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Human Endothelial Cells Effectively Costimulate Cytokine Production by, But Not Differentiation of, Naive CD4+ T Cells

Weilie Ma* and Jordan S. Pober2†

We compared costimulatory signals provided by human endothelial cells (ECs) to those provided by conventional bone marrow-derived APCs, i.e., peripheral blood-adherent mononuclear cells (PBAMCs), by measuring their effects on cytokine production by naive or memory CD4+ T cells stimulated by PHA. In these assays, ECs effectively costimulate secretion of IL-2, IFN-γ, and IL-4 from both naive and memory CD4+ T cells, quantified by ELISA or intracellular cytokine staining. ECs, which lack B7 molecules, use predominantly leukocyte-function associated Ag 3 (LFA-3) to provide costimulation. ECs are comparable to or better than PBAMCs, which use both the LFA-3 and B7 molecules, at costimulating IL-2 and IL-4 production. ECs are less effective than PBAMCs at costimulating IFN-γ production by naive T cells. ECs do not secrete IL-12, and addition of exogenous IL-12 enables ECs to costimulate IFN-γ at a level comparable to that observed with PBAMCs. ECs do not promote differentiation of naive T cells to Th1-like cells, whereas PBAMCs do. Again, addition of exogenous IL-12 enables ECs to do so. Transfection of ECs to express B7-1 or B7-2 is less effective than IL-12 supplementation for restoring these responses. These experiments suggest that a deficiency in costimulation due to lack of B7 molecule expression does not fully explain the inability of ECs to activate resting naive CD4+ T cells. The Journal of Immunology, 1998, 161: 2158 –2167.

The activation of T cells requires two types of signals (1, 2). Signal 1 is Ag dependent and is delivered by clustering the T cell Ag receptor-CD3 complex through engagement of specific foreign peptides bound to self MHC molecules on the surface of an APC. Signal 1 may be mimicked by anti-CD3 Ab or lectins (e.g., PHA) that cluster TCR complexes, bypassing the requirement for Ag. Signal 2, called costimulation, is Ag independent and may be provided by some combination of soluble factors such as cytokines, or more often, by surface ligands on the T cell that interact with their counterreceptors on the T cell. Several molecules expressed on human APCs have costimulatory functions, but the most important are B7-1 (CD80) and B7-2 (CD86), which interact with T cell CD28 (3); and LFA-3 (CD58), which interacts with T cell CD2 (4). Costimulatory signals are essential to induce maximal T cell cytokine secretion, proliferation, and induction of effector function. Engagement of the TCR without requisite costimulation often fails to induce an immune response and may instead result in a state of Ag-specific unresponsiveness, termed clonal anergy (5), or in some cases, T cell apoptosis (6).

The resting T cell population in human blood consists of roughly equal numbers of naive cells, which have not previously encountered Ag, and memory cells, which have undergone a number of changes as a consequence of previous activation. In humans, naive cells generally express the A isoform of CD45 (i.e., CD45RA), whereas memory cells generally express the O isoform (i.e., CD45RO) (7). Typically, naive cells have a more stringent requirement for costimulation than do memory cells, although these differences are probably quantitative rather than qualitative (i.e., the same costimulatory molecules appear to interact with T cells at both stages of differentiation) (8–11).

Once activated by Ag, naive CD4+ T cells may differentiate to either Th1-like or Th2-like effector cells. These two T cell subsets are defined by the cytokines they secrete (12). Th1-like cells characteristically secrete IL-2, IFN-γ, and/or lymphotoxin and serve to activate phagocyte-mediated immune responses. The signature cytokines of Th2-like cells are IL-4 and/or IL-5. IL-4 is the major inducer of IgE production by B cells and is therefore a key initiator of IgE-dependent, mast cell-mediated reactions. IL-5 activates eosinophils, the effector cells of Th2-mediated inflammatory responses. Various factors have been found to direct the differentiation of naive CD4+ T cells to Th1 vs Th2 subsets. The most potent in vitro factor is the cytokine environment. Differentiation to Th1 is promoted by the macrophage-derived cytokine IL-12 (13, 14), which also boosts IFN-γ production by activated T and NK cells (15, 16). The production of IL-12 by macrophages can be induced by various bacteria, intracellular pathogens, viruses, and activated T cells. The role of IL-12 in Th1 cell differentiation is supported by the finding that IL-12 p40 knockout mice have severely impaired Th1 responses (17). Differentiation to Th2 is promoted by IL-4, which may be produced by mast cells, basophils, and/or a population of T cells (18, 19). IL-4 knockout mice have impaired production of IgE and a paucity of IL-5-secreting T cells (20, 21). In addition to these cytokines, differentiation of naive T cells to Th1 vs Th2 may also be affected by the Ag quantity and affinity or the availability of the specific costimulatory molecules. In vivo, these factors may be influenced by the nature of the Ag, its mode of entry, and its tissue distribution (22).

Vascular endothelial cells (ECs) have been thought to act as APCs in vivo, capable of substituting for conventional bone marrow-derived accessory cells (23). Cultured human vascular ECs express class I and (in response to T cell signals) class II MHC

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3 Abbreviations used in this paper: ECs, endothelial cells; PBAMCs, peripheral blood-adherent mononuclear cells; LFA-3, leukocyte function-associated Ag 3; PE, phycoerythrin; ICE, IL-1-converting enzyme.
CD4 on ECs may account for the inability of ECs to activate naive T cells, measured as the production of IL-2 or proliferation (26, 27). In contrast, bone marrow-derived "professional" APCs, such as peripheral blood-adherent mononuclear cells (PBAMCs), are able to activate both naive and memory resting allogeneic T cells. This limited capacity of ECs to activate T cells places them in a separate category of "semiprofessional" APCs, since nonimmunologic tissue cells, such as vascular smooth muscle cells or fibroblasts, are unable to activate any resting T cell populations, although such tissue cells can productively restimulate activated T blasts (28–32). The different capability of ECs and PBAMCs for activating alloreactive T cells may reside in differences in the Ags displayed and/or in provision of costimulation. Human vascular ECs express LFA-3 but not B7 molecules, whereas human PBAMCs express both LFA-3 and B7-2 and/or B7-1 on their surfaces (33, 34). It has been proposed that the lack of B7 molecules on ECs may account for the inability of ECs to activate naive CD4+ T cells (27).

In the present study, we wished to determine whether costimulation provided by ECs was in fact deficient for full activation of naive CD4+ T cells or whether other factors might contribute to lack of responsiveness of naive T cells to Ags presented on cultured ECs. To examine this question, we activate CD4+ T cell subpopulations with PHA, bypassing signal 1, in the presence of either ECs or PBAMCs, which act as a source of signal 2. The strength of signal 2 is measured as augmented cytokine secretion. We find that costimulation provided by ECs enhances IL-2, IFN-γ, and IL-4 production from naive as well as memory CD4+ T cells, and that in general, ECs are comparable or better than PBAMCs as a source of signal 2. The principal defects observed with ECs compared with PBAMCs are that ECs are less effective costimulators of IFN-γ production by naive CD4+ T cells and that ECs are unable to promote differentiation of naive cells into Th1 effector cells. These defects can be remedied by supplementing the EC cocultures with soluble IL-12, a cytokine produced by activated monocytes and that in general, ECs are comparable or better than PBAMCs as a source of signal 2.

**Materials and Methods**

**Cell isolation and culture**

Human ECs were isolated from umbilical veins and serially cultured on human plasma fibronectin-coated tissue culture plastic (Falcon, Lincoln Park, NJ) in 20% FCS, medium 199, and 2.5 mM l-glutamine (all from Life Technologies, Grand Island, NY), and EC growth factor (Collaborative Biomedical Products, Bedford, MA) as previously described (35). For the experiments described in this report, ECs were used at passage level 3 to 5, because such cultures are free of contaminating CD45+ leukocytes by immunofluorescence microscopy yet stain uniformly positive for EC markers (von Willebrand factor and CD31). PBAMCs were obtained by leukapheresis from adult volunteer donors and further purified by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, NC) according to the manufacturer's instructions. Isolated PBAMCs were washed three times in HBSS (Mg2+- and Ca2+-free) and either used immediately or suspended in 10% DMEM and 90% heat-inactivated FCS and cryopreserved in liquid nitrogen. No differences were seen in the responses of cells recovered from cryopreservation compared with freshly isolated cells.

CD4+ T cells were isolated from PBAMCs by negative selection. In brief, PBMCs were first depleted of monocytes by adherence to human plasma fibronectin-coated petri dishes for 45 min at 37°C. Nonadherent cells were decanted and incubated with a mixture of mAb containing LB3.1 (IgG2a, anti-HLA-DR monomorphic determinant, a gift from Dr. J. Strominger, Harvard University, Cambridge, MA), B-H7 (IgG1, anti-CD8; Biosource, Camarillo, CA), and 3G8 (IgG1, anti-CD16; a gift from Dr. J. Unkeless, Mt. Sinai School of Medicine, New York, NY) at saturating conditions for 30 min at 4°C. The cells were then washed three times with RPMI 1640 (Life Technologies)/5% FCS to remove excess Ab and further enriched by magnetic immunodepletion using goat anti-mouse IgG-bound Dynabeads (Dynal, Lake Success, NY) according to the manufacturer's instruction. The purity of the isolated CD4+ T cells was determined by direct immunofluorescence labeling with anti-CD4, anti-CD8, anti-CD3, and anti-CD16 (for NK cells), anti-CD19 (for B cells), and anti-CD45R (for monocytes) (all Abs from Coulter, Keneesaw, GA), and FACs analysis using a FACSort running LYSIS II software (Becton Dickinson, Mountain View, CA). The CD4+ T cell populations used in these studies typically consisted of >90% CD4+ cells and contained no detectable (<1%) CD8+, CD14+, CD16+ or CD19+ cells. Naïve CD4+ T cells (CD45RA+CD45RO-) were negatively selected by adding mAb UCHL.1 (IgG2a, anti-CD45RO, a gift of Dr. P. Beverley, University of London, U.K.) into the mAb mixture, and memory CD4+ T cells (CD45RO+CD45RA-) were enriched by adding mAb B-C15 (IgG1, anti-CD45RA; Biosource, Camarillo, CA) into the mAb mixture. The purity of CD45RA+ or CD45RO+ cells ranged from 90 to 97%.

PBAMCs were purified by incubating PBMCs for 3 h on human plasma fibronectin-coated plates at 37°C, followed by removal of nonadherent cells by two washes with HBSS. PBAMCs were incubated in RPMI/10% FCS at 37°C in a 5% CO2 incubator until use, usually within 1 h of purification. These populations typically consisted of a mixture of ~80% CD14+ monocytes and a smaller number (10–20%) of CD19+ B cells; few if any T cells remained in these preparations.

**Primary costimulation assay**

Tissue culture round-bottom 96-well plates (Falcon) were coated with human plasma-derived fibronectin and then seeded with 20,000 ECs or PBAMCs or were mock seeded with cell-free medium. Purified T cells (200,000) of defined subpopulations were added to each well in a final volume of 200 μl of RPMI 1640 medium containing 10% FCS, 2.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The medium was changed and supplemented with fresh medium of PHA, B-C15, or IL-12, neutralizing mAb, IgG1 from PharMingen, San Diego, CA; IgG2b negative control from Dako, Glostrup, Denmark; K16/16 (nonbinding IgG1), a gift from Dr. D. Mendrick, Brigham and Women’s Hospital, Boston, MA; and CTLA4-Ig fusion protein (anti-CD80 and anti-CD86) and control human Ig, gifts from Dr. G. Gray, Repligen, Cambridge, MA. All Abs were used at concentrations optimized for maximal functional inhibition, usually 10 to 50 μg/ml, as determined in preliminary experiments.

mAb used for intracellular cytokine staining were MQ1-17H12 (anti-IL-2 receptor a-chain/p55 subunit, IgG1, used at 20 μg/ml), a gift from Dr. T. Waldmann, National Institute of Health, Bethesda, MD; TS2/9 (anti-CD4, IgG2a, PE conjugated); all were obtained from PharMingen. Inhibitory mAb used in the costimulation studies were anti-TAC (anti-IL-2 receptor c-chain/p55 subunit, IgG1, used at 20 μg/ml), a gift from Dr. T. Waldmann, National Institute of Health, Bethesda, MD; TS2/9 (anti-CD4, IgG2a, PE conjugated); all were obtained from PharMingen. mAb used for intracellular cytokine staining were MQ1-17H12 (anti-IL-2 receptor c-chain/p55 subunit, IgG1, used at 20 μg/ml), a gift from Dr. T. Waldmann, National Institute of Health, Bethesda, MD; TS2/9 (anti-CD4, IgG2a, PE conjugated); all were obtained from PharMingen. Human CD80 and CD86 cDNA expression constructs and empty control vectors were kind gifts from Dr. A. Bothwell, Yale University, New Haven, CT (37).

**Diffenitation assay**

Tissue culture 24-well plates (Falcon) were coated with human plasma-derived fibronectin and then seeded with 120,000 ECs or PBAMCs or were mock seeded with cell-free medium. Purified naïve CD4+ T cells (800,000) were added in 800 μl of RPMI 1640/10% FCS containing 2 μg/ml PHA.
This was referred to as the primary culture. In IL-12 supplementation experiments, human rIL-12 (R&D Systems) was added to indicated cultures at a final concentration of 4 ng/ml. A 100-μl sample of the medium was collected at 24 h of culture and measured for cytokine production. After 72 h of culture, T cells were collected and washed twice with RPMI 1640/5% FCS, then rested for another 72 h in 5 ml of RPMI 1640/10% FCS. The cells were recollected, washed, and then resus- pended in staining buffer and subjected to two-color FACs analysis using E. The fixed cells were then washed once with staining buffer (Dulbecco’s PBS with 1% FCS) and then fixed in 200 μl fixation buffer (Dulbecco’s PBS with 4% paraformaldehyde) for 20 min or overnight at 4°C. The fixed cells were then washed once with staining buffer, once with permeabilization buffer (Dulbecco’s PBS with 1% FCS, 0.1% saponin), then incubated for 30 min at 4°C with a pair of mAbs: FITC-conjugated anti-IL-2 mAb and PE-conjugated anti-IFN-γ mAb, or FITC-conjugated anti-IFN-γ mAb and PE-conjugated anti-IL-4 mAb. One microliter of each mAb was used per test. After two washes with permeabilization buffer and one wash with staining buffer, the cells were resus- pended in staining buffer and subjected to two-color FACs analysis using FACSort running LYSIS II software.

**Intracellular cytokine staining**

For characterization and enumeration of cytokine producers in the primary cultures, purified naive or memory CD4^+ T cells were activated by PHA alone or by PHA in the presence of either ECs or PBAMCs for 15 h. Monensin (2 μM) was added for the last 8 h. For analysis of cytokine producers in secondary cultures, CD4^+ T cells were activated by 10 ng/ml PMA plus 1 μM ionomycin in the presence of 2 μM monensin for 4 h. The same conditions were chosen to optimize detection based on preliminary experiments. In both cases, the harvested CD4^+ T cells were washed once with staining buffer (Dulbecco’s PBS with 1% FCS) and then fixed in 200 μl fixation buffer (Dulbecco’s PBS with 4% paraformaldehyde) for 20 min or overnight at 4°C. The fixed cells were then washed once with staining buffer, once with permeabilization buffer (Dulbecco’s PBS with 1% FCS, 0.1% saponin), then incubated for 30 min at 4°C with a pair of mAbs: FITC-conjugated anti-IL-2 mAb and PE-conjugated anti-IFN-γ mAb, or FITC-conjugated anti-IFN-γ mAb and PE-conjugated anti-IL-4 mAb. One microliter of each mAb was used per test. After two washes with permeabilization buffer and one wash with staining buffer, the cells were resus- pended in staining buffer and subjected to two-color FACs analysis using a FACSort running LYSIS II software.

**Results**

**ECs costimulate CD4^+ T cells to produce IFN-γ and IL-4, as well as IL-2**

IFN-γ-treated human ECs can stimulate allogeneic resting CD4^+ T cells to proliferate (39). However, IFN-γ-treated ECs stimulate fewer CD4^+ T cells to secrete IL-2 than do allogeneic professional APCs, such as PBAMCs (40). It is not known whether this difference reflects a more limited capacity to present alloantigens (e.g., fewer or different peptide-MHC complexes) or provision of less effective costimulation or both. To address this question, we experimentally separated costimulation from Ag presentation by using a polyclonal mitogen, PHA, to simulate signals provided by Ag recognition. The effects of the costimulation provided by ECs vs PBAMCs were then compared by measuring the extent to which these cells can augment the production of IFN-γ and IL-4, as well as IL-2, as a function of varying PHA concentrations. PHA-treated CD4^+ T cells in the absence of accessory cells produced little measurable cytokine (Fig. 1). Coculture of PHA-treated CD4^+ T cells with ECs markedly increased the production of all three cytokines, whereas coculture of PHA-treated CD4^+ T cells with PBAMCs significantly increased the production of IL-2 and IFN-γ, but not IL-4 (Fig. 1). The concentration of PHA that resulted in optimal costimulation by ECs or PBAMCs for all three cytokines was 3 μg/ml; this concentration was used in all subsequent studies.

**ECs costimulate naive as well as memory CD4^+ T cell production of IL-2, IFN-γ, and IL-4**

We and others have found that human ECs are able to activate IL-2 secretion by allogeneic resting memory T cells but are not able to activate secretion by resting naive T cells (26, 27). In contrast, PBAMCs can activate both resting naive and memory T cells to secrete IL-2. To study whether ECs can provide costimulation to naive or memory T cells, CD4^+ T cells were further separated into naive (CD45RO^-) or memory (CD45RA^-) subpopulations. As expected from experiments using total CD4^+ T cells, neither PHA- treated naive nor memory CD4^+ T cells produced much cytokine in the absence of accessory cells (Figs. 2 and 3). Interestingly, ECs were able to provide effective costimulation for production of all three cytokines by both naive and memory CD4^+ T cells. In these experiments, memory cells secreted significantly higher levels of all three cytokines, especially IFN-γ and IL-4, than did naive cells, such that memory cell responses (Fig. 3) constituted the majority of the response seen with total CD4^+ T cells (Fig. 1). Surprisingly,
in light of the allogeneic responses observed previously, ECs were fully comparable to PBAMCs in providing costimulation for IL-2 secretion by naive CD4⁺ T cells (Fig. 2). Indeed, compared with PBAMCs, ECs consistently provided better costimulation to both naive and memory CD4⁺ T cells for the production of IL-4. However, PBAMCs generally provided better costimulation to naive CD4⁺ T cells for the production of IFN-γ (Fig. 2), although this difference was not observed in memory T cell responses (Fig. 3).

We extended these observations of whole cell populations to the single-cell level by use of an intracellular cytokine staining technique (Fig. 4 and Table I). In these experiments, PHA alone, in the absence of accessory cells, activated few cytokine producers in naive or memory CD4⁺ T cell populations (Table I). In the presence of ECs or PBAMCs, distinct populations of cytokine producers were activated. We found that the number of cytokine producers that were activated was roughly proportional to the net amount of cytokines detected in the total culture supernatant (compare Table I with Fig. 2 and Fig. 3). Also consistent with the ELISA results, ECs costimulated more IL-4 producers and fewer IFN-γ producers in the naive CD4⁺ T cell population and comparable or greater numbers of all three cytokine producers in the memory CD4⁺ T cell population than did PBAMCs (Table I).

**Costimulation provided by ECs for IFN-γ and IL-4 production is partly mediated by enhanced IL-2 production**

The experiments described above indicate that costimulation provided by ECs increases IFN-γ and IL-4 as well as IL-2 production by CD4⁺ T cells. However, it is possible that IFN-γ and IL-4 production may increase indirectly through IL-2-mediated effects (41, 42). To determine to what extent the increased IL-2 contributes to the observed costimulation of IFN-γ and IL-4, mAb (anti-TAC) to the IL-2R was added to block the response to IL-2 in the cultures. This mAb reduced IFN-γ production by 50% and IL-4 production by 75% when either naive or memory CD4⁺ T cells were cocultured with ECs, and it inhibited secretion of these cytokines even more when T cells were cocultured with PBAMCs. Inhibition of IL-2 signaling did not appear to affect the production of IL-2 itself, indicating that the effect was specific and not due to toxicity. However, inhibition in the presence of ECs was never
FIGURE 4. Representative pattern of intracellular cytokine staining in a costimulation assay. Examples of dot plots obtained from memory CD4+ T cells activated by PHA in the presence of ECs are shown. After activation, memory CD4+ T cells were stained by: A, control mAb for IL-2 and IFN-γ; B, mAb for IL-2 and IFN-γ; C, control mAb for IFN-γ and IL-4; and D, mAb for IFN-γ and IL-4. Percentage of cytokine producers activated was equal to percentage of cells that were reactive with specific anti-cytokine mAb minus percentage of cells that were reactive with the appropriate control mAb.

Table I. ECs costimulate production of IL-2, IFN-γ, and IL-4 by naive and memory CD4+ T cells

<table>
<thead>
<tr>
<th></th>
<th>% of Cytokine Producers Activated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>Naive CD4+ T cells</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>0.47</td>
</tr>
<tr>
<td>ECs + PHA</td>
<td>2.77</td>
</tr>
<tr>
<td>PBAMCs + PHA</td>
<td>2.07</td>
</tr>
<tr>
<td>Memory CD4+ T cells</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>2.3</td>
</tr>
<tr>
<td>ECs + PHA</td>
<td>6.3</td>
</tr>
<tr>
<td>PBAMCs + PHA</td>
<td>3.59</td>
</tr>
</tbody>
</table>

a Purified naive or memory CD4+ T cells were activated by PHA alone or in the presence of either ECs or PBAMCs for 15 h. Monensin was present for the last 8 h of the activation. The cells were then washed and stained for FACS analysis. Percentage of cytokine producers activated was equal to percentage of cells that were reactive with certain cytokine mAb minus percentage of cells that were reactive with the appropriate control mAb.

b One of two similar experiments using two different donors.

c One of three similar experiments using two different donors.
memory T cells by ECs, but can provide additional signals beyond those provided by LFA-3.

ECs do not secrete IL-12, a PBAMC-derived cytokine with marked effect on IFN-\(\gamma\) production

IL-12 is a potent inducer of IFN-\(\gamma\) production and Th1 differentiation (13, 14). Activated PBAMCs but not ECs produce IL-12 (Table II). As predicted, neutralizing mAb to IL-12 did not inhibit IFN-\(\gamma\) production by naive or memory CD4\(^+\) T cells using ECs as accessory cells, whereas the same Ab inhibited about 80% of IFN-\(\gamma\) produced by naive or memory CD4\(^+\) T cells using PBAMCs as accessory cells (Fig. 8). These results suggest that lack of IL-12 in the EC cultures could contribute to the diminished IFN-\(\gamma\) secretion observed with naive CD4\(^+\) T cells compared with cultures with PBAMCs as accessory cells. To further investigate the effects of IL-12 on IFN-\(\gamma\) production, we added IL-12 into the cocultures of naive or memory CD4\(^+\) T cells with ECs. Addition of IL-12 to such cocultures significantly increased IFN-\(\gamma\) production by naive CD4\(^+\) T cells, but did not strongly affect the production of IL-2 or IL-4 (Fig. 9). Similar results were obtained when IL-12 was added to cocultures of memory CD4\(^+\) T cells with ECs (data not shown).

ECs do not support naive CD4\(^+\) T cell differentiation into Th1 cells

Upon activation, naive CD4\(^+\) T cells may differentiate into either Th1-like or Th2-like effectors depending on their environment. We

Table II. Activated PBAMCs but not ECs secrete IL-12

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ECs</th>
<th>PBAMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>LPS (10 (\mu)g/ml)</td>
<td>Undetectable</td>
<td>12</td>
</tr>
<tr>
<td>IFN-(\gamma) (1000 U/ml)</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>LPS + IFN-(\gamma)</td>
<td>Undetectable</td>
<td>153</td>
</tr>
<tr>
<td>Human CD40L trimer (10 (\mu)g/ml)</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Human CD40L trimer + IFN-(\gamma)</td>
<td>Undetectable</td>
<td>7</td>
</tr>
<tr>
<td>CD4(^+) T (2 (\times) 10(^5) cells/well) + PHA</td>
<td>Undetectable</td>
<td>12</td>
</tr>
</tbody>
</table>

\(\text{ECs and PBAMCs were treated with different reagents for 24 h. IL-12 secreted into the supernatant was then measured by ELISA.}\)

PBAMCs as accessory cells (Fig. 8). These results suggest that lack of IL-12 in the EC cultures could contribute to the diminished IFN-\(\gamma\) secretion observed with naive CD4\(^+\) T cells compared with cultures with PBAMCs as accessory cells. To further investigate the effects of IL-12 on IFN-\(\gamma\) production, we added IL-12 into the cocultures of naive or memory CD4\(^+\) T cells with ECs. Addition of IL-12 to such cocultures significantly increased IFN-\(\gamma\) production by naive CD4\(^+\) T cells, but did not strongly affect the production of IL-2 or IL-4 (Fig. 9). Similar results were obtained when IL-12 was added to cocultures of memory CD4\(^+\) T cells with ECs (data not shown).

ECs do not support naive CD4\(^+\) T cell differentiation into Th1 cells

Upon activation, naive CD4\(^+\) T cells may differentiate into either Th1-like or Th2-like effectors depending on their environment. We

FIGURE 6. PBAMCs but not ECs use B7 molecules to costimulate production of IL-2, IFN-\(\gamma\), and IL-4 by naive and memory CD4\(^+\) T cells. A combination of CTLA-4Ig fusion protein and mAb reactive to B7-2 (IT2.2) were added to: A, cocultures of naive CD4\(^+\) T cells; and B, cocultures of memory CD4\(^+\) T cells. Cytokines were measured by ELISA after 24 h. Results from three independent experiments were shown. Percentage of inhibition was calculated as stated in Materials and Methods.

FIGURE 7. B7-expressing ECs show increased costimulation for IL-2, IFN-\(\gamma\), and IL-4 production by naive CD4\(^+\) T cells. Naive CD4\(^+\) T cells were activated by PHA in the presence of ECs transfected with control empty vector or ECs transfected with either B7-1 or B7-2. After 24 h, cytokines secreted into the supernatant were measured by ELISA. Results shown are representative of four experiments with three different donors.

FIGURE 8. Neutralizing mAb to IL-12 inhibits IFN-\(\gamma\) secretion in cocultures with PBAMCs but not in cocultures with ECs. mAb to IL-12 was added to: A, cocultures of naive CD4\(^+\) T cells; and B, cocultures of memory CD4\(^+\) T cells. After 24 h, cytokines were measured by ELISA. Results from three independent experiments are shown. Percentage of inhibition was calculated as stated in Materials and Methods.
endothelial costimulation of naive CD4+ T cells

next assessed whether ECs could promote differentiation of activated naive CD4+ T cells to Th1 or Th2 effector cells. To examine this question, purified naive CD4+ T cells were initially activated by PHA in the presence of PBAMCs. IL-12 (4 ng/ml) was added to coculture with ECs. Cytokines secreted into the supernatant were measured by ELISA. Results shown are representative of four experiments with three different donors.

We next investigated whether ECs actively inhibited the differentiation or merely failed to promote differentiation to Th1 cells by adding human IL-12 into the primary cultures. Addition of IL-12 into the primary cultures with ECs significantly increased IFN-γ production during the secondary stimulation in response to PMA and ionomycin (Fig. 10A). In contrast, the primary culture with PBAMCs resulted in increased IFN-γ production in the secondary culture (Fig. 10, A and B), while neither accessory cell type appeared to augment IL-4 production in the secondary culture (data not shown). This suggests that PBAMCs but not ECs support Th1 differentiation.

In a final series of experiments, we investigated whether CD4+ T cells were activated by PHA alone or in the presence of either ECs or PBAMCs. IL-12 (4 ng/ml) was added to cells cultured with PHA in the absence of accessory cells or in the presence of accessory cells. After 72 h, the lymphocytes were recovered, washed, and rested for 72 h, and then restimulated in an accessory cell-independent manner by PMA plus ionomycin. The cytokines produced in the secondary culture were measured at 24 h. Naive cells costimulated with ECs or PBAMCs as accessory cells in primary cultures produced comparable levels of IL-2 in secondary culture, although this level was not significantly higher than that produced by naive T cells treated with PHA in the absence of accessory cells (Fig. 10, A and B). This effect was greater than that observed by adding IL-12 into the primary cultures with no accessory cells. In contrast to the effects of IL-12, transfection of ECs to express B7-1 or B7-2 did not significantly increase Th1 type differentiation (Fig. 10A). These data suggest that lack of IL-12 in the cocultures with ECs can explain the decreased level of Th1 differentiation compared with that in cocultures with PBAMCs.

Results from intracellular cytokine staining are consistent with the ELISA results and show that costimulation provided by PBAMCs but not by ECs in the primary culture supported Th1 differentiation and increased the IFN-γ secretors from 0.46% in the absence of accessory cells and 0.33% in the presence of ECs, to 3.39% in the presence of PBAMCs (Table III). Further, IL-12 addition to the primary culture with ECs increased IFN-γ secretors to 3.63% in the secondary culture (Table III).

In a final series of experiments, we investigated whether CD4+ T cells were initially activated by PHA in the presence of ECs, and then in a secondary culture, these CD4+ T cells were activated again by PHA in the presence of either ECs or PBAMCs. Such CD4+ T cells secreted significantly more IFN-γ in the secondary culture with PBAMCs than with ECs, suggesting that they were still responsive to the IL-12 produced by PBAMCs (Fig. 11A). To confirm that increased IFN-γ production in the coculture with PBAMCs is due to the production of IL-12 by PBAMCs, neutralizing mAb to IL-12 was added to secondary cultures with PBAMCs. Upon mAb addition, the production of IFN-γ in the secondary coculture with PBAMCs was decreased to the same level as in the coculture with ECs (Fig. 11B). The simplest interpretation of these experiments is that ECs fail to induce naive CD4+ T cells to differentiate into Th1 cells because they are unable to secrete IL-12. However, such T cells remain responsive to IL-12.

Discussion

In humans, microvascular endothelial cells constitutively express both class I and class II MHC molecules (43). Since the lumen of
a capillary is narrower than the diameter of a resting T cell, circulating T cells are likely to encounter Ags displayed on the EC surface. Therefore, the key question is not whether ECs can present Ags but, rather, what is the result of such an encounter. Previous studies from our laboratory and others have suggested that class II-expressing cultured human ECs (i.e., after IFN-γ pretreatment) are able to stimulate T cell proliferation in response to alloantigens or recall Ags (39, 44, 45). More recent experiments suggest that only memory (CD45RO+) CD4+ T cells respond to alloantigens presented by ECs, measured by production of IL-2 (26, 27). This has led to the suggestion that ECs are unable to provide adequate costimulation to activate naive T cells, perhaps because human ECs lack expression of B7-1 and B7-2 (27).

We and others have previously shown that cultured human ECs can provide effective costimulation for IL-2 and IFN-γ production by CD4+ T cells (34, 40, 46). These results were confirmed in the present studies and extended to show that ECs can also provide significant costimulation for IL-4 production by CD4+ T cells. These effects of ECs are fully comparable to those of PBAMCs. To study whether ECs can effectively costimulate naive as well as memory CD4+ T cells, we separated total CD4+ T cells into naive and memory T cell subsets. Our results show that the total CD4+ T cell response is dominated by memory cells, since these cells produced much more cytokines than naive cells. However, contrary to expectations, our results show that ECs also provide effective costimulation for naive CD4+ T cells.

In experiments using naive CD4+ T cells, we found that ECs actually provided better costimulation for IL-2, less effective costimulation for IFN-γ, and comparable or better costimulation for IL-2 production than did PBAMCs. These cytokine measurements were supported and extended by individual T cell assays using intracellular cytokine staining technique and FACS analysis, which showed that ECs appeared to costimulate more IL-2 secretors, more IL-4 secretors, and fewer IFN-γ secretors among naive CD4+ T cells than did PBAMCs. However, we cannot exclude the additional possibility that increased cytokine production is also due to more cytokine being produced by each individual activated CD4+ T cell. Surprisingly, even in the naive T cell population, few T cells made both IFN-γ and IL-4. Similar results for human peripheral blood T cells have been seen by others (47). This lack of detection of double cytokine producers may imply that the intracellular cytokine staining technique is too limited in sensitivity to accurately phenotype low cytokine producers, including most naive T cells.

Costimulation is largely dependent on cell surface ligands, especially B7 molecules, and in humans, on LFA-3. A key molecular difference between ECs and PBAMCs is that ECs express LFA-3 but not B7 molecules, whereas PBAMCs express both LFA-3 and B7 molecules. Both B7-1 and B7-2 costimulate through CD28. Costimulation through CD28 increases IL-2 transcription as well as stabilizing IL-2 mRNA, resulting in a markedly increased level of IL-2 secretion by newly activated T cells (48, 49). Emerging evidence also shows that a major role of CD28 signaling may be to prevent apoptosis during T cell activation (6). LFA-3 is expressed widely on hemopoietic and nonhemopoietic tissues and has been identified in humans as the major ligand for CD28 expressed on all T cells. Costimulation through CD28 results in enhanced T cell proliferation and IL-2 transcription (4). However, in some circumstances, CD28 costimulation appears to deliver more potent signals than CD28 costimulation, i.e., CD28 costimulation prevents anergy, whereas CD28 costimulation can actually reverse anergy (50).

In the present study, we find from mAb blocking experiments that LFA-3 may contribute not only to IL-2 transcription but also to augmentation of IFN-γ and IL-4. Surprisingly, the effect of LFA-3 on IL-4 is more clear-cut in experiments using ECs as accessory cells where B7 molecules are also present. Our transfection experiments show that both signals could augment the results obtained using ECs as accessory cells. These data are consistent with previous results showing that B7-transfected fibroblasts can boost anti-EC allogeneic responses through trans-costimulation (27). However, our data suggest that lack of costimulation through B7 is not an adequate explanation for the failure of human ECs to activate allogeneic naive CD4+ T cells.

Why are ECs less able to provide costimulation than PBAMCs for IFN-γ production by naive CD4+ T cells? IFN-γ production can be regulated by cytokine costimulation as well as surface molecule costimulation. IL-12 and IFN-γ-inducing factor (IGIF) are two cytokines that augment IFN-γ production (16, 51, 52), and there is a synergistic effect on IFN-γ production between IGIF and IL-12 (53). Reagents are not yet available for direct measurement of IGIF. However, IGIF mRNA was detected in total RNA of ECs by RT-PCR (our unpublished observations). IGIF,
synthesized as a precursor peptide, has to be cleaved by IL-1-converting enzyme (ICE) to become active (54, 55). Thus the ICE-inhibitory peptide YVAD can inhibit active IGF1 production. In preliminary experiments, we have observed that addition of YVAD inhibited IFN-γ production by naive CD4+ T cells cultured with either ECs or PBAMCs by ~30 to 40%. On the other hand, IL-12 was detected only in the supernatant of CD4+ T cells cocultured with PBAMCs but not with ECs. Accordingly, neutralizing anti-IL-12 mAb did not inhibit IFN-γ production in cocultures with ECs, whereas the same mAb inhibited IFN-γ production by 75% in cocultures with PBAMCs. Consistent with a recent report of others (46), we found that IL-12 had little effect on IFN-γ production by cultured CD4+ T cells in the absence of ECs, but that addition of IL-12 to cocultures of T cells with ECs could augment IFN-γ production by as much as sevenfold. This suggests that ECs can enhance responsiveness of naive CD4+ T cells to IL-12.

Why are ECs better able than PBAMCs to provide costimulation for IL-4 production by naive CD4+ T cells? LFA-3 appeared to be a less important costimulator for IL-4 production than for IL-2 or IFN-γ, since anti-LFA-3 mAb had inconsistent effects on IL-4 production in cocultures with ECs. IL-4 production is very dependent on IL-2 and was reduced by ~80% in cocultures with ECs or PBAMCs by anti-IL-2R mAb. However, increased IL-4 production in cocultures with ECs is unlikely to be wholly dependent on enhanced IL-2 production, since even in experiments in which ECs costimulated similar levels of IL-2 production as PBAMCs, the ECs still costimulated more IL-4 production than PBAMCs. Increased IL-4 production is also probably not a result of less IFN-γ production, since a blocking anti-IFN-γ receptor mAb did not increase IL-4 production in cocultures with PBAMCs (our unpublished observation). It has been shown that mouse IL-6 can induce IL-4 production by naive CD4+ T cells (56) and that human ECs produce more IL-6 than PBAMCs in cultures. However, anti-IL-6 mAb failed to reduce IL-4 production in cocultures with ECs to the same level as in cocultures with PBAMCs (our unpublished observations). Thus, further studies will be needed to determine which signals presented by ECs constitute the effective costimulation of IL-4.

The most profound defects in EC costimulation that we have observed is the inability of ECs to promote Th1 differentiation. This was not remedied by transfection of ECs to express B7-1 or B7-2, but could be rescued by addition of exogenous IL-12. Thus, the lack of IL-12 secretion appears to be the major deficiency of ECs as accessory cells compared with bone marrow-derived professional APCs.

Although our current studies have concentrated on the capacity of ECs to provide accessory functions to naive CD4+ T cells, our data also show that ECs appear superior to PBAMCs as accessory cells for the activation of memory CD4+ T cells. This capacity of ECs is consistent with a proposed Ag-presentation function. We would suggest that memory T cells passing luminal capillaries in peripheral tissues would readily sample the Ags present in those tissues as displayed in the form of peptide-MHC complexes on the luminal EC surface. Recognition of Ag by a circulating T cell could rapidly up-regulate adhesion by activation of integrins such as LFA-1 (CD11a/CD18), favoring T cell egress at the site where foreign Ag is displayed. In other words, presentation of Ag by ECs could improve the efficiency of immune surveillance for foreign peptides by selective recruitment of Ag specific T cells. Why are naive cells unaffected by their interaction with ECs? Our data presented here suggest that lack of costimulation due to the absence of B7 molecules is unlikely to be a complete explanation. Perhaps the differences in adhesion molecules expressed by naive cells simply do not allow the T cell-EC interaction to persist long enough for a signal to be delivered. In our in vitro system, the need for adhesion is bypassed by PHA cross-linking of the T cell to the accessory cell surface.

In conclusion, ECs do not express B7 molecules and do not produce IL-12, yet provide effective costimulation for cytokine production by naive as well as memory CD4+ T cells. These results suggest that a deficiency in costimulation, specifically the lack of B7 molecules, does not fully explain the inadequacy of ECs to activate resting naive CD4+ T cells. However, the deficiency in IL-12 may impede the capacity of ECs to promote IFN-γ secretion or Th1 differentiation. We hypothesize that lack of adhesion between ECs and naive CD4+ T cells may be the major factor that prevents interactions between these two cell types. This may account for why naive T cell activation and differentiation occurs primarily in secondary lymphoid organs, outside of the bloodstream, where professional APCs are available to provide more effective interaction.

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References


IL-4 are required for in vitro generation of IL-4-producing cells. J. Exp. Med. 172:921.


