Human Placental Pericytes Poorly Stimulate and Actively Regulate Allogeneic CD4 T Cell Responses

Cheryl L. Maier, Jordan S. Pober

Objective—Cell-mediated immune responses in peripheral tissues begin with T cell infiltration through endothelial cell (EC) microvessels and accumulation in the perivascular space occupied by pericytes (PC). Here, we investigate how human T cells interact with PC.

Methods and Results—We compared human placental PC with autologous umbilical vein EC. Cultured PC express lower levels of major histocompatibility complex (MHC) and positive costimulatory molecules but higher levels of negative costimulatory molecules than do EC. Unlike EC, interferon-γ-treated MHC class II-positive PC (PC+) cannot stimulate resting allogeneic CD4 T cell proliferation or cytokine production. Instead, coculture of resting CD4 T cells with PC+ induces CD25 expression and renders T cells unresponsive to restimulation by EC+ from the same donor. PC cultured across a semi-permeable membrane decrease alloreactive CD4 T cell proliferation to EC+, an effect enhanced by pretreatment of PC with interferon-γ and partially reversed by interleukin-10 and transforming growth factor-β neutralization, but do not induce anergy.

Conclusion—Human placental PC are poorly immunogenic and negatively regulate CD4 T cell responses through contact-dependent and contact-independent mechanisms. (Arterioscler Thromb Vasc Biol. 2011;31:183-189.)

Key Words: endothelium ■ immune system ■ leukocytes ■ microcirculation ■ vascular biology

The effector phase of T cell–mediated immune responses begins with recruitment of T cells through postcapillary venules.2,3 Such microvessels are composed of an endothelial cell (EC) lining supported by a network of perivascular cells, called pericytes (PC).3,4 Venular EC inducibly display luminal adhesion molecules and chemokines that mediate recruitment of circulating effector memory T cells (reviewed in Choi et al5). In addition, human EC basally express both class I and class II major histocompatibility complex (MHC) molecules in situ,6,7 mostly likely in response to circulating interferon (IFN)-γ.8 T cell recognition of MHC molecules on EC in vitro triggers chemokine-independent transendothelial migration of effector memory T cells.9,10 In vivo, transmigrated T cells typically remain in a perivascular location, in close proximity to PC, for extended periods of time.

CD4 T cell activation requires 2 signals: antigen, composed of self-MHC–foreign peptide complexes or, in allogeneic settings, of nonself MHC–peptide complexes; and antigen-independent costimulators that positively or negatively influence responses. Recognition of antigen in the absence of costimulation can cause anergy such that CD4 T cells are unable to respond to subsequent antigenic stimulation.12 Human EC can act as “semiprofessional” antigen-presenting cells, stimulating approximately 20% to 40% as many resting T cells to proliferate and elaborate cytokines as do monocytes or B lymphoblastoid cells.13-15 This quantitative difference in accessory cell function is largely due to the fact that human EC lack costimulators that engage CD28 on T cells, namely CD80 and CD86, and CD28 signals are essential for the activation of naive T cells. Consequently, EC are able to activate only alloreactive memory T cells, whereas classical antigen-presenting cells, which do express CD80 and CD86, can activate both alloreactive naive and memory T cells.10 EC do express other costimulators that are specific for the activation of memory T cells, namely CD58 (lymphocyte function-associated antigen [LFA]-3), CD40, CD275 (inducible T cell costimulator [ICOS] ligand), CD137L (41BB ligand), and CD252 (Ox40 ligand).14-16 Vascular smooth muscle cells, which lack CD275 and CD252, as well as CD80 and CD86, are unable to activate either naive or memory T cells.17 In contrast to EC, many stromal cell types lacking antigen-presenting cell capabilities inhibit T cell responses. For example, undifferentiated bone marrow–derived mesenchymal stem cells (MSC) and tissue-derived mesenchymal progenitor cells inhibit lymphocyte responses by nutrient consumption or production of inhibitory factors.18,19 Specifically, interleukin (IL)-10, transforming growth factor (TGF)-β, and prostaglandin E2 (PGE2) contribute to human MSC-mediated T cell suppression.20,21 Human aortic smooth muscle cells inhibit T cell responses22 by depleting L-tryptophan through the activity of indoleamine 2,3-dioxygenase (IDO).23 Immune-inhibitory abilities may characterize all mesenchymal cell types.24 However, immune functions of differentiated PC, anatomically positioned to exert modulatory effects on perivascular T cells, have not been previously examined.
The principal reason for the lack of information about PC immunology is that human PC have not been readily available for study. Recently, we developed a method for the isolation of human PC from placental microvessels, allowing comparison of the immunologic characteristics of PC to those of EC isolated from the same donor.\textsuperscript{25} We report here that PC are not immunogenic and actively regulate allosereactive CD4 T cell responses.

Methods

Cells and Reagents

Human placental PC, umbilical vein EC, and peripheral blood CD4 T cells were obtained following institutional review board–approved protocols. Placental PC were cultured by explant outgrowth from microvessel fragments recovered after enzymatic digestion of minced placental tissue, umbilical vein EC were harvested with collagenase treatment, and CD4 T cells were purified from leukapheresis collections by positive immunoselection. Detailed procedures are presented in the Supplemental Methods, available online at http://atvb.ahajournals.org. Placental PC express characteristic markers NG2, CD90 (Thy-1), CD146, and α-smooth muscle actin and lack contamination by cells expressing CD31 or CD34 (EC markers) or CD45 (leukocytes). Human brain PC were purchased from ScienCell and confirmed to express NG2, CD146, and α-smooth muscle actin. PC and EC were used between subcultures 2 and 6. Conditions for cultures and cocultures have been reported previously\textsuperscript{26} and are described in the Supplemental Methods, as are sources and concentrations of all cytokines, reagents, and antibodies.

Statistical Analysis

Statistical analyses were performed using the appropriate parametric or nonparametric tests as indicated.

Results

PC Express an Immunophenotype Distinct From That of EC

We compared placental PC and autologous human umbilical vein EC for expression of immunologically significant surface molecules under basal and cytokine-stimulated conditions. Like EC, PC expressed MHC class I molecules but not class II molecules under basal culture conditions; both class I and class II MHC molecule expression were induced following treatment with IFN-γ (Table). However, MHC molecule expression was lower on PC than EC. Neither PC nor EC expressed the costimulatory molecules CD80 or CD86, but both expressed positive costimulatory molecules CD58, CD40, CD275, CD137L, and CD252, although PC expression levels were somewhat lower. PC lacked glucocorticoid-induced TNFR-related protein (GITR) ligand, an inhibitor of regulatory T cell (Treg) function, which was expressed basally on EC and increased by treatment with IFN-γ or tumor necrosis factor. PC more highly expressed the inhibitory molecules CD274 (programmed death [PD]-L1) and CD273 (PD-L2) than did EC, especially following treatment with IFN-γ. Unlike EC, PC did not express the adhesion molecules CD106 (vascular cell adhesion molecule-1) or E-selectin, but they did express similar levels of CD54 (intercellular adhesion molecule-1), which was further induced on both cell types following cytokine treatment (Table). Overall, compared with umbilical vein EC, human placental PC displayed an immunophenotype expected to be less likely to activate T cell responses.

Table. Flow Cytometric Analysis of Immunologically Significant Cell Surface Proteins on Autologous Pericytes and Endothelial Cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pericytes</th>
<th>Endothelial Cells</th>
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<tr>
<td></td>
<td>Basal</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>2000</td>
<td>5000</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CD80 (B7-1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD86 (B7-2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD40</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>CD58 (LFA-3)</td>
<td>190</td>
<td>111</td>
</tr>
<tr>
<td>CD275 (inducible T cell costimulator ligand)</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>CD137L (41BB ligand)</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>CD252 (Ox40 ligand)</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>GITR ligand</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>CD274 (PD-L1)</td>
<td>150</td>
<td>830</td>
</tr>
<tr>
<td>CD273 (PD-L2)</td>
<td>290</td>
<td>800</td>
</tr>
<tr>
<td>CD106 (VCAM-1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD54 (ICAM-1)</td>
<td>40</td>
<td>1850</td>
</tr>
<tr>
<td>CD62E (E-selectin)</td>
<td>0</td>
<td>0</td>
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</table>

PC and EC were isolated from the umbilical vein and treated with vehicle control, 50 ng/ml IFN-γ for 72 hours, or 10 ng/ml tumor necrosis factor (TNF) for 24 hours. Results are expressed in arbitrary units of fluorescence as the corrected median fluorescence intensity (MFI) after subtracting the MFI of the isotype control from the MFI of the specifically stained cells. Similar results were seen with cells isolated from 4 donors. VCAM indicates vascular cell adhesion molecule; ICAM, intercellular adhesion molecule.

PC+ Do Not Effectively Stimulate Allogeneic CD4 T Cells

We directly compared the ability of placental PC and umbilical vein EC to induce allogeneic CD4 T cell responses in vitro. Peripheral blood CD4 T cells were cultured with allogeneic IFN-γ pretreated (MHC class II molecule–expressing) vascular cells (designated PC+ or EC+) as well as with untreated (MHC class II molecule–negative) cells (designated PC− and EC−). CD4 T cells cocultured with allogeneic PC+ produced very little IL-2 or IFN-γ compared with cocultures with EC+ (Figure 1A). Neither cytokine was detected in cocultures of CD4 T cells with allogeneic PC− or EC−. A similar percentage of CD4 T cells cocultured with PC+ or EC+ expressed the activation markers CD69 at 24 hours and CD25 at 72 hours (Figure 1B), which were absent on freshly isolated CD4 T cells or CD4 T cells cocultured with PC− or EC−. A greater percentage of CD4 T cells cocultured with EC+ than with PC+ were in cell cycle or were dying at day 3 assessed by propidium iodide staining (Figure 1C). In contrast to CD4 T cells cocultured with allogeneic EC+, CD4 T cells cocultured with allogeneic PC+ did not proliferate after 7 days, as assessed by carboxyfluorescein succinimidyl ester dilution (Figure 1D) or 5-bromo-2′-deoxyuridine incorporation (data not shown). The limited ability of PC+ compared with EC+ to activate allogeneic CD4 T cells was a consistent feature of multiple donor combinations (Figure 1E).
The induction of CD69 and CD25 suggests that CD4 T cells do recognize class II MHC molecules on PC and therefore might be able to restimulate activated T cells, which have minimal need for positive costimulation. We generated activated T cells by culturing CFSE-labeled CD4 T cells with allogeneic EC for 7 days and collecting CFSE low CD4 T cells by cell sorting. Activated CD4 T cells proliferated in response to coculture with PC autologous to the primary stimulation EC but not to unrelated (“third-party”) PC, assessed by 5-bromo-2'-deoxyuridine labeling (Figure 2A). However, secondary proliferation to autologous PC was less than the restimulation response to autologous EC (Figure 2A). Such differences were consistent over multiple donors (Figure 2B).

To directly assess whether the difference between PC and EC arises from differences in costimulation, we compared cocultures of vascular cells and CD4 T cells stimulated by the polyclonal-activating lectin phytohemagglutinin-L (PHA-L), an assay that is independent of MHC class II molecules but relies on costimulation provided by accessory cells. PC or PC required increased concentrations of PHA to stimulate maximal cytokine production and proliferation by CD4 T cells compared with EC or EC (Figure 3A). Furthermore, the maximum level of these responses was less in cocultures with PC than EC at saturating concentrations of PHA. As expected, no difference in CD25 induction was observed, because this response is largely mediated by T cell receptor engagement by PHA. We specifically assessed the role of negative costimulation provided by PD-1 ligands by means of antibody blocking, again using PHA to amplify the response. Addition of neutralizing antibodies to PD-1 ligands increased CD4 T cell proliferation to PC, but proliferation was still less than that observed in parallel EC cocultures at the same dose of PHA (Figure 3B). Thus, engagement of PD-1 contributes to, but does not fully explain, the poor accessory functions of PC. We conclude that the net balance of costimulation provided by PC is less than that provided by EC, likely resulting from quantitative differences in multiple different costimulators.

**PC** Induce CD4 T Cell Anergy

Because PC induced expression of the activation markers CD69 and CD25 on CD4 T cells, we investigated whether T cells demonstrate any functional change as a consequence of...
coculture with PC+. CD4 T cells were briefly cultured with PC+ for 24 hours and then restimulated with either EC+ from the same donor as the PC+ or third-party EC+. In these secondary cocultures, the response to matched EC+ resulted in less IL-2, IFN-γ, and T cell proliferation compared with secondary cocultures restimulated by third-party EC+ (Figure 4A and 4B). This functional alteration depends on recognition of class II molecules on PC because no inhibition was seen in primary cultures with PC+ instead of PC+, and inclusion of a human MHC class II (human leukocyte antigen-DR) blocking antibody in the primary coculture significantly decreased the degree of secondary inhibition (Figure 4C). In contrast, blocking PD-1 ligands in the primary culture had little effect (not shown).

Recognition of HLA-DR on PC+ could induce a degree of alloantigen-specific clonal anergy in CD4 T cells or could induce alloantigen-specific Treg cells capable of inhibiting responses in secondary cultures. We assessed Treg induction by immunophenotyping and by functional suppression assays, finding that coculture with PC+ neither induced T cell expression of characteristic Treg markers (CD4+ CD25+CD127loFoxp3+) nor rendered T cells capable of suppressing all responses of freshly isolated T cells to EC+ (data not shown). We also saw no evidence for the Treg-induced cytokines IL-10 or TGF-β or any effect of neutralizing these inhibitory cytokines (data not shown). We next examined CD4 T cell expression of several genes associated with human T cell anergy, specifically GRAIL, ITCH, and CBL-B.26,27 Activated CD25+ CD4 T cells cocultured with PC+ for 72 hours expressed increased levels of GRAIL, ITCH, and CBL-B mRNA and decreased levels of Foxp3 mRNA compared with T cells cocultured with EC+ (Figure 4D). These experiments suggest that recognition of class II MHC molecules on allologeneic PC+ renders CD4 T cells clonally anergic.

**PC+ Inhibit CD4 T Cell Proliferation Across a Semi-Permeable Membrane**

Because many stromal cell populations reportedly modulate T cell responses via the secretion of diffusible inhibitory factors, we investigated whether PC− or PC+ could affect T cell responses independent of cell contact. Freshly collected PC+ conditioned medium showed a small inhibitory effect on CD4 T cell proliferation to EC+, but the effect was inconsistent and the factor(s) appeared unstable (data not shown). In contrast, PC− or PC+, but not EC− or EC+, cultured across semi-permeable membranes from allologeneic EC/CD4 T cell cocultures, consistently inhibited CD4 T cell proliferation (Figure 5A), and PC+ were more potent than PC−. Inhibition was similarly observed when CD4 T cells were cocultured with EC+ and the superantigen toxic shock syndrome toxin-1 in the presence of PC− or PC+ across semi-permeable membranes (data not shown).

We examined several mechanisms used by other stromal cell types to inhibit T cell responses. We were particularly interested
in those mediators that were IFN-γ inducible in PC, because PC⁺ demonstrated greater inhibition. IFN-γ treatment of PC increases mRNA levels of the tryptophan metabolizing protein IDO (Supplemental Figure Ia), an enzyme known to be involved in human smooth muscle cell and dendritic cell inhibition of T cell responses via depletion of the essential amino acid L-tryptophan.²³,²⁸,²⁹ However, PC⁺ expression of IDO was not greater than IDO expression by donor-matched EC⁺ (Supplemental Figure Ib), which do not suppress T cell proliferation, and neither addition of exogenous tryptophan nor addition of the IDO-antagonist 1-methyltryptophan reverses the inhibitory effect of PC⁺ in cocultures (Figure 5B). We also investigated whether PC might deplete cocultures of L-arginine, as human embryonic stem cells may suppress T cell responses by arginase-1–mediated L-arginine depletion.³⁰ Arginase-1 was not detected in cell lysates from either PC⁺ or PC⁺, but arginase-2 and the arginine uptake membrane transporter CAT2B were expressed and increased in PC⁺ compared with PC⁺ (Supplemental Figure II). However, the addition of exogenous L-arginine did not abrogate the inhibitory effect of PC⁺ in semi-permeable membrane assays (Figure 5C). We also examined PC production of several mediators known to suppress T cell proliferation, namely nitric oxide (NO), PGE2, TGF-β, and IL-10. Whereas inducible nitric oxide synthase (iNOS) mRNA levels were increased in PC⁺ versus PC⁺, iNOS protein was not detected by immunoblotting, and increased NO was not detected in the media of PC⁺ or PC⁺ cultures (Supplemental Figure IIIa to IIIc). Furthermore, treatment of semi-permeable membrane cocultures with the specific iNOS inhibitor 1400W failed to reverse the inhibition (Figure 5D). PGE2 synthesis from arachidonic acid occurs constitutively by the action of COX-1 and inducibly through COX-2. COX-2 expression was slightly increased in PC⁺ versus PC⁺ (data not shown), and PC⁺ produced increased levels of PGE2 compared with PC⁺ (Supplemental Figure IVa). However, medium from EC⁺/CD4 T cell cocultures with PC⁺ or PC⁺ across a semi-permeable membrane contained less PGE2 at 24 hours than medium from cocultures with EC across a semi-permeable membrane (Supplemental Figure IVb). Furthermore, suppression of CD4 T cell proliferation was not reversed by treatment of cocultures with the cyclooxygenase inhibitor

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Figure 4. CD4 T cells cultured with PC⁺ become desensitized to secondary allogeneic stimulation. Allogeneic CFSE-labeled CD4 T cells were cocultured with PC⁺ for 24 hours then restimulated by EC⁺ autologous to the primary-stimulation PC⁺ (matched EC⁺) or by third-party EC⁺. A, Media were collected after 24 hours of restimulation and analyzed for the presence of IL-2 and IFN-γ by ELISA. B, CD4 T cell proliferation after 7 days of restimulation, normalized to secondary proliferation responses of T cells cultured initially with EC⁺. C, The enhanced suppression to matched EC⁺ in secondary cultures was largely reversed by inclusion of an antibody blocking HLA-DR in the primary PC⁺ coculture. D, Activated CD4 T cells cocultured with PC⁺ expressed increased GRAIL, ITCH, and CBL-B transcripts and decreased FoxP3 transcripts compared with T cells cocultured with EC⁺. Shown are representative results from 1 of 8 experiments in A and B, 1 of 2 experiments in D, and pooled data from 4 experiments in C; *P<0.05, **P<0.01; Mann-Whitney test (B); t test (C and D).
indomethacin (Figure 5E). Although neither TGF-β nor IL-10 appeared to be induced in PC by IFN-γ treatment, addition of neutralizing antibodies to IL-10, TGF-β, or both significantly relieved the inhibition of CD4 T cell proliferation caused by PC⁺ across a semi-permeable membrane (Figure 5F). However, the effect of neutralizing both cytokines was neither additive nor complete, suggesting that although IL-10 and TGF-β contribute to the inhibitory milieu, perhaps acting through the same pathway, other mechanisms must contribute as well.

We examined whether the presence of PC⁺ across a semi-permeable membrane renders T cells less responsive to restimulation, as was observed in cocultures with cell-cell contact. T cells recovered from PC⁻ and PC⁺ semi-permeable membrane cocultures responded similarly to control (EC semi-permeable membrane) T cells in secondary stimulation assays to EC⁺ (Supplemental Figure V). In other words, PC⁺ inhibited T cell responses across a semi-permeable membrane but direct contact was necessary to induce clonal anergy.

Finally, we addressed whether placental PC are unique in their immunologic properties by investigating the immunogenicity of PC isolated from another tissue source. Human brain vascular PC were assessed for MHC, costimulatory, and adhesion molecule expression under basal and IFN-γ stimulated conditions (Supplemental Table I). Brain PC expressed a relatively similar immunophenotype as compared with placental PC except that not all brain PC expressed class II MHC molecules following IFN-γ treatment. Like T cells cocultured with placental PC⁺, CD4 T cells cocultured with brain PC⁺ did not produce IL-2 (Supplemental Figure VIa) or proliferate (Supplemental Figure VIc), despite induced CD25 expression (Supplemental Figure VIb). Brain PC also inhibited alloreactive CD4 T cell proliferation to EC⁺ across a semi-permeable membrane (Supplemental Figure Vld). Although these observations do not permit generalization to all PC, they do suggest that placental PC are not unique in their immunomodulatory functions.

Discussion

Here, we describe the ability of PC to modulate CD4 T cell responses, highlighting the regulatory effects of PC on alloreactive CD4 T cell responses to EC. In contrast to immunogenic EC⁺, PC⁺ do not fully stimulate allogeneic CD4 T cells, likely because of a number of factors, including lower expression of MHC and positive costimulatory molecules and higher expression of inhibitory molecules, including PD-1 ligands. Nevertheless, class II MHC molecules on PC⁺ are recognized by alloreactive T cells, as evidenced by induced CD25 expression on resting T cells, proliferation of preactivated T cells, and decreased ability of lymphocytes to respond to subsequent allostimulation by the same donor. We failed to find evidence for induction of Tregs but did see increased expression of genes associated with clonal anergy. We also found that PC, like other populations of stromal cells, decrease T cell proliferation via contact-independent mechanisms mediated across a semi-permeable membrane, including elaboration of IL-10 and TGF-β. Secondary responses of T cells recovered from semi-permeable membrane assays are distinct from those cultured in direct contact with PC⁺ in that responses on restimulation are unaffected in the absence of cell contact.

A number of stromal cells originating from a mesenchymal lineage reportedly modulate lymphocyte responses. Of these, MSC are the best described.18,19 Some investigators have proposed that PC are the resident cell population giving rise to MSC isolated from various tissues.31 Like PC, cultured MSC express MHC class II molecules following treatment with IFN-γ but do not stimulate T cell proliferation. MSC are thought to be poorly immunogenic because of a lack of CD80 and CD86, though it remains to be investigated whether or not MSC express other costimulatory molecules sufficient for memory T cell activation. Similarly to our findings, MSC inhibit T cell proliferation by both cell contact–dependent and cell contact–independent mechanisms in vitro.21,32 No single dominant mechanism for
MSC-mediated lymphocyte suppression has been identified, and inhibition is believed to result from various mechanisms that depend on the antigenic stimulus and include tryptophan depletion by IDO, production of PGE2, and elaboration of inhibitory cytokines. Although we found evidence for the last of these mechanisms in our semi-permeable membrane system, the effects of neutralizing TGF-β and IL-10 were incomplete and showed no effects in direct coculture experiments. The source of these inhibitory cytokines is not entirely clear, because neither cytokine was significantly detected in the culture medium of direct T cell/EC cocultures. Taken together, our results suggest that PC-mediated inhibition is multifactorial.

PC are heterogeneous, varying among different vascular beds. It should be noted that our tissue source of PC, placenta, may have influenced our findings; nevertheless, our studies using commercially available PC from human brain demonstrate that the functions described are not unique to placental PC. It remains to be seen whether they can be generalized to PC from all vascular beds.

In summary, we have provided the first evidence of an immunoregulatory function of differentiated PC isolated from a peripheral tissue and extended the properties of vascular supporting cells to include desensitization of lymphocyte responses to subsequent stimulation. Future studies are needed to address the ability of differentiated PC isolated from other tissues, especially adult peripheral tissues, to similarly inhibit T cell responses, and to address the ability of PC to modulate T cell responses in vivo.

Acknowledgments
We thank Louise Benson, Gwen Arrington-Davis, and Lisa Gras for expert assistance in EC culture.

Sources of Funding
This work was funded by grants from the National Institutes of Health (R01-HL051014 to J.S.P. and T32-GM-007205 to C.L.M.).

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2011;31:183-189; originally published online November 4, 2010;
doi: 10.1161/ATVBAHA.110.217117

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Methods:

Isolation and culture of human cells

All human cells were obtained from deidentified donors under protocols approved by the Yale Human Investigation Committee. PC were isolated from human placentas obtained after vaginal delivery or caesarean section of healthy full-term infants as described previously (1). In brief, placental tissue was washed in PBS, mechanically minced with surgical scissors, and digested with 3mg/ml collagenase D (Sigma-Aldrich, St. Louis, MO) in Hanks Balanced Salt Solution for 2 hours at 37°C on an orbital shaker. The digested suspension was passed through a 100µm mesh filter, then the flow-through passed through a 40µm filter and resultant microvessels plated in tissue culture plates with Medium 199 (Gibco, Grand Island, NY) plus 20%FBS, 2mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin (all from Invitrogen, Carlsbad, CA). Outgrowth cells were confirmed to express NG2, CD90, CD146, and α-SMA by flow cytometry (>95%), and did not contain contaminating CD31, CD34, CD14, or CD45 expressing cells. Human EC were isolated by collagenase treatment of umbilical veins of the same donors as placental PC and cultured as described (1). At subculture 2 EC uniformly express CD31 and von Willebrand factor and are free of cells expressing CD45. Human brain vascular PC were purchased from ScienCell (Carlsbad, CA) and were cultured per the manufacturer’s instructions; these cells were confirmed to uniformly express NG2, CD146, and α-SMA by flow cytometry.

Human peripheral blood CD4+ T cells were isolated by positive selection using magnetic bead separation (Invitrogen) of nonadherent PBMC from human leukapheresis products as described (2) and were >98% positive for CD4 by flow cytometry.
Cytokines, reagents, and antibodies:

For cell surface staining of cultured EC and PC, cells were exposed to 50ng/ml IFN-γ (Invitrogen) 10ng/ml TNF-α (R&D Systems, Minneapolis, MN), or mock-treated for the times specified in the text. Confluent monolayers were washed twice with PBS, incubated with trypsin-EDTA (Invitrogen) until detached, and quenched with M199/20% FBS. Suspended cells were collected and washed with PBS. For directly conjugated antibody staining, cells were incubated with 5ug/ml primary antibody or isotype control diluted in 1% BSA/PBS/0.5% NaNH3 (staining buffer) for 30 minutes at 4°C. For unconjugated antibody staining, cells were incubated with 5ug/ml primary antibody or isotype control for 1 hour at 4°C, then washed with PBS and stained with a species appropriate fluorescently-labeled secondary antibody for 1 hour at 4°C. Immunostained cells were washed with PBS, resuspended in cold staining buffer and analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) using FlowJo analysis software (TreeStar, Ashland, OR). The directly conjugated antibodies used in these analyses were human-reactive CD45, HLA-A,B,C, HLA-DR, CD40, CD80 (B7-1) and CD86 (B7-2) (Beckman Coulter, Brea, CA, USA), GITR ligand and E-selectin (R&D Systems), CD58 (LFA-3), CD274 (PD-L1), CD273 (PD-L2), CD137L (41BB ligand), CD134L (Ox-40 ligand), CD106 (VCAM-1), and CD54 (ICAM-1) (BD Pharmingen, San Diego, CA, USA). The PE-labeled anti-human ICOS ligand mAb was a gift from H. W. Mages (Forschungs Institut for Molekulare Pharmacologie, Berlin, Germany).

For lymphocyte immunostaining, CD4 T cells were incubated with 5ug/ml directly conjugated antibody diluted in staining buffer for 30 minutes at 4°C, washed with PBS, then resuspended in staining buffer and analyzed using an LSRII flow
cytometer and FlowJo analysis software. The specific antibodies used were human-reactive CD4 and CD69 (eBiosciences, San Diego, CA), and CD25 and HLA-DR (Beckman Coulter). In assays where CD4 T cells were pre-labeled with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Molecular Probes, Eugene, OR), proliferation was assessed by flow cytometry for CFSE dilution. In assays where unlabeled CD4 T cells were pulsed with bromodeoxyuridine (BrdU, BD Pharmingen), cells were stained for appropriate surface molecules as described above, and then stained for incorporated BrdU following the manufacturer’s suggested protocol accompanying the BrdU Flow Kit (BD Pharmingen). Propidium iodide (PI) staining for cell cycle analysis was performed by incubating ethanol-fixed T cells with 50ug/ml PI (Invitrogen) plus 100ug/ml RNAse A (Sigma) in PBS and analysis by flow cytometry.

Antibodies used in direct and in transwell coculture assays included PD-L1 and PD-L2 blocking antibodies (10ug/ml, eBiosciences), and IL-10 (10ug/ml eBiosciences) and TGF-β (5ug/ml, Abcam, Cambridge, MA, USA) neutralizing antibodies, added at the start of the coculture. The anti-human HLA-DR blocking antibody (clone LB3.1; gift from J. Strominger, Harvard University, Cambridge, MA) was used at 30ug/ml.

**Direct cocultures:**

CD4 T cells were added to wells of a 24-well plate in RPMI plus 10% FBS containing confluent monolayers of allogeneic accessory cells (PC or EC) at a T cell:accessory cell ratio of 30:1. Where indicated PC and EC were pre-treated with 50ng/ml IFN-γ (Invitrogen) for 72 hours, to induce MHC class II molecule expression. In all experiments IFN-γ-pre-treated PC and EC (abbreviated PC⁺ or EC⁺) were confirmed to
express HLA-DR (Beckman Coulter) by flow cytometry prior to addition of lymphocytes. Cocultures with untreated MHC class II molecule negative cells (PC^- and EC^-) were carried out in parallel. CD4 T cells were harvested at the times indicated in the text and proliferation was assessed after 7 days of coculture, except in assays where PHA-L (Sigma-Aldrich) was added and proliferation was assessed at day 2 or 3 as indicated. In some experiments, CD4 T cells were prelabeled with 250nM CFSE (Molecular Probes, Eugene, OR, USA) prior to coculture.

Activated CD4 T cells were generated by coculture of CFSE-labeled CD4 T cells with allogeneic EC^+ for 7 days and collection of CFSE^low CD4 T cells by sterile sorting using a FACS Aria (BD Biosciences). Sorted cells were washed, rested for 2 days, and added to wells containing either EC^+ or PC^+, autologous to the EC^+ used in primary coculture, or third party PC^+. BrdU was pulsed at 10uM and CD4 T cells were harvested and stained for incorporated BrdU after 4 days of restimulation.

For secondary cocultures, CD4 T cells were first cocultured with EC^+ or PC^+ and various blocking antibodies as indicated in the text, then collected at the times indicated and restimulated by coculture with EC^+ from the same donor or third party EC^+ for 7 days. To assess suppression, PC^+-stimulated T cells were mixed with freshly isolated autologous CD4 T cells at increasing concentrations (1:4, 1:2, 1:1) and restimulated by EC^+ from the same donor (as the PC) or third party EC^+ for 7 days to assess suppressive ability.

*Transwell cocultures:*
For transwell experiments, PC or EC were plated on gelatin-coated semi-permeable membranes of 0.4-um pore size transwell inserts (Falcon, San Jose, CA). Where indicated cells in transwells were pre-treated with 50ng/ml IFN-γ for 72 hours, then washed twice with PBS and once with RPMI 1640. CFSE pre-labeled CD4 T cells were added directly to wells containing EC⁺ and transwells, containing PC⁻, PC⁺, EC⁻ or EC⁺, were inserted above.

Where indicated, transwell cocultures were treated with 24.5uM L-tryptophan daily, 200uM 1-methyl-D-tryptophan on day 1, 1.7mM L-arginine daily, 25mM 1400W daily, 1uM Indomethacin daily, or 100uM aspirin on day 1 (Sigma-Aldrich). Doses were chosen based on published reports and were confirmed by dose response assays to effectively abrogate the targeted effect (data not shown). In transwell assays using superantigen, 10ng/ml TSST-1 (Toxin Technology, Sarasota, FL, USA) was added at the beginning of the coculture and T cells were harvested on coculture day 4.

To test the effects of transwell cocultures on T cell restimulation, unlabeled CD4 T cells were collected after 6 days of primary transwell coculture, rested for two days, then washed and cocultured with EC⁺ either from the same donor as in primary culture or a third party donor. BrdU was added to secondary cocultures at 10uM and CD4 T cells were harvested and stained for BrdU incorporation after 4 days of restimulation.

**ELISAs**

For determination of mediator production by PC and EC in the absence of T cells, cultured cells were treated with IFN-γ or vehicle control (PBS/0.1%BSA) for 72 hours, washed twice with HBSS (Invitrogen), and given fresh M199/20%FBS. After 24 hours
media samples were collected and assayed for IL-10, TGF-β (eBiosciences), and PGE2 (R&D) by ELISA. For measurement of cytokine production in cocultures with T cells, media was collected after 24 hours of coculture and assayed for IL-2, IFN-γ, IL-10, TGF-β (all from eBiosciences), and PGE2 (R&D) using specific ELISA kits and following the manufacturer’s instructions.

**Quantitative RT-PCR:**

To assess mRNA levels in PC under basal or IFN-γ stimulated condition, cultured cells were treated with 50ng/ml IFN-γ (Invitrogen) or vehicle control for the times specified in the text. To assess mRNA levels in T cells activated by EC⁺ or PC⁺, CD4 T cells were cocultured with accessory cells for 3 days, then CD25⁺ (activated) CD4 T cells were collected by sterile cell sorting on a FACS Aria (BD Biosciences). Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommended protocol. RNA was quantified by a spectrophotometer and equal amounts were used to generate cDNA using TaqMan reverse transcriptase reagents (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. For iNOS, ITCH, CBL-B, and Foxp3 transcripts, PCR was performed using pre-developed TaqMan primers and probes and normalized to GAPDH (Assays-on-Demand; Applied Biosystems). For all other transcripts, real-time quantitative PCR was performed on cDNA template using the primer sequences listed below and SYBR Green master mix (Bio-Rad, Hercules, CA). Samples were run in triplicate on an iCycler and data analyzed using its system interface software (Bio-Rad). Each target gene was normalized using GAPDH as a reference value. The following
previously validated primer pairs were used: (5’-3’): IDO (forward) 5’-
GGACAATCAGTAAGAGGTACCA-3’, (reverse) 5’-
GGCAGATGTGGTAGCAATGAA-3’; Arginase-I (forward) 5’-
TGGAAACTTGGCATGGAC-3’, (reverse) 5’-AAGTCCGAAACAAGCCAA-3’;
Arginase-II (forward) 5’-GACACTGCCCAGACCTTTGT-3’, (reverse) 5’-
CGTTCCATGACCTTCTGGAT-3’; CAT2B (forward) 5’-
CCCAATGCCTCGTGAATCTA-3’, (reverse) 5’-TGCCACTGCACCCGATGACAA-3’;
GRAIL (forward) 5’-ACACGAATTTCACGGTGCC-3’, (reverse) 5’-
GATGGATCTTGTCTGCGAAGG-3’; GAPDH (forward) 5’-
GAAGGTGAAGGTCGGAGTC-3’, (reverse) 5’-GAAGATGGTGATGGGATTTC-3’.

Immunoblotting:

Cultured cells were treated with 50ng/ml IFN-γ (Invitrogen) or vehicle control for
the times indicated in the text. Media was aspirated from cell culture plates and the plates
were put on ice and washed with cold HBSS (Invitrogen). Ice-cold lysis buffer (50mM
Tris, pH 7.4, 300mM NaCl, 10% glycerol, 3mM EDTA, 1mM MgCl2, 20mM β-
glycerophosphate, 25mM NaF, 1% Triton X-100, 25µg/ml leupeptin, 25µg/ml pepstatin,
and 3µg/ml aprotinin) was added and lysates were collected using a cell scraper (BD
Biosciences). Samples were vortexed and centrifuged at 10,000 x g for 10 minutes at
4°C. The protein concentration of each sample was quantified using a Bio-Rad protein
assay kit. Twenty micrograms of total protein from each sample was heated in SDS
sample buffer and separated by SDS-PAGE. Proteins were transferred overnight onto
nitrocellulose membranes and then western blots performed using mAbs reactive with
human IDO (Millipore, Billerica, MA, USA) or β-actin (Sigma-Aldrich), or a polyclonal Ab reactive with human and mouse iNOS (Cayman Chemical, Ann Arbor, MI, USA), followed by a species-appropriate HRP-conjugated secondary Ab (Jackson ImmunoResearch, West Grove, PA, USA) and by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

**Statistical analysis:**

Raw values quantifying the percentage of proliferating CD4 T cells were converted to normalized proliferation values by referencing to the positive control. In stimulation assays with pre-activated allogeneic CD4 T cells (collected by sorting, as described above), the positive control was the percentage of proliferating CD4 T cells following restimulation by EC⁺ from the same donor as used in priming. In assays where CD4 T cells were cultured first in direct contact with PC⁺ and then restimulated by matched or third party EC⁺, the positive control was the percentage of proliferating CD4 T cells restimulated by matched or third party EC⁺ after being first cultured in direct contact with EC⁺. In transwell assays, the positive control was the percentage of proliferating CD4 T cells in CD4/EC⁺ cocultures in the presence of EC⁺ across a transwell and the appropriate control treatment. Positive control values, representing alloreactive proliferation, typically ranged between 4-8% of the total CD4 T cell population. Statistical analysis was performed in consultation with Yale’s WM Keck Facility using either non-parametric or parametric (t-tests and ANOVA) as appropriate. Statistical significance is indicated by *p<0.05 and **p<0.01.
**Supplemental Figures:**

**Supplemental Figure I**

**IFN-γ induces IDO expression in PC.** (A) Quantitative RT-PCR was used to measure IDO transcript levels in PC following control treatment (PC*) or treatment with IFN-γ (PC+) for 24h. Values represent fold-increase in IDO mRNA after normalization to GAPDH. (B) IDO protein expression detected by western blot in cell lysates of EC or PC after 48h of control or IFN-γ treatment. One of three independent experiments with similar results.
IFN-γ induces PC expression of enzymes involved in L-arginine consumption.
Quantitative RT-PCR was used to measure arginase-II and CAT2B transcript levels in PC following control treatment (PC-) or treatment with IFN-γ (PC+) for 24h. Values represent fold-increase of specified mRNA after normalization to GAPDH. Arginase-I mRNA was not detected. One of two independent experiments with similar results.
**IFN-γ-treatment of PC induces iNOS mRNA but not iNOS protein expression.** (A) Quantitative RT-PCR was used to measure iNOS transcript levels in PC following control treatment (PC⁻) or treatment with IFN-γ (PC⁺) for 24h. Values represent fold-increase in iNOS mRNA after normalization to GAPDH. (B) iNOS protein expression detected by western blot in cell lysates from EC or PC after 48h of control or IFN-γ treatment, or from mouse macrophages. (C) Nitric oxide detected in culture medium alone or in media collected from PC cultures stimulated with vehicle control (PC⁻) or with IFN-γ (PC⁺) for 72h. One of two independent experiments with similar results.
Supplemental Figure IV


derives PGE2 production by PC, yet levels are less than PGE2 production by EC. (A) PGE2, measured by ELISA, in culture media of EC and PC under basal (-) and IFN-γ-stimulated (+) conditions or (B) in media from CD4/EC+ cocultures in the presence of EC, PC−, or PC+ across a transwell ([EC], [PC], [PC+]). One of two independent experiments with similar results.
Supplemental Figure V

**Restimulation responses of alloreactive CD4 T cells cultured in the presence of PC\(^+\) across a transwell are not inhibited.** CD4 T cells were collected after 6 days of primary transwell coculture, rested for two days, then washed and restimulated with EC\(^+\) either from the same donor as in primary culture or a third party donor. BrdU was added to secondary cocultures and CD4 T cells were harvested and stained for BrdU incorporation after 4 days of restimulation. Proliferation of restimulated CD4 T cells from primary CD4/EC\(^+\) cocultures with EC\(^+\) across a transwell served as the positive control used to generate normalized proliferation values. Values represent normalized proliferation to matched or third party EC\(^+\) pooled from three independent experiments; ns = not significant.
Supplemental Table I. Flow cytometric analysis of surface proteins on human brain vascular pericytes.*

<table>
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<th>Marker</th>
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<td>HLA-DR</td>
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<td>CD62E (E-selectin)</td>
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* PC were treated with vehicle control or 50ng/ml IFN-γ for 72h, then collected and labeled as described in Supplemental Methods. Results are expressed in arbitrary units of fluorescence as the corrected mean fluorescence intensity (MFI) after subtracting the MFI of the isotype control from the MFI of the specifically stained cells.

† Unlike PC isolated from placenta, brain PC do not uniformly express HLA-DR following IFN-γ treatment; however, a subset of brain PC, approximately 18% of the total population and thus poorly reflected by MFI, express HLA-DR after IFN-γ stimulation.
Supplemental Figure VI

**Human brain PC poorly stimulate allogeneic CD4 T cells and inhibit alloreactive CD4 T cell proliferation to EC⁺.** (A-C) CFSE-labeled CD4 T cells were cocultured with brain PC⁻ or PC⁺, or umbilical vein EC⁻ or EC⁺ from an unrelated donor. (A) Media was collected and analyzed for the presence of IL-2 by ELISA after 24h. (B) Cocultured CD4 T cells were analyzed by flow cytometry for expression of CD25 at 72h. (C) CD4 T cell proliferation assessed after 7 days of coculture by CFSE dilution. (D) CFSE-labeled CD4 T cells were cocultured with EC⁺ in the presence of EC⁺ (control) or PC⁺ across a
transwell and CD4 T cell proliferation assessed after 7 days of coculture. Representative results from one of two experiments in (A). In (B-C) pooled data from two independent experiments.
Supplemental References: