleaved by a panel of restriction enzymes into a series of smaller fragments. AsL/Pvu restriction enzyme digestion of pBS:EBNAC3 produced a fragment of EBNA3C encoding aa 376 to 668, designated E3CAP. Subsequent restriction enzyme digestion and ligation of an oligonucleotide stop codon linker at the AccI site (aa 544) in ESCAP generated the ESC-AAP construct. After EBNA3C fragment isolation and T4 DNA polymerase repair, EBNA3C was inserted into the Smal site of pGEX3x, resulting in in-frame fusions of GST with the EBNA3C constructs starting at the amino acid positions noted above. Escherichia coli strain DH10B was transformed with each of the truncated pGEX EBNA1 and EBNA3C fusion constructs (see above), and clones containing inserts in the proper orientation were isolated and confirmed by appropriate restriction enzyme digestion. These were then electroporated into E. coli strain BL21 for purification of GST in parallel with T4 DNA polymerase repair. EBNA3C gene insert were also electroporated into BL21 for purification of GST in parallel with recombinant EBNA1 and EBNA3C. Expression of appropriately sized protein upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction of several BL21 clones was confirmed by Coomassie blue staining and immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel (27).

For preparative purposes, 100- to 200-mL cultures of rich medium plus ampicillin were inoculated with recombinant EBNA1 and EBNA3C or control pMal and pGEX BL21 clones, allowed to grow to an OD600 of ~0.5, and induced with 0.1 mM IPTG at 37°C for 3 h. Bacterial lysis may occur more readily in liquid culture, particularly for recombinant proteins with bacterial toxicity, clones from 100-mL cultures were grown under ampicillin selection on a bacterial plate, the entire contents of which were used to inoculate liquid cultures, thus minimizing the time required for growth before induction.

Bacterial lysates containing protease inhibitors (10 to 20 mL of lysis buffer [1 mg of lysozyme/mL, 50 mM Tris, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride {pH 8.0 to 8.5}] were clarified by centrifugation at 38,000 x g for 30 min and purified over glutathione-resin (Pharmacia) columns. Column construction, protein binding, column washes, and 4°C for 30 min and purified over glutathione-resin (Pharmacia) columns. Column construction, protein binding, column washes, and column elution followed the manufacturers protocols except for additional recoveries and modifications. Columns were washed extensively with phosphate-buffered for glutathione-resin until the wash flowthrough OD280 was <0.010. Recombinant antigens were eluted with approximately 10 mL of elution buffer (10 mM glutathione, 150 mM NaCl, 30 mM HEPES) collected in 1-mL fractions. Fractions containing protein, as determined by the Bradford protein assay, were pooled and quantitated by Bradford assay and SDS-PAGE (10% gel) analysis. Columns were stored and regenerated according to the manufacturer’s recommendations.

Lymphocyte cultures and the generation of CD4+ T-lymphocyte effectors. Polyclonal CD4+ T-lymphocyte effectors specific for EBNA1 or EBNA3C were generated from PBMC following stimulation with the indicated concentration of exogenous recombinant EBNA1 or EBNA3C (see below). PBMC from EBV+ donors were incubated with exogenous EBNA3C at a density of 8 x 105 to 1 x 106/mL and then cultured in 24-well tissue culture plates (Falcon) at 2 x 106 to 2.5 x 106/mL in 2 mL of AIM-V (Gibco) without any supplementation at 37°C and 5% CO2 for 10 to 14 days. The cultures were replenished with fresh AIM-V as necessary (usually 1 week post-initiation). The EBNA1- and EBNA3C-specific cultures were stimulated every 10 to 14 days with autologous, irradiated PBMC pulsed with the indicated concentration of recombinant EBNA1 or EBNA3C (4 h of incubation). Responding cells were stimulated at a density of 0.5 x 106 to 1 x 106/well with irradiated APC (1 x 105 to 1.5 x 105/well) in 24-well plates containing 2 mL of AIM-V supplemented with 50 U of human recombinant interleukin-2 (IL-2; Chiron, Emeryville, Calif.) per mL and 20% (vol/vol) supernatants from the MLA-144 cell line (47). Approximately every 4 to 5 days (twice per round of stimulation), the lines were fed with fresh AIM-V supplemented with IL-2 and MLA-144 supernatants. Antigen specificity of the cultures has been ensured by periodic short-term proliferation assays and cytokine assays (see below).

Proliferative responses of bulk PBMC cultures. PBMC were isolated from heparinized blood samples or buffy coats by Ficoll-Hypaque density centrifugation (see above). PBMC were seeded into 96-well plates (2 x 105/well) in AIM-V (Gibco). (A preliminary comparison with RPMI medium [Gibco] supplemented with 10% autologous human or fetal bovine serum demonstrated improved results for the serum-free AIM-V through decreased background proliferation.) Serial dilutions of antigen were added to the wells in triplicate, and triplicate wells containing no antigen were included as a control. Wells containing PBMC alone in media provide the background proliferation used to determine stimulation indices (SI), and phytohemagglutinin (PHA) was used for the positive control. The controls were incubated at 37°C and 5% CO2 for 5 to 6 days, with an additional of [3H]thymidine (1 μCi/mL) on days 4 to 5, and then harvested onto glass fiber filters. Incorporated [3H]thymidine, an indicator of cell proliferation, was assayed with a Packard 9600 direct beta counter or with a model 1205 Betaplate beta counter (Wallace Inc., Gaithersburg, Md.). Results of proliferation assays with bulk PBMC were expressed as SI (ratio of counts per minute of experimental values/background control per minute), and a specific proliferative response significantly above background (SI > 3) was considered significant. Results of proliferation assays with antigen-specific responder cells derived from in vitro stimulation with recombinant EBV antigens were expressed as tritium incorporation in counts per minute (cpm) (see below).

EBNA1- and EBNA3C-stimulated PBMC cultures (see above) undergoing multiple rounds of in vitro stimulation were periodically assayed in standard proliferation assays to confirm antigen specificity. Approximately 4 days after feeding with IL-2, responding T cells from these cultures (1 x 105 wells) were cultured in triplicate in 96-well round-bottom plates in AIM-V at 37°C and 5% CO2 with 10 μg/ml autologous, irradiated APC (LCL or PBMC) that had been incubated with antigen. Control groups containing no antigen, no responders, or no APC served as negative controls, while cultures stimulated with PHA were included as positive controls. Cells were pulsed with [3H]thymidine (1 μCi/mL) at 48 h for 16 h and subsequently harvested as described above.

Determination of MHC restriction by inhibition of proliferation with monoclonal antibodies. MHC restriction of the proliferative responses was analyzed by inhibiting proliferative responses with appropriate monoclonal antibodies according to established protocols (20a, 59a). Briefly, inhibitory anti-class I (W6/32) or anti-HLA-DR (PL15), anti-HLA-DO (D1H10), and anti-HLA-DR (PLA) monoclonal antibodies (provided by J. Sung, UVA) were preincubated with bulk peripheral blood lymphocytes (PBL) (or irradiated APC) corresponding to a final concentration of 5 to 25 μg/ml 0.5 h before pulsing with antigen (and adding responder cells). Cultures were incubated and pulsed with [3H]thymidine according to the proliferation assay described above. Alternatively, samples of supernatant were collected from replicate cultures at the indicated day poststimulation (usually 7 days to 10 after the third round of stimulation). In some cases, supernatants were periodically collected from EBNA1- and EBNA3C-specific responder cultures at the indicated times poststimulation and assayed for cytokine production by ELISA.

Cytokine production was determined by analyzing culture supernatants for the production of gamma interferon (IFN-γ; Endogen), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-13, and IL-5 (Pharmingen, San Diego, Calif.), using commercial reagents according to the ELISA protocol recommended by the manufacturer. Results are expressed as the average concentration of cytokine in the supernatant samples from replicate cultures. The sensitivity of the assays in the range of 0.010 to 0.020 ng/ml.
association with an array of MHC class II alleles (P. Steigerwald-Mullen, unpublished observations).

Figure 2 shows the proliferative response to EBNA1 and EBNA3C of PBMC from two unrelated donors of different HLA type. Lymphocytes from both donors proliferated in response to both antigens in a dose-dependent fashion (Fig. 2). The EBV antigen specificity is indicated by the absence of significant response (i.e., SI ≥3) from these PBMC populations to equimolar concentrations of GST fusion tag controls (from parental GST expression vectors lacking EBV gene inserts which were induced and purified in parallel with the EBV recombinant GST-antigen fusion products). In companion assays (Fig. 3), proliferative responses of PBMC from donor MK to both EBNA1 and EBNA3C were found to be inhibited (>80%) by addition of monoclonal antibodies directed to human MHC class II molecules but not by antibodies to MHC class I molecules, indicating that donor MHC class II-restricted CD4+ T lymphocytes were proliferating in response to the soluble EBV latent protein antigens.

As described below (see Table 1), significant EBNA1- and EBNA3C-specific proliferative responses were observed with PBMC from multiple unrelated individuals. This observation is in keeping with the high frequency of latent EBV infection (>85%) in the human population (49). These results along with the high incidence of immunoglobulin G antibody to EBNA1 and the easily inducible CD8+ T-lymphocyte response to EBNA3 in the peripheral blood of EBV-infected individuals (29, 49, 50) suggest that the EBNA1- and EBNA3C-specific proliferation and cytokine production described in this report most likely reflects the in vitro response of CD4+ memory T lymphocytes to these soluble EBV latent protein antigens. To further ensure that the vigorous proliferation response of PBL (particularly to EBNA1) was not due to a mitogenic or superantigen-like property of this protein, we examined the response of lymphocytes from three EBV-negative donors (i.e., umbilical cord derived PBMC from three neonates). As Fig. 4 demonstrates, all three lymphocyte sources mounted a vigorous response to stimulation with the mitogen PHA, but the PBMC failed to mount a response to EBNA1 over a range of concentrations. By contrast, the EBV-seropositive lymphocyte donor, MK, mounted a vigorous proliferative response to both EBNA1 and the mitogen PHA (Fig. 4). Both the EBV specificity and substantial magnitude of EBNA1- and EBNA3C-specific re-

FIG. 2. Representative ex vivo proliferation assays illustrating EBNA1- and EBNA3C-specific memory responses of PBMC from two EBV-positive donors, MK and donor BC1. PBL (2 × 10^5/well) were cultured in triplicate with the indicated concentrations of recombinant EBNA1 (E1GA) (top panels) or EBNA3C (E3C:AAP) (bottom panels) GST fusion proteins in standard 5-day proliferation assays as described in Materials and Methods. Equimolar concentrations of the GST fusion tags with no EBV gene inserts served as a control. Results are expressed as SI.

FIG. 3. MHC class II restriction of EBNA1- and EBNA3C-proliferative responses. EBNA1 (top)- and EBNA3C (bottom)-specific responders from donor MK were assayed in standard 3-day proliferation assays against irradiated autologous APC that had been preincubated with E1GA or E3C:AAP antigen (black bars) or control GST protein (open bars) in the presence of blocking antibodies (10 μg/ml, final concentration) specific for either HLA-DP (PL15), HLA-DQ (193F10), or HLA-DR (PL8) as detailed in Materials and Methods.
responses in EBV-positive donors strongly suggest that these latent antigens are reactivating memory (or recall) T-lymphocyte responses from PBMC of healthy, persistently infected EBV carriers.

**Divergent effector phenotypes and cytokine profiles of EBNA1- and EBNA3C-specific responses.** Having established that EBNA1- and EBNA3C-specific memory CD4\(^+\) T lymphocytes responses are reactivated from PBMC of EBV-positive donors using exogenous recombinant antigens, we analyzed the cytokine profile of the effector T lymphocytes responding to these antigens to gain insight into the regulatory roles of these populations in EBV immunity. Accordingly, EBNA1- and EBNA3C-specific responses from a panel of three unrelated EBV-positive donors were initially assayed for the secretion of the cytokines IFN-\(\gamma\) (type 1), IL-4 (type 2), and IL-5 (type 2), representative of the cytokine profiles of type 1 and type 2 CD4\(^+\) T-cell subsets, respectively. Because of technical limitations with IL-4 detection by ELISA in initial studies, an analysis of IL-13 production in response to EBNA1 and EBNA3C was performed as a surrogate for the type 2-associated cytokine IL-4, since IL-13 has similar effects as IL-4 and is reportedly secreted in parallel with IL-4 (15, 67). In subsequent assays, GM-CSF production was quantitated in parallel with the prototype type 1 and type 2 cytokines IFN-\(\gamma\) and IL-5 as an indicator of overall lymphocyte responsiveness since GM-CSF secretion has been reported to be independent of type 1 or type 2 polarization of effector T lymphocytes (34, 62).

Representative cytokine responses of EBNA1- and EBNA3C-specific lymphocyte cultures, established from PBMC of three unrelated EBV-positive donors, are shown in Fig. 5. A striking difference was observed in the cytokine profiles of reactivated memory T lymphocytes responsive to EBNA1 and EBNA3C in the three donors. Despite donor-to-donor variability in the quantity of cytokine secretion, CD4\(^+\) T lymphocytes from all donors produced the type 2-associated cytokines, IL-5 and IL-13, as well as IFN-\(\gamma\) in response to EBNA1. This contrasts with the type 1-type cytokine profile displayed for EBNA3C-specific responses, as evident by IFN-\(\gamma\)-secretion by EBNA3C-specific T lymphocytes and the absence of significant levels of IL-5 and/or IL-13 (Fig. 5). These data are representative of three or more independent experiments using PBMC from these donor samples over a 6- to 18-month period. The comparable levels of IFN-\(\gamma\) (and GM-CSF [see below]) secretion as well as the similar magnitude of proliferative responses (Fig. 2) exhibited by EBNA1- and EBNA3C-specific lymphocyte cultures for each donor suggest comparable levels of T-lymphocyte stimulation by the two antigens. These findings argue against a difference in antigen potency or memory cell frequency as the cause of the distinctly different cytokine profiles of EBNA1- and EBNA3C-specific T lymphocytes.

**Maturation of CD4\(^+\) effector cytokine profiles in vitro.** Figure 6 illustrates the time course of cytokine secretion in supernatants of lymphocyte cultures established from PBMC of EBV-positive donor MK following successive rounds of stimulation with EBNA1 or EBNA3C antigen. Production of the type 2 cytokine IL-5 by the EBNA1-specific T lymphocytes was
detected only after several rounds of in vitro stimulation with the EBNA1 antigen. IL-5 production by EBNA1-stimulated cultures was reproducibly observed only after three rounds of in vitro stimulation with this antigen. In contrast, the response to EBNA3C was characterized by a type 1-type cytokine secretion profile throughout multiple rounds of in vitro restimulation with EBNA3C antigen. It is important to note that GM-CSF and IFN-γ were produced by PBMC cultures stimulated with either EBNA1 or EBNA3C during the first round of in vitro antigenic stimulation, and production of these cytokines was stably maintained for EBNA3C-specific cultures through multiple rounds of in vitro stimulation. It should also be noted that even though the production of IFN-γ by EBNA3C-specific T cells during the second round of in vitro stimulation was reproducibly lower than the IFN-γ response during the third round of stimulation, the level of IFN-γ production (i.e., 2 to 3 ng/ml) was still substantial. Furthermore, with progressive expansion of these EBNA3C-specific cells after several rounds of restimulation, significant IL-5 production was not detected.

The finding that EBNA1-specific T lymphocytes (like EBNA3C-specific T lymphocytes) produced GM-CSF and IFN-γ after one round of in vitro antigenic stimulation and sustained the production of these two cytokines at increasing levels after successive rounds of restimulation in vitro is also noteworthy. This result along with the finding of vigorous and comparable proliferative responses to EBNA1 and EBNA3C (Fig. 2) suggests that EBNA1-specific T lymphocytes are present at a high frequency in EBV carriers and are comparably responsive over successive rounds of antigenic stimulation. Thus, the requirement for multiple rounds of in vitro stimulation with EBNA1 for type 2 (i.e., IL-5) cytokine expression most likely reflects a maturation of the type 2 cytokine-producing effector cell phenotype among EBNA1-specific T lymphocytes in response to antigen rather than the progressive expansion during in vitro stimulation of EBNA1-specific CD4+ T lymphocytes initially present at a low frequency in peripheral blood.

Antigen dose influences the magnitude of cytokine production but not the cytokine profile of EBV latent antigen-specific T lymphocytes. Antigen dose has been reported to influence the type 1/type 2 polarization of effector responses derived from naive lymphocyte precursors (reviewed in reference 12). However, the effect of antigen dose on the type of cytokines produced by activated effectors in response to antigenic stimulation is not well defined (12). It was therefore of interest to determine whether the dose of EBNA1 or EBNA3C used to elicit cytokine production from activated effector T lymphocytes (i.e., following several rounds of in vitro antigenic stimulation) influenced the type 1/type 2 polarization of these cells. To address this issue, we analyzed the cytokine response of activated effector T lymphocytes to a range of EBNA1 or EBNA3C doses.

Figure 7 depicts representative dose-response curves from such an analysis for two unrelated donors. Cytokine production by EBNA1- or EBNA3C-specific activated (effector) lymphocytes derived from donor MK and BC6 PBMC was determined using autologous LCL pulsed with EBNA1 or EBNA3C over a range of antigen concentrations. The activated lymphocytes harvested and analyzed 5 days after the third round of in

![Antigen Concentration (μM)](image_url)
vitro stimulation with homologous antigen (see Materials and Methods). While the magnitude of cytokine response to EBNA1- or EBNA3C-pulsed LCL varied in a dose-dependent manner, the cytokine profile of the EBNA1- or EBNA3C-specific effector T cells was not affected by the antigen dose used to elicit cytokine secretion from the activated T cells. Both EBNA1- and EBNA3C-specific effector lymphocytes responded in an antigen-dose dependent fashion and demonstrated comparable antigen responsiveness, as indicated by the magnitude of IFN-γ and GM-CSF production. Importantly, IL-5 production by EBNA1-specific effectors was directly proportional to the antigen dose used to elicit the response. Thus, the selective production of IL-5 by EBNA1-specific CD4+ T-lymphocyte effectors was quantitatively but not qualitatively altered by the dose of antigen used in vitro to elicit cytokine production. It is noteworthy that the distinct type 2 and type 1 cytokine profiles, displayed by EBNA1- and EBNA3C-specific effectors, respectively, were observed at the same antigen dose (0.25 μM) used for in vitro stimulation (i.e., reactivation) of memory lymphocytes. In addition, the absence of a significant cytokine response by EBNA1-specific effector T lymphocytes to either the GST fusion tag control antigen or the irrelevant EBNA3C antigen confirms the EBNA1 specificity of the differentiated effector cells in vitro.

The above analysis suggests that the dose of EBNA1 used to elicit cytokine responses from effector lymphocytes influenced the magnitude of the IL-5 cytokine response but not the commitment of these T effectors to a type 2-like cytokine response. However, since antigen dose could affect the ex vivo differentiation of memory lymphocytes to polarized effectors, it was important to determine if differences in the antigen dose used to activate and restimulate EBNA1-specific memory lymphocytes in vitro could account for the preferential IL-5 production by EBNA1-specific effectors. To address this issue, EBNA1- or EBNA3C-specific memory lymphocytes were activated and restimulated at one of three fixed antigen concentrations corresponding to a 25- to 50-fold range of antigen dose. The effector lymphocytes elicited over these antigen doses were then assayed for cytokine secretion in response to autologous LCL APC pulsed with various concentrations of EBNA1 or EBNA3C. These results, summarized in Fig. 8, indicated that the range of antigen doses capable of generating antigen-specific responses in vitro has minimal effects on the observed type 1/type 2 polarization of effector phenotypes. The magnitude of induction of EBNA1-specific effectors and the concomitant production of cytokines were directly proportional to the dose of antigen used for memory lymphocyte stimulation. However, the type 2 polarization of these effector lymphocytes (i.e., the production of IL-5) was independent of the antigen dose used for in vitro stimulation of memory T lymphocytes. Likewise, EBNA3C-specific effectors generated from memory cells stimulated over a 50-fold range of stimulating antigen concentration demonstrated dose-dependent, antigen-specific GM-CSF and IFN-γ responses without significant IL-5 production. Analysis of the lymphocyte cell surface phenotype of the responding cells in culture by flow cytometry revealed that >80% of the viable cells in culture at the time of cytokine elicitation with the EBV antigens were CD4+ (data not shown).

Absence of influences of recombinant antigen expression systems on the resulting effector phenotypes. Since the recombinant EBNA1 and EBNA3C antigens were expressed in eukaryotic and prokaryotic expression systems, respectively, it was important to evaluate the contribution of the antigen source on the cytokine profile of the latent antigen-specific T lymphocytes. For this purpose, recombinant EBNA1 protein was expressed in the prokaryotic GST fusion system used for EBNA3C. Technical limitations (see Materials and Methods) in the expression of the Gly-Ala repeat-deleted EBNA1 antigen in prokaryotes necessitated the use of deletion constructs for expression of prokaryotic EBNA1 (Fig. 1). These prokaryotic EBNA1 constructs, E1AB and E1NX, represent a more limited region of EBNA1 than the constructs expressed in the baculovirus system. Specifically, E1AB encodes EBNA1 aa 1 to 92, and E1NX represents aa 1 to 39 and 420 to 641, as in-frame C-terminal fusions with GST.

Figure 9 illustrates the cytokine responses of EBNA1- or EBNA3C-specific effectors derived from PBMC of three unrelated EBV-positive donors following in vitro stimulation with prokaryotically expressed EBNA1 or EBNA3C antigens. Again, within individual donors, comparable levels of antigen responsiveness of EBNA1- and EBNA3C-specific lymphocytes were indicated by the similar levels of GM-CSF and IFN-γ secretion. Significant IL-5 production was evident in cultures of EBNA1-stimulated lymphocytes from all three donors when EBNA1 antigen of prokaryotic origin was used. Thus, the dichotomy in CD4+ effector phenotypes between EBNA1- and EBNA3C-specific memory T lymphocytes is independent of the source of EBNA1 antigen.

Summary of EBNA1- and EBNA3C-specific effector cytokine responses for an extended donor panel. In view of the striking difference in the cytokine response of EBNA1- and EBNA3C-specific T lymphocytes among several unrelated donors, it was of interest to determine if this difference in cytokine profile was exhibited by a larger panel of donors. We therefore extended this analysis of EBNA1- and EBNA3C-specific CD4+ T-lymphocyte effector responses to include a total of 13 randomly selected individuals. For each PBMC donor, GM-CSF, IFN-γ, and IL-5 cytokine production by EBNA1- and EBNA3C-specific effector T lymphocytes were assessed after the third round of in vitro antigenic stimulation. Representative results for each donor (along with HLA class II typing where determined) are reported in Table 1. Of the 13 donors evaluated, 10 demonstrated responses to both EBNA1 and EBNA3C. In all cases, only EBNA1-specific T-lymphocyte effectors from these donors secreted significant levels of the type 2 cytokine IL-5. EBNA3C-specific T lymphocytes from these donors exhibited a type 1 pattern with little or no IL-5 production. Comparable results were obtained in two or more independent in vitro analyses using PBMC from donors of known HLA type. Results from three randomly selected donors (BC7, BC9, and BC10), were obtained after a single in vitro analysis of the cytokine response to EBNA1 and EBNA3C.

Two of the 13 donors (BC2 and BC4) failed to respond to EBNA1 or EBNA3C on two independent occasions. These individuals are most likely EBV negative, but serum samples were not available from these donors to establish EBV seronegativity (see Materials and Methods). The EBNA1-stimulated culture initiated from one donor (BC8 [not shown]) failed to respond after the second round of in vitro stimulation, precluding a comparison of EBNA1- and EBNA3C-specific effector cytokine phenotypes. EBNA3C-specific effector T lymphocytes from this donor did, however, produce significant levels of IFN-γ and GM-CSF but no IL-5 (not shown).

DISCUSSION

In this report, we have examined the proliferative and cytokine responses of CD4+ memory T lymphocytes from a panel of randomly selected, unrelated EBV-positive donors to two EBV latent antigens, EBNA1 and EBNA3C. Both antigens elicit proliferative T-lymphocyte responses. However, CD4+...
memory T lymphocytes to EBNA1 and EBNA3C exhibit distinctly different cytokine profiles after stimulation with antigen in vitro. While EBNA3C-specific T lymphocytes produce a cytokine profile consistent with a type 1-type polarization (i.e., high levels of IFN-γ and low levels of IL-5 or IL-13), the response of EBNA1-specific T lymphocytes is characterized by production of the prototypic type 2 cytokines IL-5 and IL-13. However, since EBNA1-specific CD4+ T cells produce both the type 2 cytokines IL-5 and IL-13 and the type 1 cytokine IFN-γ, these responding T cells should be formally classified as Th0-like effector T cells. Importantly, the distinct cytokine secretion profiles of EBNA1- and EBNA3C-specific effectors is independent of donor HLA type and not influenced by the source or dose of antigen used for in vitro reactivation of memory T lymphocytes.

We could routinely demonstrate a vigorous proliferative response to EBNA1 by PBMC from multiple EBV-positive donors ex vivo (Fig. 2 and 5). Since EBNA1-specific proliferative responses were not detected in umbilical cord-derived PBMC (Fig. 4) or in two adult PBMC donors (Table 1), this robust response to EBNA1 is unlikely due to a superantigen or mitogenic effect of EBNA1. However, as we do not have serologic confirmation that the two nonresponding individuals represented in Table 1 were EBV seronegative, the failure of these individuals to respond to EBNA1 or EBNA3C may be due to other reasons. Overall, these findings suggest that there is a

FIG. 8. Antigen dose dependence of in vitro induction of memory EBNA1- and EBNA3C-specific cytokine responses. Various doses of the EBNA1 or EBNA3C were used to activate and restimulate memory T lymphocytes. PBMC from donor MK were stimulated in vitro with one of three fixed doses of EBNA1 or EBNA3C corresponding to a 25- to 50-fold range of antigen and restimulated with the same antigen dose to generate activated EBNA1- and EBNA3C-specific effectors (see Materials and Methods). The resulting EBNA1- or EBNA3C-specific effectors were then assayed for cytokine secretion 24 h after stimulation with the indicated dose of EBNA1 or EBNA3C antigen (x axis). IFN-γ and IL-5 production by T lymphocytes in response to EBNA1 or EBNA3C along with GST controls is shown. GM-CSF responses are omitted for clarity, but they directly paralleled IFN-γ secretion.
The explanation for the preferential Th0 cytokine response to lymphocytes to activation-induced cell death (11, 65, 66), as EBNA1.

FIG. 9. Representative EBNA1- and EBNA3C-specific cytokine responses of PBMC from three unrelated donors, MK (A), BC6 (B), and BC5 (C), after in vitro stimulation with the prokaryotic EBNA1 antigen E1NX or E1AB or with the prokaryotic EBNA3C antigen. The response to the eukaryotic (baculovirus) EBNA1 construct (E1GA) was included for donor BC6. After the third round of in vitro stimulation with antigen, equivalent numbers of EBNA1- or EBNA3C-specific effectors were analyzed for IFN-γ, GM-CSF, and IL-5 production in response to 24-h stimulation. The response to the control GST antigen was at background levels in all instances and is excluded for clarity.

The high frequency of EBNA1-specific CD4+ memory T lymphocytes circulating in the blood of healthy viral carriers. Therefore, in contrast to the defective presentation of EBNA1 via the MHC class I processing pathway (9, 32, 50), processing and presentation of EBNA1 through the MHC class II presentation pathway is intact in vivo and ex vivo (16, 21, 27, 28, 46). The secretion of type 2 cytokines by EBNA1-specific T lymphocytes in vitro required repeated exposure to this antigen. These results parallel findings in other systems where the induction of a type 2 cytokine response, particularly IL-5 production, required several rounds of stimulation of memory lymphocytes in vitro by antigen (7, 11, 14, 58). It is, therefore, likely that the circulating EBNA1-specific CD4+ T lymphocytes in the peripheral blood in vivo and those that are responding to EBNA1 antigen in vitro are primed memory cells and not a circulating pool of activated type 2 cytokine-producing effectors. Indeed, the low frequency of B lymphocytes latently infected with EBV (1 in 10^5 circulating B cells [60]) in healthy carriers is consistent with the expected low probability of isolating activated CD4+ T lymphocyte effectors producing type 2 cytokines ex vivo from the peripheral blood. Furthermore, the stability of the type 1-type cytokine profile for EBNA3C-specific lymphocytes over an identical in vitro antigen restimulation protocol argues against selective expansion of lymphocytes producing type 2 cytokines because of the particular in vitro culture conditions used herein, because of a preferential susceptibility of type 1 cytokine-secreting CD4+ T lymphocytes to activation-induced cell death (11, 65, 66), as the explanation for the preferential Th0 cytokine response to EBNA1.

Although EBNA1- and EBNA3C-specific T lymphocytes differed in the capacity to secrete type 2 cytokines in response to antigen, they produced comparable amounts of IFN-γ in response to antigen. The IFN-γ production by EBNA1-specific T-cell populations directly paralleled the production of GM-CSF and was not inversely proportional to IL-5 production. This suggests that the IFN-γ production was related to the frequency of antigen-responsive memory T lymphocytes and was independent of type 2 cytokine production. Similar analyses of CD4+ T-lymphocyte responses to antigens in both humans and mice have, likewise, found a reciprocal relationship between IFN-γ production and the production of type 2-like cytokines by antigen-specific T lymphocytes (25, 52, 53, 56, 58, 59).

The type 1/type 2 polarization of CD4+ T-lymphocyte responses against viral antigens has been suggested to play a significant role in the virus-mediated regulation of the host responses to experimental murine, as well as some human, viral infections. For example, disease progression in human immunodeficiency virus infection (2, 17), susceptibility to recurrent herpes simplex labialis episodes (57), and the development of chronic hepatitis B (36–38) and hepatitis C (48, 63) virus infections have all been reported to be associated with the development of type 2-like responses and/or a reduction in proinflammatory type 1-like responses. Similarly, in several experimental murine viral infections, the development of a type 1 response during infection is associated with viral clearance, while the development of type 2 responses or the administration of type 2 cytokines delays viral clearance and exacerbates disease (8, 24, 40, 41, 45). However, demonstration of the selective inductions of a type 2 response to particular viral antigens has been mainly limited to experimental infections in the murine model (37, 38, 45, 58, 59). To our knowledge, this is the first demonstration of the selective induction of differentially polarized cytokine responses by memory T cells to a specific viral antigen in an unselected human population after natural infection.

The mechanism(s) underlying the preferential triggering of a polarized cytokine response by EBNA-specific memory T lymphocytes is unknown. A number of factors, including antigen dose, epitope selection, antigen persistence, and cytokine milieu, have been implicated in regulating CD4+ T-lymphocyte differentiation and the preferential production of type 1 or type 2 cytokine responses (reviewed in references 1, 12, 41, 43, and 54). Our finding that the polarized cytokine response of EBNA1- or EBNA3C-specific T cells was independent of the antigen dose used for the induction of memory T cells or for the elicitation of cytokine production by effector T lymphocytes argues against regulation of responsiveness at the level of antigen dose. Similarly, the demonstration of preferential Th1/Th2-like cytokine response to EBNA1 by CD4+ T lymphocytes from the majority of randomly selected individuals of various HLA types argues against the selection of type 2-like epitopes by the disparate MHC class II molecules displayed by these individuals as the mechanism for the type 2 cytokine response exhibited by EBNA1-specific T lymphocytes from these donors.

Several unique features of the host response to EBNA1 and/or its role in EBV persistence in vivo could account for the difference in cytokine polarization between the EBNA1-responding T cells and the T cells responding to EBNA3C. First, unlike EBNA3C and other EBV latent antigens, EBNA1 does not elicit a CD8+ CTL response because of its ability to inhibit its own processing and presentation to CD8+ T cells (32, 33). There is evidence from several systems suggesting that the induction of antigen-specific CD8+ T-lymphocyte responses may promote the differentiation of CD4+ T lymphocytes along
the type 1 pathway (22, 23, 59). In a similar manner, the induction of CD4<sup>+</sup> T-lymphocyte responses by EBNA1 in the absence of a concomitant CD8<sup>+</sup> T-lymphocyte response (which is generated in the response to EBNA3C and several other EBV latent antigens [50]) could result in the preferential differentiation of activated EBNA1-specific CD4<sup>+</sup> T lymphocytes along the Th<sub>0</sub>/Th<sub>2</sub> pathway. Our preliminary studies to date do not suggest a role for CD8<sup>+</sup> T lymphocytes in regulating CD4<sup>+</sup> T lymphocytes responding to EBNA1 in the type 1 pathway (22, 23, 59). In a similar manner, the induction of CD4<sup>+</sup> T-lymphocyte responses by EBNA1 in the absence of a concomitant CD8<sup>+</sup> T-lymphocyte response (which is generated in the response to EBNA3C and several other EBV latent antigens [50]) could result in the preferential differentiation of activated EBNA1-specific CD4<sup>+</sup> T lymphocytes along the Th<sub>0</sub>/Th<sub>2</sub> pathway. Our preliminary studies to date do not suggest a role for CD8<sup>+</sup> T lymphocytes in regulating CD4<sup>+</sup> T lymphocytes responding to EBNA1 in the type 1 pathway (22, 23, 59).

Alternatively, the differences in the cytokine responses to these two EBV latent antigens may reflect differences in the site or level of expression of these two proteins in latently infected healthy carriers. Available evidence indicates that continuous low-level EBNA1 expression is required for viral genome persistence and that EBNA1-expressing latently infected B lymphocytes are the likely reservoir for EBV in vivo (49, 60). By contrast, EBNA3C expression is down-regulated in B lymphocytes during persistent EBV infection (49, 60). Thus, it is possible that continuous EBNA1 expression in infected individuals mimics the chronic low-dose antigenic stimulation proposed for the type 2 polarization of CD4<sup>+</sup> T lymphocytes directed to allergens (12). Whether low-level antigen persistence or the absence of a regulatory CD8<sup>+</sup> T-lymphocyte response accounts for the unique character of the CD4<sup>+</sup> T-lymphocyte response to EBNA1, our results suggest that EBNA1-specific memory T lymphocytes are precommitted to Th<sub>0</sub>/Th<sub>2</sub> polarization at the time of reexposure to this antigen in vitro.

Because of the apparent qualitative difference in the in vitro response of PBMC from disparate donors to these two EBV latent antigens, it is tempting to speculate on the potential biological significance of the, albeit in vitro, findings reported here. Specifically the production of anti-inflammatory type 2 cytokines by EBNA1-specific CD4<sup>+</sup> effector T lymphocytes could contribute to EBV persistence in asymptomatic carriers through downregulation of the host inflammatory/immune response against the latently infected B-lymphocyte reservoir in carriers. This speculation must be tempered, however, for several reasons. First, little or no information is available on the cytokine response of T lymphocytes from asymptomatic carriers responding to other latency-associated EBV proteins (e.g., EBNA2, -3A, -3B, and -LP). It will be necessary to directly demonstrate that these other latent EBV antigens induce, like EBNA1, a preferential CD4<sup>+</sup> type-1 cytokine response in EBV carriers before a unique role of EBNA1 in maintaining EBV persistence in vivo can be validated. Second, as reported here, the CD4<sup>+</sup> T-lymphocyte response to EBNA1 includes the production of proinflammatory cytokine IFN-γ. The potential interplay of proinflammatory and anti-inflammatory cytokines produced by EBNA1-specific CD4<sup>+</sup> T lymphocytes in maintaining EBV persistence is not as yet clear but must be taken into account in considering the role of EBNA1 in regulating EBV persistence and latency in vivo.

In spite of the above caveats and the limitations inherent with in vitro data, the potential contribution of anti-inflammatory cytokines produced by CD4<sup>+</sup> T lymphocytes responding to EBNA1 along with the ability of this protein to inhibit CD8<sup>+</sup> T-lymphocyte response suggests a novel role for EBNA1 in promoting the escape of latently infected B lymphocytes from immune surveillance. For example, since type 2 cytokines have

### Table 1: Cytokine response of EBNA1- and EBNA3C-specific T lymphocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>DRB1</th>
<th>DRB3</th>
<th>DRB4</th>
<th>DRB5</th>
<th>DOA</th>
<th>DQB</th>
<th>Cytokine synthesis&lt;sup&gt;a&lt;/sup&gt; (ng/ml)</th>
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<sup>a</sup>—, locus not expressed; ?, allele not typed.

<sup>b</sup>Average concentration of the indicated cytokine in duplicate cultures. ND, not determined; NR, nonresponder (donor failed to demonstrate proliferative and/or cytokine responses against both EBNA1 and EBNA3C).
been reported to promote the growth and survival of EBV-infected B lymphocytes (3–6), a type 2 cytokine response to EBNA1 would also enhance viral persistence by sustaining the in vivo reservoir of latently infected B cells. In contrast, upon reactivation or the expression of the EBV latent antigen EBNA3C, the induction of both a CD8+ CTL response and a proinflammatory CD4+ type 1 cytokine response would result in the elimination of virally infected cells. This response in turn would limit virus replication and suppress the development of potentially lethal EBV-driven lymphoproliferative disease.

Although the pathogenesis of EBV-associated malignancies remains a subject of speculation, individuals with EBV-positive malignancies, such as Hodgkin’s lymphoma and Burkitt’s lymphoma, have demonstrable EBV-specific immune responses despite an inability to eradicate the tumor (18–20, 28). Indeed, viral and human IL-10 has been implicated in a local suppression of EBV-specific immune recognition of EBV-positive Hodgkin’s lymphomas and nasopharyngeal carcinomas (19, 31, 44, 64). In this context, it is noteworthy that many EBV-positive malignancies demonstrate a latency II pattern of EBV gene expression (49), in which latent nuclear antigen expression is restricted to EBNA1, with variable levels of the latent membrane proteins. Although the full spectrum of the host immune response during EBV latency in vivo has not been examined, our observations reinforce the view that there exists a complex and dynamic balance between the viral gene products and the host immune system responding to these gene products which simultaneously promotes EBV persistence and regulates the replication of the transforming virus.

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