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[1]

Thomas J. Dengler, David R. Johnson, and Jordan S. Pober[2]

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$^+$, CFSE^{low} 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. *The Journal of Immunology,* **2001, 166: 3846–3854.**

Alloreactive CTL specific for graft vascular endothelium have been isolated from biopsies of acutely rejecting heart and kidney transplants (1, 2). Such CTL may be the effectors of subintimal endothelialitis (cell-mediated vascular rejection), a potential precursor lesion of chronic graft rejection (transplant vasculopathy) (3, 4). Previous studies from our laboratory have demonstrated that stimulation of CD8$^+$ T cells with allogeneic human endothelial cells (EC)[3] in vitro generates EC-selective CTL with atypical characteristics, including weak cytotoxic activity and undetectable IFN-γ secretion into the cell supernatant (5). These findings have been substantiated further by analysis of EC-specific CTL clones (6). More recent studies of EC-stimulated CTL demonstrated additional differences between EC-specific CTL and conventional B lymphoblastoid cells (BLC)-stimulated CTL. Specifically, we found that following stimulation with EC the resulting CTL displayed an immature, persistently activated phenotype, expressing high levels of CD69, CD25, and CD62L, but low intracellular levels of perforin. Moreover, the analysis of cell division in cocultures of CD8$^+$ cells with EC or BLC with CFSE revealed that the absolute number of alloactivated CTL was markedly lower with EC stimulation despite similar input cell numbers. The lower effector cell number per culture appeared to explain the apparently reduced level of assayable cytotoxicity in EC-specific CTL cultures (7).

These findings raised several new questions concerning the basis of the relatively limited expansion of EC-stimulated CTL. The markedly reduced (10- to 25-fold) absolute number of alloactivated CTL observed following stimulation with EC compared with stimulation with BLC could theoretically be accounted for by several, nonexclusive explanations. First, in purified CD8$^+$ T cell populations the precursor frequency could be lower for EC-stimulated CTL than for BLC-stimulated, conventional CTL. Second, in cocultures of CD8$^+$ T cells with EC, less net cell expansion may occur over a given culture period due to either decreased cell proliferation or an increased rate of cell death in the cultures. Third, the presence of regulatory or suppressor CD8$^+$ T cells (i.e., unusual, perforin-negative cell types, expressing CD57, CD56 or reduced levels of CD28) (8–11) in EC-stimulated cocultures could also reduce the magnitude or extent of CTL differentiation.

To examine these questions we needed to analyze the stimulation of CTL by EC in primary culture at a clonal level, e.g., by comparing the responses of individual CD8$^+$ T cells that give rise to CTL by means of limiting dilution analysis (LDA) after EC and BLC stimulation of allogeneic CD8$^+$ T cells. Pilot experiments demonstrated that a conventional cytotoxicity-based LDA was not

Interdepartmental Program in Vascular Biology and Transplantation, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT 06510

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[2] Address correspondence and reprint requests to Dr. Jordan S. Pober, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510. E-mail address: jordan.pober@yale.edu

[3] Abbreviations used in this paper: EC, endothelial cells; LDA, limiting dilution analysis; BLC, B lymphoblastoid cells.

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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 alloresponsive 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 alloimmune 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). Naive and memory subsets of T cells were isolated from the CD8-selected population by further negative selection. $CD8^+$ T cells were incubated with anti-CD45RA (B-C15; 1 μl/10^7 cells) or anti-CD45RO mAb (UCHL-1; 5 μl/10^7 cells; both mAb from BioSource, Camarillo, CA), respectively, for 30 min at 4°C. After three washes cells were incubated with magnetic beads coated with goat anti-mouse IgG antiserum (Dynal) for 30 min at 4°C. Cell not attached to beads were recovered during application of a magnet; negatively selected populations thus obtained were routinely >95% double positive for CD8 and either CD45RA or CD45RO, respectively, and were >99% viable.

EC were isolated from umbilical veins by enzymatic digestion and maintained in culture as previously described (14). EC cultures were free from detectable $CD45^+$ contaminating leukocytes and uniformly expressed von Willebrand factor and CD31. BLC obtained from the same donors as the EC were generated by EBV immortalization of cord blood mononuclear cells (5). After 6–8 wk in culture, BLC lines were uniformly CD19 positive. EC and BLC from the same donor were used as stimulator cells in cocultures and as target cells in cytotoxicity assays. When BLC were used as stimulator cells in cocultures, they were pretreated with mitomycin C (50 μg/ml in PBS, 30 min; Sigma-Aldrich, St. Louis, MO) to prevent proliferation. This treatment did not affect the outcome of cultures stimulated with EC and was routinely omitted.

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% CO_2 at 37°C. The medium for coculture consisted of RPMI 1640 supplemented with 10% human AB 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 again 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 matching 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 $CD8^+$ cells. $CD8^+$ T cells cultured in the absence of any stimulator cells, but receiving identical IL- 2 supplementation, were used as negative controls for the highest and lowest input 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, and 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 directly PE-conjugated mouse anti-human CD8, CD25, CD69, $\gamma\delta$ TCR, 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 with 1% paraformaldehyde in PBS. FACS gates were set to identify viable cells, and a minimum of 5000 gated events/sample were collected. To perform intracellular staining for flow cytometric analysis, cells were washed twice in ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. After washing twice, cells were incubated for 15 min in permeabilization solution consisting of 1% FCS, 0.1% saponin (Sigma-Aldrich), and 0.1% sodium azide. Cells were then incubated with primary unconjugated mouse anti-human perforin mAb (T Cell Science, Woburn, MA) in permeabilization solution for 1 h at 4°C. After washing twice in permeabilization solution, cells were incubated with PE- conjugated goat anti-mouse IgG antiserum (Jackson Research, Minneapolis, MN) for 1 h at 4°C. Alternatively, cells were incubated with PE-conjugated mouse anti-human IFN-γ Ab (PharMingen). Cells were finally washed twice in permeabilization solution and once in PBS/1% BSA and stored in PBS/1% BSA until analysis by flow cytometry as above.

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 Biostatistics 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 $CD8^+/CFSE^{high}$, 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 ($CD8^+/CFSE^{low}$). Fig. 1 shows examples of two-color FACS plots from typical cultures, illustrating the presence and size of the populations of unstimulated $CD8^+/CFSE^{high}$ cells and alloactivated $CD8^+/CFSE^{low}$ cells at different input cell numbers. At sufficiently low input cell numbers, the culture becomes negative, i.e., no $CD8^+/CFSE^{low}$ cells can be detected while, in most cases, residual nonactivated cells ($CD8^+$ $CFSE^{high}$) are still seen. $CD8^+$ T cells freshly labeled with CFSE and unlabeled cells are shown for comparison. The intensity of CFSE fluorescence in alloactivated $CD8^+/CFSE^{low}$ 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 $CFSE^{low}$ 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 $CD8^+/CFSE^{low}$ cells were considered positive, and only cultures that did not show any $CD8^+/CFSE^{low}$ cells were considered negative. This was possible because in all experiments there were at least ~10 $CD8^+/CFSE^{low}$ 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), $CFSE^{high}$ 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. 2*A*.

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 $CD8^+/CFSE^{low}$ 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 $CD8^+/CFSE^{low}$ 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. 2*B*, 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. 2*C*. Initial experiments revealed a wide variation of such burst sizes even in cultures raised against the same stimulator cells (Fig. 2*C*).

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

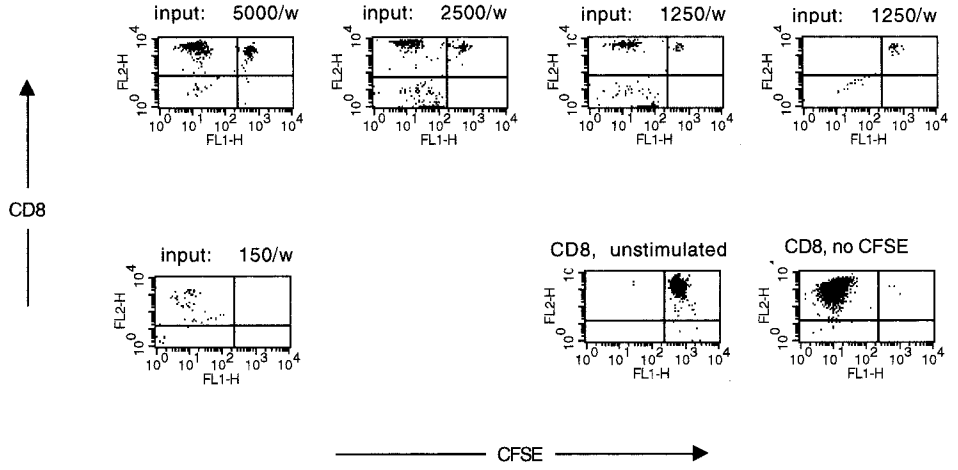

FIGURE 1. Two-color fluorescence analysis of $CD8^+$ T cells harvested from BLC-stimulated cocultures. $CFSE^{low}$ $CD8^+$ cells (*upper left quadrant*) represent alloactivated $CD8^+$ T cells, i.e., CTL. The *top row* shows an example of a serial dilution of $CD8^+$ T cells stimulated with EC resulting 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 ($CFSE^{low}$ $CD8^+$ cells present; *third panel*) and one negative culture (no $CFSE^{low}$ $CD8^+$ 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 ($CD8^+$ $CFSE^{high}$; *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

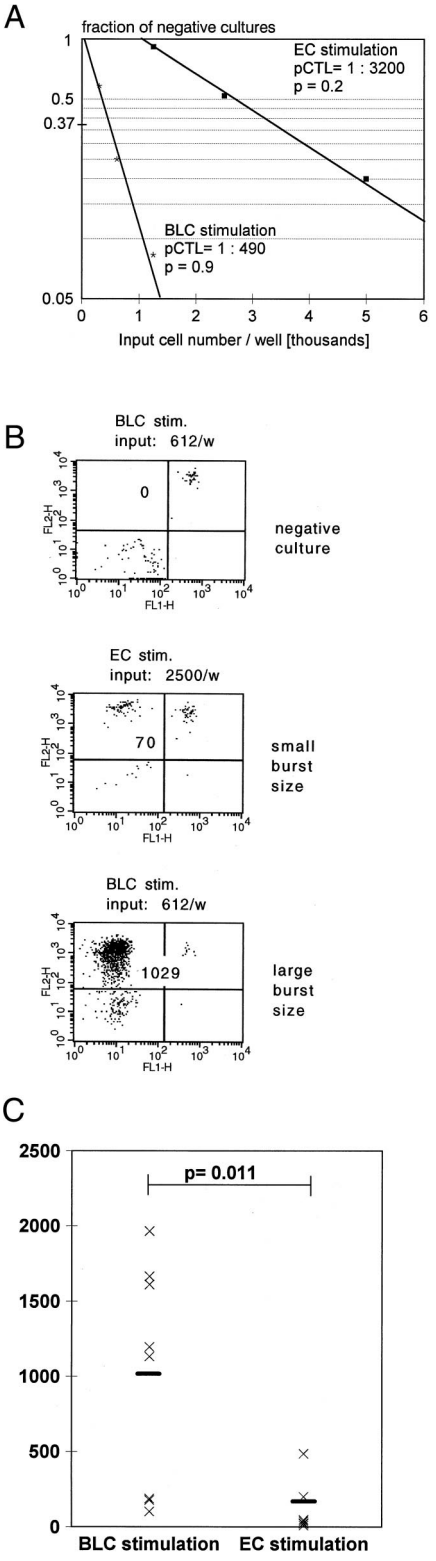

FIGURE 2. *A*, Computer-assisted determination of CTL precursor frequencies by CFSE-based LDA; Expt. 4 of Table I is shown as a representative example. The fraction of negative cultures (no alloactivated, i.e., $CFSE^{low}$ $CD8^+$ T cells, detectable by flow cytometry) is plotted against input $CD8^+$ 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 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). *C*, Quantitative comparison of clonal burst sizes after BLC or EC stimulation of $CD8^+$ T cells. Burst sizes of alloactivated $CD8^+$ T cells from microcultures with input cell numbers at or below the calculated precursor frequency (i.e., containing a mean of ≤1 precursor cell) were determined as described above. Burst sizes were plotted, and statistical differences between BLC- and EC-stimulated cultures were calculated using nonparametric *U* test. Results are from Expt. 4 in Table I.

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. 2*C*; 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 naïve, 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

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)*

Stimulation	pCTL Frequency	Probability[a]	Burst Sizes (at 1 pCTL/well)	p for Difference in Burst Sizes
Expt. 1				
BLC	1:130 (50–210)[b]	0.68	22, 26, 28, 65, 110, 338, 616	$p < 0.05$
EC	1:1000 (710–1290)	0.71	8, 10, 12, 15, 16, 20, 21, 314	
Expt. 2				
BLC	1:3300 (2100–4500)	0.2	80, 190, 229, 327, 435, 513, 1006, 1322	$p < 0.05$
EC	1:17,675 (12,575–22,775)	0.33	17, 21, 24, 36, 92, 201, 379, 429	
Expt. 3				
BLC	1:600 (285–915)	0.6	25, 30, 36, 84, 86, 90, 120, 2300	p = NS
EC	1:28,000 (22,180–33,820)	0.6	10, 13, 47, 51, 95, 156, 300	
Expt. 4				
BLC	1:490 (310–670)	0.9	104, 181, 191, 1029, 1199, 1612, 1666, 1965	$p < 0.02$
EC	1:3200 (2520–3880)	0.2	10, 22, 25, 27, 39, 70, 203, 212, 486	
Expt. 5				
BLC	1:440 (235–645)	0.2	Burst sizes not determined	
EC	1:3200 (1825–4575)	0.48		

[a] Probability of "single-hit" kinetics of CTL generation. Values of $p > 0.05$ identify single hit.
[b] Range of precursor frequencies are in parentheses.

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. 3*B*). 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 CD25^{low} and CD69^{low} (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 $\gamma\delta$ 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.

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)*

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

[a] Burst sizes are shown separately in Fig. 3, *A* and *B*.
[b] Probability of "single-hit" kinetics of CTL generation. Values of $p > 0.05$ identify single hit.
[c] Range of precursor frequencies are in parentheses.
[d] In EC-stimulated cultures of naive CD8$^+$ T cells, with maximal input cell numbers of 100,000/well, no alloactivation could be detected.

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 (CD25^{high}, CD69^{high}, CD62L^{high}), 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

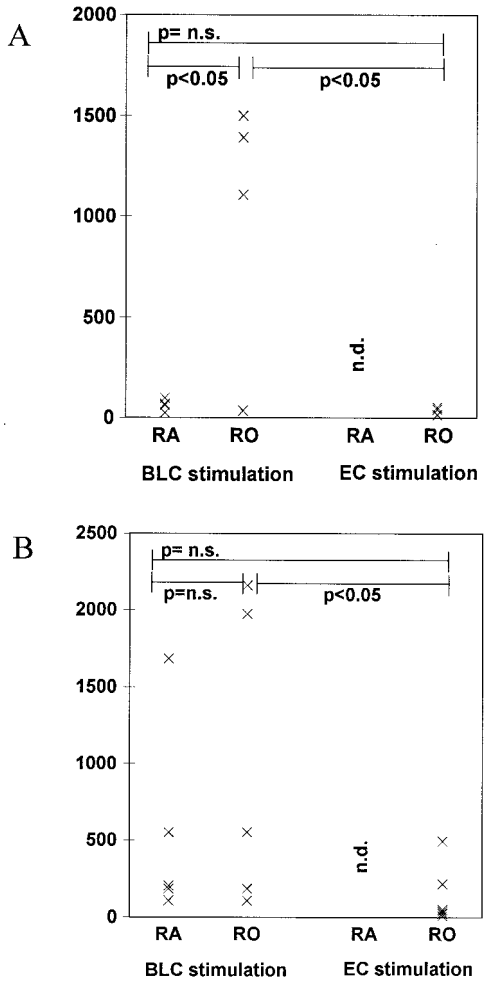

FIGURE 3. Burst size comparison of cultures of naive (CD45 RA^+) or memory (CD45 RO^+) $CD8^+$ T cells stimulated with BLC or EC, respectively. The results of two independent experiments are shown. No alloactivated $CD8^+$ T cells can be detected in cocultures of naive $CD8^+$ T cells stimulated with EC (n.d., not detectable). Burst sizes of EC-stimulated cultures of memory cells were always significantly smaller than after stimulation with corresponding BLC. In Expt. A, burst sizes in BLC-stimulated cocultures of memory (RO) $CD8^+$ T cells were significantly larger than those in cultures of naive (RA) cells; in Expt. B this difference was not significant. RA, naive $CD8^+$ T cells; RO, memory $CD8^+$ T cells; n.s., not significant.

cultures, i.e., lower CTL precursor frequencies, limited proliferation, or generation of regulatory cells could not be evaluated in the polyclonal systems in which this phenomenon was first observed.

In the present study we developed a new form of LDA, based on FACS analysis and dilution of the intracellular dye CFSE as an indicator of cell activation and division, in primary cultures of $CD8^+$ T cells using either BLC or EC as stimulator cells. The assay provided markedly enhanced sensitivity for the detection of CTL as well as an opportunity to evaluate clonal growth and phenotype. The three principal findings of our study are that the precursor frequency for CTL in purified $CD8^+$ T cell populations stimulated with EC is significantly lower than that in BLC-stimulated cultures (despite the genetic identity of the EC and BLC stimulators), that the net expansion of alloreactive clones is significantly smaller after stimulation of memory $CD8^+$ cells with EC compared with BLC stimulation, and that EC do not appear to stimulate regulatory $CD8^+$ T cells, as judged by surface phenotype. Specifically, CTL precursor frequencies in EC-stimulated $CD8^+$ 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 $CD8^+$ $CFSE^{low}$ 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 $CFSE^{low}$, i.e., alloactivated and expanded, cells. In some instances at very low $CD8^+$ input cell numbers the nonactivated $CFSE^{high}$ cells were even lost completely from the cultures, leaving only alloactivated $CFSE^{low}$ 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 expansion, 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 noncytotoxic $CD8^+$ T cells, including regulatory or suppressor $CD8^+$ T cells, NK T cells, or $\gamma\delta$ 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

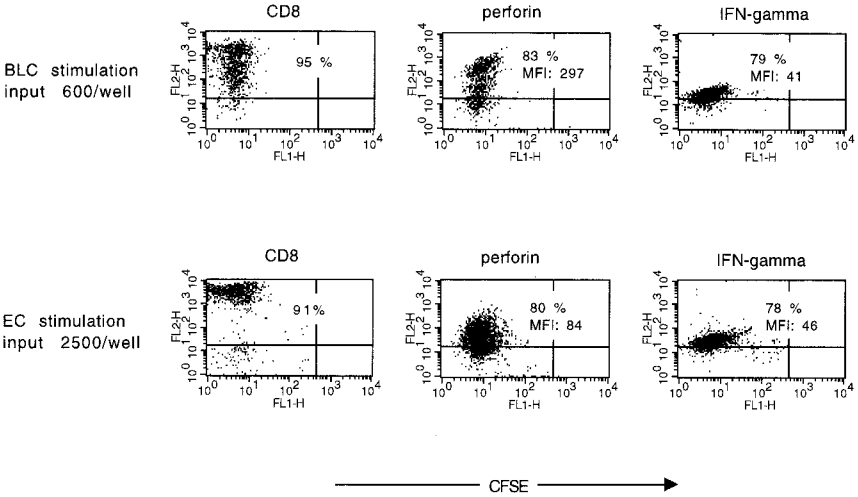

FIGURE 4. Expression of perforin and IFN-γ in BLC- or EC-stimulated CD8$^+$ T cells. Two-color flow cytometric analysis of T cells from microcultures with CD8 input cell numbers at or below the calculated precursor frequency (i.e., containing a mean of $\leq$1 precursor cell). CFSE^{low} CD8$^+$ cells represent alloactivated cells (CTL; *upper left quadrant* in CD8 panels). Perforin and IFN-γ staining is shown for BLC- and EC-stimulated cultures. Numbers indicate the percentage of CFSE^{low} cells expressing CD8, perforin, or IFN-γ, for perforin and IFN-γ the mean fluorescence intensity (MFI) is also shown for CFSE^{low} cells that stain positively for the respective Ag (*upper left quadrant*). Note that the expression level for perforin is higher in BLC-stimulated cells, while the proportion of positive cells is comparable. A representative result is shown for 10 analyzed microcultures for each type of stimulator cell.

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^{low}) CD8$^+$ cells following EC or BLC stimulation did not show significant populations of $\gamma\delta$ 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 CD56 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.

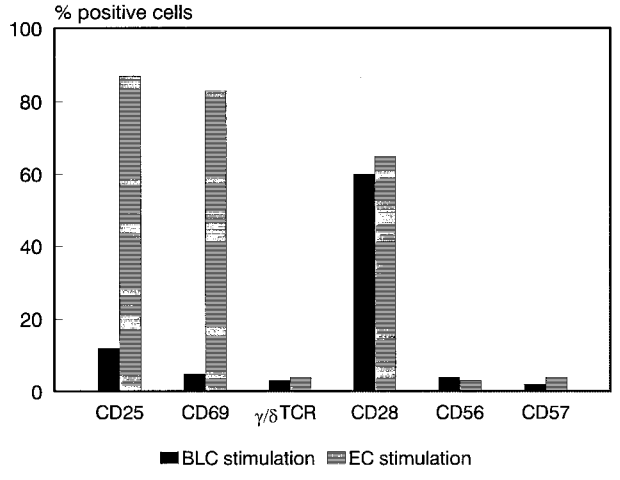

FIGURE 5. Surface Ag expression on alloactivated CD8$^+$ T cells after stimulation with BLC or EC. Cells recovered from individual microcultures at or below the calculated precursor frequency (i.e., containing a mean of $\leq$1 precursor cell) were pooled and analyzed by two-color flow cytometry for CFSE and the indicated surface Ags. Expression levels of the respective surface Ags on alloactivated (CFSE^{low}) cells only are shown. Note significant differences in CD25 and CD69 expression as described before (see text). A representative result is shown of 10 analyzed microcultures for each type of stimulator cell.

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$^+$ 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 precursor 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 allostimulation by EC would activate a (partly) separate, smaller subset 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 experimentally 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. Alternatively, as has been reported for tubular epithelial cell-specific alloreactive CTL, specificity may be determined by cell type-specific 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 estimates of the relative sizes of alloactivated cell populations in individual microcultures can be derived by counting reactive cells in wells set up at input numbers likely to contain at most one precursor clone (clonal burst sizes). Overall, the burst sizes detected in BLC-stimulated cultures were significantly larger than in cultures 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. 2*C* and 3). Such variations in cell expansion were also observed between replicate cultures of identical responder/stimulator combinations (Table I). This differential cell expansion may be due to different levels 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 expansion 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 response than memory T cells. As EC are only capable of alloactivating 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 naive and memory $CD8^+$ T cells confirmed the inability of allogeneic EC stimulators to activate naive T cells (Table II), the large clone 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 (potentially 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, including an immature activation phenotype, reduced capacity for clonal expansion, and target cell selectivity. Similar to other tissue-specific CTL (17, 18) the generation of EC-selective CTL might represent an independent, tissue-specific alloimmune process. During generalized graft rejection EC can also be damaged by conventional 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 arteritis 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. Characterization and therapeutic inhibition of EC-selective CTL should advance our understanding of the pathogenesis of immune-mediated vascular pathology and allow the development of novel therapeutic strategies.

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References

1. Jutte, N. H., C. J. Knoop, P. Heijse, A. H. Balk, B. Mochtar, F. H. Claas, and W. Weimar. 1996. Human heart endothelial-cell-restricted allorecognition. *Transplantation 62:403.*
2. Deckers, J. G., M. R. Daha, S. W. Van der Kooij, and F. J. Van der Woude. 1998. Epithelial- and endothelial-cell specificity of renal graft infiltrating T cells. *Clin. Transplant. 12:285.*
3. 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.*
4. Dong, C., D. Redenbach, S. Wood, B. Battistini, J. E. Wilson, and B. M. McManus. 1996. 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 regulate cytolytic T cell differentiation. *J. Immunol. 161:4679.*
6. Biedermann, B. C., and J. S. Pober. 1999. Human vascular endothelial cells favor clonal expansion of unusual alloreactive CTL. *J. Immunol. 162:7022.*
7. Dengler, T., and J. Pober. 2000. Human vascular endothelial cells stimulate memory but not naive $CD8^+$ T cells to differentiate into CTL retaining an early activation phenotype. *J. Immunol. 164:5146.*
8. Sadat-Sowti, B., P. Debre, L. Mollet, L. Quint, F. Hadida, V. Leblond, G. Bismuth, and B. Autran. 1994. An inhibitor of cytotoxic functions produced by $CD8^+CD57^+$ T lymphocytes from patients suffering from AIDS and immunosuppressed bone marrow recipients. *Eur. J. Immunol. 24:2882.*
9. Quan, C. P., S. Watanabe, F. Vuillier, R. Pires, T. Matsuo, M. Stanislawski, J. Pillot, and J. P. Bouvet. 1993. Purification and partial amino acid sequence of suppressive lymphokine from a $CD8^+$ $CD57^+$ human T hybridoma. *Immunology 78:205.*
10. Liu, Z., S. Tugulea, R. Cortesini, and N. Suciu-Foca. 1998. Specific suppression of T helper alloreactivity by allo-MHC class I-restricted $CD8^+CD28^-$ T cells. *Int. Immunol. 10:775.*
11. Ciubotariu, 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. Immunol. 161:5193.*
12. 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.*
13. Kerksiek, K. M., and E. G. Pamer. 1999. T cell responses to bacterial infection. *Curr. Opin. Immunol. 11:400.*

14. Pober, J. S., M. P. Bevilacqua, D. L. Mendrick, L. A. Lapierre, W. Fiers, and M. A. Gimbrone, Jr. 1986. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J. Immunol. 136:1680.*
15. Song, H. K., H. Noorchashm, Y. K. Lieu, S. Rostami, S. A. Greeley, C. F. Barker, and A. Naji. 1999. Tracking alloreactive cell division in vivo. *Transplantation 68:297.*
16. Weston, S. A., and C. R. Parish. 1990. New fluorescent dyes for lymphocyte migration studies: analysis by flow cytometry and fluorescence microscopy. *J. Immunol. Methods 133:87.*
17. Hadley, G. A., E. A. Rostapshova, and S. T. Bartlett. 1996. Dominance of tissue-restricted cytotoxic T lymphocytes in the response to allogeneic renal epithelial cell lines. *Transplantation 62:75.*
18. Heath, W. R., and L. A. Sherman. 1991. Cell-type-specific recognition of allogeneic cells by alloreactive cytotoxic T cells: a consequence of peptide-dependent allorecognition. *Eur. J. Immunol. 21:153.*
19. Warren, E. H., P. D. Greenberg, and S. R. Riddell. 1998. Cytotoxic T-lymphocyte-defined human minor histocompatibility antigens with a restricted tissue distribution. *Blood 91:2197.*
20. 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. I. Frequency and phenotype of human T helper lymphocytes that can react to allogeneic endothelial cells. *J. Immunol. 148:3753.*
21. Moretta, A., G. Pantaleo, L. Moretta, M. C. Mingari, and J. C. Cerottini. 1983. Quantitative assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T cell populations. *J. Exp. Med. 158:571.*
22. Jooss, J., T. H. Eiermann, H. Wagner, and D. Kabelitz. 1989. Interleukin 2 production by alloantigen-stimulated $CD4^+$ and $CD8^+$ human T cell subsets: frequency of HLA class I or class II-reactive precursor cells and clonal specificity of activated T cells. *Immunobiology 179:366.*
23. Kabelitz, D., W. R. Herzog, B. Zanker, and H. Wagner. 1985. Human cytotoxic T lymphocytes. I. Limiting-dilution analysis of alloreactive cytotoxic T-lymphocyte precursor frequencies. *Scand J. Immunol. 22:329.*