Published in final edited form as:

Transpl Immunol. 2020 February ; 58: 101261. doi:10.1016/j.trim.2019.101261.

An *in vitro* model of antibody-mediated injury to glomerular endothelial cells: upregulation of MHC class II and adhesion molecules

Nancy A. Wilson^a, James Dylewski^b, Kenna R. Degner^c, Megan A. O'Neill^c, Shannon R. Reese^a, Luis G. Hidalgo^c, Judith Blaine^b, Sarah E. Panzer^a

^aDivision of Nephrology, Department of Medicine, University of Wisconsin-Madison, Madison, WI, USA

^bDivision of Renal Diseases and Hypertension, Department of Medicine, University of Colorado-Denver, Aurora, CO, USA

^cDivision of Transplantation, Department of Surgery, University of Wisconsin-Madison, Madison, WI, USA

Abstract

Chronic active antibody-mediated rejection is a major cause of allograft failure in kidney transplantation. Microvascular inflammation and transplant glomerulopathy are defining pathologic features of chronic active antibody-mediated rejection and are associated with allograft failure. However, the mechanisms of leukocyte infiltration and glomerular endothelial cell injury remain unclear. We hypothesized MHC class II ligation on glomerular endothelial cells (GEnC) would result in upregulation of adhesion molecules and production of chemoattractants. A model of endothelial cell activation in the presence of antibodies to MHC classes I and II was used to determine the expression of adhesion molecules and chemokines. Murine GEnC were activated with IFN γ , which upregulated gene expression of β 2-microglobulin (MHC class I), ICAM1, VCAM1, CCL2, CCL5, and IL-6. IFN γ stimulation of GEnC increased surface expression of MHC class I, MHC class II, ICAM1, and VCAM1. Incubation with antibodies directed at MHC class I or class II did not further enhance adhesion molecule expression. Multispectral imaging flow cytometry and confocal microscopy demonstrated MHC molecules co-localized with the adhesion molecules ICAM1 and VCAM1 on the GEnC surface. GEnC secretion of chemoattractants, CCL2 and CCL5, was increased by IFN γ stimulation. CCL2 production was further enhanced by incubation with sensitized plasma. Endothelial activation induces *de novo* expression of MHC class II molecules and increases surface expression of MHC class I, ICAM1 and VCAM1, which are all co-localized together. Maintaining the integrity and functionality of the glomerular endothelium is necessary to ensure survival of the allograft. IFN γ stimulation of GEnC

Corresponding author: Sarah E. Panzer, MD, University of Wisconsin-Madison, Division of Nephrology, Department of Medicine, 1685 Highland Avenue, Madison, WI 53705, USA, sepanzer@medicine.wisc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Declarations of interest: none

propagates an inflammatory response with production of chemokines and co-localization of MHC and adhesion molecules on the GEnC surface, contributing to endothelial cell function as antigen presenting cells and an active player in allograft injury.

Keywords

glomerular endothelial cell; chemoattractant; adhesion molecule; major histocompatibility complex

1. Introduction

Chronic active antibody-mediated rejection (cABMR) contributes to the majority of late renal allograft failures [1–4]. Endothelial cells represent the key interface between the recipient's cellular and humoral effector components and the donor organ. Endothelial cells within the allograft are able to display peptide antigens on major histocompatibility complex (MHC) molecules that can be bound by T cell receptors on circulating effector memory T cells, promoting microvascular inflammation and allograft rejection without the direct involvement of professional antigen presenting cells [5]. The glomerular endothelium is commonly injured during cABMR and transcriptional studies confirm endothelial injury portends poor clinical outcomes, such as allograft failure [6–8]. Endothelial cell injury promotes formation of double contours of the glomerular basement membrane, which serves as a key diagnostic feature of transplant glomerulopathy and cABMR [9]. Modest gains have been made in the treatment of cABMR but gaps remain in our understanding of the mechanisms that promote allograft failure.

Donor specific antibodies recognize MHC class I or class II antigens and bind to the microvascular endothelium [10], making this the first site in the pathogenesis of rejection. In particular, MHC class II antibodies are associated with adverse clinical outcomes, such as patient mortality, antibody mediated rejection, transplant glomerulopathy and allograft dysfunction [11–14]. Higher mean serum creatinine and higher levels of proteinuria are observed in patients that develop anti-MHC class II antibodies, such as to HLA-DQ, along with reduced graft survival [15]. Elegant studies have been done to determine the effect of anti-MHC class I antibody binding on endothelial cells from large vessels [16–22]; however, there is less data defining how MHC class II cross-linking on microvascular endothelial cells trigger functional changes leading to microvascular inflammation [18]. Microvascular endothelial cells differ from macrovascular endothelial cells in that they have organ-specific functions and show phenotypic heterogeneity [23, 24]. Endothelial cells derived from large vessels do not behave the same as microvascular endothelial cells, such as those found in the glomerulus [25].

Cytokines from the recipient's effector lymphocytes, particularly interferon gamma (IFN γ), induce expression of surface molecules on endothelial cells that render them targets of antibodies or T cells [26, 27]. Endothelial cells are an important source of CCL5 (C-C motif chemokine ligand 5 (also known as RANTES, regulated upon activation, normal T cell expressed and secreted)) [28], a potent chemoattractant for inflammatory cells such as memory T cells, monocytes [29], and eosinophils [30]. Endothelial cells are also a source of

CCL2 (C-C motif chemokine ligand 2 (also known as MCP-1, monocyte chemotactic protein-1)), a regulator of the recruitment and differentiation of monocytes and macrophages [31]. Compared to normal tissues, the expression levels of CCL2 and CCL5 are higher in renal tissues with chronic renal allograft dysfunction [32]. An understanding of the interaction between microvascular endothelial cells, chemoattractants, inflammatory cytokines, expression of adhesion molecules, and MHC molecules could lead to critical new interventions to prevent chronic allograft rejection and failure.

2. Objective

In this study, we utilized murine glomerular endothelial cells (GEnC) to examine activation by the effector cytokine IFN γ and further stimulation with anti-MHC class I antibody, anti-MHC class II antibody, or plasma from sensitized mice. We hypothesized MHC class II ligation on GEnC would result in upregulation of adhesion molecules and production of chemoattractants and thus would enhance the ability of endothelial cells to contribute to the alloimmune response.

3. Materials and methods

3.1 Glomerular endothelial cell culture

All animal experiments were performed under National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Kidneys were harvested from B57 Bl/6 mice after intracardiac perfusion with supersaturated iron oxide. Kidney tissue was subjected to serial collagenase digestion, magnetic selection, and differential sieving in order to isolate glomeruli cells from other kidney tissue. Then the glomeruli were subjected to a second collagenase digestion and differential sieving to dissociate the cells from the glomerular basement membrane. The glomerular cells were allowed to replicate before selective isolation using von Willebrand coated Dynabeads. Isolated GEnC were stained with von Willebrand factor (endothelial cell marker) and WT1 (podocyte marker) to ensure the isolated cells were, in fact, GEnC and not podocytes. After purity and viability were established, GEnC were transformed using a thermosensitive SV40 T antigen and underwent puromycin selection to ensure transformation as previously described [33]. GEnC phenotype following transformation was verified by repeat staining and imaging for von Willebrand factor and WT1. GEnC were cultured in DMEM with 20% FBS, 100 μ g/ml of endothelial cell growth supplement (Sigma), 100 μ g/ml of penicillin/streptomycin (Gibco) and 75 U/ml IFN γ (Calbiochem). After immortalization, glomerular endothelial cells were grown at 33°C for the undifferentiated (growth-permissive) condition and then at 37°C for the differentiated (growth-restricted) condition for one week.

3.2 Generation of sensitized plasma

Balb/c mice (MHC haplotype H2^d) were given an intraperitoneal injection with 4×10^6 splenocytes from C57 Bl/6 mice (MHC haplotype H2^b). Plasma from the sensitized Balb/c mice was harvested 21 days later. Flow cross-match was performed using C57 Bl/6 splenocytes as targets to demonstrate donor antibody specificity as described previously

[34]. Briefly, C57 Bl/6 splenocytes were incubated with plasma, visualized with anti-CD3 (BioLegend, clone 17A2), anti-CD45R (BD Pharmingen, clone RA3-6B2), anti-IgG (BioLegend, clone Poly4053), anti-IgM (BD Pharmingen, clone R6-60.2), anti-IgG1 (BD Horizon, clone A85-1), anti-IgG2a (BioLegend, clone RMG2a-62), anti-IgG2b (R&D Systems, Cat N: F0133), anti-IgG2c (Southern Biotech, Cat N: 1079-02). Cells were gated for singlets and CD3+ cells, then the mean fluorescence intensity (MFI) was determined for each isotype. Plasma was also collected from non-sensitized Balb/c mice.

3.3 Endothelial cell incubation with anti-MHC I antibody, anti-MHC II antibody, sensitized plasma, or non-sensitized plasma

Differentiated GEnC were grown at 37°C for one week and cells were then stimulated with IFN γ for 48 hours to upregulate surface MHC class I and II. Following IFN γ stimulation, GEnC were incubated with 1000 ng/ml of anti-MHC class I antibody (BioLegend, clone 28-8-6), 1000 ng/ml of anti-MHC class II antibody (BioLegend, clone 25-9-17), 10% sensitized plasma, or 10% non-sensitized plasma in culture media. After 24 hours of incubation, cells and supernatant were harvested.

3.4 Gene expression analysis

Briefly, supernatant was removed, cells were washed with 1 $\times$ DPBS (Corning), then Trizol was added directly to the cells and placed into a DNase, RNase-free tube and processed according to the manual for the RNeasy kit (Qiagen). RNA concentrations were determined using a Nanodrop 1000. GEnC gene expression was analyzed with the RT2 Profiler PCR array for murine endothelial cells (Qiagen) and acquired by real-time PCR system (Applied Biosystems 7500 Fast Block). For the RT2 Profiler PCR assay, fold change was calculated using the delta delta C_T calculation between the gene of interest and housekeeping genes as a reference. Calculations were performed on the Qiagen web portal at <http://www.qiagen.com/geneglobe>.

To confirm the RT2 Profiler results, RT-PCR analysis was performed on the genes with the highest positive fold-change demonstrated by RT2 Profiler analysis. cDNA was obtained using Superscript IV First strand Synthesis system (Invitrogen). TaqMan gene expression assays (ThermoFisher) were run on a real-time PCR system (Applied Biosystems 7500 Fast Block) for CCL2 (Mm00441242), CCL5 (Mm01302427), β 2-microglobulin (Mm00437762), VCAM1 (Mm01320970), and ICAM1 (Mm00516023). Ribosomal s26 (Mm02601831) was used as a housekeeping gene for normalization. Similar to RT2 Profiler data, fold changes were calculated using the delta delta C_T method.

3.5 Chemoattractant determination

A multiplex bead-based assay (Bio-Rad, Bio-PLEX) was used to measure protein levels of CCL2 and CCL5 in GEnC culture supernatants. Manufacturer directions for the kit were followed. Data were acquired on a Luminex 200 instrument and analyzed with Xponent software version 4.3.

3.6 Flow cytometry for surface molecule expression

Accutase (Innovative Cell Technologies) was used to remove GEnC from the plate using primarily EDTA chelation of Mg^{2+} and Ca^{2+} to remove cells, resulting in minimal digestion of cell surface antigens. After washing, 250,000 GEnC were aliquoted into cluster tubes and stained for CD146 (Miltenyi, Cat N: 130-102-230), ICAM1 (BioLegend, clone YN1/1.7.4), VCAM1 (BioLegend, clone 429 (MVcam.A), MHC class I (BioLegend, clone 28-8-6), MHC class II (Miltenyi, Cat N: 130-102-139) or P-selectin (Miltenyi, Cat N: 130-105-538). Following staining, GEnC were washed, fixed with 2% paraformaldehyde in PBS, and analyzed by flow cytometry (BD LSR Fortessa or Accura). Flow analyses were performed in FlowJo version 10.

3.7 Surface molecule co-localization by multispectral imaging-based flow cytometry

GEnC were stained in a similar manner as described above. Multispectral flow cytometry images were acquired on the ImageStream C flow cytometer (Amnis/EMD Millipore, Seattle, WA, USA) with INSPIRE version 4.1 software. A total of 5,000 singlets were collected at $40\times$ magnification. Laser settings were 30 mW 488 nm and 100 mW 642 nm. Stains were read in the following channels: Brightfield channel 1 (488 nm laser – 457/45 nm), ICAM1 (FITC) was on Ch02 (488 nm laser – 533/55 nm), MHC class I (PE) was on Ch03 (488 nm laser – 577/35 nm), MHC Class II (APC) was on Ch05 (642 nm laser – 702/85 nm) and VCAM1 (PECy7) was on Ch06 (488 nm laser – 762/35 nm).

3.8 Analysis of imaging flow cytometry data

Data were processed using ImageStream Data Exploration and Analysis Software (IDEAS) version 6.2 (Amnis/EMD Millipore). A compensation matrix was derived using singly stained GEnC. First, cells were gated for in-focus cells (Gradient RMS_M01_Ch01 versus frequency, values >55 Gradient RMS_M01_Ch01 were considered to be in-focus, gate R1) and then for singlets (Aspect Ratio_M01 versus Area_M01, gate R2). Subsequently, double positive cells were identified as MHC class I⁺/ICAM1⁺, MHC class I⁺/VCAM1⁺, MHC class II⁺/ICAM1⁺, or MHC class II⁺/VCAM1⁺. The IFN γ treatment group was used as the determinate for setting double positive gates, since this was the population to which other treatment groups are compared. Next, the Intensity Feature was utilized to compare levels of surface molecules under different experimental conditions (Supplemental Table 1, and Results section 4.4).

Co-localization was determined for double positive populations (if present) and gated for ICAM1 versus MHC class I or MHC class II, or VCAM1 versus MHC class I or MHC class II. From these double positive populations, the Bright Detail Similarity was calculated to assess degree of co-localization, using the co-localization wizard (Supplemental Table 1, and Results section 4.5).

3.9 Confocal microscopy

GEnCs were cultured in MatTek dishes (MatTek Corporation) with or without 250 units IFN γ in media. Cells were fixed 48 hours after treatment with 4% paraformaldehyde then permeabilized with 0.1% Tween and blocked with 10% BSA (Fisher Scientific). Antibodies used were anti-MHC class I (10 μ g/ml; clone M1/42.3.89.8, Biocell), anti-MHC class II (5

µg/ml; clone NIMR-4, Abcam), anti-ICAM1 (4 µg/ml; Cat N: PA5-96365, Invitrogen), or anti-VCAM1 (4 µg/ml; clone EPR5047, Abcam). Rat IgG (10 µg/ml; Cat N: I-4000, Vector) and rabbit IgG (4 µg/ml; Cat N: I-1000, Vector) served as isotype controls. Primary antibodies were incubated at 4°C overnight. Subsequently, cells were incubated with goat-anti-rat Alexa Fluor 488 (2 µg/ml; Cat N: A11006, Invitrogen) and goat-anti-rabbit Alexa Fluor 647 (3 µg/ml; Cat N: A21246, Invitrogen). Cells were counterstained with DAPI (0.33 µg/ml; Cat N: D3571, Invitrogen) then covered with PBS and imaged immediately. Multichannel images were acquired using the Nikon A1R Eclipse Ti confocal microscope and Elements software (Version 4.5). All images were acquired with a 60X/NA 1.4 (pinhole 1.2 AU) objective with oil immersion with sequential scanning at the lowest possible laser power. Analysis was performed in Fiji/ImageJ NIH software. Images presented are representative of three non-overlapping images per condition from three independent experiments.

3.10 Statistics

Data are provided as mean $\pm$ standard error of the mean (SEM). Two-tailed Student's t-test was used for unpaired comparisons. One-way analysis of variance (ANOVA) was used for multiple comparisons. GraphPad Prism software version 8 was used for statistical analysis (GraphPad Software, La Jolla, CA, USA). P value less than 0.05 was considered to be statistically significant.

4. Results

4.1 Endothelial genes were upregulated by IFN γ stimulation of GEnC

To determine the effect of IFN γ stimulation on endothelial gene expression, GEnC were stimulated with IFN γ and transcriptional analysis was performed with a gene array focused on endothelial cell biology. Transcriptional analysis indicated the genes upregulated by IFN γ included chemoattractants (CCL2, CCL5), MHC proteins (β 2-microglobulin), and adhesion molecules (ICAM1, VCAM1) (Fig. 1). Results of the gene array were confirmed by RT-PCR (Supplemental Figure 1).

4.2 Production of chemoattractants by GEnC was increased by IFN γ stimulation and further increased by incubation with plasma

To examine the effect of IFN γ stimulation on protein production of chemoattractants, GEnC were incubated with IFN γ and a bead-based multiplex assay was used to determine protein concentrations in the cell culture supernatants. Based on the gene transcript results, we chose to analyze protein production of CCL2 and CCL5 by GEnC (Fig. 2). Similar to the transcriptional analysis, GEnC protein production of CCL2 and CCL5 was upregulated by activation with IFN γ compared to media controls ($p=0.04$ and $p<0.0001$, respectively) (Fig. 2 A,B).

In order to test the ability of ligation of MHC class I or II to alter GEnC chemoattractant production, surface expression of MHC class I and II was induced by IFN γ incubation. GEnC were then incubated with purified antibody directed against MHC class I or II or with plasma from non-sensitized or sensitized mice. The sensitized plasma contained donor-

specific antibodies, as demonstrated by positive flow cross-match data (Supplemental Figure 2). Incubation with IFN γ significantly increased GEnC production of CCL2 and CCL5 compared to incubation with media (Fig. 2). Subsequent incubation of GEnC with anti-MHC class I or anti-MHC class II antibodies did not further increase CCL2 or CCL5 levels compared to IFN γ stimulated GEnC. However, GEnC incubated with 10% sensitized or non-sensitized plasma further increased the amount of CCL2 production compared to GEnC stimulated with IFN γ alone ($p < 0.0001$ for both) (Fig. 2A). The observation that plasma (sensitized or non-sensitized) increased CCL2 production beyond levels when GEnC were incubated with anti-MHC class I or II antibodies alone may be due to other inflammatory mediators (cytokines or complement proteins) or non-MHC antibodies present in plasma. CCL5 production was increased by IFN γ stimulation, but was not further increased by anti-MHC antibodies or plasma (Fig 2B).

4.3 Surface expression of MHC class I, MHC class II, ICAM1, and VCAM1 on GEnC was upregulated after IFN γ activation

Surface expression of MHC class I, MHC class II, ICAM1, and VCAM1 following IFN γ incubation was determined by flow cytometry (Fig. 3). MHC class I was present on GEnC at baseline and increased by IFN γ stimulation (Fig. 3A). MHC class II was not present on the surface of GEnC at baseline, but was induced with incubation with IFN γ (Fig. 3B). IFN γ stimulation also increased GEnC surface expression of ICAM1 and VCAM1 compared to baseline (Fig. 3 C,D). Expression of CD31 and P-selectin was not observed, consistent with other microvascular endothelial cells [25]. The endothelial cell marker CD146 was present on GEnC at baseline and remained unchanged by IFN γ incubation.

4.4 Multispectral imaging flow cytometry demonstrated adhesion molecules on GEnC were not further enhanced by incubation with anti-MHC antibodies

Multispectral imaging flow cytometry was used to visualize surface MHC class I and II and adhesion molecules on GEnC. Surface expression of MHC class I, ICAM1, and VCAM1 are all identified on the GEnC in the media control group; however, MHC class II was not expressed in the media control group. After activation of GEnC with 48 hours of IFN γ exposure, MHC class II appeared on the GEnC surface and the expression of MHC class I, ICAM1 and VCAM1 was increased (Fig. 4A). The Intensity Feature of the multispectral imaging flow cytometry software was used to quantify the level of surface expression. Surface expression of all four molecules was significantly increased after IFN γ stimulation compared to media controls ($p < 0.0001$ for MHC I, MHC II, ICAM1, and VCAM1) (Fig. 4). Surface expression of MHC and adhesion molecules were not further enhanced by incubation with anti-MHC I antibody, anti-MHC II antibody, or sensitized plasma.

4.5 Multispectral imaging flow cytometry demonstrated co-localization of MHC class I, MHC class II, ICAM1, and VCAM1 on the GEnC surface

Endothelial cells can act as antigen presenting cells [5, 35] and also upregulate adhesion molecules upon stimulation to attract macrophages, NK cells, and T cells. Markey *et al.* used imaging flow cytometry to visualize the immunological synapse between dendritic cells and T cells [36]. We hypothesized activation of microvascular endothelial cells would similarly result in co-localization of MHC and adhesion molecules. The Bright Detail Similarity

Median (BDSM) software feature and multispectral imaging flow cytometry was used to quantify the extent of co-localization. Double positive populations used to compute BDSM comprised >90% of the parent singlet population, except in the unstimulated group, where MHC class II was not expressed (Supplemental Table 2). BDSM measures the correlation of the bright details between image pairs and hence the co-localization. A BDSM value >1 indicates the two surface molecules are co-localized. In media control cells (unstimulated), MHC I co-localized with both ICAM1 (BDSM=2.5, Table 1) as well as VCAM1 (BDSM=2.4). There was not co-localization of adhesion molecules with MHC class II in media controls, as MHC class II was not expressed on unstimulated media control cells (Fig. 5A). After IFN γ activation for 48 hours, GEnC expressed MHC class II, which co-localized with both ICAM1 (BDSM=2.4) and VCAM1 (BDSM=2.3) (Fig. 5B). MHC class I also co-localized with ICAM1 (BDSM=2.7) and VCAM1 (BDSM=2.5) on IFN γ activated GEnC. Median average deviation (MAD) is a statistical measure of the spread of the data. The MAD scores showed the variability of the BDSM was reasonably low, indicating consistent co-localization across treatment groups. In summary, MHC class I, MHC class II (once upregulated), co-localized with ICAM1 and VCAM1 on IFN γ activated GEnC. Co-localization did not change with ligation of MHC class I or MHC class II molecules, nor did it change when stimulated with 10% sensitized plasma by multispectral imaging flow cytometry.

4.6 Confocal microscopy confirmed co-localization of adhesion molecules and MHC molecules on IFN γ -stimulated GEnC

In order to confirm co-localization of MHC molecules and adhesion molecules, expression of MHC class I, MHC class II, ICAM1, and VCAM1 was determined by scanning confocal microscopy (Fig. 6, n=3). Expression of MHC class I was low, but detectable, on GEnCs under basal conditions (Media Control) (Figure 6Ai,Bi and Supplemental Figure 3). As expected, expression of MHC class II was extremely low in unstimulated GEnCs (Figure 6Ci,Di). Both MHC class I and MHC class II expression increased upon stimulation with IFN γ (Figure 6 Aii, Bii, Cii, Dii). An increase in ICAM1 (Figure 6 A,C) and VCAM1 (Figure 6 B,D) expression was observed with IFN γ stimulated GEnC compared to media control. The percent VCAM1 that co-localized with MHC class I and MHC class II was significantly increased after IFN γ treatment ($p=0.004$ for both) (Fig. 6E). There was a trend toward increased co-localization for ICAM percent co-localization with MHC class I ($p=0.06$) and MHC class II ($p=0.10$) (Fig. 6E). Data were obtained from three non-overlapping images per condition from three independent experiments. Individual channel images are shown in Supplemental Figure 3.

5. Discussion

In this study, we utilized a model of microvascular glomerular endothelial cells activated by the effector cytokine IFN γ and antibodies directed against MHC antigens to alter cellular expression of adhesion molecules and chemokines. Imaging flow cytometry and confocal microscopy demonstrated co-localization of surface MHC molecules and adhesion molecules on GEnC. Upon exposure to IFN γ , the expression of MHC I and adhesion molecules are further enhanced and the surface expression of MHC class II is induced.

Stimulation with IFN γ increased the production of chemokines (CCL2 and CCL5) by GEnC.

Using multispectral imaging flow cytometry, we observed the adhesion molecules and MHC molecules co-localized; this formed a region that may serve as a site of potential interaction with immune cells, not unlike that observed on dendritic cells [36]. As multispectral imaging flow cytometry provides a two-dimensional view to determine co-localization, it is possible that the molecules happen to overlap in the observed image and are not actually interacting. Therefore, to confirm co-localization we also performed confocal microscopy. Confocal microscopy allows the visualization of surface molecules in the same plane. Confocal microscopy confirmed co-localization of MHC and adhesion molecules on the GEnC surface. There were low levels of MHC class I detected under basal culture conditions by confocal microscopy, whereas MHC I was observed in basal conditions by multispectral imaging flow cytometry. Reasons for this include the differences in two-dimensional versus three-dimensional imaging and antibodies targeting fixed versus native epitopes in the different techniques. The co-localization of MHC and adhesion molecules on the GEnC surface could serve as a site of antigen presentation by endothelial cells and interaction with T cells. Although we observed co-localization between MHC and adhesion molecules in this study, we acknowledge that further studies are required to determine the degree of protein-protein interaction. Stimulation with IFN γ further enhanced and strengthened the components by inducing MHC II expression, as well as upregulating expression of ICAM1 and VCAM1. In concert, CCL2 and CCL5 production are increased to enhance leukocyte recruitment. These findings indicate the renal microvasculature is not only a site of injury in rejection, but also promotes allograft inflammation.

The mechanisms of endothelial cell injury that lead to recruitment of intragraft leukocytes is of clinical importance, as intragraft leukocytes are associated with severity of rejection, development of interstitial fibrosis, and allograft loss [37–41]. Activated endothelial cells have a greater capacity for leukocyte recruitment, as we demonstrated in the current study, through the enhanced expression of MHC molecules, adhesion molecules, and chemokines. Recent studies demonstrated activated endothelial cells can amplify alloimmunity and allograft injury; we and others demonstrated activated microvascular endothelial cells produce inflammatory mediators, such as CCL5 and IL-6 [42, 43]. We observed IFN γ enhanced GEnC production of CCL2 and CCL5 compared to basal levels. Plasma (sensitized and non-sensitized) further enhanced CCL2 production, but not CCL5 production by GEnC. The sensitized plasma was confirmed to contain donor-specific antibodies by flow cross-match and we presume that these were primarily anti-MHC antibodies; however, this does not rule out the presence of non-MHC antibodies or other inflammatory mediators in plasma, as well. CCL5 is known to promote T cell recruitment, and increased levels of intragraft CCL5 are associated with intragraft leukocytes, interstitial fibrosis and tubular atrophy, and recurrent acute rejection [44–47]. IL-6 is known to promote B cell maturation to plasma cells and induce T follicular helper cells [48]. Blockade of IL-6 reduces alloantibody production by plasma cells, induces T regulatory cells, and inhibits T follicular helper cells in animal transplant models [49] and has demonstrated benefit in clinical trials of transplant patients [50, 51]. Of note, microvascular endothelial cells can promote proliferation and polarization of T cells to pro-inflammatory Th17 cells via IL-6 secretion

from the microvascular endothelial cells and abrogate differentiation of T regulatory cells [42, 43]. Taken together, these findings support the role of endothelial cells not only as targets of the immune system in rejection, but also as active players in influencing the immune response and a therapeutic target in transplantation.

Endothelial cells are found throughout the body and are differentiated for the tasks required for the location of each vascular bed [25]. As such, endothelial cell behavior and response to stimuli vary in different organs and vascular beds. Much research has been done using larger vessel endothelial cells, human aortic endothelial cells and human umbilical vein endothelial cells (HUVEC). Upregulation of P-selectin as well as expression of CD31 has been reported in aortic endothelial cells and HUVEC following stimulation with anti-MHC antibody [16, 18, 52]; however, these molecules were not observed on microvascular GEnC in this study. This is in agreement with other studies using microvascular endothelial cells [25]. Similarly, the extent of CCL2 production by microvascular endothelial cells is unclear. The monocyte chemoattractant CCL2 is clearly increased in the kidney during renal endothelial microvascular injury. However, it is the peritubular capillary endothelial cells that have a strongly positive signal for CCL2, as well as the periglomerular region, not in the glomeruli themselves [53]. These data are consistent with the notion that there are waves of leukocytes that enter different vascular beds at different times. T cells enter the glomeruli early, but macrophage infiltration occurs several days after the initial microvascular injury in the glomeruli. In contrast, in the tubulointerstitium, T cells enter later during the injury process, and macrophages are present both early and late at this site [53]. This is in agreement with our data that after 48 hours of stimulation with IFN γ , CCL5, a potent chemoattractant for T cells, was highly upregulated at the gene and protein level. CCL2 was less upregulated at the gene expression level by IFN γ activated GEnC at the early time point. It is possible that other cells, such as peritubular capillary endothelial cells, are producing CCL2 to bring in a wave of macrophages at the later time points [53].

As with all cell culture-based studies, this work is limited by being an *in vitro* approximation of the allograft endothelium. Kidneys are complex structures, in particular the structure of the glomerulus involves multiple cell types. Two dimensional monoculture models cannot fully reflect this complexity; however, we believe that a strength of our study is the use of microvascular glomerular endothelial cells, as this highlights the differences in responses of endothelial cells to inflammatory stimuli from various organs and vascular bed types.

MHC and adhesion molecule co-localization on the cell surface, would be predicted to be easily recognized by allo-reactive CD8⁺ T cells and thus facilitate the rejection process at this site. This would help explain the early glomeruli infiltration in rejecting kidney allografts. The same may hold true in antibody-mediated rejection, where antibody-bound MHC class I aggregates together with adhesion molecules and is thus ready to facilitate an immune synapse with NK cells recognizing antibody through their Fc-gamma receptors and also complement activation. Therefore, an endothelial cell-T cell interaction composed of MHC and adhesion molecules could be a critical target of intervention in allograft rejection.

This work describes a model of microvascular injury to GEnC. Imaging flow cytometry and confocal microscopy demonstrated adhesion molecules and MHC molecules co-localized on

the GEnC surface. Activation of GEnC enhanced production of CCL2 and CCL5. Maintaining the function and integrity of the allograft endothelium is essential to ensure graft survival. The site of endothelial cell-T cell interaction and the production of chemokines by endothelial cells represent potential sites for therapeutic intervention in transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by the NIH grant K23 DK122136 and the American Society of Nephrology John Merrill Grant in Transplantation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

We acknowledge the University of Wisconsin Carbone Cancer Center (UWCCC) Flow Cytometry Core, which is supported by the Cancer Center Support Grant NIH P30 CA014520. We would like to thank Lauren Nettenstrom, SCYM (ASCP), for her technical expertise and contributions to Image Stream data analysis. We also thank Dana Clark, MA, for her editorial assistance.

We acknowledge the William S. Middleton Memorial Veterans Hospital confocal microscopy working group for use of the Nikon A1R Eclipse Ti confocal microscope.

Abbreviations:

cABMR	chronic active antibody-mediated rejection
ANOVA	one-way analysis of variance
BDSM	bright detail similarity median
CCL	C-C motif chemokine ligand
FITC	fluorescein isothiocyanate
GEnC	glomerular endothelial cell
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
MAD	mean absolute deviation
MHC	major histocompatibility complex
VCAM	vascular cell adhesion molecule

Reference list

- [1]. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M, Diagnosis and management of antibody-mediated rejection: current status and novel approaches, *Am J Transplant* 14(2) (2014) 255–71. [PubMed: 24401076]
- [2]. Loupy A, Hill GS, Jordan SC, The impact of donor-specific anti-HLA antibodies on late kidney allograft failure, *Nat Rev Nephrol* 8(6) (2012) 348–57. [PubMed: 22508180]
- [3]. Redfield RR, Ellis TM, Zhong W, Scalea JR, Zens TJ, Mandelbrot D, Muth BL, Panzer S, Samaniego M, Kaufman DB, Astor BC, Djamali A, Current outcomes of chronic active antibody mediated rejection - A large single center retrospective review using the updated BANFF 2013 criteria, *Hum Immunol* 77(4) (2016) 346–52. [PubMed: 26867813]
- [4]. Sellares J, de Freitas D, Mengel M, Reeve J, Einecke G, Sis B, Hidalgo L, Famulski K, Matas A, Halloran P, Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence., *Am J Transplant* 12(2) (2012) 388–99. [PubMed: 22081892]
- [5]. Pober JS, Merola J, Liu R, Manes TD, Antigen Presentation by Vascular Cells, *Front Immunol* 8(2017) 1907. [PubMed: 29312357]
- [6]. Kamal L, Broin PO, Bao Y, Ajaimy M, Lubetzky M, Gupta A, de Boccardo G, Pullman J, Golden A, Akalin E, Clinical, Histological, and Molecular Markers Associated With Allograft Loss in Transplant Glomerulopathy Patients, *Transplantation* 99(9) (2015) 1912–8. [PubMed: 25675205]
- [7]. Loupy A, Lefaucheur C, Vernerey D, Chang J, Hidalgo LG, Beuscart T, Verine J, Aubert O, Dupleumortier S, Duong van Huyen JP, Jouven X, Glotz D, Legendre C, Halloran PF, Molecular microscope strategy to improve risk stratification in early antibody-mediated kidney allograft rejection, *J Am Soc Nephrol* 25(10) (2014) 2267–77. [PubMed: 24700874]
- [8]. Hidalgo LG, Sis B, Sellares J, Campbell PM, Mengel M, Einecke G, Chang J, Halloran PF, NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection, *Am J Transplant* 10(8) (2010) 1812–22. [PubMed: 20659089]
- [9]. Haas M, Loupy A, Lefaucheur C, Roufosse C, Glotz D, Seron D, Nankivell BJ, Halloran PF, Colvin RB, Akalin E, Alachkar N, Bagnasco S, Bouatou Y, Becker JU, Cornell LD, van Huyen JPD, Gibson IW, Kraus ES, Mannon RB, Naesens M, Nickleit V, Nickerson P, Segev DL, Singh HK, Stegall M, Randhawa P, Racusen L, Solez K, Mengel M, The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials, *Am J Transplant* 18(2) (2018) 293–307. [PubMed: 29243394]
- [10]. Mannam VK, Lewis RE, Cruse JM, The fate of renal allografts hinges on responses of the microvascular endothelium, *Exp Mol Pathol* 94(2) (2013) 398–411. [PubMed: 22710034]
- [11]. Cole RT, Gandhi J, Bray RA, Gebel HM, Morris A, McCue A, Yin M, Laskar SR, Book W, Jokhadar M, Smith A, Nguyen D, Vega JD, Gupta D, De novo DQ donor-specific antibodies are associated with worse outcomes compared to non-DQ de novo donor-specific antibodies following heart transplantation, *Clin Transplant* 31(4) (2017).
- [12]. Willicombe M, Blow M, Santos-Nunez E, Freeman C, Brookes P, Taube D, Terasaki Epitope Mismatch Burden Predicts the Development of De Novo DQ Donor-Specific Antibodies and are Associated With Adverse Allograft Outcomes, *Transplantation* 102(1) (2018) 127–134. [PubMed: 28731902]
- [13]. Haas M, Mirocha J, Reinsmoen NL, Vo AA, Choi J, Kahwaji JM, Peng A, Villicana R, Jordan SC, Differences in pathologic features and graft outcomes in antibody-mediated rejection of renal allografts due to persistent/recurrent versus de novo donor-specific antibodies, *Kidney Int* 91(3) (2017) 729–737. [PubMed: 28104301]
- [14]. Issa N, Cosio FG, Gloor JM, Sethi S, Dean PG, Moore SB, DeGoe S, Stegall MD, Transplant glomerulopathy: risk and prognosis related to anti-human leukocyte antigen class II antibody levels, *Transplantation* 86(5) (2008) 681–5. [PubMed: 18791449]
- [15]. DeVos JM, Gaber AO, Knight RJ, Land GA, Suki WN, Gaber LW, Patel SJ, Donor-specific HLA-DQ antibodies may contribute to poor graft outcome after renal transplantation, *Kidney Int* 82(5) (2012) 598–604. [PubMed: 22622504]

- [16]. Valenzuela N, Hong L, Shen X, Gao F, Young S, Rozengurt E, Kupiec-Weglinski J, Fishbein M, Reed E, Blockade of p-selectin is sufficient to reduce MHC I antibody-elicited monocyte recruitment in vitro and in vivo., *Am J Transplant* 13(2) (2013) 299–311. [PubMed: 23279566]
- [17]. Valenzuela N, Mulder A, Reed E, HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging FcγRs., *J Immunol* 190(12) (2013) 6635–50. [PubMed: 23690477]
- [18]. Valenzuela N, Reed E, Antibodies in Transplantation: The Effects of HLA and Non-HLA Antibody Binding and Mechanisms of Injury., *Methods Mol Biol* 1034 (2013) 41–70. [PubMed: 23775730]
- [19]. Zhang X, Reed E, Effect of antibodies on endothelium., *Am J Transplant* 9(11) (2009) 2459–65. [PubMed: 19775314]
- [20]. Zhang X, Rozengurt E, Reed E, HLA class I molecules partner with integrin β4 to stimulate endothelial cell proliferation and migration., *Sci Signal* 3(149) (2010) ra85. [PubMed: 21098729]
- [21]. Zhang X, Valenzuela N, Reed E, HLA class I antibody-mediated endothelial and smooth muscle cell activation., *Curr Opin Organ Transplant* 17(4) (2012) 446–51. [PubMed: 22710387]
- [22]. Salehi S, Sosa RA, Jin YP, Kageyama S, Fishbein MC, Rozengurt E, Kupiec-Weglinski JW, Reed EF, Outside-in HLA class I signaling regulates ICAM-1 clustering and endothelial cell-monocyte interactions via mTOR in transplant antibody-mediated rejection, *Am J Transplant* 18(5) (2018) 1096–1109. [PubMed: 29045076]
- [23]. Risau W, Development and differentiation of endothelium, *Kidney Int Suppl* 67 (1998) S3–6. [PubMed: 9736244]
- [24]. Holmen C, Christensson M, Pettersson E, Bratt J, Stjerne P, Karrar A, Sumitran-Holgersson S, Wegener's granulomatosis is associated with organ-specific antiendothelial cell antibodies, *Kidney Int* 66(3) (2004) 1049–60. [PubMed: 15327398]
- [25]. Craig LE, Spelman JP, Strandberg JD, Zink MC, Endothelial cells from diverse tissues exhibit differences in growth and morphology, *Microvasc Res* 55(1) (1998) 65–76. [PubMed: 9473410]
- [26]. Ho AW, Wong CK, Lam CW, Tumor necrosis factor-α up-regulates the expression of CCL2 and adhesion molecules of human proximal tubular epithelial cells through MAPK signaling pathways, *Immunobiology* 213(7) (2008) 533–44. [PubMed: 18656701]
- [27]. Viemann D, Goebeler M, Schmid S, Nordhues U, Klimmek K, Sorg C, Roth J, TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells, *J Leukoc Biol* 80(1) (2006) 174–85. [PubMed: 16617158]
- [28]. Devergne O, Marfaing-Koka A, Schall TJ, Leger-Ravet MB, Sadick M, Peuchmaur M, Crevon MC, Kim KJ, Schall TT, Kim T, et al., Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells, *J Exp Med* 179(5) (1994) 1689–94. [PubMed: 7513016]
- [29]. Schall TJ, Bacon K, Toy KJ, Goeddel DV, Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES, *Nature* 347(6294) (1990) 669–71. [PubMed: 1699135]
- [30]. Kameyoshi Y, Dorschner A, Mallet AI, Christophers E, Schroder JM, Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils, *J Exp Med* 176(2) (1992) 587–92. [PubMed: 1380064]
- [31]. Boels MGS, Koudijs A, Avramut MC, Sol W, Wang G, van Oeveren-Rietdijk AM, van Zonneveld AJ, de Boer HC, van der Vlag J, van Kooten C, Eulberg D, van den Berg BM, DHT II, Rabelink TJ, Systemic Monocyte Chemotactic Protein-1 Inhibition Modifies Renal Macrophages and Restores Glomerular Endothelial Glycocalyx and Barrier Function in Diabetic Nephropathy, *Am J Pathol* 187(11) (2017) 2430–2440. [PubMed: 28837800]
- [32]. Yan Q, Jiang H, Wang B, Sui W, Zhou H, Zou G, Expression and Significance of RANTES and MCP-1 in Renal Tissue With Chronic Renal Allograft Dysfunction, *Transplant Proc* 48(6) (2016) 2034–9. [PubMed: 27569940]
- [33]. Jat PS, Sharp PA, Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature, *Mol Cell Biol* 9(4) (1989) 1672–81. [PubMed: 2542774]

- [34]. Wilson NA, Bath NM, Verhoven BM, Ding X, Boldt BA, Sukhwal A, Zhong W, Panzer SE, Redfield RR 3rd, APRIL/BLyS blockade reduces donor specific antibodies in allosensitized mice, *Transplantation* (2019).
- [35]. Al-Soudi A, Kaaij MH, Tas SW, Endothelial cells: From innocent bystanders to active participants in immune responses, *Autoimmun Rev* 16(9) (2017) 951–962. [PubMed: 28698091]
- [36]. Markey KA, Gartlan KH, Kuns RD, MacDonald KP, Hill GR, Imaging the immunological synapse between dendritic cells and T cells, *J Immunol Methods* 423 (2015) 40–4. [PubMed: 25967948]
- [37]. Girlanda R, Kleiner DE, Duan Z, Ford EA, Wright EC, Mannon RB, Kirk AD, Monocyte infiltration and kidney allograft dysfunction during acute rejection, *Am J Transplant* 8(3) (2008) 600–7. [PubMed: 18294156]
- [38]. Bergler T, Jung B, Bourier F, Kuhne L, Banas MC, Rummele P, Wurm S, Banas B, Infiltration of Macrophages Correlates with Severity of Allograft Rejection and Outcome in Human Kidney Transplantation, *PLoS One* 11(6) (2016) e0156900. [PubMed: 27285579]
- [39]. Brasen JH, Khalifa A, Schmitz J, Dai W, Einecke G, Schwarz A, Hallensleben M, Schmidt BMW, Kreipe HH, Haller H, von Vietinghoff S, Macrophage density in early surveillance biopsies predicts future renal transplant function, *Kidney Int* 92(2) (2017) 479–489. [PubMed: 28359537]
- [40]. Toki D, Zhang W, Hor KL, Liuwantara D, Alexander SI, Yi Z, Sharma R, Chapman JR, Nankivell BJ, Murphy B, O'Connell PJ, The role of macrophages in the development of human renal allograft fibrosis in the first year after transplantation, *Am J Transplant* 14(9) (2014) 2126–36. [PubMed: 25307039]
- [41]. Deteix C, Attuil-Audenis V, Duthey A, Patey N, McGregor B, Dubois V, Caligiuri G, Graff-Dubois S, Morelon E, Thaumat O, Intragraft Th17 infiltrate promotes lymphoid neogenesis and hastens clinical chronic rejection, *J Immunol* 184(9) (2010) 5344–51. [PubMed: 20357253]
- [42]. Lion J, Tabin C, Cross AR, Robledo-Sarmiento M, Mariotto E, Savenay A, Carmagnat M, Suberbielle C, Charron D, Haziot A, Glotz D, Mooney N, HLA Class II Antibody Activation of Endothelial Cells Promotes Th17 and Disrupts Regulatory T Lymphocyte Expansion, *Am J Transplant* 16(5) (2016) 1408–20. [PubMed: 26614587]
- [43]. Cross AR, Lion J, Poussin K, Assayag M, Taupin JL, Glotz D, Mooney N, HLA-DQ alloantibodies directly activate the endothelium and compromise differentiation of FoxP3(high) regulatory T lymphocytes, *Kidney Int* (2019).
- [44]. Kruger B, Boger CA, Obed A, Farkas S, Hoffmann U, Banas B, Fischereder M, Kramer BK, RANTES/CCL5 polymorphisms as a risk factor for recurrent acute rejection, *Clin Transplant* 21(3) (2007) 385–90. [PubMed: 17488389]
- [45]. Ruster M, Sperschneider H, Funfstuck R, Stein G, Grone HJ, Differential expression of beta-chemokines MCP-1 and RANTES and their receptors CCR1, CCR2, CCR5 in acute rejection and chronic allograft nephropathy of human renal allografts, *Clin Nephrol* 61(1) (2004) 30–9. [PubMed: 14964455]
- [46]. Pattison J, Nelson PJ, Huie P, von Leutichau I, Farshid G, Sibley RK, Krensky AM, RANTES chemokine expression in cell-mediated transplant rejection of the kidney, *Lancet* 343(8891) (1994) 209–11. [PubMed: 7507196]
- [47]. Dikow R, Becker LE, Schaier M, Waldherr R, Gross ML, Zeier M, In renal transplants with delayed graft function chemokines and chemokine receptor expression predict long-term allograft function, *Transplantation* 90(7) (2010) 771–6. [PubMed: 20697328]
- [48]. Chavele KM, Merry E, Ehrenstein MR, Cutting edge: circulating plasmablasts induce the differentiation of human T follicular helper cells via IL-6 production, *J Immunol* 194(6) (2015) 2482–5. [PubMed: 25681343]
- [49]. Kim I, Wu G, Chai NN, Klein AS, Jordan S, Anti-interleukin 6 receptor antibodies attenuate antibody recall responses in a mouse model of allosensitization, *Transplantation* 98(12) (2014) 1262–70. [PubMed: 25286051]
- [50]. Choi J, Aubert O, Vo A, Loupy A, Haas M, Puliyaanda D, Kim I, Louie S, Kang A, Peng A, Kahwaji J, Reinsmoen N, Toyoda M, Jordan SC, Assessment of Tocilizumab (Anti-Interleukin-6 Receptor Monoclonal) as a Potential Treatment for Chronic Antibody-Mediated Rejection and

- Transplant Glomerulopathy in HLA-Sensitized Renal Allograft Recipients, *Am J Transplant* 17(9) (2017) 2381–2389. [PubMed: 28199785]
- [51]. Vo AA, Choi J, Kim I, Louie S, Cisneros K, Kahwaji J, Toyoda M, Ge S, Haas M, Puliyaanda D, Reinsmoen N, Peng A, Villicana R, Jordan SC, A Phase I/II Trial of the Interleukin-6 Receptor-Specific Humanized Monoclonal (Tocilizumab) + Intravenous Immunoglobulin in Difficult to Desensitize Patients, *Transplantation* 99(11) (2015) 2356–63. [PubMed: 26018350]
- [52]. Valenzuela NM, Mulder A, Reed EF, HLA class I antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial P-selectin and, depending on subclass, by engaging FcγRs, *J Immunol* 190(12) (2013) 6635–50. [PubMed: 23690477]
- [53]. Panzer U, Steinmetz OM, Reinking RR, Meyer TN, Fehr S, Schneider A, Zahner G, Wolf G, Helmchen U, Schärli P, Stahl RA, Thaiss F, Compartment-specific expression and function of the chemokine IP-10/CXCL10 in a model of renal endothelial microvascular injury, *J Am Soc Nephrol* 17(2) (2006) 454–64. [PubMed: 16382019]

Highlights

- Glomerular endothelial cells increased surface expression of MHC (classes I and II), ICAM1, and VCAM1 in response to IFN γ .
- MHC and adhesion molecules co-localized on the glomerular endothelial cell surface, which was strengthened by IFN γ stimulation.
- Activated glomerular endothelial cells increased the production of CCL2 and CCL5.
- Activated glomerular endothelial cells can promote microvascular inflammation in the allograft.

Fig. 1. Adhesion molecule genes and chemokine genes were upregulated in GEnCs exposed to IFN γ .

RNA from GEnC activated by IFN γ was compared to RNA from GEnC in the media control group. Upregulated genes included chemoattractants, such as CCL5 and CCL2; MHC proteins, such as β 2-microglobulin; inflammatory cytokines, such as IL-6; and adhesion molecules, including VCAM1 and ICAM1. This experiment was done a single time. Confirmatory RT-PCR analysis is shown in Supplemental Figure 1.

Fig. 2. IFN γ stimulation upregulated chemokine protein production by GEnC.

After stimulation with IFN γ , GEnC were further challenged with antibody directed against MHC class I, antibody directed against MHC class II, sensitized plasma, or non-sensitized plasma. Supernatants were analyzed for chemoattractants (CCL2 and CCL5) in a multiplex bead assay. (A) IFN γ stimulation significantly increased CCL2 production compared to GEnC incubated with media control ($p=0.04$). Incubation with sensitized or non-sensitized plasma further increased CCL2 stimulation compared to IFN γ stimulation alone ($p<0.0001$, for both). (B) IFN γ stimulation of GEnC significantly increased CCL5 production compared to media control ($p<0.0001$). Incubation with anti-MHC class I antibody, anti-MHC II antibody, sensitized plasma, or non-sensitized plasma did not increase production of CCL5 beyond levels with IFN γ stimulation. There was less CCL5 production with plasma incubation compared to IFN γ ($p=0.04$), but these values were still above CCL5 production by GEnC incubated with media alone (media versus sensitized plasma $p<0.0001$, media versus non-sensitized plasma $p=0.003$). Data presented as mean $\pm$ SEM. Data were compared using ANOVA with multiple comparisons. The data represent four individual experiments, with each condition done in duplicate.

Fig. 3. GEnC surface expression of MHC and adhesion molecules was upregulated by $\text{IFN}\gamma$ activation.

(A-D) Cells were analyzed for surface expression of MHC class I, MHC class II, ICAM1, and VCAM1 by flow cytometry. (A) Surface expression of MHC class I was present at baseline in media controls (medium gray) and further increased after $\text{IFN}\gamma$ stimulation (dark gray). (B) However, MHC class II was not expressed on the surface in media control GEnC, as indicated by complete overlap with isotype control (light gray). MHC II was expressed on GEnC surface after activation with $\text{IFN}\gamma$ (dark gray). (C, D) There was basal expression of ICAM1 and VCAM1, which was further enhanced after incubation with $\text{IFN}\gamma$. Representative data is shown from an experiment that was repeated 12 times.

Fig. 4. Surface adhesion molecule expression was not further enhanced by GEnC exposure to anti-MHC antibodies.

(A) Imaging flow cytometry demonstrated MHC class I, MHC class II, ICAM1 and VCAM1 were all upregulated following IFN γ stimulation. Representative images are shown. (B, C) Surface expression of MHC class I and II was significantly upregulated by IFN γ activation ($p < 0.0001$ for both). GEnC surface expression of MHC molecules was not further increased by incubation with anti-MHC antibodies. Intensity is expressed in arbitrary units (a.u.) (D, E) A similar pattern of surface expression was seen for ICAM1 and VCAM1. Both adhesion molecules were upregulated after IFN γ activation ($p < 0.0001$ for both), but not further enhanced by incubation with anti-MHC antibodies. Statistical analyses were performed using ANOVA and multiple comparisons analysis. The experiment was performed four times. Data represent mean $\pm$ SEM.

Fig. 5. Imaging flow cytometry demonstrated co-localization of MHC and adhesion molecules on the GEnC surface.

Staining for MHC molecules is indicated by red areas, areas of adhesion molecules are in green, and areas of co-localization are yellow. (A) Co-localization (yellow) of MHC class I with adhesion molecules (ICAM1 and VCAM1) is shown in media controls. MHC class II is not expressed on unstimulated cells and therefore only adhesion molecules are seen on the surface (green). (B) Cells activated with IFN γ have increased surface expression of all four molecules. Imaging flow cytometry demonstrated MHC and adhesion molecules are co-localized on the GEnC surface, indicated by yellow areas. Representative images are shown. The experiment was performed four times and in each experiment 1000 images were collected for each condition.

Fig. 6. Confocal microscopy confirms co-localization of MHC and adhesion molecules on the surface of GEnC.

Staining for MHC molecules is indicated by areas in red, areas of adhesion molecules are in green, areas of co-localization are yellow, and nuclei are indicated by DAPI staining in blue. Scale bar represents 30 μ m (A*i*, B*i*) There were low levels of MHC class I detectable on media control treated GEnCs (red), but MHC class I was clearly detectable on cells stimulated with IFN γ (A*ii*, B*ii*). (C*i*, D*i*) There were barely detectable levels of MHC class II without IFN γ stimulation (red), but MHC class II was increased upon IFN γ stimulation (C*ii*, D*ii*). (A, B, C, D) Adhesion molecules were present on GEnC in both media control and IFN γ stimulated conditions. (A*ii*, B*ii*, C*ii*, D*ii*) Treatment with IFN γ increased surface expression of MHC molecules (red) and co-localization between MHC molecules and adhesion proteins (yellow). (E) There was a significant increase in co-localization between MHC molecules and VCAM1. Data were obtained from three non-overlapping images per condition from three independent experiments. Graphical data presented as mean $\pm$ SEM. Statistical analyses were performed using an unpaired t-test. Individual channel images are shown in Supplemental Figure 3.

Table 1

Co-localization of MHC and adhesion molecules by multispectral imaging flow cytometry.

		BDSM		MAD	
		MHC I	MHC II	MHC I	MHC II
<i>Media control</i>	ICAM1	2.5	0	0.26	0
	VCAM1	2.4	0	0.08	0
<i>IFNγ</i>	ICAM1	2.7	2.4	0.44	0.15
	VCAM1	2.5	2.3	0.21	0.38
<i>IFNγ + anti-MHC I Ab</i>	ICAM1	2.7	2.5	0.46	0.22
	VCAM1	2.5	2.4	0.22	0.18
<i>IFNγ + anti-MHC II Ab</i>	ICAM1	2.7	2.4	0.43	0.17
	VCAM1	2.5	2.4	0.21	0.15
<i>IFNγ + sensitized plasma</i>	ICAM1	2.7	2.4	0.42	0.19
	VCAM1	2.5	2.4	0.22	0.17

Ab, antibody; BDSM, bright detail similarity median; ICAM, intercellular adhesion molecule; IFN γ , interferon gamma; MAD, median average deviation; VCAM, vascular cell adhesion molecule