Endothelial Cells Modify the Costimulatory Capacity of Transmigrating Leukocytes and Promote CD28-mediated CD4⁺ T Cell Alloactivation

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Summary

Activated vascular endothelial cells (ECs) express major histocompatibility complex (MHC) class II molecules in vitro and in vivo in acute and chronic allograft rejection. However, human ECs may be limited in their ability to effectively activate CD4⁺ T cells, because they do not express members of the B7 family (CD80 and CD86) of costimulatory molecules. In this study, we show that ECs promote the full activation of CD4⁺ T cells via trans-costimulatory interactions. By reverse transcriptase polymerase chain reaction, Western blot, and FACS® analysis, we could not detect the expression of CD80 and CD86 on activated ECs and found minimal expression on purified CD4⁺ T cells. In contrast, both CD80 and CD86 were expressed in allogeneic CD4⁺ T cell–EC cocultures. Expression of CD86 peaked at early times between 12 and 24 h after coculture, whereas CD80 was not expressed until 72 h. Addition of anti-CD86 but not anti-CD80 monoclonal antibodies to cocultures inhibited IL-2 production and the proliferation of CD4⁺ T cells to allogeneic donor human umbilical vein ECs (HU-VECs), as well as to skin and lung microvascular ECs. Furthermore, we found that interferon-γ–activated ECs but not untreated ECs induced mRNA and cell surface expression of CD80 and CD86 on CD4⁺ T cells, and these T cells were functional to provide a trans-costimulatory signal to allologous CD4⁺ T cells. Blockade of MHC class II and lymphocyte function–associated antigen 3 but not other EC cell surface molecules on IFN-γ–activated ECs inhibited the induction of CD86 on CD4⁺ T cells. Transmigration of purified populations of monocytes across EC monolayers similarly resulted in the induction of functional CD86, but also induced the de novo expression of the cytokines interleukin (IL)-1α and IL-12. In addition, EC-modified monocytes supported enhanced proliferation of allogeneic and autologous CD4⁺ T cells. Taken together, these data define the ability of the endothelium to modify CD4⁺ T cells and monocytes for trans-costimulatory events. This unique function of the endothelium in alloimmune T cell activation has functional consequences for the direct and the indirect pathways of allorecognition.

Key words: endothelium • T lymphocyte • monocyte • allorecognition • transplantation

Recognition of alloantigen (donor major or minor histocompatibility antigens) by CD4⁺ T cells is the initiating event that ultimately leads to graft rejection. Recipient CD4⁺ T cells may recognize either intact allo-MHC class II molecules on donor cells (known as direct allorecognition) or peptides derived from allo-MHC molecules, shed from the allograft, and subsequently processed and presented bound to MHC class II on recipient APCs (known as indirect allorecognition) (1). Irrespective of the pathway of allorecognition, full activation of naive or previously activated CD4⁺ T cells requires a second signal that may be provided by soluble factors such as cytokines, but more often is provided by cell surface costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) (2, 3). These molecules are expressed by professional APCs and bind the counterreceptors CD28 and cytolytic T lymphocyte–associated antigen (CTLA)4 expressed by CD4⁺ T cells. Ligation of
CD28 is necessary for maximal CD4+ T cell cytokine production, proliferation, and prevention of activation-induced apoptosis (4, 5). In contrast, ligation of CTLA4 delivers a negative signal to CD4+ T cells (6).

It is well established that activated endothelial cells (ECs) express class II MHC molecules in vitro and in vivo in rejecting allografts, and may therefore provide antigen-dependent signals to CD4+ T cells for direct activation (7–11). An unresolved issue is whether ECs can provide the costimulatory signals required to fully activate CD4+ T cells (12–16). Current evidence suggesting a role for ECs in CD4+ T cell activation is based heavily on in vitro studies, and only a few studies have provided indirect evidence in vivo (17, 18). The majority of in vitro studies show that class II–expressing ECs can induce IL-2 production and alloproliferation of bulk populations of CD4+ T cells, an effect largely dependent on costimulation by endothelial lymphocyte function–associated antigen (LFA)-3 (12–15). However, inhibition of CD2-2-LFA-3 interactions with anti–LFA-3 mAbs only partially inhibits EC-induced CD4+ T cell cytokine production and alloproliferation, suggesting a role for other costimulatory molecules in this process. Although B7–dependent costimulation has been demonstrated in allogeneic mouse EC–induced T cell activation (19) and xenogenic pig EC–induced human T cell activation (20, 21), firm conclusions have not been made as to whether CD28–B7 interactions are functional in the activation of human CD4+ T cells by human ECs. This issue is relevant for a proactive role of ECs in rejection, because activation of naïve CD4+ T cells (induction of primary immune responses) and the activation of previously activated CD4+ T cells is dependent on CD28-mediated costimulation (22, 23). Indeed, in the absence of CD28-mediated signals, naïve CD4+ T cells may be rendered refractory to further stimulation by antigen (clonal anergy) (24), or reactivation responses may be qualitatively different (25). Furthermore, recent studies have demonstrated a requirement for B7 costimulation at the local site of inflammation (26). In these studies we investigate the role of CD28–B7 interactions in human EC–induced CD4+ T cell alloactivation. Our data provide new insight into the ability of ECs to modify leukocytes to promote direct (and perhaps indirect) allore cognition and define novel mechanisms by which allograft ECs may promote transplant rejection.

Materials and Methods

Reagents and Antibodies. Antibodies used in these studies include seven anti-CD86 mAbs; IT2.2 and FU N-1 clone (PharMingen), anti–human CD86 (Serotec), anti–human CD86 (B7-F3, gift from P. Linsley, Bristol Myers Squibb, Princeton, NJ), H2F 3D1, HA5 2B7, HA3 1F9 (gifts from V.J. Kuchroo, Brigham and Women's Hospital, Boston, MA), anti–human CD80 (PharMingen), anti–human CD25 (PharMingen, San Diego, CA); anti–human LFA-3 (I6E, gift from P. Hochman, Biogen Inc., Cambridge, MA), anti–human CD40 (220; a gift from D. Hollenbaugh, Bristol Myers Squibb), anti–human OX 40 (Ancell Corp.), anti–human intercellular adhesion molecule (ICAM)-1 (R R 1/1, a gift from T.A. Springer, Center for Blood Research, Harvard Medical School, Boston, MA), anti–HLA-DR (LB3.1, a gift from A.H. Lichtman, Brigham and Women's Hospital), and negative control mouse IgG (K16/16, a gift of M. Giribone, Brigham and Women's Hospital). Human CTLA4 Ig and control fusion protein were gifts from Dr. Peter Linse (Bristol Myers Squibb). Cytokines used were recombinant human IFN-γ (Genzyme) and TNF-α (a gift from Biogen Inc.). Other reagents used included recombinant soluble CD154 (a gift from D. Hollenbaugh), LPS (Sigma Chemical Co.), and PHA (Sigma Chemical Co.).

Cell Isolation and Culture. Endothelial cells were isolated from human umbilical cords as previously described (27) and were grown in M 199 medium (BioWhittaker) containing 10% FCS (GIBCO BRL), EC growth supplement, 1% penicillin/streptomycin, 1-glutamine, and heparin. Cultured cells were harvested in trypsin/ethylene diaminetetraacetic acid (Sigma Chemical Co.) and subcultured for use at passages 2–4. Saphenous vein ECs were a gift from Dr. P. Libby (Brigham and Women's Hospital) (28). Single donor, dermal, and lung microvascular ECs were purchased from Clonetics.

Cell membrane fractions of ECs or CD4+ T cells were prepared as previously described (29). In brief, untreated or IFN-γ–treated ECs (2–4 × 106) were harvested by gentle scraping, washed, and resuspended in lysis buffer containing 0.25 M sucrose, 10 mM Tris (pH 7.4), 10 mM NaCl, 0.1 M MgCl2, and 1 mM PMSF. The cells were lysed by homogenization and were centrifuged at 1,000 rpm for 15 min. Supernatants were centrifuged at 100,000 × g for a further 30 min. All manipulations were performed at 4°C. Pellets were resuspended in RPMI and added directly to CD4+ T cells (106) in 96-well plates. For select experiments, cell membranes were prepared from unactivated or mitogen-activated CD4+ T cells (107 cells/condition).

PBMCs were isolated by Ficoll–Hypaque gradient centrifugation from blood obtained from healthy volunteers. CD4+ T cells were isolated from PBMCs by positive selection using anti–CD4+–coated magnetic beads (Dynal Inc.) according to the manufacturer's instructions. Magnetic beads were subsequently removed using Detachabead (Dynal Inc.). In some experiments CD4+ T cells were further purified by negative depletion of CD14 and HLA-DR expressing cells using a CD14-coated microbead column (MiniMACS separation column; Miltenyi Biotec) and panning on anti–HLA-DR (LB3.1) coated plastic culture dish respectively. The purity of the CD4+ T cells using these methods was 98 and 99.7%, respectively. Purity was assessed by double stain FACS® analysis for CD3 and CD4 cell surface markers. Purified cells were unactivated as assessed by the lack of spontaneous proliferation, IL-2 and IFN-γ production, and CD25 cell surface expression, as previously described (30). Human monocytes were isolated from plateletpheresis residues by centrifugation on density gradients (LSM; Organon Teknika), followed by counterflow centrifugation elutriation (31). Monocytes isolated by this technique are >90% pure and are relatively unactivated as determined by minimal alterations in cell surface activation markers. In some experiments, monocytes were isolated from PBMCs by positive selection using CD14-coated microbeads (MiniMACS separation column; Miltenyi Biotec).

CD80–, CD86–, and neomycin-transfected Chinese hamster ovary (CHO) cells (a gift from Dr. G. Freeman, Dana Farber Cancer Institute, Boston, MA) were cultured in collagen-coated tissue culture flasks in complete RPMI with 10% FCS. Cells were harvested by trypsinization and fixed in 0.4% PFA before addition to EC–CD4+ T cell cocultures.

CD4+ T cell–EC Coculture. Primary cultures of ECs (passages
3–4) were treated with IFN-γ (1,000 U/ml) for 72 h to upregulate class II MHC. IFN-γ-treated ECs (5 × 10⁴/well) were then irradiated (1,750 rads) and cocultured with resting CD4+ T cells (5 × 10⁴/well) in 96-well cell culture plates in a final volume of 200 µl. Additional cells or reagents were added as indicated. Coculture supernatants were taken at days 3 and 5 for cytokine analysis by specific ELISA. Proliferation was assessed after 6 d by [³H]thymidine incorporation for the last 18 h of coculture. Cells were harvested by an automated cell harvester and incorporated radioactivity was assessed by a Beckman Betamax counter.

In separate experiments, we examined whether ECs modify CD4+ T cells to express functional CD86. CD4+ T cells were cultured on IFN-γ-treated human umbilical vein EC (HUVEC) monolayers for 24–72 h and then reisolated by positive selection using CD4-coated magnetic beads (Dynal Inc.). These cells were termed “EC-modified” CD4+ T cells. EC-modified CD4+ T cells (10⁴) were irradiated (1,750 rads) and cocultured with resting autologous CD4+ T cells (5 × 10⁴) in the presence of suboptimal doses of PHA (0.3 µg/ml). Coculture supernatants were taken at 24 h for specific ELISA. Proliferation was assessed after 3 d of coculture as described above.

Transmigration Assay. Transmigration assays were performed using a protocol modified, as follows, from one described previously (32). HUVECs were seeded at 2 × 10⁵ cells/cm² on collagen-coated, 8-µm-pore size polycarbonate tissue culture Transwell inserts (Costar Corp.) and were cultured for 5 d to attain confluence. Confluency was assessed by exclusion of Transwell inserts (Costar Corp.) and were cultured for 5 d to upregulate MHC class II. Purified, resting monocytes (5 × 10⁴) were added onto the transwell and allowed to transmigrate through the filters. Transmigrated cells were counted and RNA was isolated from 2 × 10⁵ cells 12 h after transmigration. Induction of monocyte CD86 and cytokine mRNA expression was determined by semiquantitative reverse transcriptase (RT)-PCR and RNase protection assay.

Isolation of RNA and RT-PCR Analysis. Total RNA was prepared using the Ultrascript RNA isolation system (Biotecx) according to the manufacturer’s instructions, and was quantified by spectrophotometry. CDNA was prepared by reverse transcription of 5 µg of RNA using random hexamer primers (100 ng/µl) and Moloney murine leukemia virus reverse transcriptase (50 µM/µl) (Stratagene) in a 50-µl reaction. 10 µl of CDNA was used for each PCR amplification reaction. PCR was performed with Taq DNA polymerase using the buffer supplied by the manufacturer (Boehringer Mannheim). The PCR primers were: human CD80, sense: 5'-CAT CAC GGA GGG TCT TCT AC-3' and antisense: 5'-AGG ATC TTG GGA AAC TGT TGT-3'; and human anti-CD86, sense: 5'-AGG ACA AGG GCT TGT ATC AA-3' and antisense: 5'-ATT GCT CGT AAC ATC AGG GA-3'. The PCR conditions were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 35 cycles. PCR products were analyzed by ethidium bromide staining in 1.5% agarose gels using standard techniques.

RNase Protection Assays. RNase protection was performed using the Riboquant™ Multi-Probe RNase Protection assay system (PharMingen). RNA was isolated as described above. 32P-labeled probes were synthesized from the hCK-2 human cytokine multi-probe template set and were hybridized overnight with RNA samples in hybridization buffer according to the manufacturer’s instructions. Samples were digested with RNase and T1 RNase and protected probes were purified and were run on a 5% acrylamide gel in 0.5% TBE buffer. Human control RNA and a dilution of the probe set (serving as size markers) were run in parallel. The gel was absorbed onto filter paper, dried, and exposed onto Kodak photographic paper at −70°C for 24 h.

Cytokine Assays. IL-2 was assessed by specific ELISA. Primary and secondary antibodies were purchased from Genzyme and were used according to the recommended protocol. In brief, 96-well flat-bottomed ELISA plates (Falcon; Becton Dickinson Labware) were coated with primary antibody overnight at 4°C. Blocking was then performed with 4% BSA in PBS for 2 h at 37°C and neat coculture supernatants or standards were added to each well in duplicate for 1 h at 37°C. After the incubation, secondary biotinylated anti–IL-2 mAb was added and the ELISA was developed using avidin alkaline phosphatase (Sigma Chemical Co.) and phosphatase substrate (Sigma Chemical Co.). In between each step, the plates were washed in PBS with 0.01% Triton X-100. Plates were read at 405 nm in an E-Max ELISA plate reader (Molecular Devices).

Flow Cytometry. Cell suspensions of CD4+ T cells, ECs, or monocytes were analyzed by direct immunofluorescence. In brief, 1–2 × 10⁶ cells were incubated with FITC- or PE-conjugated mAbs at 4°C for 30 min and were fixed in 1% PFA. Stained cells were then analyzed by FACSScan® (Becton Dickinson). Monocytes were preincubated with buffer containing 20% non-A non-B human serum before flow cytometry to block non-specific Fc receptor binding and optimize specific binding. Western Blotting. Western blot was performed on 5 × 10⁶ cells per condition. Cells were lysed in PBS containing 1% NP-40, and protease-inhibitor (Boehringer Mannheim) and lysates were separated by standard 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Blots were blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween 20 and 2% BSA before incubation with optimal concentrations of primary antibody diluted in TBS/0.1% Tween 20 for 12 h at 4°C. After four washes in TBS/0.1% Tween 20, blots were incubated in peroxide-conjugated goat anti–mouse secondary antibody (Jackson ImmunoResearch Labs.) at a 1:2,500 dilution in TBS for 4 h at 4°C. The blots were then washed and developed by chemiluminescence (Amersham Inc.).

Results

Expression of CD80 and CD86 in CD4+ T Cell–EC Co-cultures. To investigate a role for CD80 and CD86 in CD4+ T cell–EC interactions, we initially examined the expression of these molecules by resting or activated CD4+ T cells, and ECs alone or after coculture of both cell types (Fig. 1). Our findings were that TNF-α, IFN-γ, IL-1, or soluble CD40 ligand (CD40L)-stimulated HUVECs do not express CD80 or CD86 mRNA by RT-PCR; likewise resting CD4+ T cells do not express CD80 and express variable and low levels of CD86 mRNA. In contrast, we found a marked expression of both CD80 and CD86 mRNA in CD4+ T cell–HUVEC cocultures. CD86 mRNA was detected as early as 6 h and was maximal by 72 h of coculture, whereas CD80 mRNA was only expressed in cells harvested at 72 h of coculture (Fig. 1 A). CD86 protein was detected by Western blot in 72-h CD4+ T cell–HUVEC cocultures but not in resting CD4+ T cells or...
TNF-α, IFN-γ, or soluble CD40 ligand–activated HUVECs (Fig. 1 B). By FACS® analysis, anti-CD80 mAbs, seven anti-CD86 mAbs, and CTLA4 Ig did not bind to resting or cytokine-activated HUVECs, saphenous vein ECs, dermal microvascular ECs, or lung microvascular ECs although positive control CD80- and CD86-transfected CHO cells consistently demonstrated high levels of binding (Fig. 1 C and data not shown). Thus, human ECs express neither CD80 nor CD86, but both these molecules are induced in CD4+ T cell–EC cocultures.

Function of CD86 and CD80 in CD4+ T Cell–Allogeneic EC Cocultures. We next wished to determine the function of CD86 and CD80 in CD4+ T cell–EC interactions. Purified CD4+ T cells were rested for 48 h and were cocultured with either HUVECs, saphenous vein ECs, dermal microvascular ECs, or lung microvascular ECs, untreated or treated with IFN-γ for 72 h to upregulate class II MHC. Anti-CD86 mAbs, anti-CD80 mAbs, or control isotype antibodies were added to cocultures as indicated by each experiment (Fig. 2). We found that anti-CD86 mAbs consistently inhibited IFN-γ-treated HUVEC-induced CD4+ T cell alloproliferation (Fig. 2 A). Maximal inhibition with anti-CD86 mAbs was variable (20–50%) and was less than that observed by blocking CD2-LFA-3 interactions with
Human ECs Induce CD86 Expression by CD4+ T Cells. Since we find that human ECs do not express CD86, we next wished to assess CD4+ T cell CD86 expression and function in our model. Previous studies have shown that activated CD4+ T cells may express both CD80 and CD86 (33, 34). We also confirmed that CD86 was induced de novo on CD4+ T cells with IFN-γ treatment (Fig. 3 A). Furthermore, to determine whether EC-induced CD86 expression by CD4+ T cells was dependent on cell contact, we generated cell membranes from ECs and incubated them with CD4+ T cells. Membrane preparations from IFN-γ-activated ECs but not resting ECs induced CD4+ T cell CD86 mRNA expression by RT-PCR, consistent with the interpretation that induction of CD4+ T cell CD86 is mediated by a cell surface molecule(s) on activated ECs (Fig. 3 B). However, when membranes generated from unactivated or activated CD4+ T cells were incubated with ECs, they failed to induce EC expression of CD86. As a positive control, activated CD4+ T cell membranes enhanced EC E-selectin expression (Fig. 3 C).

Functionality of EC-induced CD4+ T cell CD86. Having established that ECs induce CD86 expression on CD4+ T cells, we next assessed function. CD4+ T cells were cultured with IFN-γ-treated ECs and were reisolated by positive selection after 24 h of coculture. These EC-modified CD4+ T cells were then irradiated and cocultured either alone or with resting autologous CD4+ T cells in the presence of submitogenic doses of PHA (0.3 μg/ml). Although resting CD4+ T cells alone and EC-modified CD4+ T cells alone failed to proliferate to low dose PHA, coculture of both cell types resulted in enhanced proliferation, which was inhibited by ~50–80% by anti-CD86 mAbs (Fig. 4 A). This suggests that induced CD86 on EC-modified CD4+ T cells provides an effective costimulatory signal in trans to resting CD4+ T cells. Moreover, the proliferative responses of CD4+ T cells in this trans-costimulation assay were less than those induced by ECs (Fig. 2 and data not shown). We note that this is consistent with the ability of ECs to provide additional costimulatory signals such as LFA-3-dependent signals as reported by others (13, 35).

To determine the molecular basis for EC modification of CD4+ T cell costimulatory activity, we incubated CD4+ T cells with IFN-γ-treated ECs in the absence or presence of anti-ICAM-1, anti-LFA-3, anti-HLA-DR mAbs, anti-CD40 mAbs, anti-ox40 mAbs, or isotype control antibodies for 24 h. EC-modified CD4+ T cells were then reisolated and irradiated, and were added to resting CD4+ T cells in the presence of submitogenic doses of PHA as described above. We found that anti-LFA-3 and anti-HLA-DR, but not anti-CD40, anti-ox40, or control antibody, inhibited the subsequent CD4+ T cell costimulatory effect (Fig. 4, B and C, and data not shown). Anti-CD40 antibodies also failed to inhibit T cell-T cell
trans-costimulation (Fig. 4 C). Thus, induction of functional CD86 on CD4+ T cells is in part dependent on interactions between CD4+ T cells and EC class II MHC and LFA-3.

Finally, to confirm functionality of CD86-dependent trans-costimulation for direct allorecognition (when signal one is provided by alloantigen on ECs), CD4+ T cells were cocultured with IFN-γ-treated ECs in the presence of increasing numbers of CHO cells transfected with CD86. Mock-transfected CHO cells were used as a negative control. CD86-transfected, CHO cells enhanced EC-induced CD4+ T cell alloprediction (Fig. 5 A) and IL-2 production (data not shown) in a dose-dependent manner that is inhibited by anti-CD86 mAbs and CTLA4 Ig (Fig. 5 B). This data confirms that CD4+ T cells can receive CD86 costimulation in trans when signal one is provided by alloantigen on ECs. EC-induced CD86 trans-costimulation is mediated by CD3+ CD4+ T Cells and Not by Contaminating CD4+ Dendritic Cells. It has been reported that a subpopulation of peripheral blood CD4+ cells are HLA-DR+ and CD3+ myeloid derived dendritic cells (36). We found that ~2% of our CD4+ cells were CD3+, and therefore we wished to confirm that the CD68-dependent trans-costimulation described above is indeed due to CD86 expressed by CD4+ T cells and not to dendritic cell CD86. We further purified our CD4+ cells by negative selection for HLA-DR and CD14 expressing cells (as described in Materials and Methods). The resulting cells were CD4+CD3+HLA-DR−T cells (Fig. 6).
A) and express low levels of CD86. However, consistently after coculture with IFN-γ-treated ECs, these T cells exhibit augmented CD86 expression (Fig. 6 B). Furthermore, these T cells provide effective trans-costimulation to autologous T cells in the presence of low dose mitogen (Fig. 6 C). Trans-costimulation is mediated in part by CD86 (with 25–75% inhibition observed with anti-CD86 mAbs) but also involves other cell surface molecules including LFA-3 (data not shown and Fig. 4). There is no antigen-dependent component to the T cell–T cell proliferative response, since anti–HLA-DR antibody (LB3.1) failed to inhibit proliferation with concentrations of mitogen (PHA, 0.3 μg/ml) used in our model. However, in the absence of mitogen or at low doses of mitogen (PHA < 0.1 μg/ml), anti–HLA-DR partially inhibits proliferative responses.

ECs Induce Functional Costimulatory Activity in Transmigrating Monocytes. We next wished to determine whether ECs modify the costimulatory and antigen presenting capacity of monocytes. Recent studies suggest that transmigration of monocytes across ECs promotes their differentiation into dendritic cells (37, 38). Indeed, a novel function of ECs may be to enhance the antigen presenting and costimulatory function of infiltrating APCs in the course of alloimmune inflammatory reactions and rejection. Monocytes were isolated by elutriation in order to obtain a relatively unactivated cell population. Cells were then allowed to transmigrate across resting or 72-h IFN-γ-treated confluent EC monolayers in transwells as described in Materials and Methods. After 12 h, cells were harvested from the lower chamber of the transwell and RNA was isolated for analysis by RT-PCR and RNase protection. We found that transmigration of monocytes across IFN-γ-treated ECs, and to a lesser extent resting ECs, resulted in the induction of monocyte CD86 mRNA expression by RT-PCR (data not shown) and enhanced CD86 protein expression by FACS® analysis (Fig. 7 A). Transmigration
IFN-γ-treated ECs, CD14+ monocytes were reisolated by positive selection and cultured with allogeneic or autologous CD4+ T cells at fixed responder/stimulator ratios. We found that EC-modified monocytes consistently induced greater proliferation of allogeneic CD4+ T cells than did resting monocytes (Fig. 8 A), an effect inhibited by CTLA4 Ig and anti–HLA-DR antibodies (Fig. 8 B). CTLA4 Ig caused a greater percentage of inhibition of CD4+ T cell proliferation induced by EC-modified monocytes, consistent with enhanced expression of CD86 by these cells. Thus, ECs augment the capacity of monocytes to provide costimulatory signals to CD4+ T cells activated by direct allorecognition. Furthermore, EC-modified monocytes induced proliferation of autologous CD4+ T cells. This suggests that ECs may donate alloantigen to monocytes for presentation to autologous CD4+ T cells via the indirect pathway of allorecognition.

Discussion

Microvascular ECs express cell surface molecules that mediate both the recruitment into and the activation of leukocytes within vascularized solid organ transplants. Thus, it is proposed that interactions between CD4+ T cells and microvascular graft ECs are critical for rejection (12). Antigen-dependent activation of CD4+ T cells is initiated by interactions between the TCR and foreign peptide in association with MHC class II molecules. ECs express MHC class I and class II molecules and provide antigen-dependent signals to T cells in vitro (7–12) and in vivo (17, 18). However, the ability of human endothelium to provide effective costimulatory signals for full CD4+ T cell activation is more controversial (12–16). Surprisingly, only endothelial LFA-3 has been identified to possess costimulatory function with little if any contribution of other human EC cell surface molecules. Endothelial LFA-3 interacts with T cell CD2 and initiates a series of activation responses in CD4+ T cells that result in IL-2, IL-4, and IFN-γ production (10, 15, 39). Since naive and previously activated CD4+ T cells are dependent on CD28 signaling for effective activation (22), we wished to examine in more detail the role of CD28–B7 interactions in EC-induced CD4+ T cell activation. Our results provide insight into how graft ECs may modify infiltrating leukocytes for provision of CD28-mediated costimulation in trans and promote CD4+ T cell activation via direct and indirect allorecognition.

In these studies, we confirm that human ECs do not express CD80 or CD86 mRNAs, nor protein assessed by RT-PCR, Western blotting, and FACS® analysis, respectively. However, we do find that CD86 is induced and is functional in CD4+ T cell–EC interactions due to its expression on CD4+ T cells and the ability of these cells to deliver CD86 mediated costimulatory signals in trans. Indeed, we demonstrate induction of expression of CD86 on CD4+ T cells after coculture with ECs. Blockade of CD4+ T cell CD86 using several anti-CD86 mAbs caused a variable but...
consistent inhibition of CD4+ T cell proliferation and IL-2 production when alloantigen was presented to CD4+ T cells by several different human microvascular ECs. Furthermore, combined blockade of LFA-3 and CD86 results in additive inhibition of CD4+ T cell activation, suggesting that these molecules participate in parallel pathways of CD4+ T cell activation. We interpret these data to suggest that ECs, in addition to providing direct costimulatory signals to CD4+ T cells, predominantly via LFA-3, may promote CD28-dependent trans-costimulation by the induction of CD86 on CD4+ T cells. The ability of ECs to modify leukocytes for provision of trans-costimulatory signals probably provides an additional mechanism whereby ECs regulate inflammatory responses.

Although PHA-activated CD4+ T cells have been reported previously to express both CD80 and CD86, the functional importance of CD4+ T cell expression of these molecules is controversial. Azuma et al. have shown that CD4+ T cell clones expressing CD80 are able to stimulate T cell cytokine production and proliferation in a mixed lymphocyte reaction (33). Furthermore, Jeannin et al. reported that human effector T cells express CD86, and may costimulate naive T cell responses (34). In contrast, it has been reported that CD4+ T cell CD86 may be nonfunctional due to reduced posttranslational glycosylation (40). Our studies clearly demonstrate that CD4+ T cell CD86 is functional and may provide CD28-mediated costimulatory signals in trans to autologous T cells when signal one is provided by mitogen or alloantigen on ECs. The high purity of our CD4+ T cell preparations and the high expression of CD86 on CD4+ T cells after coculture with ECs suggests that CD86-mediated costimulation was provided by CD4+ T cells and not low numbers of contaminating CD86+ dendritic cells. To confirm this, we depleted our CD4+ T cell population of HLA-DR- and CD14-expressing cells to eliminate contaminating CD4+ APCs (36). The resulting highly purified CD4+ T cells proliferated to IFN-γ-treated ECs, expressed enhanced levels of CD86 after 72 h.
coculture with IFN-γ-treated ECs and provide functional T cell–T cell trans-costimulation. We note that EC stimulation of CD4+ T cells resulted in a discrete population of CD86 expressing CD4+ T cells, which may represent allo-activated CD4+ T cells. Although resting CD4+ T cells may express low levels of CD86, we suggest that this level is insufficient to provide effective costimulation. Indeed, anti-CD86 reagents fail to inhibit CD4+ T cell activation when stimulation is provided by mitogen. Moreover, anti–class II MHC mAbs and anti–LFA-3 mAbs inhibit the ability of ECs to modify CD4+ T cells to provide CD86-mediated costimulation. Consistent with these findings, we found by FACS analysis that LFA-3 fusion protein and low doses of PHA additively promote CD86 protein expression in purified CD4+ T cells (data not shown). Therefore, EC LFA-3 may provide costimulation to CD4+ T cells via two distinct mechanisms. First, LFA-3 may directly costimulate cytokine production and CD4+ T cell proliferation via interactions in cis (35); and second, LFA-3 may promote trans-costimulation via the induction of CD86 on T cells. Although CD40 signals induce CD80 and CD86 expression on B cells (41), monocytes, and dendritic cells (42), we find that stimulation of CD40L–CD40 interactions does not inhibit EC induction of functional CD4+ T cell CD86. This is consistent with the low levels of CD40 expression on resting and activated CD4+ T cells. Similarly, OX40L–OX40 interactions do not appear to be functional in EC induction of CD86 expression by CD4+ T cells. Consistent with these observations, neither anti-CD40 mAbs nor anti-CD40L mAbs inhibit alloactivation of CD4+ T cells by IFN-γ-treated ECs (data not shown).

Our findings that allogeneic ECs can fully activate bulk populations of CD4+ T cells are similar to those reported by other groups (13, 15, 35, 43). However, they are different from those reported by Marelli-Berg et al., in which ECs were found to have limited costimulatory function (16). A common finding of all groups is that addition of B7-dependent costimulation in trans reconstitutes the ability of ECs to activate T cells and monocytes, which may suggest that ECs donate alloantigen to monocytes for indirect activation of CD4+ T cells. Our new findings provide evidence for a unique function of the endothelium in allograft rejection in the direct activation of CD4+ T cells. Moreover, by inducing CD86 and cytokine expression, human ECs modify the costimulatory capacity of infiltrating CD4+ T cells and monocytes, which may provide costimulation in trans to CD4+ T cells. Our new findings provide evidence for a unique function of the endothelium in allograft rejection in the direct activation of CD4+ T cells. In addition, the modification of monocytes by ECs may provide a mechanism whereby ECs promote indirect allorecognition.
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