Tissue-Specific Microvascular Endothelial Cells Show Distinct Capacity To Activate NK Cells: Implications for the Pathophysiology of Granulomatosis with Polyangiitis

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The relevance of tissue specificity of microvascular endothelial cells (MECs) in the response to inflammatory stimuli and sensitivity to immune cell–mediated injury is not well defined. We hypothesized that such MEC characteristics might shape their interaction with NK cells through the use of different adhesion molecules and NK cell receptor ligands or the release of different soluble factors and render them more or less vulnerable to NK cell injury during autoimmune vasculitis, such as granulomatosis with polyangiitis (GPA). To generate a comprehensive expression profile of human MECs of renal, lung, and dermal tissue origin, we characterized, in detail, their response to inflammatory cytokines and to proteinase 3, a major autoantigen in GPA, and analyzed the effects on NK cell activation. In this study, we show that renal MECs were more susceptible than lung and dermal MECs to the effect of inflammatory signals, showing upregulation of ICAM-1 and VCAM-1 on their surface, as well as release of CCL2, soluble fractalkine, and soluble VCAM-1. Proteinase 3–stimulated renal and lung MECs triggered CD107a degranulation in control NK cell. Notably, NK cells from GPA patients expressed markers of recent in vivo activation (CD69, CD107a), degranulated more efficiently than did control NK cells in the presence of renal MECs, and induced direct killing of renal MECs in vitro. These results suggest that, upon inflammatory conditions in GPA, renal MECs may contribute to the recruitment and activation of NK cells in the target vessel wall, which may participate in the necrotizing vasculitis of the kidney during this disease. The Journal of Immunology, 2014, 192: 3399–3408.
CCL2, soluble fractalkine (sFKN), soluble (s)ICAM-1, and sVCAM-1 were quantified by ELISA (R&D Systems) in cell-free supernatants, according to

For CD107a-degranulation assays, MECs were incubated with NK cells at a 2:1 E:T ratio for 4 h, after which cells were collected. Mortality of ECs was determined using Via-Probe (BD Biosciences) or Red Dead Cell Stain (Life Sciences), CD107a-FITC, CD94-FITC, DNAM-1–FITC, Ki67-PE (BD Biosciences), CD107a-FITC, CD94-FITC, DNAM-1–FITC, Ki67-PE (BD Biosciences), NKG2C-allophycocyanin, NKG2A-allophycocyanin (R&D Systems), and NKG2D-FITC (eBioscience, San Diego, CA). Cells were fixed, and analyzed by flow cytometry.

For cytotoxicity experiments, MECs were incubated with NK cells at a 2:1 E:T ratio for 4 h, after which cells were collected. Mortality of ECs was determined using annexin V and 7-aminoactinomycin D (7-AAD) staining after gating on CD105+ cells.

NK cell purification and functional assays
NK cells were freshly purified from PBMCs by negative selection using magnetic microbead separation (STEMCELL Technologies, Vancouver, BC, Canada) with purity > 95% and were stimulated overnight with 50 ng/ml IL-15 and 20 U/ml IL-2.

For CD107a-degranulation assays, MECs (2–5 × 10^6 per flat-bottom well) were stimulated overnight with TNF-α and/or PR3 or left untreated, after which supernatants were removed, cells were replenished with fresh medium, and NK cells were added at a 1:1 E:T ratio for 4 h. PMA (50 ng/ml) plus ionomycin (0.25 μg/ml; both from Sigma-Aldrich) was used as a positive control. For coculture experiments, IL-2–stimulated NK cells were incubated for 4 h with cell-free supernatants from 48 h resting or stimulated MECs. FITC-conjugated anti-human CD107a (diluted 1/25; clone H4A3; BD Biosciences) was added directly. After 1 h at 37˚C in 5% CO₂, monensin (6 μg/ml; Sigma-Aldrich) was added for an additional 3 h. Cells were stained with CD56-Pacific, CD3-Pacific Blue Abs, fixed, and analyzed by flow cytometry.

For cytotoxicity experiments, MECs were incubated with NK cells at a 2:1 E:T ratio for 4 h, after which cells were collected. Mortality of ECs was determined using annexin V and 7-aminoactinomycin D (7-AAD) staining after gating on CD105+ cells.

Statistical analysis
All statistical tests were performed using Instat 3 software (GraphPad, La Jolla, CA). Comparisons between two groups were analyzed by the nonparametric Mann–Whitney U test, and comparisons between more than two groups were analyzed by nonparametric ANOVA (Kruskal–Wallis test), followed by the Dunn posttest. Correlation between two variables was determined using the Spearman coefficient (rho). Two-sided p values < 0.05 were considered significant.

Results
Basal phenotype of tissue-specific MECs
As a first step to compare the ability of MECs of different tissue origins to mediate adhesion, recruitment, and activation of NK cells, we performed a detailed phenotypic characterization of primary MECs derived from renal glomerular, lung, and dermal tissues. To provide an element of comparison with published data, we also analyzed HMEC-1, an immortalized cell line derived from dermal MECs (16), which represents an established EC model and is used frequently for in vitro studies.

In pilot experiments, we evaluated whether MEC phenotype was modified by culture conditions. As previously described for HMEC-1 (17), we observed phenotypic changes of primary MECs based on the confluence state, the time from plating to confluency, and the number of cell divisions. Although the phenotype of a given MEC type remained homogeneous overall among repeated experiments, expression levels of some surface molecules varied. Thus, in the following analysis, MECs were always analyzed in the same conditions (passages 3–10, subconfluency), and only molecules showing reproducible expression levels in at least four independent experiments were considered.

We first analyzed expression of molecules allowing NK cell adhesion to ECs through integrin-dependent (ICAM-1, VCAM-1)
or integrin-independent (endoglin, FKN) interactions. As shown in Fig. 1A, all MECs significantly expressed ICAM-1 (CD54) (albeit expression was lower in dermal MECs than in others) and endoglin (CD105), weakly expressed FKN (CX3CL1), and did not express VCAM-1. MECs did not express HLA class II molecules, in line with their resting status, and expressed high, but variable, levels of HLA class I molecules.

We next determined whether tissue-specific MECs expressed ligands for activating or inhibitory NK cell receptors (Fig. 1B). Overall, tissue-specific MECs similarly displayed high levels of PVR and nectin-2 (DNAM-1 ligands). Expression of NKG2D ligands was not strictly superimposable on all MECs. Thus, MICA and MICB molecules were found on dermal and renal MECs but were detected only weakly on lung MECs. ULBP-2 was expressed on all MECs, whereas ULBP-1 and ULBP-3 were barely detectable. It must be noted that ULBP-3 is sensitive to trypsin treatment (its levels on HMEC-1 cells were higher when cells were detached with EDTA only, whereas expression of other NKG2D ligands remained unchanged; data not shown). Because tissue-specific MECs grew on fibronectin-coated flasks and could not be detached without trypsin, the low levels of ULBP-3 on these cells must be considered with some caution. Last, tissue-specific MECs weakly expressed HLA-E (CD94/NKG2A ligand) and did not express CD48 (2B4 ligand) or HLA-G (data not shown).

Altogether, our results show that, in basal conditions, primary MECs originating from skin, lung, and kidney do not show striking differences in the pattern of expression of molecules involved in adhesion and activation of NK cells.

**Phenotype of tissue-specific MECs after stimulation by inflammatory cytokines**

ECs upregulate expression of adhesion molecules upon activation by inflammatory cytokines (1, 18, 19). Thus, we compared the responsiveness of tissue-specific MECs to various cytokines that might be present in their vicinity as a result of local or systemic inflammation.

IFN-α, IL-2, IL-12, IL-17, and IL-18 did not mediate any reproducible phenotype modification, apart from the known IFN-α-induced expression of HLA class I molecules (data not shown). In contrast, IFN-γ and TNF-α significantly modified MEC phenotype, as reported in different EC lines (20–24), but the magnitude of these modifications varied, depending on their tissue origin (Fig. 2). IFN-γ alone induced de novo expression of HLA class II molecules more strongly on dermal MECs than on renal and lung MECs. IFN-γ also upregulated HLA class I on renal and lung MECs, as well as increased ICAM-1 levels on renal MECs only. TNF-α stimulation upregulated HLA class I and ICAM-1 levels on renal and dermal MECs. It also induced de novo expression of VCAM-1, in particular on dermal MECs.

Combining TNF-α and IFN-γ had a clear synergistic effect on ICAM-1 and VCAM-1 expression in renal MECs and induced FKN expression in lung MECs. Finally, none of the tested cytokines reproducibly modified expression of ligands of NK cell receptors on the different MECs.

Taken together, our results confirm that stimulation by TNF-α and/or IFN-γ significantly modifies the phenotype of ECs and further demonstrate that the extent and specificity of these changes vary, depending on the endothelium origin.

**FIGURE 1.** Basal phenotype of tissue-specific MECs. Cell surface expression of adhesion molecules (A) and ligands of NK cell receptors (B) on resting HRGMECs, HPMECs, and HDMECs compared with HMEC-1 cells. Data shown are representative of at least four independent experiments. Open graphs represent staining with isotype-matched control Abs. Numbers indicate MFIR.
Production of inflammatory cytokines and chemokines by tissue-specific MECs

Upon activation, ECs release soluble factors acting in an autocrine or paracrine way to favor leukocyte recruitment and amplification of the inflammatory response. Therefore, we compared the amount of cytokines (IL-1β, IL-6, IL-15) and chemokines (IL-8, sFKN, CCL2) in cell-free supernatants of resting or cytokine-activated MECs of different tissue origin.

IL-1β and IL-15 were not reproducibly detected in any condition (data not shown). IL-6, IL-8, and CCL2 were constitutively produced by resting MECs, as previously described in HUVEC and HMEC-1 lines (20, 25, 26). However, their levels differed depending on the MEC origin (Fig. 3). Thus, lung MECs constitutively produced high levels of IL-6 and IL-8 but relatively low amounts of CCL2. Compared with lung MECs, renal MECs released ~10-fold less IL-6 and IL-8 and 10-fold more CCL2. Dermal MECs produced minimal amounts of IL-6 but released IL-8 and CCL2 at similar levels as renal MECs. Of note, only dermal MECs released sFKN in basal conditions.

Following TNF-α or IFN-γ stimulation, tissue-specific MECs also behaved distinctly. TNF-α increased IL-6 and IL-8 production by all MECs, with lung MECs still releasing the highest levels...
of both cytokines. TNF-α increased CCL2 levels in lung MECs and enhanced sFKN levels in dermal MECs. IFN-γ alone did not augment the production of IL-6, IL-8, or sFKN. However, combining TNF-α and IFN-γ had a clear synergistic effect on sFKN production by renal and lung MECs, as previously reported in HUVECs and intestinal MECs (27).

Soluble forms of adhesion molecules (sICAM-1, sVCAM-1) have been found in the culture supernatant of cytokine-treated ECs (17, 19, 28). These soluble molecules may act as competitive inhibitors blocking adhesion to inflamed vascular endothelium, or they may modify leukocyte activation (29–33). The robust induction of ICAM-1 and VCAM-1 on renal and dermal MECs upon cytokine stimulation prompted us to investigate whether their soluble forms were released in parallel. Significant amounts of sICAM-1 were detected in supernatants of all MECs in basal conditions, and levels increased upon stimulation by TNF-α and/or IFN-γ. Surprisingly, although VCAM-1 was not expressed at the surface of resting MECs, sVCAM-1 molecules were found in supernatants of resting renal MECs and, to a lesser extent, dermal MECs. Furthermore, stimulation by TNF-α, alone or combined with IFN-γ, strongly increased sVCAM-1 release by all MECs, with the highest levels produced by renal MECs.

Altogether, these results demonstrate that MECs produce distinct amounts of cytokines, chemokines, and soluble adhesion molecules depending on their tissue origin (Table I). In particular, renal MECs release high levels of CCL2, sFKN, and sVCAM-1 molecules upon cytokine stimulation, whereas lung MECs mostly produce IL-6 and IL-8.

**PR3 stimulation induces distinct modifications in tissue-specific MECs**

Accumulated data indicate that PR3 may participate in the local inflammatory response in GPA. PR3 can activate HUVECs, inducing expression of ICAM-1 and VCAM-1, which results in enhanced adhesion of neutrophils and monocytes (34–37). Because PR3 can bind to ECs through protease-activated receptor 2 (PAR-2), we first characterized PAR-2 expression on tissue-specific MECs by flow cytometry (Fig. 4A). PAR-2 was weakly expressed on dermal and renal MECs but was not detected on lung MECs and HMEC-1. Moreover, TNF-α enhanced expression of PAR-2 on renal ECs, as previously reported in HUVECs (38, 39), but it had no effect on lung ECs (Supplemental Fig. 1).

We next determined whether tissue-specific MECs showed distinct susceptibility to PR3 stimulation (Fig. 4B, 4C). PR3 treatment alone had only minor effects on the expression of surface or soluble molecules by the different MECs. We next determined whether combining PR3 with a low concentration of TNF-α further modified the MEC phenotype. Compared with changes induced by PR3 or TNF-α alone, the combined treatment increased ICAM-1 and VCAM-1 expression on renal MECs. We also analyzed whether PR3 treatment modified the release of soluble factors by tissue-specific MECs (Fig. 4D). PR3 slightly enhanced IL-6 release by
interindividual NK cell variability was observed. Thus, we aimed to determine whether a soluble factor present in those MEC supernatants was responsible for NK cell degranulation. Expression of CD107a was not correlated with levels of IL-6, IL-8, CCL2, sFKN, sICAM-1, or sVCAM-1 in MEC supernatants. Moreover, incubating purified NK cells with any of these soluble factors did not induce CD107a expression (data not shown). Whether NK cell degranulation is related to the combined effect of those soluble molecules or to a so far unidentified factor produced by PR3-stimulated MECs remains to be determined.

**NK cells from GPA patients are activated and prone to kill renal MECs**

Prompted by these results, we next determined the phenotype and functional characteristics of NK cells in a series of patients with diffuse GPA vasculitis and glomerulonephritis. No gross abnormality in NK cell distribution was observed, with total NK cells and relative proportions of CD56bright CD16– and CD56dim CD16+ NK cell subpopulations comparable to those in healthy controls. Overall, expression of Nkp30, Nkp44, Nkp46, NKG2D, DNAM-1, CD94, NKG2C, NKG2A, and KIRs was similar in GPA patients and controls (data not shown). Of note, increased basal expression of CD69 and CD107a, but not Nkp44, was observed on NK cells from GPA patients, suggesting that these cells were recently activated (Fig. 6).

Determination of the lytic ability of patients’ NK cells. Like control NK cells, NK cells from GPA patients efficiently degranulated in the presence of tissue-specific MECs, in particular renal MECs. Furthermore, patients’ NK cells showed significantly greater CD107a degranulation in the presence of PR3- or TNF-α-stimulated renal MECs compared with control NK cells, in line with their increased basal expression of CD107a (Fig. 7A, 7B).

To determine whether NK cells actually mediate EC lysis, we exposed renal MECs to purified NK cells from controls or GPA patients for 4 h and then quantified EC death. Control NK cells did not induce significant mortality among renal MECs. In contrast,

### Table I. Summary of modifications induced in inflammatory-stimulated tissue-specific MECs

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+, present; –, absent; ++, present in high amount; ±, present in low amount; =, no change; †, increased; ††, decreased; †††, strong increase.
FIGURE 4. PR3 induces distinct modifications on tissue-specific MECs. (A) Flow cytometry analysis of PAR-2 surface expression on resting renal, lung, and dermal MECs and HMEC-1 cells. Data shown are representative of three independent experiments. (B) Changes in cell surface expression of ICAM-1 and VCAM-1 on HRGMECs, HPMECs, HDMECs, and HMEC-1 cells that were left untreated (bottom, thin dotted line) or were exposed to PR3 (5 μg/ml; shaded graphs), TNF-α (200 U/ml; bold line), or both (dashed line). Data are representative of three independent experiments. (C) Results are represented as a heat map indicating fold increase (FI) change in expression of the indicated molecule related to the baseline expression on untreated cells. (D) Concentrations of IL-6, IL-8, CCL2, sFKN, sICAM-1, and sVCAM-1 in cell-free supernatants of MECs that were left untreated (-) or were exposed to PR3 (5 μg/ml) and/or TNF-α (200 U/ml) for 48 h. The medium was collected poststimulation, and each factor was quantified by ELISA. Data are mean ± SEM of at least three independent experiments in each cell line. †p = 0.05, ††p = 0.01, †††p = 0.001.
NK cells from GPA patients efficiently killed renal MECs (80% target cell mortality at 2:1 E:T ratio, almost 100% at higher ratios) (Fig. 7C). Taken together, these results show that NK cells from GPA patients can efficiently mediate renal EC lysis, which may participate in kidney damage during this disease.

**Discussion**

ECs sit at the frontier between circulating leukocytes and inflamed tissues; as such, they play an important role as a gateway for lymphocyte infiltration. EC injury occurs in the pathogenesis of diverse pathologic conditions, ranging from vascular and immune diseases to cancer and after immunotherapy with cytokines. To elucidate interactions between ECs and their microenvironment, the use of adequate cellular models reflecting the physiology is required. However, most in vitro models that provide information about EC interactions involve HUVECs or HMEC-1 and, thus, are limited by the lack of organ specificity. Considering that GPA vasculitis primarily occurs in defined target organs, in particular lungs and kidneys, we wondered whether tissue-specific characteristics of MECs might govern the nature or strength of interactions with NK cells in an inflammatory situation. Our results indicate that ECs from a given origin present their own response profile to inflammatory cytokines, reflecting the specificity and susceptibility of the tissue from which these cells originate. In particular, renal MECs appeared to be more susceptible than lung and dermal MECs to the effect of TNF-α and IFN-γ, which mediated upregulation of ICAM-1 and VCAM-1 adhesion molecules on their surface, as well as increased the production of chemokattractants, such as CCL2, sFKN, and sVCAM-1. CCL2 and sFKN are known to play an important role in the recruitment of NK cells to inflamed tissues (42, 43), and sVCAM-1, which can bind NK cells with high affinity (32), is found in the sera of GPA patients and correlates with disease activity (44). Altogether, these renal MEC characteristics may contribute to a preferential recruitment and activation of immune effector cells in the inflamed kidney vessel wall. At variance, TNF-α and IFN-γ did not modify expression of adhesion molecules on lung MECs, but they induced a strong release of IL-6 and IL-8. These inflammatory mediators are known to drive lung-inflammatory cascades by increasing lung EC permeability and potentiating neutrophil recruitment (45); thus, they are relevant to the pathophysiology of GPA, which is characterized by granulomatous inflammation of the respiratory tract.

We next aimed at determining how tissue-specific MECs behave in the presence of PR3, which was proposed to be deleterious for vascular ECs, either directly or through binding of PR3-ANCA.

**FIGURE 5.** Tissue-specific ECs distinctly activate NK cell degranulation. (A) Cell-cell contact: control NK cells were incubated for 4 h at a 1:1 ratio with tissue-specific MECs that were left untreated or stimulated overnight with PR3 (5 μg/ml) and/or TNF-α (200 U/ml). (B) Cell-free supernatants: Control NK cells were incubated for 4 h with cell-free supernatants from resting tissue-specific MECs or ones that were stimulated overnight. Data are mean fold increase (± SEM) of CD107a-expressing NK cells in the indicated stimulating condition relative to the percentage of NK cells expressing CD107a in the absence of stimulation (healthy controls, n = 4). Stimulation with PMA and ionomycin (P/I) is shown as positive control. ***p = 0.001.

**FIGURE 6.** NK cells from GPA patients show a recently activated phenotype. Freshly isolated NK cells from 22 healthy controls (●) and 16 GPA patients (▼) were compared for surface expression of CD69, CD107a, and NKp44 activation markers. *p = 0.03, **p = 0.005.
PR3 may be released locally at inflammatory sites from cytokine-primed neutrophils and then bind to ECs, leading to their activation. Thus, it may contribute to leukocyte transvasation across the endothelium by providing chemotactic and activating stimuli. PR3 was reported to activate epithelial and nonepithelial cells via PAR-2, a G protein–coupled receptor that is activated following proteolytic cleavage of its N-terminal exodomain by serine proteases (46). PAR-2 activation augments inflammatory and profibrotic pathways through the induction of genes encoding inflammatory cytokines/chemokines, such as IL-6 and IL-8 (37), and extracellular matrix proteins (47). Furthermore, PAR-2 expression is substantially upregulated after induction of inflammation (48), which may provide an amplification loop for EC activation. Indeed, we observed that TNF-α enhanced PAR-2 expression on renal MECs. Moreover, PR3 combined with TNF-α upregulated membrane expression of ICAM-1 and VCAM-1 on renal MECs, which may further increase contact stability with immune cells and amplify the inflammatory cascade in the affected kidneys in GPA. Of note, lung MECs (PAR-2−/−) showed distinct susceptibility to PR3 treatment, which enhanced IL-6 release but, at the same time, strongly decreased CCL2 production, suggesting that PR3 binding to distinct receptors can mediate different effects in different cell types.

Therefore, we analyzed the potential consequences of inflammatory-induced modifications of tissue-specific MECs on NK cell activation and function. We found that PR3-treated renal and lung MECs released soluble factors that increased NK cell degranulation, but we could not identify which factor was responsible for this effect. Interestingly, PR3 was shown to induce phenotypic and functional maturation of monocyte-derived dendritic cells, which became fully competent APCs for the stimulation of PR3-specific CD4+ T cells (49). Recent data show that IL-32, a proinflammatory cytokine produced by vascular ECs, can be activated by PR3 and, thus, might participate in immune-mediated responses in GPA (50). Alternatively, IL-15–mediated mechanisms might be involved. Indeed, we observed that TNF-α increased expression of IL-15Ra on renal, but not lung, MECs (data not shown). Although we could not reproducibly detect IL-15 production by TNF-α–stimulated MECs, we (8) previously reported high serum IL-15 levels and strong expression of IL-15 in the kidney of GPA patients with active glomerulonephritis. Interestingly, it was demonstrated recently that increased IL-15/IL-15Ra expression on hantavirus-infected ECs was responsible for NK cell activation and killing of ECs (H.G. Ljunggren and M. Braun, personal communication). Experiments are underway to determine whether the combined effects of PR3, IL-32, and IL-15 favor NK cell activation in our experimental model. In line with this hypothesis, we observed that NK cells from GPA patients exhibited a recently activated phenotype ex vivo and were more prone than control NK cells to degranulate in vitro in the presence of PR3− or TNF-α–stimulated renal MECs. Furthermore, NK cells from GPA patients, but not from healthy controls, were able to mediate direct killing of renal MECs, which might represent another mechanism of vascular injury in GPA.

Altogether, our results suggest that PR3 binding to ECs, together with local production of inflammatory cytokines/chemokines, triggers NK cell activation and cytolytic ability against vascular endothelium, in particular of renal origin. It is likely that complex intercellular communication between NK cells and the endothelium is involved. In addition to the pathophysiological role of PR3 binding to PR3-ANCA, PR3 binding to renal MECs could instruct them to induce NK cell activation and further promote the chronic kidney inflammation in GPA.
Disclosures
The authors have no financial conflicts of interest.

References


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