Preferential Utilization of the Perforin/Granzyme Pathway for Lysis of Epstein–Barr Virus-Transformed Lymphoblastoid Cells by Virus-Specific CD4⁺ T Cells

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In this report, we show that Epstein–Barr virus (EBV)-infected lymphoblastoid cell lines (LCL) express Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 and that LCL are lysed following engagement of these receptors by agonist Fas and TRAIL receptor-specific monoclonal antibodies (MAb). We also show that EBV-specific CD4⁺ T cells mediate bystander lysis of susceptible targets through both the Fas/Fas ligand (FasL) and the TRAIL pathways, but find that the dominant mechanism of lysis following cognate, HLA class II-restricted recognition of LCL is the perforin/granzyme pathway. Killing of LCL by EBV-specific CD4⁺ T cells was strongly inhibited by concanamycin A, an agent that elevates granule pH, resulting in accelerated destabilization and degradation of perforin. In contrast, blocking anti-FasL MAb showed only limited inhibition of LCL killing. Blocking anti-TRAIL MAb had no effect on lysis of LCL by EBV-specific CD4⁺ T cells. We further show that culture of EBV-specific CD4⁺ T cells in the presence of interleukin 4 markedly abrogates effector cytotoxic function against LCL through direct depletion of intracellular perforin, with no evidence of a Th1 to Th2 shift in patterns of cytokine expression.

Key Words: Epstein–Barr virus; CD4⁺ T cell; Fas; TRAIL; perforin; interleukin-4.

INTRODUCTION

Epstein–Barr virus (EBV) is a B-lymphotropic herpesvirus that is the causative agent of infectious mononucleosis. Following primary infection, EBV persists as an asymptomatic infection in 95–99% of adults worldwide. The site of latent infection is the resting B cell (Miyashita et al., 1997; Babcock et al., 1998). EBV is also closely associated with the aggressive and rapidly lethal post-transplant B cell lymphoproliferative disorders (PTLD) that arise in immunosuppressed organ allograft recipients (Hanto et al., 1985; Nalesnik, 1996). There is a broad consensus that EBV-specific CD8⁺ cytotoxic T cell responses play a central role in control of asymptomatic infection in healthy adults, by virtue of their ability to lyse proliferating B cells expressing viral antigens associated with malignant transformation (Khanna et al., 1996; Richardson et al., 1996). EBV-specific CD8⁺ T cell responses are inhibited in cyclosporin A-treated transplant patients (Crawford et al., 1981; Gaston et al., 1982), who are accordingly at high risk of developing PTLD. In addition to the widely documented EBV-specific CD8⁺ cytotoxic T cell response, CD4⁺ T cells capable of lysing EBV-transformed B lymphoblastoid cell lines (LCL) have also been described (Misko et al., 1984; Rencher et al., 1994), but otherwise little is known of EBV-specific CD4⁺ T cell function or their potential contribution to EBV-specific immunosurveillance and control of PTLD.

Two major mechanisms account for most T-cell-mediated cytotoxicity in vitro (Kagi et al., 1994). The first pathway involves granule exocytosis and pore formation in the target cell membrane through extracellular Ca²⁺-dependent polymerization of perforin, a lytic protein with homology to the C9 component of the membrane attack complex of complement. This is accompanied by release of granzymes (serine proteases) responsible for downstream caspase activation and subsequent DNA fragmentation, resulting in target cell apoptosis. The perforin/granzyme pathway of cytotoxicity is the principal modus operandi for CD8⁺ T cells. The second pathway is mediated by T cell Fas ligand (FasL) engagement of Fas expressed by target cells. Binding of Fas recruits the Fas-associated death domain (FADD) and caspase-8 (also known as FLICE, or FADD-like interleukin-1β-converting enzyme), leading to the formation of the death-inducing signaling complex (DISC). Formation of the DISC results in activation of other downstream caspases, notably caspase-3 and caspase-6, and the death substrate poly(adenosine diphosphate)-ribose polymerase, ultimately inducing efficient cell death (for review, see Peter and Krammer, 1998). Utilization of the Fas/FasL pathway of target cell lysis is thought to be...
favored by CD4$^+$ T cells (Shresta et al., 1998), and a recent report has indicated that EBV-specific CD4$^+$ T cells can induce apoptosis in LCL via the Fas/FasL pathway (Wilson et al., 1998). However, human virus-specific CD4$^+$ T cells can also utilize the perforin/granzyme pathway of cytotoxicity (Gagnon et al., 1999; Yasukawa et al., 1999).

Recent work has revealed that T cell induction of target cell apoptosis may involve other receptor-mediated pathways, the most notable of which is tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (for review, see Griffith and Lynch, 1998). TRAIL has attracted considerable attention by virtue of its preferential ability to induce apoptosis in malignant or transformed cells, but not in normal cells (Griffith and Lynch, 1998), and because of its potential as an anti-tumor agent in vivo (Walczak et al., 1999). TRAIL is a type II membrane protein of the TNF family that has the ability to induce apoptosis in tumor cell lines (Wiley et al., 1995; Pitti et al., 1996). TRAIL has significant homology with other family members, notably FasL and TNFα. Four membrane-bound TRAIL receptors have been identified to date, of which TRAIL-R1 and -R2 are pro-apoptotic receptors that possess death domains homologous to those of Fas and TNF receptor-1. In contrast, TRAIL-R3 lacks a cytoplasmic domain and has a glycosphospholipid anchor in the cell membrane, and TRAIL-R4 has a truncated cytoplasmic domain; neither of these receptors is able to transmit a death signal. It has thus been suggested that TRAIL-R3 and -R4 act as anti-apoptotic decoy receptors for TRAIL and that target cell susceptibility to TRAIL-induced apoptosis may be at least in part regulated by differential expression of the four TRAIL receptors. In practice, however, target cell susceptibility to TRAIL-induced apoptosis cannot readily be correlated with differential patterns of TRAIL receptor expression (Griffith and Lynch, 1998), although expression of TRAIL-R1 and/or TRAIL-R2 is a requirement.

To assess the contribution of CD4$^+$ T cells to immunosurveillance, and their potential for control of EBV-driven PTLD, we have explored the mechanisms of EBV-specific cytotoxicity against in vitro transformed LCL. In this report, we show that LCL express both Fas and TRAIL-R2 and are vulnerable to lysis following engagement of Fas and TRAIL-R2. However, we find that EBV-specific CD4$^+$ T cells are strongly dependent on the perforin/granzyme pathway for HLA class II-restricted lysis of LCL and do not utilize FasL or TRAIL, although they are capable of mediating non-HLA-restricted lysis of bystander target cells by both these pathways. Finally, we show that perforin-mediated lysis of LCL is abrogated following culture of EBV-specific CD4$^+$ T cells in the presence of interleukin-4 (IL-4). The reduction in lysis was not a consequence of an IL-4-induced bias toward a Th2 response, but was apparently due to a direct, cytokine-induced depletion of intracellular perforin stores.

RESULTS

Expression of Fas and TRAIL receptors by LCL

As CD4$^+$ T cells are considered to lyse target cells primarily through receptor–ligand induction of apoptosis, we used flow cytometry to examine LCL for expression of Fas and TRAIL receptors. All three LCL tested expressed Fas at higher levels than Jurkat cells, which were used as a positive control (not shown). K562 cells (a negative control) did not express Fas. LCL from donors 1 and 3 expressed barely detectable levels of the pro-apoptotic receptor TRAIL-R1, whereas expression of TRAIL-R1 by LCL from donor 2 was not detected (Fig. 1). A second pro-apoptotic receptor, TRAIL-R2, was expressed at significant levels by LCL from donors 2 and 3, but only at very low levels by LCL from donor 1 (Fig. 1). Jurkat cells expressed TRAIL-R2 and a low level of TRAIL-R1. Expressions of the anti-apoptotic decoy receptors TRAIL-R3 and -R4 by LCL or Jurkat cells was not detected (not shown).

Lysis of LCL following engagement of TRAIL receptors and Fas

$^{16}$Cr-labeled LCL were incubated in a 16-h cytotoxicity assay with varying levels of agonist anti-Fas monoclonal antibodies (MAb). All three LCL were susceptible to lysis following Fas engagement, as were Jurkat cells, which are known to be sensitive to Fas-mediated apoptosis (Fig. 2). K562 cells, which do not express Fas, were not lysed by the anti-Fas MAb. LCL were also sensitive to lysis by plate-bound agonist MAb specific for TRAIL-R2 (Fig. 3). In contrast, plate-bound agonist anti-TRAIL-R1 MAb induced minimal levels of LCL or Jurkat cell lysis and induced only a small increment in lysis when used in combination with anti-TRAIL-R2 (Fig. 3). The sensitivity of LCL to lysis following engagement of pro-apoptotic TRAIL receptors thus correlated with the observed patterns of surface expression of TRAIL-R1 and TRAIL-R2 (see Fig. 1).

Expression of FasL and TRAIL by EBV-specific CD4$^+$ T cells

Expression of FasL was tested by flow cytometry following CD4$^+$ T cell activation. FasL expression was not detectable following antigen stimulation with LCL (not shown), but all three CD4$^+$ T cell lines expressed FasL following activation with anti-CD3 MAb (Figs. 4A–4F). In contrast, we were unable to detect TRAIL expression by flow cytometry, regardless of the activation state of the T cells (not shown).

Lysis of LCL by EBV-specific CD4$^+$ T cells

To explore the mechanisms used by CD4$^+$ T cells for lysis of target LCL, 16-h cytotoxicity assays were conducted in the presence of blocking MAb specific for FasL or TRAIL or in the presence of concanamycin A (CMA),
an agent that specifically inhibits the perforin/granzyme pathway of target cell lysis (Kataoka et al., 1996). CMA, an inhibitor of vacuolar H\textsuperscript{+} ATPase, promotes destabilization and degradation of perforin through blockade of granule acidification. For all three CD4\textsuperscript{+} T cell lines, we found that CMA strongly inhibited LCL killing. In contrast, anti-FasL MAb did not inhibit lysis by donor 1 CD4\textsuperscript{+} T cells, and the low level of inhibition of donor 2 and donor 3 CD4\textsuperscript{+} T cells by anti-FasL MAb was not statistically significant (Fig. 5). Anti-TRAIL MAb had no effect on the lysis of LCL by CD4\textsuperscript{+} T cells from donors 1 and 3, and the low level of inhibition of CD4\textsuperscript{+} T cells from donor 2 was not statistically significant (Fig. 5B). These results show that the perforin/granzyme pathway is the dominant mechanism of LCL killing by EBV-specific CD4\textsuperscript{+} T cells.

Are EBV-specific CD4\textsuperscript{+} T cells capable of using the FasL and TRAIL pathways of lysis?

Although we found that LCL could be lysed following engagement of Fas or pro-apoptotic TRAIL-R1 and -R2, CD4\textsuperscript{+} T cells preferentially used the perforin/granzyme pathway for lysis of LCL. These observations raised the possibility that FasL and TRAIL were insufficiently expressed by the CD4\textsuperscript{+} T cells following activation by LCL (see above) or that FasL and TRAIL were unable to deliver an apoptotic signal. To test the ability of CD4\textsuperscript{+} T
cells to induce cell death via FasL or TRAIL, we conducted bystander cytotoxicity assays against Jurkat cells, a target that is susceptible to cell-mediated killing by these mechanisms (Kayagaki et al., 1999). Because Jurkat cells do not express HLA class II molecules, and do not induce alloresponsive CD4+ T cell activation (data not shown), cytotoxicity assays against this target were carried out in the absence or in the presence of plate-bound anti-CD3 MAb (to deliver a surrogate activation signal).

Resting (i.e., nonactivated) CD4+ T cells induced significant lysis of Jurkat cells in a 16-h 51Cr-release assay (Fig. 6A). In contrast with the cytotoxicity assays against LCL, killing of Jurkat cells was not inhibited by CMA, indicating that the perforin/granzyme pathway was not a component of bystander lysis. However, cytotoxicity was strongly inhibited by blocking anti-TRAIL MAb and was slightly inhibited by blocking anti-FasL MAb (Fig. 6A). Lysis of Jurkat cells was markedly enhanced following CD4+ T cell activation by anti-CD3, but CMA again failed to inhibit bystander lysis (Fig. 6B). In this instance, anti-FasL MAb strongly inhibited killing, whereas anti-TRAIL MAb had no inhibitory effect on lysis of Jurkat cells. Essentially the same results were observed for all three CD4+ T cell lines. Collectively, these results indicate that EBV-specific CD4+ T cells are capable of using both FasL and TRAIL for target cell lysis and further suggest that preferential utilization of these pathways may be related to the activation status of the T cells.
EBV-specific CD4⁺ T-cell-mediated lysis of LCL is inhibited by IL-4

Two independent studies have demonstrated elevated levels of IL-4 in the serum of solid organ transplant patients (Mathur et al., 1994; Martinez et al., 1995). The presence of IL-4 may favor development of noncytotoxic Type 2 (Th2) CD4⁺ T cell responses at the expense of Type 1 (Th1) responses with cytotoxic potential (Romagnani, 1991; Seder and Paul, 1994; Carter and Dutton, 1996), thus raising the possibility that IL-4 may abrogate EBV-specific CD4⁺ T cell cytotoxicity. To test this proposal, we cultured CD4⁺ T cells in the absence or in the presence of recombinant human IL-4 (50 U/ml) and subsequently examined their ability to lyse LCL. A marked reduction in killing was observed for the CD4⁺ T cell line derived from the heart transplant patient (donor 3) and also for a CD4⁺ T cell line from one of the normal donors (Fig. 7). Only short periods of IL-4 treatment (1–3 weeks) were necessary to achieve this effect. However, the reduction in cytotoxic function was not the result of an IL-4-induced Th2 bias, at least as revealed by analysis of intracellular IFNγ, IL-4, and IL-13 expression at the single-cell level. When compared with matched, conventional cultures, CD4⁺ T cells grown in the presence of IL-4 displayed an essentially unchanged pattern of intracellular cytokine expression (Fig. 8).

IL-4 treatment of CD4⁺ T cells results in depletion of intracellular perforin stores

As IL-4-mediated inhibition of cytotoxicity was apparently not a consequence of a Th2 bias in the CD4⁺ T cell response, we asked whether IL-4 had a direct effect on cytotoxic function. As the perforin/granzyme pathway is the major pathway for CD4⁺ T cell lysis of LCL (see above), we looked at intracellular perforin levels in CD4⁺ T cells cultured in the absence or in the presence of IL-4. Flow cytometric analysis clearly showed that culture of donor 3 CD4⁺ T cells in the presence of IL-4 resulted in a marked reduction of intracellular perforin expression (Fig. 9).

DISCUSSION

In this report, we show that target LCL express Fas and that LCL can be lysed following engagement of Fas by agonist MAb. This observation supports and extends earlier studies showing that proliferation of LCL was
inhibited by anti-Fas (Falk et al., 1992). We also find that LCL expressed TRAIL-R2 (and to a lesser extent TRAIL-R1) and that LCL can be lysed following engagement of these receptors by agonist MAb. In contrast, however, we find that the dominant mechanism for HLA class II-restricted lysis of LCL by EBV-specific CD4\(^+\) T cells is the perforin granzyme pathway, even though EBV-specific CD4\(^+\) T cells are capable of utilizing both the Fas/FasL and the TRAIL pathways for non-HLA-restricted spontaneous or activation-dependent bystander lysis of sensitive target cells.

One of the more intriguing findings was that EBV-specific CD4\(^+\) T cells were capable of lysing sensitive bystander targets (Jurkat cells) via TRAIL without a requirement for T cell activation. Furthermore, blocking assays with Mab specific for TRAIL and FasL, respec-

**FIG. 8.** Intracellular expression of IFN\(_\gamma\), IL-4, and IL-13 by EBV-specific CD4\(^+\) T cells (donor 3) cultured in the absence (A and B) or in the presence (C and D) of 50 U/ml IL-4. T cells were activated with PMA and ionomycin prior to permeabilization with saponin and staining for intracellular cytokine expression, as described under Materials and Methods. Control, i.e., nonactivated, CD4\(^+\) T cells failed to stain for cytokine expression (not shown).

**FIG. 9.** Intracellular perforin expression by EBV-specific CD4\(^+\) T cells cultured in the absence (A) or in the presence (B) of 50 U/ml IL-4. CD4\(^+\) T cells from donor 3 were cultured with IL-4 for 19 days. Intracellular perforin was measured by flow cytometry following saponin permeabilization of the cells.
tively, showed that activation of CD4⁺ T cells with plate-bound anti-CD3 MAb abrogated TRAIL-mediated lysis of Jurkat cells and favored lysis by the Fas/FasL pathway. Utilization of the Fas/FasL pathway in bystander lysis by activated CD4⁺ T cells correlated with our observation that anti-CD3-mediated CD4⁺ T cell activation resulted in elevated expression of FasL. Although in this instance we were unable to detect TRAIL expression by flow cytometry, our finding that nonactivated CD4⁺ T cells preferentially utilize the TRAIL pathway for bystander lysis is in accord with earlier observations that TRAIL is constitutively expressed by human CD4⁺ T cells and that spontaneous cytotoxicity of resting human CD4⁺ T cell clones against sensitive targets can be blocked by anti-TRAIL MAb, but not by anti-FasL MAb (Kayagaki et al., 1999).

The observation that EBV-specific CD4⁺ T cells show a strong preference for utilization of the perforin/granzyme pathway for HLA class II-restricted lysis of LCL accords with other reports on the cytotoxic function of human CD4⁺ T cells (Lewinsohn et al., 1998; Rivoltini et al., 1998; Gagnon et al., 1999; Yasukawa et al., 1999) and contrasts with the well-documented bias in favor of the Fas/FasL pathway for mouse CD4⁺ T cells (Shresta et al., 1998). Collectively, our findings and those of other investigators suggest that the perforin/granzyme pathway may be dominant for human CD4⁺ T cells. The EBV-specific CD4⁺ T cell lines described in this study exhibited a broad phenotypic range of cytokine expression, including Th1 T cells expressing IFNγ but not IL-4, Th2 T cells expressing IL-4 but not IFNγ, and an intermediate phenotype expressing both IFNγ and IL-4 (see Fig. 8). By virtue of the polyclonal nature of these lines, perforin/granzyme-mediated cytolysis cannot readily be attributed to any particular CD4⁺ T cell subset. Clonal analysis of EBV-specific CD4⁺ T cells may help to resolve this question.

While there is no doubt that EBV-specific CD8⁺ cytotoxic T cell responses play a crucial role in immunosurveillance, these results suggest that CD4⁺ T cell responses may also be important. The preferential use of the more potent perforin/granzyme pathway for lysis of LCL strongly supports the proposal that EBV-specific CD4⁺ T cell responses against transformed B cells contribute to immunosurveillance and maintenance of an asymptomatic carrier state in the healthy host. The use of the perforin/granzyme pathway assumes further significance in light of the finding that EBV-specific CD4⁺ T cells were ineffective in cytotoxicity assays against LCL in which this pathway was inhibited by CMA, notwithstanding the demonstration that LCL could be lysed following engagement of Fas and TRAIL-R2 receptors and that EBV-specific CD4⁺ T cells were capable of lysing sensitive bystander targets via the Fas/FasL and TRAIL pathways. Our findings contrast with the observations of Wilson et al. (1998), who found that growth of LCL could be inhibited by EBV-specific CD4⁺ T cells through induction of Fas/FasL-mediated apoptosis, thus suggesting a potential role for Fas/FasL interactions in CD4⁺ T-cell-mediated immunosurveillance. The apparent discrepancy between our results and those of Wilson et al. may be attributable to their use of phorbol myristate acetate (PMA)-activated LCL as stimulators for their CD4⁺ T cells. PMA treatment results in increased expression of lytic cycle antigens and may also modify cytokine secretion and expression of accessory and adhesion molecules by LCL. All of these factors may influence the CD4⁺ T cell response. In comparison with the EBV-specific CD4⁺ T cell responses we describe against conventional LCL, CD4⁺ T cells stimulated with PMA-treated LCL showed very low cytotoxic responses against both PMA-activated and untreated LCL (Wilson et al., 1998), suggesting that PMA-activated LCL preferentially induce a noncytotoxic response, at least as measured in a 5-h ¹¹¹In-chromium release assay.

Although we found that LCL were sensitive to lysis following engagement of Fas or TRAIL receptors by agonist MAb, LCL were resistant to EBV-specific CD4⁺ T cell cytolyis in the presence of CMA, even in extended 16-h ¹¹¹In-chromium-release assays. These results indicate that CD4⁺ T cells are unable to compensate for the loss of the perforin/granzyme pathway through utilization of the Fas/FasL or TRAIL pathways, even though these mechanisms were found to be functional in bystander cytotoxicity assays against sensitive Jurkat target cells. We also found that anti-CD3-activated CD4⁺ T cells (which express significant levels of FasL; see Fig. 4) do not lyse LCL in extended 16-h ¹¹¹In-chromium-release assays conducted in the presence of CMA (not shown). It is thus apparent that agonist MAb against Fas deliver a more potent apoptotic signal than FasL-expressing EBV-specific CD4⁺ T cells. The relative resistance of LCL to CD4⁺ T-cell-mediated lysis via FasL or TRAIL may be due to overexpression of FLICE-inhibitory protein, which has the capacity to inhibit apoptosis following engagement of both Fas and TRAIL receptors (French and Tschopp, 1999). Nevertheless, the ability of MAb engagement of TRAIL-R2 to overcome the resistance of LCL to receptor–ligand-induced apoptosis suggests that recombinant soluble TRAIL may have therapeutic potential for PTLD. Recombinant soluble TRAIL has been effective in inhibiting tumor formation by human mammary adenocarcinoma cells in a SCID mouse model and does not incur the hepatotoxicity associated with recombinant soluble FasL or TNFα treatment (Walczak et al., 1999).

Perhaps the most remarkable observation from this study is the finding that culture of EBV-specific CD4⁺ T cells in the presence of IL-4 resulted in a marked abrogation of cytotoxicity against LCL. IL-4-induced inhibition of CD4⁺ T cell cytotoxic function did not correlate with a change in pattern of cytokine expression, but was apparently due to depletion of intracellular perforin stores.
This finding is of particular interest in light of reports describing elevated serum IL-4 levels in immunosuppressed solid organ allograft recipients (Mathur et al., 1994; Martinez et al., 1995). Collectively, these observations suggest that IL-4 may play a direct role in abrogation of EBV-specific T cell immunosurveillance in high-risk individuals under cyclosporin A (CsA) therapy. It should also be noted that CsA can inhibit FasL expression (Nakajima and Oka, 1996), thus short-circuiting a potential alternative pathway of lysis in vivo.

**MATERIALS AND METHODS**

**Stimulator/target cells**

EBV-transformed LCL were established by infection of peripheral blood lymphocytes (PBL) with the B95.8 strain of EBV in the presence of 1 μg/ml CsA and maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 3 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-4} M 2-mercaptoethanol (RPMI/10), as described (Nazaruk et al., 1998), except that in some cases the medium was supplemented with 10% human AB (HuAB) serum (Gemini Bio-Products, Calabasas, CA) (RPMI/10Hu) rather than 10% FCS. Jurkat cells and K562 cells were maintained in RPMI/10.

**EBV-specific CD4^+ T cell lines**

CD4^+ T cell lines (and matching LCL; see above) were derived from two healthy adult individuals (donors 1 and 2) and one heart transplant patient (donor 3), who was under CsA immunosuppressive therapy (100 mg, oral, twice daily). The blood level of CsA at the time of blood drawing was 167 ng/ml. CD4^+ T cells were purified from PBL by positive selection with anti-CD4-coupled Dynabeads (Dynal A.S., Lake Success, NY). A total of 3 × 10^7 PBL were incubated with 2 × 10^7 anti-CD4-Dynabeads in 2 ml PBS/2% HuAB serum at 4°C for 1 h. Dynabead-rosetted CD4^+ T cells were magnetically separated, washed five times with cold PBS/2% HuAB serum, and subsequently recovered by incubation with 60 μl CD4-Detachabead reagent (Dynal A.S.) in 150 μl RPMI/10Hu. Purified CD4^+ T cells (3 × 10^6/ml) were cocultured with autologous, irradiated (7500 cGy) LCL (2 × 10^5/ml) in RPMI/10Hu for 9–11 days, after which period the T cells were restimulated with irradiated LCL. Recombinant human IL-2 (50 U/ml; provided by the Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD) was added to the cultures at this time. EBV-specific CD4^+ T cells were subsequently maintained by restimulation with irradiated LCL every 14–21 days, with interim 50–70% changes of fresh medium plus IL-2 every 2 to 4 days. LCL used for CD4^+ T cell stimulation were maintained in RPMI/Hu, to avoid stimulation of CD4^+ T cells specific for bovine serum protein. The purity of the CD4^+ T cells was continuously monitored by flow cytometric analysis. In all experiments, CD4^+ T cells were 98–100% pure, with no detectable contamination by CD8^+ T cells.

**Definition of CD4^+ T cell specificity for EBV**

The specificity of the CD4^+ T cell lines was confirmed in proliferation assays against LCL and EBV-uninfected normal, activated B lymphoblasts. Briefly, CD4^+ T cells proliferated strongly in response to stimulation with autologous LCL maintained in medium supplemented with FCS or HuAb serum, but failed to recognize HLA class II-mismatched allogeneic LCL. In addition, responses against autologous LCL could be markedly inhibited (50–80% reduction in stimulation indices) in the presence of blocking anti-HLA class II MAb.

Most important, CD4^+ T cells failed to mount proliferative or cytotoxic responses against autologous, normal (i.e., non-EBV-transformed) B lymphoblasts activated with anti-CD40 MAb and IL-4 in the presence of CDw32-transfected L cells (Banchereau et al., 1991), as previously reported (Fu and Cannon, 2000). CDw32 encodes an IgG Fc receptor, which cross-links the anti-CD40 MAb, thereby delivering an activation signal to the B cells. Flow cytometric analysis indicated that the normal B lymphoblasts were phenotypically comparable to LCL. Like LCL, normal B lymphoblasts expressed high levels of CD23 and adhesion molecules CD54 (ICAM-1) and CD58 (LFA-3). Finally, the anti-CD40-activated B lymphoblasts expressed high levels of CD86 and HLA-DR, indicating that they would be excellent antigen-presenting cells (as are LCL, which also express CD86 and HLA-DR). Collectively, this analysis shows that the normal B lymphoblasts are fully activated and phenotypically comparable to LCL.

**Flow cytometry**

FITC-conjugated anti-Fas MAb were from Becton-Dickinson (San Jose, CA). For detection of FasL expression, we used NOK-2 MAb (Kayagaki et al., 1995). TRAIL expression was detected with RIK-2 MAb (Kayagaki et al., 1999) or M180 MAb (Immunex Corp., Seattle, WA). MAb specific for TRAIL receptors were also kindly provided by Immunex Corp. FITC-conjugated goat anti-mouse IgG was from Sigma (St. Louis, MO). For staining of surface markers, cells were washed once in phosphate-buffered saline (PBS) and incubated with primary antibody for 30 min on ice, after which they were washed twice with cold PBS. Where unconjugated primary antibodies were used, the cells were incubated for a further 30 min on ice with FITC-conjugated secondary antibodies. After two further washes with PBS, the cells were fixed with 2% paraformaldehyde in PBS. FITC-conjugated anti-perforin MAb and a FITC-conjugated isotype control were from PharMingen (San Diego, CA). For intracellular staining of perforin, cells were washed and permeabilized by incubation in PBS plus 1% bovine serum albumin.
(BSA) and 0.5% saponin (S-7900; Sigma) for 10 min at room temperature. After being stained for 30 min at room temperature, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, washed once with PBS plus 0.5% BSA, and fixed with 2% paraformaldehyde in PBS. Flow cytometric analysis was conducted with a FACScan (Becton-Dickinson), using LYSIS II software (Becton-Dickinson) and WinMDI 2.7 software (kindly made available by Joe Trotter, The Scripps Research Institute, La Jolla, CA).

Cytotoxicity assays

Cytotoxicity against LCL and Jurkat cells was tested in standard 6- or 16-h 3[1]Cr-release assays in 96-well microtiter plates at indicated effector:target ratios, as described (Nazaruk et al., 1998). The role of perforin in cell-mediated cytotoxicity was assessed by the addition of 8 nM CMA (Sigma). The NOK-2 anti-FasL MAb (10 μg/ml) was used to inhibit Fas/FasL-mediated lysis, and the RIK-2 anti-TRAIL MAb (10–20 μg/ml) was used to inhibit TRAIL-mediated cytotoxicity. Where appropriate, microwells were precoated with anti-CD3 MAb (50 μg/ml OKT3 in PBS, 50 μl/well overnight at 4°C).

Intracellular cytokine expression

T cells were rested for at least 14 days following antigen stimulation before activation with PMA and iomycin, as described (Nazaruk et al., 1998; Fu and Cannon, 2000). Briefly, T cells (7.5 × 10^5/ml) were incubated with 50 ng/ml PMA and 500 ng/ml iomycin at 37°C for 6 h in RPMI/10%Hu. Brefeldin A (10 μg/ml) was added for the final 3 h of incubation. Control, nonactivated cultures were incubated with the addition of Brefeldin A only. The cells were harvested, washed, and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, after which they were washed once with PBS and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin for 10 min at room temperature. MAb used were FITC-conjugated anti-IFNγ and phycoerythrin (PE)-conjugated anti-IL-4 and anti-IL-13, respectively (all from Becton-Dickinson), using LYSIS II software (Becton-Dickinson) and WinMDI 2.7 software (kindly made available by Joe Trotter, The Scripps Research Institute, La Jolla, CA).

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