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$CD4^+$ T-Cell Effectors Inhibit Epstein-Barr Virus-Induced B-Cell Proliferation

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In immunodeficient hosts, Epstein-Barr virus (EBV) often induces extensive B-cell lymphoproliferative disease and lymphoma. Without effective in vitro immune surveillance, B cells infected by the virus readily form immortalized cell lines. In the regression assay, memory T cells inhibit the formation of foci of EBV-transformed B cells that follows recent in vitro infection by EBV. No one has yet addressed which T cell regulates the early proliferative phase of B cells newly infected by EBV. Using new quantitative methods, we analyzed T-cell surveillance of EBV-mediated B-cell proliferation. We found that $CD4^+$ T cells play a significant role in limiting proliferation of newly infected, activated $CD23^+$ B cells. In the absence of T cells, EBV-infected $CD23^+$ B cells divided rapidly during the first 3 weeks after infection. Removal of $CD4^+$ but not $CD8^+$ T cells also abrogated immune control. Purified $CD4^+$ T cells eliminated outgrowth when added to EBV-infected B cells. Thus, unlike the killing of EBV-infected lymphoblastoid cell lines, in which $CD8^+$ cytolytic T cells play an essential role, prevention of early-phase EBV-induced B-cell proliferation requires $CD4^+$ effector T cells.

In the absence of effective T-cell-mediated immune surveillance, Epstein-Barr virus (EBV) behaves as a potent human tumor virus. For example, recipients of solid-organ transplants maintained on immunosuppressive drug regimens manifest dramatically increased risks for developing EBV-associated lymphoproliferative disease or lymphoma (15, 16, 43). As many as one quarter of recipients of T-cell-depleted allogeneic bone marrow transplants (BMT) develop B-cell lymphomas derived from donor EBV-infected B cells (R. S. Shapiro, A. Chauvenet, W. McGuire, A. Pearson, A. W. Craft, P. McGlave, and A. Filipovich, Letter, N. Engl. J. Med. **318:**1334, 1988). Patients with AIDS develop Burkitt's lymphoma, non-Hodgkin's B-cell lymphoma, and central nervous system lymphoma, many of which are EBV related (23). In addition, children with a variety of congenital T-cell immunodeficiencies are prone to developing fatal lymphoproliferative disease and EBV-related lymphoma (60).

Adoptive immunotherapy can restore T-cell surveillance that limits the proliferation of EBV-infected B cells in some immunodeficient hosts. Transfers of EBV-specific cytotoxic T-cell lines (CTLs) or lymphocytes of donor origin can arrest EBV lymphoproliferative disease in BMT recipients (17, 29, 41, 42, 57). Several assay systems have explored the identity of the effector cells active against EBV-transformed B cells. Engraftment of SCID mice with human peripheral blood mononuclear cells (PBMCs) from EBV-seropositive donors (hu-PBL-SCID mice), for example, results in the development of polyclonal EBV-transformed B-cell tumors of human origin, while infusion of T-cell lines raised against EBV-transformed lymphoblastoid cell lines (LCLs) from the donor of the PBMCs can prevent these tumors (6, 7, 27, 36, 44, 46, 50, 56, 59). These in vivo data together with in vitro experiments indicate that memory T cells are required to regulate outgrowth of EBV-associated lymphomas. However, the crucial T effectors in the protective response are not well characterized.

Many experiments focus on the role of cytotoxic $CD8^+$ T cells in the cell-mediated immune response to EBV. $CD8^+$ T cells make up the majority of "atypical" mononuclear cells characteristic of primary EBV infection, manifested as infectious mononucleosis (2, 68). $CD8^+$ T cells derived from mononucleosis patients recognize antigenic epitopes of both latent and lytic-cycle EBV proteins (9, 20, 51, 67). New techniques such as enzyme-linked immunospot assays and fluorescence-activated cell sorting (FACS) staining with tetrameric major histocompatibility complex (MHC) class I-peptide complexes reveal a surprisingly high frequency of circulating $CD8^+$ T cells specific for individual EBV proteins in healthy seropositive carriers (63). In vitro stimulation of PBMCs from EBV-seropositive donors with an autologous LCL yields cytotoxic $CD8^+$, HLA class I-restricted T-cell clones recognizing the same panel of epitopes, which fall predominantly within the EBNA 3A, -B, and -C and LMP2 latency proteins as well as lytic-cycle epitopes such as BZLF1 and BMLF1 (51). In vivo experiments show that transfer of $CD8^+$ T cells raised against human LCLs can prevent formation of tumors in hu-PBL-SCID mice after infusion of LCLs from the same individual (50). Virtually all of the published experiments investigating the activity of $CD8^+$ T cells employ fully transformed LCLs expressing the full panel of EBV latent antigens as the antigen-presenting cells and as targets for cytotoxic T cells (9, 26, 50, 54, 67).

Less is known about the $CD4^+$, HLA class II-restricted T-cell response to EBV antigens (35). Until recently, in vitro cocultivation of PBMCs with LCLs yielded rare cytotoxic $CD4^+$ T-cell lines; these CTLs recognized epitopes in the EBNA1 and EBNA2 latent antigens as well as in the BHRF1

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lytic antigen (24, 51). However, new evidence suggests that in vitro exposure to EBNA1-expressing dendritic cells or to LCLs induced to lytic-cycle expression yields $CD4^+$ EBV-reactive cytotoxic T cells (39). In vivo evidence for $CD4^+$-mediated EBV-specific activity also exists in hu-PBL-SCID mouse systems. In these mice, $CD4^+$ T-cell lines injected concurrently with LCLs reduce the incidence of lymphoma (50). Moreover, some T-cell lines effective in human adoptive immunotherapy trials have consisted of 98% $CD4^+$ T cells (17, 40, 57).

In the current functional experiments, we explore the role of memory T cells in limiting early stages of proliferation of $CD23^+$ B cells recently infected with EBV. Most previous experiments have focused on cytotoxic T-cell responses to established lymphoblastoid cell lines as opposed to freshly infected B cells. It is likely that the panel of virally encoded or cellular antigens expressed by newly infected cells at the time T cells are exerting their inhibitory effects on proliferation are different from those expressed in LCLs (64). EBV-infected PBMCs which persist in seropositive individuals in vivo express a very limited panel of latent antigens. Moreover, EBV-infected cells in immunosuppressed patients with increased EBV serum levels display a unique pattern of latency, with predominant expression of LMP1 and LMP2. Both patterns of EBV antigen expression are distinct from the latency III pattern displayed by LCLs (2, 22, 47, 48, 65). Accordingly, control of proliferation of recently infected B cells may be carried out by mechanisms physiologically distinct from those that control growth of transformed LCLs. Employing new approaches to monitor B-cell responses to EBV, our experiments yield the novel finding that memory $CD4^+$ T cells play a major role in preventing the expansion of B cells recently infected by EBV.

MATERIALS AND METHODS

Cultures of PBMCs. Cell donors were healthy individuals between 19 and 40 years of age whose EBV serologic status was ascertained by standard assays for antibodies to viral capsid antigen and EBNA (14, 32, 49). Venous blood drawn in a heparin-coated syringe was diluted with 1 volume of RPMI 1640 medium and underlaid with 1 volume of lymphocyte separation medium (Ficoll-Hypaque; ICN). The mixture was centrifuged at 1,000 rpm for 40 min in a clinical centrifuge at room temperature (RT). PBMCs were isolated from the interface of the gradient and resuspended at 10^6 cells/ml in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B. Cultures were seeded in 250-μl aliquots into 96-well U-bottomed plates for proliferation assays or in 2.5-ml aliquots into flat-bottomed 24-well culture plates (Falcon) for analysis of cell surface markers. Cultures were maintained at 37°C in 5% CO_2. After 2 weeks, either 100 μl or 1 ml of culture volume was replaced with fresh complete medium.

Preparation of stocks of infectious EBV and mock inoculum. Virus stocks were prepared from culture supernatants of the EBV-positive B95-8 cell line (34). B95-8 cells were seeded at 2×10^5 cells/ml in complete RPMI 1640 medium and held for 14 days at 37°C without refeeding. Cells were deposited at 1,500 rpm in a clinical centrifuge. Supernatant fluids harvested at this time contained approximately 10^5 transforming units/ml as measured by immortalization of human umbilical cord lymphocytes. The supernatant was adjusted to contain 10 g of NaCl and 8% (wt/vol) polyethylene glycol (PEG-8000; Sigma) per liter. The precipitate formed after overnight incubation at 4°C was collected by centrifugation at 7,500 rpm in a Sorvall centrifuge with a GS3 rotor. The pellet was resuspended at a ratio of 1 ml of complete medium per 50 ml of starting culture supernatant. Viral stocks were aliquoted and stored at −70°C. The mock inoculum was prepared in an identical manner from culture supernatant of the EBV-negative B-lymphoma cell line BJAB (25).

Infection of PBMCs. The dilution of EBV stock that would produce maximal proliferation of PBMCs in the presence of FK506 17 to 20 days after infection was determined by virus titration and measurement of [^{3}H]deoxythymidine ([^{3}H]dT) (Amersham) incorporation (55). For different virus stocks, optimal activity occurred with a 1:20 to 1:80 dilution of concentrated virus. A comparable dilution of mock inoculum was used. Thus, cultured PBMCs received an inoculum representing from a 2.5-fold concentration to a 1.6-fold dilution of the original B95-8 or BJAB culture supernatant fluid.

FK506 treatment. FK506, kindly provided by Fujisawa, Japan, was prepared as a stock solution at 10 mM in ethanol. FK506-treated PBMC cultures received a final concentration of 10 nM at 8 to 24 h prior to the addition of EBV or mock inoculum.

Proliferation assays. At intervals after infection or mock inoculation, 1 μCi of [^{3}H]dT in 50 μl of medium was added to 250-μl cultures of PBMCs in 96-well U-bottomed plates. The cells were incubated for an additional 24 h at 37°C. Cultures were collected onto glass fiber filters using an automated sample harvester (Wallac). Filters were suspended in scintillation fluid, and the incorporated radioactivity was determined with triplicate samples for each experimental condition.

Analysis of cell surface molecules. At intervals after infection or mock inoculation, 2.5-ml aliquots of cells were collected. The number of living cells was determined by trypan blue dye exclusion; viable cells were isolated on a Ficoll-Hypaque density gradient. The cells were washed once in phosphate-buffered saline (PBS) and resuspended at 10^6 cells/ml in PBS containing 5% FBS and 0.01% sodium azide. A mixture of saturating concentrations of two different fluorochrome-conjugated mouse monoclonal antibodies against human cell surface molecules was added. These antibodies included anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE), anti-CD8-PE, anti-CD16-PE, anti-CD19-Quantum Red, and anti-CD23-FITC. Murine immunoglobulin (Ig) (1 mg/ml) was included in the mixture to inhibit nonspecific binding. The reactivity of antibodies to human lymphoid cell surface molecules was compared to the reactivity of isotype controls consisting of polyclonal murine IgG1-FITC, IgG1-PE, and IgG2-Quantum Red. All antibodies were purchased from Sigma or Dako. Antibodies and cells were incubated together for 45 min on ice. The cells were washed twice in PBS and fixed in 1% paraformaldehyde in PBS. Staining was analyzed on a fluorescence-activated cell sorter (FACS; Becton Dickinson).

Analysis of EBV gene expression. Mixed PBMCs were examined for expression of latent EBNAs by immunofluoresence assays (IFA). PBMCs from cultures infected with EBV 23 days previously and cultured in the presence or absence of FK506 were isolated over a Ficoll-Hypaque gradient and resuspended at 3×10^6 cells/ml in PBS. Cells were allowed to air-dry for 25 min at RT on glass slides (PGC Scientifics) and fixed in a 2:1 acetone-methanol solution for 5 min at RT. Slides were stored at −20°C for up to 1 month. Detection of the latent EBNAs was performed by incubating cells fixed on the slides with a 1:10 dilution of human serum, which had been previously determined to contain antibodies directed against all known EBNAs (58). Cells were washed in PBS and incubated with a 1:16 dilution of freshly frozen EBV-seronegative human serum as a source of complement. Another wash in PBS was followed by incubation with a 1:40 dilution of anti-human complement conjugated to FITC (Atlantic Antibodies/Incstar). All incubations were performed for 1 h at 37°C. Negative controls consisted of cells reacted with complement and anticomplement-FITC without human serum. Fluorescence was detected under UV illumination with a Zeiss microscope.

Magnetic activated cell sorting. Cells expressing low levels of surface CD23 were separated from those expressing high levels on a magnetic bead column prior to detection of EBNA antigens by indirect IFA. Live PBMCs from cultures infected with EBV for 23 days were harvested over a Ficoll-Hypaque gradient. Cells were incubated with biotinylated anti-human CD23 for 30 min at 4°C in PBS plus 5% bovine serum albumin (BSA). Cells were washed and incubated at 10×10^7 cells/ml for 30 min at 4°C in PBS plus 5% BSA with streptavidin-conjugated 50-nm-diameter magnetic microbeads (Miltenyi Biotec). Cells were washed and loaded onto a MACS depletion ferromagnetic matrix column (Miltenyi Biotec) set within a magnetic field. Cells with negative or low CD23 expression were isolated using a 24-gauge needle under ambient conditions. Cells with high CD23 expression were recovered by removing the column from the magnetic field and actively plunging the cells through the column. The CD23 low and high fractions were air-dried on separate glass slides. The level of CD23 expression on each of the fractions was verified by costaining with avidin-PE and analysis by FACS.

Analysis of dividing cells. PBMCs isolated on a Ficoll-Hypaque gradient were washed once in PBS and resuspended at 10^7 cells/ml in PBS. They were incubated with 1 μM carboxyfluorescein diacetate succinymidyl ester (CFSE; Molecular Probes) for 10 min at 37°C and washed in ice-cold RPMI 1640 plus 10% FBS. CFSE-labeled cells were cultured and infected as described. At intervals after infection, viable cells were isolated on a Ficoll-Hypaque gradient. Lymphocyte cell surface molecules were detected in two steps. In the first step, the cells were incubated with biotinylated anti-CD3, anti-CD4, anti-CD8, anti-CD19, or

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anti-CD23 antibodies at saturating concentrations for 45 min at 4°C. Anti-CD23-biotin was obtained from Pharmingen. Anti-CD19-biotin was purchased from Dako. All other biotinylated antibodies were purchased from Sigma. Cells were washed in PBS, and in the second step, avidin-PE was added for a 1-h incubation at 4°C. Cells were fixed in 1% paraformaldehyde and analyzed by FACS.

Depletion of lymphocyte and specific T-cell subpopulations. Fresh PBMCs isolated on a Ficoll-Hypaque gradient were resuspended at 2×10^7 cells/ml in PBS plus 2% FBS. They were incubated for 30 min at 4°C with magnetic beads conjugated to murine anti-human CD3, anti-CD4, or anti-CD8 antibodies (against T-cell antigens) or to anti-human CD16 antibodies (against a natural killer cell antigen), at the concentrations recommended by the manufacturer (Dynal). Beads and bound cells were removed using magnets (PerSeptive Biosystems). $CD14^+$ $CD45^+$ monocytes were depleted by standard plastic-adherence protocols (11). The remaining cells were resuspended in complete RPMI 1640, cultured, and infected. The efficiency of depletion was determined immediately after separation by assessment of cell surface molecule expression. In the majority of experiments, less than 1.5% of the remaining final population consisted of cells targeted for depletion.

Isolation of B and T-cell populations by positive selection. PBMCs freshly isolated on a Ficoll-Hypaque gradient were resuspended at 5×10^6 to 1×10^7 cells/ml in PBS plus 2% FBS, based on suggestions of the magnetic bead manufacturer. Cells were incubated with antibody-conjugated magnetic beads using the conditions employed for negative selection. Anti-human CD4 or anti-CD19 antibody-conjugated beads and bound cells were recovered using magnets and resuspended at 10^8 cells/ml in RPMI 1640 plus 1% FBS. A detachment solution consisting of competing polyclonal goat or sheep antibodies raised against mouse Igs was added, and cells were incubated with gentle agitation for 1 h at RT. $CD3^+$ cells were recovered from the anti-CD3-conjugated beads by incubating the positively selected cell-bead mixture in RPMI 1640 plus 10% FBS at 37°C for at least 6 h, since competing antibodies were not available. Beads were removed by using magnets, and the cells that had been freed from the beads were harvested. The efficiency of isolation of each population was determined immediately after isolation by assessment of cell surface molecule expression. Selected populations were greater than 84.6% pure.

Use of CD23 expression as a marker of B cells activated by EBV. The number of $CD19^+$ $CD23^+$ cells was used as a marker of immune control over EBV-infected B cells when cultures were initiated from mixed PBMC populations (see Fig. 2). However, cultures depleted of various T-cell subsets manifest different growth properties regardless of infection by EBV. Therefore, in depleted cultures, the percentages, not numbers, of $CD19^+$ $CD23^+$ cells were used as an indicator of immune control (see Fig. 5 to 7).

RESULTS

Lack of memory T-cell activity augments proliferation in EBV-infected PBMC cultures from EBV-seropositive individuals. To analyze the role of T cells and effector mechanisms active in immune surveillance over early stages of EBV infection, we examined the effects of FK506 on EBV-induced lymphoproliferation in vitro. Most previous in vitro studies have monitored PBMC culture responses to EBV via a regression assay, in which the readout is microscopic enumeration of transformed-cell foci after 4 weeks in culture or isolation of transformed lymphoblastoid lines (37, 40). As a quantitative and rapid assay for T-cell control, we initially assessed cell proliferation in EBV-infected and mock-inoculated PBMC cultures by measuring [^{3}H]dT incorporation 17 to 18 days after inoculation (13, 26, 33, 55).

Proliferation of infected cells from EBV-seropositive donors was much increased in the presence of FK506, a well-known inhibitor of T-cell activation (10, 12). In the experiment illustrated in Fig. 1A, the incorporation of [^{3}H]dT in EBV-infected seropositive cultures was 8.7-fold greater in the presence of FK506 than in the absence of FK506. When results from 35 seropositive donors were pooled, incorporation of [^{3}H]dT in EBV-infected cultures was 5.4 ($\pm$0.9)-fold greater in the presence of FK506 than in the absence of FK506. In contrast, the proliferation of PBMCs from seronegative donors following EBV infection was high in both the presence and the absence of FK506, as shown in Fig. 1B. When results from five seronegative donors were pooled, incorporation of [^{3}H]dT in EBV-infected cultures was not any greater (1.2 [$\pm$0.2]-fold) in the presence of FK506.

To determine when T-cell activity impacts EBV-induced lymphoproliferation, kinetic analysis of [^{3}H]dT incorporation in response to EBV infection of PBMC cultures was performed. When the donors of PBMCs were seropositive, the EBV-infected cultures grown in the absence of FK506 proliferated for 7 to 10 days to a level somewhat higher than in mock-infected cultures, but thereafter the proliferation rate declined (Fig. 1C). In contrast, when the PBMC donors were EBV seronegative, the EBV-infected cultures continued to proliferate and eventually, 15 days after infection, reached the same level of [^{3}H]dT incorporation measured in EBV-infected cultures treated with FK506 (Fig. 1D). PBMC cultures from all donors infected with EBV in the presence of FK506 displayed progressively increasing proliferative activity to high levels after infection. The proliferation seen in mock-inoculated cultures was not unexpected, as the mock inoculum contains many antigens from FBS and EBV-negative cells which might cause T cells to proliferate. This proliferation was abolished by FK506 treatment (26). These experiments showed that the ablation of the T-cell response eliminates regression of early proliferation of EBV-infected PBMCs from seropositive donors just as it leads to outgrowth of foci in classical regression assays. This regression of early proliferation is reproducibly manifested 7 to 10 days after infection. This inhibition of proliferation is mediated by memory T cells, since it is not observed in cultures from EBV-seronegative donors.

Memory T-cell activity selectively prevents the outgrowth of EBV-infected proliferating $CD23^+$ B cells in PBMC cultures. CD23 has been regularly associated with latent infection and B-cell blast transformation induced by EBV (1, 8, 21, 62). To determine whether outgrowth of $CD23^+$ cells was selectively inhibited after EBV infection in vitro, we analyzed the numbers and proliferation profiles of T- and B-cell subsets in infected cultures. As illustrated in Fig. 2A, the presence of T-cell activity in PBMCs from seropositive donors dramatically decreased the number of $CD23^+$ B cells seen after EBV infection. By day 17.5 after infection of the FK506-treated cultures, in the absence of T-cell control, $CD19^+$ B cells made up 57% of the population, and notably 37% of lymphocytes were $CD23^+$ B cells. In contrast, by day 17.5, the untreated cultures consisted of 96% T cells, 3.2% $CD19^+$ B cells, and 0.6% $CD19^+$ $CD23^+$ cells. Up to 6.5 days after infection, the untreated and FK506-treated cultures were indistinguishable in expression of lymphocyte surface markers. At this time, both cultures contained a significant number of $CD19^+$ $CD23^+$ B cells, 4.4% of the culture, but these were subsequently eliminated in the presence of T-cell activity in the untreated culture. Although CD23 may also appear on activated $CD4^+$ T cells and follicular dendritic cells as well as activated B cells, in our experiments CD23 expression was not detected on any T-cell populations (data not shown) (5, 38).

The $CD19^+$ $CD23^+$ cells observed in the absence of T-cell activity are actively proliferating, as demonstrated in Fig. 2B by cells labeled with the fluorescent dye CFSE and cell surface

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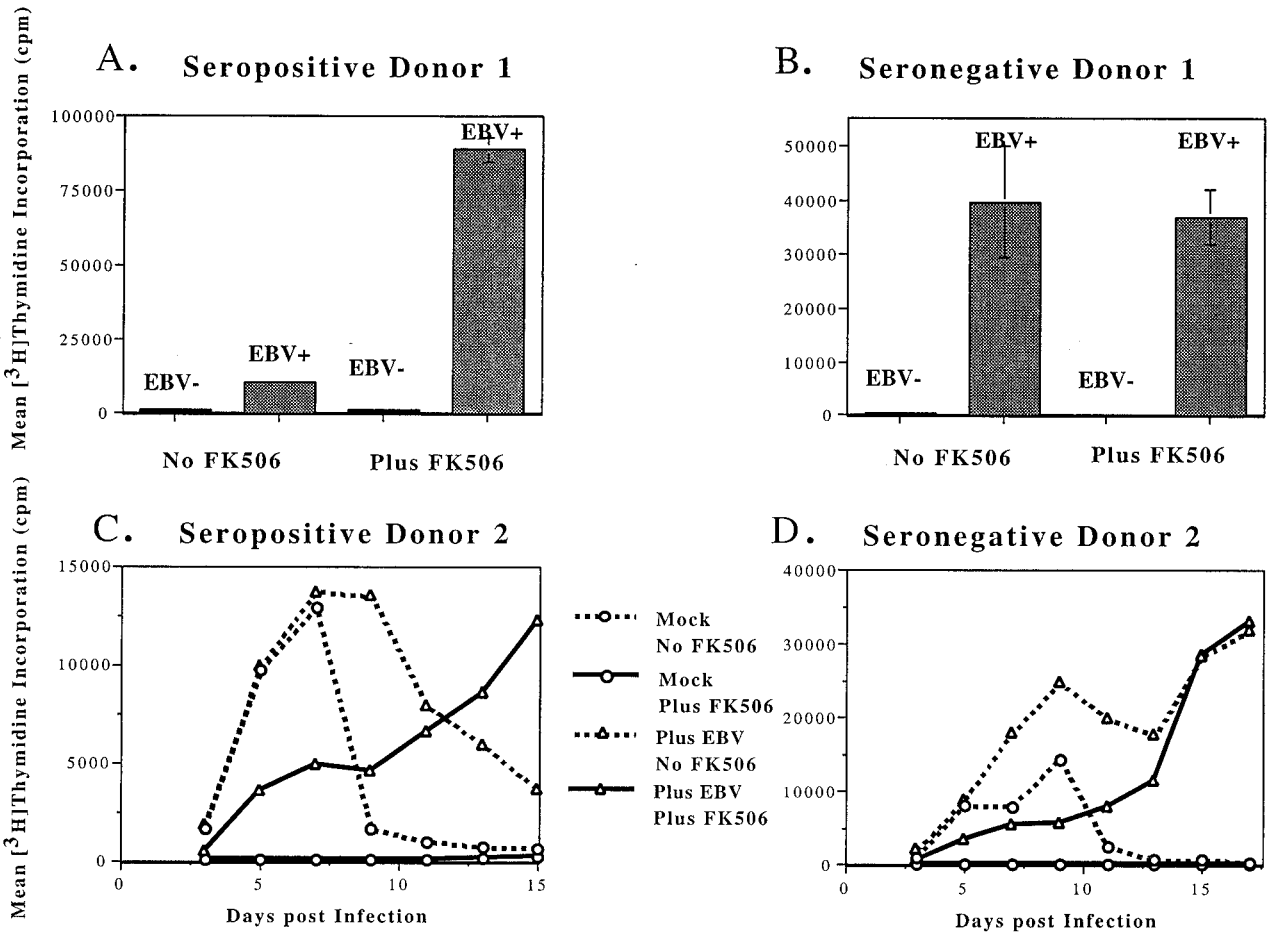

FIG. 1. Effects of FK506 on the kinetics of proliferation of uninfected and EBV-infected PBMCs as measured by incorporation of [^{3}H]dT. PBMCs from EBV-seropositive donors (A and C) or EBV-seronegative donors (B and D) were infected with EBV (shaded bars and triangles, EBV$^+$) or a control inoculum (solid bars and circles, EBV$^-$) in the presence or absence of FK506. (A and B) Seventeen days after infection, [^{3}H]dT was added to the PBMC cultures; the incorporated radioactivity was measured 24 h later. Results represent the mean ± standard error of the mean (SEM) of triplicate samples for each experimental condition. (C and D) Solid lines indicate addition of FK506; dashed lines represent PBMC cultures without FK506. [^{3}H]dT incorporation was determined at intervals from 2 to 15 days after infection.

markers. With each cell division, the CFSE dye partitions to daughter cells, and CFSE fluorescence intensity declines by half (28). At 3 days after infection, there was no difference in fluorescence profile and little cell division was evident between cultures grown in the presence or absence of FK506 (data not shown). In the absence of FK506, by 17.5 days after infection, only a CD3$^+$ T-cell population which had undergone multiple rounds of proliferation remained (Fig. 2B, left panel). However, in the presence of FK506, the CD19$^+$ and CD23$^+$ populations had undergone more than seven cell divisions, as assessed by intermediate time points where six peaks of proliferation are clearly visible (data not shown) and by dividing the intensity of the nonproliferating peak by 2^7. The majority of the surviving T cells had not proliferated (Fig. 2B, right panel). Division of significant numbers of CD3$^+$ T cells or CD23$^+$ B cells was first noted 6 to 8 days after infection (data not shown).

The CD19$^+$ CD23$^+$ cells which proliferated in the absence of memory T-cell activity expressed latent EBV proteins detected by immunoblotting and anticomplement IFAs (data not shown). At least 75 to 80% of cells present at 23 days after infection with EBV in the presence of FK506 stained brightly for EBNAs, the marker of latent viral infection. Separation of cultures into CD23-low and CD23-high cells showed that the EBNA$^+$ cells resided mainly within the CD23-high population (Table 1).

Comparison of the number of CD19$^+$ CD23$^+$ cells present 2 to 3 weeks after EBV infection in the absence and presence of FK506 reproducibly indicated the presence or absence of EBV immunosurveillance by memory T cells in culture. Using cells from four EBV-seropositive donors, there were 4- to 20-fold more CD23$^+$ B cells in cultures treated with FK506 than in untreated cultures (data not shown). Thus, memory T cells act to prevent rapid proliferation of EBV-infected CD23$^+$ B cells in the first 3 weeks after infection in addition to preventing eventual outgrowth of foci and EBV-infected LCLs.

Control over proliferation of CD23$^+$ B cells is dependent on cell density, as are established regression assays. Our data showing a role for memory T cells in preventing early CD23$^+$ B-cell proliferation are consistent with the crucial role for T cells in preventing outgrowth demonstrated in regression assays. We therefore asked whether control over proliferation of

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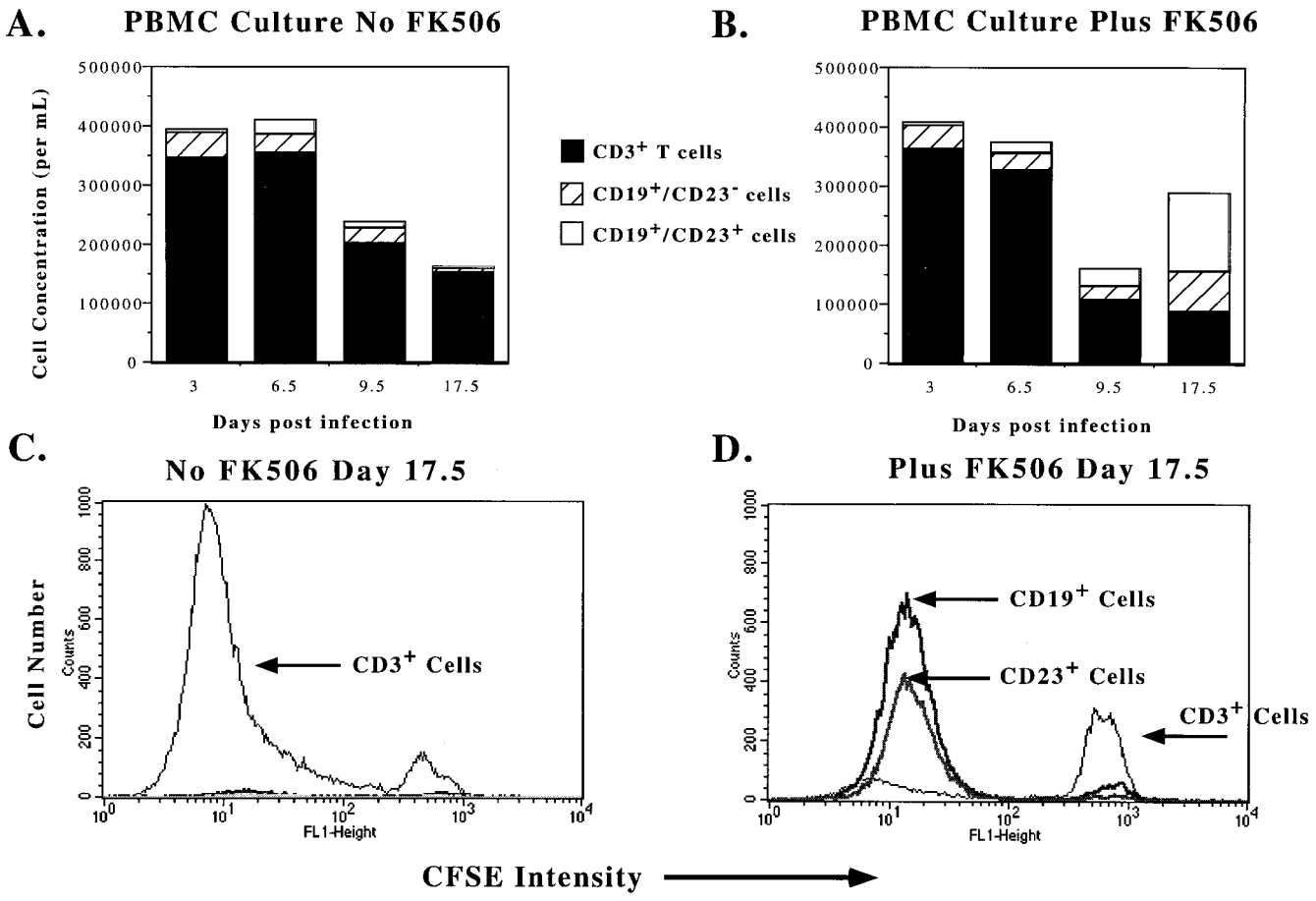

FIG. 2. Composition and proliferation of PBMC cultures infected with EBV in the absence or presence of FK506. (A and B) PBMCs from an EBV-seropositive donor were infected with EBV. The numbers of $CD3^+$ (black), $CD19^+$ (hatched), and $CD23^+$ (white) cells were determined by FACS four times after infection with EBV in the absence (A) or presence (B) of FK506. (C and D) PBMCs from an EBV-seropositive donor were incubated with CFSE dye and then infected with EBV in either the absence (C) or presence (D) of FK506. Living cells were analyzed for CD3, CD19, and CD23 expression and the intensity of CFSE fluorescence 3 and 17.5 days after infection. Progressive rounds of cell division are reflected in sequential halvings of fluorescence intensity and a shift to the left in the profile of CFSE staining.

$CD23^+$ B cells was likewise dependent on the cell density at which infected cultures are initiated; this density dependence is a classic feature of the regression phenomenon when assessed by formation of foci of transformed lymphocytes. In cultures derived from EBV-seropositive donors, regression is noted at high starting cell density and is eliminated or reduced at low starting cell densities (31, 52). It is not known whether this dependence on high cell density reflects a requirement for a critical number of memory T cells per culture, a need for cell-to-cell contact, or other factors such as cytokine concentrations.

TABLE 1. Expression of latent EBV nuclear antigen EBNA correlates with expression of CD23[a]

Cells	Treatment or subpopulation	% Reactive cells		
		Anti-EBNA	Anticomplement	Anti-CD23
PBMCs	No FK506	8	2–3	2
	Plus FK506	75–80	<1	55
CD23 selected	Low CD23	5	5	
	High CD23	60	5	

[a] All cells were derived from seropositive donors, analyzed 23 days postinfection. EBNA and complement were analyzed by immunofluorescence microscopy. CD23 was analyzed by FACS.

We found that inhibition of proliferation assessed by the number of $CD19^+$ $CD23^+$ cells was also dependent on starting cell density (Fig. 3). By day 16, there were only 3 to 5% $CD19^+$ $CD23^+$ cells when cultures were initiated at 6×10^5 cells/ml or higher in the absence of FK506. However, when cultures were initiated at 2×10^5 cells/ml, the cultures contained 44% $CD19^+$ $CD23^+$ cells on day 16. In the presence of FK506, more than 20% of the infected population were $CD19^+$ $CD23^+$, even if the cells were initially seeded at the highest cell density (10^6 cells/ml). This fraction rose to 44% when the cells were seeded at the lowest cell density; this indicates that even in the presence of FK506, there may be some residual immune activity. Thus, the ability of memory T cells to regulate $CD23^+$ B-cell proliferation after new EBV infection is diminished as initial culture density decreases.

Depletion of particular T-cell subsets enables outgrowth of $CD23^+$ B cells. While FK506 inactivated the entire T-cell population, depletion of T-cell subsets allowed us to ask which T

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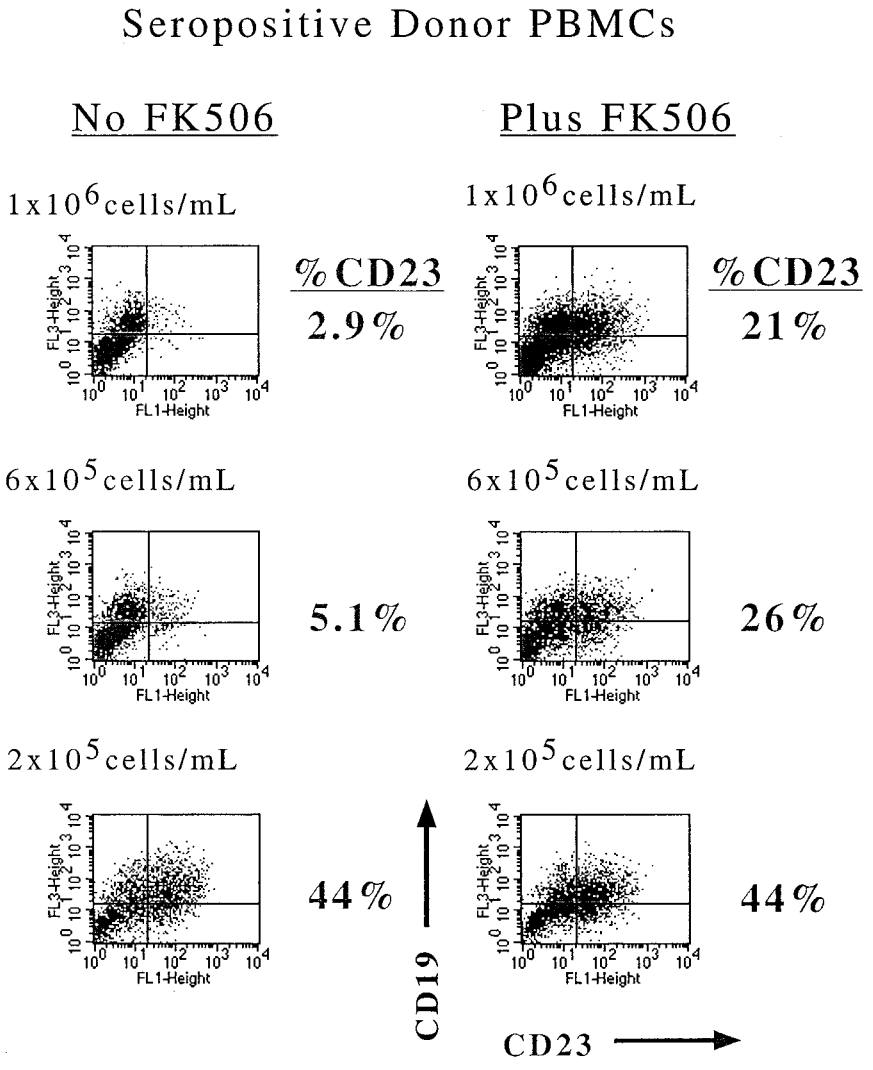

FIG. 3. Effect of initial cell density on the proportion of $CD23^+$ B cells in PBMC cultures infected with EBV in the presence and absence of FK506. FACS analysis of expression of CD19 (vertical axis) and CD23 (horizontal axis) in PBMC cultures initiated at starting densities varying between 10^6 and 2×10^5 cells/ml. All cultures were derived from the same EBV-seropositive individual, simultaneously infected with EBV, and analyzed 16 days after infection.

cells were crucial in early stages of EBV-specific immune control. As shown in Fig. 4, depletion of $CD4^+$ but not of $CD8^+$ T cells had an impact on $CD23^+$ B-cell appearance. Initially, cultures of nondepleted PBMCs or cultures depleted of $CD3^+$, $CD4^+$, or $CD8^+$ cells by magnetic bead selection contained very low fractions of $CD23^+$ B cells, fewer than 1.1% of the cultures. However, 5 days after infection there began a rapid progressive increase in the fraction of $CD23^+$ B cells in the CD3-depleted cultures which was not observed in nondepleted PBMC cultures (Fig. 4A). Not surprisingly, $CD3^+$ cells were necessary for immune control. There was a slight increase in the percentage of CD23-positive cells in CD3-depleted cultures treated with FK506. This is likely secondary to FK506's ability to block calcineurin-phosphatase activity in cells other than T cells and its ability to enhance B-cell viability (3, 18).

A rapid increase in the fraction of $CD23^+$ B cells also occurred in EBV-infected cultures depleted of $CD4^+$ cells (Fig. 4B). Thus, $CD4^+$ cells appeared to be necessary for control over early stages of $CD23^+$ B-cell generation. Surprisingly, regression was maintained in the CD8-depleted cultures, which were indistinguishable from PBMC cultures by CD23 expression 13 days after infection (Fig. 4B). The addition of FK506 to CD8-depleted cultures eliminated immune control (data not shown). This indicated that CD8 depletion per se was not inhibitory to EBV-induced lymphoproliferation.

Comparison of CD3-depleted, CD4-depleted, and CD8-depleted PBMC cultures derived from a single EBV-seropositive donor confirmed that $CD4^+$ cells were necessary and sufficient to inhibit the accumulation of $CD23^+$ B cells, while $CD8^+$ cells on their own could not (Fig. 4C). Outgrowth of $CD23^+$ B cells occurred in all 10 trials of depletion of $CD3^+$ cells, in 9 of 10 trials of depletion of $CD4^+$ cells, and in none of 14 trials of depletion of $CD8^+$ cells (data not shown). Depletion of $CD3^+$ enhanced both the rate and extent of increase in the $CD23^+$ fraction over that seen in depletion of $CD4^+$ cells, suggesting some role for residual $CD3^+$ $CD4^-$ cells.

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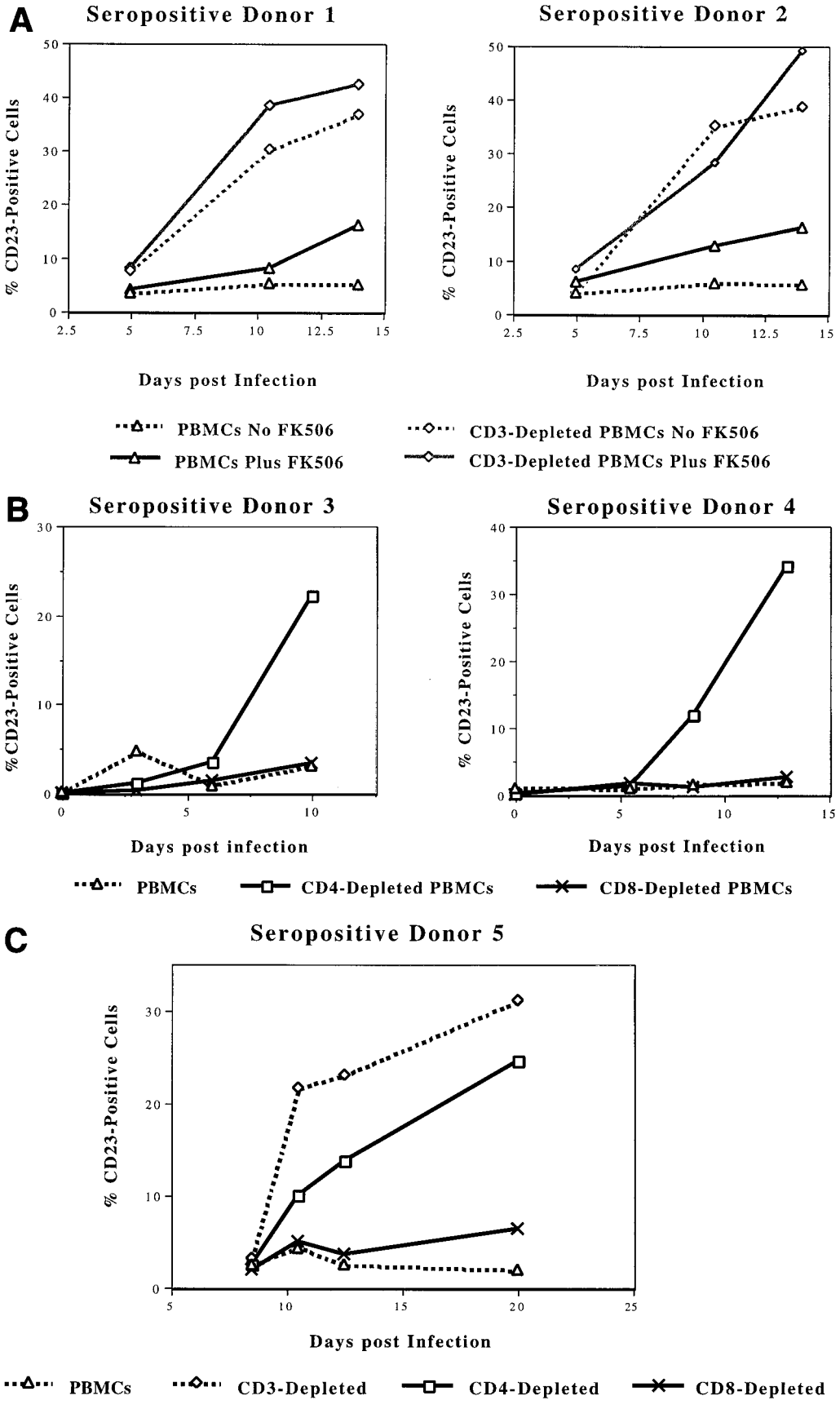

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Cultures of PBMCs depleted of $CD14^+$ $CD45^+$ monocytes-macrophages maintained the capacity to control outgrowth of $CD23^+$ B cells. In these cultures, less than 0.9% of the residual mononuclear cells were $CD14^+$ $CD45^+$. By contrast, more than 1.8% of the cells in CD4-depleted cultures in which immune control was abrogated were $CD14^+$ $CD45^+$. Depletion of $CD14^+$ $CD45^+$ cells did not alter the capacity of CD8-depleted cultures to control $CD23^+$ cell proliferation. This demonstrated that loss of immune control correlated with depletion of $CD4^+$ T cells and not $CD4^+$ $CD14^+$ $CD45^+$ monocytes or macrophages. Likewise, depletion of $CD16^+$ natural killer cells did not affect immunosurveillance (data not shown).

CD8-depleted and PBMC cultures are similarly affected by cell density. It was unknown whether CD8-depleted cultures could maintain immune control at initial culture densities lower than 10^6 cells/ml. Figure 5 demonstrates that cultures depleted of more than 97% of $CD8^+$ T cells and cultures of mixed PBMCs inhibit proliferation of $CD23^+$ B cells equally well at various initial culture densities. There were fewer than 10% $CD23^+$ B cells in mixed PBMC and $CD8^+$ T-cell-depleted cultures initiated at the two higher cell densities, 10^6 cells/ml and 2×10^5 cells/ml; there was a loss of immune control in both cultures seeded at very low starting cell densities, such as 4×10^4 cells/mL. In contrast, when cultures depleted of $CD3^+$ and $CD4^+$ T cells were initiated at all three cell densities, $CD23^+$ B cells represented more than 50% of the cell population at the time of staining.

$CD23^+$ B cells in cultures depleted of T-cell subsets have different proliferation profiles. While PBMC and CD8-depleted cultures reduced the proportion of $CD23^+$ B cells in culture, it was unknown whether the proliferation profiles of the few $CD23^+$ cells that did exist in those cultures resembled the profiles of $CD23^+$ cells grown in the absence of immune control. Data from CFSE-labeled cultures showed that $CD8^+$ cell depletion does not alter the early proliferation profile of $CD23^+$ B cells from that seen in PBMC cultures. However, $CD3^+$ or $CD4^+$ cell depletion allows rapid appearance and proliferation of $CD23^+$ B cells (Fig. 6). At 8.5 days after infection, all cultures contained a low fraction (1.9 to 3.2%) of $CD23^+$ B cells with similar proliferation profiles. After 4 more days (day 12.5), there was a five- to sevenfold increase in the fraction of $CD23^+$ cells in the CD3- and CD4-depleted cultures, with multiple $CD23^+$ cell divisions, as reflected by the large peaks at low fluorescence intensity. During the same interval in PBMC and CD8-depleted cultures, there was little change in the fraction of $CD23^+$ cells or in the CFSE staining profile. $CD23^+$ cells from PBMC and CD8-depleted cultures that did divide did not undergo as many rounds of proliferation as those from CD3- and CD4-depleted cultures. This confirms that the ability to prevent early $CD23^+$ B-cell proliferation is maintained in the absence of $CD8^+$ cells and is particularly manifest between 7 and 12 days after EBV infection in vitro.

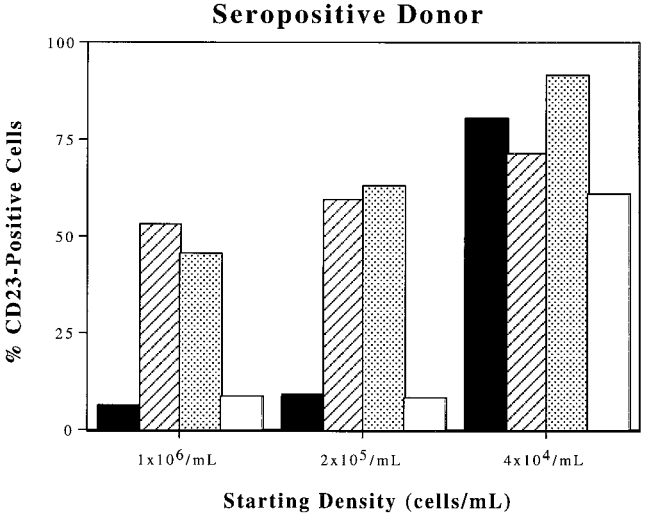

FIG. 5. Effect of depletion of T cells on the proportion of $CD23^+$ B cells in EBV-infected cultures initiated at different cell densities. PBMC cultures (solid bars) are compared with CD3-depleted (hatched bars), CD4-depleted (stippled bars), and CD8-depleted (white bars) cultures, all derived from the same EBV-seropositive donor. Cultures were infected with EBV and initiated at the indicated cell densities. Cultures depleted of CD3-, CD4-, and CD8-expressing cells contained contaminating residual populations of less than 1.1% $CD3^+$ cells, less than 0.6% $CD4^+$ cells, and less than 0.8% $CD8^+$ cells, respectively, at the time of initiation. Results represent cells analyzed 18 days after infection. ■, PBMCs; ▨, CD3 depleted; ▩, CD4 depleted; □, CD8 depleted.

Positively selected $CD3^+$ and $CD4^+$ T-cell populations exert control over proliferation of EBV-infected $CD23^+$ B cells. We explored whether purified $CD4^+$ T cells are capable of exerting EBV-specific immune control by adding positively selected T cells to EBV-infected target cells. This was necessary because depletion of $CD8^+$ cells yields a population enriched for $CD4^+$ cells but contains other cells potentially wielding EBV-specific immune activity. In the experiment illustrated in Fig. 7, the infected target cells were CD3-depleted PBMCs. Addition of increasing numbers of either $CD3^+$ or $CD4^+$ cells to EBV-

FIG. 4. Effect of depletion of T cells on the proportion of $CD23^+$ B cells in EBV-infected cultures. At the indicated times, cultures of EBV-infected cells originally drawn from EBV-seropositive donors were harvested and analyzed by FACS for the fraction of $CD23^+$ B cells. (A) Effect of depletion of $CD3^+$ cells. Cultures depleted of CD3-expressing cells prior to infection were initiated at 2×10^5 cells/ml and compared with cultures of PBMC initiated at 10^6 cells/ml. Both groups of cells were cultured in the presence (solid lines) and absence (dashed lines) of FK506. Following $CD3^+$ cell depletion, cultures from donor 1 contained fewer than 3.3% $CD3^+$ cells at 5 days after infection, and cultures from donor 2 contained fewer than 0.8% $CD3^+$ cells. (B) Effect of depletion of $CD4^+$ and $CD8^+$ cells. PBMC cultures were initiated at 10^6 cells/ml; cultures depleted of $CD4^+$ cells were initiated at 4×10^5 cells/ml; and cultures depleted of $CD8^+$ cells were initiated at 8×10^5 /mL. CD4-depleted and CD8-depleted cultures from donor 3 contained fewer than 1.4% $CD4^+$ cells and fewer than 0.7% $CD8^+$ cells, respectively. CD4-depleted and CD8-depleted cultures from donor 4 contained fewer than 4.2% $CD4^+$ cells and fewer than 0.5% $CD8^+$ cells, respectively, measured 3 days after infection. (C) Effect of depletion of different T-cell subpopulations in an experiment utilizing PBMCs from one seropositive donor. All cultures were simultaneously infected with EBV and initiated at the same starting cell density of 2×10^6 cells/ml. Cultures depleted of CD3-, CD4-, and CD8-expressing cells contained less than 0.9% $CD3^+$ cells, less than 0.5% $CD4^+$ cells, and less than 1.4% $CD8^+$ cells respectively, at 8.5 days after infection. Triangles, PBMCs; diamonds, CD3-depleted cells; squares, CD4-depleted cells; and X's, CD8-depleted cells.

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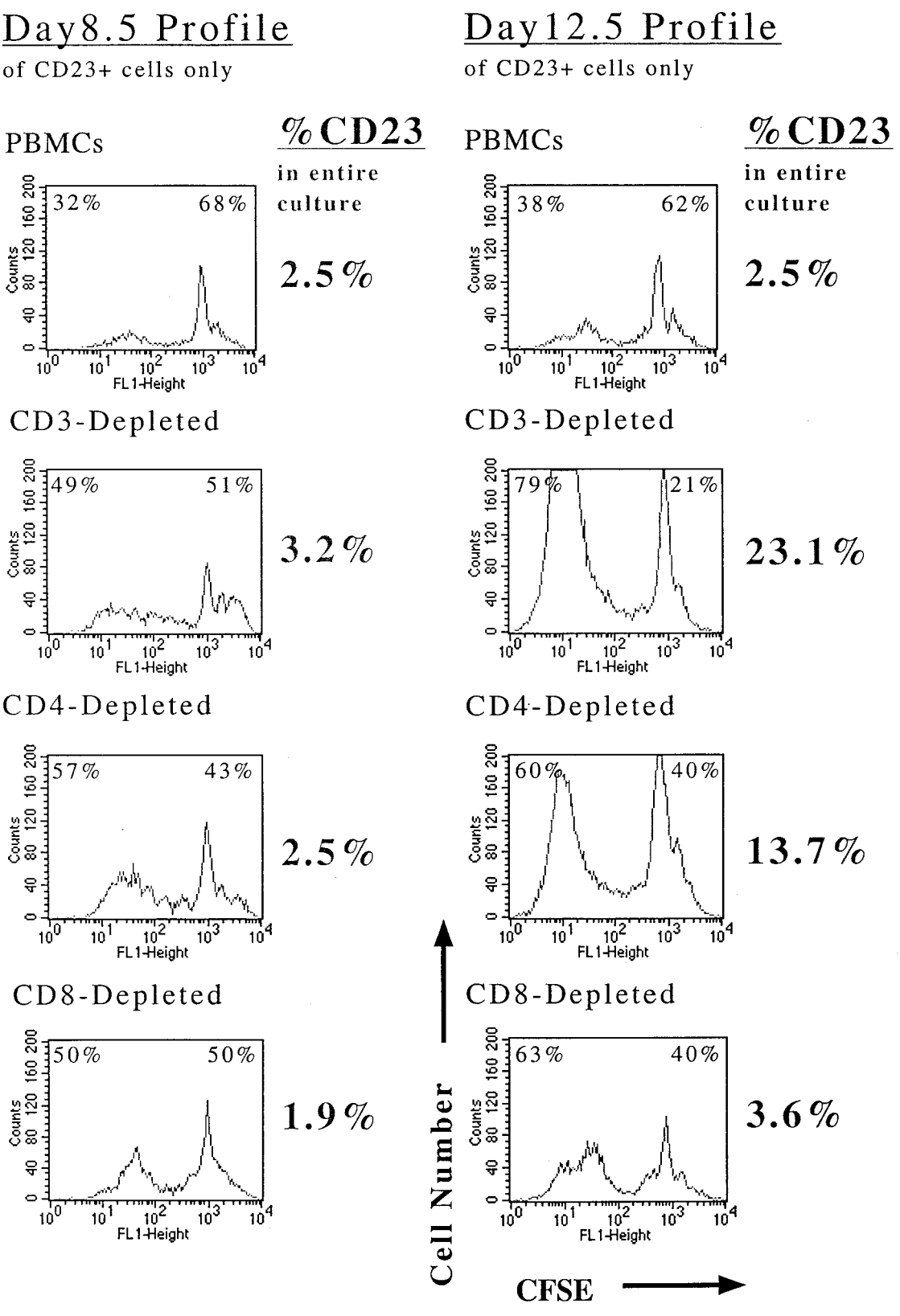

FIG. 6. Effect of depletion of different T-cell subsets on the proportion of dividing $CD23^+$ cells. Mixed PBMC cultures or cultures that had been drawn from an EBV-seropositive donors and depleted of CD3-, CD4-, or CD8-expressing cells were incubated with CFSE and infected with EBV. At 8.5 and 12.5 days after infection, $CD23^+$ B cells in the cultures were selectively gated and analyzed for CFSE fluorescence intensity (horizontal axis). Cultures depleted of CD3-, CD4-, and CD8-expressing cells contained contaminating residual populations of less than 0.9% $CD3^+$ cells, less than 0.5% $CD4^+$ cells, and less than 1.4% $CD8^+$ cells, respectively, at 8.5 days after infection. Percentages in the upper left corner of each proliferation profile represent the percentage of $CD23^+$ B cells in that culture which have undergone proliferation. Percentages in the upper right corner of each profile represent the percentage that have not yet undergone proliferation.

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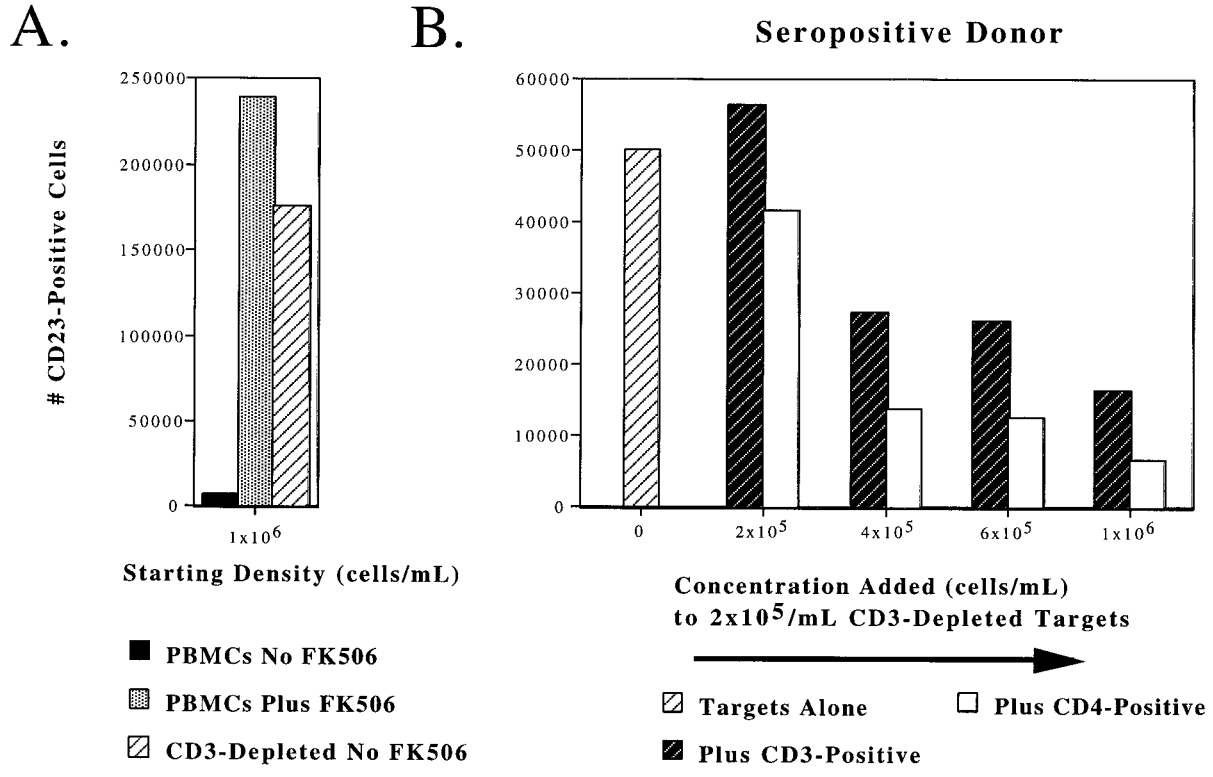

FIG. 7. Effect of addition of T-cell subpopulations on the number of $CD23^+$ B cells. (A) EBV-infected cells were either PBMCs or CD3-depleted PBMCs at 2×10^6 cells/ml. (B) EBV-infected target cells were CD3-depleted PBMCs (white hatched bar) seeded at 2×10^5 cells/ml to which were added increasing numbers of purified, positively selected CD3- (dark hatched bars) or CD4-expressing (white bars) T cells. There were fewer than 0.4% contaminating $CD19^+$ B cells in the purified $CD3^+$ population and less than 0.3% $CD8^+$ and 0.2% $CD19^+$ cells in the purified $CD4^+$ population. The cultures were analyzed for the number of $CD23^+$ B cells 17.5 days after infection. Cells in all cultures were initially derived from the same EBV-seropositive donor.

infected target cells resulted in a decrease in the number of $CD23^+$ B cells remaining at 18 days after infection (Fig. 7). Positively selected $CD3^+$ cells appeared to be less effective than equal numbers of $CD4^+$ cells. The damping effect of T cells on the number of $CD23^+$ B cells was strongly manifested when 4×10^5 or more T cells were added to 2×10^5 CD3-depleted cells per ml. This ability of $CD4^+$ T cells to exert control over the fraction of $CD23^+$ B cells present 17 days after EBV infection was also demonstrated when $CD19^+$ cells were infected and used as targets (data not shown).

DISCUSSION

$CD4^+$ T cells inhibit EBV-induced B-cell proliferation. In this study we analyzed the capacity of memory T cells to abrogate the proliferation of $CD23^+$ B cells newly infected by EBV. A novel finding of our study is that $CD4^+$ T cells taken directly from the peripheral blood of EBV-seropositive donors contribute significantly to preventing the early stages of EBV-induced lymphoproliferation. In the absence of $CD4^+$ T cells, $CD8^+$ T cells do not inhibit proliferation of $CD23^+$ B cells. In cultures depleted of $CD4^+$ T cells, the fraction of $CD23^+$ B cells increases markedly, and the majority of these cells actively proliferate between days 6 and 12 after EBV infection (Fig. 4B, 4C, and 6). This increase in $CD23^+$ B cells does not appear in PBMC cultures or in cultures depleted of $CD8^+$ cells within the first 3 weeks after infection. Even at high initial starting cell densities, such as 10^6 cells/ml, depletion of $CD4^+$ T cells promotes an increase in $CD23^+$ B cells, while depletion of $CD8^+$ cells does not allow outgrowth of $CD23^+$ B cells at the same starting cell density (Fig. 5 and 6). Moreover, after addition to EBV-infected purified target cells, purified $CD4^+$ T cells are sufficient to prevent the outgrowth of $CD23^+$ B cells (Fig. 7). These experiments suggest that $CD4^+$ T cells themselves act as effectors of regression as well as helpers of a $CD8^+$ T-cell-mediated response.

Advances in assessing functional immune control of proliferation. Our conclusions that $CD4^+$ T cells separated from $CD8^+$ T cells are competent to mediate immune control of EBV-induced B-cell activation and proliferation exploit powerful quantitative tools for studying early events following EBV infection. We have employed assays based on proliferation of the total culture ($[^3H]$dT incorporation), the composition of the culture (the number and fraction of $CD23^+$ B cells), and analysis of the number of cells in the culture that have undergone cell division (CFSE dye dilution). All of these assays permit a more accurate, quantitative, and reproducible assessment of the T-cell response to new EBV infection in vitro than does the subjective enumeration of clumps of putatively transformed cells. Moreover, these assays allow monitoring of the kinetic evolution of cell populations responding to EBV infection and indicate the time between 6 and 12 days postinfec-

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tion as the time of major T-cell expansion and activity (Fig. 3, 5, and 7).

Similarities of findings in assays based on CD23$^+$ B-cell proliferation and morphologic changes. Our assays, which enumerate CD23$^+$ B cells within the first 2 to 3 weeks after EBV infection, may not measure the same events as the classical regression assay. Nonetheless, our quantitative assay system reproduces many of the findings of the classical regression assay, in which foci of transformed cells are counted under a microscope. Both assays show that (i) immune surveillance occurs only when target cells for EBV infection are obtained from EBV-seropositive individuals (Fig. 1), (ii) T cells are necessary to prevent outgrowth, and inclusion of a T-cell immunosuppressant, such as FK506, blocks the capacity of memory T cells to mediate immune surveillance (Fig. 1 to 4), and (iii) immune surveillance over EBV-induced lymphoproliferation in cultures derived from EBV-seropositive individuals is exquisitely sensitive to the density at which the culture was initiated (Fig. 3 and 5) (53). These observations are consistent hallmarks of the regression phenomenon. Thus, our CD23$^+$ B-cell assay could yield quantitative assessment of patient immune status in clinical disease states, just as classical regression assays have been used to gauge the vigor of cell-mediated immunity to EBV in immunocompromised patients (70). Preliminary results indicate that our assay based on CD23$^+$ B-cell proliferation can be used to monitor the acquisition of effective T-cell surveillance after primary EBV infection manifested clinically as infectious mononucleosis (data not shown).

Reconciliation of CD4$^+$ T-cell activity with published cytotoxic activity of CD8$^+$ T cells against EBV-infected targets. That CD4$^+$ T cells can act as both cytotoxic effectors and immune helpers and thus play a significant role in preventing early proliferation of activated CD23$^+$ EBV-infected lymphoblasts does not preclude contributions to immunosurveillance by other immune cells, including CD8$^+$ T cells. Our data show that CD8$^+$ T cells by themselves, in the absence of CD4$^+$ T cells, are unable to prevent CD23$^+$ B cell proliferation in the 2 to 3 weeks following EBV infection in vitro. However, it is likely that CD8$^+$ T cells are active in the presence of CD4$^+$ T cell help, and our experiments do not rule out such activity in a primary mixed PBMC culture. There is no question that CD8$^+$ T cells are highly reactive and cytotoxic towards EBV-transformed LCLs (9, 20, 51, 67). In fact, HLA class I-restricted (putatively CD8$^+$) T cells have been isolated from cultures in the process of regression and shown to inhibit the growth of already established LCLs (54). However, this experiment does not address whether these same T cells are responsible for inducing regression in the primary cultures. Our experiments investigate the nature of the immune response in regulating B cells in the early stages of EBV infection and transformation, not the immune response of CTLs to immortalized LCLs. Newly infected B cells and fully transformed LCLs are different targets for cell-mediated immunity. Newly infected cells undergoing EBV-induced proliferation likely express different cellular and viral antigens than already immortalized cells and may be subject to surveillance by different types of T cells (64). The design of our experimental system, in terms of the nature of the EBV-infected targets, the initial resting state of the fresh memory T cells, the time of analysis, and the CD23$^+$ B-cell readout, differs from those of assays used previously. Thus, our findings on the role of CD4$^+$ T cells do not contradict the established ability of CD8$^+$ T cells to exert immune surveillance over EBV infection. Both types of T cells appear to be important.

Increasing evidence for the role of CD4$^+$ T cells in EBV and other viral infections. In summary, our work using a physiologically relevant, functional, in vitro assay system solidifies the role of CD4$^+$ T cells in an in vitro EBV-specific immune response. Quantitative tools to analyze this role have far-reaching implications, because CD4$^+$ CTLs can recognize both latent and lytic-cycle antigens and can be generated from both naive and memory populations (19, 51, 61). While the antigenic targets have not yet been identified, reactivation of lytic virus in LCLs can generate a CD4-dominated rather than a CD8-dominated CTL response (69). However, CD4$^+$ CTL are not restricted to lytic-cycle antigens, since Munz et al. have found that EBNA1, a latent EBV protein which is poorly recognized by CD8$^+$ CTL because of impaired MHC class I loading of EBNA1, is particularly important in the CD4$^+$ CTL response (4, 39). CD4$^+$ CTLs can act through perforin-mediated cytotoxicity or via a Fas-Fas ligand-mediated pathway (19, 69). Our study leaves open the possibility that EBV-specific CD4$^+$ T cells may function by a cytolytic or an antiproliferative mechanism. Specifically, preliminary studies indicate that gamma interferon is preferentially produced by CD4$^+$ cells in the presence of virus versus the presence of a mock inoculum. Other preliminary findings indicate that two clones of EBNA1-specific CD4$^+$ CTLs which secrete gamma interferon also inhibit CD23$^+$ B-cell outgrowth in our assay. Moreover, CD4$^+$ T cells have been found to exert a protective effect in vivo following infection by respiratory syncytial virus, lymphocytic choriomeningitis virus, and herpes simplex virus in mice (30, 45, 66). Thus, the CD4$^+$ T-cell-mediated inhibition of EBV-induced proliferation demonstrated here may prove a crucial component of the in vivo immune response to EBV.

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