Human Endothelial Cells Induce and Regulate Cytolytic T Cell Differentiation[1]

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We compared the capacity of cultured human endothelial cells (EC) vs B lymphoblastoid cells (BLC) from the same donor to stimulate allogeneic CD8$^+$ T cells to differentiate into CTL, assaying for allorestricted cytotoxicity, T cell growth, IFN-γ secretion, and perforin expression. The input cell number affected specificity and potency of the resulting CTL. At low input ($<10^5$ cells/well), anti-EC CTL were rarely detected. At high input ($>10^6$ cells/well), anti-EC CTL developed that displayed unrestricted, low-titer killing and an unstable phenotype. At intermediate input ($1.0–2.5 \times 10^5$ cells/well), classical class I MHC-restricted, CD8$^+$, and perforin-positive anti-EC CTL developed with reproducible frequencies. However, under all conditions EC were less efficient stimulators than BLC from the same donor. Anti-EC CTL did not kill BLC, whereas anti-BLC CTL killed BLC and EC from the same donor with comparable efficiency. When CD8$^+$ T lymphocytes were grown in the presence of EC and BLC together, the differentiation of anti-BLC CTL was completely suppressed, while the anti-EC response was intact. The inhibition of the allogeneic anti-BLC CTL response was independent of T cell-EC contact, and proliferation of CD8$^+$ T cells was inhibited by EC-conditioned medium. We conclude that EC are competent but less efficient activators of CTL differentiation than are BLC and that EC actively regulate differentiation and/or expansion of allospecific CTL.   *The Journal of Immunology,* 1998, 161: 4679–4687.

Recent analyses of human kidney allograft biopsies have shown a strong correlation between the presence of infiltrating CD8$^+$ cytolytic lymphocytes and histopathologic rejection (1). Moreover, the presence of transcripts for proteins associated with lymphocyte cytolytic function, namely granzyme B, perforin, and Fas ligand, also correlates strongly with acute cellular rejection (2). Most cytolytic lymphocytes are CD8$^+$ T cells that specifically recognize short peptides bound to class I MHC molecules displayed on the surface of a target cell. Normally, each CTL is specific for one antigenic structure formed by a specific foreign (e.g., viral protein-derived) peptide associated with a self-allelic form of a class I MHC molecule. Alloreactive CTL arise from cross-reactions of self MHC-restricted CTL with complexes formed by peptides and non-self (allogeneic) class I MHC molecules expressed on graft cells (3, 4). The same peptide-MHC molecular complex must be recognized both during the differentiation of a CTL from a "preCTL",[3] i.e., a resting CD8$^+$ T cell commonly found in blood that lacks cytolytic function (5, 6) and during the delivery of a lethal hit to a target cell by a mature CTL (7, 8). During the effector phase, mature allospecific CTL may recognize their target peptide-MHC molecule complexes on the surface of essentially any cell type, enabling CTL to lyse a wide variety of graft cells. However, during the differentiation process, preCTL require additional signals (costimulation) that may be provided only by specialized APC such as dendritic cells (9–12). The necessity for costimulation of preCTL by such "professional" APC may help explain the apparent requirement for graft-derived "passenger leukocytes" to initiate allograft rejection in rodents (13).

It has been proposed that human endothelial cells (EC) may substitute for "passenger leukocytes" as initiators of the host anti-graft response (14, 15). Human EC are competent in culture to present allogeneic class I MHC molecules directly to resting CD8$^+$ memory T cells, leading to cytokine secretion and proliferation (16, 17). Recently, CTL have been recovered from rejecting allografts that appear to recognize EC specifically, i.e., they can lyse donor EC but not donor leukocytes (18, 19). Because each mature CTL must recognize the same Ag as its preCTL progenitor, this finding implies that donor EC stimulated the differentiation of such CTL from precursors. However, these data do not rule out the possibility that professional APC may also have played a role (e.g., as "trans-costimulators" (20)).

The simplest way to test the sufficiency of human EC to promote CTL differentiation is to coculture EC with preCTL. To date, such experiments have given conflicting results. Clayberger et al. (21), using human foreskin microvascular EC as stimulators, found that PBMC differentiated into nonspecific (i.e., nonallorestricted) CD8$^+$ killers, whereas BLC under the same conditions stimulated the development of class I MHC-restricted CTL. In contrast, Pardi and Bender (22) observed that human foreskin microvascular EC were able to stimulate allospecific CTL differentiation from purified CD8$^+$ T cell precursors, but no comparison to BLC or other professional APC were made. The present study was undertaken to assess the relative capacity of cultured EC to promote allogeneic CD8$^+$ T cell differentiation in comparison to BLC derived from the same donor as the EC. In addition, we wished to test the hypothesis that stimulation of CD8$^+$ T cells by allogeneic EC would lead to the emergence of CTL reactive with EC but not BLC targets.

Materials and Methods

Cytokines, drugs, and Abs

Recombinant human IL-2, IFN-γ, and IL-12 were purchased from R&D Systems (Minneapolis, MN). Mitomycin C, indomethacin, N^ω-nitro-L-arginine methyl ester (L-NAME) and N^ω-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma (St. Louis, MO). Neutralizing Abs reactive with TGF-β (used at a final concentration of 5 μg/ml) were obtained from R&D Systems. OKT8 hybridoma cells were obtained from American Type Culture Collection (Manassas, VA), and mAbs (IgG2a) were affinity purified from crude ascites using HiTrap Protein G according to the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). K1616, a nonbinding control Ig (IgG1), was purified in the same way.

Cell isolation

Human EC were isolated from individual umbilical veins as previously described (16) and cultured at 37°C and 5% CO_2-humidified air in Medium 199 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY), 50 μg/ml endothelial cell growth supplement (Collaborative Research/Becton Dickinson, Bedford, MA), and 100 μg/ml heparin (Sigma). In the experiments performed in this study, EC were used at passage levels 3 to 9. Such EC cultures have been found to be devoid of $CD45^+$-contaminating leukocytes, yet retain uniform von Willebrand factor positivity.

BLC were prepared from cord blood harvested from the same individual as the EC. Cord blood mononuclear cells were isolated by density gradient centrifugation using lymphocyte separation medium (LSM, Organon Teknika, Durham, NC). A portion of these cells were used for serologic tissue typing (courtesy of Dr. M. I. Lorber, Yale Tissue Typing Laboratory, New Haven, CT), and the remainder were used for the generation of BLC by EBV transformation with the EBV secreting cell line 95.8 (a generous gift from Dr. G. Miller, Yale Medical School, New Haven, CT) as described (23). BLC lines were cultured at 37°C and 5% CO_2-humidified air in RPMI 1640 (Life Technologies) in 10% FCS with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

$CD8^+$ T cells were purified from PBMC isolated from healthy volunteer donors by leukapheresis and density gradient centrifugation. A portion of the cells were tissue typed serologicly for HLA class I molecule expression (courtesy of the Yale Tissue Typing Laboratory) and the remainder, about 2 to 3 × 10^9 PBMC per donor, were suspended in ice cold FCS/10% DMSO, aliquoted, and stored in liquid nitrogen until use. Thawed PBMC were washed in RPMI 1640/5% FCS, and $CD8^+$ lymphocytes were positively selected using anti-CD8 Ab-coated magnetic beads (Dynabeads M450-CD8, Dynal, Lake Success, NY) according to the manufacturer's instructions. Briefly, PBMC were suspended at 2 × 10^7 cells/ml in the same medium and incubated with 1.2 × 10^7/ml Dynabeads for 30 min at 4°C. Beads with attached $CD8^+$ cells were washed five to six times until the supernatant was clear. The Detachabead solution (Dynal) was added to the suspension, which was then incubated for 45 to 60 min at room temperature. The recovery was 70 to 75% of the total input $CD8^+$ T lymphocytes with >96% $CD8^+$ expression determined by FACS analysis (see below) and >99% viability assessed by trypan blue exclusion. No discrete contaminating populations of $CD4^+$, $CD19^+$, $HLA-DR^+$, or $CD16^+$ cells were detected. In pilot experiments, $CD8^+$ T cells purified by negative selection (for HLA-DR, CD16, and CD4) were never >85 to 90% $CD8^+$. However, no significant differences were noted between $CD8^+$ CTL generated from positively or negatively selected cells. Because of the higher degree of purity, all of the experiments reported in this paper used positively selected populations. For FACS analysis, cells were washed in PBS/1% BSA and incubated with directly conjugated mAbs (isotype matched, FITC- or phycoerythrin-conjugated control mAbs, or mouse anti-human HLA-DR, HLA class I (W6/32), CD45, CD25, CD16, CD19, CD14, CD4, CD8, and CD3 Abs, all from Immunotech, Westbrook, ME) according to the manufacturer's instructions. Stained cells were washed three times with PBS/1% BSA, and then fixed in 2% paraformaldehyde in PBS and analyzed within 24 h using a FACSort (Becton Dickinson, San Jose, CA). All staining, washing, and incubation steps were conducted on ice.

CTL differentiation

Both EC and BLC were used as stimulator cells for CTL differentiation from $CD8^+$ preCTL. EC were suspended from confluent cultures using trypsin-EDTA and were seeded at confluent density into 96-well round-bottom microtiter plates (for limiting dilution analysis and microcultures), 24-well plates (for bulk cultures), or 6-well plates for Transwell experiments (all plasticware from Falcon, Becton Dickinson, Bedford, MA) 1 to 2 days before the cocultures were started. Where indicated, EC were fixed

with 1% paraformaldehyde for 10 min at room temperature, extensively washed, and rested for 10 min before cocultures were initiated. BLC were washed in HBSS, and mitomycin C was added at 50 μg/ml final concentration. Cells were incubated at 37°C for 20 min and washed three times with RPMI 1640/5% FCS. After resting for 1 h at 37°C, cells were again washed three times and then added to wells at numbers comparable to confluent EC.

Cocultures were initiated by addition of $CD8^+$ T cells from donors allogeneic to the EC or BLC. Purified $CD8^+$ T cells were suspended in RPMI 1640, 10% human type AB serum (Irvine Scientific, Santa Ana, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and added to confluent EC monolayers or BLC at the indicated input cell number. For limiting dilution analysis, $CD8^+$ input cell numbers were titered over a broad range (250,000 to 2,500 cells/well) to observe the different response patterns against EC and BLC, respectively. Microcultures were typically initiated with 36 replicates (2 times 18 replicates for testing against stimulator vs third party cell line or EC vs BLC) per test group. When the allospecificity of CTL was tested, 200,000 $CD8^+$ T cells/well were used with EC stimulators and 50,000 $CD8^+$ T cells/well were used with BLC stimulators. When EC and BLC were compared for the efficiency with which they stimulate CTL differentiation, cocultures were initiated with the same $CD8^+$ T cell input number (100,000 cells/well) for both cell types. Bulk cultures were initiated in replicate cultures in 24-well plates at input cell numbers of 1 to 4 × 10^6 $CD8^+$ T cells/well. Transwell cultures were performed using 6-well culture inserts (PET, pore size 0.4 μm, Falcon). $CD8^+$ T cells (2 × 10^6) were added together with BLC to the upper compartment of the culture insert and confluent living or fixed EC were grown in the bottom of the 6-well plate. Where indicated, pharmacologic inhibitors of cyclooxygenase (5 μM indomethacin) or nitric oxide synthase (5 mM L-NAME) were included in the cultures.

In all cases, cocultures were fed with fresh medium containing IL-2 at a final concentration of 5 ng/ml after 3 days. After 7 days, 150 μl of supernatant per well was removed from the microcultures, pooled, and frozen at −70°C for cytokine measurements. Fresh medium containing IL-2 (100 μl/well) was added, and the $CD8^+$ T cells were transferred to fresh EC or BLC. The bulk cultures were suspended and collected into tubes, spun down, resuspended in fresh medium containing IL-2 and transferred to fresh EC or BLC. The cultures were fed again after 10 days in medium supplemented with IL-2. Where indicated, fresh medium was replaced by a 1:1 mixture of fresh and conditioned medium. After 2 wk, supernatant was harvested, pooled, and frozen for cytokine measurements. The medium was replaced by Medium 199, 5% FCS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cells were used for cytotoxicity assays.

In selected experiments, proliferation of anti-BLC CTL was measured as previously described (16). In brief, 10^5 $CD8^+$ T cells/well were incubated with mitomycin C-treated BLC in quadruplicate. After 78 h, 1 μCi/well [³H]thymidine (NEN Life Science Products, Boston, MA) in complete medium was added. Cells were harvested after an additional 18 h using a 96-well harvester (Tomtec, Orange, CT) and counted on a Microbeta scintillation counter (Wallac, Gaithersburg, MD). The specific cpm were corrected for background (mitomycin C-treated BLC), typically <200 cpm.

Cytotoxicity assay

Cytotoxicity was measured by a calcein fluorescence release assay performed according to published methods (24). In brief, target EC were grown in flat-bottom 96-well plates to confluence (2 × 10^4 cells/well). Cells were incubated with 20 μM calcein-AM (Molecular Probes, Eugene, OR) in Medium 199 and 5 mM HEPES for 30 min at 37°C. The medium was replaced by complete EC growth medium, and cells were rested overnight. After 8 to 12 h, cells were washed twice with Medium 199, 5% FCS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Alternatively, target BLC were harvested from suspension culture, washed once with Medium 199 and 5 mM HEPES, and incubated with 20 μM calcein-AM for 30 min at 37°C. The cells were washed three times, counted, and added to the microculture wells at numbers comparable to confluent EC.

Effector cells from microcultures were suspended and 150 μl/well transferred onto calcein-loaded target cells. The effector cell number (mean of cell counts from three randomly chosen replicate wells) was counted with a hemocytometer and the average E:T ratio calculated. Effector cells from bulk cultures were washed once, counted, and added at titered E:T ratios in duplicates. Twelve replicate wells/plate were incubated with lysis buffer (50 mM sodium borate, 0.1% Triton X-100, pH 9.0, maximal release). After 4-h incubation at 37°C, supernatant (75 μl/well) was carefully removed and transferred into a flat-bottom 96-well plate. Released calcein was measured using a fluorescence multiwell plate reader (Cytofluor 2,

Perspective Biosystems, Framingham, MA; excitation wavelength 485 nm, emission wavelength 530 nm). Percent specific killing was calculated as [(release sample − spontaneous release)/(maximal release − spontaneous release)] × 100%. Spontaneous release was generally <25%.

IFN-γ ELISA

IFN-γ was measured in tissue culture supernatants with a sandwich ELISA using a commercially available Ab pair (monoclonal mouse anti-human IFN-γ, MAB285, and biotinylated polyclonal goat anti-human IFN-γ, BAF285, both from R&D Systems) according to the manufacturer's instructions. IFN-γ was measured either in the pooled and frozen coculture supernatants after 7 or 14 days, or in the individual microculture supernatants from cytotoxicity assays. In the latter case, the medium was replaced after harvesting the supernatant from the CTL assay to measure calcein release, and the cultures further incubated overnight at 37°C. Supernatant (100 μl) from six replicate microculture wells were then transferred directly to the precoated ELISA plate.

Perforin staining

Fresh CD8+ T cells or CTL from cocultures were fixed with freshly prepared, ice cold 4% paraformaldehyde for 10 min and spun onto gelatin-coated glass slides (Cytospin 2, Shandon, Pittsburgh, PA). After washing the slides in PBS, cells were permeabilized and blocked with 0.1% saponin (Sigma) in PBS supplemented with 1% BSA, 5% normal human serum, and 5% normal goat serum for 10 min at room temperature. Monoclonal mouse anti-human perforin (1/250, T-Cell Diagnostics, Cambridge, MA) and rat anti-human CD8 Abs (1/250, MCA351, Serotec, Washington, DC) were added, and cells were incubated for 60 min at room temperature. An isotype-matched (IgG2b) nonbinding mouse mAb (Cappel, Organon Teknika) was used as control. After the slides were washed for 10 min with PBS/0.1% saponin, polyclonal Cy2-conjugated goat anti-mouse and Texas red-conjugated goat anti-rat Abs (both from Jackson ImmunoResearch Laboratories Inc, West Grove, PA) were added for 30 min. After the slides were washed for 10 min, they were air dried and embedded in aqueous mounting medium. Cells were examined and photographed with a fluorescence microscope (Microphot FXA, Nikon, Tokyo, Japan). For optimal staining of perforin granules, it appeared critical to stain cells immediately after fixation.

Statistical analysis

Data analysis of limiting dilution experiments was performed according to likelihood maximization using a computer program kindly provided by Dr. C. Orosz (Ohio State University, Columbus, OH). Results from different groups were compared with ANOVA or with the Wilcoxon test.

Results

Differentiation of CTL in response to allogeneic EC vs BLC

We compared the capacity of cultured EC and BLC, derived from the same donor, to stimulate the differentiation of allogeneic peripheral blood CD8+ T cells into CTL. Preliminary experiments confirmed the observation of Pardi and Bender (22) that the addition of small amounts of exogenous IL-2 was necessary to develop CTL activity in vitro. Subsequently, IL-2 (5 ng/ml) was introduced to all cocultures at day 3 and replenished at restimulation (day 7) and at subsequent refeeding (day 10).

At limiting dilutions of purified CD8+ T cells/well (Fig. 1A), the frequency of a positive response against allogeneic BLC stimulators conformed to "single hit" and suggested an allogeneic precursor CTL frequency ranging from 1/8,000 to 1/80,000 in different experiments. In contrast, the frequency of a positive response against allogeneic EC stimulators was very low and generally did not fulfill the criteria for "single hit" interactions. In the single instance when they did, limiting dilution analysis suggested very low precursor frequencies of <1/500,000.

At input cell numbers of between 0.5 and 1 × 10^5 CD8+ T cells/well, the majority of replicate microcultures stimulated with allogeneic BLC developed significant cytotoxicity against the stimulator cell line, whereas most of the microcultures stimulated with EC were negative (Fig. 1B). However, at intermediate input cell numbers (e.g., at 2 × 10^5 CD8+ T cells/well) both anti-BLC and anti-EC CTL could be reproducibly generated. Such anti-EC
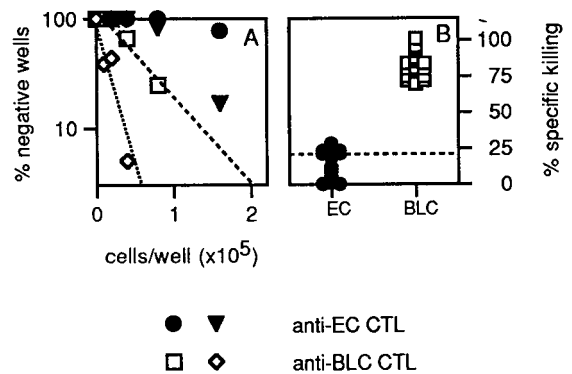

FIGURE 1. Comparison of the allogeneic CTL response to EC vs BLC at low CD8+ T cell input numbers. CD8+ T cells were cocultured with EC or BLC for 2 wk in microculture replicates. The resulting CTL populations were tested for cytolytic activity in a fluorescence release assay. Each individual microculture was scored as positive (test result > spontaneous release + 3 SD) or negative. *A*, CTL were grown at limiting dilutions in the presence of EC or BLC as stimulators. After 2 wk, the effector cells were tested for cytotoxicity against the stimulator EC or BLC line. Regression curves for 2 typical anti-BLC CTL cultures (dashed, dotted lines) conforming to "single hit" criteria (*p* > 0.05) are shown, suggesting precursor frequencies of 1 in 16,000 (95% confidence interval: 1 in 12–24,000) and 1 in 79,000 (95% confidence interval: 1 in 58–122,000). Anti-EC CTL cultures did not typically fulfill "single hit" criteria (*p* < 0.002). *B*, Percent specific killing by CTL microcultures (*n* = 18) generated at low input cell number of 10^5 CD8+ T cells/well in a microculture system against EC or BLC.

CTL displayed allospecificity, i.e., anti-EC CTL grown and tested under these conditions killed the stimulator with much higher frequency than they killed an unrelated third party EC line (Fig. 2*A*). This degree of allospecificity in such anti-EC cultures was comparable to that measured with anti-BLC CTL (Fig. 2*B*).

At high input CD8+ T cell numbers (4 × 10^6), i.e., in a bulk culture system, EC stimulated the differentiation of unrestricted CTL that showed no specificity for the stimulator line (Fig. 3*A*).
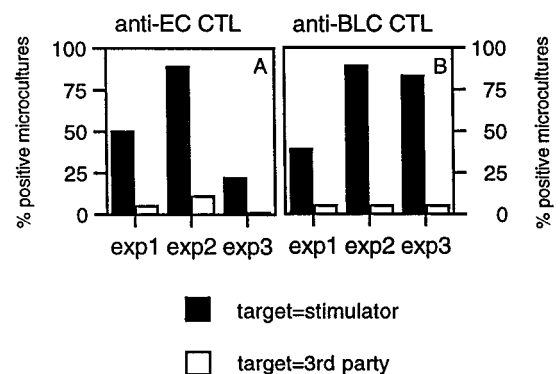

FIGURE 2. Comparison of the allogeneic CTL response to EC vs BLC at intermediate CD8+ T cell input numbers. CD8+ T cells were cocultured with EC or BLC for 2 wk in microculture replicates. The resulting CTL populations were tested for cytolytic activity in a fluorescence release assay. Each individual microculture was scored as positive (test result > spontaneous release + 3 SD) or negative. *A*, Percent positive microcultures when anti-EC CTL were grown in microculture replicates (input cell number 2 × 10^5 CD8+ T cells/well) and were tested against the stimulator or a third-party EC line. *B*, Percent positive microcultures when anti-BLC CTL were grown in microculture replicates (input cell number 0.5 × 10^5 CD8+ T cells/well) and were tested against the stimulator or a third-party BLC line.
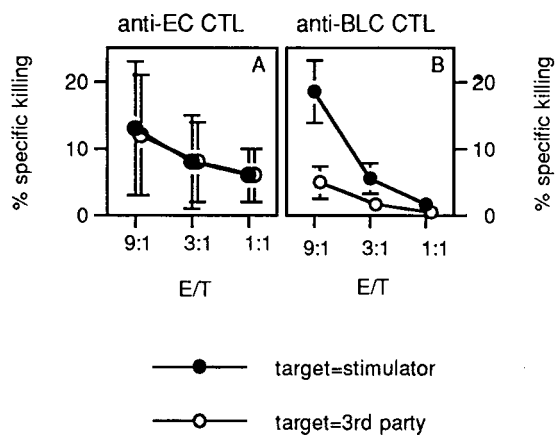
FIGURE 3. Comparison of the allogeneic CTL response to EC vs BLC at high CD8$^+$ T cell input numbers. CD8$^+$ T cells were cocultured with EC or BLC for 2 wk in bulk cultures (24-well plates). The resulting CTL populations were tested for cytolytic activity in a fluorescence release assay. The effector cells were counted and added to target cells at the indicated E:T ratio. *A*, Percent specific killing when anti-EC CTL from bulk cultures (input cell number 4 $\times$ 10^6 CD8$^+$ T cells/well) were tested for cytotoxicity against the stimulator or a third-party EC line. Values are mean $\pm$ SD from four independent experiments performed with cells from two different donor pairs. *B*, Percent specific killing when anti-BLC CTL from bulk cultures (input cell number 4 $\times$ 10^6 CD8$^+$ T cells/well) were tested for cytotoxicity against the stimulator or a third-party BLC line. Values are mean $\pm$ SD from four independent experiments performed with cells from two different donor pairs.

Among different experiments, the same CD8$^+$ T cell responder/EC stimulator combination led to results that were highly variable and unpredictable when assayed at 2 wk. Longer-term bulk cultures (i.e., greater than 2 wk) of CD8$^+$ T cells stimulated by allogeneic EC frequently stopped growing or lost cytotoxic activity, or both. Furthermore, the cytotoxicity of anti-EC bulk cultures did not appropriately titer with varying effector number and sometimes actually decreased at increasing E/T ratios. The inclusion of mAbs to CD8, which strongly inhibits killing by long-term anti-BLC CTL lines (data not shown), actually enhanced killing by anti-EC CTL and revealed titerable effector cells (Fig. 4*A*). BLC, on the other hand, routinely stimulated titerable allospecific CTL responses under the identical bulk culture conditions (Fig. 3*B*). Such CD8$^+$ CTL lines could be stably propagated for several weeks, often increasing in potency (data not shown).

Microcultures of anti-EC CTL, grown at intermediate density and which individually displayed allospecificity, lost significant killing when pooled. Like anti-EC bulk cultures, these pooled anti-EC CTL also failed to titer, i.e., they did not show significant killing even at high E:T ratios (Fig. 4*B*). In contrast, anti-BLC CTL from pooled microcultures continued to kill significantly, consistent with the behavior of bulk cultures of anti-BLC CTL. This result suggests that EC but not BLC may stimulate a CD8$^+$ "suppressor" population, although we have not shown that these regulatory T cells are alloantigen or Id specific.

EC stimulate conventional, class I MHC-restricted CTL differentiation

In the experiments described above, we identified a set of conditions involving an intermediate number of input preCTL, which led to the differentiation of allospecific anti-EC CTL responses. We proceeded to characterize these effector cells more fully. To determine whether these allospecific cultures displayed class I MHC restriction, we compared killing of third party EC lines shar-
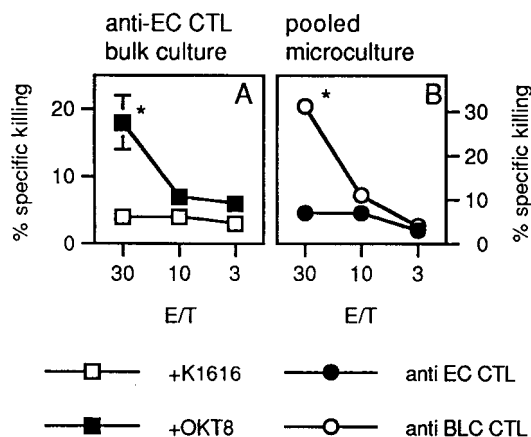

FIGURE 4. Atypical behavior of anti-EC CTL. *A*, CD8$^+$ T cells were cocultured with EC for 2 wk in bulk cultures (24-well plates, input cell number 1 $\times$ 10^6 cells/well). Anti-EC CTL were tested for cytotoxicity against the stimulator EC line at the indicated E:T ratio in the presence of a control Ab (K1616) or an anti-CD8 Ab. Shown are mean $\pm$ SEM of nine independent experiments with cells from six different donors. *, Significantly different from the cytotoxicity in the presence of control Abs ($p <$ 0.05). *B*, CD8$^+$ T cells were cocultured with EC (input cell number 2 $\times$ 10^5 CD8$^+$ T cells/well) or BLC (input cell number 0.5 $\times$ 10^5 CD8$^+$ T cells/well) for 2 wk in microculture replicates. Before testing of cytotoxicity vs EC or BLC, respectively, the effector cells were pooled, counted, and added to the target cells at the indicated E:T ratio. Pecent specific killing is expressed as mean $\pm$ SEM of four independent experiments with cells from four different donors. *, Significantly different from the cytotoxicity of pooled anti-EC CTL ($p <$ 0.05).

ing *HLA-A* or *-B* alleles with the stimulator cell line with third party cells sharing no *HLA-A* or *-B* alleles. As may be seen in Figure 5, cross-reactivity was detectable only in cultures that share class I alleles, indicative of MHC restriction.

We next analyzed the phenotype of the anti-EC CTL. The purified T cells used in this study were >96% CD8$^+$ and almost uniformly perforin negative at the onset of the coculture. After 2 wk, the vast majority of lymphoid cells continued to express CD8. However, nearly 20% of these CD8$^+$ anti-EC CTL now expressed perforin, slightly fewer than the percentage of cells in anti-BLC CTL cultures that expressed this cytolytic molecule (Figs. 6, *B* and *D* and 7*C*). Perforin appeared to be relevant for CTL functions
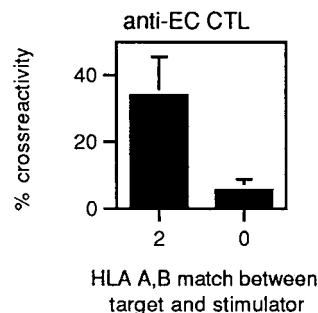

FIGURE 5. Allospecific anti-EC CTL show class I MHC restriction. CD8$^+$ T cells were cocultured with allogeneic EC in microculture replicates. After 2 wk, the CTL were tested for cytotoxicity against the stimulator or a third-party EC line with complete *HLA-A* or *-B* mismatch or sharing two *HLA-A* or *-B* alleles. Percent cross-reactivity is expressed as (% positive microcultures against third party EC line/% positive microcultures against stimulator EC line) $\times$ 100%. Values are mean $\pm$ SEM from four independent experiments with cells from eight different donors ($p <$ 0.05).

FIGURE 6. Antiendothelial CTL are CD8$^+$ and express perforin. *A* to *D*, CD8$^+$ T cells were cocultured with EC or BLC for 2 wk in microculture replicates. Small aliquots of effector cells were removed before the cytotoxicity assay was performed and stained for CD8 (Texas red) and perforin (Cy2). Morphology (phase contrast) and immune phenotype of anti-EC (*A* and *B*) and anti-BLC (*C* and *D*) CTL are shown. *E* and *F*, When anti-BLC CTL were grown for 2 wk in the upper compartment of a Transwell in the presence of EC in the lower compartment, perforin expression was strongly suppressed. Arrow, detached surviving EC.
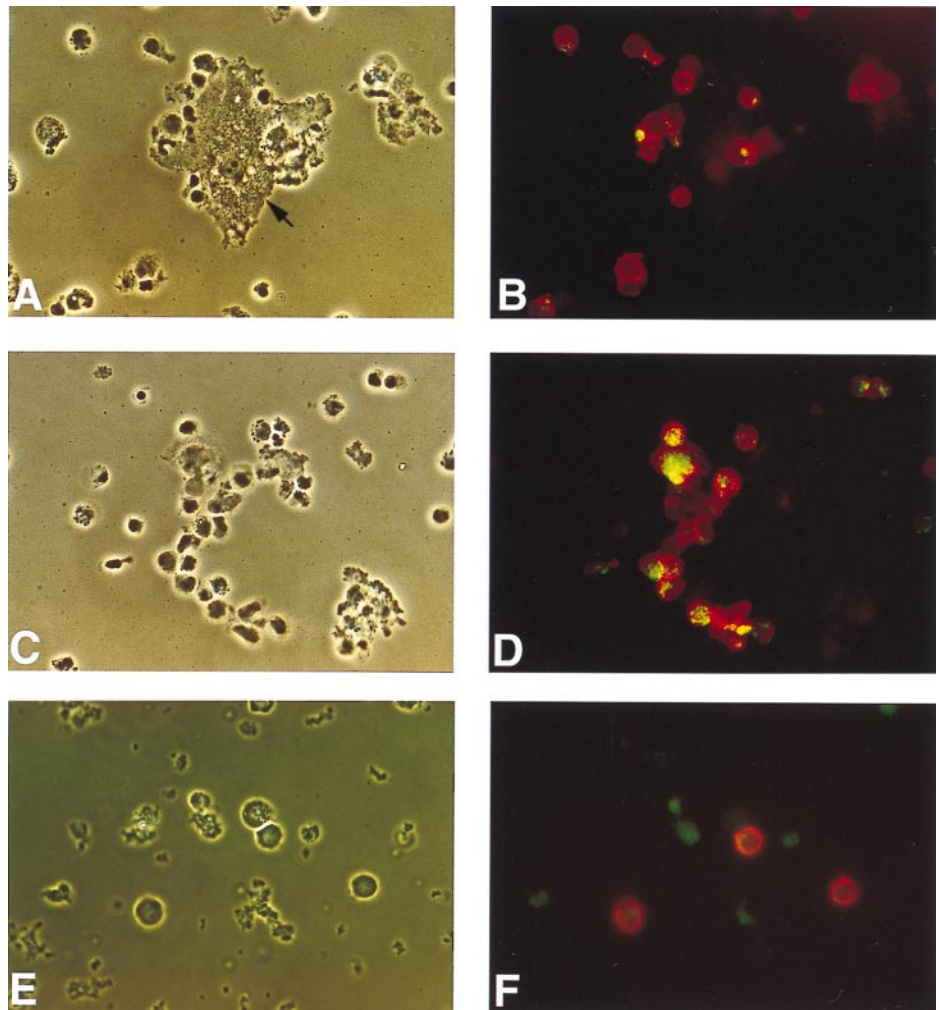
because cytotoxicity against the stimulator cell line was never observed in cultures that lacked perforin expression.

EC are inefficient stimulators of CTL growth and IFN-γ secretion

Although EC could stimulate allospecific CTL differentiation, they appeared to be quantitatively less efficient stimulator cells than BLC from the same individual donor (Fig. 1*B*). We further analyzed differences between the capacities of EC and BLC by assessing several other parameters of the CD8$^+$ T cell response (Fig. 7). CD8$^+$ T cell growth was consistently less when stimulated with allogeneic EC than with BLC (Fig. 7*A*). Growth in both cases was absolutely dependent upon exogenous IL-2. However, IL-2 alone in the absence of BLC or EC was unable to support CD8$^+$ T cell proliferation or even survival, because the T cell number fell precipitously by 2 wk to 4 ± 2% of input cells. Furthermore, growth differences between cultures stimulated by BLC or by EC did not correlate with the fraction of IL-2Rα (CD25) expressing cells, which were 21 ± 6% and 19 ± 8%, respectively.

Differences between EC and BLC were even more pronounced when IFN-γ secretion was assayed. In the 7 day coculture supernatant, IFN-γ was not detectable in cocultures with EC but reproducibly reached over 100 pg/ml when BLC were used as stimulators (Fig. 7*B*). At this point, cell numbers in the microcultures grown against EC and against BLC were still comparable, and IFN-γ levels were not adjusted to CTL number.

We attempted to improve the efficiency with which EC stimulate CTL differentiation and IFN-γ production by various manipula-

tions. First, BLC are known to produce IL-12, a 70-kDa heterodimeric glycoprotein originally identified as differentiation factor for CTL and NK cells that increases IFN-γ secretion (25, 26). Therefore, we added rIL-12 to the culture medium to determine whether this manipulation would promote anti-EC CTL differentiation. As shown in Figure 8, IL-12 actually reduced the frequency of CTL development, although IL-12, as expected, increased IFN-γ secretion in the same cultures (data not shown). Variations in the timing or concentration of IL-12 addition did not improve the efficacy of this cytokine in our system. Second, BLC are also known to express high levels of class I MHC molecules, which might also influence the outcome of the cocultures. Therefore, we pretreated the EC for 24 h with IFN-γ (27) before the CD8$^+$ T cells were added, a manipulation that increased class I MHC expression six- to sevenfold (data not shown). However, this approach also failed to increase the frequency of CTL differentiation by EC (Fig. 8).

EC stimulate anti-EC-specific CTL and regulate the anti-BLC response

The observation that EC are less efficient than BLC as stimulators of allospecific CTL responses could either arise from a deficiency of activating signals or from provision of inhibitory signals or both. To address this question, we performed a mixing experiment by culturing CD8$^+$ T cells with EC + BLC and compared the results to cultures stimulated with EC or BLC alone. Cell growth, IFN-γ secretion during the culture period, and percent perforin-positive cells was similar for CD8$^+$ T cells cocultured with EC or
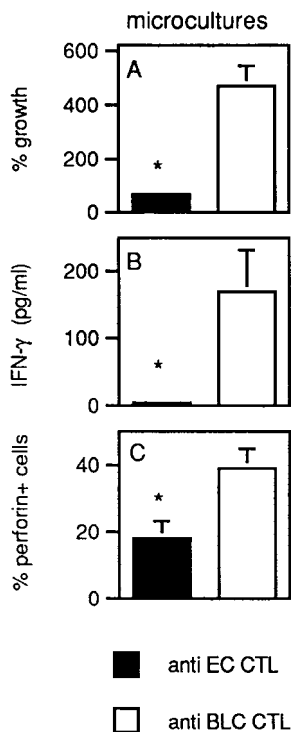
FIGURE 7. Cell growth, IFN-γ secretion during the coculture, and perforin expression in anti-EC and anti-BLC CTLs. CD8^{+} T cells were cocultured with EC or BLC for 2 wk in microculture replicates. *A*, After 2 wk, effector cells were counted, cell counts were compared with input cell numbers, and percent cell growth calculated. Values are mean ± SEM from 11 independent experiments with cells from nine different donors. *, Significantly different from cultures with BLC as stimulators ($p < 0.001$). *B*, After 1 wk in culture, supernatant was removed and pooled, and IFN-γ concentration was measured by ELISA. Values are mean ± SEM from eight independent experiments with cells from seven different donors. *, Significantly different from cultures with BLC as stimulators ($p < 0.05$). *C*, After 2 wk, a small aliquot of CTL was double stained for CD8 and perforin. Double positive cells were counted and normalized to the total number of CD8^{+} cells. Values are mean ± SEM from seven independent experiments with cells from six different donors. *, Significantly different from cultures with BLC as stimulators ($p < 0.05$).

FIGURE 8. Effects of IL-12 addition to the cocultures or induction of increased MHC molecule expression on EC on the efficiency of EC as stimulators of CTLs. CD8^{+} T cells were cultured with EC for 2 wk in microculture replicates. After 2 wk, the resulting CTL populations were tested for cytolytic activity against the stimulator cell line in a fluorescence release assay. Each individual microculture was scored as positive (test result > spontaneous release + 3 SD) or negative and the result expressed as percent positive microcultures. Control microcultures ($n = 18$) were grown in the presence of IL-2 (5 ng/ml) only. Eighteen replicate cultures were grown in the presence of IL-12 (5 ng/ml), cytokine that is known to induce CTL differentiation. In 18 replicate cultures, EC were pretreated with IFN-γ (1000 U/ml) for 24 h before the coculture was initiated to increase class I MHC expression. Shown are the results of one of four experiments with similar outcomes. Dashed line, spontaneous release + 3 SD.

ence may reflect the reduced number of effector cells generated in cultures containing EC. The most consistent observation was that CTL produced less IFN-γ when cultured overnight with EC compared with culture with BLC, irrespective of the stimulator cell type (EC, BLC, or EC + BLC) used to generate the CTL (Fig. 9, *D–F*, dark bars).

To determine whether the failure to detect anti-BLC CTL in mixed cocultures resulted from the generation of suppressor cells by EC + BLC stimulators, we mixed CD8^{+} CTL stimulated by EC + BLC with those stimulated by BLC alone in the effector phase. In this case, anti-EC CTL did not inhibit the cytotoxic activity of anti-BLC CTL when they were pooled and tested on BLC targets (data not shown). This experiment suggests that EC-mediated inhibition of the anti-BLC response appears to occur during the differentiation of CTL from preCTL and not during the CTL assay.

Analysis of the inhibitory effect of EC upon generation of anti-BLC CTLs

In a final series of experiments, we analyzed the basis of the inhibitory effects mediated by EC on anti-BLC CTL differentiation. To determine whether cell contact was required for EC to inhibit the development of anti-BLC CTL we used a Transwell system. When anti-BLC CTL were grown in the upper compartment of a Transwell, the presence of EC in the lower compartment strongly reduced CTL growth (Fig. 10*A*). The resulting anti-BLC CTL expressed little perforin (Fig. 6*F*), were essentially unable to kill BLC targets (Fig. 10*B*), and produced lesser amounts of IFN-γ during the effector phase (Fig. 10*C*) compared with control anti-BLC CTL generated in the absence of EC. Fixing the EC in the lower compartment with 1% paraformaldehyde before the initiation of the Transwell coculture markedly reduced the extent of growth inhibition, and the resulting anti-BLC CTL secreted more IFN-γ during the effector phase and were as effective killers as the control CTL grown in the absence of EC (data not shown). The

EC + BLC, and all of these parameters were dramatically less than in cultures of CD8^{+} T cells cocultured with BLC (Table I). Consistent with our previous results, EC were less efficient than BLC as stimulators of CTL in these experiments (compare Fig. 9, *A* (dark bars) and *B* (open bars), respectively). The frequency of microcultures with CTL activity stimulated by EC + BLC was similar to that using EC stimulators alone. While anti-BLC CTL generally lysed both BLC and EC, anti-EC CTL appeared specific for EC and often did not kill BLC from the same donor. CTL from the mixed cocultures, grown in the presence of EC and BLC, were also able to lyse EC and failed to kill BLC (Fig. 9*C*). In other words, in the mixed cocultures EC appear to inhibit the anti-BLC CTL response while simultaneously stimulating an anti-EC-specific response.

We also measured IFN-γ secretion during the effector phase, i.e., in the supernatants of the CTL assay, using CTL generated by EC stimulators, by BLC stimulators, or by EC + BLC stimulators. Anti-BLC CTL produced significant amounts of IFN-γ when cultured overnight with BLC (Fig. 9*E*, open bar). CTL grown in the presence of EC or EC + BLC produced less IFN-γ when cultured overnight with BLC (Fig. 9, *D* and *F*, open bars), but this differ-
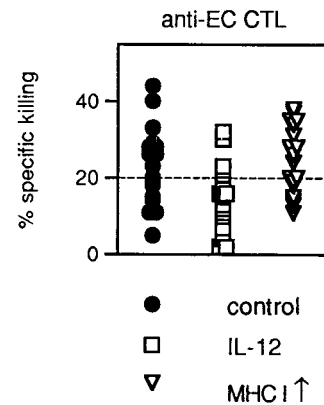
Table I. *CTL grown in the presence of EC, BLC, or EC + BLC*[a]

Stimulating Cell	EC	BLC	EC + BLC
Anti-EC cytotoxicity (% positive microcultures)[b]	16 ± 9^c	63 ± 17	18 ± 14^c
Anti-BLC cytotoxicity (% positive microcultures)[b]	3 ± 2^c	71 ± 13	0 ± 0^c
Cell growth (% initial cell count)[d]	68 ± 10^c	468 ± 76	92 ± 16^c
IFN-γ (pg/ml)[e]	0 ± 0^c	168 ± 62	14 ± 5^c
Percent perforin/CD8 positive cells[f]	18 ± 5^c	39 ± 6	22 ± 5^c

[a] CD8^{+} T cells were cocultured with EC or BLC for 2 wk in microculture replicates. IFN-γ was measured in culture supernatant after 7 days. Cytotoxicity, cell growth, and perforin expression were determined after 2 wks. Shown are mean ± SEM.
[b] Seven experiments, eight donors.
[c] Significantly different (p < 0.05) from BLC.
[d] Eleven experiments, nine donors.
[e] Eight experiments, seven donors.
[f] Seven experiments, six donors.

addition of fixed EC into mixed cocultures with CD8^{+} T cells and BLC also failed to inhibit CTL differentiation (data not shown). In other words, metabolically active EC are required to inhibit anti-BLC CTL differentiation and growth, and this inhibition is independent of EC-T cell contact.

Experiments transferring EC-conditioned medium to anti-BLC CTL cultures suggested that EC may secrete an inhibitor of CTL differentiation, but even repetitive addition of conditioned medium over the 2-wk culture period reduced killing by about 30% (data not shown) compared with much stronger inhibition observed by EC across Transwells. Because EC appeared to inhibit growth of

anti-BLC CTL (Fig. 11A) as well as function, we attempted to demonstrate the presence of a soluble inhibitor in a simpler, short-term assay. We found that CD8^{+} T cell proliferation to BLC was detectable on day 3 and peaked at day 4 of culture. Three-day EC-conditioned medium was tested for its effects on CD8^{+} T cell proliferation to BLC. As shown in Figure 11B, such EC-conditioned medium inhibited allostimulated CD8^{+} T cell proliferation by 30% when added to CD8^{+} T cell/BLC cocultures. In contrast,
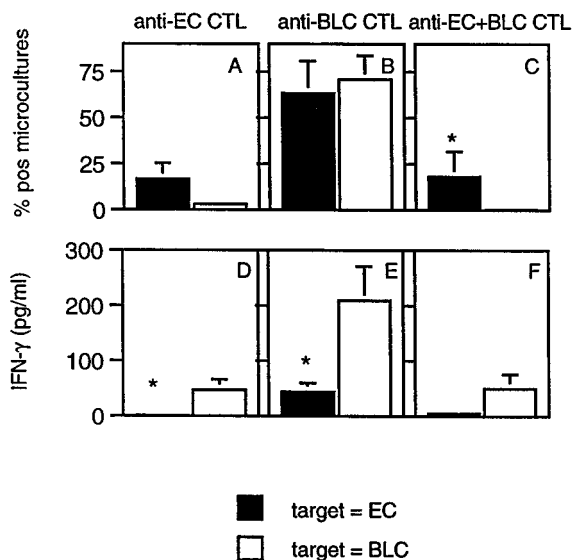

FIGURE 9. EC regulate CTL differentiation and inhibit production of IFN-γ by CTLs. CD8^{+} T cells were cocultured with EC, BLC, or EC + BLC for 2 wk in microculture replicates. After 2 wk, the resulting CTL populations were tested for cytolytic activity against EC or BLC in a fluorescence release assay. Each individual microculture was scored as positive (test result > spontaneous release + 3 SD) or negative and the result expressed as percent positive microcultures. CTL that were grown in the presence of allogeneic EC (A), BLC (B), or EC + BLC (C) in microculture replicates were tested for cytotoxicity against EC or BLC from the same donor. Values are mean ± SEM from seven independent experiments with cells from eight different donors. IFN-γ was measured in the 24-h cell culture supernatants from the CTL assay. The effector cells originally stimulated by EC (D), BLC (E), or EC + BLC (F), which were tested for cytotoxicity against EC or BLC, continued to secrete cytokines overnight. Values are mean ± SEM from five independent experiments with cells from seven different donors. *, Significantly different from the effect against BLC (p < 0.05).
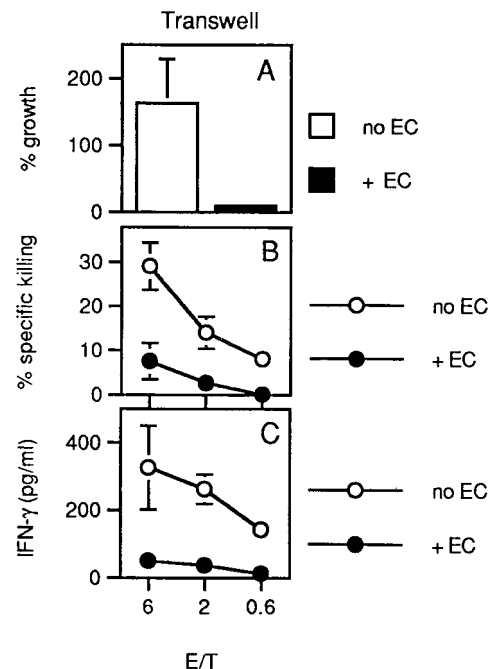

FIGURE 10. Inhibition of CTL differentiation and growth by EC is independent of cell contact. 10[6] CD8^{+} T cells were cocultured with allogeneic BLC in the upper compartment of a Transwell tissue culture insert for 2 wk. EC were either present or absent in the lower compartment of the Transwell system. A, After 2 wk, anti-BLC CTL in the upper compartment were counted and cell counts were compared with input cell number and expressed as percent growth in the absence or presence of EC cultured in the lower chamber. Shown are mean ± SD from five independent experiments using cells from five different donors. B, Anti-BLC CTL grown in the absence or in the presence of EC in the lower chamber were added at the indicated E:T ratios to BLC as target cells and the results expressed as percent specific killing. Shown are mean ± SEM from five independent experiments with cells from five different donors. C, IFN-γ was measured in the 24-h cell culture supernatants from the CTL assay described in B. Shown are mean ± SEM values from three experiments with cells from four different donors.
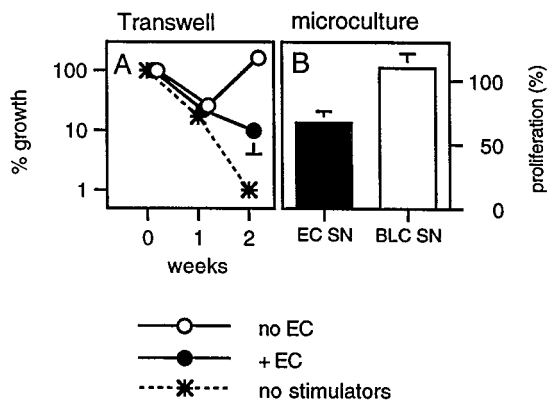
FIGURE 11. EC-conditioned medium inhibits CTL proliferation. *A*, A total of 10^6 CD8$^+$ T cells were cocultured with allogeneic BLC in the upper compartment of a Transwell tissue culture insert for 2 wk. EC were either present or absent in the lower compartment of the Transwell system. Growth of anti-BLC CTL in the absence or presence of EC in the lower chamber of a Transwell insert was quantified by cell counting, normalized to the initial cell number, and expressed as percent growth. Stars, number of surviving CD8$^+$ T cells incubated without stimulator cells. Shown are mean ± SD of five experiments with cells from four different donors. *B*, Conditioned medium from EC and BLC cultures was harvested after 3 days, aliquoted, and frozen at −70°. CD8$^+$ T cells (10^5 cells/well) were grown with BLC in microculture replicates and the culture medium mixed 1:1 with conditioned medium from EC or BLC. After 78 h, [^{3}H]thymidine (1 μCi/well) was added to the cultures and incorporation measured after an additional 18 h. Results are expressed as percent of cpm of control cultures incubated with culture medium mixed 1:1 with mock-conditioned medium incubated in the absence of cells. Shown are mean ± SD from three experiments, each performed in quadruplicate.

conditioned medium from BLC slightly enhanced T cell proliferation under the same conditions. This degree of growth inhibition, if sustained, could explain the profound inhibitory effects on growth found in the Transwell experiments (Fig. 11*A*), where a 10-fold difference in cell numbers was observed after 2 wk. The inhibitory activity produced by EC does not appear to be TGF-β-mediated because neutralizing Abs reacting with this molecule did not prevent EC-mediated inhibition in coculture experiments with mixed stimulators (EC + BLC), nor did it boost the anti-EC CTL response (data not shown). We also ruled out NO and prostaglandin as potential candidates for EC inhibitory activity because neither L-NAME (5 mM) nor indomethacin (5 μM) were able to reduce the level of inhibition of anti-BLC differentiation produced by EC in a Transwell system.

Discussion

Host CTL are major effectors and graft EC are important target cells of acute allograft rejection (1, 2, 28, 29). Therefore, we studied the capacity of EC to induce allospecific CTL. Our studies have led to three major conclusions. First, we find that EC are sufficient to stimulate the differentiation of preCTL into functional, allogeneic, class I MHC-restricted CD8$^+$ and perforin-positive CTL. This result is a confirmation and extension of the previous results of Pardi and Bender (22). Such anti-EC CTL cultures are able to lyse stimulator EC but not BLC from the same donor, whereas anti-BLC CTL lyse both cell types. These in vitro data are consistent with the recovery of EC-specific or selective CTL from rejecting human allografts. We do not know the basis of the specificity or selectivity for EC targets. It is not likely to be increased sensitivity of EC to killing because anti-BLC CTL show no such preference. Rather, it is likely to reside in the target Ag (e.g., an

EC-specific protein-derived peptide) or in an unusual requirement of such CTL for signals from an EC-specific accessory molecule during the killing phase. Because our CTL cultures are not pure, it will be necessary to clone these cells before further characterization is possible.

The second major conclusion from our study is that EC are inefficient stimulators of CTL compared with BLC. This conclusion was suggested by the limiting dilution experiments and from the quantitative comparison of positive microcultures at low input cell numbers. In general, EC are less efficient than BLC at promoting CD8$^+$ T cell growth, IFN-γ secretion, and acquisition of cytolytic functions, but equally effective at inducing IL-2Rα (CD25) expression (16). The failure to observe "single hit" responses at low input cell number could imply either that additional stimulator signals were needed (e.g., because of lack of costimulators) or that responder cells needed to interact (e.g., by provision of stimulatory cytokines) (30). Four manipulations, namely addition of exogenous IL-12, up-regulation of class I MHC molecule expression, neutralization of TGF-β, or addition of BLC, did not appear to help. However, in a pilot experiment, inclusion of irradiated PBMC feeder cells from the responder did allow input CD8$^+$ T cell differentiation to conform to a "single hit" response, supporting the notion of missing or inadequate cytokines.

The third conclusion is that EC regulate CTL responses. This result comes both from the bulk culture experiments and from the mixed stimulator, the Transwell, and the conditioned medium experiments. The bulk culture experiments suggest that EC induce regulatory T cells that inhibit allospecific killing and/or promote allo-unrestricted killing. This observation may explain the previous results of Clayberger et al. (21). Further evidence for such regulatory T cells is provided by the observation that anti-CD8 mAbs increase the potency of anti-EC bulk cultures and that mixing of anti-EC CTL microcultures does not result in high titer allospecific killing. Such regulatory T cells may be "suppressor" cells that act in the effector phase, but we have no evidence that they are alloantigen or Id specific. The observation that anti-CD8 mAbs increase killing also implies that it does not block killing by anti-EC CTL.

The mixed stimulator, conditioned media, and Transwell experiments all point to the fact that EC inhibit the ability of BLC to stimulate differentiation of preCTL into CTL by elaboration of a stable secretory activity present in EC-conditioned medium. We do not know if this activity is a single factor or even if it is a protein, but Ab neutralization suggests that it is not TGF-β1, a well-characterized immunosuppressant made by cultured EC (31). EC-conditioned medium is much less efficient than EC in Transwell cultures, suggesting that the inhibitory activity has a relatively short half-life. We ruled out both NO and prostaglandins as candidate molecules for this inhibitor. Anti-EC CTL emerge in settings where EC completely suppress the anti-BLC response, e.g., in the mixed-stimulator experiments. This observation further indicates that anti-EC CTL may differ from anti-BLC CTL by more than simply Ag specificity, i.e., EC-specific CTL may not be inhibited by the EC-secreted activity. Alternatively, it may suggest that EC provide costimulation that overcomes the inhibitory effect of this suppressor molecule but are unable to do so in "trans", i.e., when BLC are stimulating the TCRs of the responding CD8$^+$ T cells.

Our findings have two potentially important implications for acute allograft rejection. First, they suggest that the generation of anti-EC CTL may differ from the generation of CTL reactive with passenger leukocytes. If such anti-EC CTL mediate acute vascular rejection in vivo, a lesion that often portends a therapy-resistant form of graft rejection (29) remains to be shown. If the development of anti-EC CTL is, in fact, the harbinger of therapy-resistant

rejection, then inhibition of this response may require alternative therapies, potentially testable in our in vitro model. Second, the capacity of EC to suppress conventional CTL differentiation may point to a natural EC-derived immunosuppressant that can be added to the armamentarium of antirejection therapy.

Acknowledgments

References

1. Meehan, S. M., R. T. McCluskey, M. Pascual, F. I. Preffer, P. Anderson, S. F. Schlossman, and R. B. Colvin. 1997. Cytotoxicity and apoptosis in human renal allografts: identification, distribution and quantitation of cells with a cytotoxic granule protein GMP-17 (TIA-1) and cells with fragmented nuclear DNA. *Lab. Invest. 76:639.*

2. Strehlau, J., M. Pavlakis, M. Lipman, M. Shapiro, L. Vasconcellos, W. Harmon, and T. B. Strom. 1997. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc. Natl. Acad. Sci. USA 94:695.*

3. Sherman, L. A., and S. Chattopadhyay. 1993. The molecular basis of allorecognition. *Annu. Rev. Immunol. 11:385.*

4. Smith, K. D., E. Huczko, V. H. Engelhard, Y. Y. Li, and C. T. Lutz. 1997. Alloreactive cytotoxic T lymphocytes focus on specific major histocompatibility complex-bound peptides. *Transplantation 64:351.*

5. Lu, P., J. A. Garcia-Sanz, M. G. Lichtenheld, and E. R. Podack. 1992. Perforin expression in human peripheral blood mononuclear cells. *J. Immunol. 148:3354.*

6. Kummer, J. A., A. M. Kamp, T. M. Tadema, W. Vos, J. L. M. Meijer, and C. E. Hack. 1995. Localization and identification of granzymes A and B-expressing cells in normal human lymphoid tissue and peripheral blood. *Clin. Exp. Immunol. 100:164.*

7. Berke, G. 1995. The CTL's kiss of death. *Cell 81:9.*

8. Henkart, P. A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity 1:343.*

9. Mescher, M. F. 1995. Molecular interactions in the activation of effector and precursor cytotoxic T lymphocyts. *Immunol. Rev. 146:177.*

10. Azuma, M., and L. L. Lanier. 1995. The role of CD28 costimulation in the generation of cytotoxic T lymphocytes. *Curr. Top. Microbiol. Immunol. 198:59.*

11. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*

12. Parra, E., A. G. Wingren, G. Hedlund, T. Kalland, and M. Dohlsten. 1997. The role of B7-1 and LFA-3 in costimulation of CD8+ T cells. *J. Immunol. 158:637.*

13. Stuart, F. P., T. Garrick, A. Holter, A. Lynch, and E. Bastien. 1971. Delayed rejection of renal allograft in the rat and dog by reduction of passenger leukocytes. *Surgery 70:128.*

14. Fabre, J. W. 1982. Rat kidney allograft model: was it all too good to be true? *Transplantation 34:223.*

15. Pober, J. S., C. G. Orosz, M. L. Rose, and C. O. S. Savage. 1996. Can graft endothelial cells initiate a host anti-graft immune response? *Transplantation 61: 343.*

16. Epperson, D. E., and J. S. Pober. 1994. Antigen-presenting function of human endothelial cells: direct activation of resting CD8 T cells. *J. Immunol. 153:5402.*

17. Adams, P. W., H. S. Lee, W. J. Waldman, D. D. Sedmak, C. J. Morgan, J. S. Ward, and C. G. Orosz. 1992. Alloantigenicity of human endothelial cells. *J. Immunol. 148:3753.*

18. Jutte, N. H. P. M., C. J. Knoop, P. Heijse, A. H. M. M. Balk, B. Mochtar, F. H. J. Claas, and W. Weimar. 1996. Human heart endothelial-cell-restricted allorecognition. *Transplantation 62:403.*

19. Jutte, N. H. P. M., C. J. Knoop, P. Heijse, A. H. M. M. Balk, B. Mochtar, F. H. J. Claas, and W. Weimar. 1997. Cytotoxicity of graft-derived lymphocytes: specific for donor heart endothelial cells? *J. Heart Lung Transplant. 16:209.*

20. Marelli-Berg, F. M., R. E. G. Hargreaves, P. Carmichael, A. Dorling, G. Lombardi, and R. I. Lechler. 1996. Major histocompatibility complex class II-expressing endothelial cells induce allospecific nonresponsiveness in naive T cells. *J. Exp. Med. 183:1603.*

21. Clayberger, C., T. Uyehara, B. Hardy, J. Eaton, M. Karasek, and A. M. Krensky. 1985. Target specificity and cell surface structures involved in the human cytolytic T lymphocyte response to endothelial cells. *J. Immunol. 135:12.*

22. Pardi, R., and J. R. Bender. 1991. Signal requirements for the generation of CD4+ and CD8+ T cell responses to human allogeneic microvascular endothelium. *Circ. Res. 69:1269.*

23. Tosato, G. 1994. Generation of Epstein-Barr-Virus (EBV)- immortalized B cell lines. In *Current Protocols in Immunology.* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. John Wiley & Sons, Inc., New York, Vol. 7, pp. 22.1–22.3.

24. Lichtenfels, R., W. E. Biddison, H. Schulz, A. B. Vogt, and R. Martin. 1994. CARE-LASS (calcein-release assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. *J. Immunol. Methods 172:227.*

25. Sartori, A., X. Ma, G. Gri, L. Showe, D. Benjamin, and G. Trinchieri. 1997. Interleukin-12: an immunoregulatory cytokine produced by B cells and antigen-presenting cells. *Methods 11:116.*

26. Mehrotra, P. T., D. Wu, J. A. Crim, H. S. Mostowski, and J. P. Siegel. 1993. Effects of IL-12 on the generation of cytotoxic activity in human CD8+ T lymphocytes. *J. Immunol. 151:2444.*

27. Johnson, D. R., and J. S. Pober. 1990. Tumor necrosis factor and immune interferon synergistically increase transcription of HLA class I heavy and light chain genes in vascular endothelium. *Proc. Natl. Acad. Sci. USA 87:5183.*

28. Solez, K., R. A. Axelsen, H. Benediktsson, J. F. Burdick, A. H. Cohen, R. B. Colvin, B. P. Croker, D. Droz, M. S. Dunnill, P. F. Halloran, et al. 1993. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int. 44:411.*

29. Colvin, R. B., A. H. Cohen, C. Saiontz, S. Bonsib, M. Buick, B. Burke, S. Carter, T. Cavallo, M. Haas, A. Lindblad, et al. 1997. Evaluation of pathologic criteria for acute renal allograft rejection: reproducibility, sensitivity and clinical correlation. *J. Am. Soc. Nephrol. 8:1930.*

30. Lefkovits, I., and H. Waldmann. 1979. *Limiting Dilution Analysis of Cells in the Immune System.* Cambridge University Press, Cambridge, U.K.

31. Hannan, R. L., S. Kourembanas, K. C., Flanders, S. J. Rogelji, A. B. Roberts, D. V. Faller, and M. Klagsbrunn. 1988. Endothelial cells synthesize basic fibroblast growth factor and transforming growth factor β. *Growth Factors 1:7.*