Tissue engineering is a promising approach to address organ shortages currently limiting clinical transplantation. “Off-the-shelf” engineered vascularized organs will likely use allogeneic endothelial cells (ECs) to construct microvessels required for graft perfusion. Vasculogenic ECs can be differentiated from committed progenitors (human endothelial colony forming cells or HECFCs) without risk of mutation or teratoma formation associated with reprogrammed stem cells. Like other ECs, these cells basally express both class I and class II major histocompatibility complex (MHC) molecules, bind donor-specific antibody (DSA), activate alloreactive T effector memory cells, and initiate rejection in the absence of donor leukocytes. We report here that CRISPR/Cas9-mediated dual ablation of β2-microglobulin and CIITA in HECFC-derived ECs eliminates both class I and II MHC expression while retaining EC functions and vasculogenic potential. Importantly, dually ablated ECs no longer bind human DSA or activate allogeneic CD4+ effector memory T cells and are resistant to killing by CD8+ alloreactive cytotoxic T lymphocytes in vitro and in vivo. Despite absent class I MHC molecules, these ECs do not activate or elicit cytotoxic activity from allogeneic natural killer cells. These data suggest that HECFC-derived ECs lacking MHC molecule expression can be utilized for engineering vascularized grafts that evade allorejection.
Progenitor-Derived Human Endothelial Cells Evade Alloimmunity by CRISPR/Cas9-Mediated Complete Ablation of MHC Expression

Jonathan Merola¹, Melanie Reschke², Richard W. Pierce³, Lingfeng Qin¹, Susann Spindler¹, Tania Baltazar⁴, Thomas D. Manes⁴, Francesc Lopez-Giraldez⁵, Guangxin Li¹, Laura G. Bracaglia², Catherine Xie⁴, Nancy Kirkiles-Smith⁴, W. Mark Saltzman³, Gregory T. Tietjen¹, George Tellides¹, Jordan S. Pober⁴

¹Departments of Surgery, ³Pediatrics, and ⁴Immunobiology, Yale School of Medicine, New Haven, CT, USA
²Department of Biomedical Engineering, Yale School of Engineering and Applied Science, New Haven, CT, USA
⁵Yale Center for Genome Analysis, Department of Genetics, Yale University, New Haven, CT, USA

Running Title: Ablation of MHC Expression on Human Endothelial Cells

Conflict of Interests: The authors have declared no conflicts of interest.

Correspondence:
Jordan S. Pober, M.D., Ph.D.
Department of Immunobiology
Yale School of Medicine
10 Amistad Street, Room 401D
New Haven, CT 06520-8089
Tel: (203) 737-2292
Fax: (203) 737-2293
E-mail: jordan.pober@yale.edu

Keywords: human endothelial colony forming cells; alloreactivity; effector memory T cells; alloantibody; NK cells
ABSTRACT

Tissue engineering may address organ shortages currently limiting clinical transplantation. “Off-the-shelf” engineered vascularized organs will likely use allogeneic endothelial cells (ECs) to construct microvessels required for graft perfusion. Vasculogenic ECs can be differentiated from committed progenitors (human endothelial colony forming cells or HECFCs) without risk of mutation or teratoma formation associated with reprogrammed stem cells. Like other ECs, these cells can express both class I and class II major histocompatibility complex (MHC) molecules, bind donor-specific antibody (DSA), activate alloreactive T effector memory cells, and initiate rejection in the absence of donor leukocytes. CRISPR/Cas9-mediated dual ablation of β2-microglobulin and CIITA in HECFC-derived ECs eliminates both class I and II MHC expression while retaining EC functions and vasculogenic potential. Importantly, dually ablated ECs no longer bind human DSA or activate allogeneic CD4+ effector memory T cells and are resistant to killing by CD8+ alloreactive cytotoxic T lymphocytes in vitro and in vivo. Despite absent class I MHC molecules, these ECs do not activate or elicit cytotoxic activity from allogeneic natural killer (NK) cells. These data suggest that HECFC-derived ECs lacking MHC molecule expression can be utilized for engineering vascularized grafts that evade allorejection.
INTRODUCTION

Organ transplantation is the most effective therapy for patients afflicted with end-stage failure of the heart, lungs, kidney, liver, or intestine, but its current application is significantly limited by the lack of available allografts (1-4). Over 110,000 patients are on transplant waiting lists, while only 35,000 organ transplants occur annually in the US (5). Bioengineered replacement organs have the potential to address this shortage. Endothelial cell (EC)-lined microvessels will be required for adequate perfusion of the engineered donor tissue. Circulating endothelial colony-forming cells (ECFCs), which are abundant in umbilical cord blood, display substantially greater replicative potential than vessel wall-derived ECs and readily differentiate into functional ECs with capacity to self-organize into microvessels (6). As committed progenitors, ECFCs do not differentiate into other cell types, retain normal chromosome number, and unlike induced pluripotent stem cells, do not give rise to teratomas (7). These attributes make ECFCs an attractive cell source for constructing vascularized engineered tissues (8).

Critically ill patients may need “off-the-shelf” replacement organs pre-assembled using cells from allogeneic sources (9). Responses to allogeneic natural organs can provide important insights as to how recipient immune systems will respond. Broadly, transplant rejection may be precipitated by donor-reactive antibodies or host T cell-mediated responses. Nearly 15% of transplant candidates on the waiting list harbor circulating antibodies reactive with non-self allelic forms of class I and class II major histocompatibility complex (MHC) molecules (designated as “panel reactive antibodies” (PRA)), capable of precipitating graft rejection (10, 11). Graft recipients may also develop
donor-specific antibodies (DSA) de novo after transplantation, most often reactive with class II MHC molecules on graft ECs (12). Binding of PRA or DSA to ECs leads to the activation of complement and deposition of membrane attack complexes (MAC) on the EC surface. MAC does not cause EC lysis but instead triggers proinflammatory signaling cascades that enhance recruitment and activation of alloreactive T cells promoting rejection (13). Of clinical significance, the presence of a DSA is highly correlated with late graft loss (14).

T cell-mediated rejection most frequently occurs within the first post-transplant year and is initiated by effector memory T cells (T_{EM}) that recognize graft MHC molecules, each complexed to one of a large number of different peptides (15),(16). Recognition of graft MHC molecules on human ECs by circulating T_{EM} induces transendothelial recruitment. Infiltrating CD8^{+} T_{EM} differentiate into cytotoxic T lymphocytes (CTL) and mediate acute rejection (17, 18). CTL differentiation from CD8^{+} T_{EM} is aided by activated CD4^{+} T_{EM}; these cells release interleukin-2 (IL-2) and other mediators, which promote the survival and expansion of CTL (19). Collectively, graft class I and II MHC molecules on ECs serve as targets of alloantibody and T-cell mediated recognition that precipitate rejection responses. Importantly, ECs in an engineering tissue may represent the only graft cell type capable of initiating cellular rejection (20).

Since the expression of MHC molecules is a necessary property of ECs to trigger both humoral and cellular rejection, loss of MHC expression should diminish the capacity of ECs to cause these processes. We previously used Clustered-Regular-Interspaced-
Palindromic-Repeats, and its associated nuclease CRISPR-associated protein 9 (CRISPR/Cas9), to generate stable biallelic gene disruption in ECs derived from ECFCs, that could then be clonally expanded (21). CRISPR/Cas9 targeting of the class II transactivator (CIITA) in ECs eliminated class II MHC expression and significantly diminished activation of CD4+ memory T cells (19). However, CIITA\textsuperscript{null} ECs were only partially protected from vascular destruction, as cytotoxicity is principally mediated by CD8+ CTL cells recognizing class I MHC. Here, we extended this approach to target β2-microglobulin, an invariant component of the class I MHC complex, both alone and in combination with CIITA. We find that dual CRISPR/Cas9 targeting of β2-microglobulin and CIITA eliminates class I and II MHC expression in human ECs derived from ECFCs while retaining core EC functions, including the capacity to form microvessels. As expected, dually class I and class II MHC-null ECs exposed to high titer PRA sera show markedly reduced antibody binding and markedly diminished capacity to activate allogeneic CD8+ and CD4+ T\textsubscript{EM} proliferative responses. Despite loss of class I MHC molecule expression, β2-microglobulin\textsuperscript{Null} ECs do not show susceptibility to allogeneic NK cells. Furthermore, tissue-engineered and perfused microvessels generated from β2-microglobulin\textsuperscript{Null}+CIITA\textsuperscript{Null} ECs in immunodeficient mouse hosts are resistant to destruction by adoptively transferred allogeneic (to the ECs) peripheral blood mononuclear cells (PBMC) in vivo. These data suggest that tissue-engineered constructs perfused through vessels lined by allogeneic ECs lacking MHC molecules will be significantly less prone to rejection.
RESULTS

**CRISPR/Cas9-Mediated Genetic Targeting of β2-microglobulin and CIITA in Human Endothelial Cells**

We previously described a method for high efficiency biallelic genomic disruption of the class II transactivator (CIITA) gene locus in ECFC-derived ECs (21). Resultant EC clonal populations were unable to express class II MHC molecules following human interferon (IFN)-γ treatment (21). To evaluate the effects of ablating class I MHC expression, we targeted β2-microglobulin, an invariant component of the class I MHC complex. We targeted exonic regions of either the (i) β2-microglobulin locus alone, (ii) CIITA locus alone, (iii) both β2-microglobulin and CIITA loci, or (iv) the adeno-associated viral integration site 1 (AAVS1) locus, using the latter as a control target that would not affect class I or II MHC expression. Modified cultures were treated with human interferon (IFN)-γ for 72 hours to both maximize class I MHC expression and restore class II MHC expression, reversing down-modulation associated with cell culture. Flow cytometry was used to sort viable cells lacking class I and/or II MHC expression, which represented 40-50% of the population in cells targeted for a single locus and 5-20% in dually β2-microglobulinNull+CIITANull ECs (Supplementary Figure 1). Subsequent re-treatment with IFN-γ failed to re-induce class I and HLA-DR expression in β2-microglobulin and CIITA targeted cells, respectively. Dually targeted cells, similarly showed absence of both class I MHC and HLA-DR following IFN-γ treatment. Control cells transduced with an irrelevant guide strand vector targeting AAVS1 expressed high levels of class I MHC and HLA-DR, the predominant class II locus expressed in ECs (Figure 1A). Further characterization of surface and total MHC heavy and light chain expression after IFN-γ treatment, as assayed
by intracellular staining and flow cytometry, revealed an absence of both class I MHC components in cells targeted with β2-microglobulin (Figure 1B) and all three human class II loci in cells targeted with CIITA (Figure 1C). Single-cell derived colonies were expanded from ECs transduced with guide strands targeting the β2-microglobulin and/or CIITA loci. Genomic DNA from each expanded colony was isolated. Sequencing of polymerase chain reaction (PCR) amplicons obtained from the regions surrounding targeted protospacer adjacent motif at both β2-microglobulin and CIITA exonic loci confirmed biallelic mutation (Figure 1D).

Transplanted hepatocytes derived from β2-microglobulinNull mice into wild type recipients demonstrated their capacity to endocytose exogenous β2-microglobulin and reconstitute class I MHC in trans (22). We therefore evaluated the ability of β2-microglobulinNull human ECs to reconstitute class I MHC expression after culture with human serum, with or without recombinant β2-microglobulin protein supplementation and observed no rescue of class I MHC expression in β2-microglobulinNull ECs under these conditions (Supplementary Figure 2A). β2-microglobulin normally pairs with class I heavy chains during the process of peptide loading in the endoplasmic reticulum (ER). In the absence of β2-microglobulin, accumulation of unpaired class I heavy chains could potentially trigger an unfolded protein response. We evaluated this possibility by assessing expression of CCAAT-enhancer-binding protein homologous protein (CHOP), a major transcription factor of the ER stress response. We observed no significant expression of CHOP in control or β2-microglobulinNull cells (Supplementary Figure 2B). CHOP was
readily detected following treatment of cells with thapsigargin, a positive control for induction of ER stress.  

**RNA Sequencing and Functional Characterization of CRISPR/Cas9-Modified Endothelial Cells**

RNA sequencing was performed to compare the transcriptome of cells targeted with a control guide RNA (AAVS1) to β2-microglobulinNull, CIITANull and dually ablated β2-microglobulinNull+CIITANull EC following treatment with IFN-γ. Differential gene expression analysis revealed 57 significantly differentially expressed genes (FDR-adjusted $p<0.05$) with a fold change $\geq 4$ when comparing β2-microglobulinNull, CIITANull and dually ablated β2-microglobulinNull+CIITANull EC to the control EC (AAVS1) (Table 1 and 2). As expected, loss of known CIITA-regulated genes were significantly underrepresented in cells ablated of CIITA (Fisher’s exact test $p<0.0001$) when compared to the AAVS1-targeted EC control (23). Only one gene encoding the elastin microfibril interface-locate protein 1 (EMILIN1), was significantly downregulated in β2-microglobulinNull ECs compared to the AAVS1-targeted EC control. This gene was also downregulated in both CIITANull and dually ablated β2-microglobulinNull+CIITANull. EMILIN1 expression was similar in control guide strand-treated cells and untransduced cells, suggesting that its downregulation is not a general feature of lentiviral transduction or of Cas9 activity despite being caused by totally unrelated guide strands in the MHC-targeted cells. The gene encoding EMILIN protein is found on chromosome 2 in humans, not physically linked to the locations of genes encoding β2-microglobulin (chromosome 15) or CIITA (chromosome 16) and knowledge of its expression and function in ECs is unknown.
Several functional phenotypic characteristics were compared between ECs edited with a control guide RNA (AAVS1), β2-microglobulinNull ECs, CIITANull ECs, and combined β2-microglobulinNull+CIITANull ECs. All four EC types exhibited similar morphology and junctional staining patterns of PECAM-1 (CD31) and VE-Cadherin (CD144), as evaluated by confocal microscopy (Figure 2A). Moreover, monolayer cultures of all four EC types formed junctions with comparable barriers (Figure 2B) that were similarly disrupted after treatment with thrombin or tumor necrosis factor (TNF)-α, as assessed by electrical cell impedance sensing (ECIS) (Figure 2C). All four EC types normally upregulated the adhesion molecules ICAM-1 and PD-L1 in response to treatment with IFN-γ, indicative of preserved activation responses (Figure 2D). These attributes suggest that β2-microglobulinNull+CIITANull ECs retained core endothelial phenotypic functions in vitro.

ECs will self-assemble into perfusable vessels, an attribute critical for their use in tissue engineering. To assess if this ability was retained in our Cas9-modified ECs, control (AAVS1), β2-microglobulinNull, and dually ablated β2-microglobulinNull+CIITANull ECs were suspended in a collagen/fibronectin protein gel matrix and implanted subcutaneously in the abdominal wall of C.B-17 severe combined immunodeficient (SCID)/beige (bg) mice. Two weeks later, the animals were injected with human IFN-γ every other day for five days to induce MHC molecule re-expression (17, 18). Mice were sacrificed and collagen gels were harvested 15 minutes after intravenous injection of fluorescently-labeled Ulex europeanus agglutinin (UEA), which is reactive with human blood group H antigen expressed on all human ECs. Fluorescence microscopy revealed formation of perfused (UEA+) human EC-lined vessels in all three groups. Control ECs showed expression of
both class I and class II MHC molecules. Similar to in vitro behavior, no class I MHC expression was detectable in β₂-microglobulinNull cells and neither class I nor II MHC molecules were expressed by dually ablated β₂-microglobulinNull+CIITANull ECs (Figure 2E). Thus MHC-ablated ECs retain core endothelial characteristics in vivo, making them useful for tissue engineering.

_Alloantibody-Mediated Responses of CRISPR/Cas9-Modified Endothelial Cells_

Circulating antibodies recognizing non-self class I and II MHC molecules that predispose patients to rejection of allogeneic grafts are present in “pre-sensitized” transplant candidates (~15% of waitlist candidates) (10, 11). Some of these individuals have high titers of PRA, recognizing over 80% of all tested HLA specificities. Binding of alloantibody to graft ECs triggers complement activation and deposition of terminal complement MAC on EC membranes. Biopsies from such rejecting grafts often show uninjured ECs despite the presence of bound antibody and MAC (24, 25). In cell culture and in humanized mouse models, MAC is endocytosed, transferred to endosomes, and triggers non-canonical nuclear factor-κB (NF-κB) signaling (13). Sera pooled from highly sensitized patients (high PRA serum) were added to control vector transduced, β₂-microglobulinNull, CIITANull, and dually ablated β₂-microglobulinNull+CIITANull ECs under conditions that support complement activation. Compared to control ECs, β₂-microglobulinNull and CIITANull cells exhibited a reduction in alloantibody binding (Figure 3A), complement deposition (Figure 3B) and complement-mediated non-canonical NF-κB signaling (Figure 3C). Dually ablated β₂-microglobulinNull+CIITANull ECFCs showed nearly complete elimination of alloantibody binding and complement deposition. Signaling
through the complement-dependent non-canonical NF-κB pathway was markedly diminished but not completely absent. Residual signaling may have been induced by cytokines present in the circulation of some individuals or by binding of alloantibody reactive with targets other than MHC molecules (26).

**T Cell Responses to CRISPR/Cas9-Modified Endothelial Cells**

Human allografts can be rejected when CD8\(^+\) and/or CD4\(^+\) circulating effector memory T (T\(_{EM}\)) cells recognize non-self class I and II MHC molecules expressed on graft ECs (16, 27). We therefore tested the ability of IFN-\(\gamma\)-treated control vector transduced, \(\beta_2\)-microglobulin\(^{\text{Null}}\), CIITA\(^{\text{Null}}\) and dually ablated \(\beta_2\)-microglobulin\(^{\text{Null}}\)+CIITA\(^{\text{Null}}\) ECs to activate allogeneic CD8\(^+\) and CD4\(^+\) T\(_{EM}\) cells in vitro. As expected IFN-\(\gamma\) pre-treated control ECs induce proliferation in a measurable subpopulation of allogeneic CD4\(^+\) and CD8\(^+\) T\(_{EM}\) cells. Compared to control co-cultures, CD8\(^+\) T\(_{EM}\) cells showed a marked reduction in proliferation when co-cultured with \(\beta_2\)-microglobulin\(^{\text{Null}}\) and \(\beta_2\)-microglobulin\(^{\text{Null}}\)+CIITA\(^{\text{Null}}\) cells. Similarly, a lack of proliferation of CD4\(^+\) T\(_{EM}\) cells was noted when co-cultured with CIITA\(^{\text{Null}}\) and \(\beta_2\)-microglobulin\(^{\text{Null}}\)+CIITA\(^{\text{Null}}\) cells (**Figure 4A**). Proliferating CD4\(^+\) T\(_{EM}\) co-cultured with class II MHC expressing ECs expressed the activation marker HLA-DR. However, this activation marker was not expressed when CD4\(^+\) T\(_{EM}\) were co-cultured with CIITA\(^{\text{Null}}\) and \(\beta_2\)-microglobulin\(^{\text{Null}}\)+CIITA\(^{\text{Null}}\) cells (**Figure 4B**). Thus the absence of MHC molecules on ECs prevents TCR-mediated activation and expansion of alloreactive T cells.
Cell-mediated rejection of allografts is largely mediated by CTL clones that recognize different peptides complexed to non-self allelic forms of class I MHC molecules expressed on target cells. To evaluate whether the absence of class I MHC was sufficient to abrogate CTL-mediated cytotoxicity, alloreactive T cell clones were raised on wild type ECs (17, 18) and subsequently co-cultured with control vector transduced, β2-microglobulinNull, CIITANull or dually ablated β2-microglobulinNull+CIITANull ECs from the same donor. Absence of class I MHC was sufficient to nearly eliminate T cell killing in vitro (Figure 4C).

**NK cell Responses to CRISPR/Cas9-Modified Endothelial Cells**

NK cells are innate lymphoid cells that use the same mechanisms to kill target cells as CTL. Almost all NK cells express an activating low affinity receptor for certain IgG isotypes and thus kill target cells to which relevant IgG isotypes are bound. NK cells can also kill targets when activated by cytokines or by engagement of activating receptors with target cell ligands. However, many NK cells also express inhibitory receptors that recognize certain self-allelic forms of HLA-B and -C molecules and lack of class I MHC molecules on allogeneic hematopoietic cells or some tumor cells results in NK cell activation and killing (28). Since class I MHC molecules will be absent on β2-microglobulinNull and β2-microglobulinNull+CIITANull ECs, we examined if the absence of class I MHC on human ECs conferred greater capacity for NK activation by culturing allogeneic CD3-CD56*CD335*CD16+ NK cells with on β2-microglobulinNull and β2-microglobulinNull+CIITANull ECs as compared to co-cultures with control ECs or the K562 leukemic cell line, a known NK cell target used as a positive control. Release of TNF-α
and IFN-γ as well as cytotoxicity by human NK was observed when cultured with K562 cells. However, no enhancement of cytokine release was detected in class I absent cells compared to control ECs (Figure 5A) and no significant NK-mediated cytolysis was observed upon co-culture with β2-microglobulinNull ECs compared to control, despite an intact capacity of NKS to lyse ECs bound by alloantibody (Figure 5B). Therefore the absence of MHC I on ECs is not sufficient to trigger NK-mediated activation and cytolysis.

Failure to activate allogeneic NK cells could result from absence of activating ligands on ECs. To investigate this further, the transcriptional abundance of known NK cell ligands was evaluated using RNA sequencing data obtained previously (Table 1). From these we identified expression of HLA-E, MICA/MICB, CD111/Nectin-1, CD112/Nectin-2, and CD155/PVR. We then compared ECFC-derived ECs as well as adult dermal ECs (HDMECs) under both resting conditions and following 48 hours of treatment with interferon-γ or TNF-α to simulate inflammatory conditions. K562 cells, known NK-cell targets, were included as a comparator. As shown in Figure 5C, both ECFCs and HDMECs had similar responses to these pro-inflammatory cytokine, exhibiting increased levels of HLA-E, CD111, CD112 and CD155 compared to K562 cells, while HLA-G was highly expressed by K562 cells but not the ECs. The function of these NK ligands may be both activating or inhibitory depending on their receptor engagement. For example, CD112 may be activating when engaging with DNAM-1, but inhibitory when engaging with TIGIT (29, 30). Similarly HLA-E may activate NK cells when engaging with NKG2C and inhibitory when binding NKG2A (31). Thus our expression data indicate that while
ECs express some potentially activating NK ligands, they are not sufficient for ECs to activate NK cells in the absence of MHC I.

To determine whether NK cells are simply ignorant of or transiently inhibited by ECs or if they are rendered anergic by their interactions with ECs, we co-cultured isolated NK cells with either control (sgAAVS1) ECs, class I knockout (β2-MNull) ECs or no target cells, harvested them 6 h later and then added them back to K562 NK-cell targets to assess if they could still be activated. Remarkably, pre-incubation of NK cells with β2-MNull ECs but not control ECs prevents interferon-γ release in the presence of K562 NK cell targets (Figure 5D). The mechanism of how β2-MNull ECs inactivate NK cells while MHC I sufficient ECs do not is unclear, but this observation has identified an important question for future investigation.

**In vivo characterization of Allogeneic Lymphocyte-Mediated Responses to CRISPR/Cas9-Modified Endothelial Cells**

Recapitulating alloresponses in mouse models is challenging both due to the lack of alloreactive memory cells in animals maintained in clean vivaria and the absence of class II MHC molecules on mouse ECs necessary to activate alloreactive CD4+ T effector cells (16, 27). Assessment of human alloresponses to ECs in vivo can be modeled by embedding human ECs in a collagen-fibronectin protein gel and implanting the neotissue subcutaneously in an immunodeficient C.B-17 SCID/bg mouse, allowing a human microvascular network to establish over a period of 10-14 days, followed by adoptive transfer of PBMCs allogeneic to the ECs (32). The transfer of human PBMCs results in
microvascular destruction over a subsequent period of 10 days that is allospecific (33-35). To assess the susceptibility of CRISPR/Cas9-modified ECs to T cell-mediated responses, control (sgAAVS1), \( \beta_2 \)-microglobulin\(^\text{Null} \), or \( \beta_2 \)-microglobulin\(^\text{Null} \)+CIITA\(^\text{Null} \) ECs were embedded in a collagen-fibronectin gel and implanted subcutaneously in C.B-17 SCID/bg. Mice implanted with gels were then subjected to saline injection or adoptive transfer of human PBMC from a donor allogeneic to the ECs. In concordance with in vitro experiments, destruction of synthetic microvessels formed from \( \beta_2 \)-microglobulin\(^\text{Null} \), or \( \beta_2 \)-microglobulin\(^\text{Null} \)+CIITA\(^\text{Null} \) was not observed while completed destruction of microvessels was observed in gels containing control EC (Figure 6).

**DISCUSSION**

In this report we demonstrate that CRISPR/Cas9-targeting of \( \beta_2 \)-microglobulin and CIITA in HECFC-derived ECs eliminates class I and II MHC expression and can generate functional EC that evade alloimmune responses. MHC-absent ECs no longer bind human alloantibody, activate allogeneic CD4\(^+ \) effector memory T cells and are resistant to killing by CD8\(^+ \) alloreactive cytotoxic T lymphocytes *in vitro* and *in vivo*. Moreover, the absence of MHC I does not confer susceptibility to allogeneic natural killer cells. Collectively these data demonstrate the potential utility of these ECs as cellular components in engineering vascularized grafts.

The capacity of a graft to perform its vital functions is reliant upon its ability to obtain inflow of nutrients and oxygen as well as yield a circulatory outflow to the host. In human tissues, these properties are facilitated by capillary beds lined by ECs that serve as the
intermediaries between the vascular space and interstitial tissue. Tissue-engineered epithelialized skin grafts lacking vascularized capillaries can promote healing but characteristically slough due to ischemia. In absence of EC however, they do not sensitize the host to alloantigens expressed on the stromal or parenchymal cells. In contrast, natural human skin is vigorously rejected in a process that targets the EC-lined blood vessels (36). Acute organ graft rejection is largely a cell-mediated process and class I and II MHC expressed on graft cell luminal surface are the principal proteins recognized by both circulating alloreactive TEM cells. Class I molecules are the principal target of CTL, the primary effectors of cell-mediated rejection. Preformed alloantibodies directed against graft class I and class II MHC molecules, when present, may also contribute to acute rejection. Late graft loss is most commonly attributed to chronic rejection and de novo formation of donor specific antibodies, usually targeting graft class II MHC molecules, is a major risk factor. The ECs lining graft vessels are the primary cells that express both class I and class II MHC molecules. Potent immunosuppressive therapies are necessary to make clinical organ transplantation feasible. The fundamental premise of this work is that ECs lacking class I and II MHC molecules, even from allogeneic sources, are likely to reduce the potency of rejection, permitting a reduction in the level of immunosuppression required and thus sparing patients from the side effects of these agents.

Tissue engineering will require fashioning of organs that do not provoke strong allogeneic responses. ECs in this process may be differentiated from autologous stem cells or from allogeneic cell sources. Only allogeneic sources will allow off-the-shelf assembly of
organs, but technical advances, such as 3D-bioprinting may close the gap. The absence of dendritic cells in such grafts will reduce alloreactivity. However, unlike the experimental situation in rats, where removal of dendritic cells produces immunological ignorance, acute rejection will still occur in humans (37),(38). The accepted explanation for this difference is that human graft ECs, unlike rat ECs, can initiate rejection by presentation of non-self MHC molecules to circulating T_{EM}. Out data demonstrate that ablation of MHC molecules from human ECs is possible by dual targeting of β2-microglobulin and CIITA, providing protection from alloimmunity without altering key EC functions.

Interestingly, β2-microglobulinNull microvessels were completely resistant to destruction by allogeneic PBMC in our in vivo humanized mouse model of allorejection despite continued expression of HLA class II molecules and a retained capacity to activate alloreactive CD4+ T cells. Coupled with previously published observations that CIITANull EC conferred only partial protection from allogeneic PBMC, these findings suggest that T cell-mediate cytotoxicity is solely conferred by CD8+ T lymphocytes, but whose alloimmune potential is significantly augmented in the presence of CD4+ T lymphocytes (19). While other studies have suggested that CD4+ T lymphocytes may directly mediate cytotoxic responses (CD4+ CTL) in allorejection, they do not appear to function in our model (39, 40).

Our study found that dual ablation of β2-microglobulin and CIITA markedly reduced but did not complete eliminate binding of alloantibody. The presence of serum cytokines such as interleukin-1, are capable of inducing NF-κB signaling in absence of alloantibody and
may account for the presence of p100 to p52 conversion in ECs unexposed to allosensitized serum (26). Moreover, binding of non-HLA antibodies, which can be directed against antigens including MICA/MICB, angiotensin receptor, perlecan and collagen as well as mediate both antibody-mediated acute and chronic rejection (41) may account for residual alloantibody binding and NF-κB signaling noted in evaluation of alloantibody responses to MHC-absent EC. Whether significant non-HLA responses result de novo or as a consequence of significant ischemia/reperfusion injury however is unknown. Whether such responses will limit the ability of MHC-absent ECs to exhibit complete immune ignorance is also unknown and a topic worthy of future investigation.

CRISPR/Cas9 targeting of β2-microglobulin to eliminate class I MHC molecules has recently been successfully employed in mouse tumor lines for the purposes of generating transplantable tumors across mouse strains (42). As with prior experiments using hematopoietic cell lines, some tumor cells ablated of β2-microglobulin lose susceptibility to CTL recognition but become prone to NK cell cytotoxicity (28, 43). However, NK responses to vascularized tissues may require activation signals to initiate responses, as has been observed in MHC-deficient tumors (44). Our findings are consistent with observations that solid organ allografts are not rejected in mice by NK cells in the absence of alloreactive T cells that provide activating cytokines or alloantibody that binds to graft ECs even though NK cells can control infections in the same animals (45, 46). These experiments contradict the widely expressed view that “absent self” is a sufficient trigger for NK-mediated rejection, but the original laws of transplantation formulated by George Snell distinguished between failure of a F1 offspring of two different inbred parents to
reject tissue grafts from either parent with active rejection of hematopoietic cell grafts from the same parent by the same offspring (47). We know that NK cells are responsible for killing the hematopoietic grafts yet fail to reject the tissue graft. We do not know the mechanisms by which ECs avoid activating alloreactive NK cells and our finding that ECs lacking class I MHC may anergize NK cells was unexpected. Wild type ECs are, as expected, are resistant to NK cells but do become susceptible to NK cell-mediated killing when coated with alloantibody. Thus, the lack of MHC molecule expression by β2-microglobulinNull ECs may actually provide dual protection from both NK cells as well as CTL. The humanized mouse model we have used to test EC resistance to CTL in vivo does not have functional human or mouse NK cells. Newer humanized mouse models currently in development may overcome this limitation and enable in vivo testing of more complete human alloimmune responses to composite tissue grafts (48).

Use of ECs, differentiated from cord blood ECFCs (formerly known as endothelial progenitor cells, a terminology abandoned because many publications conflated ECFCs with pro-angiogenic monocytes) has recently garnered interest as a promising tool for vascularization of injured tissue due to their augmented proliferative capacity and ability to form microcapillaries in vivo (49, 50). Other investigators have modified induced pluripotent stem (iPS) cells in a similar approach (51). While CRISPR/Cas genetic modification of ECFCs has the potential to introduce off-target mutations, the technology for such engineering is steadily improving with better engineered reagents. While the presence of off target mutagenesis was not evaluated by whole genome sequencing of modified cells in this study, we found that modification of MHC-dependent genes in
ECFCs produces few unexpected transcriptional changes and does not alter their characteristic functional phenotype. Engineering of committed endothelial progenitors may be safer than modifying inducible pluripotent stem cells as an EC source because of the absence of deleterious mutations introduced during the dedifferentiation and re-differentiation process and the absence of a risk for teratoma formation (52).

Utilization of non-immunogenic ECs could have significant therapeutic potential in two major areas in addition to the role for tissue engineering emphasized in our study; (i) as a directed cellular therapy to promote revascularization of an ischemic tissue; and (ii) as a vehicle for efficient delivery of gene products in the circulation. Therapeutic revascularization has the potential to significantly mitigate damage from acute ischemic events including myocardial infarction, stroke, retinal ischemia and peripheral limb ischemia (53-56). Targeted application of a cellular suspension into affected tissues in rodent animal models have demonstrated improvement in organ function and increased vascularization of affected tissues, particularly when co-implanted with host myeloid cells or smooth muscle cells (57, 58). In addition to aiding in vasculogenesis, secretion of soluble regulators governing tissue repair may present additional benefits. For example, engraftment of pancreatic islets under the renal capsule of NOD/SCID mice in the presence of ECFC-derived ECs promoted beta cell proliferation and improved glycemic control and C-peptide production in these animals (59). These effects are likely due to paracrine provision of signals by the ECs. Recent studies have also evaluated the utility of ECs as vehicles for exogenous gene delivery. Transfected ECFCs carrying modified coagulation factor VIII were able to engraft in NOD/SCID mice and efficiently secrete
factor VIII intro the circulation to achieve therapeutic levels relevant for treatment of hemophilia within 5 months (60). Genetically engineered ECFCs optimized for delivery of erythropoietin and embedded in collagen/fibrin gels in immunodeficient mice have shown the ability to augment hematocrit, red blood cell counts and splenic extramedullary erythropoiesis for treatment of nephrectomy-induced anemia (61).

In summary, we have shown that the alloimmunogenic properties of ECs may be eliminated by ablation of MHC molecules, enhancing their utility for vascularization of human tissue engineered constructs as well as for promoting tissue regeneration or therapeutic protein delivery (62).

METHODS

Culture of Human ECs derived from Cord-Blood ECFCs

Late outgrowth endothelial colony forming cells were collected from cord blood and cultured as described previously (63). Colonies of proliferating, differentiated cells were typically identified at 10 days at which time the medium was changed to EGM2MV Complete Medium (Lonza, Walkersville, MD) for expansion and propagation.

CRISPR/Cas9-Mediated Ablation of β2-microglobulin and CIITA

Guide RNA sequences in the exonic regions of the β2-microglobulin and CIITA gene loci were identified using the design software available at crispr.mit.edu. Sequences with perfect matches in the human genome and at least 3 mismatches to the nearest coding off-target sequences were selected and used to create IDT gBlock segments (IDT
Technologies, Coralville, IA) containing XhoI restriction site, U6 promoter, sgRNA target sequences and NheI restriction site in consecutive order from the 5’ to 3’ end. gBlocks were subsequently cloned into pLX-sgRNA (produced by Lander & Sabatini and available through Addgene as plasmid #50662), which contained a blasticidin resistance gene. Guide strand targeting sequences included GAGTAGCGCGAGCACAGCTA for targeting of the β2-microglobulin gene locus, GATATTGGCATAAGCCTCCC for targeting of the CIITA gene locus, and GGGGCCACTAGGGACAGGAT for targeting of the AAVS1 gene locus (used as a control). A separate lentiviral vector encoding tetracycline-inducible FLAG-Cas9 and a puromycin resistance gene (pCW-Cas9, produced by Eric Lander & David Sabatini, and available through Addgene as plasmid #50661) was used to create TetOn-Cas9-EC.

pCW-Cas9 or (AAVS1, β2-microglobulin or CIITA-targeting) pLX-sgRNA lentiviral vector plasmids were co-transfected with psPAX2 (produced by Didier Trono, available as Addgene plasmid #12260) and CMV VSV-G containing plasmid pMD2.G, psPAX2 (produced by Didier Trono, available as Addgene plasmids #12260 and #12259) packaging and envelope plasmids into human 293T (ATCC, Manassas, VA) cells in a 1:1 mixture of plasmids and a calcium phosphate-based transfection buffer (4mg NaCl, 190μg KCl, 50μg Na2HPO4, 2.5mg HEPES, 500μg Glucose, 125μmol CaCl2 in 500μL, pH=7.05). Lentiviral supernatant was collected at 48 and 72 hours and filtered through a 0.45µm filter. Polybrene (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 8 µg/mL pCW-Cas9 and either AAVS1, β2-microglobulin or CIITA-targeting pLX-sgRNA supernatant were simultaneously co-transduced into ECs for an 8-hours incubation at
MOIs of 30 and 20, respectively (Lenti-X p24 Lentiviral Titration Kit, Clontech, Mountain View, CA). Following a 16-hour recovery period, a second 8-hour transduction was carried out, after which transduced ECs were selected with 1μg/ml puromycin (Invitrogen, Carlsbad, CA) for 72 hours. AAVS1, β2-microglobulin, CIITA or β2-microglobulin and CIITA Cas9-ECs subsequently underwent a second selection with 10μg/ml blasticidin (Sigma-Aldrich, St. Louis, MO) for 5 days, performed concurrently with induction of TetOn-Cas9 with 1μg/ml doxycycline (Sigma-Aldrich, St. Louis, MO) for 10 days.

**Flow Cytometry and Isolation of CRISPR/Cas9-modified Endothelial Cells**

After antibiotic selection and Cas9-induction with doxycycline, ECs were cultured in the presence of 50ng/mL IFN-γ (Gibco, Gaithersberg, MD) and collected after treatment with trypsin (TrypLE Express, Invitrogen). Cells were co-stained with directly conjugated eFluor450 Viability dye (Invitrogen), FITC-anti-HLA-A,-B,-C (Clone W6/32, Biolegend) and Alexa647-anti-HLA-DR (Clone L243, Biolegend). β2-microglobulin and CIITA loss-of-function was identified by the presence of live cells that increased expression of HLA-DR and HLA-A,-B,-C (in the case of the AAVS1 control vector), increased expression of HLA-DR and not HLA-A,-B,-C (in the case of β2-Microglobulin) or increased expression of HLA-A,-B,-C and not HLA-DR (in the case of CIITA) or no expression of either (in the case of β2-microglobulin and CIITA dual targeted cells), with the positive threshold defined by fluorescence minus one staining (**Supplementary Figure 1**). These gates were used to collect β2-microglobulin+/CIITA+/ β2-Microglobulinnull, CIITAnull, and β2-microglobulinnull+CIITAnull EC using a low-pressure 100 μm nozzle on a FACS sorter (BD Biosciences, FACS AriaII). Single cells were deposited into each well of a C-96 flat-
bottom plate containing EGM2MV Complete Medium supplemented with 5μM of the ROCK-selective inhibitor Y-27632 (Sigma-Aldrich). EGM2MV Complete Medium was then exchanged every 48 hours and colonies were serially expanded into larger vessels over the course of 2 to 3 weeks.

For functional assays and immune assays that did not involve RNA sequencing or deep sequencing, CRISPR/Cas9 transduced cells that did not exhibit biallelic loss-of-function were isolated by antibody depletion. Unsorted, pooled β2-microglobulin+/+/CIITA+/+, β2-microglobulinnull, CIITAnull, or β2-microglobulinnull+CIITAnull EC were treated for 72 hours with 50ng/mL IFN-γ and incubated with 10μg/mL control antibody (MOPC-173, Biolegend), anti-HLA-A,-B,-C (Clone W6/32, Biolegend), anti-HLA-DR (Clone L243, Biolegend) or both anti-HLA-A,-B,-C and anti-HLA-DR, respectively. Positive cells were depleted using pan-mouse IgG Dynabeads (Invitrogen), according to the manufacturer’s protocol.

**PCR and Deep CRISPR/Cas9 Sequencing**

Genomic DNA was isolated from clonally expanded EC in a C-96 well plate using 200μL QuickExtract DNA Extraction Solution (Epicentre, Madison, WI). A DNA fragment flanking both sides of the target sequence and Cas9 protospacer adjacent motif was amplified by PCR (Platinum PCR SuperMix High Fidelity, Invitrogen) according to the manufacturer’s protocol. Forward and reverse primer pairs used to evaluate a 280-base pair fragment of the β2-microglobulin gene locus were CTGGCTTGGAGACAGGTGAC and GAAGTCACGGAGCGAGAGAG, respectively. Forward and reverse primer pairs used
to evaluate a 180-base pair fragment of the CIITA gene locus were AGACACCATCAACTGCGACC and CGTGGCTCATGATGAATGGG, respectively. PCR amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and submitted for deep CRISPR sequencing at the MGH CCIB DNA Core (Cambridge, MA).

**RNA Sequencing**
Sorted and clonally expanded CRISPR/Cas9-modified EC colonies were grown to confluence on a C-12 well plate and treated with 50ng/mL IFN-γ (Gibco, Gaithersberg, MD) for 96 hours. RNA was extracted and purified using a RNeasy Mini kit (Qiagen), in which an on-column DNase treatment was included. Six clonally derived colonies, derived from two EC donors, were included for each of the following conditions: β2-microglobulin+/+/CIITA+/+, β2-microglobulinnull, CIITAnull, and β2-microglobulinnull+CIITAnull. Twenty-four strand-specific sequencing cDNA libraries (6 per condition) were produced from purified total RNA samples by the Illumina TruSeq stranded protocol. The libraries underwent 101bp pair-end sequencing using Illumina HiSeq 4000 according to Illumina protocols, generating an average of 33 million paired-end reads per library. For each read, we trimmed the first 6 nucleotides and the last nucleotides at the point where the Phred score of an examined base fell below 20 using in-house scripts. If after trimming the read was shorter than 45-bp, the read was discarded. Trimmed reads were mapped to the human reference genome (hg19) with a known transcriptome index (UCSC Known Gene annotation) with Tophat v2.1.1 (64) using the very-sensitive preset, first strand library type, and providing the corresponding gene model annotation. Only reads that mapped
to a single unique location within the genome, with a maximum of two mismatches in the anchor region of the spliced alignment, were reported in these results. We used the default settings for all other Tophat options. Tophat alignments were then processed by Cuffdiff (Cufflinks v2.2.1;(65)) to obtain differential gene expression using first strand library type, providing gene model annotation and the genome sequence file for detection and correction of sequence-specific bias that random hexamer can cause during library preparation. P-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg procedure (66). Sequencing and analysis results were deposited in NCBI's Gene Expression Omnibus (GEO) under accession number GSE130443.

**Immunofluorescence Microscopy**

ECs were grown to confluence on gelatin-coated glass coverslips in C24 well plates and fixed in 95% ethanol for 30 minutes. Fixed monolayers were incubated overnight in 5 μg/mL mouse anti-human CD31 (Clone WM59, BioLegend) and CD144 (Clone BV9, BioLegend) diluted in Tris-buffered solution/0.2% Triton-X-100/5% normal donkey serum. Donkey Alexa 488 and Alexa 594 anti-mouse secondary antibodies were used to detect the primary antibody. Coverslips were mounted for analysis in ProLong mounting medium (Invitrogen) and randomly selected fields were collected by a Zeiss Axiovert fluorescence microscope with an ORCA-ER digital camera (Hamamatsu Photonics).

**Electrical Cell-Substrate Impedance Sensing Assays**

ECs were grown to confluence on 96-well plates (8W20idf PET, Applied Biophysics). Transendothelial electrical resistance of ECFCs were assessed by a 96-well electrical

26
cell-substrate impedance sensor (Model Z-θ, Applied BioPhysics). Confluent cell monolayers were stimulated with 10ng/mL TNF-α (Invitrogen) or 1 U/L thrombin (Invitrogen). Data was acquired using a Z-θ instrument with ECIS software (Applied BioPhysics).

Flow Cytometry Analysis and Western Blot Characterization of Endothelial Cells

Colonies expanded from single cell clones were further evaluated for the presence of class I and II MHC by surface and intracellular staining for class I heavy chain (Clone EMR8-5, Abcam) and class II heavy chain (Clone XD5.A11 ascites, gifted by Peter Cresswell, Yale University) following fixation and permeabilization (Intracellular Fixation and Permeabilization Kit, BioLegend). ECs were also characterized for expression of HLA-DQ (BioLegend, Clone Tu169), and HLA-DP (Abcam, Clone B7/21) by flow cytometry, according to manufacturer’s protocol. β2-microglobulin<sup>+/+</sup>/ CIITA<sup>+/+</sup>, β2-microglobulin<sup>null</sup>, CIITA<sup>null</sup>, or β2-microglobulin<sup>null</sup>+/CIITA<sup>null</sup> EC were also co-stained with Pacific Blue-conjugated anti-CD54 (BioLegend, Clone HA58) and APC-conjugated anti-CD274 (BioLegend, Clone 29E.2A3). Stained cells were evaluated in triplicate using 4 independent donors with a minimum of 10<sup>5</sup> live cells per sample acquired on an LSR II (BD Biosciences). All assays were carried out following 72 hours of treatment with 50ng/mL IFN-γ (Gibco, Gaithersburg, MD).

To determine whether β2-microglobulin<sup>null</sup> EC were able to reconstitute class I MHC in <i>trans</i> cells were cultured for 72 hours in the presence of EGM2MV media (Lonza) supplemented with 15% human ABO serum (Sigma Aldrich) and 5 μg/mL of recombinant
human β2-microglobulin (Abcam). ECs were characterized for expression of HLA-A-B,-C (Clone W6/32, BioLegend) by flow cytometry as described above.

The presence of an unfolded protein response was examined by Western blot by collecting 10⁵ EC treated with vehicle, 50ng/mL IFN-γ (Gibco) for 72 hours or 2μM Thapsigargin (Sigma Aldrich) for 6 hours were lysed in 50 microliters of Laemmelli buffer (BioRad). 10 microliters were loaded into a 12% SDS gel and transferred onto PVDF membrane overnight. Membranes were blocked with 5% BSA in TBS and incubated with 1 μg/mL anti-human CHOP antibody (Cell Signaling, Clone L63F7) overnight at 4°C and a 100 ng/mL horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson Laboratories) at room temperature for 2 hours and visualized using Femto Western Maximum Sensitivity Substrate (ThermoFisher).

Alloantibody Assays

ECs were stimulated for 72 hours with 50ng/mL IFN-γ (Gibco) and plated to confluence in a C12 well. In experiments evaluating alloantibody binding to ECs, cells were treated with either 25% unsensitized human ABO serum (Sigma-Aldrich) or 25% high titer (cPRA >80%) panel reactive antibody serum isolated and pooled from multiple allosensitized transplant candidates diluted in gelatin veronal buffer. In compliance with Health Insurance Portability and Accountability Act (HIPPA) regulations, cPRA >80% sera, as determined by Luminex single antigen testing (One Lambda, Canoga Park, California), were collected and pooled from multiple individuals by the Yale-New Haven Hospital Histocompatibility Laboratory. Following a 2 hours incubation at 37°C, cells were
harvested and stained with 5μg/mL FITC-anti-human IgG (Abcam), mouse anti-human C4d (Clone A213, Quidel). Cells stained with the latter antibody were then incubated with 5μg/mL Alexa647-anti-mouse antibody (Life Biotechnologies). Samples were analyzed on an LSR II flow cytometer (BD Biosciences). To evaluate non-canonical NF-κB signaling, 10^5 Cas9-modified cells treated with gelatin veronal buffer or 25% high titer cPRA serum were lysed in 100 microliters of Laemelli buffer (BioRad). 15 microliters were loaded into a 5-20% SDS gel and transferred onto PVDF membrane overnight. Membranes were blocked with 5% BSA in TBS and incubated with 1 μg/mL anti-human p100/p52 antibody (Cell Signaling, Clone 18D10) overnight at 4°C and a 100 ng/mL horseradish peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 1 hour and visualized using Femto Western Substrate (ThermoFisher). Densitometry was quantified from scanned images using ImageJ.

*Isolation of Human CD4 and CD8 Effector Memory T Cells*

Peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis from anonymized healthy human volunteers and enriched by density centrifugation using Lymphocyte Separation Medium (MP Biochemicals, Santa Ana, CA) according to the manufacturer’s protocol, and subsequently cryopreserved in liquid nitrogen after resuspension in 90% fetal bovine serum (VWR)/10% DMSO (J.T. Baker).

PBMCs were thawed and CD4^+ and CD8^+ T lymphocytes were isolated using a Dynabeads CD4^+ or CD8^+ Positive Isolation Kit (Invitrogen, Carlsbad, CA). Removal of naïve T cells, central memory T cells, activated and CD4^+CD8^+ lymphocytes was
achieved by negative selection following incubation with mouse anti-CD45RA (Clone H100, 5μg/mL), anti-CCR7 (Clone G04387, 10μg/mL) anti-CD62L (Clone DREG-56, 5μg/mL), anti-HLA-DR (Clone L243, 5μg/mL), anti-CD25 (Clone BC96, 5μg/mL) and either CD4 (Clone SK3, 5μg/mL) or CD8 (Clone SK1, 5μg/mL) (all from Biolegend) and subsequent depletion using pan-mouse IgG beads (Invitrogen). Isolated $T_{EM}$ were routinely $>95\%$ CD45RO$^+$CCR7$^-$CD62L$^-$HLA-DR$^-$CD25$^-$CD4$^+$ or CD45RO$^+$CCR7$^-$CD62L$^-$HLA-DR$^-$CD25$^-$CD4$^+$CD8$^+$ (Supplementary Figure 3). In lymphocyte assays, $T_{EM}$ were cultured with ECs in RPMI 1640 medium (Gibco) with 10% FBS (VWR), 2% L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Invitrogen).

**Mixed Endothelial Cell-Lymphocyte Proliferation and Activation Assays**

ECs were plated at confluence ($10^5$ cells/well) into C-24 well tissue culture plates and stimulated with 50ng/mL IFN-γ (Gibco, Gaithersberg, MD) for 72 hours. Unfractionated PBMC or purified CD4$^+$ or CD8$^+$ $T_{EM}$ were labeled at 37°C using the Cell Trace Violet Cell Proliferation Kit (Invitrogen). Labeled CellTrace Violet$^+$ effector cells were then added to well containing EC-monolayers at an effector:target ratio of 30, respectively. Co-culture was maintained for 7 days in 1mL of RPMI 1640 medium (Gibco) with 10% FBS (VWR), 2% L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Invitrogen). Lymphocytes were subsequently co-stained with an eFluor780 viability dye (eBioscience), APC anti-CD4 (Clone SK3, BioLegend), anti-CD8 (Clone SK1, BioLegend), anti-HLA-DR (Clone L243, BioLegend), and analyzed by flow cytometry (LSR II, BD Biosciences).
Generation of Cytotoxic T Lymphocytes and Cytotoxicity Assays

CD8+ lymphocytes were isolated from PBMC using Dynabeads CD8 Positive Isolation Kit (Invitrogen) and co-cultured with allogeneic ECs in C24 well plates at a responder-stimulator ratio of 20 in RPMI 1640 medium (Gibco) with 10% FBS (VWR), 2% L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Invitrogen). The medium was supplemented with IL-2 (Gibco) to a final concentration to 6 U/mL on day 3. On day 7, the medium was changed and CTL were transferred to fresh stimulator cells from the same stimulating donor. The medium was supplemented with IL-2 (Gibco) to a final concentration of 6U/mL on day 10. T lymphocytes were re-isolated sing Dynabeads CD8 Positive Isolation Kit (Invitrogen) on day 14 and used for cytotoxicity assays.

Approximately 2 x 10^4 EC were stimulated for 72 hours with 50ng/mL IFN-γ (Gibco) in a C-96 well U-bottom microtiter plate (Corning). Target ECs were labeled with 2 μM calcein AM for 30 minutes (Invitrogen) and differentiated CTL raised on donor-matched ECs were added in 100μL RPMI medium (without phenol-Red) supplemented with 5mM HEPES at 10, 3, 1, and 0.3 to 1 effector:target ratios. Following 4 hours of co-culture, 75μL of supernatant was collected and read on a fluorescent plate reader. Eight replicates were used for each condition. The percentage of cytotoxicity was defined as (dead-experimental)/(dead-live), where live corresponds to the value of calcein-loaded ECs without CTL, experimental is with CTL and dead denotes ECs lysed with 1% Triton-X 100 (Sigma-Aldrich).

Isolation of Human Natural Killer Cells, ELISAs and Cytotoxicity Analysis
Natural killer cells were isolated from thawed PBMCs using an EasySep Human NK Cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada), according to the manufacturer's protocol. The NK-enriched cell population was further depleted of remaining CD3+ lymphocytes with CD3 Dynabeads (Invitrogen). Isolated natural killer cells (NK) were routinely >90% CD3-CD56+ and expressed low levels of CD335 (Supplementary Figure 3).

ECs were stimulated for 72 hours with 50ng/mL IFN-γ (Gibco) in a C-24 well microtiter plate (Corning). Purified, unactivated, human NK cells were then co-cultured with each respective EC cell type or K562 lymphoblasts (ATCC) in RPMI 1640 medium (Gibco) with 10% FBS (VWR), 2% L-glutamine (Sigma-Aldrich), and 1% penicillin-streptomycin (Invitrogen) at an effector:target ratio of 20 for 6 hours in a volume of 0.2mL. Supernatants were collected and run in duplicate using human TNF-α or IFN-γ ELISA kits (Invitrogen) according to the manufacturers protocol.

For cytotoxicity assays, confluent monolayers of ECs were stimulated for 72 hours with 50ng/mL IFN-γ (Gibco) in a C-96 well plate (Corning). A set of β2-microglobulin+/+ / CIITA+/+ were incubated for 15 minutes with 25% high PRA serum to serve as a positive control. All ECs were labeled with 2μM calcein AM for 30 minutes (Invitrogen) and co-cultured with purified NK cells for 6 hours at an effector:target ratio of 20. Release of fluorescent dye was measured using a plate reader (BioTek) in bottom reading mode. The percentage of cytotoxicity was defined as (live – experimental)/(live – lysed) × 100, where
live is the value of calcein-loaded ECs without CTL, experimental is with CTL, and lysed is the number of lysed cells treated with 1% Triton-X 100 (Sigma-Aldrich).

Wild type ECFCs and adult human dermal microvascular cells were compared after 48 hours of treatment with either 10 ng/mL TNF-α (R&D Systems, Minneapolis, MN), 50 ng/mL IFN-γ (Gibco) or PBS with resting K562 for expression of NK ligands by staining with APC-HLA-E (Clone 3D12), APC-HLA-G (Clone 87G) APC-MICA/MICB (Clone 6D4), PE-CD111 (Clone R1.302), APC-CD112 (Clone TX31), APC-CD155 (Clone SKII.4) or with an appropriately conjugated isotype control (all from BioLegend), according to the manufacturer’s instruction. The effects of TNF-α or IFN-γ were confirmed by staining for PE-VCAM-1/CD106 (Clone STA) and APC-PD-L1/CD274 (Clone B7-H1), respectively, antigens known to be upregulated in EC. Human dermal microvascular cells were isolated as previously described (67).

In an assay performed to distinguish immune inhibition from induction of anergy of NK cells, isolated NK cells were cultured on confluent monolayers of β2-Microglobulin+/+ /CIITA+/+, β2-microglobulinnull or no target cells at a concentration of 10⁶ cells/mL for 6 hours in RPMI/10% FBS medium. NK cells were then harvested and co-cultured with 2 × 10⁵ K562 cells in a C48 at an effector:target ratio of 5 or a well lacking target cells in a total volume of 0.2mL. Supernatant was harvested at 6 hours and run in duplicate using a human IFN-γ ELISA kit (Invitrogen) according to the manufacturers protocol. This was performed in two independent donor pairs.
Synthetic Microvessel Formation and In vivo Implantation

Human microvessels comprised of either β2-microglobulin+/+/CIITA+/+, β2-microglobulinnull, CIITAnull, β2-microglobulinnull+CIITAnull EC were embedded in a collagen/fibronectin gel and implanted subcutaneously in the abdominal wall of the 6-8 week old female C.B-17 SCID/bg mouse, as previously described (32). In brief, CRISPR/Cas9-modified ECs were suspended in rat tail type I collagen (Corning), human plasma fibronectin (EMD Millipore) and M199 medium (Gibco) in a 0.5mL cellular suspension and polymerized at 37°C for 30 minutes prior to implantation. In one experiment, each mouse received a single implant of either β2-microglobulin+/+/CIITA+/+, β2-microglobulinnull, CIITAnull, β2-microglobulinnull+CIITAnull EC. In a separate experiment, each mouse received two gel implants to compare β2-microglobulin+/+/CIITA+/+ and β2-microglobulinnull+CIITAnull EC. Fourteen days after implantation one gel was harvested from each mouse to confirm vessel formation 10 minutes after intravenous inoculation with fluorescein-\textit{Ulex europeanus} agglutinin (Vector Laboratories), which labels perfused human vessels. After confirmation of vessel formation, implanted mice were either inoculated with PBS or 120 million PBMC. Animals were euthanized approximately 10 days after inoculation and EC-containing protein gels were fixed in 10% neutral buffered formalin (Sigma) and embedded in paraffin. Sections of 5μm thickness were cut and immunoperoxidase staining for human CD31 (Clone JC/70A, Dako) was performed with a hematoxylin counterstain (Vector Laboratories). Tiled microscope images of stained tissue sections were taken in bright field mode using an EVOS FL Auto2 imaging system (Thermo Fisher Scientific). 40-60 randomly selected sections sampling all part of the tissue block were evaluated per animal. Whole sections were imaged using a 40x objective (Olympus...
UPlanSApo, 0.95 NA), with an image pixel size of 0.175µm. A MATLAB code was employed to quantify the vessel to gel area ratio in each section. Briefly, the collagen gel region was first defined in the raw image using the MATLAB function `roipoly` and gel area was calculated by multiplying with the squared pixel size. The green image channel was subtracted from the red channel resulting in an image with the highest intensity for stained vessels. The gel region was then binarized by defining a threshold and holes in adjacent regions were filled. The vessel area was defined as the number of nonzero pixels in the resulting image multiplied by the squared pixel size. The vessel number was quantified by the number of vessels staining positive for CD31+ normalized to the total gel area. 3-6 animals per group were evaluated to observe statistical differences.

**Statistical Analysis**

Data are expressed as mean +/- one standard error of the mean. Continuous variables were compared using Student's t test (2-tailed) or analysis of variance, as appropriate. Analyses were conducted with GraphPad Prism v7.0 and statistical significance was claimed when p<0.05.

**Study Approval**

Protocols involving collection and experimentation with human cells were approved by the Yale Human Investigation Committee. All animal protocols were approved by the Yale Institutional Animal Care and Use Committee.
AUTHOR CONTRIBUTIONS

JSP and JM conceived the study and wrote the manuscript. JM, WMS, GTT, GT and JSP aided in the experimental design. JM, RWP, LQ, TB, TDM, GL, LGB and NKS conducted the experiments. JM, MR, SS, FLG, CX performed critical analyses. JM and JSP wrote the manuscript and all other authors provided critical revisions.

ACKNOWLEDGEMENTS

This work is supported by grants from the National Institutes of Health: R01-HL085416 to JSP and WMS as well as UL1-TR000142 and T32-DK007276 to JM. This work was also supported by the HPC facilities operated by the Yale Center for Research Computing and the Yale Center for Genome Analysis, as well as NIH grant 1S10OD018521-01, which helped fund the cluster. We thank Gwendolyn Davis-Arrington for assistance with endothelial cell isolation. We acknowledge Zuzanna Tobiasova and Ewa Menet for help with FACS sorting as well as Peter Cresswell for providing anti-human MHC class II heavy chain antibodies.

REFERENCES

memory and correlates with the risk of posttransplant rejection.


39.


50.


51.


52.


53.


54.


55.


56.


57.


58.


59.


60.


61.


62.


63.


64.


65.


66.


67.

Figure 1. CRISPR/Cas9 biallelic disruption of β2-microglobulin and CIITA eliminates class I and II MHC expression in human cord-blood endothelial cells. (A) Clonally expanded colonies of sorted Cas9-ECFC transduced with targeting guide strands targeting β2-microglobulin, CIITA or a control gene locus (AAVS1). (B) Surface class I MHC heavy chain and total class I MHC heavy chain assayed by surface and intracellular flow cytometry reveals absence of expression in β2-microglobulinnull ECFCs. (C) Total class II MHC heavy chain expression, HLA-DQ and HLA-DP expression is absent in CIITAnull ECFCs. (D) Biallelic indel mutations noted in β2-microglobulin and CIITA gene loci in cells transduced with targeting guide strands. Representative plots shown from 3 unrelated donors shown as mean ± SEM.
Figure 2. CRISPR/Cas9 Ablation of β2-microglobulin and CIITA Does Not Alter Core Endothelial Cell Functional Characteristics. (A) Confocal microscopy exhibiting similar junctional VE-Cadherin (CD144) and PECAM-1 (CD31) staining and cell morphology. Scale bar=20μm. (B) Formation of equivalent barriers overtime as measured by transepithelial electrical resistance (TEER). (C) Barriers are equally disrupted in response to thrombin (1 U/L) and TNF-α (10 ng/mL) and (D) Upregulation of ICAM-1 and PD-L1 in response to IFN-γ (50ng/mL, 48 hours) in MHC-ablated compared to control (AAVS1) ECs. (E) Vessel formation preserving pre-implantation MHC expression in Cas9-modified ECs 2 weeks following suspension in collagen/fibronectin gel and implantation in an immunodeficient mouse in vivo. Scale bar=50μm. Representative of 4 independent donors.
Figure 3. β2-microglobulin\textsuperscript{Null}+CIITA\textsuperscript{Null} ECs Evade Alloantibody Mediated Binding, Complement Deposition and Complement-Dependent NF-κB Signaling. Cas9-modified ECs were co-cultured with 25% High PRA Serum in gelatin veronal buffer for 2 hours and surface (A) human IgG (B) human C4d were assessed by flow cytometry (2 donors). (C) Immunoblot of Cas9-Modified ECs co-cultured with 25% high PRA serum shows diminished non-canonical NF-κB signaling, characterized by processing of p100 to p52, in β2-microglobulin\textsuperscript{Null}+CIITA\textsuperscript{Null} compared to control (AAVS1) ECs. Densitometry exhibiting mean ± SEM relative p52/β-actin expression of 3 independent endothelial donors and high titer cPRA pooled serum pairs run in triplicate.
Figure 4. Ablation of class I and II MHC Expression Abrogates CD4⁺ and CD8⁺ T Cell Proliferation, CD4⁺ Activation, and CD8⁺ Cytotoxicity. (A) CIITA⁺Null and β₂-microglobulin⁺Null⁺CIITA⁺Null ECs exhibit a reduction in CD4⁺ TEM proliferation and β₂-microglobulin⁺Null and β₂-microglobulin⁺Null⁺CIITA⁺Null ECs exhibit a reduction in CD8⁺ TEM proliferation compared to control (sgAAVS1) ECs. (B) CD4⁺ TEM co-cultured with CIITA⁺Null ECs fail to express activation markers. (C) Cytotoxic T lymphocytes raised on donor-matched wild type EC fail to kill β₂-microglobulin⁺Null EC compared to control ECs as measured by calcein AM release. Representative of 3 independent donor pairs shown as Mean ± SEM.
Figure 5. Absence of class I MHC on human endothelium does not enhance natural killer cell activation or cytotoxicity. (A) IFN-γ and TNF-α ELISA of supernatant in co-cultures of human NK cells with MHC absent ECs. No enhancement of cytokine release was observed in β2-microglobulinNull cells compared to control ECs. (B) Cytotoxicity measured by calcein AM release with human alloantibody bound endothelial cells included as a positive control. Representative of 4 independent donor pairs (Mean ± SEM). (C) Expression of NK cell ligands in human cord-blood derived ECs (ECFC), adult human dermal endothelial cells (HDMEC) and leukemic K562 NK cell targets under resting conditions and 48 hours of IFN-γ or TNF-α. VCAM-1 and PD-L1 expression are included as control antigens known to be upregulated in EC in the presence of TNF-α or IFN-γ, respectively. Representative of 2 independent donors. (D) NK release of IFN-γ on secondary exposure of K562 cells following 6 hours of primary co-culture with either control (sgAAVS1), β2-microglobulinNull, or no target cells. Absence of IFN-γ release on exposure to K562 cells following β2-microglobulinNull EC co-cultures suggests β2-microglobulinNull EC induce NK cell anergy. Representative of 2 independent donors.
Figure 6. β₂-microglobulin<sup>Null</sup> and β₂-microglobulin<sup>Null</sup>+CIITA<sup>Null</sup> ECs evade allogeneic PBMC destruction in a humanized mouse model. (A) Quantification of CD31<sup>+</sup> vascular destruction in control, β₂-microglobulin<sup>Null</sup> and β₂-microglobulin<sup>Null</sup>+CIITA<sup>Null</sup> EC relative to saline-injected mice. Each data point represents the area of CD31<sup>+</sup> vasculature normalized over the area of the collagen gel in a paraffin-embedded 5 μm section. A minimum of 30 sections were quantified per collagen gel and at least 3 collagen gels were used per sample. (B) Representative explant of collagen gels containing vasculature from CRISPR/Cas9 modified ECFC. Scale bar=100 μm (C) Representative images of vascularized collagen gels composed of control (sgAAVS1) β₂-microglobulin<sup>Null</sup> and β₂-microglobulin<sup>Null</sup>+CIITA<sup>Null</sup> ECs in immunodeficient mice injected with vehicle of allogeneic PBMC. Scale bar=500 μm. 3-6 animals used in each group. CD31<sup>+</sup> immunohistochemical staining is shown in representative sections demonstrating the presence of vessels in animals treated with vehicles and the in those lacking class I MHC, but not those harboring class I MHC in animals engrafted with allogeneic PBMC.
## TABLES

<table>
<thead>
<tr>
<th>CRISPR/Cas9 Targeted Locus/Loci</th>
<th>Gene</th>
<th>Locus</th>
<th>spAAVS1 Gene Expression (FPKM)</th>
<th>CRISPR/Cas9 Targeted Gene Expression (FPKM)</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Microglobulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DQA1</td>
<td>chr6:67101782-67129310</td>
<td>89.8</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRA</td>
<td>chr6:67101782-67129310</td>
<td>1049.6</td>
<td>0.8</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DMB</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>814.0</td>
<td>0.1</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DOA</td>
<td>chr6:67101782-67129310</td>
<td>421.5</td>
<td>0.6</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>chr6:67101782-67129310</td>
<td>73.2</td>
<td>0.2</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>chr6:67101782-67129310</td>
<td>299.5</td>
<td>2.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>chr6:67101782-67129310</td>
<td>68.3</td>
<td>0.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>chr6:67101782-67129310</td>
<td>550.0</td>
<td>6.3</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>β-Microglobulin+CIIA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC02A1</td>
<td>chr3:13365159-133748920</td>
<td>35.1</td>
<td>2.5</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>ITGB4</td>
<td>chr7:33717515-33753899</td>
<td>1.1</td>
<td>0.2</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>EMLIN1</td>
<td>chr2:27301434-27309265</td>
<td>1.5</td>
<td>0.3</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>NQR1</td>
<td>chr1:153651163-153666468</td>
<td>2.1</td>
<td>0.4</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>PALM</td>
<td>chr1:1670962-1743830</td>
<td>5.8</td>
<td>1.1</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>ASS1</td>
<td>chr9:13332093-133378661</td>
<td>12.1</td>
<td>2.4</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>CCL14</td>
<td>chr2:33717515-33753899</td>
<td>13.8</td>
<td>2.7</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>GALNTL2</td>
<td>chr2:16216183-16272153</td>
<td>4.4</td>
<td>0.9</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>PKHD1L1</td>
<td>chr1:110374705-110543500</td>
<td>0.9</td>
<td>0.2</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>SELENBP1</td>
<td>chr1:151367795-151345164</td>
<td>4.3</td>
<td>0.9</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>FHOD3</td>
<td>chr1:33877701-33630018</td>
<td>0.5</td>
<td>0.1</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>TSPYL5</td>
<td>chr8:9285713-9290176</td>
<td>5.2</td>
<td>1.2</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>IL33</td>
<td>chr9:12515765-1257983</td>
<td>5.7</td>
<td>1.3</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>BGN</td>
<td>chrX:152700346-152775004</td>
<td>210.8</td>
<td>49.7</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>IGFBP2</td>
<td>chr2:27301434-27309265</td>
<td>13.3</td>
<td>3.2</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Genes significantly upregulated and FDR-adjusted p<0.05 with CRISPR/Cas9-targeting compared to control vector (AAVS1) targeted ECs with fold change > 4 after stimulation with IFN-γ (50 ng/ml) for 96 hours. Expression values are fragments per kilobase of exon per million fragments mapped (FPKM).
<table>
<thead>
<tr>
<th>CRISPR/Cas9 Targeted Locus/Loci</th>
<th>Gene</th>
<th>Locus</th>
<th>sgAAVS1 Gene Expression (FPKM)</th>
<th>CRISPR/Cas9 Targeted Gene Expression (FPKM)</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2-Microglobulin</td>
<td>NCAM2</td>
<td>chr21:22370632-22912517</td>
<td>0.1</td>
<td>1</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>SCUBE1</td>
<td>chr22:43590228-43730394</td>
<td>0.1</td>
<td>1.2</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>TNNT1</td>
<td>chr19:55644160-55660060</td>
<td>0.1</td>
<td>1.4</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>POU4F1</td>
<td>chr13:79173229-79177695</td>
<td>0.1</td>
<td>0.6</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>HOXB9</td>
<td>chr17:46698518-46703835</td>
<td>0.2</td>
<td>1.6</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>GREM1</td>
<td>chr15:33010204-33026870</td>
<td>0.2</td>
<td>1.7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>PAPPA</td>
<td>chr6:118916070-119164600</td>
<td>0.3</td>
<td>1.2</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>EEF1A2</td>
<td>chr20:62119385-62130505</td>
<td>0.6</td>
<td>2.4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CiITA</td>
<td>COL6A2</td>
<td>chr21:47518032-47552763</td>
<td>0.2</td>
<td>3.8</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>SIM1</td>
<td>chr6:100836749-100911551</td>
<td>0</td>
<td>0.6</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>NCAM2</td>
<td>chr21:22370632-22912517</td>
<td>0.1</td>
<td>0.9</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>TAF7L</td>
<td>chrX:100523240-100548059</td>
<td>0.1</td>
<td>0.6</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>GREM1</td>
<td>chr15:33010204-33026870</td>
<td>0.2</td>
<td>2.2</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2-Microglobulin+CiITA</td>
<td>MMP20</td>
<td>chr11:102447585-10246683</td>
<td>0</td>
<td>0.4</td>
<td>inf</td>
</tr>
<tr>
<td></td>
<td>LARGE</td>
<td>chr22:33659061-34315416</td>
<td>0</td>
<td>1.1</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>PAPPA</td>
<td>chr1:176432306-176811970</td>
<td>0</td>
<td>0.8</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>POU4F1</td>
<td>chr13:79173229-79177695</td>
<td>0.1</td>
<td>0.5</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>SERPINB2</td>
<td>chr18:61554938-61577124</td>
<td>1.1</td>
<td>8.5</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>AFF3</td>
<td>chr2:102163715-100769307</td>
<td>0.4</td>
<td>3.1</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>FAM129A</td>
<td>chr1:184760165-184943862</td>
<td>1.9</td>
<td>10</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>LPXN</td>
<td>chr11:58294343-58345639</td>
<td>7.1</td>
<td>34.7</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>KIT</td>
<td>chr4:55524094-55606881</td>
<td>0.2</td>
<td>0.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 2. Genes significantly downregulated (FDR-adjusted p<0.05) with CRISPR/Cas9-targeting compared to control vector (AAVS1) targeted ECs with fold change > 4 after stimulation with IFN-γ (50 ng/ml) for 96 hours. Expression values are fragments per kilobase of exon per million fragments mapped (FPKM).