Metabolic regulation of differentiation and maturation: from induced pluripotent stem cell to endothelial cell

Es - Tiemeier, G.L. van

Chapter 5

Immunogenicity of human iPSC-derived endothelial cells

This Thesis (in preparation for submission)

Gesa L. Tiemeier, Lonneke H. Gaykema, Dominique de Jong, Cathelijne W. van den Berg, Bernard M. van den Berg, Arnaud Zaldumbide, Ton J. Rabelink

Abstract

Human induced pluripotent stem cell derived endothelial cells (hiPSC-ECs) are promising candidates for the engineering of microvasculature in tissue constructs due to the unlimited proliferation capacity of hiPSCs. Since creation of sufficient autologous hiPSC-ECs for every patient is unfeasible, the possibility of biobanking allogeneic hiPCS-ECs is currently being explored. Studies have shown that hiPSC-ECs are less mature compared to primary human endothelial cells (hECs) in multiple aspects, which could be an advantage in the context of immunogenicity. This is the first study characterizing the immunogenicity of hiPSC-ECs by assessing surface expression of histocompatibility markers, such as the human leukocyte antigens (HLA), and complement inhibiting factors. At a functional level, we tested differences in an allogeneic PBMC activation assay.

We found significant differences in HLA expression between hiPSC-ECs and primary hECs. When stimulated with IFN-γ and IL-1β, HLA-A,B,C expression was significantly higher in hiPSC-ECs compared to primary hECs, whereas the proportion of cells expressing HLA-DR was significantly lower. PBMC co-cultures suggested that hiPSC-ECs induce less allogeneic $CD4^+$ and $CD8^+$ T cell and NK cell activation compared to primary hECs, but activation levels were low in both groups in general. Compared to primary hECs, hiPSC-ECs expressed similar levels of complement inhibitors CD46 and CD59.

Although we observed significant differences between primary hECs and hiPSC-ECs in HLA expression and PBMC activation, it is still unclear whether hiPSC-ECs are better protected from allogeneic rejection, which needs further *in vivo* research.

Introduction

In end–stage renal disease (ESDR), the only curative treatment is organ transplantation. Due to worldwide donor organ shortages [1, 2], patients need to undergo dialysis as renal replacement therapy, which drastically reduces the quality of life[3, 4]. Bioengineered tissues have the potential to address the worldwide allograft shortage. Currently, the greatest challenge in tissue engineering is perfusion[5]. Tissue constructs of a clinically relevant size exceed the diffusion limit of 100-200 μm[6]. A recent topic of debate is which cell type is the most suitable source for microvascular engineering. Primary, patient-derived human endothelial cells (ECs) would be the best option in terms of safety and functionality, but availability of these cells is limited since primary hECs can only be derived from explanted organs or umbilical veins at birth. Moreover, expansion capacity of these cells is restricted by their limited life span and proliferation *in vitro* [7]. Another possibility is the use of endothelial colony forming cells (ECFCs). These circulating endothelial progenitor cells isolated from adult blood have an exceptional proliferative capacity and maintain endothelial characteristics during long-term in vitro expansion[8, 9]. However, success rates for ECFCs isolations are limited and high inter- and intra-donor variability have been reported [8, 10, 11].

Yet another option for microvascular engineering is the use of induced pluripotent stem cell derived endothelial cells (iPSC-ECs). iPSCs exhibit an almost unlimited proliferative capacity and have the potential to differentiate towards any cell type of the human body. Although multiple endothelial-like characteristics, such as functional blood vessel formation, have been demonstrated in hiPSC-ECs [12-16], they are not fully mature in many aspects as described previously [13, 17, 18].

Initially, iPSCs were generally assumed to be immune-privileged, but several studies have indicated otherwise. Despite negligible immunogenicity *in vitro* [19], some *in vivo* studies demonstrated rejection of undifferentiated hiPSCs in syngeneic mice and humanized mouse models [20-22]. In contrast, terminally differentiated murine iPSC-ECs are well tolerated by syngeneic recipients [22, 23], which is promising for clinical application. In this context, hiPSC-ECs immaturity could potentially be a great advantage if it also extends to the immunogenicity of these cells.

Important for immunogenicity are the cell surface major histocompatibility complexes (MHC), in humans also known as human leukocyte antigens (HLA). Class I molecules, among which HLA-A,-B and -C, are expressed by virtually all cell types and engage with $CD8^+$ T cells to present intracellular proteins [24]. Absence of HLA class I

can trigger NK cell activation [25]. Expression of HLA class II molecules, including HLA-DR, -DP and -DQ, is on the other hand mostly reserved to so-called antigen presenting cells (APC), for instance dendritic cells (DCs), for presentation of extracellular proteins to $CD4^+$ T cells [24]. In allogeneic transplantation, the donor immune system can be activated by 'non-self' antigens, predominantly HLA molecules, or by the absence of 'self' antigens. HLA proteins are highly polymorphic and differences of only a few amino acids can be sufficient to provoke graft rejection [24].

As the physical border between graft and host, via flowing blood, ECs play a key role in the alloimmune response [26]. In inflammatory conditions, primary ECs are activated and act as "semi-professional" APCs. Activated ECs express HLA class II proteins and T cell co-stimulation factors like CD58 and CD86. Consequently, activated ECs can directly activate allogeneic T cells, surpassing the involvement of dendritic cells [26-30]. On the contrary, mature ECs also have inhibitory effects on the immune system, for instance by the expression of complement inhibitors [31], which protect ECs from complement damage. Given the overall immaturity, we hypothesize all these functions might be reduced in hiPSC-ECs.

A reduction in immunogenicity of the vascular inner lining could result in a prolonged graft survival. Some *in vitro* evidence has been published supporting a low alloreactive immunogenicity in other hiPSC derived cell types [32-34], which suggests this could be the case for hiPSC-ECs as well.

In this study, we aim to characterize hiPSC-ECs immunogenicity by studying HLA expression, allogeneic T cell and NK cell activation and complement activation. Based on earlier publications about other iPSC derived cell lines[32-34], we hypothesize that hiPSC-ECs express less HLA proteins than primary hECs and are therefore less able to activate alloreactive T-cells. Because of the overall immaturity of hiPSC-ECs, we expect a reduction in complement inhibitor expression compared to primary hECs, which, besides the reduced glycocalyx expression, might detrimentally affect the complement inhibitory surface properties.

Materials and methods

hiPSCs

The hiPSC cell line NCRM1 was obtained from RUCDR (Rutgers University Centre for Dermal Research) (reprogramming of CD34+ cord blood using episomal vectors). LUMC0072iCTRL01 (LUMC0072) and LUMC0099iCTRL04 (LUMC0099) cell lines were generated by the LUMC iPSC core facility from fibroblasts on mouse embryonic fibroblasts (MEFs) using a Simplicon RNA Reprogramming Kit (Millipore-Merck, Amsterdam, the Netherlands) and ReproRNA (Stem Cell Technologies, Köln, Germany) respectively, as described previously by Yoshioka et al. [60], and further cultured in TeSR-E8 medium (Stem Cell Technologies), defined by Chen et al. (2011) [61].

hiPSC-EC cell differentiation

hiPSCs (NCRM1, LUMC0072 and LUMC0099) were differentiated towards hiPSC-ECs using the protocol of Orlova et al[62]. In short, hiPSCs were cultured in TeSR-E8 medium on Matrigel-coated (Corning Inc., Corning, NY) flasks. Four days after seeding (day 0) the medium was changed to BSA polyvinylalcohol essential lipids (BPEL) medium (Stem Cell Technologies) supplemented with Bone Morphogeneic Protein 4 (R&D Systems, Minneapolis, MN), activin A (Miltenyi Biotec., Leiden, the Netherlands), CHIR99021 (a small-molecule inhibitor of glycogen synthase kinase-3β)(Tocris Bioscience, Bristol, UK) and vascular endothelial growth factor (VEGF; Miltenyi Biotec) to induce mesoderm development. On day 3, the medium was changed to BPEL supplemented with TGF-β pathway small-molecule inhibitor SB431542 (Tocris Bioscience) and VEGF. This medium was refreshed on day 7 and 9. hiPSC-ECs were isolated on day 10 by positive selection on CD31 with anti-CD31 antibody-coupled magnetic beads (Dynabeads, Miltenyi Biotec). After isolation, hiPSC-ECs were cryopreserved until further use.

Before starting each experiment, hiPSC-ECs were thawed and cultured in endothelial cell serum free medium (EC-SFM) (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with platelet-poor plasma serum (1% vol/vol) (Biomedical Technologies Inc., Tewksbury, MA), 50 µg/ml VEGF-165 (R&D Systems), 100 µg/ml basic fibroblast growth factor (bFGF) (Miltenyi Biotech), 100 IU/ml penicillin and 100 µg/ml streptomycin (from now referred to as full EC-SFM) on porcine gelatin coated plates for approximately 4 days.

Primary hECs

Human microvascular ECs (hMVECs) were purchased from Cell Systems (ACBRI-128, Kirkland, WA). Upon delivery, cells were at passage 3 and cryopreserved in CSC Cell Freezing Medium. hMVECs were expanded in Endothelial Cell Growth Medium 2 (EGM2) (PromoCell, Heidelberg, Germany) supplemented with the Growth Medium 2 Supplement Pack (PromoCell) and 100 IU/mL penicillin and 100 µg/mL streptomycin (from now referred to as full EGM2), and cryopreserved at passage 7.

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords (UCs). First, the UC was rinsed with Phosphate Buffered Saline (PBS)(Gibco, Thermo

Fisher Scientific) to wash out remaining blood. Then, the UC was filled with trypsin and incubated at 37°C for 20 minutes. Afterwards, the UC was emptied and trypsin was neutralized with 20% Fetal Bovine Serum (Bodinco, Alkmaar, the Netherlands) in PBS. Subsequently, the UC was filled with PBS and rubbed to loosen the cells. PBS containing the cells was collected. Cells were then cryopreserved until experimental use.

For each experiment, primary hECs were thawed and cultured in full EGM2 on gelatin coated flasks for approximately 24 hours. 3 days before the start of the experiment, medium was changed to full EC-SFM to create the same culture conditions as for the hiPSC-ECs.

Cell culture

Primary hECs and hiPSC-ECs were enzymatically passaged onto gelatin coated 6-well plates at a density of 120,000 and 140,000 cells per well, respectively. hiPSCs were seeded on recombinant human vitronectin (rhVTN)(Thermo Fisher Scientific) coated plates. The seeding density of hiPSCs was estimated separately for each experiment, aiming for 60-80% confluence at the moment of harvesting. Primary hECs and hiPSC-ECs were cultured in full EC-SFM and hiPSCs in E8 medium (Gibco, Thermo Fisher Scientific) supplemented with 0.5% Pen/Strep for 48 hours. To measure both activated and resting endothelium, the conditions included cytokine supplementation with 1000 IU/mL recombinant human IFN-γ (rhIFN-γ) (R&D systems) and 2 ng/mL rhIL-1β (Sigma-Aldrich, Saint Louis, MO) (referred to as stimulated), or no additional supplementation (referred to as unstimulated). After the 48 hours, cells were detached by TrypLE Express (Gibco, Thermo Fisher Scientific) and were subsequently resuspended in 10% vol/vol Fetal Bovine Serum (FBS) (Bodinco, Alkmaar, the Netherlands) in Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Thermo Fisher Scientific) (from now referred to as FACS buffer) and used for flow cytometry measurements.

PBMC co-cultures

Human peripheral blood mononuclear cells (PBMCs) were separated from whole blood of a healthy individual by Ficoll-Hypaque density gradient and frozen until further use.

Primary ECs and hiPSC-ECs were seeded on a gelatin coated 96-wells plate at a density of 30,000 cells/well in full EC-SFM and hiPSCs at a density of 10,000 cells/well in E8 medium, all supplemented with 1000 IU/mL rhIFN-γ (R&D systems) and 2 ng/mL rhIL-1β (Sigma-Aldrich) for 24- (48h co-cultures) or 48 hours (5 and 24h co-cultures) to stimulate HLA expression. Before addition of PBMCs, cultures were thoroughly washed to remove all supplemented IFN-γ. PBMCs were thawed and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fischer Scientific) supplemented with 10% vol/vol FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin. 400,000 PBMCs were added to each well. The supplemented RPMI 1640 medium was then diluted 1:1 with E8 (hiPSCs co-culture) or full EC-SFM. Next to the co-cultures, a positive and negative control were included of respectively PBMCs stimulated with 5 µg/mL polyhydroxyalkanoate (PHA) and PBMCs without any stimulation. All conditions were executed in triplicate. Co-cultures with 1 million PBMCs per well were also tested but these did not result in significantly different results.

After 5, 24 or 48 hours, plates were centrifuged and supernatant was collected from each well separately and stored at -80°C. IFN-γ concentration in supernatant was measured with the Human IFN Gamma Uncoated ELISA Kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's protocol. Non-adherent cells were harvested by resuspending them in FACS buffer. Triplicates were pooled and used for flow cytometry measurements.

Flow cytometry measurements

Immunostainings were performed by incubating cells for 30 minutes on ice with the antibodies listed in Table 1 diluted in FACS buffer. For staining of primary hECs, hiPSC-ECs and hiPSCs, 100.000 cells were used per sample and for staining of PBMCs 200,000. Anti-HLA-A,B,C and -HLA-DR antibodies were combined in one cocktail whereas CD46 and CD59 binding antibodies were used for single stains. To check whether the measured HLA-DR expression in hiPSC-ECs could be explained by dedifferentiation, a single experiment was conducted in which next to HLA-A,B,C and HLA-DR, cells were also stained for endothelial cell marker Vascular Endothelial Growth Factor Receptor 2 (VEGFR2). For the PBMC staining, all relevant antibodies (CD4, CD8, CD56, CD69 and CD25) were used in a single cocktail.

In all experiments, compensation controls and isotype controls were included. In case of the PBMC measurements, the isotype control cocktail consisted of all antibodies for the identification markers (CD4, CD8 and CD56) combined with mouse IgG2a/PE and mouse IgG1/FITC.

Table 1: Antibodies used for flow cytometry measurements

Target	Label	Clone	Manufacturer	Dilution
hHLA-A,B,C	PE	W6/32	Dako, Glostrup, Denmark	1:100
hHLA-DR	APC	G46-6	BD Biosciences, Franklin Lakes, NJ	1:100
hCD46	PE	8E2	eBioscience, Thermo Fisher Scientific	1:100
hCD59	FITC	OV9A2	eBioscience, Thermo Fisher Scientific	1:50
hCD4	APC-Cy7	RPA-T4	BD Biosciences, Franklin Lakes, NJ	1:400
hCD8	Pacific Blue	RPA-T8	BD Biosciences, Franklin Lakes, NJ	1:500
hCD56	APC	HCD56	BioLegend, San Diego, CA	1:50
hCD69	FITC	L78	BD Biosciences, Franklin Lakes, NJ	1:5
hCD25	PE	M-A251	BD Biosciences, Franklin Lakes, NJ	1:5
hVEGFR2	FITC	FAB357F	R&D Systems, Minneapolis, MN	1:20
Isotype	**Label**	**Clone**	**Manufacturer**	**Dilution**
mIgG2a, κ	PE	G155-178	BD Biosciences, Franklin Lakes, NJ	1:5
mIgG1, κ	FITC	MOPC-21	BioLegend, San Diego, CA	1:500
mIgG2a, κ	APC	G155-178	BD Biosciences, Franklin Lakes, NJ	1:5

After staining, cells were washed twice with FACS buffer. PBMCs were then resuspended in FACS buffer with viability dye 7-AAD (1:50) (BD Biosciences). Primary hECs, hiPSC-ECs and hiPSCs were resuspended in FACS buffer without 7-AAD. Instead, a single stain of 7-AAD has been performed on these cell types to check whether the forward and side scatter gating was sufficient to exclude dead cells.

Flow cytometry measurements were executed with the BD FACSCanto™ flow cytometry system and processed in FlowJo. Gating strategies are presented in Supplementary figure 1 and 2. Gates were determined separately for each cell type. Results were corrected for spectral overlap by compensation based on single stain controls.

HLA-A,B,C, CD46 and CD59 expression in primary hECs, hiPSC-ECs and hiPSCs were quantified as median fluorescence intensity (MFI). To correct for background signal, the MFI of the isotype control was subtracted. HLA-DR expression was quantified as proportion of cells positive for HLA-DR since it was not homogeneously expressed in primary hECs, hiPSC-ECs and hiPSCs. To this end, a HLA-DR-gate was set at the 99th percentile of the isotype control for each cell type. Cells with a fluorescence intensity higher than the gate value were considered HLA-DR positive. The expression of the positive population was then quantified as MFI, again corrected for background signal by subtracting the isotype control MFI.

In the analysis of the PBMC co-cultures, $CD4^+$ and $CD8^+$ T cells and NK cells were selected using the gates demonstrated in Supplementary figure 2. Expression of CD25 and CD69 was subsequently quantified as the percentages of cells expressing these markers in the same manner as for HLA-DR.

Statistics

Results are presented as mean ± SEM unless indicated otherwise. Differences in HLA-A,B,C, CD46 and CD59 expression between cell types were tested on significance using two-way ANOVA and Tukey's multiple comparisons test. Here, measurements of unstimulated and stimulated cells of the same cell type were considered as paired data. Since HLA-DR was only detected in stimulated cells, differences in expression between cell types were tested with ordinary one-way ANOVA and Tukey's multiple comparisons test. Read-outs of the PBMC co-cultures were tested on significance using the Student's t-test. A p-value lower than 0.05 was considered statistically significant. Statistical analyses were performed in GraphPad Prism 8.1.1.

Results

hiPSC-ECs express HLA class I proteins but show a limited expression HLA class II

Expression levels of HLA class I and class II of hiPSC-ECs compared to primary ECs (hMVECs and HUVECs) and undifferentiated hiPSCs were investigated. Cells were cultured either under normal conditions or stimulated with cytokines (IFN-γ and IL-1β) and subsequently analyzed with flow cytometry (Figure 1). Only hMVECs are presented here since similar trends were found in HUVECs.

In unstimulated conditions, hiPSC-ECs expressed similar levels of HLA class I proteins HLA-A,B,C in comparison to hMVECs (Figure 1a,c). Upon cytokine stimulation however, HLA-A,B,C was upregulated much stronger in hiPSC-ECs than in hMVECs (MFI of 62,8±1,1 versus 27,0±5,4, $p<0.0001$) (Figure 1c).

In HLA-DR expression, a different trend was observed. HLA-DR was undetectable in unstimulated hECs and iPSCs (Figure 1a,b). Upon cytokine stimulation, HLA-DR was not up regulated homogeneously, but only expressed in a fraction of the cells. In hiPSC-ECs, the population expressing HLA-DR after cytokine stimulation was significantly lower in comparison to hMVECs (13.0±4.7% versus 45.1±4.9% positive cells, $p=0.0038$) (Figure 1d). The amount of HLA-DR expressing cells in hiPSC-ECs was comparable to undifferentiated hiPSCs. Notably, when comparing the HLA-DR

expression levels in HLA-DR positive cells, the expression level (MFI) was significantly higher in hiPSC-ECs in comparison to hiPSCs (7742±1642 versus 1338±67 delta MFI, p=0.0309) (Figure 1d).

To investigate whether the low amount of cells expressing HLA-DR in hiPSC-ECs was not caused by a dedifferentiation process, a single experiment was conducted where hiPSC-ECs were stained for both HLA-DR and VEGFR2, an endothelial cell marker which is used to differentiate endothelial progenitor cells from stem cells[35]. All hiPSCs were positive for VEGF2, while two populations of HLA-DR expressing cells were observed (Supplementary Figure 3).

Comparisons between hMVECs and HUVECs are presented in Supplementary Figure 4. HUVECs were somewhat more responsive to cytokine stimulation when comparing them to hMVECs (Supplementary Figure 4a,b,e,f). This resulted in a significantly higher HLA-A,B,C expression in stimulated cells but when comparing hiPSC-EC HLA-A,B,C expression to that of HUVECs, similar results were found as when comparing them to hMVECs.

hiPSC-ECs induce less allogeneic T cell and NK cell activation than primary hECs in PBMC co-culture

To investigate whether the differences in HLA expression between hiPSC-ECs and primary hECs would result in functional differences in allogeneic T cell and NK cell activation, co-cultures with allogeneic PBMCs from 3 different donors were performed. hiPSC-EC induced T and NK cell activation was compared to hMVEC induced activation.

Activation markers CD69 and CD25 were selected as read-outs. CD69 is detectable on T cells within 1 to 4 hours of initial activation (see Supplementary Figure 5). Expression is transient and disappears after approximately 72 hours [36, 37]. The CD69 peak precedes that of CD25, which is usually seen 24-48 hours after activation[38]. In NK cells, CD69 is an early functional marker for cytotoxic activity whereas CD25 expression is believed to correlate with proliferative potential[39].

Expression of CD69 and CD25 in $CD4^+$ and $CD8^+$ T cells and in NK cells was measured after 5, 24 and 48 hours of co-culture. Additionally, supernatant from the co-cultures were collected and analyzed for IFN-γ concentration. Complete data of all conducted PBMC co-cultures and technical controls are presented in Supplementary Figure 6-1 and 6-2. After 48h, the largest differences between co-cultures were found between hMVEC and hiPSC-EC stimulated PBMCs (Figure 2).

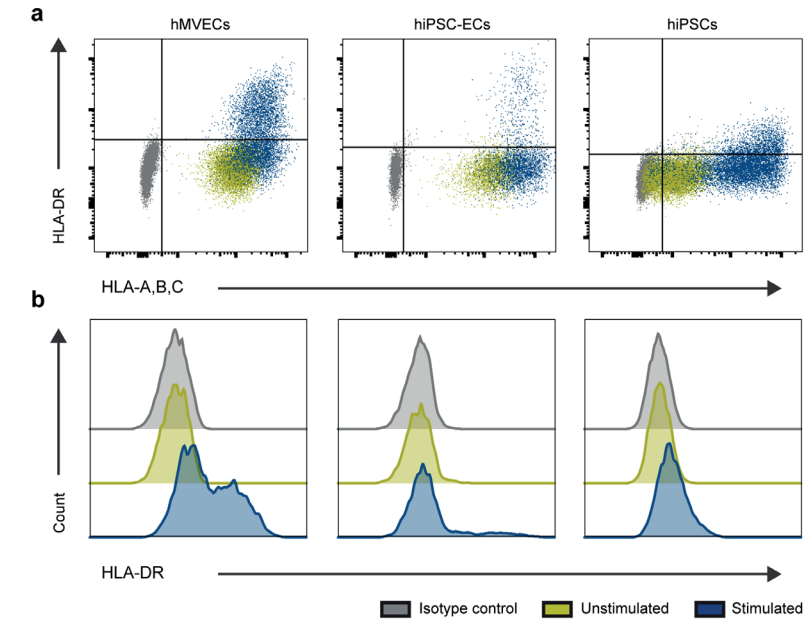


Figure 1: hiPSC-ECs express HLA class I proteins but show a limited expression of HLA class II. Using flow cytometry, the binding of anti-HLA-A,B,C/PE and anti-HLA-DR/APC antibody to hMVEC, hiPSC-ECs and hiPSCs was measured. Cells were either cultured under normal conditions (unstimulated) or stimulated with IFN-γ and IL-1β for 48h (stimulated). Representative data is demonstrated in a dot plot showing HLA-A,B,C and HLA-DR expression (a), and as histograms showing the HLA-DR distribution (b). (c,d) Quantification of HLA-A,B,C and HLA-DR expression for unstimulated (-) and cytokine stimulated (+) hMVECs, hiPSC-ECs and hiPSCs (n=3). HLA-A,B,C was quantified as median fluorescent intensity (MFI) corrected for background signal by subtracting the MFI of the isotype control (c). HLA-DR expression was only detectable in stimulated cells. The expression was then quantified as percentage of cells with a higher HLA-DR/PE signal than the isotype control. This subpopulation was then further charachterized on expression levels as delta MFI, the median fluorescence intensity of the positive population, corrected by the isotype control. * p≤0.05, ** p≤0.01, **** p≤0.0001.

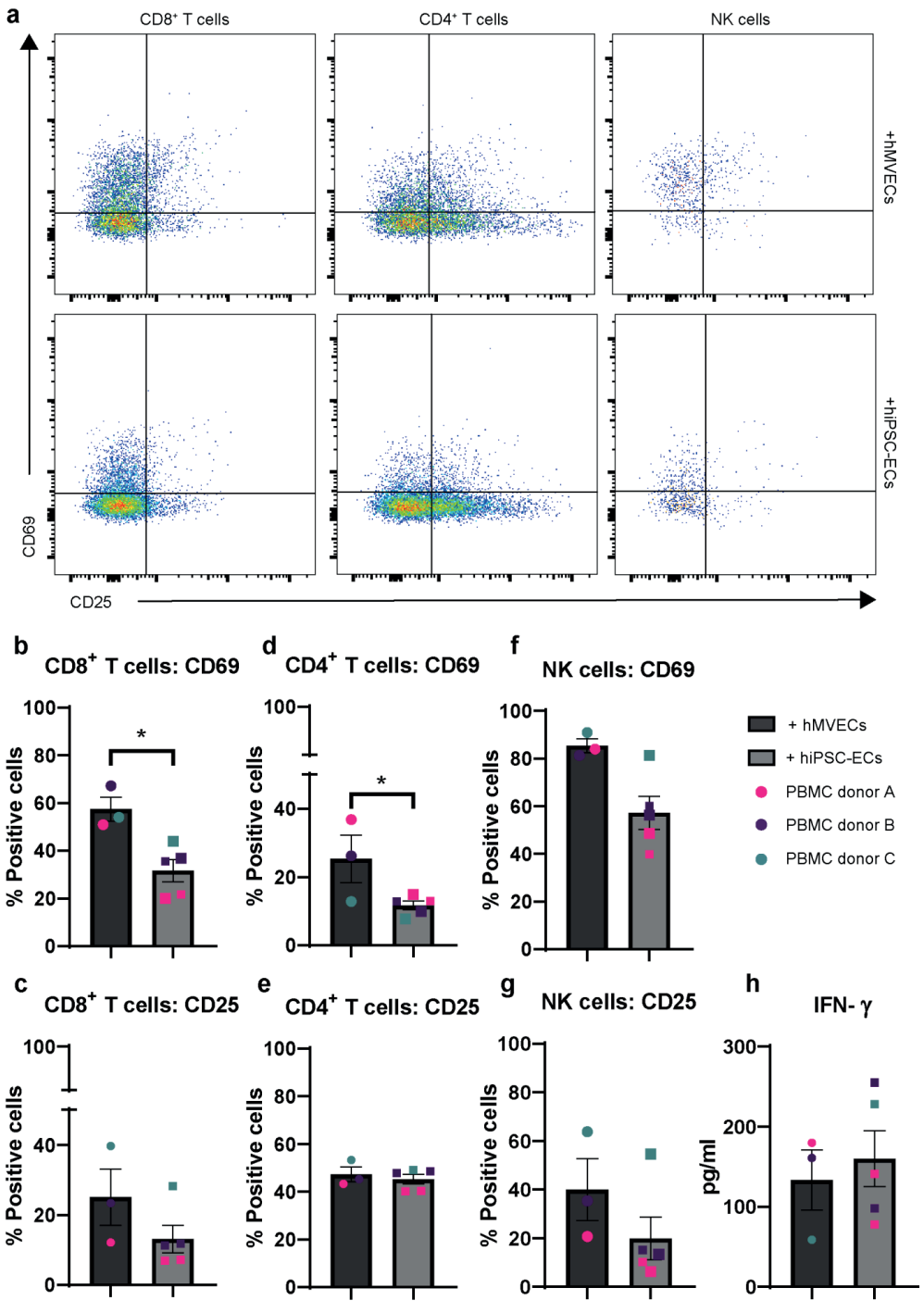


Figure 2: In allogeneic PBMC co-culture assays, hiPSC-EC stimulation resulted in lower activation of T cells and NK cells compared to hMVECs stimulation. Activation of Human PBMCs were assessed by flow cytometry after co-culture with either hMVECs or hiPSC-ECs for binding of anti-CD69/FITC and anti-CD25/PE antibody. Target cells were cultured to confluence in 96-wells and stimulated with IFN-γ and IL-1β for 24h before adding PBMCs at a concentration of 400.000 cells/well. Cells and supernatants were harvested after 48 hours of co-culture. (a) Representative flow cytometry data is presented in dot plots. (b-g) Results were quantified as percentages of positive cells, using the 99th percentile of the isotype control as the cut-off gate (n=3). 3 PBMC donors and 3 hiPSC-EC lines were tested. PBMCs of donor A and B were each co-cultured with 2 different hiPSC-EC lines. (h) IFN-γ concentrations in supernatant were measured using ELISA. * p≤0.05.

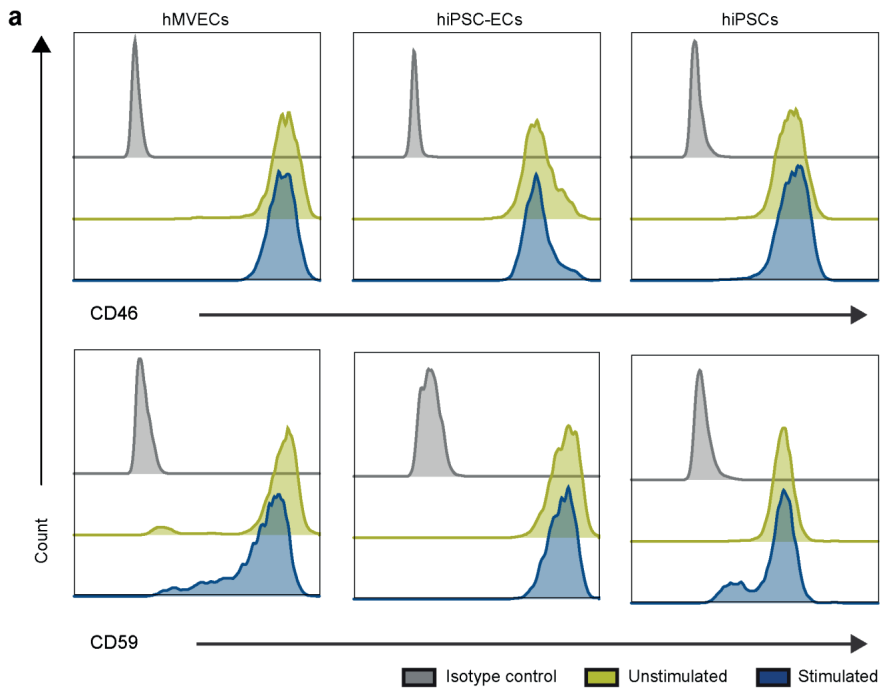
Overall, hiPSC-ECs induced a lower PBMC activation than primary hECs. In CD8$^+$ T cells, both CD69 and CD25 expression levels were higher for PBMCs co-cultured with hMVECs, though only CD69 resulted in significant differences (57.4±5.0% in co-culture with hMVECs versus 31.6±4.6% with hiPCS-ECs, p=0.0439) (Figure 2b,c). This trend was confirmed by the CD25 expression on HLA-A2 reactive CD8$^+$ T cell clone co-cultures (Supplementary Methods, Supplementary Figure 7). In CD4$^+$ T cells, a similar trend in CD69 expression was seen (Figure 2d). However, CD25 expression in CD4$^+$ T cells was very comparable between hMVEC and hiPSC-EC stimulation (Figure 2e). Although no significant difference was observed in NK cell activation, the expression of both CD69 and CD25 was lower in hiPSC-EC stimulated cells (Figure 2f,g).

No differences were found in IFN-γ concentration between supernatant from hMVEC and hiPSC-EC PBMC co-cultures (Figure 2h). Remarkably, PBMC co-cultures with undifferentiated hiPSCs resulted also in high levels of IFN-γ production, comparable to the positive control of PHA stimulated PBMCs (Supplementary Figure 6-1e).

hiPSC-ECs express similar levels of complement inhibitors compared to primary hECs

Besides T and NK cell activation, complement binding donor-specific HLA antibodies (DSAs) play an important role in graft rejection rates. To investigate the potency of hiPSC-ECs to inhibit the complement system, hiPSC-EC expression of complement inhibitors CD46 and CD59 was measured and compared to hMVECs and undifferentiated hiPSCs (Figure 3). HUVECs were also included in this assay, but no significant differences were found between HUVECs and hMVECs (Supplementary Figure 4).

Although compared to hMVECs, the expression of both CD46 and CD59 was higher in hiPSC-ECs in unstimulated and in stimulated conditions, no significant differences



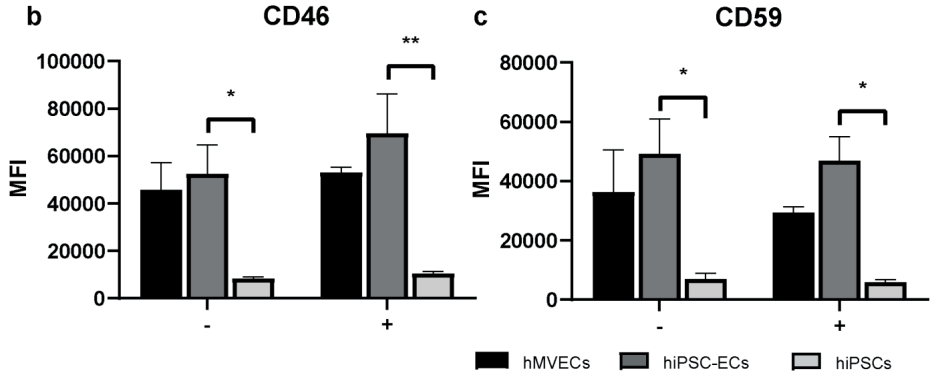


Figure 3: hiPSC-ECs express similar levels of complement inhibitors as hMVECs. Single stains with anti-CD46/PE and anti-CD59/FITC antibody were performed on hMVECs, hiPSC-ECs and hiPSCs and analyzed by flow cytometry. Cells were either cultured under normal conditions (unstimulated (-)) or stimulated with IFN-γ and IL-1β for 48h (stimulated (+)). (a) Representative data is demonstrated in histograms. (b,c) Presented MFIs are corrected for background signal by subtraction of the isotype control MFI (n=3). * p≤0.05.

were found (Figure 3b,c). Cytokines stimulation had little effect on all cell types. Compared with unstimulated cells, expression of CD46 in stimulated cells was slightly - but not significantly – upregulated (Figure 3b). CD59 on the other hand was downregulated in a small fraction of cells after cytokine stimulation (Figure 3a,c). Expression of both complement inhibitors was significantly higher in hiPSC-ECs compared to undifferentiated hiPSCs (Figure 3b,c).

Discussion

he future clinical use of allogenic hiPSC-EC is greatly dependent on their immunogenicity. This is the first study characterizing hiPSC-ECs immunogenicity in an allogeneic context. We found significant differences in HLA expression and PBMC activation compared to primary hECs whereas complement inhibitor expressions were comparable.

HLA-A,B,C expression in hiPSC-ECs was similar to primary ECs in cytokine free conditions. However, in contrast to earlier studies on other hiPSC derived cell types[32-34], HLA-A,B,C expression was higher in a small subset of hiPSC-ECs than in their primary counterpart after cytokine stimulation. This is an critical finding in context of allogeneic transplantation.

Higher HLA-A,B,C expression on hiPSC-ECs in an inflammatory environment could potentially have a negative effect on graft rejection rates through several mechanisms. Besides $CD8^+$ T cell activation, it has been proposed that HLA-A,B,C plays an important role in T cell recruitment by endothelial cells [40, 41]. Whether differences in T cell activation and recruitment are present between hiPSC-ECs and primary hECs is not only dependent on HLA-A,B,C, but also on a wide range of co-stimulatory factors, leukocyte adhesion molecules and chemoattractants. Given the known general immaturity of hiPSC-ECs, one could hypothesize that the expression of these proteins typically expressed by hECs is lower in hiPSC-ECs. Previous work by Rosa *et al.* has demonstrated this for VCAM-1 [13].

Moreover, higher HLA-A,B,C expression could make hiPSC-ECs more vulnerable to DSA deposition, potentially leading to higher rates of antibody mediated rejection (AMR), a major cause of allograft failure[42-44]. Next to complement injury, deposition of anti-HLA antibody has been shown to induce pro-inflammatory cytokine production and cell surface expression of P-selectin in hMVECs[45, 46]. To conclude whether hiPSC-ECs are indeed more susceptible to DSA deposition needs further research.

In contrast to HLA-A,B,C, HLA-DR was not detectable in all tested cell lines under neutral conditions. After stimulation with IFN-γ and IL-1β, both hiPSC-ECs and primary hECs partially expressed HLA-DR but the proportion of HLA-DR positive cells was much lower in hiPSC-ECs than primary hECs. This finding seemed not to be related to differentiation or dedifferentiation, since all hiPSC-ECs continued to express VEGF2. Therefore, we suggest that not all hiPSC-ECs have fully matured in HLA-DR expression.

Allogenic $CD4^+$ T cell activation might be reduced in hiPSC-ECs compared to primary hECs because of the lower HLA class II expression. Whether this will have a significant impact on rejection rates is questionable since activated ECs are only one of several APC types.

Still, it is uncertain whether the direct comparison between cultured hMVECs and hiPSC-ECs is completely valid. For hMVECs, cytokine stimulated conditions are most representative of an *in vivo* situation since HLA class I and II expression is downregulated in culture but restored after IFN-γ stimulation[47]. However, for hiPSC-ECs, it is unknown which *in vitro* conditions are most representative of an *in vivo* situation. The allogeneic PBMC co-cultures suggested that hiPSC-ECs were less potent to activate $CD4^+$ and $CD8^+$ T cells than primary hEC. The observed lower $CD4^+$ T cell activation was in line with our expectations based on HLA-DR expression. For $CD8^+$ T cells on the other hand, results were quite surprising, since $CD8^+$ T cells activation is mediated through HLA-A,B,C, which was expressed more strongly in hiPSC-ECs. This data implies that hiPSC-ECs differentially express co-stimulatory or co-inhibitory molecules involved in T cell activation. Since undifferentiated hiPSCs lack the expression of several co-stimulatory factors, even after cytokine stimulation[19], it is not unlikely that hiPSC-ECs express only intermediate levels of these proteins. However, to our knowledge, no studies investigating expression of these factors in hiPSC-ECs have been published yet.

NK cell activation was also lower in co-culture with hiPSC-ECs. Since NK cells are regulated by a balance of many inhibitory and activating signals, it is hard to pinpoint which underlying mechanisms play a role in the observed differences between hiPSC-ECs and hMVECs. A possible explanation could be the higher HLA-A,B,C expression in hiPSC-ECs, since HLA-A,B,C functions as inhibitory NK cell receptor ligand. However, inhibitory Killer cell Ig-like Receptors (KIRs), are highly polymorphic and can differentiate between several HLA allotypes [48, 49]. For instance, KIRs can distinguish two general groups of HLA-C, the most prominent inhibitory ligand, whereas only "self" HLA-C will provide an inhibitory signal. Since the HLA haplotypes of the PBMC donors are unknown, it cannot be assumed that differential expression of HLA-A,B,C was the cause of the differences in NK cell activation. The relatively low presence of heparan sulfate (HS glycosaminogycans (GAGs) is protective for NK cell activation by hiPSC-EC. hiPSC-EC. HS GAGs are ligands to several activating NK receptors[50, 51], and it has been shown that the glycocalyx of hiPSC-ECs contains less heparin sulfate compared to that of hMVECs[17].

In contrast to the flow cytometry measurements, observed IFN-γ concentrations did not differ between hiPSC-ECs and hEC stimulation. Compared to the positive control, measured concentrations in the co-cultures with hiPSC-ECs and hEC were very low. PBMCs consist of more than just T and NK cells and general IFN-γ concentrations therefore provide limited information on which cell types contribute to IFN-γ production.

Although the PBMC co-culture assay gives a general impression of hiPSC-ECs potency to activate allogeneic PBMCs, it should be noted that the assay knows many technical limitations, such as the relatively high baseline activation in PBMC-only cultures. Moreover, large donor-variations were observed, which calls for more repetitions. Furthermore, PBMCs are highly sensitive for the amount of target cells present, which might have slightly deviated between cell lines. Since hiPSC-EC and primary hECs were seeded in the same densities days before PBMC addition and in general, we observed a slower growth in hiPSC-ECs compared to primary hECs, it is likely that cell counts differed between cell types.

As mentioned earlier, complement is another key player in graft rejection. CD46 and CD59 inhibit complement activation at different points in the cascade [52-54] and protect endothelial cells from complement-mediated injury[55]. Our work demonstrated that hiPSC-ECs express similar levels of complement inhibitors CD46 and CD59 compared to hMVECc, which is favorable since complement inhibitors could protect cells from DSA related complement damage. This hypothesis is supported by a study by Michielsen et al. that found associations between promoter polymorphisms of CD46 and CD59 and donor kidney survival [56].

Conclusions and futher directions

The most important finding in our study is the differential expression of HLA class I and class II in hiPSC-ECs compared to primary hECs. How these differences translate to allogeneic rejection responses needs further research. It is advisable to conduct T cell proliferation assays, in which PBMCs are stained with CSFE and cultured in the

presence of hiPCS-ECs or primary hECs for 5-7 days. The longer time co-culture will more likely establish results that are significantly different from the negative control. Characterization of co-stimulatory and inhibitory factors might explain the observed low $CD8^+$ T cell activation by hiPSC-ECs. Also functional assays looking into donor specific antibody deposition and complement activation, similar as experiments described by Pober *et al*. (2019)[57], would provide more insight. Pro-inflammatory cytokine secretion would also be an interesting topic of research. Since hiPSC-EC lack functional Weibel-Palade bodies[8], secretory vesicles which normally store IL-8[58], IL-8 secretion is likely reduced. Lower secretion of pro-inflammatory signaling molecules would protect hiPSC-ECs from inflammation and hereby rejection.

Since allograft rejection is highly complex and hard to fully model *in vitro,* ultimately, *in vivo* studies are needed. To this end, vascular organoids could be transplanted into a humanized mouse models, using the method described by Wimmer et al (2019)[59]. Lymphocyte infiltration, antibody formation and deposition, lymphocyte phenotyping and tissue damage in organoids would provide a more comprehensive overview of which rejection mechanisms might be altered in hiPSC-ECs in comparison with primary hECs. Since immune-mediated endothelial damage could lead to graft loss in the long term, a reduction in endothelial immunogenicity would be favorable. However, endothelial functions like barrier formation and antithrombotic regulation are indispensable when engineering microvasculature.

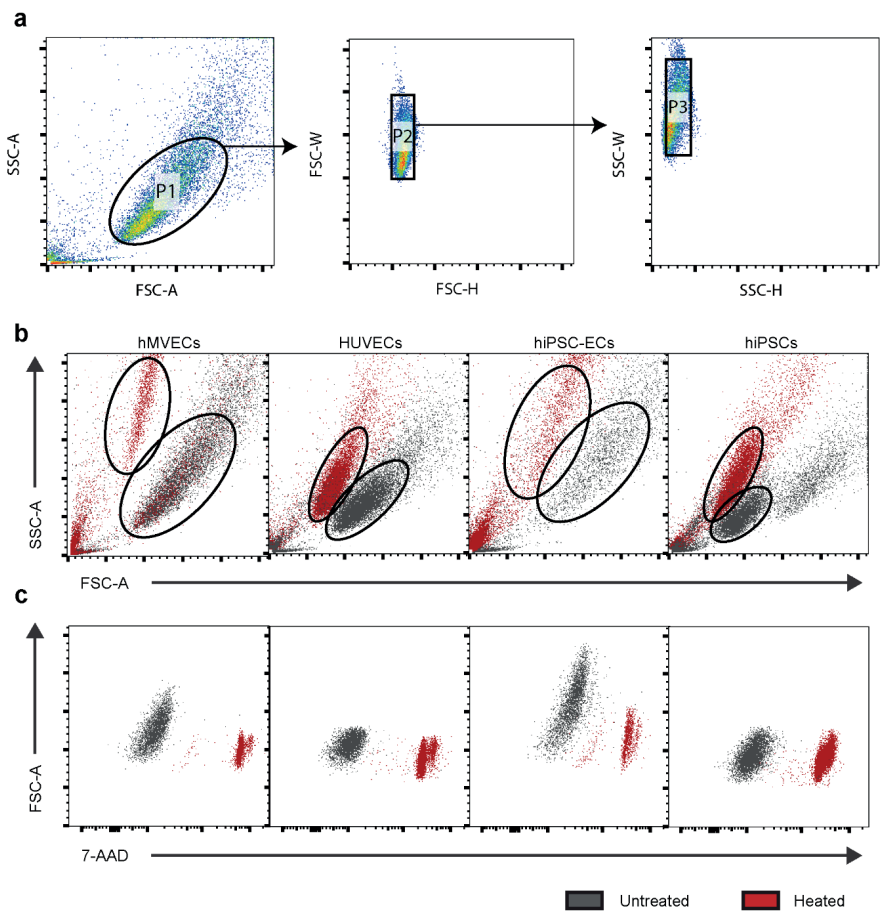
References

1. System, U.S.R.D., *2018 USRDS annual data report: Epidemiology of kidney disease in the United States*. 2018, United States Renal Data System, National Institutes of Health: Bethesda, MD.
2. Haase-Kromwijk, B., et al., *Jaarverslag 2018 Nederlandse Transplantatiestichting*. 2019, NTS.
3. Valderrábano, F., R. Jofre, and J.M. López-Gómez, *Quality of life in end-stage renal disease patients.* American Journal of Kidney Diseases, 2001. **38**(3): p. 443-464.
4. Fukuhara, S., et al., *Health-related quality of life among dialysis patients on three continents: The Dialysis Outcomes and Practice Patterns Study.* Kidney International, 2003. **64**(5): p. 1903-1910.
5. Min, S., I.K. Ko, and J.J. Yoo, *State-of-the-Art Strategies for the Vascularization of Three-Dimensional Engineered Organs.* Vascular specialist international, 2019. **35**(2): p. 77-89.
6. Jain, R.K., et al., *Engineering vascularized tissue.* Nature Biotechnology, 2005. **23**(7): p. 821-823.
7. Bouïs, D., et al., *Endothelium in vitro: A review of human vascular endothelial cell lines for blood vessel-related research.* Angiogenesis, 2001. **4**: p. 91-102.
8. Martin-Ramirez, J., et al., *Establishment of outgrowth endothelial cells from peripheral blood.* Nature Protocols, 2012. **7**(9): p. 1709-1715.
9. Medina, R.J., et al., *Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities.* BMC Med Genomics, 2010. **3**(18).
10. de Jong, A., et al., *Variability of von Willebrand factor-related parameters in endothelial colony forming cells.* J Thromb Haemost, 2019. **17**(9): p. 1544-1554.
11. Paschalaki, K.E. and A.M. Randi, *Recent Advances in Endothelial Colony Forming Cells Toward Their Use in Clinical Translation.* Frontiers in medicine, 2018. **5**: p. 295-295.
12. Orlova, V.V., et al., *Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts.* Arterioscler Thromb Vasc Biol, 2014. **34**(1): p. 177-86.
13. Halaidych, O.V., et al., *Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells.* Stem Cell Reports, 2018. **10**(5): p. 1642-1656.
14. Samuel, R., et al., *Generation of functionally competent and durable engineered blood vessels from human induced pluripotent stem cells.* PNAS, 2013. **110**(31): p. 12774-12779.
15. Bezenah, J.R., et al., *Assessing the ability of human endothelial cells derived from induced-pluripotent stem cells to form functional microvasculature in vivo.* Biotechnology and bioengineering, 2019. **116**(2): p. 415-426.
16. Clayton, Z.E., et al., *Induced pluripotent stem cell-derived endothelial cells promote angiogenesis and accelerate wound closure in a murine excisional wound healing model.* Bioscience reports, 2018. **38**(4): p. BSR20180563.
17. Tiemeier, G.L., et al., *Closing the Mitochondrial Permeability Transition Pore in hiPSC-Derived Endothelial Cells Induces Glycocalyx Formation and Functional Maturation.* Stem Cell Reports, 2019. **13**(5): p. 803-816.
18. Rosa, S., et al., *Functional characterization of iPSC-derived arterial- and venous-like endothelial cells.* Scientific Reports, 2019. **9**(1).
19. Lu, Q., et al., *Negligible Immunogenicity of Induced Pluripotent Stem Cells Derived from Human Skin Fibroblasts.* PLOS ONE, 2014. **9**(12): p. e114949.
20. Zhao, T., et al., *Immunogenicity of induced pluripotent stem cells.* Nature, 2011. **474**(7350): p. 212-215.
21. Zhao, T., et al., *Humanized Mice Reveal Differential Immunogenicity of Cells Derived from Autologous Induced Pluripotent Stem Cells.* Cell Stem Cell, 2015. **17**(3): p. 353-359.
22. de Almeida, P.E., et al., *Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance.* Nature Communications, 2014. **5**.
23. Guha, P., et al., *Lack of Immune Response to Differentiated Cells Derived from Syngeneic Induced Pluripotent Stem Cells.* Cell Stem Cell, 2013. **12**(4): p. 407-412.

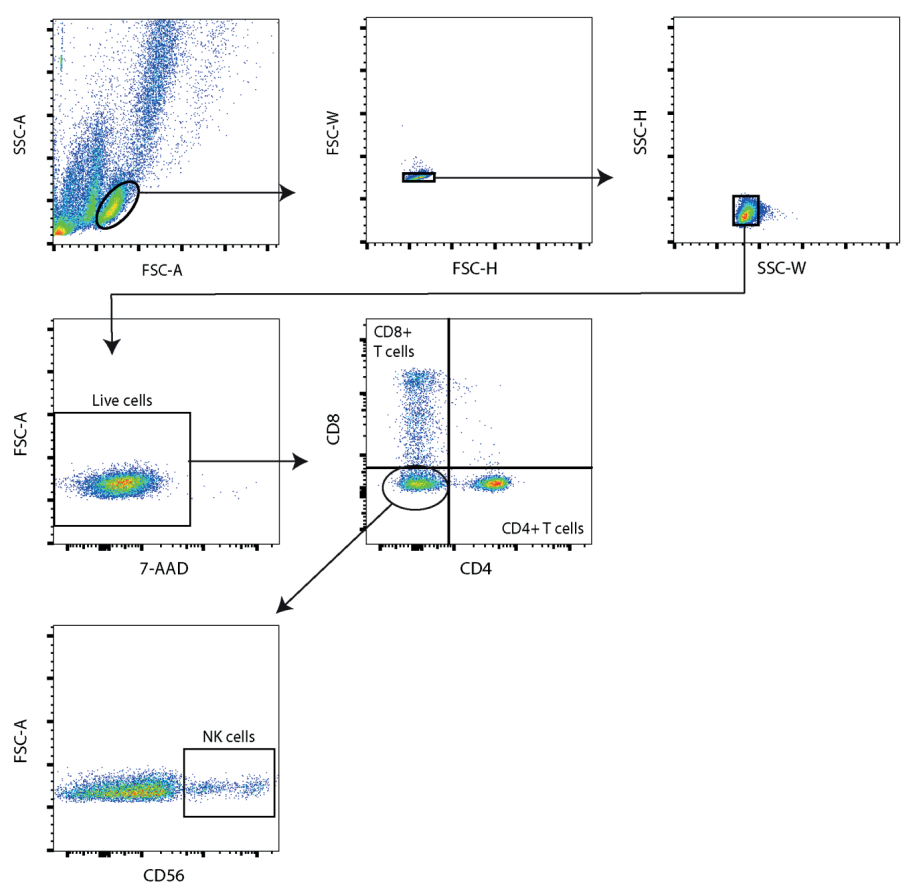
24. Nankivell, B.J. and S.I. Alexander, *Rejection of the Kidney Allograft.* New England Journal of Medicine, 2010. **363**(15): p. 1451-1462.
25. Koenig, A., et al., *Missing self triggers NK cell-mediated chronic vascular rejection of solid organ transplants.* Nature Communications, 2019. **10**(1): p. 5350.
26. Al-Lamki, R.S., J.R. Bradley, and J.S. Pober, *Endothelial cells in allograft rejection.* Transplantation, 2008. **86**(10): p. 1340-1348.
27. Shiao, S.L., et al., *Human effector memory CD4+ T cells directly recognize allogeneic endothelial cells in vitro and in vivo.* J Immunol, 2007. **179**(7): p. 4397-4404.
28. Taflin, C., et al., *Human endothelial cells generate Th17 and regulatory T cells under inflammatory conditions.* PNAS, 2011. **108**: p. 2891-6.
29. Epperson, D.E. and J.S. Pober, *Antigen-presenting function of human endothelial cells. Direct activation of resting CD8 T cells.* The Journal of Immunology, 1994. **153**(12): p. 5402-5412.
30. Seino, K., et al., *CD86 (B70/B7–2) on endothelial cells co-stimulates allogeneic CD4+T cells.* International Immunology, 1995. **7**(8): p. 1331-1337.
31. Hindmarsh, E.J. and R.M. Marks, *Complement Activation Occurs on Subendothelial Extracellular Matrix In Vitro and Is Initiated by Retraction or Removal of Overlying Endothelial Cells.* The Journal of Immunology, 1998. **160**(12): p. 6128-6136.
32. Kimura, T., et al., *Limited Immunogenicity of Human Induced Pluripotent Stem Cell-Derived Cartilages.* Tissue engineering. Part A, 2016. **22**(23-24): p. 1367-1375.
33. Ozaki, M., et al., *Evaluation of the immunogenicity of human iPS cell-derived neural stem/progenitor cells in vitro.* Stem Cell Research, 2017. **19**: p. 128-138.
34. Kim, E.-M., G. Manzar, and N. Zavazava, *Human iPS cell–derived hematopoietic progenitor cells induce T-cell anergy in in vitro–generated alloreactive CD8+ T cells.* Blood, 2013. **121**(26): p. 5167-5175.
35. Peichev, M., et al., *Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors.* Blood, 2000. **95**(3): p. 952-958.
36. Ziegler, S.F., F. Ramsdell, and M.R. Alderson, *The activation antigen CD69.* STEM CELLS, 1994. **12**(5): p. 456-465.
37. Simms, P.E. and T.M. Ellis, *Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation.* Clinical and diagnostic laboratory immunology, 1996. **3**(3): p. 301-304.
38. Reddy, M., et al., *Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function.* Journal of Immunological Methods, 2004. **293**(1): p. 127-142.
39. Clausen, J., et al., *Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells.* Immunobiology, 2003. **207**(2): p. 85-93.
40. Marelli-Berg, F.M. and S.J. Jarmin, *Antigen presentation by the endothelium: a green light for antigen-specific T cell trafficking?* Immunology Letters, 2004. **93**(2): p. 109-113.
41. Lozanoska-Ochser, B. and M. Peakman, *Level of major histocompatibility complex class I expression on endothelium in non-obese diabetic mice influences CD8 T cell adhesion and migration.* Clinical and experimental immunology, 2009. **157**(1): p. 119-127.
42. Loupy, A., et al., *Subclinical Rejection Phenotypes at 1 Year Post-Transplant and Outcome of Kidney Allografts.* Journal of the American Society of Nephrology, 2015. **26**(7): p. 1721.
43. Lefaucheur, C., et al., *Antibody-mediated vascular rejection of kidney allografts: a population-based study.* The Lancet, 2013. **381**(9863): p. 313-319.
44. Valenzuela, N.M. and E.F. Reed, *Antibodies in transplantation: the effects of HLA and non-HLA antibody binding and mechanisms of injury.* Methods in molecular biology (Clifton, N.J.), 2013. **1034**: p. 41-70.
45. Valenzuela, N.M., et al., *Blockade of p-selectin is sufficient to reduce MHC I antibody-elicited monocyte recruitment in vitro and in vivo.* American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 2013. **13**(2): p. 299-311.

46. Naemi, F.M.A., et al., *Anti-Donor HLA Class I Antibodies: Pathways to Endothelial Cell Activation and Cell-Mediated Allograft Rejection.* Transplantation, 2013. **96**(3).
47. Cines, D.B., et al., *Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders.* Blood, 1998. **91**(10): p. 3527-3561.
48. Ichise, H., et al., *NK Cell Alloreactivity against KIR-Ligand-Mismatched HLA-Haploidentical Tissue Derived from HLA Haplotype-Homozygous iPSCs.* Stem cell reports, 2017. **9**(3): p. 853-867.
49. Benjamin, J.E., S. Gill, and R.S. Negrin, *Biology and clinical effects of natural killer cells in allogeneic transplantation.* Current opinion in oncology, 2010. **22**(2): p. 130-137.
50. Barrow, A.D., C.J. Martin, and M. Colonna, *The Natural Cytotoxicity Receptors in Health and Disease.* Frontiers in Immunology, 2019. **10**: p. 909.
51. Tremblay-McLean, A., et al., *Expression of ligands for activating natural killer cell receptors on cell lines commonly used to assess natural killer cell function.* BMC Immunology, 2019. **20**(1): p. 8.
52. Dho, S.H., J.C. Lim, and L.K. Kim, *Beyond the Role of CD55 as a Complement Component.* Immune network, 2018. **18**(1): p. e11-e11.
53. Lachmann, P.J., *The control of homologous lysis.* Immunology Today, 1991. **12**(9): p. 312-315.
54. Liszewski, M.K. and J.P. Atkinson, *Complement regulator CD46: genetic variants and disease associations.* Human Genomics, 2015. **9**(1).
55. Sartain, S.E., N.A. Turner, and J.L. Moake, *TNF Regulates Essential Alternative Complement Pathway Components and Impairs Activation of Protein C in Human Glomerular Endothelial Cells.* The Journal of Immunology, 2016. **196**(2): p. 832.
56. Michielsen, L.A., et al., *Association Between Promoter Polymorphisms in CD46 and CD59 in Kidney Donors and Transplant Outcome.* Frontiers in Immunology, 2018. **9**: p. 972.
57. Merola, J., et al., *Progenitor-derived human endothelial cells evade alloimmunity by CRISPR/Cas9-mediated complete ablation of MHC expression.* JCI Insight, 2019. **4**(20).
58. Romani de Wit, T., et al., *Von Willebrand factor targets IL-8 to Weibel–Palade bodies in an endothelial cell line.* Experimental Cell Research, 2003. **286**(1): p. 67-74.
59. Wimmer, R.A., et al., *Human blood vessel organoids as a model of diabetic vasculopathy.* Nature, 2019. **565**(7740): p. 505-510.
60. Yoshioka, N., et al., *Efficient Generation of Human iPSCs by a Synthetic Self-Replicative RNA.* Cell Stem Cell, 2013. **13**(2): p. 246-254.
61. Chen, G., et al., *Chemically defined conditions for human iPSC derivation and culture.* Nature Methods, 2011. **8**(5): p. 424-429.
62. Orlova, V.V., et al., *Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells.* 2014. **9**(6): p. 1514-1531.

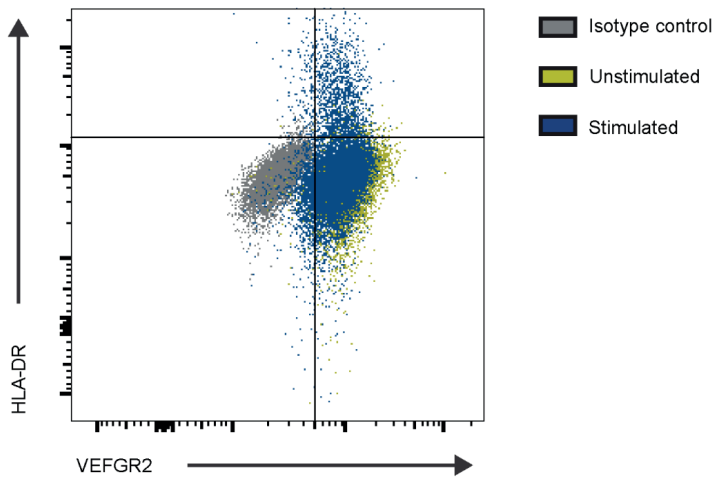
Supplementary Figures



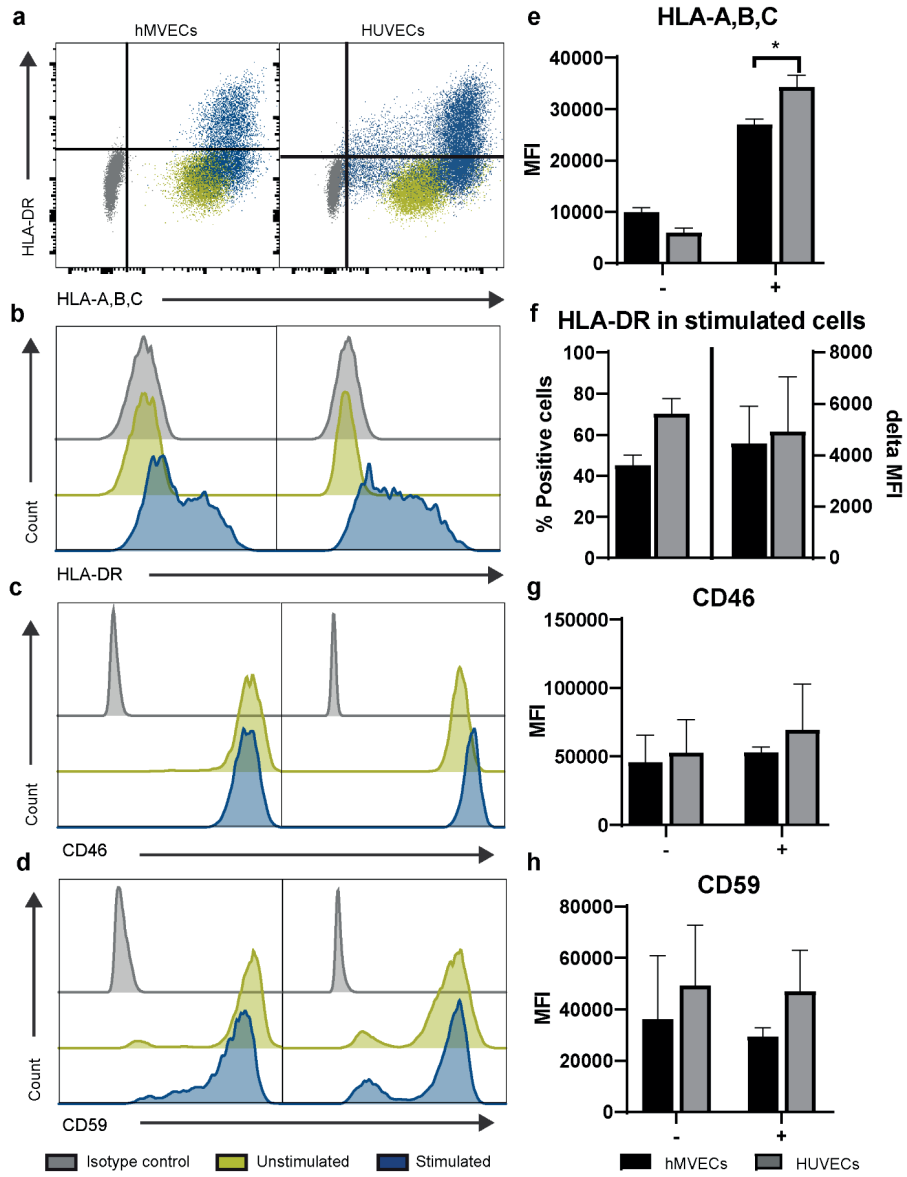
Supplementary Figure 1: Gating of hECs, hiPSC-ECs and hiPSCs. (a) Single cells were selected based on three hierarchical gates using forward and side scatter. To check whether dead cells played a role in the performed experiments, single stains with viability dye 7-AAD were performed on cells that underwent the same procedure as the ones used for quantification (untreated) and on cells that were kept on 60°C for 30 minutes prior 7-AAD staining. (b) Heated cells clearly shifted in the forward and side scatter plots. (c) Gated untreated cells showed no 7-AAD staining, indicating that the single cells selection did not include dead cells.



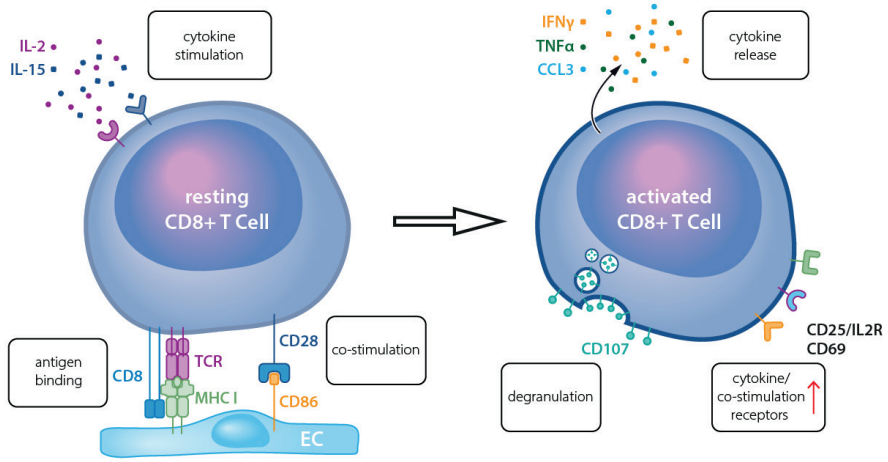
Supplementary Figure 2: PBMC gating strategy. Single cells were first selected using three hierarchical gates based on forward and side scatter properties. Then, dead cells were excluded based on 7-AAD staining. The cut-off gate was based on a positive control of cells that were kept at 60°C for 30 minute prior to staining. Then, from the live cells, $CD4^+$ and $CD8^+$ T cells were selected. From the $CD4^-/CD8^-$ population, NK cells were selected based on CD56 staining.



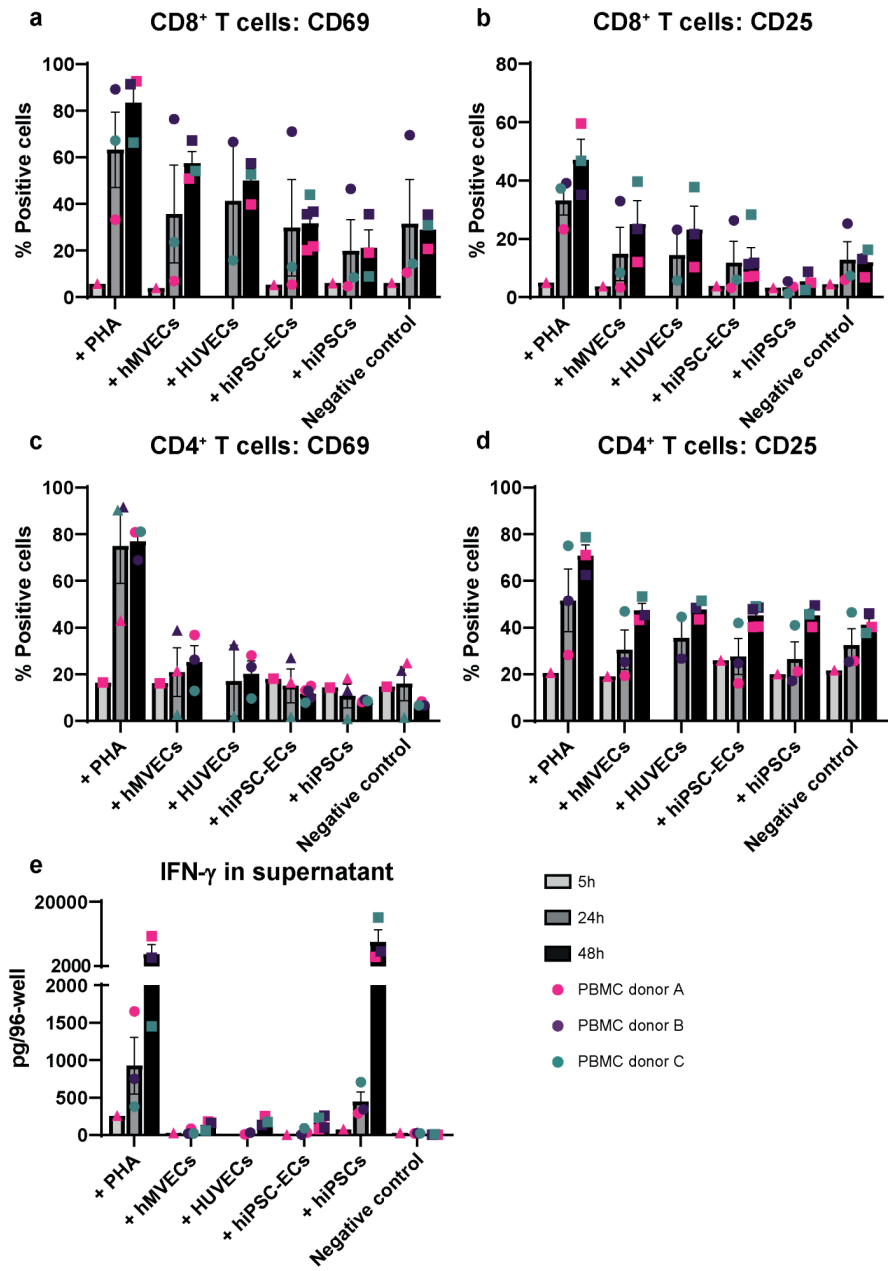
Supplementary Figure 3: The subpopulation of hiPSC-ECs expressing HLA-DR after cytokine stimulation shows similar expression in VEGFR2 compared to the rest of the population. To check whether the HLA-DR expressing subpopulation in hiPSC-ECs was different in terms of endothelial differentiation, VEGFR2 expression was measured. Corresponding data is demonstrated in a dot plot. Cells were either cultured under normal conditions (unstimulated) or stimulated with IFN-γ and IL-1β for 48h (stimulated).



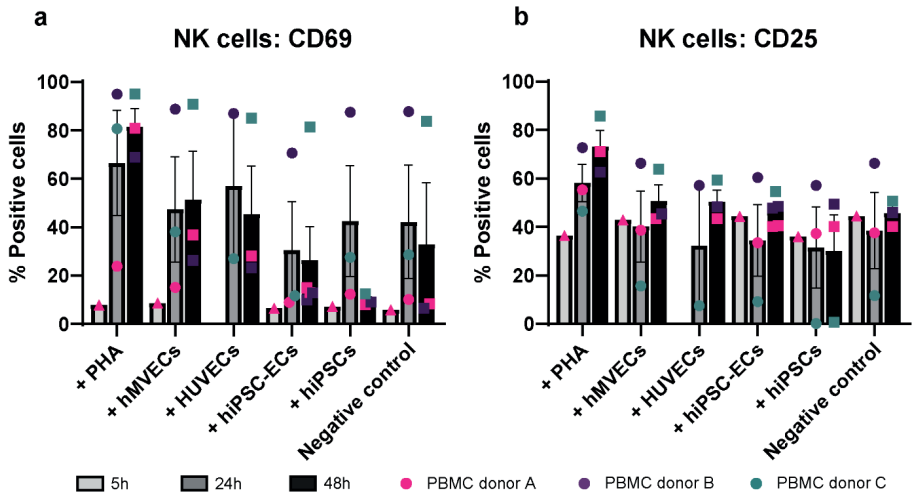
Supplementary Figure 4: Comparison between primary ECs hMVECs and HUVECs. HUVECs were also included in the measurements as a second primary EC type. (a-d) Representative data of the quantified surface markers on ECs is shown in histograms. (e-h) Corresponding quantifications as described earlier are presented (n=3). * $p \leq 0.05$.



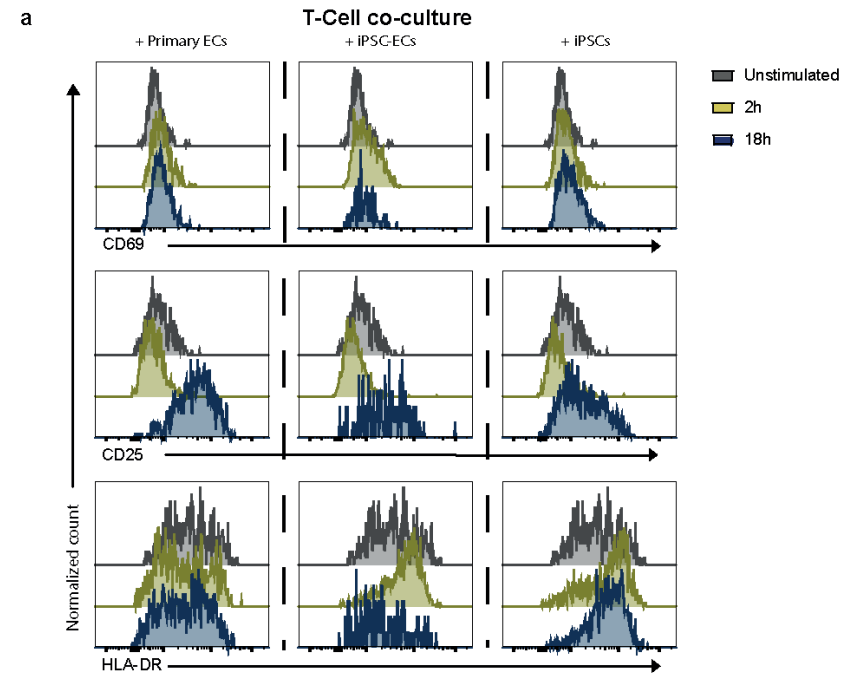
Supplementary Figure 5: Schematic overview of CD8⁺ T cell activation. For T cell activation, binding of the T cell receptor (TCR) to its target presented by MHC proteins is necessary. CD8⁺ T effector memory cells can directly be activated by antigen binding alone. Activation can be enhanced by several cytokines and by co-stimulation factors expressed on the target cell, for instance by binding of CD86 on the target cell to CD28 on the T cell. For naive T cells, co-stimulation through the CD28 receptor is necessary for activation. Once activated, perforin containing granules are released and CD107a is transiently expressed on the cell membrane. Activated T cells produce multiple cytokines and chemokines, including IFN-γ, TNF-α and CCL3. Also, several cytokine and co-simulation receptors are upregulated in activated CD8⁺ T cells. (*Original figure, made in cooperation with Manon Zuurmond)*

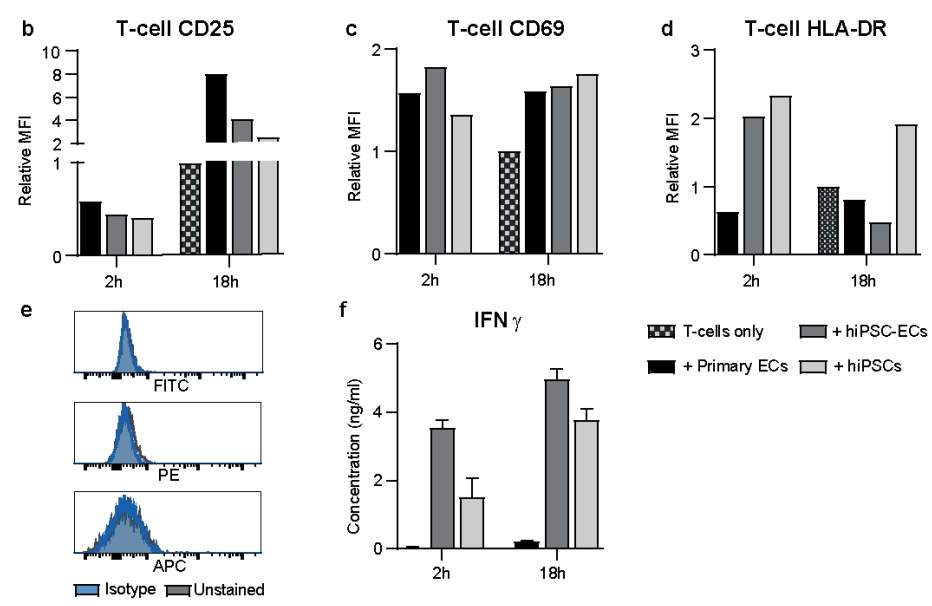


Supplementary Figure 6-1: Complete data from PBMC co-cultures: T cells and IFN-γ. The PBMC co-cultures were optimized using different time points. Here, all measurements of T cells and IFN-γ are presented after 5h (n=1), 24h (n=3) and 48h (n=3) of co-culture. Also, the data from the positive control (PBMCs + 5 µg/mL PHA) and the negative control (PBMCs only) are included.



Supplementary figure 6-2: Complete data from PBMC co-cultures: NK cells. The PBMC co-cultures were optimized using different time points. Here, all measurements of NK cells are presented after 5h (n=1), 24h (n=3) and 48h (n=3) of co-culture. Also, the data from the positive control (PBMCs + 5 µg/mL PHA) and the negative control (PBMCs only) are included.





Supplementary Figure 7: Anti-HLA-A2 CD8⁺ T cell clone co-culture. (a) Human CD8⁺ T-cell clones targeting HLA-A2 were assessed by flow cytometry after co-culture with either HUVECs, hiPSC-ECs or hiPSCs for binding of anti-CD69/FITC, anti-CD25/PE and anti-HLA-DR/APC antibody. Target cells were cultured to confluence and stimulated with IFN-γ and IL-1β for 48 hours before adding T-cells at a concentration of 2 million cells/ml. Cells were harvested after 2 or 18 hours of co-culture. The measurements are presented in histograms. (b-d) Median fluorescent intensity (MFI) was corrected for background signal by subtracting the MFI of the isotype control (n=1). Corrected MFIs were then normalized to the biological negative control of T-cells without target cell stimulation. The measured normalized expression levels of the T-cell activation markers (b) CD69, (c) CD25 and (d) HLA-DR are presented. (e) Isotype controls showed no significant non-specific antibody binding. (c) At the moment of harvesting, supernatant was also collected. The concentration of IFN-γ was measured by ELISA (average±SEM based on biological triplicates). The full experiment was performed once.

Supplementary Methods

Alloreactive CD8⁺ T cell clone co-culture

Human peripheral blood mononuclear cells (PBMCs) were separated from whole blood of a healthy individual by Ficoll-Hypaque density gradient. To generate alloreactive CD8⁺ T cell clones to HLA-A2 the PBMCs were stimulated with irradiated Epstein-Barr virus (EBV)-transformed B cell line expressing HLA-A2.

Primary hECs (HUVECs) and hiPSC-ECs (LUMC0072) were seeded on a gelatin coated 96-wells plate at a density of 20.000 cells/well (HUVECs, hiPSC-ECs) in full EC-SFM and hiPSCs (LUMC0072) at a density of 10.000 cells/well in E8 medium, all supplemented with 1000 UI/ml rhIFN-γ (R&D systems) and 2 ng/ml rhIL-1β (Sigma-Aldrich) to stimulate HLA expression for 48 hours. Before adding the T cells, cultures were thoroughly washed to remove all rhIFN-γ.

T cell clones were thawed and resuspended in 2x full T cell medium, consisting of Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Thermo Fisher) supplemented with 8% FBS , 2mmol/mL glutamine, 50 CU/mL rhIL-2 and 10 ng/mL rhIL-15 (full T cell medium) at a concentration of 2 million cells/mL. Then, 50µL of EC-SFM (hMEVCs, iPSC-ECs) or E8 (iPSCs) without cytokines and 50µL of T cell suspension was added to each well. Next to that, a biological negative control was included of T-cells only. For this, the T cells were cultured on a 96 well-plate in 1x full T cell medium at a concentration of 25.000 cells/ml for 18 hours. All conditions were executed in triplicate.

2- or 18 Hours after starting the co-culture, supernatant and cells were harvested. IFN-γ concentration in supernatant was measured as described previously. Cells were detached with TrypLE and resuspended in FACS buffer. Triplicates were pooled and samples were prepared for flow cytometry analysis as described previously.

Cells were stained with CD8/PE-Cy7 (clone SK1, 1:800) (BD Biosciences), mouse anti-hCD69/FITC (clone L78, 1:20) (BD Biosciences), mouse anti-hCD25/PE (clone M-A251, 1:20 dilution) (BD Biosciences) and mouse anti-hHLA-DR/APC (clone G46-6, 1:100) (BD Bioscience) Isotype controls were included using PE mouse IgG2a, κ (clone G155-178, 1:5) (BD Biosciences) and APC mouse IgG2a, κ (clone G155-178, 1:5) (BD Biosciences). Thereafter, cells were resuspended in 7-AAD supplemented (1:50) (BD Biosciences) FACS buffer.

T cells were selected based on CD8⁺ expression. Then, the expression of the activation markers CD69, CD25 and HLA-DR was quantified as median fluorescence intensity (MFI) minus the MFI of the isotype control. MFIs were subsequently normalized for the biological negative control of T cells only.