SUPPLEMENTAL MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and microvascular ECs (HMVECs) were purchased from Lonza (Basel, Switzerland) and cultured in EBM complete medium (Lonza). ECs were used up to the sixth passage and were CD45-negative and uniformly VE-cadherin-positive (data not shown). To examine the induction of CD155 expression, ECs were treated with IFNγ (50 ng/mL; Invitrogen, Carlsbad, CA), IL-1α (10 ng/mL; NCI Biological Resources Branch Preclinical Repository), TNF-α (10 ng/mL; R&D Systems, Minneapolis, MN), and/or IL-6 (50 ng/mL; Peprotech, Rockey Hill, NJ).

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by phlebotomy with approval of the appropriate institution review boards. CD8 and CD4 T cells were isolated from PBMC by positive isolation using Dynal magnetic beads (Invitrogen) as described previously \(^1\) and were consistently greater than 98% in purity. To purify naïve (CD45RO-) and memory (CD45RO+) T cells, CD8 T cells were stained with a PE-conjugated CD45RO antibody (BD Biosciences, Franklin Lakes, NJ), and then CD45RO- and CD45RO+ cells FACSorted using a BD FACS Aria.

To examine T cell activation, CD8 T cells were co-cultured with allogeneic ECs at a 10:1 ratio in RPMI + 10% FCS as described \(^1\). In some experiments phytohemagglutinin (PHA; 3 µg/mL; Sigma, St. Louis, MO) was added. In the PHA system, the TCR is stimulated in all T cells by PHA (which overrides the TCR stimulation provided to a subset of alloreactive cells by alloantigens presented on ECs) and ECs provide co-stimulatory signals. In experiments examining allogeneic activation of T cells, CD8 T cells were cultured with ECs in the absence of PHA. When T cell – EC co-cultures were maintained for more than six days, fresh medium was...
added after six days and we have determined previously that cell death in these cultures is minimal 2. For allogeneic CD4 T cell activation, ECs were stimulated with IFNγ (50 ng/mL) for three days prior to addition of CD4 T cells in order to induce MHC class II expression. ECs were washed extensively before addition of CD4 T cells 1. In the indicated experiments, a human IFNγ neutralizing antibody (10 µg/mL; R & D Systems), IL-1 receptor antagonist (IL-1RA; 100 ng/mL; R & D Systems), a previously described CD155 neutralizing antibody that inhibits CD155 interactions with DNAM-1 (clone D171; 10 µg/mL; Thermo Scientific, Rockford, IL) 3, or a DNAM-1 neutralizing antibody that inhibits DNAM-1-mediated cell adhesion (R&D Systems) was added to the cultures. All experiments were performed at least three times with similar findings.

Flow Cytometry

Flow cytometry was performed as described 1. ECs were harvested by either trypsinization or by incubation in 3 mM EDTA. For examination of CD155 expression, ECs were incubated with a mouse CD155 antibody (Thermo Scientific) for 1 h followed by incubation with a FITC-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. In some experiments ECs were subsequently stained with a PE-conjugated VE-cadherin antibody (1 µg; eBioscience, San Diego, CA). For analysis of CD8 T cell proliferation, T cells were labelled with CFSE (0.5 µM; Sigma, St. Louis, MO) prior to coculture with ECs 1. At the indicated time points, cells were harvested and stained with a PE-conjugated CD8 antibody (BD Biosciences) prior to analysis. Staining for DNAM-1 was performed using a mouse monoclonal antibody (R&D Systems) and granzyme B using a FITC-conjugated mouse monoclonal antibody (BD Bioscience).
Supplemental Material

For intracellular cytokine staining, CD8 T cells were stimulated with ECs and PHA for three days, treated with PMA (50 ng/mL; Sigma) and ionomycin (1 µg/mL; Sigma) in the presence of brefeldin A (Sigma, St. Louis, MO) for 6 h, and then stained with a PE-conjugated CD8 antibody and FITC-conjugated IFNγ antibody as per the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ). All data was acquired on a BD FACS Aria.

RNA Analysis

Total RNA was isolated using a RNA Minikit (Qiagen, Valencia, CA) and Taqman quantitative RT-PCR performed as described ¹ using validated primer/probe sets (Applied Biosystems, Foster City, CA). Data was acquired on an ABI 7900HT iCycler.

ELISA

Cytokine concentrations in culture supernatants were examined with IFNγ (Invitrogen), IL-10 (e-Bioscience), IL-5 (e-Bioscience), and IL-17A (eBioscience) ELISA kits.

Calcein-Release Assay

Evaluation of CD8 T cell cytotoxicity was performed as we have described previously using effector T cells generated by activation with mitogen ⁴. Briefly, CD8 T cells were activated with ECs + PHA in the absence or presence of a CD155 neutralizing antibody. After three days, CD8 T cells were harvested, washed extensively to remove antibodies, and then cultured at the indicated effector:target ratios with calcein-loaded (10 µg/mL; Invitrogen) ECs in the presence of PHA. After 6 h, supernatants were harvested and calcein release quantified on a Molecular Devices Spectromax M5 at Ex = 485nm and Em = 530nm. Percent specific lysis was determined with the following formula: % Specific Calcein Release = (calcein release in sample – spontaneous release)/(total calcein release – spontaneous release).
Supplemental Material

Statistical Analysis

A Student’s t-test was performed to determine significant differences between groups. An $\alpha$-value of <0.05 was determined to be significant.

REFERENCES


