Human Vascular Endothelial Cells Stimulate a Lower Frequency of Alloreactive CD8+ Pre-CTL and Induce Less Clonal Expansion than Matching B Lymphoblastoid Cells: Development of a Novel Limiting Dilution Analysis Method Based on CFSE Labeling of Lymphocytes

Thomas J. Dengler, David R. Johnson and Jordan S. Pober

J Immunol 2001; 166:3846-3854; doi: 10.4049/jimmunol.166.6.3846

References

This article cites 23 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/166/6/3846.full#ref-list-1

Why The JI? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human Vascular Endothelial Cells Stimulate a Lower Frequency of Alloreactive CD8+ Pre-CTL and Induce Less Clonal Expansion than Matching B Lymphoblastoid Cells: Development of a Novel Limiting Dilution Analysis Method Based on CFSE Labeling of Lymphocytes

Thomas J. Dengler, David R. Johnson, and Jordan S. Pober

We have previously shown that human endothelial cells (EC) are less efficient than professional APC, e.g., B lymphoblastoid cells (BLC), at stimulating allogeneic CD8+ T cells to develop into CTL. In this study we describe FACS-based limiting dilution analyses using the dilution of the intracellular dye CFSE as an indicator of CD8+ T cell alloactivation and expansion with significantly increased sensitivity compared with conventional, cytotoxicity-based assays. In addition, this assay permits the relative size of clonal CTL populations that are generated in individual CD8+ T cell cultures to be determined (clonal burst size). We have applied this method to quantitatively compare the generation of CTL at the clonal level following stimulation of allogeneic CD8+ T cells by either BLC or HUVEC derived from the same donor. CD8+ T cells expanded by allostimulation were identified as CD8+, CFSElow cells and were categorized as CTL by the expression of intracellular perforin and IFN-γ. Precursor frequencies for EC-stimulated CTL were 5- to 40-fold (mean, 7.5-fold) lower compared with BLC-stimulated CTL (p < 0.01). Concomitantly, the average clonal burst sizes in EC-stimulated CTL cultures were significantly smaller than those in conventional CTL cultures, primarily due to the occurrence of some very large clone sizes exclusively with BLC stimulation. Although EC-stimulated CTL were generated only from the memory subset of CD8+ T cells, BLC-stimulated very large burst sizes of CTL were observed from both naive and memory CD8+ T cell precursors. These data establish that both a lower frequency of reactive precursors and more limited clonal expansion, but not regulatory T cells, contribute to the reduced capacity of EC to promote alloreactive CTL differentiation compared with that of professional APC.

sufficiently sensitive to derive precursor frequencies for EC-stimulated CTL, because the cytotoxicity remained below the detection threshold in many cultures with single clones, presumably due to the low absolute numbers of CTL that were generated (7). LDA based on IFN-γ production was also judged inadequate due to the diminished levels of production of this cytokine by EC-stimulated CTL (5). We have previously used LDA based on IL-2 production as a read-out of alloreponsive cells (12). However, recent studies with MHc tetramer staining methods, which have been enormously informative in the analysis of anti-microbial immune responses (13), have suggested that this approach may significantly underestimate the number of alloantigen-specific T cells. Unfortunately, MHc tetramer staining cannot be used in the allogeneic setting due to the large number of alloantigens and unavailability of defined MHc/peptide complexes. To overcome these problems, we developed a new FACS-based LDA method using CFSE labeling and dilution. In addition to higher sensitivity in the detection of alloactivated CTL, this method allows semiquantitative assessment of the burst sizes originating from individual precursor cells. We also demonstrate that there is sufficient expansion of clonal populations of CTL for phenotypic analysis, e.g., for identification of responders as CTL by detection of intracellular perforin and IFN-γ or various surface Ags. With this new method, we found that the lower CTL numbers that we observe after stimulation of CD8+ T cells by EC compared with stimulation by BLC are due to both a lower frequency of alloreactive precursors and reduced clonal expansion. In addition, the alloactivated CD8+ cells in these cocultures were positively identified as CTL by the detection of intracellular perforin and IFN-γ. At the same time, we found no evidence of EC-stimulated expansion of regulatory or suppressor (non-CTL) CD8+ T cells as judged by FACS analysis of surface markers. Overall, these findings further substantiate the idea that the Ag-presenting capacities of EC differ from those of professional APC.

Materials and Methods

Cell isolation

PBMC were obtained from healthy volunteers by density gradient centrifugation of leukapheresis products and were stored in liquid nitrogen as described previously (12). CD8+ T cells were isolated from PBMC by positive selection (5) using Dynabeads (Dynal, Lake Success, NY). The selected population obtained by this procedure was routinely >98% CD8+/CD3+ by flow cytometry and >99% viable, as shown by trypan blue exclusion. We have previously shown that positive selection by this method does not alter T cell responses (5). Naïve and memory subsets of T cells were isolated from the CD8-selected population by further negative selection. CD8+ T cells were incubated with anti-CD45RA (B-C15; 1 μg/10^7 cells) or anti-CD45RO mAb (UCHL-1; 5 μg/10^7 cells) and 2 μg/ml CD45RA-RO subsets were washed twice in cold PBS and then incubated in 0.25 μM CFSE (Molecular Probes, Eugene, OR) in PBS at 37°C for 15 min. Cells were then washed twice before addition to cocultures. For CFSE-based LDA, CFSE-labeled CD8+ T cells were serially diluted before addition to microcultures with either EC or BLC (20,000 stimulator cells/well), resulting in input cell numbers per well as indicated in individual experiments, usually ranging from 40,000 CD8+ T cells/well to 306 cells/well (eight 2-fold dilution steps). Twelve replicate microcultures were initiated for each dilution step of input CD8+ T cells. CD8+ T cells cultured in the absence of any stimulator cells, but receiving identical IL-2 supplementation, were used as controls for the highest and lowest cell numbers. After 14 days of coculture, CFSE-labeled CD8+ T cells were harvested from individual wells, transferred into 96-well V-bottom microtiter plates, washed once with ice-cold PBS/1% BSA, and incubated with directly PE-conjugated mouse anti-human CD8 Ab (Coulter, Miami, FL) for 1 h at 4°C. Cells were then washed twice, resuspended and fixed in 150 μl of 1% paraformaldehyde. For flow cytometric analysis all cells from individual wells were transferred into plastic tubes and analyzed using a FACSort (Becton Dickinson, San Jose, CA) and CellQuest analysis software. To ensure optimal quantitative comparison, all samples were run for exactly 1 min (on the high setting), equivalent to ~100 μl of sample, i.e., ~70% of total CD8+ T cells in the sample. Unlabeled CD8+ T cells and cells fixed immediately after CFSE labeling were used as negative and positive internal controls for staining, respectively. Determination of precursor frequencies and probability of single-hit kinetics were performed by computer-assisted statistical analysis, using least square approximation (software provided by Dr. C. Orosz, Ohio State University, Columbus, OH).

Immunophenotyping of CTL from microcultures

For flow cytometric analysis, T cells (initially labeled with CFSE) were harvested from individual microculture wells. Cells were washed twice in ice-cold PBS/1% BSA and incubated with saturating concentrations of di- or trivalent PE-conjugated mouse anti-human CD3, CD25, CD69, CD80, CD57 (all from Coulter), CD28 (PharMingen, San Diego, CA), or CD56 (Sigma-Aldrich) for 1 h at 4°C. Cells were washed twice in ice-cold PBS/1% BSA and fixed in 1% paraformaldehyde. For flow cytometric analysis all cells from individual wells were transferred into plastic tubes and analyzed using a FACSort (Becton Dickinson, San Jose, CA) and CellQuest analysis software. To ensure optimal quantitative comparison, all samples were run for exactly 1 min (on the high setting), equivalent to ~100 μl of sample, i.e., ~70% of total CD8+ T cells in the sample. Unlabeled CD8+ T cells and cells fixed immediately after CFSE labeling were used as negative and positive internal controls for staining, respectively. Determination of precursor frequencies and probability of single-hit kinetics were performed by computer-assisted statistical analysis, using least square approximation (software provided by Dr. C. Orosz, Ohio State University, Columbus, OH).

Microcultures for CTL generation

The procedure for alloreactive CTL differentiation has been described in detail previously (5). In brief, variable numbers of purified CD8+ T cells (see below) were incubated with EC or BLC stimulator cells (20,000/well each) in 96-well microculture plates (Falcon, Becton Dickinson, Bedford, MA). All cultures were maintained in 5% CO2 at 37°C. The medium for coculture consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Irvine, Santa Ana, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was further supplemented with exogenous IL-2 (National Institutes of Health, Bethesda, MD) on day 3 (final concentration, 5 ng/ml) and subsequently fed with fresh medium containing IL-2 on days 7 and 10. On day 14 CTL were harvested from individual microculture wells and subjected to further analyses described below.

LDA assays of CD8+ T cells based on CFSE dilution

Labeling of CD8+ T cells with CFSE was performed as described Previously (15, 16). In brief, total CD8+ T cells or separated CD45RA/RO subsets were washed twice in cold PBS and then incubated in 0.25 μM CFSE (Molecular Probes, Eugene, OR) in PBS at 37°C for 15 min. Cells were then washed twice before addition to cocultures. For CFSE-based LDA, CFSE-labeled CD8+ T cells were serially diluted before addition to microcultures with either EC or BLC (2,000,000 stimulator cells/well), resulting in input cell numbers per well as indicated in individual experiments, usually ranging from 40,000 CD8+ T cells/well to 306 cells/well (eight 2-fold dilution steps). Twelve replicate microcultures were initiated for each dilution step of input CD8+ T cells. CD8+ T cells cultured in the absence of any stimulator cells, but receiving identical IL-2 supplementation, were used as controls for the highest and lowest cell numbers. After 14 days of coculture, CFSE-labeled CD8+ T cells were harvested from individual wells, transferred into 96-well V-bottom microtiter plates, washed once with ice-cold PBS/1% BSA, and incubated with directly PE-conjugated mouse anti-human CD8 Ab (Coulter, Miami, FL) for 1 h at 4°C. Cells were then washed twice, resuspended and fixed in 150 μl of 1% paraformaldehyde. For flow cytometric analysis all cells from individual wells were transferred into plastic tubes and analyzed using a FACSort (Becton Dickinson, San Jose, CA) and CellQuest analysis software. To ensure optimal quantitative comparison, all samples were run for exactly 1 min (on the high setting), equivalent to ~100 μl of sample, i.e., ~70% of total CD8+ T cells in the sample. Unlabeled CD8+ T cells and cells fixed immediately after CFSE labeling were used as negative and positive internal controls for staining, respectively. Determination of precursor frequencies and probability of single-hit kinetics were performed by computer-assisted statistical analysis, using least square approximation (software provided by Dr. C. Orosz, Ohio State University, Columbus, OH).

Statistical analysis

For statistical analysis, CTL precursor frequencies and (clonal) burst sizes in different experimental groups were compared by nonparametric Mann-Whitney U test, using Primer of Biotistics analysis software (McGraw Hill, New York, NY). Differences between groups were considered significant at p < 0.05.
Results

Establishment of a FACS-based LDA method using CFSE dilution

After labeling of purified CD8⁺ T cells with CFSE, microcultures using BLC or EC as stimulator cells were initiated with addition of serially diluted CD8⁺ T cells, with input numbers ranging from 150 to 100,000 CD8⁺ cells/well. For analysis after 14 days of culture, CD8⁺ T cells recovered from individual microcultures were examined by two-color flow cytometry (CFSE and CD8-PE). Unstimulated T cells remained CD8⁺/CFSEbright, similar to the staining of input cells, and alloactivated T cells could be identified as CD8⁺ cells that show a reduction of the intensity of the CFSE fluorescence signal (CD8⁺/CFSElow). Fig. 1 shows examples of two-color FACS plots from typical cultures, illustrating the presence and size of the populations of unstimulated CD8⁺/CFSEbright cells and alloactivated CD8⁺/CFSElow cells at different input cell numbers. At sufficiently low input cell numbers, the culture becomes negative, i.e., no CD8⁺/CFSElow cells can be detected while, in most cases, residual nonactivated cells (CD8⁺ CFSEbright) are still seen. CD8⁺ T cells freshly labeled with CFSE and unlabeled cells are shown for comparison. The intensity of CFSE fluorescence in alloactivated CD8⁺/CFSElow cells from positive microcultures is very similar to that of unlabeled CD8⁺ T cells, indicating that the alloactivated CD8⁺ cells have undergone a sufficient number of cell divisions (eight or more) within the 14-day culture period, so that the CFSE signal has been diluted to background levels. In control cultures of unstimulated, IL-2-treated CD8⁺ T cells no CFSElow cells were detectable in any of the experiments, even when using as many as 100,000 input cells. Thus, for the purpose of LDA, all microcultures containing CD8⁺/CFSElow cells were considered positive, and only cultures that did not show any CD8⁺/CFSElow cells were considered negative. This was possible because in all experiments there were at least ~10 CD8⁺/CFSElow cells in a positive microwell (minimum of eight) clustered as a discrete grouping, allowing clear differentiation between positive and negative cultures. The size of the population of nondivided CD8⁺ cells also progressively decreased as input number declined, and at very low input cell numbers (<1000/well), CFSEbright cells were sometimes lost (Fig. 1, bottom row, left panel). A similar loss of nonactivated CD8⁺ cells occurred in unstimulated, IL-2-treated control cultures at low input cell numbers (not shown).

To obtain precursor frequency, the fraction of negative cultures was determined for each CD8⁺ T cell input number (usually from 12 cultures/input cell level). From these fractions of negative cultures the CTL precursor frequencies were determined using computer-assisted standard LDA statistical analysis, including probability estimated for single-hit kinetics. An example of the CTL precursor frequencies after stimulation with EC or corresponding BLC is shown in Fig. 2A.

In contrast to most other LDA approaches, the FACS-based CFSE dilution LDA additionally provides semiquantitative information on the extent of clonal expansion of CD8⁺ T cells in individual allostimulated microcultures. For this purpose, the absolute number of CD8⁺/CFSElow cells is determined in those positive cultures for which the input cell numbers are below the calculated precursor frequency, i.e., cultures in which only one alloreactive precursor cell should be present. Absolute cell numbers were obtained by counting the cells in the CD8⁺/CFSElow quadrant. Quantitative comparison between individual microculture samples was made possible by running samples of equal volume for equal amounts of time. We refer to the size of these populations arising from single precursors as a burst size, analogous to hemopoietic cell precursor behavior. In Fig. 2B, examples of two different burst sizes from cultures with a single CTL precursor are shown in comparison with a negative culture. Burst sizes for cultures arising from single CTL precursors were compared between different stimulation conditions or input cells and analyzed for statistical differences as demonstrated in Fig. 2C. Initial experiments revealed a wide variation of such burst sizes even in cultures raised against the same stimulator cells (Fig. 2C).

CTL precursor frequencies in CD8⁺ T cell populations are lower after stimulation with EC than with matching BLC

The precursor frequencies of allogeneic CTL in populations of purified, unseparated resting CD8⁺ T cells were determined after stimulation with BLC or matching EC, respectively, using the FACS-based LDA described. CTL precursor frequencies after

**FIGURE 1.** Two-color fluorescence analysis of CD8⁺ T cells harvested from BLC-stimulated cocultures. CFSElow CD8⁺ cells (upper left quadrant) represent alloactivated CD8⁺ T cells, i.e., CTL. The top row shows an example of a serial dilution of CD8⁺ T cells stimulated with EC resulting in activated populations of decreasing size as used in CFSE-based LDA assays. For 1250 input cells (calculated input cell level for a mean of ≤1 precursor/well) one positive culture (CFSElow CD8⁺ cells present; second panel) and one negative culture (no CFSElow CD8⁺ cells present; fourth panel) are shown, respectively, demonstrating the clear discrimination within the LDA system. Note that with very low input cell numbers (<1000/well) nonactivated T cells (CD8⁺ CFSEbright; upper right quadrant) are lost from the cultures (bottom row, left panel). For comparison, unstimulated CFSE-labeled CD8⁺ T cells and unlabeled cells are shown as positive and negative controls, respectively (bottom row, right panels). Input refers to cell input number per microculture well.
stimulation with BLC ranged from 1/130 to 1/3,300 with a median of 1/490; after stimulation with EC, the precursor frequencies were significantly lower, ranging from 1/1,000 to 1/28,000, with a median of 1/3,200 ($p < 0.01$). Computer-assisted statistical analysis of the LDAs confirmed single-hit kinetics for each allostimulation. The complete data from five independent LDA assays comparing stimulation of CD8$^+$ T cells from different donors with allogeneic, corresponding BLC and EC are summarized in Table I. When precursor frequencies were compared pairwise for matching BLC and EC stimulators, CTL precursor frequencies were normally 6- to 8-fold higher with BLC stimulators than with EC (range, 5.3- to 46-fold).

Clonal burst sizes of alloactivated CD8$^+$ T cells are lower after stimulation with EC than with matching BLC

In four of the five CFSE-based LDA assays described in Table I, burst sizes were directly compared between cultures stimulated with BLC or corresponding EC. In all pairwise comparisons, the average burst sizes generated by BLC stimulation were higher than those after stimulation with corresponding EC. In three of four experiments these differences reached statistical significance. A detailed analysis of the distribution of the observed burst sizes revealed that in each experiment a number of very large burst sizes could be detected exclusively in BLC-stimulated cultures, and that these large clones accounted for the significant difference in average burst size after BLC or EC stimulation. A graphic example of this dichotomous distribution of clonal burst sizes is shown in Fig. 2C; the complete data for burst size comparisons in all four experiments are compiled in Table I.

CTL precursor frequencies and clonal burst sizes in naive and memory CD8$^+$ T cells after stimulation with BLC or EC

To determine the precursor frequencies for BLC-stimulated vs EC-stimulated CTL in naive and memory CD8$^+$ T cells, CFSE-based LDA was performed with purified subsets of CD8$^+$ T cells based on the expression of the CD45 isoforms, i.e., CD45RO$^+$ cells were considered naive, and CD45RA$^+$ cells were considered memory. In two independent experiments, stimulation with EC activated only memory CD8$^+$ T cells to expand and differentiate into CTL, but not naive CD8$^+$ cells. This findings at limiting dilution conditions confirmed our previous experiments using microwell cultures (7). CTL precursor frequencies in naive or memory T cells stimulated with BLC were, on the average, 2- to 4-fold higher than with EC-stimulated memory CD8$^+$ cells (Table II). Based on the approximately equal frequencies of naive and memory cells in unseparated CD8$^+$ T cell populations (not shown), this results in an overall difference in CTL precursor frequencies between stimulation with BLC vs EC of 4- to 8-fold, corresponding well with the results of experiments performed with unseparated CD8$^+$ cell populations. In both experiments very large clonal burst sizes again only occurred after stimulation of CD8$^+$ cells with BLC, but not EC (Fig. 3). Surprisingly, there was a tendency of large clonal

amounts of sample was ascertained by suspension of harvested cells in identical volumes and identical analysis time (1 min). Examples of a negative culture, a small burst size, and a large burst size are shown from Expt. 4 (Table I).

FIGURE 2. A, Computer-assisted determination of CTL precursor frequencies by CFSE-based LDA; Expt. 4 of Table I is shown as a representative example. The fraction of negative cultures (no alloactivated, i.e., CFSE$^{low}$ CD8$^+$ T cells, detectable by flow cytometry) is plotted against input CD8$^+$ cell numbers. Precursor frequencies are derived by computer-assisted least square approximation (see text for details). The probability score for single/multiple hit kinetics is given ($p$). A single hit is indicated by values $> 0.05$. B, Determination of (clonal) burst sizes by two-color flow cytometry. Absolute numbers of alloactivated CD8$^+$ T cells (CFSE$^{low}$, CD8$^+$; upper left quadrant) in each microculture of CFSE-based LDA were measured by quadrant statistics (CellQuest). Analysis of comparable
burst sizes to occur more frequently in the memory subset of CD8+ T cells. However, this did not reach statistical significance in all experiments (Fig. 3B). Clearly, large burst sizes were not confined to the naive subset of CD8+ T cells, and the activation of naive T cells cannot explain the capacity of B cells to stimulate greater clonal expansion.

EC-alloactivated CD8+ T cells are CTL and do not express markers of regulatory or suppressor cell types

Our FACS-based CFSE dilution LDA allowed us to examine the intracellular expression of perforin and IFN-γ of clonally alloactivated CTL on the individual cell level without subjecting the CD8+ T cells to cloning procedures. In general, all CD8+ cultures stimulated by BLC or EC contained a majority of cells that expressed perforin and IFN-γ intracellularly (Fig. 4). For IFN-γ, the intracellular expression levels and the proportion of positive cells were similar after stimulation with BLC and EC. The proportion of alloactivated cells positive for perforin was also similar after BLC and EC stimulation. In contrast, perforin expression levels per individual cell (mean fluorescence intensity) were consistently higher in BLC-stimulated, conventional CTL than in EC-stimulated CTL (Fig. 4). Nevertheless, the presence of perforin in these clonal populations allows us to conclude that essentially all the alloreactive CD8+ T cells differentiate into CTL under our culture conditions. The analysis of intracellular IFN-γ and perforin expression additionally demonstrated that even on a clonal level neither BLC-stimulated nor EC-stimulated CTL populations were 100% positive for these cytokines, and that small numbers of T cells not expressing IFN-γ or perforin were consistently detectable in both BLC- and EC-stimulated cocultures.

We also examined the surface expression of several reported markers of regulatory or suppressor CD8+ T cells or NK T cells in pooled cocultures of either BLC- or EC-stimulated CTL. Consistent with our previous report analyzing bulk populations, EC-stimulated CD8+ T cells expressed high levels of CD25 and CD69 after 14 days of coculture, whereas BLC-stimulated CTL were CD25low and CD69low (Fig. 5). CD28 was expressed at comparable levels and prevalence (~70% positive cells) on CTL stimulated with BLC or EC (Fig. 5). Expression levels and frequency of CD28+ cells were also similar to those in freshly isolated or unstimulated, IL-2-treated CD8+ T cells (not shown), so that no significant reduction in CD28 levels could be detected over the course of CTL differentiation. The frequency of CD8+ cells expressing γδ TCR, CD56, or CD57 was also similar after stimulation of CD8 cultures with BLC or EC and was no different from that of unstimulated CD8+ T cells (Fig. 5). Collectively, these data do not support the idea that EC selectively promote the differentiation of CD8+ T regulatory cells.

Discussion

Cell type-selective, alloreactive CTL have been described after various forms of solid organ transplantation, and they have been implicated in tissue-specific injury during allograft rejection (17, 18) or graft-vs-host disease (19). Reports of such unconventional CTL include effectors that were found to be selectively reactive with vascular EC (1, 2). We have previously reported that EC stimulation of allogeneic peripheral blood CD8+ T cells in vitro results in the generation of EC-selective CTL that exhibit several unique features, including the persistence of an immature, activated surface phenotype (CD25high, CD69high, CD62Lhigh), reduced cytotoxic capacity, and undetectable IFN-γ secretion. EC-stimulated microcultures also exhibited a markedly reduced number of effector cells compared with replicate cultures stimulated by BLC (5, 7). The pertinent biological explanations for the reduced CTL numbers in EC-stimulated vs BLC-stimulated CTL.

Table I. CFSE-LDA of CTL precursor frequencies and burst sizes of alloactivated unseparated CD8+ T cell cultures after stimulation with BLC or EC (least squares approximations)

<table>
<thead>
<tr>
<th>Stimulations</th>
<th>pCTL Frequency</th>
<th>Probability</th>
<th>Burst Sizes (at 1 pCTL/well)</th>
<th>p for Difference in Burst Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>BLC 1:130 (50–210)</td>
<td>0.68</td>
<td>22, 26, 28, 65, 110, 338, 616</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>EC 1:1000 (710–1290)</td>
<td>0.71</td>
<td>8, 10, 12, 15, 16, 20, 21, 314</td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td>BLC 1:3300 (2100–4500)</td>
<td>0.2</td>
<td>80, 190, 229, 327, 435, 513, 1006, 1322</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>EC 1:17,675 (12,575–22,775)</td>
<td>0.33</td>
<td>17, 21, 24, 36, 92, 201, 379, 429</td>
<td></td>
</tr>
<tr>
<td>Expt. 3</td>
<td>BLC 1:600 (285–915)</td>
<td>0.6</td>
<td>25, 30, 36, 84, 90, 120, 2300</td>
<td>p = NS</td>
</tr>
<tr>
<td></td>
<td>EC 1:28,000 (22,180–33,820)</td>
<td>0.6</td>
<td>10, 13, 47, 51, 95, 156, 300</td>
<td></td>
</tr>
<tr>
<td>Expt. 4</td>
<td>BLC 1:490 (310–670)</td>
<td>0.9</td>
<td>104, 181, 191, 1029, 1199, 1612, 1666, 1965</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td></td>
<td>EC 1:3200 (2520–3880)</td>
<td>0.2</td>
<td>10, 22, 25, 27, 39, 70, 203, 212, 486</td>
<td></td>
</tr>
<tr>
<td>Expt. 5</td>
<td>BLC 1:440 (235–645)</td>
<td>0.2</td>
<td>Burst sizes not determined</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC 1:3200 (1825–4575)</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Probability of “single-hit” kinetics of CTL generation. Values of p > 0.05 identify single hit.

Table II. CFSE-LDA analysis of CTL precursor frequencies of alloactivated naive or memory CD8+ T cell cultures after stimulation with BLC or EC (least squares approximations)

<table>
<thead>
<tr>
<th>Responder/Stimulator Cells</th>
<th>pCTL Frequency</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive CD8/BLC</td>
<td>1:5300 (4125–6475)</td>
<td>0.73</td>
</tr>
<tr>
<td>Memory CD8/BLC</td>
<td>1:10,470 (7,820–13,120)</td>
<td>0.93</td>
</tr>
<tr>
<td>Naive CD8/EC</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Memory CD8/EC</td>
<td>1:21,300 (17,890–24,710)</td>
<td>0.23</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive CD8/BLC</td>
<td>1:1150 (865–1435)</td>
<td>0.8</td>
</tr>
<tr>
<td>Memory CD8/BLC</td>
<td>1:950 (640–1260)</td>
<td>0.25</td>
</tr>
<tr>
<td>Naive CD8/EC</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Memory CD8/EC</td>
<td>1:3750 (2285–5215)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Burst sizes are shown separately in Fig. 3A and B.

b Probability of “single-hit” kinetics of CTL generation. Values of p > 0.05 identify single hit.

Range of precursor frequencies are in parentheses.

In EC-stimulated cultures of naive CD8+ T cells, with maximal input cell numbers of 100,000/well, no alloactivation could be detected.
CD8+ cell populations were between 5- and 40-fold lower than those in matched BLC-stimulated cultures. The absolute number of alloactivated CD8+ CTL in EC-stimulated cultures was consistently smaller than that in BLC-stimulated CTL cultures, principally because some clones of T cells stimulated by B cells are significantly larger than those stimulated by EC. Thus, the reduced absolute numbers of CTL following stimulation by EC compared with stimulation by BLC can be accounted for by a combination of lower precursor frequencies and a reduced net CD8+ cell expansion after EC stimulation.

The findings of the current study were made possible by the use of a FACS-based LDA method that uses the detection of expansion of alloactivated CD8+ T cells by CFSE dilution, such that the presence of CD8+ CFSElow cell populations defined a positive event. The inclusion of positive and negative labeling controls and control CD8+ T cell cultures that were treated identically except for the absence of allogeneic stimulator cells allowed precise determination of positive or negative culture events, particularly so as the IL-2-treated control cultures of CD8+ T cells without stimulators were consistently negative for CFSElow, i.e., alloactivated and expanded, cells. In some instances at very low CD8+ input cell numbers the nonactivated CFSEhigh cells were even lost completely from the cultures, leaving only alloactivated CFSElow cells to be detected in the microcultures. The CFSE-LDA approach could detect even very small numbers of specifically alloactivated CD8+ T cells, which would not be detectable by conventional cytotoxicity assays due to their critical dependence on the absolute number of alloreactive CTL per culture, i.e., the E:T cell ratio, and the efficiency of target cell killing. Therefore, cytotoxicity-based LDAs underestimate the precursor frequency for CTL in settings of reduced responder cell killing, which are seen with CTL proliferation after stimulation with EC and with CD8+ T cell alloactivation in general. These considerations are largely also valid for LDA assays based on IL-2 secretion as the measured parameter. Accordingly, our original studies regarding the generation of EC-specific CTL underestimated the frequency of positive CTL cocultures; specifically, only 20–30% of cocultures were assayed as positive for cytotoxicity and seemed to follow multihit kinetics, suggesting that only CD8 cocultures containing multiple CTL precursors were able to generate sufficiently large CTL populations to display detectable cytotoxicity.

It is formally possible that the increased sensitivity of CFSE-based LDA might be due to confounding factors during coculture resulting in spuriously elevated values for CTL precursors. For example, the increase in CTL precursor frequencies might be accounted for by the proliferation of nontoxic CD8+ T cells, including regulatory or suppressor CD8+ T cells, NK T cells, or γδ TCR CD8+ T cells. In the CFSE-based LDA, such cell types would count toward CTL precursor frequency, but would remain undetected in a cytotoxicity assay. However, intracellular FACS data confirm the persistent expression of IFN-γ and perforin (Fig. 4) in all BLC- and EC-stimulated microcultures at a mean CD8+ input cell level of 1 or lower (a clonal level), identifying these cells as mature CTL by surrogate markers, because direct demonstration of cytotoxic potential is not feasible with these low effector cell numbers. The proportion of cells positive for perforin was comparable between EC- and BLC-stimulated CTL, while the expression level of perforin in individual cells was lower in EC-stimulated CTL. IFN-γ expression was comparable in both types of CTL. Notably, in both EC- and BLC-stimulated cocultures, perforin and IFN-γ expression on activated CD8+ T cells never reached 100% even in clonal populations, confirming previous findings from bulk cocultures (7). These data also suggest that the IFN-γ levels in culture supernatants of primary EC-stimulated
bulk cultures in a previous study were below the assay detection limit (5), primarily as a consequence of the markedly reduced CTL numbers in these cocultures. Methodologically, it is of note that intracellular staining for IFN-γ could be performed in the absence of Golgi export blocking substances such as monensin, most likely due to the abundance of this cytokine in CTL. This direct approach is likely to avoid the interference with quantitative (as opposed to qualitative) measurements of cytokine production introduced by secretion blockade with monensin.

Non-CTL CD8⁺ T cells might also confound the correct determination of precursor frequencies by CFSE-LDA. The phenotypic analysis of alloactivated (CFSE⁺⁺) CD8⁺ cells following EC or BLC stimulation did not show significant populations of γδ TCR T cells or CD56⁺ T cells (NK T cells). In addition, alloactivated CTL from pooled LDA cocultures did not show expression of certain reported surface markers of regulatory or suppressor CD8⁺ cells, such as CD36 or CD57, or the loss of CD28 (8–11) (Fig. 5). The absence of relevant concentrations of IL-10 and TGF-β (cytokines implicated in mediation of the effects of CD8⁺ T cells) from these cultures had been demonstrated previously (5). However, as there are currently no reliable markers for suppressor/regulatory CD8⁺ T cells, the presence of unidentified suppressor cells expressing other or no specific markers cannot be ruled out.

The use of surrogate markers of CTL was required, as direct demonstration of cytotoxicity was prevented by the limited number of effector cells generated at the clonal level, especially following stimulation with EC. Therefore, true EC selectivity of these CTL cannot be demonstrated experimentally in this setting, but based on previous findings from cocultures with higher input cell numbers it appears quite likely that such EC selectivity should be preserved at the clonal level, especially as it was demonstrated in the original description of this type of CTL that EC selectivity was lost with higher input cell numbers (5).

When comparing allostimulation of CD8⁺ T cells by BLC and corresponding EC side by side, the CTL precursor frequencies determined with EC as stimulators were 5- to 40-fold lower than those with BLC. This is a much larger difference than could be explained by the previously described finding that allogeneic EC only activate memory CD8⁺ T cells and not naive CD8⁺ T cells, because memory cells constitute 50% of the peripheral blood T cell population (7). In general, precursor frequencies found with CFSE-based LDA in this study were higher than had been reported for CD8⁺ T cell alloactivation previously. Following allostimulation of purified CD8⁺ T cells with professional APCs precursor frequencies have been reported in the range of ~1/1,000 to 1/6,000 (12, 20–23), whereas we report frequencies from 1/100 to 1/3,300 with BLC as stimulators (Table I). Data on the alloactivation of CD8⁺ T cells and CTL generation by EC are very limited; in an earlier study from our own laboratory (12) and one other report (20) precursor frequencies of 1/20,000 to 1/30,000 were reported for EC-mediated CD8⁺ T cell activation using IL-2 production as a read-out. In contrast, using CFSE-based LDA we now find CTL precursor frequencies after EC stimulation of 1/1,000 to 1/3,000 in three of five experiments (Table I). Incidentally, these increases in sensitivity correspond quite well to data obtained in microbial settings by MHC tetramer staining (13), a technology that currently cannot be applied to T cell alloactivation.

Different CTL precursor frequencies following alloactivation by different types of APC (BLC vs EC) can be accounted for in several ways. Allostimulation with EC may activate only a fraction of the same spectrum of CTL precursors activated by professional APC owing to deficiencies in either alloantigen presentation or costimulation. In this case CTL clones emerging from EC stimulation would overlap in specificity with CTL generated by BLC.
stimulation, but represent a smaller range of anti-allogeneic pre-
cursor specificities or effector cells. Alternatively, differences in
effector cell phenotype could also explain a reduced frequency of
detectable CTL following stimulation with EC. In this case allo-
stimulation by EC would activate a (partly) separate, smaller sub-
set of CTL precursors displaying specificities that do not fully
overlap with those of CTL generated by stimulation with BLC.
This latter explanation is supported by our previous experimental
findings in cocultures using higher input cell numbers that EC
stimulation generates EC-specific CTL incapable of killing BLC
targets (5, 7). However, as CTL specificities cannot be determined
eperimentally at the clonal level due to insufficient CTL numbers
(clonal burst sizes), we are unable to conclusively resolve this
question by experiment. We as yet have no clear explanation why
EC-stimulated CTL from polyclonal cocultures fail to kill BLC yet
display class I MHC restriction. It remains possible that activation
of a separate subset of CTL precursors following stimulation by
EC involves recognition of unique allo-MHC/peptide complexes,
which differ from those presented by other types of APC. Alter-
natively, as has been reported for tubular epithelial cell-specific
alloreactive CTL, specificity may be determined by cell type-spe-
cific accessory interactions, e.g., via adhesion molecules (17). In
any event, the presence of EC-specific CTL in grafts supports the
idea that cytotoxic anti-EC responses can occur independently of
alloresponses against other graft cell types.

Our new CFSE-based LDA provided semiquantitative data on
cell expansion that are generally not obtainable from other types of
LDAs. Although the exact number of alloactivated cells in the
individual microcultures cannot be determined, reasonable esti-
mates of the relative sizes of alloactivated cell populations in in-
dividual microcultures can be derived by counting reactive cells in
wells set up at input numbers likely to contain at most one pre-
cursor clone (clonal burst sizes). Overall, the burst sizes detected
in BLC-stimulated cultures were significantly larger than in cul-
tures stimulated by corresponding EC (Table I). The differences in
average clone sizes were due to the presence of some large burst
sizes following stimulation by BLC, resulting in a discontinuous,
almost bimodal spectrum of clone sizes (Table I, Figs. 2C and 3).
Such variations in cell expansion were also observed between re-
licate cultures of identical responder/stimulator combinations (Ta-
ble I). This differential cell expansion may be due to different lev-
els of costimulation received by the responding CTL precursor cell
or a greater potential for expansion of the CTL precursor subset
activated by BLC compared that activated by EC. Net cell expan-
sion is also be affected by activation-induced cell loss, which may
be differentially regulated by BLC or EC stimulators. Following
activation, naive T cells usually show a greater proliferative re-
response than memory T cells. As EC are only capable of alloacti-
vating memory CD8\(^+\) T cells (7), we at first suspected that the
occurrence of large clone sizes in BLC-stimulated cultures might
reflect the exclusive activation of naive CD8\(^+\) T cells in these
cultures. Although LDA experiments performed with purified na-
ive and memory CD8\(^+\) T cells confirmed the inability of allogeneic
EC stimulators to activate naive T cells (Table II), the large
clonal sizes could not be attributed exclusively to CD8\(^+\) T cells of
the naive subset. In fact, larger clone sizes even appeared to occur
more frequently in memory CD8\(^+\) cell populations (Table II and
Fig. 3). Thus, we currently cannot explain the conditions that give
rise to large burst sizes.

In summary, our new results extend the concept of the vascular
endothelium in an immunomodulatory cell type with properties
distinct from those of professional APC. Stimulation of CD8\(^+\) T
cells by EC appears to favor the alloactivation of smaller (poten-
tially separate?) subsets of memory CD8\(^+\) T cells than stimulation
by professional APC such as dendritic cells or B cells. The direct
demonstration of EC selectivity on a clonal level is precluded by
technical constraints, but it might be speculated, based on previous
findings in bulk cultures (7), that these precursors differentiate into
EC-selective CTL displaying a variety of unique features, includ-
ing an immature activation phenotype, reduced capacity for clonal
expansion, and target cell selectivity. Similar to other tissue-spe-
cific CTL (17, 18) the generation of EC-selective CTL might rep-
resent an independent, tissue-specific alloimmune process. During
generalized graft rejection EC can also be damaged by conven-
tional CTL, but endothelial injury occurs only in the most serious
rejection episodes. In contrast, the exclusive alloactivation of
weaker, EC-selective CTL could provide the basis for intimal ar-
teritis as a form of smoldering endothelial rejection, mediated by
these EC-selective CTL in the absence of generalized parenchymal
rejection, a scenario reminiscent of the postulated early endothelial
injury during the pathogenesis of transplant vasculopathy. Char-
acterization and therapeutic inhibition of EC-selective CTL should
advance our understanding of the pathogenesis of immune-medi-
vated vascular pathology and allow the development of novel ther-
apeutic strategies.

Acknowledgments

We are grateful to Louise Benson, Gwendolyn Davis, and Lisa Gras for
textual technical assistance with cell culture. We also thank Elise Allen
and Sarah Nikiforow for helpful suggestions on CFSE labeling.

References

Transplantation 62:403.


3. Colvin, R. B., A. H. Cohen, C. Saison, S. Bonsib, M. Bauck, B. Burke, S. Carter,
for acute renal allograft rejection: reproducibility, sensitivity, and clinical corre-

4. Dong, C., D. Redenbach, S. Wood, B. Battistini, J. E. Wilson, and
B. M. McManus. 1998. The pathogenesis of cardiac allograft vasculopathy.
Curr. Opin. Cardiol. 11:183.

5. Biedermann, B. C., and J. S. Pober. 1998. Human endothelial cells induce and


7. Dengler, T., and J. Pober. 2000. Human vascular endothelial cells stimulate mem-
ory but not naive CD8\(^+\) T cells to differentiate into CTL retaining an early

8. Sadat-Sowti, B., P. Debre, L. Mollet, L. Quint, F. Hadida, V. Leblond,
by CD8\(^+\) CD57\(^+\) T lymphocytes from patients suffering from AIDS and immu-

9. Quan, C. P., S. Watanabe, F. Vaillier, R. Pires, T. Matsuo, M. Stanislawski,
J. Pillois, and J. P. Bouvet. 1993. Purification and partial amino acid sequence of
suppressive lymphokine from a CD8\(^+\) CD57\(^+\) human T hybridoma. Immunology.
78:205.

of T helper alloreactivity by allo-MHC class I-restricted CD8\(^+\) T cells.
Int. Immunol. 10:775.

11. Cuboatuariu, R., A. I. Colovai, G. Pennesi, Z. Liu, D. Smith, P. Berlocco,
R. Cortesini, and N. Suciu-Foca. 1998. Specific suppression of human CD4\(^+\) Th
cell responses to pig MHC antigens by CD8\(^+\) CD28\(^+\) regulatory T cells. J. Im-
munol. 161:15193.


