Human Vascular Endothelial Cells Favor Clonal Expansion of Unusual Alloreactive CTL

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Human Vascular Endothelial Cells Favor Clonal Expansion of Unusual Alloreactive CTL

Barbara C. Biedermann and Jordan S. Pober

We have shown previously that cultured HUVEC or mixtures of endothelial cells (EC) and B lymphoblastoid cells (BLC) induce the differentiation of purified CD8+ PBL into allospecific, class I MHC-restricted CTL that lyse EC, but not BLC autologous to EC. Furthermore, these EC-selective CTL lines secrete little IFN-γ after target cell contact. In the present study, we have analyzed these polyclonal populations at a single cell level by cloning at limiting dilution and propagating the resulting CTL clones in the absence of EC. Phenotypically stable, alloreactive EC-selective CTL preferentially emerge from cocultures in which EC or EC + BLC are the initial stimulating cell types compared with cocultures stimulated by BLC alone (p = 0.005). Compared with BLC-stimulated CTL, EC-stimulated CTL clones often fail to secrete IFN-γ after target cell contact (p = 0.0006) and constitutively express CD40 ligand (CD40L) at rest (p = 0.0006). The absence of IFN-γ secretion does not result from a switch to IL-4 secretion. The expression of CD40L inversely correlates with the secretion of IFN-γ after target cell contact (p = 0.0001), but correlations of CD40L expression and failure to secrete IFN-γ with EC-selective killing did not reach statistical significance. We conclude that in a microenvironment in which allogeneic EC are in close contact with infiltrating CD8+ T cells, such as within a graft arterial intima, CTL subsets may emerge that display EC selectivity or express CD40L and secrete little IFN-γ after Ag contact. The Journal of Immunology, 1999, 162: 7022–7030.

Materials and Methods

Cell isolation

PBMC were obtained from healthy volunteers by density-gradient centrifugation of leukapheresis products and stored in liquid nitrogen, as described previously (3). These populations were used to isolate responder cells for the CTL differentiation cultures as well as feeder cells for cloning at limiting dilution. CD8+ T lymphocytes were isolated by positive selection (2). In brief, Dynabeads (Dynal, Lake Success, NY) coated with an anti-CD8 mAb were incubated with the PBMC suspension to bind CD8+ T cells, which were subsequently EC contact to maintain their unusual phenotypes.

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3 Address correspondence and reprint requests to Dr. Jordan S. Pober, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Ave., New Haven, CT 06510. E-mail address: Jordan.Pober@Yale.edu
4 Abbreviations used in this paper: EC, endothelial cells; BLC, B lymphoblastoid cells; CD40L, CD40 ligand; FasL, Fas ligand.
Table 1. PCR product size, primer sequences, and annealing temperature for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full-Length Size (bp)</th>
<th>Competitor Size (bp)</th>
<th>Upper Primer (5')</th>
<th>Lower Primer (3')</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3e</td>
<td>820</td>
<td>730</td>
<td>CCCATGAAACAAAAAGATGCAG</td>
<td>GGTACCACGCGAGAACGGCGCAG</td>
<td>55</td>
</tr>
<tr>
<td>Perforin</td>
<td>770</td>
<td>670</td>
<td>GTCTGTCCTCCTCGGGCGCCT</td>
<td>GACAGTCCAGCAGCTCTCCA</td>
<td>59</td>
</tr>
<tr>
<td>FasL</td>
<td>830</td>
<td>740</td>
<td>ATGCACAGGACCCCTTCAATTA</td>
<td>CCGAAAACAGTCTGGAGATT</td>
<td>54</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>770</td>
<td>680</td>
<td>ACCTTTTTGCTTAATTCCT</td>
<td>TGATACACATAGCCTGGCC</td>
<td>47</td>
</tr>
<tr>
<td>CD40L</td>
<td>770</td>
<td>700</td>
<td>ATGATCGAAGACATACACACC</td>
<td>CCAAAAGCGTGAAGCCAGT</td>
<td>50</td>
</tr>
</tbody>
</table>

Bedford, MA) at a responder to stimulator cell ratio of 2.5–7.5. All cultures were maintained at 37°C in 5% CO2, room air. In each experiment, 18–30 microculture replicates per stimulator-responder combination were initiated. The medium for coculture consisted of RPMI 1640 supplemented with 10% human AB serum (Irvine Scientific, Santa Ana, CA), 2 mM l-glutamine, 100 μM penicillin, and 100 μg/ml streptomycin. The medium was supplemented with exogenous IL-2 (R&D Systems, Minneapolis, MN) on day 3 (final concentration: 5–10 ng/ml). On day 7, the medium was changed and the CTL were transferred to fresh stimulator cultures. Cultures were again fed with fresh medium plus IL-2 on day 10. On day 14, the CTL microcultures were tested for cytotoxicity against the stimulator cell type (EC or BLC, respectively) using a calcein fluorescence release assay (5), as described below. From each experiment, the three microcultures that displayed the highest level of cytotoxicity were chosen for cloning by limiting dilution followed by published protocols (6), (7), with minor modifications as follows. The lymphocytes were counted and suspended in complete cloning medium (RPMI 1640, 10% FCS, 2 mM l-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, 20 ng/ml IL-2, and 1 μg/ml PHA-L (Sigma) at 1000, 100, or 10 cells/ml). These cell suspensions were distributed to round-bottom 96-well plates (100 μl/well), resulting in input cell numbers of 100 (24 replicates), 10 (4 replicates), and 1 (8 replicates) per well. Each well was additionally supplemented with feeder cells consisting of 50,000 irradiated (30 Gy) PBMC autologous to the responder CD8+ T-cells and 2,000 mitomycin C-treated BLC autologous to the stimulator cell. In most experiments, EC-stimulated CTL were distributed into 96-well plates that also contained stimulator EC at subconfluent density (5–10,000 cells/well). On day 7, the clones were fed with the same medium, except that PHA was not included. Beginning on day 14, cultures were inspected daily for the presence of expanding clones. These were collected into 5 ml complete cloning medium plus fresh feeder cells and expanded in tissue culture flasks. The CTL clones were maintained in culture by repetitive weekly restimulations (0.5 × 10^6 CTL/ml complete cloning medium plus feeder cells). On day 21, the cloning plates were analyzed for the final number of expanded clones per dilution to assess cloning efficiency and conformance to “single hit” responses.

Cytotoxicity assay

Cytotoxicity by CTL was measured at least 7 days after the last restimulation with feeder cells and PHA by a calcein fluorescence release assay (5), as described (2). In brief, target cells were loaded with calcein-AM (Molecular Probes, Eugene, OH), washed, and incubated with effector CTL at titrated E:T ratios (30:1, 10:1, 3:1) for 4 h at 37°C. The supernatant was then harvested and calcein release was measured using a fluorescence plate reader (Cytofluor 2; PerSeptive 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 obtained by adding medium alone; maximal release was obtained by adding lysis buffer (50 mM sodium borate, 0.1% Triton X-100, pH 9).

Cytokine measurements

IFN-γ, TNF, and IL-4 were measured in the CTL assay supernatant that was collected 18–24 h after the cytotoxicity assay was started. After harvesting the supernatant to measure calcein release, medium was replaced (RPMI 1640, 10% human serum AB, without IL-2) and the cultures were further incubated at 37°C. The supernatant was collected from all E:T ratios tested per each individual clone, pooled, and stored frozen at −70°C. Cytokine concentration was determined by an ELISA using commercially available Ab pairs (monoclonal mouse anti-human IFN-γ (MAB285), monoclonal mouse anti-human TNF (MAB610), monoclonal mouse anti-human IL-4 (MAB604), biotinylated polyclonal goat anti-human IFN-γ (BAF285), biotinylated polyclonal goat anti-human TNF (BAF210), and biotinylated mouse anti-human IL-4 (BAF204), all from R&D Systems), according to the manufacturer’s instructions.

Immunophenotyping of the CTL clones

CTL were collected for immunophenotyping at least 7 days after restimulation with feeder cells and PHA. CTL were either fixed with paraformaldehyde, spun onto gelatin-coated slides, permeabilized, and double stained for CD8 and perforin as described previously (2), or processed unfixed for FACS analysis. In the latter case, 50,000 CTL/sample were washed once with ice-cold PBS/1% BSA and incubated with saturating concentrations of directly FITC- or PE-conjugated mouse anti-human CD8, CD3, CD25, CD28 (all from Coulter Immunotech, Miami, FL), or biotinylated mouse anti-human TNF (MAB610), biotinylated polyclonal goat anti-human TNF (BAF210), and biotinylated mouse anti-human IL-4 (BAF204), all from R&D Systems).

Quantitative competitive RT-PCR

Total RNA was isolated from 5 × 10^9 resting CTL using a guanidinium isothiocyanate-based RNA isolation kit (RNaseasy mini kit; Quagen, Santa Clara, CA), according to the manufacturer’s instructions. A total of 2 μg of total RNA (final volume: 20 μl) was suspended in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 20 mM DTT, 16.5 μg/ml oligo(dT)15 (Promega) for 30 min at 30°C, heated to 95°C, and allowed to cool slowly to 4ºC. A total of 2 μl of 20X RT reaction mix was added to a total volume of 50 μl containing 0.5 mM dNTP (New England Biolabs, Beverly, MA), and 40 U RNasin (Promega, Madison, WI), and reverse transcribed with 200 U Superscript (Life Technologies) for 60 min at 45°C. After 15 min of heat inactivation at 70°C, the reaction tubes were incubated for 5 min on ice. A total of 3 U RNase H (Life Technologies) was added, and the reaction was incubated for 20 min at 37°C. A total of 80 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was added, and the samples were stored at 4°C and analyzed within 1 mo. The sequences for the genes of interest (CD3e, perforin, FasL, IFN-γ, and CD40L) were analyzed for primer annealing sites using the primer analysis software Oligo version 4.0 (National Biosciences, Plymouth, MN). To easily rule out amplification of genomic DNA, the 5’ and 3’ primer annealing sites were placed on two different exons of the gene. Table I shows the primer pairs (all 5’ to 3’ direction) that were used for PCR. All full-size templates could be amplified from cDNA obtained from PHA-stimulated PBMC, and competitor cDNA was shortened by 70–100 nucleotides applying rPCR. The competitor cDNA was amplified and the weight concentration was determined by comparison with the known amount of nucleotide size standard (Lambda DNA EII digest; New England Biolabs) on the same gel. For each competitor, a 100 pg/ml and a 100 fg/ml stock solution was prepared in TE buffer and stored at 4°C. The cDNA obtained from the CTL clones (1 μl) was mixed with the same volume of competitor cDNA, added at four different concentrations (10-fold dilutions). This template mix was amplified in a PCR reaction (final volume: 10 μl) containing 0.2 mM dNTP, 200 ng of each primer, and 10 μM Taq (Boehringer Mannheim, Indianapolis, IN) through the following protocol: 5-min initial denaturation at 95°C, then 35 cycles of denaturing 30 s at 95°C, annealing 30 s at the gene-specific annealing temperature, and elongating 1 min at 72°C, with incubating for 10 min at 72°C for final extension. The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV transillumination (Eagle Eye; Stratagene, La Jolla, CA). The resulting pictures were scanned (Scan Jet I1cx; Hewlett Packard, Bedford, MA), and the PCR amplified products were size-sequenced using the ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) with the appropriate dye-primers, and the unknown products were aligned with known nucleotide size standard (Lambda DNA EII digest; New England Biolabs) on the same gel. Each sample was run in duplicate with each primer pair, and primer and product sizes were compared using the BioNumerics Software (Applied Maths).
Palo Alto, CA), band intensities were quantified (National Institute of Health Image 1.61), and the competitor concentration of equivalent band intensity to the test samples was determined. This concentration was taken as the full-size cDNA concentration present in the sample. For each sample, the concentration of perforin, FasL, IFN-γ, and CD40L was normalized to the concentration of CD3ε (arbitrarily set to 100,000 U (8)).

TCRVβ determination

The TCRVβ profile of the clones was determined by RT-PCR, according to published methods applying a set of 22 Vβ family-specific primers (9). A total of 1 μl of cDNA was amplified in a PCR reaction (final volume: 10 μl) containing 0.2 mM dNTP, 250 nM of each primer, and 5 U/ml Taq (Boehringer Mannheim, Indianapolis, IN) through the following protocol: 5-min initial denaturation at 95°C, then 30 cycles of denaturing 30 s at 95°C, annealing 30 s at 55°C, and elongating 1 min at 72°C with final extension for 10 min at 72°C.

Statistical analysis

Data analysis of limiting dilution cloning was performed according to likelihood maximization using a computer program kindly provided by Dr. C. Orosz (Ohio State University, Columbus, OH). The outcome of various treatments between paired groups was tested for significant differences using the χ2 analysis. Results from different groups in multivariable experiments were compared by ANOVA.

Results

Cloning of CTL from EC-stimulated polyclonal lines

To generate clonal lines, primary 2-wk cultures of CD8+ T cells stimulated by EC, BLC, or EC + BLC (see Materials and Methods) were cloned by limiting dilution. The cloning conditions included irradiated PBMC as feeder cells (autologous to the responder T cells), mitomycin C-treated BLC (autologous to the stimulator cells), IL-2 (20 ng/ml), and PHA-L (1 μg/ml). Pilot experiments in the absence of PHA led to CD3εCD8−CD4− clones that failed to display cytotoxicity. Stimulator EC were usually present during the limiting dilution cloning if EC were present in the initial stimulator cultures, but their absence did not seem to influence the cloning efficiency (p = 0.71) nor change the phenotype (p = 0.30) of the emerging CTL clones. Therefore, the data from cloning in the absence and presence of EC have been pooled for purposes of statistical analysis. Overall, 66 CTL lines were cloned using cells from 10 different donors and resulting in 7 different allocombinations. With only one exception, all 66 limiting dilution clonings conformed to single hit kinetics for clonal growth (Fig. 1A). The observed frequencies were much higher than in primary cultures, reported previously (2), indicating that clonal expansion of CTL precursors had occurred during the initial 2-wk coculture. The fraction of alloreactive T cells present in the 2-wk microcultures that were capable of expansion varied between 0.2% and 25%. Surprisingly, this frequency was significantly higher for cultures stimulated with EC, which expanded least during the 2-wk primary culture than for cultures stimulated with both cell types or BLC alone (Fig. 1B). Eighteen of these CTL clones were studied by identifying the TCRVβ-chain expressed by these cells (Fig. 1C). A total of 4 of 18 CTL lines expressed two, 8 of 18 expressed one TCRVβ-chain, and 4 of 18 CTL lines were negative for all of the 20 TCRVβ families tested, consistent with expected frequencies for human CD8+ T cell clones (10). Only 2 of 18 CTL lines expressed more than two TCRVβ-chains, suggesting that they were in fact oligoclonal. For the cytolytic functional studies reported in this work, all of the cloned CTL lines (including the confirmed oligoclonal ones) were included in the analysis, but only

FIGURE 1. Generation of CTL clones. A, CTL grown in microcultures positive for cytotoxicity against the stimulator cell line were cloned by limiting dilution. On day 21, clonal growth was assessed. Percentage of negative microcultures per input cell number and frequency of growth competent cells are shown for six representative cloning experiments. These data conform to single hit events. B, The fraction of growth-competent cells in the 2-wk microcultures as a function of the initial stimulator cell type is shown. C, 18 CTL clones were analyzed for the expression of 20 TCRVβ-chain families using 22 family-specific PCR primers. With the exception of 2 lines showing 3 and 5 TCRVβ-chains, the other 18 lines appear to be true clones by this analysis.
true clones, confirmed by TCRVβ analysis, are presented in the analysis of CTL phenotype.

From 66 cloning experiments, a total of 94 T cell clones were successfully propagated. A total of 37 clones were expanded from lines stimulated by EC alone, 48 clones were expanded from lines stimulated by BLC alone, and 9 clones were expanded from lines stimulated by EC + BLC. The cytotoxicity profile versus EC or BLC targets (autologous to the original stimulator cells) of these 94 clones is shown in Fig. 2, in which the data are separated according to the initial allogeneic stimulator cell type (EC, BLC, or EC + BLC). Only 11 T cell clones of the 94 were not cytolytic for EC or BLC. These clones arose from EC (n = 6), BLC (n = 3), and EC + BLC (n = 2) lines and were not further characterized in this study. We operationally defined EC-selective clones as those that display percent specific lysis of EC ≥2× percent specific lysis of BLC. By this definition, 17 of 94 CTL clones were EC selective. Nine of these EC-selective CTL clones were derived from lines stimulated with EC alone, 48 clones were expanded from lines stimulated by EC + BLC, and, surprisingly, four arose from lines stimulated with BLC alone that had never been in contact with cultured EC before the cytotoxicity assay. As shown in Table II, the presence of EC in the initial cocultures increased the frequency of outgrowth of EC-selective CTL clones (p < 0.005). Mixed stimulator cell populations (i.e., EC + BLC) seem to be even more efficient inducers of EC-selective CTL than EC alone (not shown), although the number of clones analyzed is too small to allow separate statistical analysis. This trend is consistent with our previous observation that mixed stimulator cells accentuate EC selectivity of polyclonal CTL lines (2).

The target cell profiles support the idea that EC selectivity is a stable phenotypic trait of unconventional CTL. We have shown previously that EC suppress the expansion of conventional CTL in primary cocultures. To determine whether EC also alter the behavior of stable conventional CTL, we also examined whether the addition of EC at the time of cloning by limiting dilution could influence the phenotype of BLC-stimulated CTL clones. Seven BLC-stimulated 2-wk microcultures were cloned both in the presence or absence of EC. None of the 16 CTL clones that arose in these groups displayed an EC-selective phenotype whether or not EC were present during cloning. These experiments suggest that EC exert their selective effects during the initial differentiation of the CTL, supporting the conclusion that EC select for expansion of particular unusual but stable CTL phenotypes rather than transiently modulate the behavior of established CTL.

About 50% of the clones analyzed continued to expand for at least 8 wk. We defined such clones as being long-term CTL. We tested some of the long-term EC-selective CTL clones for NK-like, allospecific, and class I MHC-dependent killing (Fig. 3). EC-selective CTL clones did not lyse the NK cell target K562. Pooled EC were not lysed by EC-selective CTL clones, consistent with allospecificity (Fig. 3A). EC-selective CTL clones were inhibited by mAb against class I MHC and CD8 to the same extent as conventional, i.e., cell type-unrestricted CTL clones tested in parallel (i.e., by about 35–50% at E/T ratios of 30:1; Fig. 3B). Unfortunately, HLA-typed EC lines were not available to directly test class I MHC restriction of these clones. However, these data are consistent with allosreactive, class I MHC-restricted CTL that lack NK activity. The pattern of EC-selective, allosreactive, class I MHC-restricted CTL clones is also consistent with the characteristics of the 2-wk CTL lines described previously (2), some of which were tested on HLA-typed EC cultures, supporting the interpretation that allosreactive EC-selective killing exhibited by EC-stimulated CTL lines results from characteristics of individual CTL clones that have been expanded within the original cultures. Phenotype of the CTL clones

We applied FACS analysis, immunostaining, and competitive RT-PCR to analyze the phenotypes of long-term (8-wk) CTL clones. All long-term CTL clones analyzed were CD3/CD8double positive, but the level of CD8 was variable (Fig. 4B).

### Table II. EC favor the emergence of EC-selective CTL clones

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Clones (n)</th>
<th>EC Specific (%)</th>
<th>Conventional (%)</th>
<th>Nonkilling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC, EC + BLC</td>
<td>46</td>
<td>28</td>
<td>52</td>
<td>20</td>
</tr>
<tr>
<td>BLC</td>
<td>48</td>
<td>4</td>
<td>83</td>
<td>4</td>
</tr>
</tbody>
</table>

*a For all data, p = 0.0005.

*b Percent specific lysis of EC ≥2× BLC.

*c Percent specific lysis of EC <2× BLC.

*d Percent specific lysis <10%.

### Table III. EC favor the emergence of CTL clones that are not able to secrete IFN-γ after target cell contact

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Clones (n)</th>
<th>IFN-γ+ (%)</th>
<th>IFN-γ− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC, EC + BLC</td>
<td>22</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>BLC</td>
<td>21</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

*a For all data, p = 0.0006.

*b A total of more than 25 pg/ml supernatant 18–24 h after target cell lysis was assessed.

### Table IV. EC favor the emergence of CTL clones that express CD40L

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Clones (n)</th>
<th>CD40L+ (%)</th>
<th>CD40L− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>23</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>BLC</td>
<td>21</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

*a For all data, p = 0.0006.

*b Measured by FACS.

![FIGURE 3. EC-selective CTL clones are allospecific and class I MHC dependent. A, EC-selective CTL clone 122.1 was tested for cytosis of the stimulator EC, pooled third party EC, and K562, a typical NK cell target. B, EC-selective CTL clone 19.1.3. was tested for cytosis in the presence of anti-class I MHC Ab (W6/32), anti-CD8 Ab (OKT 8), and a nonbinding control Ab (K16/16). Representative of three experiments evaluating six EC-selective CTL clones.](http://www.jimmunol.org/DownloadedFrom/337x107 to 507x260)
some EC-selective CTL clones seemed to contain a CD8<sup>dim</sup> subpopulation, whereas conventional CTL clones were uniformly CD8<sup>bright</sup>. Interestingly, CD3<sup>+</sup>CD8<sup>dim</sup> cells were present in populations established as monoclonal by TCRV<sub>b</sub> analysis and probably do not represent a differential T cell population. All of the CTL clones expressed perforin<sup>+</sup> granules detectable by immunofluorescence microscopy (data not shown), and all clones tested expressed perforin mRNA (Fig. 5). FasL was detectable on only 4 of 39 resting CTL clones: 2 EC-selective clones contained a subpopulation of FasL<sup>+</sup> cells, and 2 BLC-stimulated conventional CTL clones were weakly FasL<sup>+</sup> (not shown). However, mRNA for FasL was detectable even in surface FasL<sup>−</sup> cells and varied over a broad range (Fig. 5). It is possible that such mRNA<sup>−</sup>, surface<sup>−</sup> clones contain intracellular pools of FasL protein (11), but this was not explored in the present study.

CD40L is typically thought of as a Th cell costimulatory molecule (12, 13). Surprisingly, 7 of 9 of the first EC-stimulated, EC-selective CTL clones analyzed expressed CD40L at rest, i.e., at least 7 days after the last restimulation with PHA and feeder cells (Fig. 4C). mRNA levels for CD40L were 10–100-fold higher in CTL clones that expressed CD40L on their surface than on CD40L-negative cells (Fig. 5B). CD40L was also found on some
EC-stimulated CTL clones that did not display EC selectivity, but not on BLC-stimulated CTL clones. To determine whether EC stimulators favored the emergence of CD40L+ CTL, we prospectively analyzed the next 44 CTL clones, including 23 that were stimulated by EC and 21 that were stimulated by BLC. As shown in Table IV, CTL that have been initially stimulated by EC are much more likely to express CD40L at rest than CTL that were not (p < 0.0006).

Several additional molecules were analyzed on some samples of the CTL clones produced in this study. All clones analyzed were positive for CD2 (14 conventional and 3 EC selective) and negative for TCRg-chain (4 conventional and 2 EC selective) and CD45RA (4 conventional and 2 EC selective). A total of 12 of 16 cytolytic CTL clones tested (10 conventional and 2 EC selective) were positive for Mac-1 (CD11b/CD18). A total of 3 of 7 tested CTL clones (1 conventional and 2 EC selective) expressed CD28. Only 2 of 7 (both EC selective) were CD25+ 7 days after the last restimulation, but all CTL clones (13 conventional, 1 EC selective) tested expressed Fas (CD95) on their surface.

Cytokine secretion by the CTL clones

Forty-one long-term CTL clones were analyzed for their capacity to secrete IFN-γ. All clones tested secreted IFN-γ in response to PHA (not shown). However, differences emerged when CTL clones were activated by target cells (Fig. 6). None of the 10 EC-selective clones, but 8 of 14 EC-stimulated and 16 of 17 BLC-stimulated conventional CTL clones secreted significant amounts of IFN-γ (>25 pg/ml), irrespective of the target cell in the assay (Fig. 6B). The level of secretion of IFN-γ did not correlate with cytotoxicity at the single clone level in any of the groups analyzed.

FIGURE 5.

mRNA levels for T cell activation genes in resting EC-selective and conventional CTL clones. Total RNA was isolated from resting CTL clones and reverse transcribed, and cDNA levels for various T cell activation genes were quantified by competitive PCR. For each gene of interest, the cDNA level was normalized to the amount of CD3e (arbitrarily set to be 100,000 U) present in the sample. A, A representative competitive RT-PCR analysis of two clones, one EC selective (6.1.2.) and one not (12.3.1.). B, Normalized data for 12 clones analyzed. ‐, Unidentified; n.d., not determined; b.d., below detection.
FIGURE 6. Long-term, EC-selective CTL do not secrete IFN-γ after target cell contact. A total of 41 long-term stable, cytolytic CTL clones (10 EC-selective (left panel), 31 conventional (middle and right panels)) were tested for cell type-selective target cell lysis (A) and target cell-dependent secretion of IFN-γ (B). Seventeen long-term stable CTL clones were also tested for target cell-dependent TNF secretion (C). A. For each of these clones, cell type-specific cytolyis was determined in at least two independent cytotoxicity assays performed in weekly intervals, and the mean value for each individual clone is displayed. B and C, In the second CTL assay, in which cell type selectivity was confirmed, supernatant was also harvested and analyzed for secretion of IFN-γ and TNF.

To test whether EC stimulators favored the emergence of CTL, which were poor secretors of IFN-γ, we prospectively analyzed the next 43 CTL clones, including 22 that were stimulated by EC and 21 that were stimulated by BLC. As shown in Table III CTL that have been initially stimulated by EC are much more likely to be poor IFN-γ secretors than CTL that were not (p = 0.0006).

IFN-γ mRNA levels at rest were not significantly lower in EC-selective CTL clones (p = 0.22), and they did not correlate at all with the capacity to secrete IFN-γ after target cell contact (Fig. 5B). Interestingly, we observed several clones that had IFN-γ mRNA levels >2000 CD3ε units at rest, but did not secrete measurable amounts of IFN-γ even after target cell lysis (e.g., clone 11.3.2; Fig. 5B). This suggests that additional translational or posttranslational control mechanisms of IFN-γ production must be occurring.

The pattern of TNF secretion by CTL after lysis of EC was similar to the pattern observed for IFN-γ. However, the EC-selective CTL clones produced TNF after BLC contact, indicating a partial dissociation of cytokine production and cytotoxicity in these CTL clones (Fig. 6C). These data also suggest that EC-selective clones can recognize BLC, but it was not tested whether such recognition is allorstricted. None of the CTL clones analyzed (EC selective and conventional) secreted measurable amounts of IL-4 after target cell contact (not shown). Thus, lack of IFN-γ secretion does not reflect immune deviation to a Tc2-like phenotype (14).

Correlations among EC-stimulated characteristics
In the preceding analyses, we prospectively analyzed the effects of EC upon CTL differentiation. The conclusions of our initial experiments, which held up through completion of the analyses, indicated that EC stimulation favors EC selectivity, poor IFN-γ secretion, and CD40L expression. A retrospective analysis of the initial clones suggested that these three traits were linked. In a final series of experiments, we prospectively analyzed more than 40 additional clones to determine the statistical significance of the relationships among EC selectivity, poor IFN-γ production, and CD40L expression (Table V). We found that CD40L expression correlated strongly with poor secretion of IFN-γ after target cell lysis (p = 0.0001). However, neither CD40L expression nor poor secretion of IFN-γ after target cell lysis reached statistical significance as indicators of EC selectivity (p = 0.12 and p = 0.06, respectively). Apparent dissociation of IFN-γ secretion and EC selectivity would be surprising since polyclonal CTL lines appeared to display both characteristics (2). It is more likely that these traits are linked, albeit less tightly than poor IFN-γ secretion and CD40L expression, and that if we had further increased the numbers of clones prospectively analyzed, the correlation between poor IFN-γ secretion and EC selectivity would have reached statistical significance.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Clones</th>
<th>IFN-γ (%)</th>
<th>IFN-γ (%)</th>
<th>EC Selective (%)</th>
<th>Conventional (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40L⁺⁺⁻⁻</td>
<td>13</td>
<td>23*</td>
<td>77*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L⁻⁻⁻⁻</td>
<td>30</td>
<td>83*</td>
<td>17*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ⁺⁺⁻⁻</td>
<td>27</td>
<td>4**</td>
<td>96**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ⁻⁻⁻⁻</td>
<td>8</td>
<td>25**</td>
<td>75**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L⁺⁺⁻⁻</td>
<td>10</td>
<td>20***</td>
<td>80***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L⁻⁻⁻⁻</td>
<td>25</td>
<td>4***</td>
<td>96***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A total of 25 pg/ml in supernatant was collected 18–24 h after target cell lysis was assessed.

* Percent specific lysis of EC ≥2× BLC.

* Percent specific lysis of EC <2× BLC.

* Measured by FACS.

* * p = 0.0001, ** p = 0.06, *** p = 0.12.
Discussion

Cell type-selective effector T cells may play a role in tissue-specific injury during solid organ transplant rejection (15–17) or graft versus host disease (18). EC-selective CTL have been isolated from acutely rejecting heart transplants (1). We have described previously that polyclonal CTL lines, which differentiated in the presence of EC as stimulator cells, lyse EC, but not BLC autologous to the EC. We have now cloned individual CTL from these polyclonal CTL lines to determine whether EC-selective killing is a feature of an unusual subset of CTL clones. To do this, we first developed culture conditions that allow limiting dilution and subsequent propagation of human CD3"CD8" perforin CTL clones from our primary cocultures. Our initial analysis of Ag specificity indicates that EC preferentially stimulate expansion of CTL clones that are EC selective, class I MHC dependent, and allospecific. We have not yet positively identified the restricting element that is recognized by these clones as a conventional class I MHC molecule since we did not have a panel of class I MHC-typed EC available to screen for specificity. This presents a logistical problem since EC are not immortal and each new culture must be individually typed. Nevertheless, it is likely that these clones are class I MHC restricted since we had shown previously that the polyclonal, EC-selective lines, similar to the ones used for cloning in the present study are, in fact, class I MHC restricted. We also have not yet shown that these CTL are specific for particular peptides displayed by allogeneic class I MHC molecules. The precise specificity of these clones will be investigated in future studies.

Although EC-selective CTL preferentially emerged from EC-stimulated cultures, a majority of long-term CTL clones from these same cultures actually displayed a cell type-unrestricted, i.e., conventional pattern of killing. We had noted previously that EC suppress growth of conventional CTL stimulated by BLC (2). The simplest explanation of our results is that EC also suppress growth of conventional CTL stimulated by EC, but that such clones can be expanded under the conditions of limiting dilution culture in the presence of feeder cells. In other words, the cloning conditions allow emergence of conventional CTL that were silent (or poorly expanded) in the polyclonal lines. However, we cannot rule out that the cloning conditions that we optimized for growth at limiting dilution allow some EC-selective CTL to convert to a more conventional specificity.

A major surprise of these studies was the production of four EC-selective clones from cultures that had never seen EC in vitro. In theory, EC-selective killing could arise from a target structure formed by a peptide derived from an EC-specific protein (e.g., von Willebrand factor) not synthesized by BLC (15). If so, it is hard to imagine how such clones could be activated by BLC. Alternatively, cell-selective CTL may arise from a requirement for unusual accessory or adhesive interactions that would favor killing of EC over BLC (e.g., binding to E-selectin or ICAM-2, adhesion molecules expressed on EC, but not BLC (19)). Such an accessory molecule-based explanation has recently been offered to account for cell-selective killing of renal epithelial cells by CTL (20). It is possible that the BLC-stimulated clones that display EC selectivity also fit into this category. If this explanation is true, the generation of EC-selective CTL arising from stimulation by BLC raises the possibility that some or all EC-stimulated CTL that display EC selectivity are also selective because of accessory interactions rather than cell-specific peptides. This possibility will also be explored in our future studies.

Cytolytically active CTL clones fulfill the definition of an effector T cell. Expression of Mac-1 and perforin is a recognized marker of effector CTL (21), and these molecules are also found in a majority of our EC-selective and conventional CTL clones. CD40L is also an effector cell marker, but more typically on CD4+ Th cells (12, 13). We identified CD40L as the most consistent surface marker for EC-stimulated CTL. However, the correlation of CD40L expression with EC-selective killing pattern did not reach statistical significance. Although we think these traits are probably linked, and that statistical significance would become clearer in a larger analysis, the points remain that some CD40L-expressing CTL may exhibit a conventional target cell profile, and that not all EC-selective CTL are CD40L positive. CD40L-CD40 signals have been shown to induce B cell activation and Ab isotype switching (22, 23), dendritic cell maturation (24, 25), as well as macrophage (26) and EC activation (27). Transiently expressed CD40L on CD8+ T cell clones has been shown to be functionally active (28). If EC-stimulated CTL express this important costimulatory molecule so persistently in vivo, it may well substitute for CD4+ T cells and amplify immune responses in the absence of class II MHC-restricted signals.

The second major difference between EC- and BLC-stimulated CTL was the capacity to secrete IFN-γ after target cell lysis. Long-term EC-selective CTL did not secrete IFN-γ in response to EC nor BLC. The threshold of integrated TCR activation events required for IFN-γ secretion has been reported to be orders of magnitude higher than that for cytolysis (29). Our data would conform with this model if EC-selective CTL were activated by a very rare EC-specific Ag signal, sufficient to trigger killing, but insufficient to induce cytokine secretion. However, EC-selective CTL clones can secrete TNF in response to BLC, but not EC. This observation indicates EC-selective CTL are responsive to BLC, and supports the notion that peptide recognition is not the basis of the cell type-selective killing by EC-selective CTL clones, although we did not show that TNF synthesis was actually alloantigen dependent.

The effects of EC upon CTL differentiation reported in our previous study and extended here to the clonal level have implications for both transplantation and vascular biology. For example, our data suggest that the differentiation and/or expansion of conventional CTL precursors are likely to be suppressed in a microenvironment in which EC are in close apposition to infiltrating T cells, e.g., in the intima of the arterial wall. Those CTL that do emerge may be EC selective, poorly secrete IFN-γ after activation, and constitutively express CD40L. In allografts, EC-selective CTL may mediate endothelialitis, the harbinger of therapy-resistant acute vascular rejection (30). Acute endothelialitis may evolve into chronic graft arteriosclerosis, the principal cause of cardiac and renal graft failure (31, 32). On the other hand, IFN-γ has been shown in mouse heart transplant models to be essential for intimal expansion in subacute/chronic allograft rejection, despite the fact that it is not required for acute parenchymal rejection (33). In contrast, elevated CD40L and perforin mRNA levels have been shown to be independent risk factors for acute kidney transplant rejection (34, 35). CD40L is also relevant in chronic pathobiology of the arterial intima, contributing to the formation of atheromata in hyperlipidemic mice (36), possibly triggering acute coronary syndromes in humans by promoting macrophage production of tissue factor and matrix metalloproteinases (26) and mediating arterial intimal expansion in a heterotopic heart transplantation model in mice (37). Finally, our new data suggest that coexpression of CD40L and perforin may be a useful marker to identify unusual, EC-stimulated CTL in situ in the setting of intimal disease. We conclude by noting that endothelial cells may not only be activators of circulating memory T cells, but may influence the outcome of immune reactions by mediating novel forms of immune deviation.
Acknowledgments
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References