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Human Vascular Endothelial Cells Stimulate Memory But Not Naive CD8+ T Cells to Differentiate into CTL Retaining an Early Activation Phenotype

Thomas J. Dengler and Jordan S. Pober

Endothelial cell (EC)-selective alloreactive CTL may mediate alloimmune vascular injury. In the present study, EC-selective CTL were generated in cocultures of purified human CD8+ T cells with allogeneic EC and were compared with conventional CTL against corresponding B lymphoblastoid cells (BLC). EC caused activation and expansion of memory but not naive CD8+ T cells, which differentiated into EC-selective CTL that retained high surface expression of CD69, CD25, and CD62L and displayed low intraacellular perforin content. In contrast, BLC-stimulated CTL could be generated from naive or memory CD8+ T cells and showed a more mature phenotype (low CD69, CD25, and CD62L with higher levels of perforin). The expansion of alloreactive T cells by EC stimulation was 5- to 20-fold less effective than in corresponding BLC-stimulated cultures, accounting for a reduction in the assayable cytotoxicity of individual microcultures. In these IL-2-supplemented cocultures, no effect on CTL generation or phenotype was observed by mAb blocking of costimulation provided by LFA-3, ICAM-1, or CD40, by addition of comitogenic anti-CD28 mAb, or by preactivation of EC with CD40 ligand. Cyclosporine inhibited CTL expansion and cytotoxicity similarly in both EC- and BLC-stimulated cultures but did not affect the phenotype of those CTL that did emerge. This study extends the characterization of endothelium as an immunoregulatory cell type distinct from conventional APC and may explain why graft rejection within the arterial intima, an anatomic compartment in which EC may be the primary type of APC, is separable from rejection in the graft parenchyma. The Journal of Immunology, 2000, 164: 5146–5155.

Cytotoxic T lymphocytes reactive with endothelium have been proposed to be the effectors of cell-mediated vascular rejection (endothelialitis), a potential precursor lesion of chronic graft rejection (transplant vasculopathy) (1, 2). Endothelial cell (EC)3-selective, alloreactive CTL have been isolated from endomyocardial biopsies of acutely rejecting heart transplants (3), and it is likely that such EC-selective CTL arise in response to alloantigen presented by graft EC. Previous studies from our laboratory have demonstrated that allogeneic human EC stimulators can generate EC-selective CTL with atypical characteristics, including weak cytotoxic activity and absent IFN-γ stimulators can generate EC-selective CTL with atypical characteristics, including weak cytotoxic activity and absent IFN-γ secretion (4). Further studies of EC-stimulated CTL clones confirmed these unusual features, especially EC selectivity and low production of IFN-γ, which correlated closely with constitutive surface expression of CD40 ligand (CD154) (5), a molecule that is not usually seen on CD8+ T cells.

Although our initial studies highlighted the capacity of EC to stimulate an unusual CTL response, these analyses were limited to a relatively late time point, i.e., after CD8+ T cell clonal expansion had progressed sufficiently to permit measurement of cytolysis or after cloning by limiting dilution with further propagation in culture. In the present study, we have used 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling to identify and selectively analyze proliferating lymphocytes (6), which allowed us to look at earlier events in the primary coculture system. We have used this approach to compare EC with professional APC, e.g., B lymphoblastoid cells (BLC), as stimulators of CTL by analyzing the kinetics of clonal expansion, the identity of the resting T cells that are able to respond to alloantigen by proliferation, and the phenotype of the proliferating T cell populations during their clonal expansion. We have also been able to analyze the effects of costimulation blockade, costimulatory augmentation, and cyclosporine (CsA) on both clonal expansion and CTL maturation. Our experiments show that EC stimulate a smaller number of alloreactive CD8+ T cells drawn entirely from the memory T cell subset compared with BLC, which stimulate expansion of both naive and memory alloreactive T cells. Interestingly, EC-stimulated CTL appear to retain an early activation phenotype characterized by persistent expression of CD69, CD25, and L-selectin (CD62L). In the presence of exogenous IL-2, which is required for CTL generation in these cocultures with EC or BLC stimulators, we have not detected a role for costimulation of CD8+ T cells through B7, LFA-3, ICAM-1, or CD40. CsA inhibits EC- and BLC-stimulated cocultures primarily by reducing clonal expansion despite the presence of exogenous IL-2 and does not appear to significantly affect other CTL differentiation events. We conclude that EC cause a form of immune deviation resulting in activation of unusual CTL effector populations that could be important for allogeneic responses that develop within the graft vessel wall.
Materials and Methods

Cell isolation

PBMC were obtained from healthy volunteers by density-gradient centrifugation of leukapheresis products and were stored in liquid nitrogen as described previously (7). CD8+ T cells were isolated from PBMC by positive selection (4) using Dynabeads (Dynal, Lake Success, NY). The selected population obtained by this procedure was routinely >98% CD8+/CD3+ by flow cytometry and >99% viable, as shown by trypan blue exclusion. Use of positively selected CD8+ T cells in this assay had previously been shown to yield identical results compared with negative selection (4). Naive and memory subsets of T cells were isolated from the CD8-selected population by further negative selection. CD8+ T cells were incubated with anti-CD45RA (B-C15; 1 µl/10⁶ cells) or anti-CD45RO mAb (5 µl/10⁶ cells; UCHL-1, both mAb from Biosource, Camarillo, CA), respectively, for 30 min at 4°C. After three washes, cells were incubated with magnetic beads coated with goat anti-mouse IgG antisera (Dynal) for 30 min at 4°C. Cells not attached to beads were recovered during application of a magnet; negatively selected populations obtained in this manner were routinely >95% double-positive for CD8 and either CD45RA or CD45RO, respectively, and were >99% viable.

HUVEC were isolated from umbilical cords by enzymatic digestion and maintained in culture, as described (8). EC cultures were free from detectable CD45containing leukocytes and uniformly expressed von Willebrand factor and CD31. BLC from the same donors as the HUVEC were generated by EBV immortalization of cord blood mononuclear cells (4). After 6–8 wk in culture, BLC lines were uniformly CD19-positive. HUVEC and BLC of the same donor were used as stimulator cells in cocultures and as target cells in cytotoxicity assays. When BLC were used as stimulator cells in cocultures, they were pretreated with mitomycin C (50 µg/ml in PBS, 30 min; Sigma-Aldrich, St. Louis, MO) to prevent proliferation. This treatment did not affect the outcome of cultures stimulated with HUVEC and was routinely omitted.

CTL generation

The procedure for CTL differentiation has been described in detail elsewhere (4). In brief, purified CD8+ T cells were incubated with EC or BLC stimulator cells in 96-well microculture plates (Falcon; Becton Dickinson, Bedford, MA) at responder-to-stimulator cell numbers of 200,000/20,000 for EC cocultures and 100,000/20,000 for BLC cocultures. A total of 24–48 microculture replicates were initiated for each experimental group within a given responder-stimulator combination. All cultures were maintained in 5% CO2, room air at 37°C. The medium for coculture consisted of RPMI 1640 supplemented with 10% human AB serum (Irvine Scientific, Santa Ana, CA), 2 mM t-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The medium was further supplemented with exogenous IL-2 (R&D Systems, Minneapolis, MN; or National Cancer Institute, Frederick, MD) on day 3 (final concentration, 5 ng/ml [12.5 U/ml; R&D conversion table]). On day 7, T cells were transferred to fresh stimulator cells in fresh medium containing IL-2, and cultures were fed with fresh medium containing IL-2 on day 10. On day 14, CTL were harvested from microculture wells, counted manually in a hemocytometer, and subjected to further analyses described below either from individual wells or after pooling cells from 3–12 microwells. CD8+ T cells in the absence of any stimulator cells but receiving the same IL-2 treatment were cultured in replicate wells as unstimulated controls.

In experiments involving the addition of blocking mAb, mAb were added at the initiation of cocultures at final concentrations indicated below; treatment with mAb was repeated at times of medium change on days 3, 7, and 10 for EC cocultures and 24/48 h after the last addition for BLC cocultures. For flow cytometric analysis, CD8+ T cells were labeled with CFSE (Molecular Probes) in PBS at 37°C for 15 min. Cells were again washed twice in cold PBS and then incubated in 0.25% paraformaldehyde in PBS for 45 min at 4°C. Cells were then incubated with saturating concentrations of directly PE- or FITC-conjugated mouse anti-human CD3, CD8, CD25, CD69, CD45RA/R0, CD62L, HLA-DR (all from Coulter Immunotech, Hialeah, FL; or D5, TRAP-1; Pharmingen, San Diego, CA) for 1 h at 4°C. Cells were washed twice in ice-cold PBS/1% BSA and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. After washing twice, cells were incubated for 15 min in permeabilization solution consisting of 1% FCS, 0.1% saponin (Sigma-Aldrich), and 0.1% sodium azide. Cells were then incubated with primary unconjugated mouse anti-human perforin mAb (T-Cell Sciences, Woburn, MA) in permeabilization solution for 1 h at 4°C. After washing twice in permeabilization solution, cells were incubated with PE-conjugated goat anti-mouse IgG antiserum for 1 h at 4°C. Cells were finally washed twice in permeabilization solution and once in PBS/1% BSA and stored in PBS/1% BSA until analysis by flow cytometry as above. For immunofluorescence microscopy, CTL were fixed, spun onto glass slides, permeabilized, and stained as described previously (4). Cells were examined with a Microphot FXA microscope (Nikon, Natick, MA).

Labeling studies of CD8+ T cells with CFSE

Labeling and FACS analysis of CD8+ T cells were performed as described elsewhere (6, 12). In brief, total CD8+ T cells or separated CD45RA+ and CD45RO subsets were washed twice in cold PBS and then incubated in 0.25 µM CFSE (Molecular Probes) in PBS at 37°C for 15 min. Cells were again washed twice before addition to cocultures. At indicated times, CFSE-labeled cells were collected, washed in PBS/1% BSA twice, and fixed in PBS/1% paraformaldehyde. For two-color fluorescence studies, CFSE-labeled cells were incubated with saturating concentrations of directly PE-conjugated mouse anti-human mAbs reactive with CD3, CD8, CD69, CD25, CD62L, CD45RA, CD45RO, HLA-DR, and CD154 for 30 min at 4°C, washed twice, and subjected to flow cytometric analysis. Unlabeled CD8+ T cells and cells fixed immediately after CFSE labeling were used as negative and positive internal controls for staining, respectively.

Results

T cell expansion after stimulation with EC or BLC

To analyze the extent of lymphocyte expansion after stimulation with allogeneic EC or BLC, CD8+ T cells were labeled with CFSE before addition to microwell cocultures. Fig. 1 is a representative example of two-color FACS analysis of surface CD8 labeled with PE-conjugated mAb and CFSE content after 14 days of coculture. In experiments involving CsA or mAb, CTL were washed three times before addition to cytotoxicity assays. The supernatant was harvested 12 h after addition of stimulator cells and quantitated with a fluorescence plate reader (Cytofluor 2; Perceptive Biosystems, Framingham, MA; excitation wavelength, 485 nm; emission wavelength, 530 nm). Killing was considered positive for individual microcultures if released fluorescence in a sample well exceeded the mean + 3 SD of the spontaneous release. Percent specific killing was calculated as follows: (release sample − spontaneous release)/(maximal release − spontaneous release) × 100. Spontaneous release was obtained by medium alone, and maximal release was obtained by adding lysis buffer (50 mM sodium borate and 0.1% Triton X-100 [pH 9]). Corresponding BLC or EC were used as targets for BLC-stimulated or EC-stimulated cultures, respectively. In most experiments, the cytotoxicity of EC-stimulated cultures against BLC autologous to the EC was also measured to confirm EC selectivity.

Immunophenotyping of CTL from microcultures

For flow cytometric analysis, T cells were harvested from microculture wells, and cells were further processed either from individual wells or after pooling of cells from 6–18 wells, as indicated. Cells were washed twice in ice-cold PBS/1% BSA and incubated with saturating concentrations of directly FITC- or PE-conjugated mouse anti-human CD3, CD8, CD25, CD69, CD45RA/R0, CD62L, HLA-DR (all from Coulter Immunotech, Hialeah, FL; or D5, TRAP-1; Pharmingen, San Diego, CA) for 1 h at 4°C. Cells were washed twice in ice-cold PBS/1% BSA and fixed with 1% paraformaldehyde in PBS. Samples were analyzed using FACS sorter (Becton Dickinson, San Jose, CA) and CellQuest analysis software by gating on viable cells and collecting 5000 gated events per sample. For intracellular staining for flow cytometric analysis, cells were washed twice in ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. After washing twice, cells were incubated for 15 min in permeabilization solution consisting of 1% FCS, 0.1% saponin (Sigma-Aldrich), and 0.1% sodium azide. Cells were then incubated with primary unconjugated mouse anti-human perforin mAb (T-Cell Sciences, Woburn, MA) in permeabilization solution for 1 h at 4°C. After washing twice in permeabilization solution, cells were incubated with PE-conjugated goat anti-mouse IgG antiserum for 1 h at 4°C. Cells were finally washed twice in permeabilization solution and once in PBS/1% BSA and stored in PBS/1% BSA until analysis by flow cytometry as above. For immunofluorescence microscopy, CTL were fixed, spun onto glass slides, permeabilized, and stained as described previously (4). Cells were examined with a Microphot FXA microscope (Nikon, Natick, MA).

Cytotoxicity assay

Cytotoxicity by CTL was measured by a calcein fluorescent dye release assay as described previously (4, 11). In brief, target cells were loaded with calcein-AM (Molecular Probes, Eugene, OR) and incubated with effector CTL from individual microculture wells for 4 h at 37°C. In experiments involving CsA or mAb, CTL were washed three times before addition to cytotoxicity assays. The supernatant was harvested 12 h after addition of stimulator cells and quantitated with a fluorescence plate reader (Cytofluor 2; Perceptive Biosystems, Framingham, MA; excitation wavelength, 485 nm; emission wavelength, 530 nm). Killing was considered positive for individual microculture wells if released fluorescence in a sample well exceeded the mean + 3 SD of the spontaneous release. Percent specific killing was calculated as follows: (release sample − spontaneous release)/(maximal release − spontaneous release) × 100. Spontaneous release was obtained by medium alone, and maximal release was obtained by adding lysis buffer (50 mM sodium borate and 0.1% Triton X-100 [pH 9]). Corresponding BLC or EC were used as targets for BLC-stimulated or EC-stimulated cultures, respectively. In most experiments, the cytotoxicity of EC-stimulated cultures against BLCl autologous to the EC was also measured to confirm EC selectivity.
recovered from cocultures with either type of stimulator cell, express the same intensity of CFSE fluorescence as unstimulated CD8<sup>+</sup> T cells or CD8<sup>+</sup> T cells immediately after labeling (not shown), indicating that no cell divisions have occurred in these populations. CFSE<sup>low</sup>/CD8<sup>+</sup> T cells show a fluorescence intensity comparable to that of unlabeled CD8<sup>+</sup> T cells. These populations represent cells that have been activated by alloantigen and have undergone multiple cell divisions such that there has been a dilution of the CFSE signal below the limit of detection. Although the number of cell divisions that these alloreacted CD8<sup>+</sup> T cells have undergone cannot be exactly defined, a minimum of seven to eight rounds of cell divisions is required to dilute CFSE to background levels of fluorescence. Further enhancement of the CFSE signal to detect more rounds of division was not possible because higher concentrations of CFSE inhibited CD8<sup>+</sup> T cell proliferation (not shown). The absence of cells with intermediate CFSE labeling, which are readily detectable in PHA-activated cultures (unpublished observation), is consistent with the interpretation that the alloreactive precursor populations of CD8<sup>+</sup> T cells (as low as 1:20,000 for EC stimulators) are too small to be detectable until multiple divisions have occurred (7).

Absolute cell numbers for the alloactivated and nonactivated populations of CD8<sup>+</sup> T cells were determined by multiplying the relative percentage of such cells, determined by CFSE labeling analysis, with the total number of T cells per microwell determined by counting with a hemocytometer. To facilitate comparison between EC- and BLC-stimulated microwell cocultures initiated with inputs of 200,000 and 100,000 CD8<sup>+</sup> T cells per well, respectively, absolute cell numbers are expressed as a percentage of the input cell number. Using this method, microwell cocultures of CD8<sup>+</sup> T cells stimulated with EC or corresponding BLC were followed over several time points during the 14-day coculture period (Fig. 2). On day 3 of the coculture period, no significant population of CFSE<sup>low</sup> cells was detectable either after EC or BLC stimulation (not shown). With BLC stimulators, significant numbers of alloactivated (CFSE<sup>low</sup>) T cells became detectable on day 7, expanding until day 10 and then either leveling off in absolute number (Fig. 2, Exp. A) or expanding further (Fig. 2, Exp. B) until day 14. Total numbers of expanded CD8<sup>+</sup> T cells always reached at least 100% of input cell numbers with BLC stimulation. On day 14 of coculture, essentially all microwell cultures stimulated with BLC con-

**FIGURE 1.** CD8<sup>+</sup> T cell division in BLC- or EC-stimulated cocultures analyzed by CFSE labeling. CFSE-labeled CD8<sup>+</sup> T cells were harvested from BLC- or EC-stimulated microwell cocultures and analyzed by two-color fluorescence flow cytometry as described in Materials and Methods. CFSE<sup>low</sup> cell populations represent alloactivated cells that have undergone multiple cell divisions. CFSE-labeled unstimulated CD8<sup>+</sup> T cells and unlabeled CD8<sup>+</sup> T cells were analyzed as controls. Note that CFSE fluorescence intensity of alloactivated (expanded) cells in BLC- and EC-stimulated cocultures have declined to the level of background signal obtained using unlabeled cells. One of five similar experiments is shown.

**FIGURE 2.** Expansion of CD8<sup>+</sup> T cells in cocultures stimulated with BLC or EC. Cells were harvested from cocultures containing CFSE-labeled CD8<sup>+</sup> T cells at indicated time points, counted, and analyzed by two-color flow cytometry. **Left panels.** The relative proportion of alloactivated (expanded) cells in cocultures (percentage of CFSE<sup>low</sup> cells of total CD8<sup>+</sup> T cells) was combined with the total number of cells harvested per microwell (open bars) to yield absolute numbers for alloactivated, expanded CD8<sup>+</sup> T cells (filled bars), both of which are expressed as a percentage of the original input cell number per well. **Right panels,** Proportion of BLC- or EC-stimulated coculture microwells with detectable significant cytotoxicity against corresponding target cells for respective cell expansion experiments. Two of five similar experiments are shown.
FIGURE 3. Expression of surface markers and perforin in alloactivated CD8+ T cells after 14 days of stimulation with BLC or EC. A. Expression of surface markers on alloactivated (CFSElow; cf Fig. 1 and text) cells stimulated with BLC or EC; for comparison, expression on unstimulated cells (total population) is shown. B. Two-color FACS analysis for CFSE and perforin of cells from BLC- or EC-stimulated cocultures. One representative experiment of three similar experiments is shown.

Persistently tested positive for cytotoxicity (Fig. 2, insets) with mean specific killing exceeding 30%. With EC stimulation, low but significant numbers of alloactivated, expanded CD8+ T cells only became detectable on day 10. Such cells usually expanded moderately further until day 14. However, total cell numbers of alloactivated, expanded CD8+ T cells (relative to input cell numbers) after 14 days, ~15–25% of microculture wells stimulated with EC were positive for cytotoxicity (Fig. 2, insets), and mean specific killing remained below 15%. Control experiments, demonstrating the absence of cytotoxicity of EC-stimulated CTL vs corresponding BLC, confirmed the EC specificity of killing in these cocultures as reported previously (Ref. 4 and not shown). Extension of EC-stimulated cocultures beyond day 14 by restimulation and refeeding failed to further enhance the absolute number of alloactivated cells or the extent of cytotoxicity. Indeed, in most EC-stimulated cultures the absolute and relative sizes of the expanded (CFSElow) cell pool actually contracted between day 14 and day 21 (not shown). The number of surviving, nondividing (CFSEhigh) CD8+ T cells (relative to input cell number) was not significantly different between BLC or EC stimulation (Fig. 1). These data show that BLC stimulation of CTL consistently displayed significantly greater expansion of relative and absolute numbers of alloactivated cells than EC stimulation. This greater expansion of the alloactivated population correlates with and can explain the higher degree of measured cytotoxic activity of cells recovered from BLC-stimulated cocultures.

Immunophenotype of CD8+ T cells after stimulation with BLC or EC

After 14 days of microwell coculture, recovered CD8+ T cells were analyzed by two-color flow cytometry for CFSE fluorescence levels and for expression of several surface activation markers or intracellular perforin expression. CFSE labeling of CD8+ T cells in these experiments allowed the separate analysis of surface Ags and perforin expression for unactivated (CFSEhigh) and for alloactivated, expanded (CFSElow) cells. At 14 days after stimulation with BLC, the alloactivated CD8+ T cells were almost exclusively CD45RO+ (memory phenotype) and expressed high levels of HLA-DR and very low levels of CD25 and CD69. These last two markers, indicative of early activation, had fallen to levels comparable to those of unstimulated, IL-2-treated control cells (Fig. 3A). Expression of L-selectin (CD62L) was significantly reduced with that in control cells but was not completely absent. In day 14 cultures stimulated with EC, the alloactivated, expanded CD8+ T cells (CFSElow) were also exclusively of CD45RO+ phenotype and expressed high levels of HLA-DR. In striking contrast to BLC-stimulated CD8+ T cells, alloactivated (CFSElow) T cells in EC-stimulated cultures retained high expression levels of the early activation markers CD69 and CD25. In addition, almost all activated cells were positive for CD62L, an even higher percentage than that observed in unstimulated control cells (Fig. 3A). In cocultures extended beyond 14 days, these phenotypic differences were essentially maintained (not shown); in most EC-stimulated cocultures, the number of alloactivated T cells began to decline after more than 14 days (see above). Comparisons of 10-day cultures, i.e., 3 days after restimulation, showed some expression of CD25 and CD69 in BLCC-stimulated cultures, confirming that the low levels seen in day 14 cultures did represent a decline from peak levels. However, even at day 10 the level and percentage of positive cells were still markedly lower than those in EC-stimulated cultures (not shown). In BLC- or EC-stimulated cocultures, CD154 was not detectable on alloactivated (CFSElow) cells by flow cytometry (Fig. 3A).

The expression of perforin by alloactivated (CFSElow) CD8+ T cells after stimulation with BLC or corresponding EC was qualitatively similar but differed significantly in quantitative analysis (Fig. 3B). After stimulation with either cell type, ~90% of the alloactivated CD8+ T cell population expressed detectable levels of perforin; however, the expression level per cell was markedly higher after stimulation with BLC. Nondividing CD8+ T cells in coculture and unstimulated, IL-2-treated control cells did not show detectable perforin expression (not shown).

Activation of CD45RA+ (naive) and/or CD45RO+ (memory) subsets

Although all of the alloactivated CD8+ T cells recovered at day 14 expressed CD45RO, these cells could have arisen from resting memory (CD45RO+) or resting naive (CD45RA+) subsets or both. To directly identify the subset(s) of CD8+ T cells that were activated by stimulation with BLC or EC, cocultures were set up...
memory CD8 cells were similar in both subsets, indicating that both naive and activated CD8 differ from those obtained after stimulation with EC, when alloactivated CD45RA/RO double-positive after activation. These results crowell cocultures of CD45RA cocultures were concordant with perforin expression: 100% of microwell cocultures showed detectable cytotoxicity (Fig. 4A), indicating general inhibition of the expansion/proliferation of alloactivated CTL (4). BLC also differ from EC regarding the costimulator molecules expressed by these cell types (13), and these differences are reflected in the pathways used to stimulate IL-2 production. The influence of CD8+ T cell costimulation by EC upon CTL generation was examined in cocultures using blocking mAbs. Because these cocultures were also fed routinely with exogenous IL-2, these blocking experiments examine features of T cell activation and differentiation independent of IL-2 synthesis. Inhibition of costimulation with mAbs vs LFA-3, ICAM-1, or CD40 did not influence any of the parameters analyzed after 14 days of coculture, including T cell numbers, extent of cytotoxicity, perforin positivity and expression level, and expression of surface activation markers (Fig. 5).

As the generation of CTL by stimulation with EC had consistently been less effective than by corresponding BLC, we examined whether enhanced costimulation during stimulation of CD8+ T cells could improve the CTL response. Because EC do not express the CD28 ligands CD80 and CD86, additional costimulation was provided via a comitogenic anti-CD28 mAb. The comitogenicity of the mAb used in soluble form in these studies had been confirmed in pilot experiments with purified CD8+ T cells stimulated with anti-CD3 mAb or PHA (not shown). However, addition of comitogenic concentrations of an anti-CD28 mAb did not increase the generation of CTL by stimulation with EC as assessed by cell numbers, extent of cytotoxicity, perforin positivity and expression level, and expression of surface activation markers (Fig. 6A).

An important role of CD40 ligation for activation of APC to induce CTL has been reported (14, 15). Therefore, cocultures were performed using EC preactivated via CD40 ligation for 24 h. Activity of the recombinant trimeric CD40 ligand molecule had been demonstrated in EC previously (Ref. 16) and our unpublished observations). Preactivation of EC used as stimulator cells in coculture assays had no effect on the differentiation of CTL in that all examined parameters were unchanged between untreated cocultures and cocultures after pretreatment with CD40 ligand trimer (Fig. 6B).

**Effect of CsA on CTL differentiation**

To address the influence of the immunosuppressant CsA on the differentiation of CTL after stimulation with BLC or EC, cocultures were performed in the presence of 500 ng/ml of CsA added at various time points after coculture initiation. In the presence of CsA, the absolute number of alloactivated cells (i.e., CFSElow) was markedly reduced with both types of stimulator cells (Fig. 7), suggesting general inhibition of the expansion/proliferation of alloactivated CD8+ T cells even in the presence of exogenous IL-2. In case of stimulation with BLC, the absolute size of the alloactivated (CFSElow) cell population was reduced by ~75% after 7, 10, and 14 days compared with cultures without CsA (Fig. 7, left half of panel). In EC-stimulated cultures, alloactivated CD8+ T cells in the presence of CsA could only be detected on day 14, and the absolute number of expanded (activated) cells was reduced by
with stimulating cells, thus eliminating potential selection bias and compared with conventional CTL directly from primary cocultures. Experiment of three similar experiments is shown. One representative experiment of three similar experiments is shown. CTX, Cytotoxicity.

Discussion

Cell type-selective CTL have been described after various forms of solid organ transplantation, and they have been implicated in tissue-specific injury during allograft rejection (3, 17, 18) or graft-versus-host disease (19). We have previously described the in vitro generation of EC-selective CTL (4), from which CTL clones could be derived, that exhibited atypical features including absence of IFN-γ production and CD40L (CD154) expression (5).

In the present study, EC-selective CTL were analyzed and compared with conventional CTL directly from primary cocultures with stimulating cells, thus eliminating potential selection bias and long-term culture changes that may occur during cloning procedures. Two principal findings of our study are that CD8+ T cells expanded less well after stimulation with EC than with BLC and that stimulation of CD8+ T cells by EC exclusively activated memory (CD45RO+) cells to differentiate into EC-selective CTL, whereas BLC stimulators were able to recruit both naïve and memory CD8+ T cells to differentiate into CTL. Specifically, there was a 5- to 20-fold reduction in the final absolute number of expanded (i.e., alloactivated) CD8+ T cells after EC stimulation compared with that after BLC stimulation. Therefore, on the basis of these data, the reduced cytotoxicity seen with EC-selective CTL appears to be largely due to much lower E:T ratios in these cytotoxicity assays. However, EC-stimulated CTL also expressed significantly lower amounts of perforin in individual cells (Fig. 2, Exp. B), which could also contribute to the difference in cytotoxicity.

The smaller number of expanded, alloactivated CD8+ T cells in EC-stimulated cocultures can in principle be explained either by a lower frequency of precursor cells or by fewer generations of cell division during cocultures. To account for the observed difference in CTL at the end of cocultures, precursor frequencies for BLC-stimulated cultures. Moreover, previous studies determined that the precursor frequency in CD8+ T cells comparing EC to monocyte stimulators was ~5 times lower when measured by limiting dilution analysis of IL-2 production (7). Moreover, if EC-selective CTL arise from precursors that only recognize EC-derived peptides, this precursor pool of pre-CTL may be even smaller than that determined by limiting dilution analysis. On the other hand, proliferation after stimulation with EC does appear to be
delayed compared with BLC stimulation (7), and EC reduce T cell proliferation stimulated by BLC in mixed cocultures (4). Thus, both factors may contribute to the lesser capacity of EC to stimulate alloreactive T cell populations. The inability of EC to activate naive T cells has been proposed to be due to limitations in Ag-independent adhesion compared with other, professional APC (9) or to reduced capacity of cytokine secretion, especially IL-12 (9, 20). Previous observations that supplementation of EC-stimulated cocultures with IL-12 did not improve the generation of CTL (4) render the latter alternative less likely.

CTL from EC-stimulated cultures continued to express surface markers CD69, CD25, and L-selectin (CD62L) at the end of the coculture period, representing an early, immature activation phenotype. CD69 and CD25 are generally considered early activation markers (21–23), and L-selectin is usually lost rapidly during the course of T cell activation (24–26). Also, the expression level of perforin per cell in EC-selective CTL was markedly lower than in corresponding conventional CTL. In contrast, on BLC-stimulated CTL, the expression of CD69 and CD25 had subsided, and CD62L expression, highly expressed in CD8+ T cells at the beginning of coculture, had largely been lost. The findings in BLC-stimulated CD8+ T cells are in accordance with numerous in vitro and in vivo reports showing the loss of early activation markers 2–3 days after activation of T cells by BLC or other professional APC (21–23). A direct correlation between the number of cell divisions and loss of CD25 and CD69 also has recently been reported (27). Time course of the activation phenotype on T cells after (allo)activation by EC has not been reported previously, and the basis for the retention of this early activation phenotype of EC-specific CTL is currently unknown. Several (not mutually exclusive) explanations appear possible. First, the phenotype seen on EC-stimulated CTL might represent an immature state of activation caused by slower or delayed activation of CD8+ T cells by EC, such that the EC-selective CTL lag behind their conventional counterparts in the down-regulation of early activation markers and in the synthesis of perforin. Such defective activation in EC-stimulated cocultures may be caused by differences in EC-mediated adhesion, costimulation, or cytokine production. However, the persistence of the phenotypic differences between EC- and BLC-stimulated CTL beyond 14 days of coculture seems to argue against a simple temporal lag in the EC cocultures. It also appears unlikely that the differential phenotype of EC-selective CTL compared to BLC-selective CTL is due to differences in cytokine production.

**FIGURE 6.** Exogenous enhancement of costimulation does not improve CTL differentiation by stimulation with EC. Pooled cells from 14-day cocultures of CD8+ T cells stimulated with EC in the absence and presence of enhanced costimulation were analyzed for total cell number, perforin positivity, and expression of surface activation markers (horizontal bar graph). At the same time, the proportion of coculture microwells with detectable significant cytotoxicity from indicated cocultures was determined in cytotoxicity assays (inset). A. Cocultures were performed in the absence and presence of comitogenic anti-CD28 mAb. B. Cocultures were performed in the absence and presence of 24-h pretreatment with recombinant trimeric CD40L. One representative experiment of three similar experiments is shown each. CTX, Cytotoxicity.

**FIGURE 7.** Effect of CsA on expansion of CD8+ T cells after stimulation with BLC or EC. Cells were harvested from cocultures containing CFSE-labeled CD8+ T cells in the absence and presence of 500 ng/ml CsA at indicated time points, counted, and analyzed by two-color flow cytometry. The relative proportion of alloactivated (expanded) cells in cocultures (percentage of CFSElow cells of total CD8+ T cells) was combined with the total number of cells harvested per microwell (open bars) to yield absolute numbers for alloactivated, expanded CD8+ T cells (filled bars), both of which are expressed as a percentage of the original input cell number per well. +CsA, Coculture in presence of cyclosporine A. One representative experiment of three similar experiments is shown each.

**Supplementary Figure A:** Comparison of CTL generation in EC-stimulated cocultures in the presence or absence of CsA. A. Total number of cells harvested at different time points. B. Percentage of alloactivated cells.
of CTL after stimulation with EC or BLC should be exclusively due to fewer cell divisions after EC stimulation, especially because similar differences in the expression of early activation markers could already be detected after 10 days of coculture with EC. If the differences in expansion and phenotype between the cocultures were entirely due to the number of cell divisions, the number of cell divisions at day 14 after stimulation with EC would be expected to be more equivalent to the cell divisions at day 10 after stimulation with BLC, and cell phenotypes would likely correspond. Also, the up-regulation of CD62L was only seen in EC-stimulated cultures and represents a unique feature of the EC-selective CTL phenotype. EC-derived effects could also account for the increased expression of CD69, even on nondividing (CFSE<sup>high</sup>) CD8<sup>+</sup> T cells, in EC-stimulated cocultures compared with IL-2-treated control cultures (not shown). EC have been shown to secrete IL-7 and IL-15 and could thus up-regulate the expression of CD69 and CD25 (28–31). Finally, continuing activation of CD8<sup>+</sup> T cells by alloantigen in cocultures with EC but not with BLC could account for the described phenotype. Persistent expression of early activation markers has been demonstrated on T cells in disease states associated with continuous long-term immune activation, especially HIV and CMV infection or rheumatoid arthritis (32–34). In the current study, persistent T cell activation might theoretically result from a greater persistence of EC than BLC in the cocultures such that EC would continue to activate CD8<sup>+</sup> T cells. EC stimulators (in contrast to BLC stimulators) were not treated with mitomycin for growth arrest, but pilot experiments failed to show any difference in CTL generation irrespective of mitomycin treatment (not shown). In addition, no viable EC were detectable by microscopy in coculture wells after 5 days.

Previous studies from our group and others have emphasized differences in the costimulators used by human EC and professional APC. In IL-2-supplemented EC-stimulated cultures, clonal expansion, surface phenotype, perforin positivity, and cytotoxicity toward EC were unchanged in the presence of blocking Abs to the three most relevant accessory receptor/ligand pairs involved in the interaction of EC and T cells: LFA-1/ICAM-1, CD2/CD58, and CD40/CD154. HUVEC do not express the ligands for either CD28 (B7.1 and B7.2; Ref. 10) or CD137, the human equivalent for mouse 4-1BB (ILA; T. J. Dengler, unpublished observation), and the T cell costimulatory potential of VCAM-1 and E-selectin are thought to be of minor importance (20, 35). However, exogenous costimulatory enhancement with a comitogenic anti-CD28 mAb was also without effect on CTL generation. The central role of the CD40/CD154 interaction for the priming of professional APC as stimulators of CTL has been demonstrated extensively (14, 15, 36), but we did not observe a similar effect on EC in our experiments using recombinant CD40 ligand trimer. These results of our costimulation studies are in accordance with the classic concept that T cell costimulation by accessory cells primarily induces increased IL-2 production (37), an effect that was bypassed in our experiments by exogenous IL-2.

Finally, as EC-selective CTL might play an important part in the pathogenesis of forms of rejection (e.g., endothelialitis) that are less amenable to treatment, we investigated the sensitivity of EC-selective CTL to CsA. Our results demonstrate a similar inhibition of alloactivated CD8<sup>+</sup> T cell expansion by CsA in EC- or BLC-stimulated cocultures. In BLC-stimulated cultures, the proportion of CD8<sup>+</sup> T cells expanding despite the presence of CsA increased very slightly (30% vs. 43%, respectively).

**FIGURE 8.** Effect of CsA on cytotoxicity displayed by CTL cocultures stimulated with BLC or EC. After 14 days of coculture of CD8<sup>+</sup> T cells with indicated stimulator cells, cells were harvested from individual microwells and tested for cytotoxicity against respective target cells. CsA was added to cocultures at indicated time points. Bars represent the proportion of microwells displaying significant cytotoxicity. Numbers indicate mean percentage of specific killing of all tested microwells. One representative experiment of three similar experiments is shown.

**FIGURE 9.** Effect of CsA on the expression of surface markers and perforin of alloactivated CD8<sup>+</sup> T cells after stimulation with BLC or EC. Cells were analyzed by two-color flow cytometry after 14 days of microwell coculture of CD8<sup>+</sup> T cells with respective stimulator cells in the absence or presence of 500 ng/ml CsA. Expression of surface markers and perforin in alloactivated (expanded; CFSE<sup>low</sup>; cf Fig. 1) cells stimulated with BLC or EC, for comparison, on unstimulated cells (total population) is shown. A, EC-stimulated coculture; B, BLC-stimulated coculture. One representative experiment of three similar experiments is shown in each panel.

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over time and was markedly higher than in EC-stimulated cocultures, which is again compatible with a delayed activation process in the presence of EC stimulators. Interestingly, the suppression of proliferation occurs in the presence of significant amounts of exogenous IL-2. Similar suppression of T cell growth by CsA has been reported previously (38, 39) and may be explained by more recent data indicating that CsA can inhibit T cell activation by additional mechanisms such as TGF-β production, up-regulation of the cdk inhibitor p21, and inhibition of IL-2 receptor expression (40–42). Unspecific toxic or apoptotic cell death induced by CsA can be excluded as a cause of the reduced cell numbers in our experiments on the basis of the low concentration of CsA (500 ng/ml) used and the absence of apoptotic cells. CsA also reduced cytotoxicity, primarily via the reduction in CTL numbers, and consequently reduced E:T ratios. Differentiation or maturation of CTL at the level of the individual effector cell was not affected by CsA, as indicated by normal expression of activation markers and perforin. Late treatment of cocultures with CsA seemed to have a marginal inhibitory effect on cytotoxicity, which is in line with previous reports suggesting inhibition of perforin degradation by CsA (43, 44). Taken together, EC stimulation of CD8+ T cells for CTL generation does not appear to impart any CsA resistance.

The effects of EC upon CTL activation and differentiation described here and in our previous studies have significant implications for transplantation and vascular biology. As shown earlier (4), the generation of highly cytotoxic, conventional CTL is suppressed by EC, a finding with particular pertinence for microenvironments in which EC are closely apposed to infiltrating immune cells, such as the vascular intima. On the other hand, stimulation of CD8+ T cells by EC appears to favor the differentiation to a unique population of CTL displaying a variety of characteristic features, including an immature early activation phenotype, low perforin content, reduced capacity for clonal expansion, and cell type selectivity. These results strengthen a function of vascular EC as semiprofessional APC of intermediate stimulatory capacity as previously suggested (4). Unspecific toxic or apoptotic cell death induced by CsA appears to be a temporal bridge between a CD4+ T-helper and a T-killer cell.

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