CD155 on Human Vascular Endothelial Cells Attenuates the Acquisition of Effector Functions in CD8 T Cells

Nichole K. Escalante, Anna von Rossum, Martin Lee, Jonathan C. Choy

Objective—CD155 is a cell surface protein that has recently been described to exert immune regulatory functions. We have characterized the expression of CD155 on human vascular endothelial cells (ECs) and examined its role in the regulation of T-cell activation.

Methods and Results—CD155 was expressed on resting human vascular ECs and was upregulated in an interferon-γ (IFNγ)-dependent manner. When the function of CD155 in regulating T-cell activation was examined, antibody-mediated neutralization of CD155 did not affect CD8 T-cell proliferation in response to stimulation with ECs. However, neutralization of CD155 activity or small interfering RNA-mediated inhibition of CD155 expression in ECs increased expression of IFNγ and cytotoxic effector function in activated CD8 T cells.

Conclusion—CD155 is an IFNγ-inducible immune regulatory protein on the surface of human ECs that attenuates the acquisition of effector functions in CD8 T cells. (Arterioscler Thromb Vasc Biol. 2011;31:1177-1184.)

Key Words: cytokines ■ endothelium ■ immune system ■ CD155 ■ T cell

Human vascular endothelial cells (ECs) act as semiprofessional antigen-presenting cells and, in this way, regulate immune responses by presenting antigenic peptides and cell surface proteins to T cells in peripheral tissues. This immunoregulatory property of the endothelium is established in the control T-cell responses in solid organ transplantation and may regulate immune responses in autoimmunity and some cardiovascular diseases. With regard to the expression of cell surface proteins, human ECs provide positive costimulatory signals to T cells mainly through leukocyte functional antigen-3, intercellular adhesion molecule-1, CD154, 41BB ligand, inducible co-stimulator ligand, and OX40 ligand. These signals are required for the activation of memory CD4 and CD8 T cells by ECs. In contrast, ECs also express cell surface proteins, such as PD-L1, that inhibit T cell responses. The expression of immune regulatory proteins on ECs is controlled by cytokines. In particular, interferon-γ (IFNγ) is especially pertinent to EC immunobiology as it induces the expression of both immune activating and inhibitory proteins. The precise effects of IFNγ on determining the phenotype of ECs may depend on its cooperative signaling with other cytokines or the temporal regulation of target genes. Overall, understanding the role of immune regulatory proteins on the surface of ECs is important for providing insight into the regulation of immune responses.

Activation of CD8 T cells by allogeneic human ECs induces proliferation, IFNγ secretion, and the expression of cytotoxic effector proteins, such as granzyme B. These properties of activated CD8 T cells play a pathological role in allograft rejection. Also, IFNγ production from activated T cells within arteries drives pathological smooth muscle cell proliferation in the arterial intima and vasomotor dysfunction, thereby contributing to the pathogenesis of some vascular diseases. Interleukin (IL)-10 is also produced by effector T cells that secrete IFNγ, as well as by some induced regulatory T cells. Expression of IL-10 in effector T cells develops late after T-cell activation, correlates with increased immune activation, and serves to self-regulate the associated effector T-cell response.

CD155, originally described as the poliovirus receptor, is an Ig-like cell surface protein expressed on many cell types that has recently been discovered to have immune regulatory properties. CD155 knockout mice are characterized by reduced accumulation of CD8 single-positive thymocytes and by defective humoral immune responses in response to oral vaccination. With regard to ECs, antibody-mediated neutralization of CD155 prevents monocyte and T-cell receptor–dependent effector memory T-cell transendothelial migration in vitro. Most of the immunologic effects of CD155 are mediated by its interaction with DNAX accessory molecule-1 (DNAM-1) (also called CD226) or CD96 on the surface of leukocytes. DNAM-1 is expressed on both CD8 and CD4 T cells in human peripheral blood. It contributes to T-cell activation in experimental autoimmune encephalomyelitis and in a mouse model of graft versus host disease. CD96 is expressed mainly by natural killer cells and is upregulated on T cells very late (day 6 to 9) after activation.
We have shown previously that targeting of graft endothelium by effector CD8 T cells is involved in the initiation of allograft vasculopathy, a vascular condition that is a main cause of solid organ rejection, and that ECs regulate CD8 T-cell responses in a humanized mouse model of allograft vasculopathy. In our current study, the role of CD155 on human ECs in regulating CD8 T-cell responses was examined. CD155 is expressed on resting human ECs, and its expression is upregulated through an IFNγ-mediated mechanism. CD155 on ECs does not affect proliferation of CD8 T cells stimulated by ECs, but surprisingly, it attenuates the secretion of IFNγ and the acquisition of cytotoxic effector functions. Taken together, these data identify a novel immunoregulatory role of CD155 in human ECs and suggest that it may modulate CD8 T-cell effector responses in peripheral tissues.

Materials and Methods

Cell Culture

Human umbilical vein ECs (HUVECs) and microvascular ECs (HMVECs) were purchased from Lonza (Basel, Switzerland), were used up to the sixth passage, and were CD45 negative and uniformly vascular endothelial-cadherin positive (data not shown). Peripheral blood mononuclear cells were obtained from healthy volunteers with the approval of the appropriate institutional review boards. CD8 T blood mononuclear cells were obtained from healthy volunteers with the approval of the appropriate institutional review boards. CD8 T cells were isolated from peripheral blood mononuclear cells by positive isolation and were cocultured with allogeneic ECs at a 10:1 ratio as described. All experiments were performed at least 3 times with similar findings.

Small Interfering RNA Gene Silencing

ECs were transfected with 100 pmol of nonspecific control RNA (Qiagen) or with small interfering RNA (siRNA) specific for CD155 (Dharmacon, Lafayette, CO) by Amaza nucleasection using program A-034.

Additional description of the materials and methods used can be found in the Supplemental Material, available online at http://atvb.ahajournals.org.

Results

CD155 Expression Is Induced in ECs Through an IFNγ-Dependent Mechanism

The basal and cytokine-induced expression of CD155 on ECs was examined by flow cytometry. Resting HUVECs express CD155. When HUVECs were stimulated with IFNγ, IL-1α, tumor necrosis factor-α, or IL-6 alone, there was minimal effect on CD155 expression. However, CD155 expression on HUVECs was increased after stimulation with all of the studied cytokines together (Figure 1A). The effect of cytokine combinations was then determined. IFNγ and IL-1α acted together to induce the expression of CD155 in HUVECs, but neither tumor necrosis factor-α nor IL-6 had any effect (Figure 1B). When CD155 expression was examined in untreated and IFNγ/IL-1α-treated HUVECs from 4 separate donors, IFNγ/IL-1α significantly induced CD155 expression by 2-fold (Figure 1C). Similar results were observed in HMVECs (Figure 1D). In addition to protein, CD155 mRNA expression was also induced by IFNγ/IL-1α. This was observed as early as 4 hours poststimulation and decreased at later time points (Figure 1E). The kinetics of CD155 protein expression were delayed compared with mRNA expression, being evident as early as 6 hours poststimulation and maximal at 24 to 48 hours poststimulation (Figure 1F).

IFNγ is produced by T cells in response to activation by ECs, and IL-1α is produced by ECs in response to their targeting by reactive T cells. Therefore, we investigated whether these 2 cytokines, when produced during T-cell responses, affected CD155 expression on ECs. CD8 T cells were cocultured with ECs in the presence of phytohemagglutinin (PHA). In this system, PHA stimulates T cell receptor signaling in all T cells (thereby overriding the recognition of allogeneic peptide-major histocompatibility complex by the T cell receptor), and the presence of cell surface molecules on ECs provides costimulatory signals. This leads to the activation of all T cells, and the presence of both PHA and ECs is required. This is a robust system that provides relevant information on the role of EC surface molecules in regulating T-cell activation.

ECs were cultured with CD8 T cells, and IFNγ activity was inhibited with a neutralizing antibody or IL-1 activity was inhibited by the addition of IL-1 receptor antagonist. After 3 days, cells were harvested, and 2-color flow cytometry was performed for CD155 and VE-cadherin to visualize CD155 expression on ECs. This time point was chosen because IFNγ levels in these cocultures peaks at day 3. The presence of activated CD8 T cells increased the expression of CD155 on the surface of ECs, and neutralization of IFNγ activity completely prevented the induction of CD155 (Figure 2). However, the inhibition of IL-1 signaling had no effect on CD155 expression, although IL-1 receptor antagonist was able to completely inhibit nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase 1/2 activation in response to IL-1α (data not shown). Taken together, the above findings indicate that CD155 expression is induced on the surface of ECs by activated T cells through an IFNγ-mediated mechanism. Although IFNγ can act together with IL-1 to induce CD155, molecules in addition to IL-1 act in a redundant manner during T-cell responses.

Expression of CD155 on ECs Does Not Affect CD8 T-Cell Proliferation

Resting human CD8 T cells uniformly express the CD155 ligand DNAM-1 but not CD155 (Supplemental Figure I). To investigate the role of CD155 on human ECs in regulating CD8 T-cell activation, purified CD8 T cells were initially cultured with ECs in the presence of PHA, and an isotype control antibody or a previously characterized neutralizing antibody to CD155 was added. CD8 T-cell proliferation was examined by carboxyfluorescein diacetate succinimidyl ester labeling of T cells before stimulation and then quantifying carboxyfluorescein diacetate succinimidyl ester dilution at day 6 poststimulation. There was minimal proliferation of CD8 T cells in the absence of ECs. In the presence of ECs, the majority of CD8 T cells proliferated extensively, and neutralization of CD155 did not affect the accumulation of proliferated T cells (Figure 3A). The effect of CD155 on CD8 T-cell proliferation induced by alloantigen recognition was
Figure 1. Induction of CD155 by IFNγ/IL-1α in human ECs. 

A, HUVECs were treated with IFNγ (50 ng/mL), IL-1α (10 ng/mL), tumor necrosis factor-α (TNF) (10 ng/mL), IL-6 (50 ng/mL), or all cytokines together. After 24 hours, CD155 expression was examined by flow cytometry. Red indicates isotype staining control; blue, untreated ECs; green, cytokine-treated ECs. 

B, HUVECs were treated with the cytokine combinations indicated, and CD155 expression was determined by flow cytometry after 24 hours. Red indicates isotype staining control; blue, untreated ECs; green, cytokine-treated ECs. 

C, Mean±SD of CD155 protein expression (as measured by correct mean fluorescence intensity [cMFI]) in untreated and IFNγ/IL-1α-treated HUVECs from 4 separate experiments with different donors. *P<0.02. 

D, Mean±SD of CD155 protein expression (as measured by cMFI) in untreated and IFNγ/IL-1α-treated HMVECs from 3 separate experiments. *P=0.02. 

E, HUVECs were treated with IFNγ/IL-1α, and the mRNA expression of CD155 was quantified by quantitative reverse transcription–polymerase chain reaction at various time points. Mean±SD is shown of triplicate measurements in 1 representative experiment. 

F, HUVECs were treated with IFNγ/IL-1α for the indicated time points, and CD155 expression examined by flow cytometry. Red indicates isotype staining control; blue, ECs treated for 0 hours; green, ECs treated until the indicated time point.
also examined by culturing CD8 T cells with allogeneic ECs (in the absence of PHA). A subset of reactive CD8 T cells proliferated in response to allogeneic ECs and neutralization of CD155 did not affect the proliferation of allogeneically stimulated human CD8 T cells (Figure 3B). Consistent with the lack of effect of CD155 on T-cell proliferation, neutralization of DNAM-1 also did not affect CD8 T-cell proliferation (Supplemental Figure IIA).

**CD155 on ECs Attenuates the Acquisition of CD8 T-Cell Effector Functions**

The role of CD155 in regulating the effector functions of CD8 T cells was then examined. When stimulated by ECs and PHA, CD8 T cells secreted abundant levels of IFN-γ and some IL-10. Unexpectedly, antibody-mediated neutralization of CD155 increased the levels of both cytokines (Figure 4A and 4B). The induction of IL-5 and IL-17A was not consistently detected in our experiments (data not shown). Congruent with the effects observed when CD155 activity was inhibited, neutralization of DNAM-1 also increased the production of IFN-γ by activated CD8 T cells (Supplemental Figure IIB). This was observed as early as day 1 poststimulation and was maintained at later time points. The role of CD155 in regulating the production of IFN-γ from naïve (CD45RO−) and memory (CD45RO+) CD8 T cells was also examined. Memory CD8 T cells produced more IFN-γ after activation with ECs+PHA compared with naïve CD8 T cells, and neutralization of CD155 increased the production of IFN-γ from both naïve and memory populations (Figure 4C).

In addition to cytokine secretion, neutralization of CD155 also increased significantly the expression of granzyme B in activated CD8 T cells (Figure 4D; correct mean fluorescence intensity of 29.3 ± 1.0 in activated CD8 T cells treated with an IgG control antibody compared with 37.4 ± 4.7 in activated CD8 T cells treated with a CD155 neutralizing antibody, n=3, P<0.05). When the effect of CD155 on the development of cytotoxic effector function was evaluated, CD8 T cells that were activated in the presence of a CD155 neutralizing antibody exhibited significantly greater cytotoxicity toward target ECs compared with CD8 T cells activated in the absence of CD155 neutralization (Figure 4E).

CD155 expression was then inhibited by siRNA gene silencing. ECs were transfected with a nonspecific control RNA or siRNA targeting CD155. CD155 siRNA effectively inhibited expression of CD155 mRNA and protein by more than 75% (Figure 5A and Supplemental Figure III). There was no effect of CD155 knockdown on cell viability or confluence (data not shown). When CD8 T-cell activation was analyzed, gene silencing of CD155 increased IFN-γ and IL-10 secretion by CD8 T cells (Figure 5B and 5C). The effect of CD155 on the expression
of IFNγ in activated CD8 T cells was also examined by intracellular cytokine staining. Stimulation of CD8 T cells with ECs and PHA induced the expression of IFNγ in a large subset of activated T cells. When CD155 expression was inhibited, there was no change in the size of the IFNγ-expressing population (34.1 ± 3.1% in control group compared with 36.7 ± 9.2% in siRNA group, P = not significant). However, the level of cytokine expression, as measured by correct mean fluorescence intensity, was increased significantly (Figure 5D; 123.5 ± 3.5 in control group compared with 145.5 ± 3.5 in siRNA group, P < 0.02). These results indicate that CD155 expression on ECs attenuates the level of expression of cytokines, in particular IFNγ, in effector CD8 T cells but does not affect their differentiation into effector cells.

EC regulation of T-cell activation is particularly pertinent to the control of immune responses during organ transplan-
tation. Therefore, the role of CD155 in regulating cytokine production from allogeneically stimulated T cells was examined. IFNγ was not produced by ECs in this system. Activation of CD8 T cells by allogeneic HUVECs induced the production of IFNγ from reactive T cells, and neutralization of CD155 significantly increased IFNγ secretion (Figure 6A). Similar findings were obtained when CD155 was inhibited during the activation of CD8 T cells with allogeneic HMVECs (Figure 6B). IL-10 was not detected from allogeneically stimulated CD8 T cells, either by ELISA or intracellular cytokine staining, nor was IL-5 or IL-17A (data not shown). Finally, in contrast to CD8 T cells, blockade of CD155 did not affect IFNγ secretion from CD4 T cells stimulated with allogeneic ECs (Figure 6C).

**Discussion**

We have shown that CD155 is an IFNγ-regulated cell surface protein on human ECs that, surprisingly, attenuates the acquisition of effector functions in CD8 T cells activated by ECs. To our knowledge, these findings establish a novel immune regulatory role for CD155 and may have implications for understanding the control of CD8 T-cell responses in peripheral tissues.

CD155 has been shown to be expressed basally in arterial, venular, and microvascular ECs in human tissues.15 We have confirmed the basal expression of CD155 on ECs and determined that its expression is induced through an IFNγ-mediated pathway. The level of induction is similar to that observed for some costimulatory molecules, such as 41BBL and OX40L, in ECs in response to tumor necrosis factor.3 Furthermore, signals in addition to IFNγ are required to induce CD155. Although IL-1α is able to act in concert with IFNγ to induce CD155 when the 2 cytokines are added exogenously, blockade of IL-1 activity did not affect the induction of CD155 expression in EC–T-cell cocultures, indicating that other redundant signals are present during immune responses. The identity of these redundant signals is not known. In general, very little is known about the regulation of CD155 expression. The CD155 promoter contains NF-κB and AP-2 binding sites, and overexpression of AP-2 can induce CD155 expression in vitro.26 Also, CD155 expression is induced in mouse fibroblasts through a Ras- and mitogen-activated protein kinase–mediated pathway.27 We have observed that pharmacological inhibition of NF-κB or mitogen-activated protein kinase pathways prevents CD155 induction in response to IFNγ/IL-1α (unpublished data). Many potential protein and nonprotein factors are produced by both ECs and T cells in our cocultures that could activate these pathways. These include candidates such as vascular endothelial growth factor, fibroblast growth factor, CD40L, high-mobility group box-1 protein, and nucleic acids released by injured cells. Biochemical studies may be needed to isolate the relevant factor(s). In addition to ECs, it is interesting to note that we did not observe CD155 expression on resting human CD8 T cells, although CD155 expression has been documented on resting CD8 T cells in mice.13

Systemic elimination of CD155 in mice results in a reduction in the number of mature CD8 SP thymocytes and defects in antibody class-switching in response to oral administration of antigen13,14,28. Furthermore, DNAM-1 expression on mouse CD8 T cells is needed for optimal CD8 T-cell proliferation and development of cytotoxic effector function in response to activation by B cells that overexpress CD155.29 Interestingly, DNAM-1 expression is not required for the activation of CD8 T cells by dendritic cells, indicating a cell type-specific role of this pathway in immune regulation. Our data indicate that CD155 expression on human ECs attenuates CD8 T-cell effector functions. This may be important in the downregulation or fine-tuning of T-cell responses in peripheral tissues and may have implications for the regulation of T-cell responses in solid organ transplantation or autoimmunity. Although the role of CD155-DNAM-1 interactions in solid organ transplantation is not known, recent genome-wide association studies have shown that a single-nucleotide polymorphism in DNAM-1 is associated with the development of type 1 diabetes, rheumatoid arthritis, and Wegener’s granulomatosis.10,31 The effect of this polymorphism on DNAM-1 expression or function was not experimentally addressed, but sequence analysis suggests that the susceptibility allele could reduce DNAM-1 expression.30 Indeed, Lofgren et al32 recently identified a 3-variant haplo-type in DNAM-1 that reduces its expression in lymphocytes and that confers increased susceptibility to systemic lupus erythematosus. These findings suggest that the DNAM-1 pathway complexly regulates immune responses and may have an inhibitory role in some contexts.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Neutralization of CD155 increases IFNγ secretion from allogeneically activated CD8 T cells. A and B, CD8 T cells were cultured alone or with allogeneic HUVECs (A) or HMVECs (B). No PHA was added to the cultures. After 3 days, supernatants were collected and IFNγ quantified by ELISA. As a control, ECs were also cultured alone in the absence or presence of a CD155 neutralizing antibody. Mean±SD is shown of triplicate measurements in 1 representative experiment. *P<0.01. C, CD4 T cells were cultured alone or with allogeneic IFNγ-stimulated HUVECs. After 3 days, supernatants were collected, and IFNγ was quantified by ELISA. Mean±SD is shown of triplicate measurements in 1 representative experiment. ND indicates not detected; NS, not significant.
The complex immunologic functions of the CD155-DNAM-1 pathway may relate to the need to differentially regulate the activation and differentiation of T cells within lymphoid tissues compared with peripheral tissues. Mechanistically, this may involve the use of different accessory molecules by B cells compared with ECs. For example, human ECs do not express CD80 and CD86 and provide costimulatory signals through other pathways, such as leukocyte functional antigen-3/CD2 and intercellular adhesion molecule/leukocyte functional antigen-1. The cell surface localization of CD155 could also be different in B cells compared with adherent ECs, where it is present in cell-cell junctions. CD155 may also differentially regulate separate aspects of immunity. Such context specific immune regulation has been reported for the PD-1 pathway, which inhibits CD8 T-cell responses to virus infection but is needed for the optimal development of some CD4 T-cell responses. Although CD155 and PD-L1 inhibit similar aspects of CD8 T-cell activation when expressed by the endothelium, the effects of CD155 in our experiments are not due to alterations in PD-L1 expression in ECs (unpublished data). Moreover, the dual function of endothelial CD155 in facilitating diapedesis of some leukocytes and attenuating T-cell effector functions may be analogous in some respects to the dual role of endothelial platelet endothelial cell adhesion molecule-1 in facilitating diapedesis and dampening inflammation.

To initially evaluate the role of CD155 in the regulation of CD8 T-cell responses, we performed some experiments using ECs and PHA. This system bypasses the requirement for T-cell recognition of cognate antigen presented by ECs but is still reliant on the expression of accessory molecules on ECs for T-cell activation. In this system, CD155 attenuated the production of IFNγ, IL-10, and granzyme B. Although IL-10 suppresses some effector T-cell responses, it is produced concomitantly with IFNγ in effector T cells, and its expression is likely associated with the development of effector T-cell function. In addition to effector molecules, we also examined the effect of CD155 neutralization on the expression of Foxp3. However, there was a low level of Foxp3 in all activated CD8 T cells, which is consistent with the established transient induction of Foxp3 in activated effector human T cells. CD155 neutralization did not affect this non-specific expression of Foxp3 (data not shown). In addition to mitogen-mediated stimulation, we observed similar effects of CD155 on the attenuation of IFNγ production from allogeneically stimulated activated CD8 T cells.

In summary, we have shown that CD155 is a cytokine-inducible protein on the surface of human ECs and that its expression attenuates the acquisition of effector functions in CD8 T cells. To our knowledge, this represents a novel immune regulatory function for CD155 that may have implications for the regulation of immune responses in peripheral tissues. Further understanding the role of CD155 in ECs may provide insight specifically into the regulation of T-cell responses in transplanted organs.

Acknowledgments

We thank Winnie Enns for assistance with flow cytometry.

Sources of Funding

This work was funded by an operating grant from the Canadian Institutes of Health Research and a grant-in-aid from the Heart and Stroke Foundation of British Columbia and Yukon. Dr Choy is a recipient of a Canadian Institutes of Health Research New Investigator Award.

Disclosures

None.

References


27. Hirota T, Irie K, Okamoto R, Ikeda W, Takai Y. Transcriptional acti-


CD155 on Human Vascular Endothelial Cells Attenuates the Acquisition of Effector Functions in CD8 T Cells
Nichole K. Escalante, Anna von Rossum, Martin Lee and Jonathan C. Choy

Arterioscler Thromb Vasc Biol. 2011;31:1177-1184; originally published online February 17, 2011;
doi: 10.1161/ATVBAHA.111.224162

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/5/1177

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/02/17/ATVBAHA.111.224162.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure I. DNAM-1 and CD155 expression on CD8 T cells. The expression of DNAM-1 and CD155 on resting human CD8 T cells was examined by flow cytometry. Red line: Isotype control, Blue line: DNAM-1 or CD155
Supplemental Figure II. Effect of DNAM-1 neutralization on CD8 T cell proliferation and IFNγ secretion. A. CFSE-labelled human CD8 T cells were cultured with ECs in the presence or absence of PHA. The latter condition represents allogeneically-stimulated T cells. A control IgG or DNAM-1 neutralizing antibody was added to the cultures. Cells were harvested after either 6 days (for cultures with PHA) or 12 days (for allogeneic cultures), stained for CD8, and CFSE dilution examined by flow cytometry. B. CD8 T cells were activated with ECs and PHA, and a control IgG antibody or DNAM-1 neutralizing antibody added to the cultures. Supernatant was collected at the indicated time-points and IFNγ levels quantified by ELISA. Mean ± SD of triplicate measurements. *p<0.01.
Supplemental Figure III. Effect of CD155 siRNA on CD155 protein expression. HUVECs were transfected with control or CD155 siRNA, and CD155 expression examined by flow cytometry. Green line: Staining control, Red line: Control RNA, Blue line: CD155 siRNA.
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 overides 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 IFN\(\gamma\) (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 IFN\(\gamma\) 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 co-culture 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).
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**


